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

Editors: J.L. Sherwood and C.M. Rush

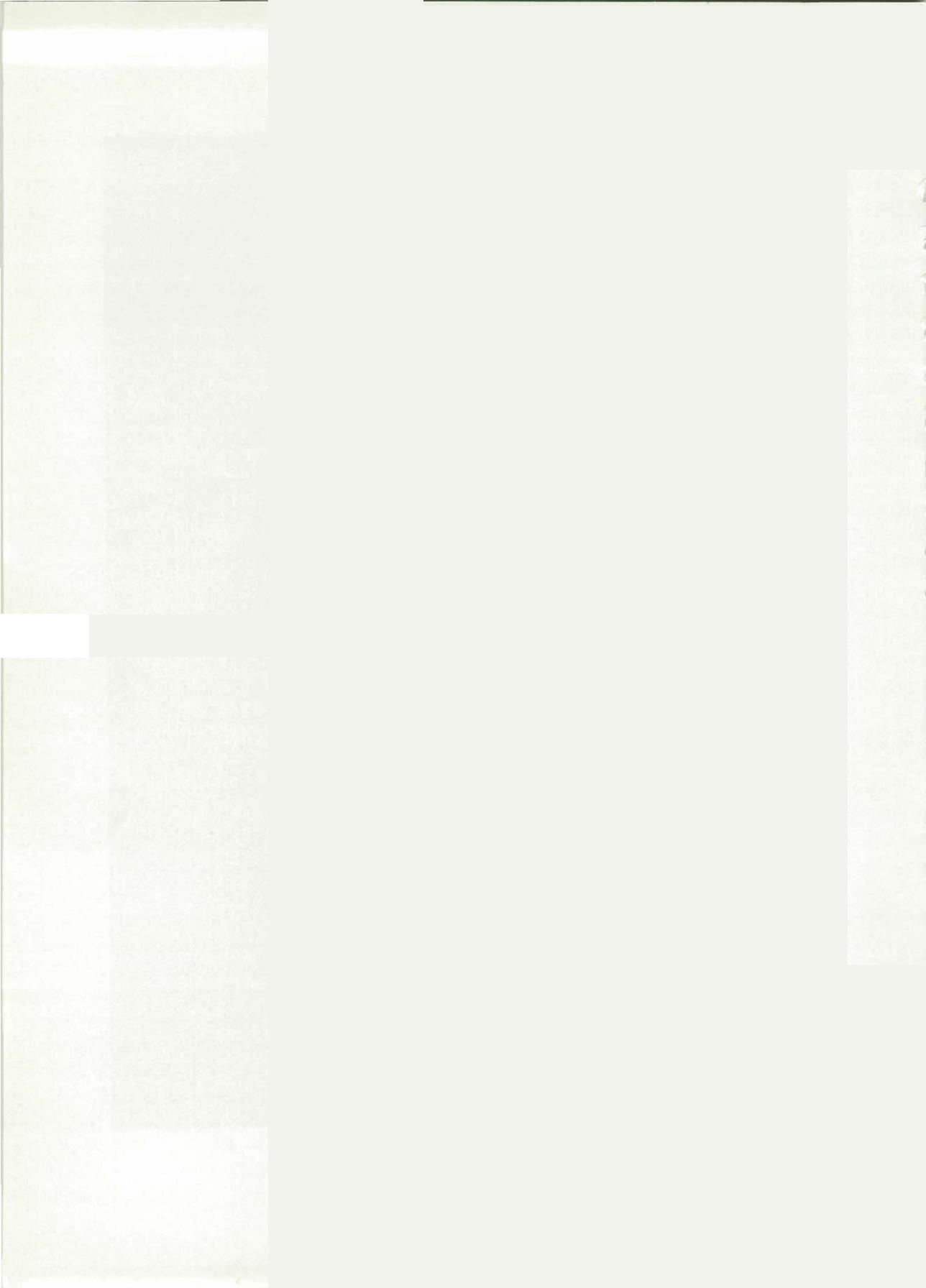West Park Conference Centre, Dundee, Scotland
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PREFACE

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) and, most recently, at The West Park Conference Centre, University of Dundee, Dundee, Scotland (1996). 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. The next symposium is scheduled for 1999.

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Contents

Characterization

6

Sequence and genome analysis

Mycology

Cytology

Detection, etiology and control

8

Special subject

BARLEY MILD MOSAIC VIRUS: DELETIONS, DUPLICATIONS AND TRANSMISSION

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Summary

The UK-M isolate of BaMMV, which was repeatedly mechanically transmitted to barley and lost the ability to be transmitted by its fungal vector, was shown previously to have a deletion of 1092 nt in the coding region of RNA2. Now, a similarly sized RNA2 deletion has been detected in a UK field sample, and a smaller deletion has been observed in experimental glasshouse-grown plants. In addition, a subpopulation of RNA2 of BaMMV UK-M has been found to have a 552 nt sequence duplication in the 3' untranslated region. The significance of these deletions and duplications for fungus transmission is being investigated by the production of RNA transcripts and cDNA clones of the different RNA2 species.

Introduction

Barley mild mosaic bymovirus (BaMMV) can cause yield losses in winter barley in Western Europe, China and Japan. It is a member of the Potyviridae family, and in common with other bymoviruses is transmitted by a soil-borne plasmodiophoromycete fungus, *Polymyxa graminis*. BaMMV is transmitted to fresh barley hosts inside zoospores and resting spores of the fungus, unlike some other fungally transmitted plant viruses (such as TNV), which are carried outside the spore/zoospore. When inside resting spores, BaMMV can persist for several years, and this, coupled with the impracticality of eradicating *Polymyxa graminis*, makes BaMMV difficult to control.

BaMMV, like other bymoviruses, has a bipartite genome of poly-A -tailed positive-sense single-stranded RNA: RNA1 of a wild-type UK isolate (UK-F) is 7276 nt, and RNA2 is 3524 nt (Peerenboom *et al*, 1996). Both RNAs encode polyproteins (257 & 98 kDa respectively) which are subsequently proteolytically processed to yield smaller functional proteins. RNA1 encodes the coat protein, and is also believed to encode an NIb polymerase, an NIa proteinase and a cytoplasmic inclusion protein. RNA2 is thought to encode two proteins: the first 25 kDa protein is similar to the helper component proteinase of the aphid-transmitted potyviruses, but the function of the second 73 kDa protein is unknown, and it has no homology with currently available protein sequences of known function.

However, one BaMMV isolate, UK-M (which was derived from the wild-type UK-F by repeated mechanical transmission) was previously found to have lost the ability to be transmitted by the fungal vector (Adams *et al*, 1988). This isolate has a 1092 nt deletion (Jacobi *et al*, 1995) in the region of RNA2 that codes for the 73 kDa protein, but is otherwise similar to RNA2 of UK-F, suggesting that the 73 kDa protein has a role in fungal transmission.

Experimental and Discussion

Recently, the RNA1's of both isolates were sequenced (Peerenboom *et al*, unpublished results), and their translated ORF's are 99.33 % similar (see Fig.1), indicating that the RNA2 deletion of UK-M is responsible for the inability of UK-M to be fungally transmitted.
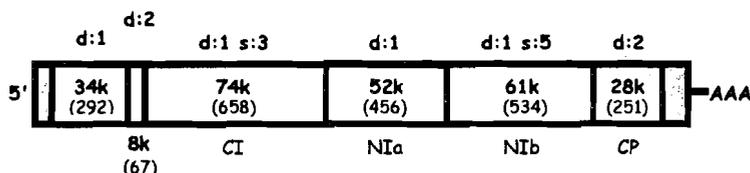
Fig. 1. Number of amino acid differences (d:) and similarities (s:) between BaMMV RNA1 of UK-F and UK-M. Figures in brackets denote the total number of amino acids in each putative protein.

BaMMV	697	ADIIDT**ERER**GDLTQFD	713
BaYMV	691	LERIAA**ERL.NTL**TAYD	706
BaMMV	813	NEMA**ASQRD**IANATREA	829
BaYMV	805	QAQI**DSQRR**AVTITEAS	821
BNYVV	463	LEEAEL**ERRER**DMTMIA	479
	549	SMPS**KTER**YVHTGIQGG	565
PCV	441	WEMIEK**ER**LKLSAEKNR	457
PMTV	242	LEITR**DERK**RQLHEVRV	258
	472	RCNVES**QR**ESAYFNELI	488
SBWMV	531	HHVDED**QKRK**NDRAIEW	547

Table 1. Common ER (glutamic acid-arginine) or QR (glutamine-arginine) motifs (underlined) identified as being likely to be on the surface (bold type) in both BaMMV, BaYMV and the capsid protein readthroughs of other fungally transmitted viruses

From January to May 1996, barley mild mosaic virus was periodically sampled from two field plots in a field near Hatherop in Southern England. An RT-PCR test, using primers designed to span the deleted region of UK-M RNA2, was used to look for the occurrence of similar deletions in the field samples. For the first two months, all samples tested retained a full-length RNA2. However, in a sample taken from one field plot in March, a proportion of RNA2 had a deletion. The RT-PCR product from this sample with the deletion was sequenced, and was shown to be a similar (but not identical) deletion to the existing UK-M deletion. This new deletion (D3) is 1095 nt long, and corresponds to nts 1644 to 2738 in UK-F RNA2. Also, in March
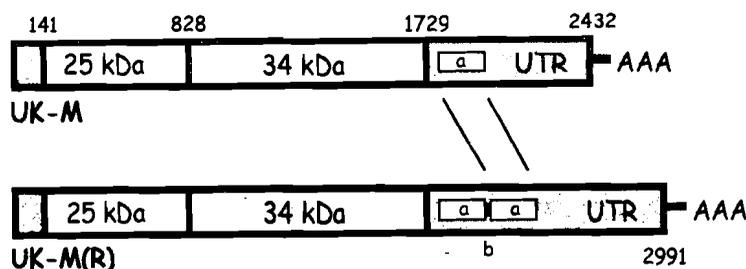
Fig. 2. Diagram showing the duplicated region in the 3' UTR of BaMMV UK-M(R) RNA2. Key: a = 552 nt duplicated region; b = 11 nt AT-rich linker.

1995 UK-F isolate maintained in some glasshouse-grown plants was seen to have developed a deletion (D2: 921 nt deletion correspon-ding to nts 1863 to 2783 in UK-F) smaller than that of UK-M, and three deletions have been previous-ly reported from field isolates of BaMMV sampled from Aschersleben and Gießen in Germany (Timpe & Kühne, 1994). Common to all deletions described so far is that they all occur within nts 1625 to 2785 (relative positions in UK-F RNA2), roughly occupying the latter two-thirds of the coding region for the 73 kDa protein. Unfortunately, it is not known whether isolates other than UK-M with these RNA2 deletions can be transmitted by the fungus.

Comparison of sequences predicted to be near the surfaces of the 73 and 70 kDa pro-teins of BaMMV and the closely related barley yellow mosaic bymovirus revealed conserved ER and QR motifs. The amino acid sequences for the BaMMV RNA2 73 kDa and the corresponding BaYMV 70 kDa proteins were compared, and any regions of homology noted. Then, using the GCG program Peptide Structure, the homologous regions that were likely to be on the surface of the eventual protein were marked. Any regions that fitted these criteria were then compared with the capsid protein readthrough products (which are thought to be involved in transmission) of other fungally transmitted viruses (BNYVV, PCV*, PMTV and SBWMV). Two conserved motifs (see Table 1) were revealed: glutamic acid-arginine (ER) and glutamine-arginine (QR). The ER motif aligned with a KTER motif in the 75 kDa readthrough product of BNYVV RNA2, which has been identified as being important for fungal trans-mission. Both motifs are within the deleted region of the non-fungally transmissible UK-M iso-late.

As well as the deletion, part of the UK-M isolate population at Rothamsted has recently been shown to have a duplication of 552 nt within the 5' untranslated region of RNA2. The duplicated sequence starts at nt 1804 and ends at nt 2355, and the duplication starts at nt 2367 and ends at nt 2920 (see Fig. 2). Between the duplicated stretches is an 11 nt AT-rich linker, and the homology between the duplicated stretches is 98 %. RT-PCR tests of the UK-M virus population at Rothamsted (see Fig. 3) have shown that this duplication is genuine, and not merely an artefact of the sequencing process. Part of the virus population was found to have the duplicated form of RNA2 (called UK-M(R)). It is unlikely that the duplicated RNA2 is linked to a particular form of RNA1, however, as it is known that RNA1 and RNA2 molecules from different BaMMV isolates can be mixed, retaining infectivity. The biological significance of the duplication is unknown. RNA viruses are highly variable, and it has been suggested that the viral RNA polymerase can 'fall off' an RNA molecule if it encounters a particularly convoluted secondary structure, and may restart copying another RNA molecule at a different position within the sequence, leading to either a deletion or a duplication.

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* It was assumed that the 39 kDa protein of PCV RNA2, which is downstream of the capsid protein, had a similar function to the capsid readthrough proteins of other furoviruses.

| 123 | UK-F | UK-M |

-1811 bp
-1282 bp
-719 bp
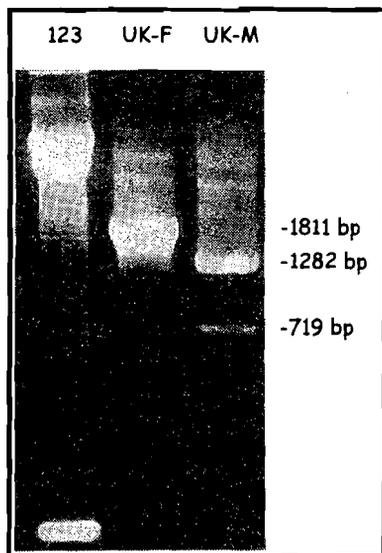
Fig. 3. Gel photograph of RT-PCR products showing 719 bp band amplified from UK-M, and the 1282 bp band amplified from UK-M(R). Also shown is the 1811 bp band amplified from UK-F using the same primers, in which there is no duplication.

To try to study the transmission process in more detail, full-length clones of both UK-F and UK-M RNA2s have been inserted into transcription vectors so that RNA transcripts can be produced from them. These transcripts have been coinoculated (because both RNAs 1 and 2 are required for infectivity) together with virus into host plants: e.g. UK-F RNA2 transcript was coinoculated with UK-M virus, so that if the transcript replicated within the host, it could be distinguished from the virus by a simple RT-PCR test. So far, no coinoculation experiments have shown the transcripts to be replicating. To avoid having to coinoculate with virus, a complete RT-PCR product of RNA1 has been cloned into pUC BM20, and will later be subcloned into the transcription vector. Then both RNA transcripts can be inoculated together without virus. If these transcripts prove to be infect-ious, alterations will be made to the 73 kDa coding region, to try to establish whether this is indeed res-ponsible for fungal transmissibility of the virus.

References

Adams, MJ; Swaby, AG & Jones, P (1988) Confirmation of the transmission of barley yellow mosaic virus (BaYMV) by the fungus *Polymyxa graminis*. Annals of Applied Biology **112** 133 – 141.

Jacobi, V; Antoniw, JF; Adams, MJ; Peerenboom, E; Steinbiß, H-H & Schell, J (1995) Cloning and sequence analysis of RNA2 of a mechanically-transmitted UK isolate of barley mild mosaic virus. Virus Research **37** 99 – 111.

Peerenboom, E., Jacobi, V., Antoniw, J.F., Schlichter, U.H.A., Cartwright, E.J., Steinbiss, H.-H. and Adams, M.J. (1996) The complete nucleotide sequence of RNA-2 of a fungally-transmitted UK isolate of barley mild mosaic bymovirus (BaMMV) and identification of amino acid combinations possibly involved in fungus transmission. Virus Research **40** 149 – 159.

Timpe, U & Kühne, T. (1994) The complete nucleotide sequence of RNA2 of barley mild mosaic virus (BaMMV). European Journal of Plant Pathology **100** 233 – 241.

SYNTHESIS, EXPRESSION AND LOCATION OF THE TOBACCO NECROSIS *NECROVIRUS* STRAIN D p7a MOVEMENT PROTEIN.

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Summary

Tobacco necrosis *Necrovirus*, strain D RNA encodes a polymerase gene, two 7 kDa proteins (p7a and p7b) and the capsid protein (CP). A 3' subgenomic (sg) RNA encodes a transframe p7a and p7b fusion protein with the p7b gene possibly being internally initiated *via* an internal 'shifty' UUUU sequence. Mapping the 5' terminus of this sg RNA and the CP sg RNA revealed a marked degree of homology downstream of the initiation sites which was maintained at the 5' terminus of the genomic RNA. Bacterially-expressed p7a protein binds single-stranded RNA and DNA but not double-stranded DNA and an antiserum raised against p7a locates it to the combined cell wall and membrane fraction of infected leaves. These results and the early transient expression of the p7a protein suggests that it is similar to other carmovirus movement proteins with which it has limited sequence similarity.

Introduction

Tobacco necrosis *Necrovirus* (TNV), a small icosahedral plant virus, has a single positive-sense RNA genome of *c.* 4 kb and a capsid composed of 180 subunits of 29 kDa coat protein (CP). The complete nucleotide (nt) sequence of the genome of TNV strain D (TNV-D; Coutts et al., 1991) reveals that it has four open reading frames (ORFs); a 5'-proximal ORF encoding a 22 kDa protein which may be read-through to produce an 82 kDa protein with the characteristics of a putative RNA-dependent RNA polymerase, two centrally located ORFs encoding two out-of-frame 7 kDa proteins (p7a and p7b) and the 3'-proximal 29 kDa CP. The genome organisation of TNV-D is similar to TNV strain A (TNV-A; Meulewaeter et al., 1990), turnip crinkle *Carmovirus* (TCV; Carrington et al., 1989) and olive latent virus 1 (OLV-1) which may be a distinct *Necrovirus* species (Grieco et al., 1996). The TNV-D p7a and p7b proteins have some sequence similarity with the corresponding ORFs of both TNV-A (8 kDa and 6 kDa respectively) and TCV (8 kDa and 9 kDa respectively; Coutts et al., 1991). These small ORFs in both TNV strains are probably expressed from a single sub-genomic (sg) RNA of *c.* 1.5 kb (Meulewaeter et al., 1990, 1992; Coutts et al., 1991) while the CP is expressed from a 1.2 kb sg RNA (Offei and Coutts, 1996). The 8 kDa and 9 kDa proteins of TCV are both required for cell-to-cell movement of the virus (Hacker et al., 1992), and the corresponding TNV proteins probably play a similar role. Here we describe the binding properties of bacterially-expressed p7a protein and comment on its possible role in virus movement with the knowledge that a number of other virus-encoded movement proteins (MPs) are known to bind ss RNA and facilitate virus transport (Citovsky et al., 1992). This class of MPs are molecular chaperones that bind the viral RNA genome and guide it to

plasmodesmata where the MP increases the size exclusion limit facilitating movement of the virus genome to adjacent cells. In TNV the two functional domains of the MP may be separated and located respectively on the p7a and p7b ORFs, and a mechanism is suggested for their expression *in vivo*. To further evaluate the function of p7a we produced a specific antiserum to the bacterially-expressed protein and investigated its subcellular location, and expression in infected leaves.

Materials and Methods

Nucleic acid purification, Northern analysis and mapping of sg RNAs by primer extension:
    Total nucleic acid extracts of TNV-D infected French bean leaves were produced and ss and ds enriched fractions isolated and probed by Northern blotting. The 5' termini of the 1.5 kb and 1.2 kb sg RNAs were mapped with suitable primers downstream of their suspected termini using both ss and ds RNA templates, by comparison with di-deoxy terminated cDNA clones of known size and genomic location following after polyacrylamide gel electrophoresis (PAGE; Offei and Coutts, 1996).
Cloning, Escherichia coli *expression and nucleic acid binding properties of p7a:*
    The p7a gene was cloned by RT-PCR using suitable primers for expression of the protein in a recombinant plasmid constructed from the T7 RNA polymerase-based vector pET-3a. The p7a protein was expressed and induced in *E.coli* with IPTG and crude extracts of the bacteria were used for Northwestern and Southwestern probing following SDS-PAGE, renaturation and transfer to nitrocellulose. The radioactive probes used were all based on the TNV-D RNA sequence and included ds or ss DNA and ss RNA types (Offei *et al.*, 1995).
Subcellular location and expression of p7a and CP:
    Proteins from healthy and TNV-D infected French bean leaves were fractionated by centrifugation as described by Lehto *et al.*, (1990). The various protein fractions, taken from plants harvested at different times post-inoculation, were subjected to Tricine SDS-PAGE, renatured and electroblotted to duplicate nitrocellulose membranes for probing with either an antiserum raised against bacterially-expressed p7a (see above) or antiserum to CP, raised against the whole virus.

Results

    Both TNV-D sg RNAs started at G residues. The larger sg RNA was 1547 nt in length (encompassing both the p7a and p7b ORFs) and had a leader sequence of 36 nt and the smaller sg RNA had a 90 nt leader upstream of the CP AUG and was 1202 nt long. Analysis of the 5' terminal locations of both sg RNAs and the previously mapped analogous sg RNAs associated with infection with TNV-A (Meulewaeter *et al.*, 1992) and OLV-1 (Grieco *et al.*, 1996) revealed a marked degree of homology downstream of the initiation sites for each RNA. This homology was maintained at the 5' termini of the three virion RNAs and could be extended to another TNV isolate (TNV-Nebraska; TNV-NE) for which partial sequence data, but not sg mapping RNA data are available (Zhang *et al.*, 1993). Part of the conserved sequence in all of the RNAs mentioned above included an invariant ACCA box, 6-9 nt downstream of the 5' terminal nt.
    Over-expression of the p7a ORF in *E. coli* following induction with IPTG resulted in the production of a protein of the expected size. Unfortunately p7a did not form insoluble aggregates after induction and could not be easily purified and the binding properties of the

protein were investigated following separation in gels, transfer to nitrocellulose and probing which showed that p7a was able to bind ss RNA and DNA but not ds DNA. These protein-nucleic acid complexes were stable at moderately high salt concentrations. Attempts to express the p7b ORF in a similar fashion were unsuccessful.

Using an antiserum raised against purified p7a, isolated by preparative PAGE, immunoblot analysis showed that the protein was detectable only in the combined cell wall and cell membrane (MCW) fraction prepared from TNV-D infected bean leaves. The p7a protein was detectable 1 day after inoculation and reached a maximum 3 days later, before declining in amount, whereas CP was not detectable until 3 days after inoculation and continued to increase in amount for a further 2 days before declining.

Discussion

The sequence similarity at the 5' termini of the genomic and sg *Necrovirus* RNAs noted here may reflect common replicase recognition signals for the corresponding minus-strand RNA. However, the positioning of potential sg RNA promoter sequences may occur either upstream or downstream of their initiation sites. If, as has been demonstrated for brome mosaic *Bromovirus* (Marsh et al., 1988), the mechanism of TNV-D sg RNA generation involves internal initiation on minus-strand templates, it is possible that upstream promoter sequences are virus-specific whereas the downstream sequences may be less specific and vary for individual viruses. The p7a protein, which is expressed from the 1.5 kb TNV-D sg RNA, has a differing affinity for ss and ds nucleic acids and a salt binding profile with RNA indicative of a stable physiological interaction *in vivo*. These features are in common with a number of well-characterised virus MPs including that from tobacco mosaic *Tobamovirus* (Citovsky et al., 1992). However while a comparisons of MPs from several different plant virus groups revealed the presence of conserved amino acid motifs these were absent from the distantly related TNV-D p7a and TCV 8 kDa proteins (28% identical; 48% similar). Based on its similarity in size, genomic location and sequence the p7a may be analogous to the TCV 8 kDa protein which is necessary for virus cell-to-cell movement (Hacker et al., 1992). Like other plant virus MPs including the TCV 8 kDa protein (Li and Morris, personal communication), TNV-D p7a was detected in the MCW fraction of infected tissue in which it is transiently expressed. This feature of transient expression may be artifactual and only reflect differential extraction efficiency of p7a from the various fractions or either, taken in concert with the lack of synchrony of CP production, a reduction in competition for binding viral RNA or a differential stability of CP and p7a in tissue extracts. Nevertheless we suspect that TNV-D (and possibly other Necroviruses) may have evolved a novel mechanism of virus movement in plants and separated the necessary functions of RNA binding and plasmodesmatal targeting into separate proteins, allowing differential regulation of levels of expression of these functions. In all of the viruses described here and in several other viruses with similar centrally located ORFs (which are as yet, in many cases, of unknown function), these genes appear to be expressed from a single RNA transcript, with no adequate method of expression of the internal ORFs having been demonstrated. A sequence responsible for producing a transframe fusion of two 3' prime-terminal ORFs, probably translated from a single sg RNA has been identified for the *Carlavirus* potato virus M (Gramstat et al., 1994). The second protein is also produced by an internal initiation mechanism. These 'shifty' sequences, consisting only of AAAA followed by a stop codon, were more efficient when mutated to UUUU and dramatically less so when mutated to GGGG or CCCC. An examination of the sequences at the

borders between p7a and the out of frame p7b ORF and the corresponding genes of TNV-A, TNV-NE, TCV and OLV-1 shows in each case sequences identical or nearly identical to the sequence giving maximum transframe fusion above (i.e. TNV-D, UUUU<u>UAA</u>; TNV-A, CUUU<u>UAA</u>; TNV-NE, CUUU<u>UAG</u>; TCV, CUUC<u>UAA</u>; OLV-I, CUUU<u>UAG</u>- in each case the stop codon is underlined). Thus a testable mechanism of expression can be proposed where, in the case of TNV-D, p7a is expressed both singly and as a translational fusion with p7b, and p7b is also expressed separately by internal ribosome entry (Offei *et al*, 1995). This would allow the levels of the three protein types to be differentially regulated for the various functions of virus movement to be fulfilled and is currently being investigated with an infectious clone of TNV-D RNA.

References

Carrington, J. C., Heaton, L. A., Zuidema, D., Hillman, B. I., and Morris T. J. (1989). The genome structure of turnip crinkle virus. *Virology* 170:219-226.

Citovsky, V., Wong, M. L., Shaw, A. L., Prasad, B. V. V., and Zambryski, P. (1992). Visualization and characterisation of tobacco mosaic virus movement protein binding to single-stranded nucleic acids. *Plant Cell* 4,397-411.

Coutts, R. H. A., Rigden J. E., Slabas, A. E., Lomonossoff, G. P., and Wise, P. J. (1991). The complete nucleotide sequence of tobacco necrosis virus strain D. *J. Gen. Virol.* 72,1521-1529.

Gramstat, A., Prufer, D., and Rohde, W. (1994). The nucleic acid-binding zinc finger protein of potato virus M is translated by internal initiation as well as by ribosomal frameshifting involving a shifty stop codon and novel mechanism of P-site slippage. *Nuc. Acids Res.* 22:3911-3917.

Grieco, F., Savino, V., and Martelli, G. P. (1996). Nucleotide sequence of the genome of a citrus isolate of olive latent virus-1. *Arch. Virol.* 141:825-838.

Hacker, D. L., Petty, I. T. D., Wei, N., and Morris, T. J. (1992). Turnip crinkle virus genes required for RNA replication and virus movement. *Virology* 186:1-8.

Lehto, K., Bubrick, P., and Dawson, W. O. (1990). Time course of tobacco mosaic virus 30 K protein accumulation in intact leaves. *Virology* 174:290-293.

Marsh, L. E., Dreher, T. W., and Hall, T. C. (1988). Mutational analysis of the core and modulator sequences of the BMV RNA 1 subgenomic promoter. *Nuc. Acids Res.* 16:981-995.

Meulewaeter, F., Seurinck, J.,and van Emmelo, J. (1990). Genome structure of tobacco necrosis virus strain A. *Virology* 177:699-709.

Meulewaeter, F., Cornelissen, M., and van Emmelo, J. (1992). Subgenomic RNAs mediate expression of cistrons located internally on the genomic RNA of tobacco necrosis virus strain A. *J. Virology* 66:6419-6428.

Offei, S. K., and Coutts, R. H. A. (1996) Location of the 5' termini of tobacco necrosis virus strain D subgenomic mRNAs. *J. Phytopathol.* 144:13-17.

Offei, S. K., Coffin, R. S., and Coutts, R. H. A. (1995). The tobacco necrosis virus p7a protein is a nucleic acid-binding protein. *J. Gen.Virol.* 76:1493-1496.

Zhang, L., French, R., and Langenberg, W. G. (1993). Molecular cloning and sequencing of the coat protein gene of a Nebraskan isolate of tobacco necrosis virus: the deduced coat protein sequence has only moderate homology with those of strain A and D. *Arch. Virol.* 132:291-305.

THE LOCALIZATION OF THE FUNCTIONAL SEQUENCE ON RNA4 OF BEET NECROTIC YELLOW VEIN VIRUS (BNYVV) RELATED TO FUNGUS TRANSMISSION BY INOCULATION WITH INFECTIOUS RNA4 AND ITS MUTANTS TRANSCRIPTED *IN VITRO*.

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Summary

The full-length RNA4 of beet necrotic yellow vein virus (BNYVV) and its mutants, including the frame-shift and deletion within the coding region, was constructed into transcriptable cDNA clones. With complementation to a BNYVV isolate containing RNA1, 2 and 3 only, the RNA4 and its mutants transcripted *in vitro*, through a propagation, were used for inoculation to sugar beets on a sand culture system. The result showed that the efficiency of BNYVV transmission among the plants by *Polymyxa betae* was highly decreased by the deletions of 346 nucleotides in the coding region of RNA4.

Introduction

Beet necrotic yellow vein virus(BNYVV) was first reported in China in 1983 (Gao, et al.). The rhizomania disease caused sever yield loss of sugarbeet in northern part of the country and is increasing in 3-4% of the area. It was observed that BNYVV RNA4 is responsible for the efficient transmission by *Polymyxa betae*(Tamada, 1989; Lemaire et al., 1988). In these experiments, BNYVV isolate containing no RNA4 is transmitted much lower frequently than the ones containing it and an isolate from Germany(G1)(Bouzoubaa, et al., 1985) with 324 nucleotides deletion within its RNA4 coding region shows similar behavior. RNA4 cDNA of a BNYVV isolate from Inner Mongolia of China(BNYVV-NM1) has been amplified and sequenced by our group(Yu et al., 1996), which has 1465 nucleotides and shows 97.1% identity with that of a French isolate(Bouzoubaa, et al., 1985). To understand the function of BNYVV RNA4 more detail, in this paper, a primary result is reported about localization of the sequence in the RNA4 related to fungal transmission.

Materials and Methods

Virus and fungus:
BNYVV used for the experiments was first isolated from the Inner Mongolia in 1991 and

propagated on sugarbeet or *Tetragonia expansa* in our laboratory respectively. The virus (BNYVV-NM1) on sugarbeet infected by viruliferous *Polymyxa betae* contain all four species of the genome RNAs, but that (BNYVV-NM3) on *T. expansa* contain only the RNA1, 2 and 3 after several passages of mechanically inoculation. *P. betae* screened by ELISA for BNYVV more than five passages of inoculation to sugarbeet was used as virus-free vector.

Construction of infectious clones for the RNA4 transcripts:

The recombinant plasmid pGBF6 of full-length BNYVV-NM1(Yu et al., 1996) was used as PCR template to amplify different RNA4 mutants. T7 promoter and a poly(T)$_{26}$ tail were included in the primers corresponding to the RNA4 5' and 3' termini respectively(P1: CCAAGCTTATTAATACGACTCACTATAGAAATCAAATCTCAAATATA; P2: CCTC TAGAT$_{(26)}$GTCAATATACTGACAGAGAACCCTATA). Three additional primers and several endonucleases were involved to create the mutated RNAs with frame-shift or internal deletion (P3: CATTTACTGCAGATGGAGAG corresponding to 375-394nt, P4: TCCATCTGCAGT AAATGTTAC corresponding to 391-371nt and P5: GCGTTAACACATAA CTCATCAGAC corresponding to 757-742nt)(figure 1). Double enzymes digestion of the cDNA of wild type RNA4 with HincII plus NdeI or StyI were filled in and religated to remove the flanking sequences, while the DNA was digested by NdeI or StyI seperately and religated with the filled-in ends to shift its coding frame. Primer 3 and primer 4, both containing a PstI site, was used for PCR amplification to modify the initiation codon of ATG to ACT. Also, an internal fragment of 346 nucleotides was deleted by PCR with primer 5 containing a HincII site.
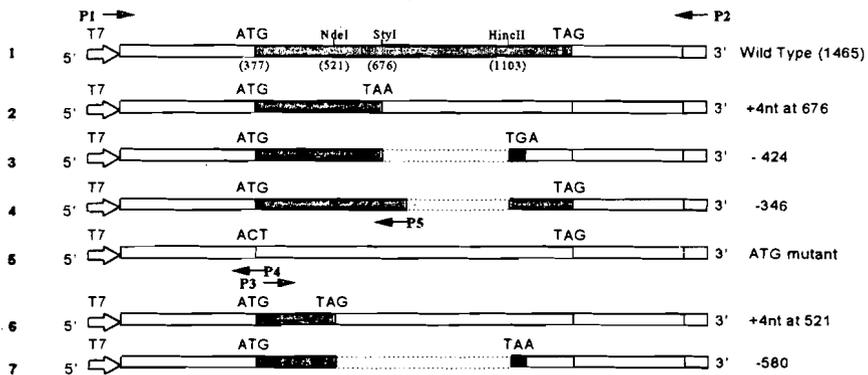
Fig.1: **Construction Strategy of BNYVV RNA4 Mutant**

Inoculation and detection:

In vitro transcripts of different RNA4 mutants were coinoculated respectively to *T. expansa* with the BNYVV-NM3 isolate together. After checking for the RNA4 seven to ten days post-inoculation, extracts from the infected *T. expansa* were used as inocula to infecte the sugarbeets

mechanically, while the virus-free *P. betae* were coinfected to the same plants. The seedlings in the test tubes with nutrient and light supplement were kept in tissue culture room or growth chamber at 25°C for 15 to 20 days before screening for the virus and the fungus by ELISA or under microscope. After then, at least 10 seedlings of sugarbeet were inoculated with the zoospores collected from each of the infected sugarbeet. Detection by ELISA were carried out 20 days later to estimate the difference in virus contents between the mutants and control.

Results

In vitro Transcription of the different cDNA clones

The constructions of mutated RNA4 were transcripted *in vitro* and capped as manufacturer's instruction. After assay by gel electrophoresis(Figure 2), they were used for plant inoculation .

Fig.2   Agarose gel electrophoresis of the viral RNAs
      a. △ 346 transcript;   b. Wild type transcript;
      c. Wild type transcript treated by DNase;
      d. Wild type template DNA;
      e. BNYVV-NM3;   f. BNYVV-NM1
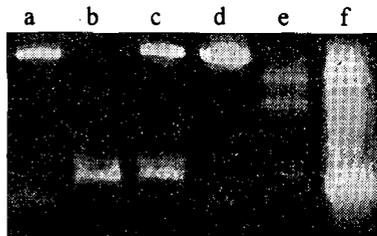
Propagation of virus complementation:

After inoculation, virus replication in *T. expansa* infected by the transcripts complementary to BNYVV-NM3 was detected by northern blot with a specific probe against to the sequence of nucleotide 1 to 521 of BNYVV-NM1 RNA4. The results showed that the RNAs transcripted from △ 424, △ 346 and wild type cDNA clone are infectious(Figure 3). Some of the sugarbeet plants inoculated with these two mutants reacted positively with BNYVV antibody by ELISA, although the viral RNAs were not detectable by northern hybridization(data not shown). The roots of these beet plants were used as inocula of viruliferous *P. betae* for reinoculation of sugarbeets.

Fig.3   Northern blot for RNA4 replication
      a-1: Wild type;   a-2: NM1 isolate;
      b-1: △ 424;   b-2: NM3 isolate;
      c-1: △ 346;   c-2: Healthy plant;
      A: Probe specific to RNA4;
      B: Probe to 3' end conserved in RNA1-4.
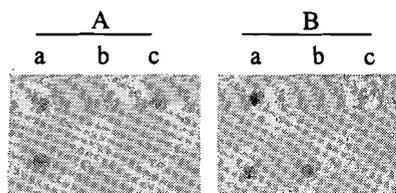
Influence of the deletion on the transmission by P. betae:

Sugarbeet plants firstly screened for *P. betae* infection under light microscope were detected for BNYVV by ELISA. The result showed that BNYVV transmission by *P. betae* was highly inhabited by the internal deletions of the two mutants, △ 424 and △ 346, virus concentration in which is at least twenty-five times lower than that of the controls (Table 1). There is no

significant difference of the influence between the lengths of the deletions, meaning that the decrease was mainly effected by the sequence removed from Δ 346.

Table 1:   ELISA tests of BNYVV in sugarbeet transmitted by *Polymyxa betae*

Coinocula		BNYVV RNAs				P. betae	
		NM3				virus/+	virus/ −
RNA4 transcript		Δ 424	Δ 346	WT	−	−	−
OD$_{410}$	1	0.00	0.01	0.50	0.03	0.55	0.01
	2	0.00	0.01	0.35	0.02	ND	0.03
	3	0.00	0.01	0.75	ND	ND	0.00
	4	0.00	0.06	ND	ND	ND	0.05
	5	ND	0.01	ND	ND	ND	ND
X		0.00	0.02	0.53	0.025	0.55	0.02

ND means not detected.

Discussion

Because of the sensitivity of the methods used in the experiments, the RNA4 can not be identified in the roots of infected sugarbeet by northern blot and the virus may exist in very low concentration in some plants infected by viruliferous vectors. The function of BNYVV RNA4 to efficient virus transmission by *P. betae* was further confirmed by this work, but the mechanism of how it works still remains unknown. To understand it, study is been carrying out with different mutants, including ATG codon modification for non-translation RNA4 transcription.

References

Bouzoubaa, S., Guilley, H., Jonard, G., Richards, K. and Putz, C. (1985). Nucleotide sequence analysis of RNA-3 and RNA-4 of beet necrotic yellow vein virus, isolates F2 and G1. *J. Gen. Virol.* 66: 1553-1564.

Gao, J.L., Deng, F., Zhai, H.Q., Liang. X.S. and Liu. Y.(1983). The occurrence of beet necrotic yellow vein virus in China. *Acta Phytopathologica Sinica* 13: 1-4.

Lemaire, O., Merdinoglu, D., Valentin, P., Putz, C., Ziegler-Graff, V., Guilley, H., Jonard, G. and Richards, K. (1988). Effect of beet necrotic yellow vein virus RNA composition on transmission by *Polymyxa betae*. *Virology* 162: 232-235.

Tamada, T. (1989). Evidence that beet necrotic yellow vein virus RNA4 is essential for efficient transmission by the fungus *Polymyxa betae*. *J. Gen. Virol.* 70: 3391-3398.

Yu, J.L, Han, C.G., Yang, L.L., Li, D.W. and Liu, Y. (1996). cDNA cloning, sequencing and expression of RNA4 from beet necrotic yellow vein virus. *Acta Micorbiologica Sinica* (Accepted).

THE GENOME ORGANIZATION AND RNA SEQUENCE OF CUCUMBER LEAF SPOT VIRUS, A TOMBUS-LIKE VIRUS

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Summary

The complete nucleotide sequence of cucumber leaf spot virus (CLSV) has been determined and the sizes and locations of predicted viral proteins deduced. The genome consists of 4432 nucleotides and contains 5 long ORFs. The 5' proximal ORF encodes a 25K product that terminates in an amber codon which may be read through to produce an 84K protein (ORF2). ORF 3 codes for the 41K capsid protein. Amino acid sequence comparisons of the CLSV CP and the coat proteins of several other small spherical plant viruses suggest that CLSV is most closely related to melon necrotic spot virus (MNSV). Interestingly, CLSV and MNSV are both transmitted by *Olpidium bornovanus*. ORFs 4 and 5 are completely overlapping at the 3' terminus and code for 27K and 17K products, respectively. The CLSV genome organization is similar to tombusviruses and nearly identical to pothos latent virus (PoLV), a proposed new,but atypical, member of the Tombus-viridae. It is proposed that CLSV and PoLV be considered strains of a new tombusvirus species.

Introduction

CLSV, currently classified as a carmovirus (Russo *et al.*, 1994), is known to infect several cultivars of *Cucumis sativis* in addition to a wide range of herbaceous hosts (Weber, 1986). The virions are icosahedral in shape and measure *ca.* 28 nm in diameter. They are comprised of a single coat protein of *ca.* 41K and contain positive-sense, single-stranded RNA of 4.4 kb (Weber, 1986). Transmission of CLSV is facilitated by the soil-inhabiting fungus, *Olpidium bornovanus* (Campbell *et al.*, 1991). To further characterize CLSV, cDNA clones covering the entire genome were sequenced. Comparisons of the CLSV genome organization and protein products with other Tombusviridae members are reported. Results of these studies suggest that CLSV represents a new tombusvirus species.

Materials and Methods

Virus propagation, purification and RNA extraction.
CLSV particles were purified from infected *Nicotiana clevelandii* or *N. benthamiana* leaves (Rochon and Tremaine, 1988; inoculum was kindly provided by Dr. R.N. Campbell) RNA was extracted from virions as described previously for CNV (Rochon and Tremaine, 1988).
cDNA cloning and sequencing
cDNA clones representing the entire CLSV genome were initially prepared using poly (A)-tailed CLSV virion RNA and oligo d(T) primers as previously described for synthesis of CNV cDNA clones (Rochon and Tremaine, 1989). Sequence information from these clones was then used to synthesize specific oligonucleotides for subsequent RT-PCR amplification, cloning and sequencing. The 5' sequence of CLSV RNA was obtained using an oligonucleotide complementary to a region near the CLSV 5' terminus and dideoxy-chain termination using MMLV reverse transcriptase. Clones were sequenced using Sequenase[TM] and/or by automated sequencing using an Applied Biosystems Prism 310 Genetic Analyzer. Sequence data was analyzed and assembled using Geneworks[TM] and Sequence Navigator[TM]. Alignments and comparsions were conducted using programs available through GCG (Devereux *et al.*, 1984).
Northern blot analysis and in vitro translation
Northern blot analysis (Rochon and Johnston, 1991) and *in vitro* translation in wheat germ extracts (Johnston and Rochon, 1990) were as described previously.

Results and Discussion

CLSV genome organization

The genome organization of CLSV as predicted from its nucleotide sequence is shown in Fig. 1. The CLSV genome contains five long ORFs encoding proteins with predicted mol. wts. of 25K, 84K, 41K, 27K and 17K. A comparison of the genome organization and predicted protein products of CLSV with those of CNV, a representative tombusvirus species, and pothos latent virus (PoLV), a newly proposed tombusvirus species, is shown in Fig.1. The CLSV genome organization is nearly identical with that of PoLV and highly similar, but distinct, from that of CNV. Moreover, the CLSV and PoLV nucleotide sequences are very similar (*ca.* 66% identical) whereas little sequence similarity occurs between CLSV and CNV (45%).
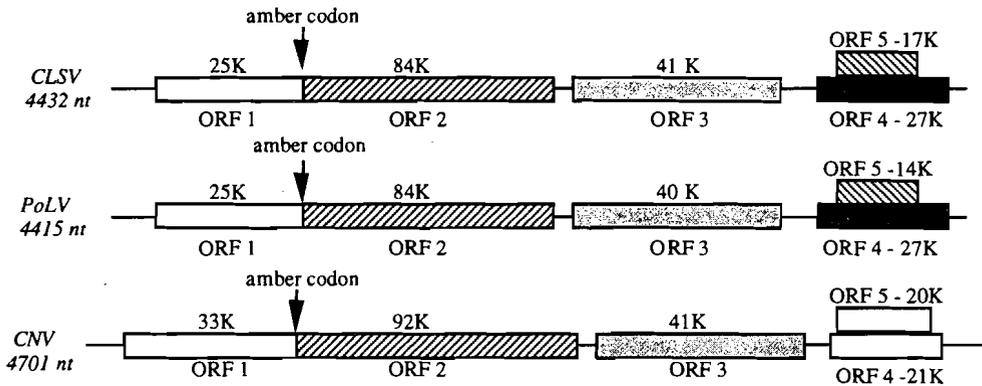
FIG. 1. Comparisons of the genome organizations of CLSV, PoLV and CNV. Shading indicates similar amino acid sequences.

ORF 2 of CLSV, CNV and PoLV encode proteins with similar amino acid sequences (Fig. 1 and Table 1) whereas similarity in ORF1 is only apparent with CLSV and PoLV. In CNV and other sequenced tombusviruses, ORFs 1 and 2 have been shown to encode proteins involved in viral RNA replication (see *Russo et al.*, 1994). The two 3' proximal nested ORFs of CLSV share a high degree of amino acid sequence similarity with those of PoLV (see Table 2) but share little or no statistically similar sequences with CNV. In the definitive tombusviruses, the proteins encoded by the overlapping ORFs have been shown to play a role in virus movement and possibly also an accessory role in viral RNA replication (see Russo *et al.*, 1994; Scholthof *et al.*, 1995). Although it is not yet possible to assign a function for the proteins encoded by the overlapping ORFs in CLSV, they may have roles analogous to those encoded by the corresponding ORFs of tombusviruses.

CLSV gene expression

Northern hybridization analyses with PoLV, CNV and other tombusviruses have demonstrated the existence of two 3'-coterminal subgenomic RNAs which serve as messenger RNAs for the proteins encoded by ORF3 and the two 3'-terminal nested ORFs (see Russo *et al.*, 1994). CLSV virion RNA and total RNA extracted from CLSV-infected *N.clevelandii* was electrophoresed and blotted and then hybridized with a labeled 3'-terminal CLSV-specific probe. In addition to the 4.4 kb genomic RNA, these experiments detected a strongly hybridizing 2.1 kb RNA species and a weakly

hybridizing 0.8 kb RNA species in both virion RNA and infected leaf extracts. *In vitro* translation experiments have shown that the 2.2 kb RNA species encodes a 41K protein and that the 0.8 kb RNA encodes 27K and 17K products. Genomic RNA encoded a 25K protein and small amounts of an approximate 84K protein (data not shown). These experiments suggest that CLSV, like PoLV and the tombusviruses, produces sgRNAs which serve as templates for the expression of its downstream ORFs.

Table 1. Percent amino acid sequence identity in ORFs 1 and 2.

	CLSV	PoLV	CNV	TBSV
CLSV	--	63	29	31
PoLV	87	--	27	29
CNV	45	46	--	89
TBSV	46	46	95	--

Values on the upper right correspond to sequence identity in ORF 1 and values on the lower left to identity in ORF 2

Table 2. Percent amino acid sequence identity in ORFs 4 and 5.

	CLSV	PoLV	CNV	TBSV
CLSV	--	78	24	25
PoLV	78	--	24	22
CNV	21	19	--	90
TBSV	23	23	75	--

Values on the upper right correspond to sequence identity in ORF 4 and values on the lower left to identity in ORF 5

Fungus transmission

We have previously demonstrated that the coat protein gene of CNV contains the determinants for the specificity of transmission by *Olpidium bornovanus* (MacLean *et al.*, 1994). In addition, we have previously noted that melon necrotic spot carmovirus (MNSV) which is also transmitted by *O. bornovanus*, shows unexpected amino acid sequence similarity with CNV in the coat protein protruding domain, suggesting a role for this portion of the coat protein in fungus transmission (Riviere *et al.*, 1990). We have examined the amino acid sequence of the CLSV coat protein for similarity to CNV and MNSV. So far, we have been unable to clearly define a particular region which is shared among these three viruses and which may be involved in fungus transmission. However, a dendogram depicting possible evolutionary relationships among these viruses and several other small spherical viruses (Fig. 2) suggests that CLSV is most closely related to MNSV and also red clover necrotic mosaic dianthovirus. A more distant but clear relationship to CNV is also evident.
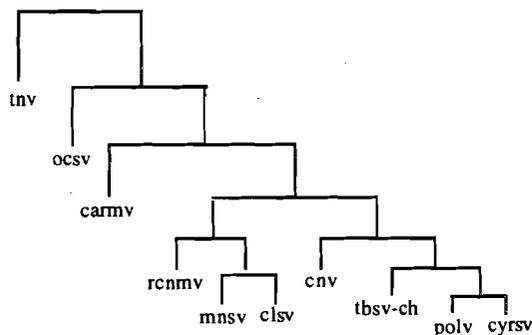
Fig. 2. Dendogram depicting relationships among coat proteins of several icosahedral viruses. The branch lengths are proportional to the distances between the respective sequences. TNV= tobacco necrosis necrovirus; ocsv=oat chlorotic stunt (unclassified) carmv= carnation mottle carmovirus; cyrsv=cymbidium ringspot tombusvirus. For remaining viruses see text. The dendogram was obtained using "Growtree" available through GCG. Sequences were from the EMBL database.

Classification

The high degree of similarity in genome organization and in nucleotide and amino acid sequence between CLSV and PoLV suggest that these two viruses represent strains of the same species. In addition, the highly similar genome organizations of CLSV and PoLV along with the conservation of sequence in ORFs 2 and 3 with those of the definitive tombusviruses suggest that CLSV and PoLV may together constitute a separate tombusvirus species. Other workers have demonstrated a high degree of nucleotide sequence similarity between tombusviruses both on the basis of direct nucleotide sequence comparisons and through hybridization analyses (Rochon and Tremaine, 1988; Hearne *et al.*, 1991; Gallitelli *et al.*, 1985; DMR, unpublished observations) . Our work with CLSV suggests that the present organization of the tombusvirus genus should be reconsidered. It is suggested that there are presently two major species of tombusviruses, those represented by CLSV and PoLV, and, as previously suggested, those represented by the cross-hybridizing members of the present tombusvirus genus (Hearne *et al.*, 1991).

References

Campbell, R.N., Lecoq, H., Wipf-Scheibel, C. & Sim, S.T. (1991). Transmission of cucumber leaf spot virus by *Olpidium radicale. J. Gen. Virol.* **72**: 3115-3119.

Campbell, R.N., Sim, S.T. & Lecoq, H. (1995). Virus transmission by host-specific strains of *Olpidium bornovanus* and *Olpidium brassicae. Eur. J. Plant Path.* **101**: 273-282.

Devereux, J., Haeberli, P. & Smithies, G. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**: 387-395.

Gallitelli, D. Koenig, R. & Hull, R. (1985). Relationships among viruses in the tombusvirus group: nucleic acid hybridization studies *J. Gen. Virol.* **66:** 1523-1531.

Hearne, P.Q. Knorr, D.A., Hillmann, B.I. & Morris, T.J. (1991). The complete genome structure and synthesis of infectious RNA from clones of tomato bushy stunt virus.*Virology* 77: 141-151.

Johnston, J.C. and Rochon, D.M. (1990). Translation of cumber necrosis virus RNA *in vitro. J. gen. Virol.* 71 2233-2241.

McLean, M.A., Campbell, R.N., Hamilton, R.I. & Rochon, D.M. (1994). Involvement of the cucumber necrosis virus coat protein in the specificity of fungus transmission by *Olpidium bornovanus. Virology,* **204**: 840-842.

Martelli, G.P., Gallitelli, D. & Russo, M. (1988). Tombusviruses. In *The Plant Viruses,* vol. 3, pp. 13-72. Edited by R. Koenig. New York: Plenum Press.

Riviere, C.J., Pot, J., Tremaine, H. & Rochon, D.M. (1989). Coat protein of melon necrotic spot carmovirus is more similar to those of tombusviruses than those of carmoviruses. J.*Gen.Virol.* **70**: 3033-3042.

Riviere, C.J. & Rochon, D.M. (1990). Nucleotide sequence and genomic organisation of melon necrotic spot virus. *J. Gen. Virol.* **71**: 1887-1896.

Rochon, D.M. & Johnston, J.C. (1991). Infectious transcripts from cloned cucumber necrosis virus cDNA: evidence for a bifunctional subgenomic mRNA. *Virology,* 181: 656-665.

Rochon, D.M. & Tremaine, J.H. (1988). Cucumber necrosis virus is a member of the tombusvirus group. *J Gen. Virol.* **69**: 395-400.

Rochon, D.M. and Tremaine, J.H. (1989). Complete nucleotide sequence of the cucumber necrosis virus genome. *Virology* **169**: 251-259.

Russo, M., Burgyan, J. & Martelli, G.P. (1984). Molecular biology of tombusviridae. *Adv. Virus Res.* **44**: 381-428.

Rubino, L., Russo, M. & Martelli, G.P. (1995). Sequence analysis of pothos latent virus genomic RNA. *J. Gen. Virol.* **76**: 2835-2839.

Scholthof, H.B., Scholthof, K-B.B. and A.O. Jackson. *Plant Cell* 7, 1157-1172 (1995).

Sanger, F., Nicklen, S., & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Nat'l. Acad. Sci., U.S.A.* **74**: 5463-5467.

Weber, I. (1986) Cucumber leaf spot virus. *CMI/AAB Description of Plant Viruses,* No. 319.

CHARACTERIZATION OF A POORLY TRANSMISSIBLE CUCUMBER NECROSIS VIRUS MUTANT

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Summary

A mutant of CNV deficient in fungus transmission (LL5) has been identified and characterized. The lowered transmissibility was found to be due to a single amino acid change (Glu to Lys) in the coat protein shell domain. LL5 particles are morphologically similar to wild type (WT) particles but show altered electrophoretic mobility on agarose gels. It is shown that LL5 particles are stable, contain intact RNA, are as infectious as wild type and accumulate to WT levels in infected plants. Preliminary *in vitro* binding assays indicate that the poor transmissibility is at least partly due to reduced ability of the virus to bind zoospores.

Introduction

Cucumber necrosis (CNV), a tombusvirus, is a small spherical virus which encapsidates a monopartite plus-sense RNA genome of approximately 4.7 kb. CNV is one of several members of the Tombusviridae family whose transmission in the soil is facilitated by zoospores of the Chytridiomycete, *Olpidium bornovanus* (see Campbell *et al.*, 1995). Transmission of CNV by *O. bornovanus* occurs in the *in vitro* manner similar to that described for transmission of tobacco necrosis virus (TNV) by *O. brassicae* (Campbell and Fry, 1966). Virus particles adsorb to the zoospore outer membrane and then enter the zoospore protoplast upon withdrawal of the flagellum. Transmission to a root cell occurs during or after the discharge of virus containing protoplasm from the encysted zoospore. Reciprocal exchanges of the coat protein genes of CNV and the non-transmissible cherry strain of tomato bushy stunt virus (TBSV-Ch) have demonstrated that the CNV coat protein contains the determinants for the specificity of fungus transmission (McLean *et al.*, 1994). In this study, we have analyzed naturally occurring CNV mutants deficient in transmission in order to further understand the role of CNV genes in the transmission process. We describe the isolation and detailed characterization of a CNV coat protein mutant which is deficient in transmission by *O. bornovanus.*

Materials and Methods

Virus purification, and characterization. All viruses were purified as described previously (Rochon and Tremaine, 1988). Electron microscopy of virus was as described (McLean *et al.*, 1994). Agarose gel electrophoresis of particles was as described (Heaton, 1992).

Transmission experiments. Purified virus was tested for *in vitro* acquisition and transmission to cucumber seedlings using *O. bornovanus* isolate SS196 originally obtained from Dr. R.N. Campbell as described previously (McLean *et al.*, 1994) For the initial screen of CNV mutants, leaves were ground with liquid nitrogen and then added to transmission buffer. Equal aliquots of leaf extracts were used for transmission.

Cloning, sequencing and in vitro mutagenesis. The LL5 coat protein gene was obtained using RT-PCR and oligonucleotides specific to regions flanking the CNV coat protein gene. The RT-PCR product was then digested and inserted into the corresponding region of pK2/M5 (see McLean *et al.*, 1994) which is a full-length infectious CNV cDNA clone. Sequence analysis, cloning procedures

and *in vitro* mutagenesis were as described previously (McLean *et al.*, 1994). *In vitro* transcription and inoculation of plants and preparation of total leaf RNA was as described (Rochon and Johnston, 1991)

Results

Isolation of CNV mutants deficient in transmissibility

CNV was serially transferred through the systemic host *Nicotiana clevelandii* by mechanical inoculation. Leaf extracts from the 15th passage were then used to inoculate the local lesion host, *Chenopodium quinoa*. Individual local lesions were isolated, and further "purified" by an additional inoculation onto *Ch. quinoa*. Virus from local lesions was amplified by back inoculation onto *N. clevelandii* and virus was tested for fungus transmissibility as previously described (Campbell *et al.*, 1991) using total leaf extracts. Briefly, leaf extracts obtained by grinding leaves in 50 mM glycine, pH 7.6 were added to zoospore suspensions (*ca.* 1 X 10^4 zoospores/ml) for an acquisition period of 15 min. and then used to inoculate roots of young cucumber cotyledons. After 6 days, inoculated roots were ground and assayed for the presence of virus by inoculation of *Ch. quinoa* and/or by ELISA using CNV polyclonal antiserum. Several CNV mutants deficient in transmissibility were identified. Some properties of four of these mutants are outlined in Table 1. One mutant, LL2 appears unable to produce coat protein and another, LLA16, contains a genomic RNA deletion in the coat protein gene. The inability of these mutants to be transmitted is consistent with the role of the coat protein in transmission. One other mutant, LLA1, was found associated with defective interfering RNAs and therefore may have been only inefficiently transmitted due to lowered levels of virus in infected leaves. Characteristics of LL5 are described in detail below.

Table 1. *Phenotypes of CNV transmission mutants*

Virus	FT*	symptoms	particles	genotype
CNV	>100	wild type	+	wild type
LL2	0	delayed	-	no CP
LL5	9	systemic	+	ΔCP
LLA1	0	attenuated	+	DI RNA
LLA16	0	delayed	ND	no CP gene

* Average number of local lesions per leaf in bioassay on Ch. quinoa following fungus transmission.

Table 2. *Transmission of LL5, LL5$_S$ and LL5$_a$*

Virus	O. bornovanus			
	*1	2	3	4
CNV	0.757	1.689	1.324	1.786
M5/LL5	0.085	0.231	0.039	0.085
M5/LL5S	0.168	0.301	0.106	0.129
M5/LL5a	1.506	0.709	0.941	1.436
no virus	0.050	0.051	0.015	0.111

* Values are absorbance at 405 nm using DAS-ELISA and a CNV polyclonal antiserum. 1-4 represent separate transmission experiments.

Characteristics of LL5

The coat protein gene of LL5 was amplified from LL5 virion RNA extracts using RT-PCR and cloned into the wild type CNV genome to determine if alterations in the LL5 coat protein gene are responsible for its reduced transmissibility. Virions produced from infections with this construct (M5/LL5) were quantified and used in a fungus transmission assay. Table 2 shows that M5/LL5 virions consistently showed either markedly reduced or no transmission demonstrating that the LL5 coat protein gene contains the alterations which reduce LL5 transmissibility. The LL5 coat protein gene was sequenced and found to contain three nucleotide substitutions relative to the CNV coat protein gene (see Fig. 1): one in the arm (a) portion of the coat protein, one in the shell (S) domain

and one in the protruding (P) domain. The arm and shell mutations resulted in amino acid changes whereas the one in the protruding domain was silent.

The arm and shell domain mutations were individually introduced into the wild type CNV coat protein gene by *in vitro* mutagenesis to determine if one or both of these nucleotide changes are responsible for the reduction in transmissibility. Table 2 summarizes the results of fungus transmission assays using virions produced from both mutants (termed M5/LL5$_a$ and M5/LL5$_S$ for the arm and shell domain mutations, respectively). It can be seen that M5/LL5$_a$ particles are transmitted as efficiently as wild type virions whereas M5/LL5$_S$ particles show reduced transmission similar to that observed with M5/LL5. These results demonstrate that the reduction in LL5 transmissibility is due to a single amino acid change in the coat protein shell domain.

Additional properties of M5/LL5s

Our results indicate that the reduction in LL5 transmissibility may be due to a structural aberration in the coat protein or virus particle (see Discussion). However, it is still possible that the loss of transmissibility is due to loss of particle integrity or to a reduced ability to infect or accumulate in infected plants. M5/LL5$_S$ particles were examined by electron microscopy and found to be morphologically identical to CNV WT particles (not shown). Particles were also examined by agarose gel electrophoresis. As can be seen in Fig. 2, M5/LL5 and M5/LL5$_S$ particles migrate slightly slower than either WT or M5/LL5$_a$ particles (see Discussion). Purified M5/LL5 and M5/LL5$_S$ particles were also assessed for their relative infectivities and stabilities on several hosts. The results of such local lesion tests indicate that these particles are equally infectious as wild type and that they retain infectivity after prolonged incubation in transmission buffer (not shown). Finally, M5/LL5 and M5/LL5$_S$ virion RNAs are intact and viral RNAs accumulate to high levels in infected plants as determined by analysis of total leaf RNA extracts (not shown).

WT   LL5   LL5,   LL5,
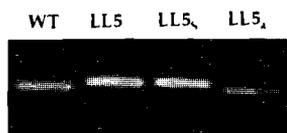
Fig. 1. Location of mutations in the LL5 CP gene.
A) Structure of CNV genome and location of the two primers (1 and 2) used to amplify the LL5 CP gene.
B) Organization of domains in the CNV CP gene. The arrows indicate the locations of mutations.

Fig. 2. Agarose gel electrophoresis of mutant virus particles. Particles were electrophoresed in 1% agarose gels in the presence of 10 mM Tris/75 mM glycine, pH 8.0 and stained with ethidium bromide as described (Heaton, 1992).

In vitro binding assays.

In vitro binding assays were developed in order to assess if the reduced ability of LL5 and LL5$_S$ to be transmitted by *O. bornovanus* is due to inability to bind zoospores or instead to some other aspect of the transmission cycle. Particles were mixed with zoospores, allowed to acquire and then washed by two low speed centrifugations. Our preliminary data indicate that LL5 and LL5$_S$ bind zoospores

with about 50% the efficiency of that of wild type. In addition, similar experiments have shown that little or no binding occurs with TBSV-Ch.

Discussion

Our results show that CNV mutants with reduced transmissibility can be found in mechanically passaged virus. However, we have not yet conducted a comprehensive analysis of the level of such mutants in passaged virus. In certain other fungally transmitted plant viruses, genomic deletions are associated with mechanical passage and the deleted variants can eventually predominate in the population (Shirako and Brakke, 1984). CNV deletion mutants do not accumulate to high levels during mechanical passage, but as is shown in Table 1, such deletion mutants can be detected.

The Glu to Lys substitution in the LL5 shell domain may reduce transmissibility by changing the charge of particles. Alternatively, or in addition, since the Glu to Lys mutation is immediately adjacent to an Asp residue known to be involved in Ca^{+2} mediated viral subunit-subunit contacts (see Harrison, 1983) it is possible that this change alters the conformation of particles.

Finally, preliminary findings with our *in vitro* binding assay show that the ability to specifically bind zoospores is an important factor in the transmission process. This is in agreement with previous electron microscopic studies (Temmink *et al.*, 1970) showing that virus only absorbs to the vector species which transmits it. Together, these studies indicate the involvement of a specific zoospore receptor in transmission by *Olpidium* spp.

References

Campbell, R.N. and Fry, P.R. (1966). The nature of the association between *Olpidium brassicae* and lettuce big vein and tobacco necrosis viruses. *Virology* **29**: 222-233.

Campbell, R.N., Lecoq, H, Wipf-Scheibel Ca nd Sim, S.T. (1991). Transmission of cucumber leaf spot virus by *Olpidium radicale*. *J. gen. Virol.* **72**: 3113-3119.

Campbell, R.N., Sim, S.T. and Lecoq, H. (1995). Virus transmission by host-specific strains of *Olpidium bornovanus* and *Olpidium brassicae*. *Eur. J. Plant Path.* **101**: 273-282.

Harrison, S.C. (1983). Virus structure: high resolution perspectives. *Adv. Virus Res.* **28**: 175-240.

Heaton, L.A. (1992). Use of agarose gel electrophoresis to monitor conformational changes of some small, spherical plant viruses. *Phytopathology* 82: 803-807.

McLean, M.A., Hamilton, R.I., and Rochon, D.M. (1993). Symptomatology and movement of a cucumber necrosis virus mutant lacking the coat protein protruding domain. *Virology* **193**: 932-939.

McLean, M.A., Campbell, R.N., Hamilton, R.I. and Rochon, D.M. (1994). Involvement of the cucumber necrosis virus coat protein in the specificity of fungus transmission by *Olpidium bornovanus*. *Virology* **204**: 840-842.

Rochon, D.M. and Tremaine, J.H. (1988).Cucumber necrosis virus is a member of the tombusvirus group. *J. gen. Virol:* **69**: 395-400.

Rochon, D.M. and Johnston, J.C. (1991). Infectious transcripts from cloned cucumber necrosis virus cDNA: evidence for a bifunctional subgenomic RNA. *Virology* **181**: 656-665.

Shirako, Y. and Brakke, M.K. (1984). Spontaneous deletion mutation of soil-borne wheat mosaic virus RNA II. *J. gen. Virol.*, **65**: 855-858.

Temmink, J.H.M., Campbell, R.N. and Smith, P.R. (1970). Specificity and site of *in vitro* acquisition of tobacco necrosisi virus by zoospores of *Olpidium brassicae.J. gen. Virol.* 9:201-213.

SIMILARITIES BETWEEN BEET SOILBORNE MOSAIC VIRUS AND BEET NECROTIC YELLOW VEIN VIRUS RNA2 NUCLEOTIDE SEQUENCE AND GENOMIC ORGANIZATION

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Summary

Studies to further define the taxonomic relationship between beet soilborne mosaic virus (BSBMV) and beet necrotic yellow vein virus (BNYVV) were conducted. Two RT-PCR products comprising 4,546 bases from BSBMV RNA2 were sequenced and data were compared with sequence from several closely related viruses. Six putative open reading frames (ORFs) were identified on BSBMV, which were nearly identical in size and position to those of BNYVV RNA2. Best fit comparisons of amino acid sequence homology from each individual ORF of BSBMV and BNYVV revealed a low of 40% identity and 62% similarity in ORF 6 and a high of 81% identity and 90% similarity in ORF 4. Sequence homology of BSBMV with other furoviruses or viruses possessing triple gene block sequences rarely exceeded 30% identity. Sequence identity of the coat protein region from two isolates of BSBMV exceeded 98% even though the two isolates are serologically distinct. Similarities between BSBMV and BNYVV suggest they represent a sub-group of the furoviruses.

Introduction

Beet soilborne mosaic virus (BSBMV) was discovered in Texas in 1986 (Liu and Duffus, 1988) and has since been identified in California, Colorado, Idaho, Nebraska, and Wyoming (Rush and Heidel, 1995). It has a host range similar to that of beet necrotic yellow vein virus (BNYVV) and is vectored by *Polymyxa betae*. Studies conducted by Wisler et al. (1994) indicated that BSBMV and BNYVV are serologically related but yet distinct. BSBMV and BNYVV are more similar to each other than to other members of the furoviruses in that most field isolates have quadripartite polyadenylated genomes, capsid protein molecular weights are similar, and probes derived from the 3' end of BNYVV hybridize with BSBMV in northern blots (Heidel et al., 1996; Rush et al., 1993). Because of these similarities, Rush et al. (1993) speculated that BSBMV might be a strain of BNYVV, but cautioned that additional work was required to determine the true taxonomic relationship between the two viruses. In an attempt to further clarify the relationship between BSBMV and BNYVV, studies to determine the nucleotide sequence and genomic organization of BSBMV RNA2 were initiated.

Materials and Methods

Virus isolates: Two BSBMV isolates, designated BSBMV-FS and BSBMV-RC, were used. Both were maintained on sugar beets naturally infected by *P. betae,* and all plants exhibited systemic symptoms. BSBMV-FS reacts normally with BSBMV polyclonal antiserum in DAS-ELISA, but BSBMV-RC gives a negative or borderline positive reaction (Rush and Heidel, 1995). However, RT-PCR products can be amplified using BSBMV-RC as a template and BSBMV specific primers (Rush et al., 1994).

RT-PCR: Oligonucleotide primers for use in reverse transcriptase-polymerase chain reactions (RT-PCR) were synthesized by the Gene Technologies Lab (Texas A&M University, College Station, TX). Primers were derived from BNYVV RNA 1-4 sequences using published data from European isolates (Bouzoubaa et al., 1986). Total RNA was extracted from sugar beet leaves systemically infected with BSBMV-FS and used as a template in combination with an oligo-dT primer for first strand BSBMV cDNA synthesis in reverse transcriptase reactions. The BSBMV-FS cDNA was then used in RT-PCR, with various combinations of the BNYVV primers. In previous studies, certain BNYVV primer combinations were shown to amplify BSBMV cDNA (Rush et al., 1994). Amplification of BSBMV-FS cDNA was carried out in 50 μl reactions using 5 μl cDNA, 10 pmol of each primer, 0.2 mM of each dNTP and 5 U *Taq* DNA polymerase in a reaction buffer provided with the enzyme (Robertson et al., 1991). PCR products were visualized after electrophoresis in a 1% agarose gel by staining with ethidium bromide.

Sequencing and analysis: Both strands of BSBMV-FS PCR products were directly sequenced at the Gene Technologies Lab using the ABI PRISIM Dye Terminator Cycle Sequencing Core Kit on the ABI 373 Automated Sequencer. Sequence data was analyzed using the BLAST program (Gish and States, 1993). Putative open reading frames (ORFs) were identified using the DNA Strider 1.2 program, and predicted starts and stops were further defined based on BNYVV RNA2 sequence in GenBank. A primer pair was developed from BSBMV-FS sequence to amplify the putative ORF1 of BSBMV-RC. This primer pair was used with BSBMV-RC RNA in RT-PCR and resulting dsDNA products of the predicted size were sequenced and compared with the BSBMV-FS sequence. The amino acid sequence from each individual ORF of BNYVV RNA2 was used for best fit analysis with BSBMV sequence and also sequence from beet soilborne virus, peanut clump virus, potato mop top virus, barley stripe mosaic virus, and *Nicotiana velutina* mosaic virus.

Westerns: Total plant extracts were denatured and resolved on 10% SDS-polyacrylamide gels followed by transfer to nitrocellulose membrane and immunoanalyses as previously described (Scholthof et al., 1995). For western blots, BSBMV antiserum was used at 1:20,000 dilution in 5% milk with TBS, pH 7.4 and 0.02% Tween-20. The secondary antibody was used at 1:5,000 as goat anti-rabbit horseradish peroxidase and developed with chemiluminescent substrates (SuperSignal CL-HRP substrates, Pierce, Inc., Rockford, IL). Exposure was for 5 seconds on X-ray film (Scholthof et al., 1995).

Results and Discussion

RT-PCR: Use of BNYVV primers with BNYVV cDNA as template gave RT-PCR products of the expected size. When BSBMV-FS cDNA was used as template, multiple RT-PCR products were formed, but usually not of the predicted size, indicating non-specific binding. Initial attempts at cloning the RT-PCR products failed, so direct sequencing of the products was initiated. The first PCR product selected for sequencing was from the 3' end of RNA2 and included the poly (A) region. This product was approximately 1,450 bp in length, excluding the poly (A) tail. Following preliminary sequencing of this product, a reverse primer was developed from the sequence near the 5' end and used in additional RT-PCR reactions with BNYVV primers. The largest product amplified in these reactions, approximately 3,500 bp, was selected for sequencing.

Sequence analysis and genomic organization of BSBMV-FS: Sequence of the two RT-PCR products overlapped and together represented 4,546 continuous nucleotides (nt) from BSBMV-FS. When this sequence was entered into the BLAST program, best fit was with BNYVV RNA2. Based on measurements from RNA gels, the 4,546 nt represents a near full-length sequence of BSBMV-FS RNA2 and differs from the reported length of BNVYY RNA2 by only 66 nt (Bouzoubaa et al., 1986).

Computer assisted analysis of potential coding capacity of BSBMV-FS sequence indicated further similarities with BNYVV RNA2. Six putative ORFs of similar size and position to those of BNYVV RNA2 were identified. Coordinates of the putative ORFs of BSBMV-FS and estimates of their translation products are shown in Table 1. The first ORF begins at nt (53) and ends with an UAG at nt 628, to give a protein of 20,953 daltons. Following the stop at 628, an in-phase coding region extends to nt 1863 to encode a predicted readthrough translation product of 75,580 daltons. These first two ORFs are analogous to the coat protein region and readthrough of BNYVV and have been detected in western blots using antiserum developed against BSBMV coat protein. The remaining four ORFs of BSBMV-FS are also similar in size and position to those on BNYVV RNA2.

Table 1. Predicted coordinates of BSBMV-FS RNA2 ORFs

ORF	First AUG (nt)	Termination (nt)	Protein (Mr)
1	53	628	20,953
2	53	1,863	75,580*
3	2,110	3,180	38,990**
4	3,180	3,536	12,602
5	3,520	3,918	14,660
6	3,946	4,308	13,719

*   Assuming readthrough of ORF 1
**   Modification of one nt in the BSBMV-FS sequence increases size of the predicted ORF 3 protein to approximately 42,000 daltons, the same as ORF3 of BNYVV RNA2.

BLAST analysis of putative BSBMV-FS ORFs showed greater amino acid sequence homology with BNYVV than with several other related viruses. The highest degree of homology was in ORF 4, which exhibited 81% identity and 90% similarity. The lowest homology was with ORF 6 which codes for a 14 kDa non-structural protein in BNYVV and had only 40% amino acid sequence identity with the analogous region in BSBMV-FS. The coat protein region of BSBMV-FS and BNYVV had 59% amino acid identity and 72% similarity. All ORFs of BSBMV-FS had regions which exhibited greater than 90% amino acid sequence homology with analogous ORFs of BNYVV. When amino acid sequence from specific ORFs of BNYVV were compared by best fit analysis to BSBMV-FS and several other related viruses, BNYVV was more similar to BSBMV-FS than to any of the other viruses (Table 2).

Table 2. Best fit comparisons between specific BNYVV ORF regions and comparative regions of several related viruses

BNYVV	21 kDa	42 kDa	13 kDa	15 kDa
BSBMV	59(72)*	74(86)	81(90)	65(78)
PCV	18(40)	30(51)	32(51)	20(44)
PMTV	19(39)	29(50)	37(56)	20(46)
NVMV	21(42)	26(53)	40(62)	23(49)
BSMV	21(43)	27(50)	42(63)	12(42)
BSBV	14(34)	29(50)	39(56)	23(47)

*   Values represent percent amino acid identity and similarity

Sequence comparisons between BSBMV isolates FS and RC: The primer pair based on sequence from BSBMV-FS ORF 1 region amplified a RT-PCR product of the predicted size when using BSBMV-RC cDNA as a template. Sequence comparisons between the two isolates indicated nucleotide homology exceeding 98%. Only four amino acid differences were detected between the two isolates. Although BSBMV antiserum gives negative or borderline positive readings in DAS-ELISA tests, the same antiserum detected BSBMV-RC in western blots.

Because of its polyadenylated quadripartite genome, BNYVV has long been considered an outlier among the furoviruses. However, with BSBMV and the serologically distinct BSBMV-RC, there are now three serologically distinct members with polyadenylated quadripartite genomes. Furthermore, the genomic organization between BNYVV RNA2 and BSBMV RNA2 is practically identical. Because of the many similarities between BSBMV and BNYVV and their differences from other furoviruses, we believe a sub-group within the furoviruses should be created with BNYVV as the "type" member.

References

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards, K., and Jonard, G. (1986). Nucleotide sequence of beet necrotic yellow vein virus RNA-2. *J. Gen. Virol.* 67:1689-1700.

Gish, W. and States, D.J. (1993). Identification of protein coding regions by database similarity search. *Nat. Genet.* 3:266-72.

Heidel, G.B., Rush, C.M., Kendall, T.L., and Lommel, S.A., French, R.C., and Manohar, S.K. (1996). Characteristics of beet soilborne mosaic virus, a furo-like virus infecting sugar beet. *Phytopathology* (submitted).

Liu, H.-Y. and Duffus, J.E. (1988). The occurrence of a complex of viruses associated with rhizomania of sugarbeet. (Abstr.) *Phytopathology* 78:1583.

Robertson, N.L., French, R., and Gray, S.M. (1991). Use of group-specific primers and the polymerase chain reaction for the detection and identification of luteoviruses. *J. Gen. Virol.* 72:1473-1477.

Rush, C. M., French, R. C., and Heidel, G. B. (1993). Texas 7 a possible strain of beet necrotic yellow vein virus. Pages 59-62 in: *Proc. Symp. Int. Work. Group Plant Viruses Fungal Vectors,* 2nd. Montreal Canada.

Rush, C.M., French, R.C., and Heidel, G.B. (1994). Differentiation of two closely related furoviruses using the polymerase chain reaction. *Phytopathology* 84:1366-1369.

Rush, C.M. and Heidel, G.B. (1995). Variation in symptomatology and serotype among furoviruses infecting sugar beets. *J. Sugar Beet Res.* 32:157.

Scholthof, K.-B. G., Scholthof, H.B., and Jackson, A.O. (1995). The tomato bushy stunt virus replicase proteins are coordinately expressed and membrane associated. *Virology* 208: 365-369.

Wisler, G.C., Liu, H.-Y., and Duffus, J. E. (1994). Beet necrotic yellow vein virus and its relationship to eight sugar beet furo-like viruses from the United States. *Plant Dis.* 78:995-1001.

BNYVV READTHROUGH DOMAIN ANALYSIS: A **KTER** MOTIF IS IMPORTANT FOR TRANSMISSION OF THE VIRUS BY *POLYMYXA BETAE*

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Summary

RNA 2 of beet necrotic yellow vein furovirus encodes the 21 kDa major coat protein at its 5' terminus and terminates with a suppressible amber stop codon allowing the synthesis of a 75 kDa readthrough (RT) protein (P75). P75 has been shown to be a minor component of the virions, located near one extremity of the particles and its 54 kDa RT domain intervenes both in virus assembly and transmission by the fungal vector, *Polymyxa betae*. A collection of in frame deletion mutants as well as alanine scanning substitutions were tested for virus encapsidation and transmission. The results suggest that the N-terminal half of the RT domain is necessary for an efficient assembly whereas the C-terminal part is dispensable for encapsidation but important for transmission. A KTER peptide motif located within this C-terminal portion has been identified as essential for fungus transmission.

Introduction

Beet Necrotic Yellow Vein Virus (BNYVV) is the causal agent of rhizomania disease and consists of four or of five distinct plus sense RNA encapsidated in rigid rod-shaped particles (for review see Richards and Tamada, 1992) transmitted by *Polymyxa betae*. RNA 1 and 2 are necessary and sufficient for infection of leaves by mechanical inoculation but RNA 3 and 4 influence virus proliferation in root tissue and vector transmission.

The 5'-proximal cistron of RNA2 directs the synthesis of the major coat protein (21K) and terminates with an amber stop codon, which can undergo readthrough (RT) into the following 54 kDa ORF (RT domain). The resulting readthrough protein (P75) is located at an extremity of the viral particle (Haberlé *et al.*, 1994) and the RT domain of this minor virion component is required for virus assembly and vector transmission (Schmitt *et al.*, 1992; Tamada and Kusume, 1991).

Introduction of deletions or alanine substitutions into the RT domain

Earlier findings have revealed that two natural isolates (2a and 2b) with deletions in the C-terminal part of the RT domain of P75 (Fig. 1), produced virions but were defective in fungus transmission. Mutations in the N-proximal portion of the RT domain, on the other hand, inhibited production of virions although viral RNA and the 21kDa coat protein accumulated properly (Schmitt *et al.*, 1992; Tamada and Kusume, 1991). Thus the RT domain contains two distinct subdomains: one near the N-terminus involved in virus assembly and one near the C-terminus, which probably interacts with the vector.

To better map these subdomains we constructed mutants carrying short in-frame deletions (Fig. 1) covering the entire RT domain with the exception of the 336 last nucleotides; these are believed to be important for the synthesis of the subgenomic RNA directing the

translation of the essential P42 (Gilmer *et al.*, 1992). Five alanine substitution or 'alanine scanning' mutants were prepared between the two *Accl* sites (positions 1413 and 1826), a region contained within the sequences deleted in isolates 2a and 2b referred to above (Fig. 1). These substitutions target clusters of charged residues potentially accessible for interaction with the fungus.
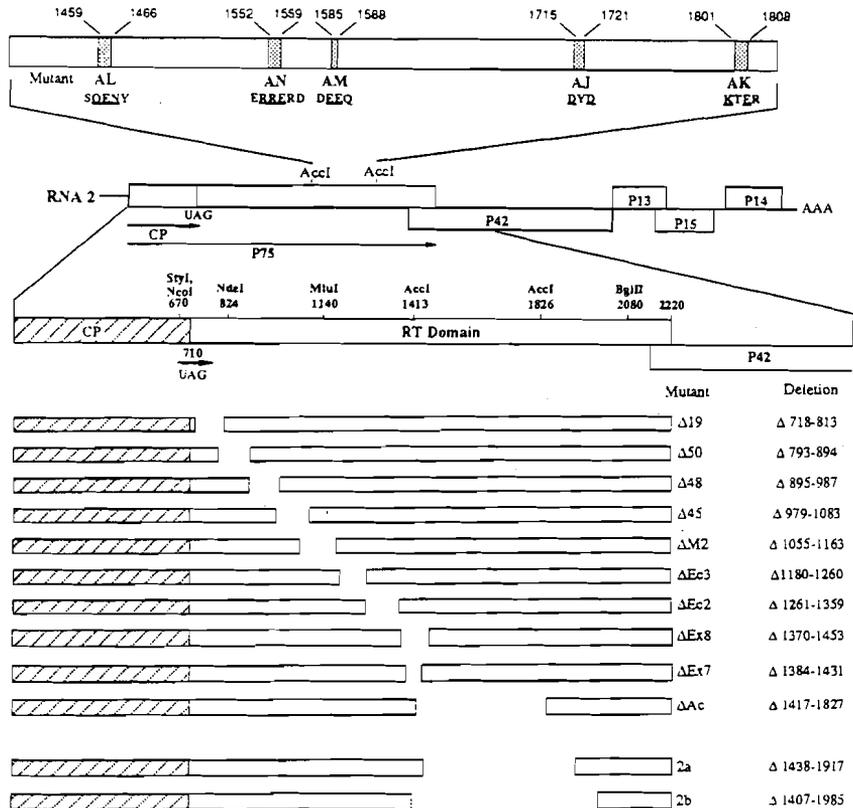
Fig. 1: Structure of BNYVV RNA 2 and the position of the alanine scanning mutations (upper) and the deletions (lower) introduced into the RT domain. The genetic map of RNA 2 is shown near the top with the portion of the map between the *Accl* sites at residues 1413 and 1826 blown up to show detail (above). The amino acids replaced by alanine in the mutants AL, AN, AM, AJ and AK are underlined. The lower portion of the figure shows the structures of the deletion mutants of which extents are given to the right. The positions of the deletions in the non-transmissible isolates 2a and 2b are shown at the bottom.

Replication and virus assembly of the RNA 2 mutants

C. *quinoa* leaves infected with the above described RNA 2 mutants were harvested 6-8 days post inoculation. For each mutant total RNA and Rnase-resistant viral RNA was isolated as described (Quillet *et al.*, 1989; Schmitt *et al.*, 1992).

Northern blot revealed that all the mutants replicated efficiently although the quantity of progeny RNA varied considerably from experiment to experiment (30 % to 100 % of the wild type controls).

As judged by the sensitivity of the progeny viral RNA to RNase extraction, all the mutants deleted in the N-proximal half of the RT domain (mutants Δ19, Δ50, Δ48, Δ45, ΔEc3, ΔEc2, ΔEx7 and ΔEx8) were poorly encapsidated. Thus the efficient assembly of BNYVV requires the entire N-terminal half of the RT domain of P75 with no (or very small) dispensable sequences, although low level of virus assembly can occur in the presence of truncated P75.

The five alanine scanning mutants (AL, AN, AM, AJ and AK), on the other hand, behaved like the ΔAc deletion mutant in that they were efficiently replicated and encapsidated.

Fungus transmission

A selection of the RT domain mutants was then tested for their capacity to be transmitted by *P. betae*. The mutants tested included the virus assembly-defective deletion mutants Δ50, ΔM2, ΔEc3, ΔEx7 and ΔEx8, the assembly-competent ΔAc mutant and the five alanine scanning mutants. They were inoculated to leaves of the systemic host *B. macrocarpa* and the roots were then used as source of virus for vector transmission experiments. Preliminary experiments were carried out to check the systemic movement of the mutants in *B. macrocarpa*; the assembly-competent mutants accumulated in the upper leaves and roots similarly to the wild type virus isolate St1234. Systemic movement of the assembly-defective mutants, on the other hand, was less efficient and slower than for wild type, presumably because of the low rate of virion formation. It should be noted than long distance movement has never been observed for mutant B25. Only inoculated *B. macrocarpa* where movement had occurred were used as source plants. The transmission tests were performed as previously described (Tamada and Kusume, 1991) and the virus content of both *B. macrocarpa* source plants and sugarbeet bait plants were assessed by ELISA.

In five separate experiments with mutant ΔAc, no virus transmission was observed to any of the 25 sugarbeet test plants, whereas 27/30 of the test plants challenged with the wild type inoculum St1234 were infected. These figures confirm the results obtained with isolates 2a and 2b and rule out the possibility that the loss of their transmissibility was due to a mutation elsewhere in the genome.

Table 1 summarises the transmission characteristics of the five 'alanine scanning' mutants. Mutant AK was never transmitted even though the virus content in the source plant was comparable to the other mutants AL, AN and AJ, which are transmissible or to the wild type control. 'Alanine scanning' substitution of charged aminoacids is believed to conserve backbone configuration and thus to allow identification of motifs that are important in their own right (Cunningham and Wells, 1989). This suggests that the KTER motif (aminoacids 553-556) modified in mutant AK is directly involved in virus-fungus contacts during transmission. Note also that in experiment 2, mutant AM was less efficiently transmitted (4/6) than were mutants AL, AN and AJ even though the virus content in the source plant was higher than for the other mutants. Thus the region targeted in mutant AM may also intervene in interaction with the fungus.

Movement of the assembly-defective mutants Δ50, ΔM2, ΔEc3, ΔEx7 and ΔEx8 from the inoculated leaves into the roots of *B. macrocarpa* was of low efficiency and only two transmission events each were obtained for ΔEc3 and ΔEx7, possibly due to the presence of rare virions which had incorporated mutated RT protein. The apparent non-transmissibility of ΔM2, ΔEx8 and Δ50 could be due to even lower efficiency of incorporation of mutated

RT protein for these mutants or. alternatively. may indicate that the eliminated sequences contain signals that are important for transmission as well as virus assembly. Additional experiments will be required to distinguish between these possibilities.

Inoculum	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Virus content of source plant*	Transmission to bait plants**	Virus content of source plant*	Transmission to bait plants**	Virus content of source plant*	Transmission to bait plants**	Virus content of source plant*	Transmission to bait plants**
St 1234	1	2/6	13	6/6	NT	NT	NT	NT
AL	0.3	3/6	5	6/6	38	6/6	NT	NT
AN	0.3	1/6	9	6/6	10	6/6	NT	NT
AM	0.1	2/6	20	4/6	NT	NT	NT	NT
AJ	1.4	3/6	5	6/6	5	6/6	NT	NT
AK	3	0/6	6	0/6	9	0/6	18	0/6

Table 1: Transmission of alanine substitution RT domain mutants from roots of *B. macrocarpa* to roots of sugarbeet by *P. betae*.
* μg virus / gm root of B. macrocarpa source plant.
** sugarbeets infected / sugarbeets tested
NT = not tested

References

Cunningham, B. and Wells, J. A. (1989). High-resolution epitope mapping oh hGH-receptor interactions by alanine scanning mutagenesis. *Science* **244**, 1081-1085.

Gilmer. D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K. and Jonard, G. (1992). Efficient cell-to-cell movement of beet necrotic yellow vein virus requires the 3' proximal genes located on RNA 2. *Virology* **189**, 40-47.

Haberlé. A.-M., Stussi-Garaud, C.. Schmitt, C.. Garaud, J.-C.. Richards, K., Guilley, H. and Jonard. G. (1994). Detection of by immunogold labelling of P75 readthrough protein near an extremity of beet necrotic yellow vein virus particles. *Archives of Virology* **134**, 195-203.

Quillet, L.. Guilley, H.. Jonard. G. and Richards, K. (1989). *In vitro* synthesis of biologically active beet necrotic vein virus RNA. *Virology* **172**, 293-301.

Richards. K. E. and Tamada. T. (1992). Mapping functions on the multipartite genome of beet necrotic vein virus. *Annual Review of Phytopathology* **30**, 291-313.

Schmitt, C. Balmori. E., Guilley. H., Richards, K. and Jonard, G. (1992). *In vitro* mutagenesis of biologically active transcripts of beet necrotic yellow vein virus RNA 2: evidence that a domain of the 75 kDa readtrough protein is important for efficient virus assembly. *Proceedings of the National Academy of Science. USA* **89**, 5715-5719.

Tamada. T. and Kusume, T. (1991). Evidence that the 75K readthrough protein of beet necrotic yellow vein virus RNA 2 is essential for transmission by the fungus *Polymyxa betae. Journal of General Virology* **72**, 1497-1504.

Tamada. T.. Schmitt, C., Saito. M.. Guilley. H.. Richards, K. and Jonard, G. (1996). High resolution analysis of the readthrough domain of beet necrotic yellow vein virus readthrough protein: a KTER motif is important for efficient transmission of the virus by *Polymyxa betae. Journal of General Virology* **77**. 1359-1367.

CHARACTERISATION OF UK ISOLATES OF BARLEY YELLOW MOSAIC VIRUS

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Summary

Several isolates of barley yellow mosaic virus (BaYMV) from different sites in the UK, including some that were virulent on European resistant winter barley cultivars (resistance-breaking strain: BaYMV-2) and some that were not, were examined by RT-PCR of the entire bipartite genome, restriction mapping, single-strand conformation polymorphism (SSCP) analysis and sequencing of selected regions. Isolates differed in their restriction digest and SSCP patterns in several regions, but in no case was the pattern able to distinguish between common and resistance-breaking strains. In regions where the nucleotide sequences were determined, there was no simple relationship between numbers of nucleotide differences and SSCP patterns. No consistent strain-related differences in nucleotide or amino acid sequences were found in the regions examined so far, including the coat protein.

Introduction

Barley yellow mosaic virus (BaYMV) is controlled by growing cultivars resistant to the virus. In Europe, resistant cultivars grown since the disease first appeared in the late 1970s have all possessed the same resistance gene, *ym4*, located on chromosome 3. Since 1988, a resistance-breaking strain, usually called BaYMV-2, has been reported from several European countries but attempts to distinguish the European common and resistance-breaking strains of BaYMV serologically have proved unsuccessful. The aim of the present work was to use molecular techniques to characterise UK isolates of BaYMV from both strains and to assess the feasibility of developing a rapid diagnostic test based on the differences detected (e.g. RT-PCR). The first experiments, using restriction digestion of RT-PCR fragments, were unable to distinguish isolates from the two strains and we therefore used single-strand conformation polymorphism analysis of PCR fragments (PCR-SSCP) in an attempt to identify the region(s) of the genome that differ between the two strains. Because sequence information was available for the isolates in some of these regions, the relationship between sequence differences and SSCP patterns was also examined.

Materials and Methods

Barley leaves with typical symptoms were collected from six different UK sites in the spring of 1994 (isolates 1-3) or 1995 (isolates 4-6). Isolates 1 and 4 were common strain isolates from fields in use for cultivar trials and where resistant (*ym4*) cultivars remained uninfected. The remaining isolates were from resistant (*ym4*) cultivars. Total leaf and BaYMV RNAs were obtained from all samples by maceration in TLES buffer (100 mM Tris-HCl, pH 8.0, 100 mM LiCl, 10 mM EDTA, 1% SDS), followed by phenol-chloroform extraction, LiCl precipitation and washing with ethanol (Shi *et al.*, 1995).

First-strand cDNAs were reverse transcribed from BaYMV RNA1 and RNA2 using oligonucleotide primers complementary to sequences at the 3' ends of RNA1 and RNA2. Different parts of the viral cDNA1 and cDNA2 were then amplified by PCR, using primer pairs designed

from published and unpublished sequence data to scan virtually all of RNA1 (nt 31-7632) and RNA2 (nt 31-3585) (Shi *et al.*, 1996). Successful amplification of fragments of the expected size was confirmed by electrophoresis through 1% or 1.5% agarose gels.

For SSCP, 1-5 µl dsDNA (PCR product) were mixed with an equal volume of single-strand loading buffer (20 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol, in formamide), heated in a thermal cycler at 100°C for 10 min, then immediately cooled on ice and loaded onto a 10% or 12% polyacrylamide gel (20 x 16 cm; 1.5 mm thick in a vertical gel tank). The gel was run at 240 V, 40 mA (unless stated otherwise) at room temperature overnight, then silver stained.

PCR fragments were cloned using the vectors pCR II (InVitrogen) or pT7Blue (Novagen), following the manufacturers' protocols. Sequenase (version 2.0) DNA sequencing kits (USB) were used as recommended by the supplier.

Results

Restriction mapping of RNA2:

A detailed restriction map of each RNA2 was prepared for the 1994 BaYMV isolates 1, 2 and 3, using 48 restriction endonucleases and three RT-PCR fragments covering almost the entire RNA. Isolates 1 and 3 were identical and differed at only four sites from isolate 2 (Shi *et al.*, 1995). All UK isolates had about 70% of sites in common with BaYMV from Germany and about 60% with BaYMV from Japan (Shi *et al.*, 1995).
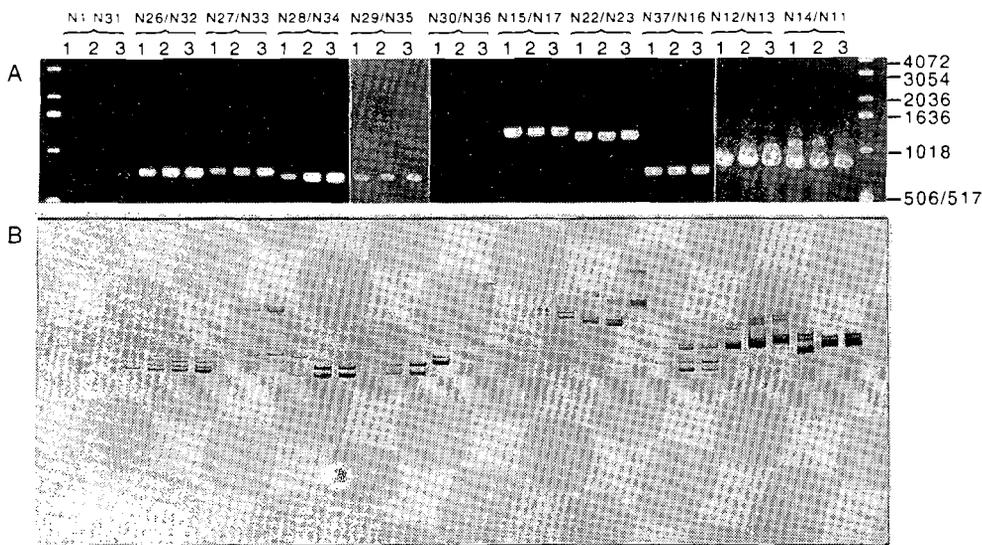
Fig. 1 RT-PCR products (A) and SSCP analysis (B) of fragments amplified from RNA1 of BaYMV isolates 1-3.

SSCP patterns and known strain differences:

RT-PCR amplification products of c.800-1400 bp, covering almost all of RNA1 and RNA2 of BaYMV isolates 1, 2 and 3 were obtained (Figs 1A and 2A). The SSCP patterns (Figs 1B and
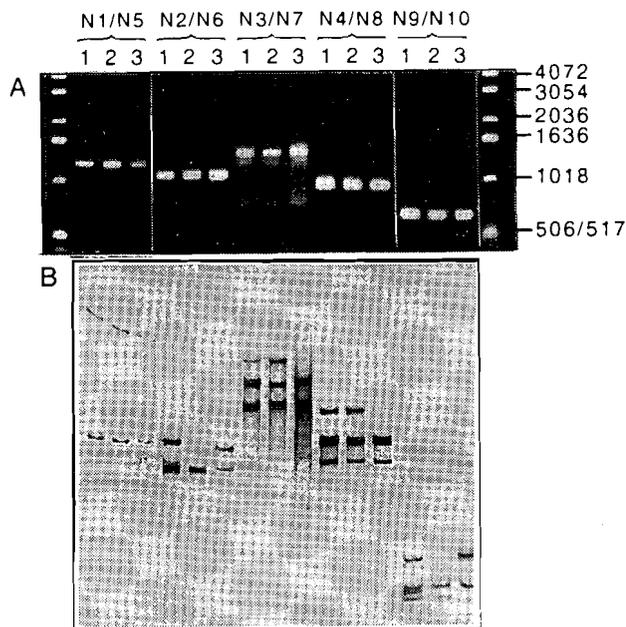
Fig. 2 RT-PCR products (A) and SSCP analysis (B) of fragments amplified from RNA2 of BaYMV isolates 1-3.

Fig. 3 SSCP analysis of fragments amplified from the 3'-terminus of the CI region on RNA1 of all six isolates of BaYMV using primers N30/N36 of Shi *et al.* (1996).

2B) showed differences between the common strain (isolate 1) and the two resistance breaking strain isolates (2 and 3) in several RT-PCR fragments from RNA1, but not in those from RNA2. When SSCP was repeated using all six isolates, for the regions N27/N33, N28/N34 and N14/N11, isolates 2-6 were identical to one another but differed from isolate 1 (results not shown). Only in the 3'-end of the CI gene (N30/N36) was the SSCP pattern of UK common strain isolates (1 and 4) consistently different from resistance-breaking isolates, but there were also differences in the SSCP patterns within each group of isolates. Thus, resistance-breaking isolates 2 and 3 were very different from 5 and 6, while the two common strain isolates (1 and 4) also differed from one another (Fig. 3).

SSCP patterns in relation to known sequence differences and gel conditions:

Sequence data from the 5'-terminus of the NIa region of BaYMV isolates 1, 2 and 3 showed 11 nucleotide differences within about 250 bases. To examine the effects of gel running conditions on SSCP patterns and hence the sensitivity of the method, we amplified this fragment from all six UK isolates. In an SSCP gel run at 260 V at room temperature for 2 h, all isolates showed the same unseparated bands. By contrast, when the gel was run at 80V overnight, the SSCP patterns of isolates 2 and 6 were very similar to each other, but the other isolates were distinct (Shi *et al.*, 1996). Under the latter conditions, a difference of only 2 out of 250 nucleotides between isolates 2 and 3 had a small effect on the SSCP pattern but, once again, no difference consistently related to resistance-breaking was detected. Comparisons were also made between SSCP patterns and known nucleotide differences between the isolates in several other regions. The results (Table 1) show that, whereas a difference of 2 or 3 nucleotides sometimes resulted
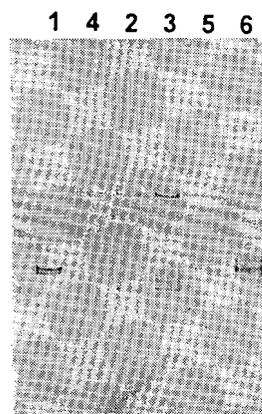
Table 1. Numbers of nucleotide differences between amplified regions of BaYMV isolates 1-3: **bold numbers** show differing SSCP patterns

Region	RNA	nts[†]	Isolates compared		
			1/2	1/3	2/3
5'-terminus, NIa	1	240	**11**	**10**	**2**
NIa	1	1316	29	26	20
5'-terminus, NIb	1	1235	**20**	**13**	**11**
3'- terminus, NIb	1	766	11	12	7
Coat protein	1	910	8	7	1
5'-terminus, 70kDa	2	671[‡]	**6**	**3**	**7**

[†], fragment size, excluding the primer regions at the 5'- and 3'- ends of the PCR products.

[‡], sequence data available only for 621 nts.

in a changed SSCP pattern, relatively large numbers of differences did not necessarily do so.

Discussion

SSCP analysis clearly has potential as a rapid, powerful tool for distinguishing virus isolates, but it cannot be guaranteed that isolates with minor sequence differences will necessarily yield different SSCP patterns. This can only be determined by experiment. The isolates tested here had differing SSCP patterns in several regions but in no case was this linked to resistance breaking, the only known biological difference between them. Furthermore, there was no simple relationship between sequence differences and SSCP patterns; differences of only 2 or 3 nucleotides gave different SSCP patterns, whereas differences of as many as 29 nucleotides did not. This was not simply due to the size of the fragment amplified.

The two UK strains of BaYMV are clearly very similar. To identify their differences and develop diagnostic methods to distinguish them, it seems that complete sequence determination will be needed.

References

Shi, N., Zhu, M., Chen, J., Stratford, R., Wilson, T.M.A., Antoniw, J.F., Foulds, I.J., MacFarlane, S.A. and Adams, M.J. (1995) Molecular characterisation of UK isolates of barley yellow mosaic bymovirus. *Virus Res.* **38**, 193-204.

Shi, N., Chen, J. Wilson, T.M.A., MacFarlane, S.A., Antoniw, J.F. & Adams, M.J. (1996). Single-strand conformation polymorphism analysis of RT-PCR products of UK isolates of barley yellow mosaic virus. *Virus Res.*, in press.

STRAIN DIFFERENCES BETWEEN ISOLATES OF BARLEY YELLOW MOSAIC VIRUS IN CHINA

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Summary

About 90 European or Japanese barley cultivars were grown for 2-5 years at eight sites in China infested with barley yellow mosaic virus (BaYMV). On the basis of cultivar response, there were several different virus strains present at these sites, and these were probably all different to the two reported from Europe and the six from Japan. Molecular differences between isolates from these sites were shown by single strand conformation polymorphism (SSCP) of PCR products, scanning the full length of both RNA1 and RNA2 and by sequencing regions coding for the coat protein and the 5'-end of the 70kDa protein on RNA2. Restriction mapping suggested that each site had at least two different virus populations.

Introduction

Resistance to barley yellow mosaic virus (BaYMV) in current European and Japanese cultivars is expressed as immunity to the virus (there are no symptoms, and no virus can be detected within the plants) and its use has revealed the presence of different virus strains. Six strains have been identified in Japan (Kashiwazaki *et al.*, 1989), whereas in Europe, where resistant cultivars have all possessed the same resistance gene (*ym4*), a resistance-breaking strain (BaYMV-2) is becoming increasingly important. BaYMV has been an increasing problem in Eastern China from the mid-1970s and there is some evidence that cultivars respond differently at different sites. Field experiments were therefore done to identify Chinese BaYMV strain differences, to relate them if possible to strains reported in Japan and Europe and to identify sources of resistance to Chinese BaYMV isolates. Molecular studies were also done on virus isolates from the same sites.

Materials and Methods

Field experiments:
Barley seeds of about 90 cultivars were kindly provided by W. Friedt, Giessen, Germany; S. Kashiwazaki, Tsukuba, Japan; W. Huth, Braunschweig, Germany; and P. R. Aldis, Docking, UK. Eight heavily infected experimental sites were chosen, well distributed within the area where the disease is known to occur in China. Experiments were sown in each of 5 years (1990-4) at Rudong (RU) , 4 years (1991-4) at Shanghai (SH) and Yancheng (YC) and 2 years (1993-4) at Funing (FU), Haian (HA), Jiashan (JI), Nantong (NA) and Yangzhou (YZ) (Fig. 1). Only 31 cultivars were sown in the first three years, but most or all cultivars were sown on all sites in 1993 and 1994. Plots of *c.* 20 seeds of each cultivar were sown in the autumn; there were single plots in

1990-2, but three replicates in 1993 and 1994. Every tenth row was a Chinese susceptible control cultivar. Plants were examined in the spring and the numbers with typical mosaic symptoms recorded. In ELISA tests, virus was detected only where symptoms were present, so results are shown as % plants affected.

Molecular studies:

Leaves with typical symptoms were collected from the 8 experimental sites and also from Hangzhou (HZ) and Ningbo (NI). Total leaf and virus RNAs were obtained from all samples as described by Shi *et al.* (1995). First-strand cDNAs were reverse transcribed from RNA1 and RNA2 using oligonucleotide primers complementary to sequences at the 3' ends of RNA1 and RNA2. Different parts of the viral cDNA1 and cDNA2 were then amplified by PCR, using primer pairs designed from published and unpublished sequence data to scan virtually all of RNA1 (nt 31-7632) and RNA2 (nt 31-3585) (Shi *et al.*, 1996). Amplification of fragments of the

Fig.1. Map showing sites of samples and experiments

expected size was confirmed by electrophoresis through agarose gels. For single strand conformation polymorphism (SSCP), 1-5 μl dsDNA (PCR product) were denatured, loaded onto a 10% or 12% polyacrylamide gel and run at 240 V, 40 mA at room temperature overnight (Shi *et al.*, 1996). PCR fragments were cloned using the vectors pCR II (InVitrogen) or pT7Blue (Novagen), following the manufacturers' protocols. Sequenase (version 2.0) DNA sequencing kits (USB) were used as recommended by the supplier.

Results and Discussion

The following 38 cultivars were susceptible at all Chinese sites: **European:** Atem, Golden Promise, Triumph, Zarina, Chestnut, Fallon, Finesse, Gerbel, Gypsy, Halcyon, Hoppel, Igri, Magie, Mallard, Marinka, Maris Otter, Medallion, Monix, Panda, Plaisant, Puffin, Sonja, Sprite, Team, Tipper, Zest, **Japanese:** Akagi Nijo 1, Amagi Nijo, Asahi 5, New Golden, Nitta Nijo 1, Yashio Golden, Asahi 9, Kinonig 3, Nirakei 31, **Other:** Gobernadoras, Hiproly, Turkey Naked 2. The following 18 cultivars (with their known resistance genes) appeared to be resistant at all sites: Akashinriki, Anson, Asama Mugi, Chikurin Ibaraki 2, Chosen (*ym3*), Energy (*ym4*), Hagane Mugi (*ym3*), Iwate Mensury 2 (*ym3*) , Kanto Nijo 19, Kashima Mugi, Kogoshima Kobai 1, Mihori Hadaka 3 (*Ym2*), Mokusekko 3 (*Ym+?*), Muju Covered 2, Oita Nejire, Shinano 1, Suifi, Toranoo Sai 1, Touhoku Kawa 73. The European cv. Energy is interesting because this is one of a group of *ym4* cultivars that were usually susceptible at some of the sites (see below), so it seems possible that this cultivar carries some additional resistance. Amongst the Japanese cultivars, Chosen, Hagane Mugi, Iwate Mensury 2 and Mokusekko 3, are reported to be resistant to all local strains.

The remaining cultivars appeared to respond differently at different sites. These included 19 European *ym4* cultivars (Asorbia, Athene, Banjo, Brunhild, Express, Firefly, Frances, Franka, Gaulois, Jana, Mimosa, Nixe, Palomino, Romanze, Sonate, Target, Torrent, Venus, Waveney),

ANALYSIS OF RNA1 OF WHEAT SPINDLE STREAK MOSAIC BYMOVIRUS (WSSMV)

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Summary

A cDNA clone complementary to the 5'terminal part of RNA1 of wheat spindle streak mosaic virus (WSSMV) from Southern France has been cloned and sequenced. The investigated cDNA clone consists of 3050 nt. The missing 23 nt of the very 5'end were determined by sequencing of 5'RACE products. The sequence shows an overlapping region with the published 3'terminal half of RNA1 (Sohn et al., 1994) and therefore the complete RNA1 sequence of 7050 nt can be assembled. The 5'non-translated region (NTR) is 170 nt in length. One large open reading frame (ORF) of the complete RNA1 (7167 nt) coding for a putative polyprotein of 2389 amino acids was found. Because of the amino acid sequence homology to other bymoviral and potyviral sequences the N-terminal part of the polyprotein is supposed to comprise a P1 and a P2 protein which show similarities to the P3 and $6K_1$ protein of potyviruses. Furtheron, it encodes the N-terminal part of the cytoplamic inclusion protein (CI) which has a putative helicase function. Comparisons of WSSMV RNA1 encoded N-terminal amino acid sequences with corresponding bymoviral and potyviral regions confirm the marginal homology to potyviruses and the very close relationship of WSSMV to BaYMV.

Introduction

Yellow mosaic disease is caused by soil-borne viruses and can lead to serious damage in wheat crops. Slykhius (1970) identified a soil-borne virus in wheat grown in Ontario, Canada, which was designated as wheat spindle streak mosaic virus (WSSMV). It has also been reported in the USA, India, France, Italy and China. In Japan the causal agent of a yellow mosaic disease is called wheat yellow mosaic virus (WYMV). As WSSMV and WYMV show very similiar features, so far they were considered to be strains of the same virus (Usugi and Saito, 1979). WSSMV is a member of the genera bymovirus. These viruses are, unlike potyviruses but similar to furoviruses, transmitted by the soil-borne fungus *Polymyxa graminis*. Their genome comprises two positive sense, single-stranded, polyadenylated RNAs. To understand the organization of the genome we cloned and sequenced RNA1 of WSSMV. The 3'part of RNA1 was published elsewhere (Sohn et al., 1994). Here we present the sequence of the 5'part of the same isolate purified from the same field material to complete the sequence information of RNA1.

Material and Methods

Virus purification, RNA isolation and cDNA synthesis
Virus was purified from infected wheat leaves (cv. Aramon) collected from a field in Southern France. Viral RNA isolation, cDNA first and second strand synthesis were performed as described previously (Sohn et al., 1994).

Sequencing and 5'RACE reaction

DNA sequence was determined by the dideoxynucleotide triposphate chain termination method (Sanger et al., 1977) using a Applied Biosystems sequencing machine (ABI). Subclones for the analysis of overlapping nucleotide sequences of both strands were obtained by means of different restriction enzymes. In order to gain the extreme 5'terminus a 5'RACE procedure was performed as described by Bertioli and Burrows (1995) with the following modifications. After using oligo 5840 (5' GTGGGATAGTGAATATGG 3') for reverse transcription a T-tailing reaction was done. For the PCR reaction 50 ng of oligo 5674 (5' CGCGGATCC T_{14}) and oligo 5841 (5' GCTCAGCTACAGCTGTTGAGG 3') were used with an annealing temperature of 55°C. The achieved PCR fragment was cloned into the vector pBSK (Stratagene). Five independent clones were sequenced. Sequence data were assembled and analysed using the UWGCG software package of Devereux et al. (1984).

Results and Discussion

The cDNA clone pGEMWI47 (3050nt) and the 5' RACE products which extend the sequence by 23 nt were sequenced. Alignment with the sequence of the cDNA clone containing the 3'terminal part of RNA1 (Sohn et al., 1994) reveals an overlapping region. Fig.1 shows the nucleotide (2924 nt) and amino acid sequence of the 5' terminal part of RNA1, which can directly be assembled with the published sequence of the 3'end to the complete WSSMV RNA1 consisting of 7570 nt. Analysis of the distribution of translation initiation and termination codons revealed the presence of one long ORF beginning with an start codon ATG (170 to 172) and terminating with UAA stop codon (7337 to 7339) in the plus strand. The large ORF is 7167 nt long and codes for a putative polyprotein of 2389 amino acids with a calculated molecular weight of 267 kD. Based on sequence comparison with other bymo- and potyviruses putative processing sites (Fig.1) were identified.
The 5'non-translated region consists of 170 nt and has a high A/T and a low G content typical for bymo- and potyviruses (Riechmann et al., 1992). The very 5'end of RNA1 show in the approximately first 50 nt high similarities to other bymoviral 5'NTRs which might reflect a common mechanism to stabilize the 5'end of the viral RNA.
The 5'terminal part of RNA1 codes for a P1 and a P2 protein which shows similarities to the P3 and $6K_1$ protein of potyviruses, repectively and it encodes the N-terminal part of the cylindrical inclusion protein (CI). The function of the N-terminal two proteins is unknown. The CI protein is part of the cylindrical inclusion of the pinwheel type found in infected cells. A helicase function is postulated because of the high amino acid homology with other helicases. Furtheron, the sequence of the CI protein includes a conserved NTP-binding motif consisting of the amino acids GXXGXGKS (X=any amino acid) and DE (Fig.1) which is found in RNA helicases (Gorbalenyaet al., 1988). In conclusion, the genome organization of the WSSMV RNA1 is identical to the one of the other bymoviruses.

Table 1 shows the percentages of identical amino acids found by comparisons of the amino acid sequences of WSSMV RNA1 and corresponding protein sequences of the fungus-transmitted BaYMV (X 69757 ,D 01099) and BaMMV (L 49381, X 90904) and the aphid-transmitted TEV (M 15239) and PVY (A 08776) as the type member of potyviruses. As expected the highest homologies were found between WSSMV and BaYMV (68-88%, Table1) while the degree of homology to BaMMV is lower (33-58%). This could explain the cross reaction of BaYMV protein derived antisera with WSSMV proteins (Sohn et al., 1995). The analysis of the 5' part of RNA1 confirm the close relationship found so far between WSSMV and BaYMV.

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AAAAATAAAACCGACCATACACATCAAAACACACAAACCAACACAACATCAACCTACCTTCATATGCGCATCTTATAGTGCACATC 84
TTCTCGTATCTCTGAAACTCCTCTTCATTCCATTCTGAGGACTTTCAAACTTCACTCGTTTGTCTACGTATTTCACAATCCCAC 168
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      M  E  A  Q  G  T  A  S  P  M  N  F  S  A  N  F  M  A  P  E  L  F  Y  S  S  N  V  K  -
AAAGCTTAAGGCGATTTTCAAATGTCGTAGCACTACCCGCGTTATTGACGCAATAGCCAATGACTTTGAACTTGTTGCTTTCCT 336
  K  L  K  A  I  F  K  C  R  S  T  T  R  V  I  D  A  I  A  N  D  F  E  L  V  A  F  L  -
CATTTTGAGTCCGGCGCATCTTATGCAGTTGGAGACAACACTTCGCAATGAAATAAACTCCACTGTTGTACCACTGGCAAACTC 420
  I  L  S  P  A  H  L  M  Q  L  E  T  T  L  R  N  E  I  N  S  T  V  V  P  L  A  N  S  -
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  D  A  S  F  E  T  V  A  V  L  K  T  A  L  D  G  L  R  Y  H  F  G  A  T  T  L  E  K  -
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  L  Q  ↓A  R  S  A  N  E  K  E  K  K  L  M  M  L  L  A  S  A  V  G  I  T  Y  L  F  D  -
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  A  Ï  F  L  **D  E  A**  H  D  V  K  E  H  S  L  V  F  E  S  I  C  N  K  F  T  S  V  R  -
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  T  L  R  L  D  R  R  R  V  T  N  N  E  R  K  Q  R  I  G  R  A  G  R  L  K  A  G  Y  -
TGCCATCACTTGTGGGGATGTTGACACTGAAGTTAATGTTGTTTCACCAGATGTGCTGTATGGTGCCGCGCTTCTGAGTTTTAA 2520
  A  I  T  C  G  D  V  D  T  E  V  N  V  V  S  P  D  V  L  Y  G  A  A  L  L  S  F  K  -
ACACAATGTGCCCTTCTATATGAACCATACTTTCGAAAGCTCGTGGCTTGATAGCATCACAAAGGCGCAAGCTGAGACGATGTC 2604
  H  N  V  P  F  Y  M  N  H  T  F  E  S  S  W  L  D  S  I  T  K  A  Q  A  E  T  M  S  -
AATTTTCAAACTACCGATATTTCTGACGCGAGATCTGGTTAATGCAGATGGTTCCGTTGCGAAGGATTTTCTCGACGTTCTTAA 2688
  I  F  K  L  P  I  F  L  T  R  D  L  V  N  A  D  G  S  V  A  K  D  F  L  D  V  L  K  -
GAAGCATCAATTCACCACGAGCGATGTGAAGCAAGCACCAAGTTCCACAGCGAAGCACATCTTCCCTACATGGCCATCCTATTT 2772
  K  H  Q  F  T  T  S  D  V  K  Q  A  P  S  S  T  A  K  H  I  F  P  T  W  P  S  Y  F  -
CGCCCTGCATCAAGCAATCCACTATGGGATAATAAGGACAACATTCCGAGTGAACTCATGAACACAAGAATCCCATTCTCTGT 2856
  A  L  H  Q  A  I  H  Y  G  D  N  K  D  N  I  P  S  E  L  M  N  T  R  I  P  F  S  V  -
GTCAACTCTGTCGAAATTTGACTGGCCAGCTTTGGCACTTGCTTGCGAGAAATACCGCTCAACTGTAA 2924
  S  T  L  S  K  F  D  W  P  A  L  A  L  A  C  E  K  Y  R  S  T  V
```

Fig1. The nucleotide sequence and derived amino acid sequence of the 5'terminal part of RNA1 of WSSMV. The predicted processing sites of the polyprotein were indicated by an arrow. Amino acids of the NTP-binding motif of the CI protein are shown in bold letters.

Table 1. Percentage of identical amino acids found in the N-terminal regions of the WSSMV RNA1-encoded polyprotein with corresponding regions of bymoviral and potyviral polyproteins

	% of identical amino acids of WSSMV regions and			
region	BaYMV	BaMMV	PVY	TEV
P1	73	33	24	17
P2	88	36	20	15
CI	78	45	29	23

The knowledge of the sequences allows now the construction of a full-length cDNA clone which will help to improve the understanding of the transmission process and the virus-vector interactions. In spite of these remarkably high similarities WSSMV and BaYMV have different hosts (Slykhuis, 1970; Jackson et al., 1976). As known so far WSSMV has never been found in barley and vice versa BaYMV has never been detected in wheat, independent of infection with the natural vector *Polymyxa graminis* or after mechanically inoculations. This indicates that the difference in the host range might be due to differences in the sequence. Comparative experiments with infectious clones and the exchange of sequence parts of BaYMV and WSSMV will be a tool to answer this question.

References

Bertioli D. J. and Burrows P. R. (1995) A simple RACE method based on CTAB precipitation. Methods Mol. Cell. Biol. **5**: 118-121.

Devereux J., Haeberli P. and Smithies O. (1984) A comprehensive set of sequence analysis programms for the VAX. Nuc. Acids Res. **12**: 387-395.

Jackson A. O., Bracker C.E and Shaner G. (1976) Mechanical transmission, varietal reactions and electron mircoscopy of a desease in Indiana with properties of Wheat spindle streak mosaic virus. Plant Dis. Rep. **60**: 202-206.

Gorbalenya A. E., Koonin E. V., Donchenko A. P. and Blinov V. M. (1988) A conserved NTP-binding motif in putative helicases. Nature **33**:22.

Riechmann J. L., Lain S. and Garcia J. A. (1992) Highlights and prospects of potivirus molecular biology. J. Gen. Virol. **73**: 1-16.

Sanger F., Nicklen S. and Coulson A. R. (1977) DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74**: 5463-5467.

Slykhuis, J.T. (1970). Factors determining the development of wheat spindle streak mosaic caused by a soil-borne virus in Ontario. Phytopath. **60**: 319-3315.

Usugiu T.; Saito Y. (1979) Relationships between wheat yellow mosaic virus and wheat spindle streak mosaic virus. Ann. Phytopath. Soc. Japan **45**: 397-400.

Sohn, A., Schenk P., Signoret P., Schmitz G., Schell J. and Steinbiss H.-H. (1994) Sequence analysis of the 3´terminal half of RNA1 of wheat spindle streak mosaic virus. Arch. of Virology **135**: 279-292.

Sohn, A., Schenk P., Hamacher J., Signoret P. and Steinbiss H.-H. (1995) Comparison of wheat spindle streak mosaic virus (WSSMV) and barley yellow mosaic virus (BaYMV): two closely related bymoviruses. agronomie **15**: 427-431.

EVIDENCE THAT BEET NECROTIC YELLOW VEIN VIRUS RNA-5 IS INVOLVED IN SYMPTOM DEVELOPMENT OF SUGAR-BEET ROOTS

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Summary

Beet necrotic yellow vein virus (BNYVV) RNA-5 consisted of 1342-1347 nucleotides and contained a single ORF encoding a 26 kDa protein. Out of 42 isolates, which were collected throughout Hokkaido, 11 isolates contained RNA-5. BNYVV field isolates containing RNA-5 and those lacking RNA-5 were referred to as the F and N types, respectively. When sugar-beet seedlings inoculated by virus-carrying *Polymyxa betae* were grown in field, the F type isolates induced more severe symptoms and yield reduction in sugar-beet roots than the N type isolates. The severity of symptom (yield loss) paralleled the susceptibility of the cultivars. However, the laboratory isolate containing RNA-5 but lacking RNA-3 caused more severe damage to a partially resistant cultivar than to susceptible cultivars. This isolate showed scab-like symptoms on roots, differed from those caused by isolates containing RNA-3. Thus, RNA-5 and RNA-3 seem to play important, but different, roles in symptom development in sugar-beet roots.

Introduction

Rhizomania is a severe disease of sugar beet caused by beet necrotic yellow vein virus (BNYVV) which is transmitted by the soil-inhabiting fungus *Polymyxa betae* (Richards & Tamada, 1992). The disease has now been distributed in many sugar beet growing countries and is economically very important, because it causes not only a severe loss in root yield and sugar content but also because it is difficult to control (Asher, 1993).

Genetic resistance is the most promising approach to the control of rhizomania, and therefore several resistant cultivars such as Rizor have been developed and grown in many areas of the rhizomania-infested regions of the world (Asher, 1993). Resistance of such resistant cultivars has been reported to be caused by restriction of virus multiplication and/or translocation (Giunchedi *et al.* 1987; Paul *et al.*, 1992; Tuitert *et al.*, 1994).

The genome of beet necrotic yellow vein virus consists generally of four RNA species; RNA-1 and RNA-2 are required for viral RNA replication, assembly and virus movement, whereas RNA-3 and RNA-4 are needed for disease development and spread in nature (Richards & Tamada, 1992). RNA-3 facilitates the multiplication and/or spread of the virus in root tissue (Tamada *et al.*, 1990, Jupin *et al.*, 1991, Koenig *et al.* 1991), and RNA-4 is important for efficient vector transmission (Tamada & Abe, 1989). However, some Japanese isolates contain RNA-5 which is present together with RNA-3 and RNA-4 (Tamada *et al.* 1989). In this paper, we described the nuclotide sequence of RNA-5 and have tested the effect of RNA-5 on symptom development and sugar yield in susceptible and partially resistant cultivars under field conditions.

Materials and Methods

Virus isolates: The laboratory isolates of BNYVV S-345 (RNA-1+2+3+4+5). S-34

(RNA-1+2+3+4), S-45 (RNA-1+2+4+5) and S-4 (RNA-1+2+4) described by Tamada *et al.* (1989, 1990) were used. Four field isolates S44, T101, K80 and M87 were used to compare their disease development. Isolates D, SH1, S43 and R83 were used to analyse the sequence of RNA-5.

Sequencing: Nucleotide sequences were determined as described (Kiguchi *et al.*, 1996).
Detection of BNYVV: BNYVV infection and virus content in sugar-beet roots were determined by ELISA (Tamada & Abe, 1989).

Detection of BNYVV RNA: BNYVV RNAs were analysed by agarose gel electrophoresis and Northern blot hybridization using digoxigenin-labelled DNA probes specific to each of RNA-3, RNA-4 and RNA-5.

Fungus inoculation tests: *P. betae* cultures carrying each of BNYVV isolates were produced and maintained in rootlets of sugar-beet seedlings which were grown in special test tubes in a growth cabinet with a 16 h light and 8 h dark cycle (Tamada *et al.*,1989). Fungal inoculation was done by pouring the crude homogenate of infected rootlets or zoospore suspensions into test tubes, in which the seedlings were grown. For zoospore inoculation, a zoospore suspension was obtain by collecting water drained from bottom of test tubes into which nutrient solution had been poured.

Field tests: Virus inoculation was done by pouring zoospore suspensions of virus-carrying *P. betae* to sugar-beet seedlings grown in paper pots. Virus-inoculated and non-inoculated control seedlings were transplanted to the field on the end of May and were cultivated in accordance with the standard commercial practice. At harvest on the end of October, groups of 40 roots per plot were tested on the yield parameters. If necessary, sugar-beet root tips were individually sampled and the virus concentrations were examined by ELISA.

Results

The nucleotide sequence of RNA-5: The sequences of RNA-5 from D, SH1, S43 and R83 were at least 98 % identical and from 1342 to 1347 nucleotides in length, excluding the poly(A) tail (Kiguchi *et al.*,1996). Each contained a single ORF encoding a polypeptide of 26KDa. The coding sequence was bordered by a long leader of 443 to 448 nucleotides and a 3'-terminal non-coding region of 215 nucleotides. No sequence identity was found between RNA-5 and either RNA-3 or RNA-4, except for the 5'-terminal nine residues and for about the 3'-terminal 200 residues.

Geographical distribution of BNYVV isolates with RNA-5: Japanese field isolates, which were collected from single fields in different areas in Hokkaido, were tested on RNA component by agarose gel electrophoresis and Northern blot hybridization. Out of 42 field isolates tested, 31 isolates contained four RNA species (RNA-1+2+3+4) and the other 11 isolates contained four RNAs plus RNA-5. There were no apparent differences between these field isolates in the size of each of five RNAs. BNYVV isolates containing RNA-5 and those without RNA-5 were referred to as the F and the N type, respectively.

Comparison of the F and N types in disease development: Effect of the F and N types of BNYVV on sugar content and root yield in sugar-beet roots was tested using three different cultivars. The results are shown in Fig. 1. The symptom severity was strongly reflected in the yield reduction. With increasing susceptibility of the cultivars, sugar yield decreased. The F type isolates caused a much more severe reduction in sugar yield than the N type isolates in susceptible and resistant cultivars. In a partially resistant cultivar, the F type virus caused about 80 % loss in yield, whereas the N type virus caused only about 20 % loss. This indicates that the F type virus causes a severe damage to the resistant sugar-beet cultivar.

Effect of RNA-5 on the disease development: BNYVV laboratory isolates containing

combinations of each of RNA-3 and RNA-5 were tested. Similar results to above experiments were obtained from experiments using S-345 and S-34 (Fig. 2). However, it is interesting to note that S-45 caused more severe loss in the resistant cultivar than in the susceptible cultivar. In addition, this yield loss by S-45 was much greater than that by S-34 or even by S-345. In this case, the virus was detected from almost all roots (data not shown). On the other hand, in resistant plants inoculated with S-34, the virus was detected in about 70 % of roots, and the virus content was much lower, whereas in susceptible cultivars the virus was detected in all roots, in which the virus content was high (data not shown).

Isolates S-34 and S-345 caused typical rhizomania symptoms such as stunting and abnormal proliferation of fine rootlets, whereas isolates S-45 (with RNA-5 but without RNA-3) caused scab-like symptoms.

When inoculated with S-4 which lacks either RNA-3 or RNA-5, sugar yield of roots in two cultivars were almost the same as those of non-inoculated plants (data not shown).

Sugar-beet cultivar	BNYVV type*	BNYVV isolate	Sugar yield (kg/10a)		
			500	1,000	
Suscepptible "Monohikari"	F	S 44	0		
		T 101	0		
	N	K 80	9		
		M 87	9		
	—	Control			100
Susceptible "Ema"	F	S 44	0		
		T 101	13		
	N	K 80	30		
		M 87	21		
	—	Control			100
Partially resistant "Rizor"	F	S 44	11		
		T 101	25		
	N	K 80	80		
		M 87	85		
	—	Control			100
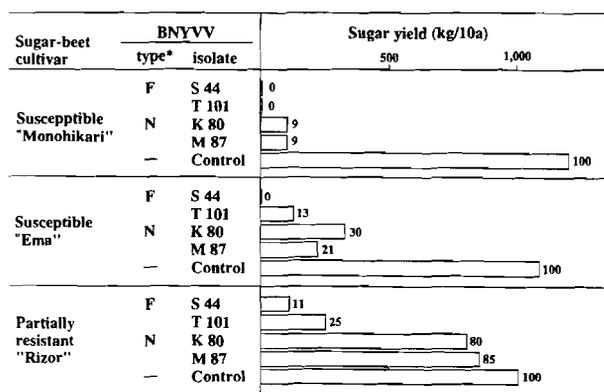
Fig. 1. Effect of BNYVV isolates belonging to the F and N types on the sugar yield in roots of sugar-beet plants.

* F-type: RNA-1+2+3+4+5, N-type: RNA-1+2+3+4

Sugar-beet cultivar	Virus isolate	RNA content	Sugar yield (kg/10a)	
			500	1000
Susceptible "Mono-ace"	S-345	1+2+3+4+5	11	
	S-34	1+2+3+4	24	
	S-45	1+2+4+5	60	
	Control			100
Partially resistant "Rizor"	S-345	1+2+3+4+5	41	
	S-34	1+2+3+4	77	
	S-45	1+2+4+5	14	
	Control			100
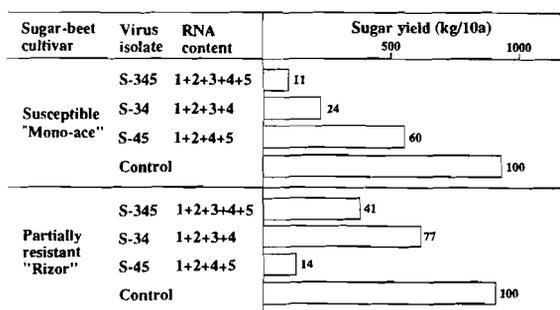
Fig. 2. Effect of BNYVV RNA-3 and RNA-5 on the sugar yield in roots of sugar-beet plants.

Discussion

Although BNYVV usually contains four species of RNA-1 to RNA-4, we have found that about 25 % Japanese isolates contained RNA-5, and that the isolates with RNA-5 were widely

distributed throughout Hokkaido, although the distribution seems to be limited.

We have determined the complete nucleotide sequence of BNYVV RNA-5, which consisted of 1342-1347 nucleotides and contained a single ORF encoding a 26 KDa protein. The genome organization of RNA-5 is very similar to that of RNA-3 and RNA-4, but no sequence identity was found between these RNAs, except for the 5'-terminal nine residues and for about the 3'-terminal 200 residues.

In a previous paper (Tamada et al., 1990), we have shown that isolates with RNA-3 cause rhizomania symptoms in roots and RNA-3 is strongly associated with abnormal proliferation of fine rootlets. In addition, this paper showed that the isolates containing RNA-5 (F type) induce more severe symptoms (yield loss) than those lacking RNA-5 (N type), indicating that RNA-5 is involved in symptom severity of BNYVV. Especially it is interesting to find that the isolate containing RNA-5 but lacking RNA-3 has a much more severe damage in the resistant cultivar than in the susceptible cultivar. Thus, RNA-5 and RNA-3 seem to play important, but different, roles in symptom development in sugar-beet roots.

References

Asher, M. J. C. (1993) Rhizomania. In *The Sugar Beet Crop: Science into practice*. pp 311-346. Edited by D. A. Cooke & R. K. Scott, Chapmann & Hall, London.

Giunchedi, L., De Biaggi, M. & Poggi Pollini, C. (1987). Correlation between tolerance and beet necroptic yellow vein virus in sugar-beet genotypes. Phytopath. Mediterr. 26, 23-28.

Jupin, I., Tamada, T. & Richards, K. (1991). Pathogenesis of beet necrotic yellow vein virus. Semin. Virol. 2: 121-129.

Kiguchi, T., Saito, M., & Tamada, T. (1996). Nucleotide sequence analysis of RNA-5 of five isolates of beet necrotic yellow vein virus and the identity of a deletion mutant. J. Gen. Virol. 77: 575-580.

Koenig, R., Jarausch, W., Li, Y., Commandeur, U., Burgermeister, W., Gehrke, M. & Luddecke, P. (1991). Effect of recombinant beet necrotic yellow vein virus with different RNA compositions on mechanical inoculation of sugarbeets. J. Gen. Virol. 72: 2243-2246.

Paul, H., Henken,, B. & Alderlieste, M. F. J. (1992). A greenhouse tests for screening sugar-beet (*Beta vulgaris*) for resistance to beet necrotic yellow vein virus (BNYVV). Netherlands J. Plant. Pathol. 98: 65-75

Richards,, K. E. & Tamada, T. (1992) Mapping functions on the multipartite genome of beet necrotic yellow vein virus. Ann. Rev. Phytopath. 30: 291-313.

Tamada, T. & Abe, H. (1989). Evidence that beet necrotic yellow vein virus RNA-4 is essential for efficient transmission by the fungus *Polymyxa betae*. J. Gen. Virol. 70: 3391-3398.

Tamada, T. Shirako, Y., Abe, H., Saito, M., Kiguchi, T. & Harada, T. (1989). Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA components. J. Gen. Virol. 70: 3399-3409.

Tamada, T., Saito, M., Kiguchi, T. & Kusume, T. (1990). Effect of isolates of beet necrotic yellow vein virus with different RNA components on the development of rhizomania symptoms. Proc. 1st Symp. Int. Work. Group Plant Viruses Fungal Vectors, pp.41-44. Edited by R. Koenig. Stuttgart: Eugen Ulmer.

Tuitert, G., Muster-van Oorschot, P. M. S. & Heijbroek, W. (1994). Effect of sugar beet cultivars with different levels of resistance of beet necrotic yellow vein virus on transmission of virus by *Polymyxa betae*. Europ. J. Plant Pathol. 100: 201-220.

COMPARATIVE MOLECULAR ANALYSES OF SEVERAL BNYVV AND BSBMV-RELATED FUROVIRUSES INFECTING SUGARBEET

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Summary

The relationships between five isolates of BNYVV from the United States and four isolates which are serologically identical to the BSBMV were evaluated based on the reactivity of molecular probes, the size and number of the RNAs, and polyadenylation. The BNYVV isolates were virtually identical to one another. The BSBMV-related isolates differed from each other by the size and number of RNAs, the reactivity with specific molecular probes, and different symptoms on indicator plants. All isolates tested were polyadenylated and had at least three RNAs. Thus, the BSBMV-related isolates, because of their polyadenylation and number of RNAs, more closely resemble BNYVV than the type member of the furovirus group, wheat soil-borne mosaic virus (WSBMV). It is suggested that these viruses of sugarbeet belong to the Benevirus group, as proposed by Dolja et al. (1994).

Introduction

Previous studies at the USDA-ARS in Salinas have compared several beet necrotic yellow vein virus (BNYVV) isolates (two from California, one each from Colorado, Idaho, and Nebraska) and several isolates which are serologically identical to beet soil borne mosaic virus isolates 1 and 2 (BSBMV-1 and -2; two each from Texas and Nebraska) using immunodiffusion, ELISA, western blot analysis, host range and reverse transcriptase-polymerase chain reaction (RT-PCR) (Wisler et al., 1994). The purpose of this project is to better understand the diversity of BNYVV and related furoviruses infecting sugarbeet. This understanding is crucial to the accurate and sensitive detection of these viruses, particularly in light of the regulatory restrictions involved in Rhizomania of sugarbeet. The primary conclusions from our previous research are that virtually all BNYVV isolates studied in the U.S. are identical to one another, suggesting a single introduction into the United States. In contrast, all of the isolates which were originally thought to be identical to BSBMV-1 and -2 from Texas based on serological identity of the coat protein, are a heterogeneous group and are more likely to have originated here in the United States. Recent molecular analyses of the furoviruses addressed in this study support previous results.

Materials and Methods

RNA isolation and Northern blot analysis: All BNYVV and BSBMV isolates were propagated in, and virions were purified from, *Chenopodium quinoa* plants as described by Lecoq and Pitrat for zucchini yellow mosaic virus (1987). Purified virion preparations (400 µg) were lyophilized in a 10% sucrose solution at a 1:1 ratio (v:v). The virion RNAs were extracted by resuspension in 356 µl diethylpyrocarbonate (DEPC)-treated water, 2 µl Proteinase K (20 mg/ml), 40 µl of 10% SDS, and 2 µl of RNasin (40 U/µl, Promega, Madison, WI). After a 30 min incubation at 37 °C, the solution was extracted twice using an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by ethanol precipitation. After precipitation, the RNA was solubilized in DEPC-treated water, denatured in glyoxal and dimethyl sulfoxide (DMSO) (McMaster and Carmichael, 1977) and analyzed on a

1% agarose gel in Loen's buffer (0.07 M Trizma base, 2 mM EDTA, 0.07 M NaH$_2$PO$_4$, pH 7.8). The 0.24-9.5 Kb RNA ladder (BRL, Grand Island, NY) was used for size standards. After staining with ethidium bromide (0.5 µg/ml), agarose gels were soaked in 50 mM NaOH for 10 min followed by neutralization in 20X SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) for 20 min. RNAs were transferred to positively-charged nylon membranes (Boehringer Mannheim) overnight using an Optiblot Transfer unit (Scotlab, Shelton, CT). Membranes were washed for 2 min in 2X SSC and UV-crosslinked (120,000 µJ/cm^2) using a Hoefer UVC 500 Crosslinker (San Francisco, CA).

   cDNA cloning: Freshly prepared virion RNA from BSBMV-2 (4 µg) used as the template for cDNA synthesis. Subsequent steps were performed according to the Superscript Plasmid System for cDNA Synthesis and Plasmid Cloning (BRL, Grand Island, NY). The MAX Efficiency DH5α Competent Cells (BRL) were used for transformation and propagation of the recombinant plasmids. cDNA clones were used to generate digoxigenin-UTP-labeled transcripts using the DIG-Labeling and Detection Kit (Boehringer Mannheim, Indianapolis, IN). Hybridizations, washes, incubation with anti-DIG alkaline phosphatase, and development with Lumi-Phos 500 were performed according to manufacturer's instructions. Chemiluminescence was detected on X-ray film. Clones of BNYVV were obtained from the American Type Culture Collection (ATCC) and were labeled similarly. To determine polyadenylation of virus isolates, a biotinylated-oligo-dT probe (Promega) was used also in a Northern analysis, with streptavidin as the substrate. Sequencing reactions were performed by the University of Florida DNA Sequencing Facility, Gainesville, FL. Sequence analyses were made using the Wisconsin Genetics Computer Group (GCG) analysis programs.

Results

Two BNYVV isolates and four isolates serologically identical to BSBMV-1 and -2 were purified, their RNA's extracted and analyzed for determination of the number and relative sizes of their RNAs (Fig. 1). These isolates were also tested for polyadenylation of the 3' end of the RNAs. All of the isolates tested were polyadenylated (Fig. 2), and thus belong to the BNYVV subgroup, which has been suggested by Dolja et al., (1994) to be termed the Benevirus group. In addition, all isolates contained at least three RNAs (Fig. 1, Fig. 2), again more like BNYVV than WSBMV, which has only two RNAs.
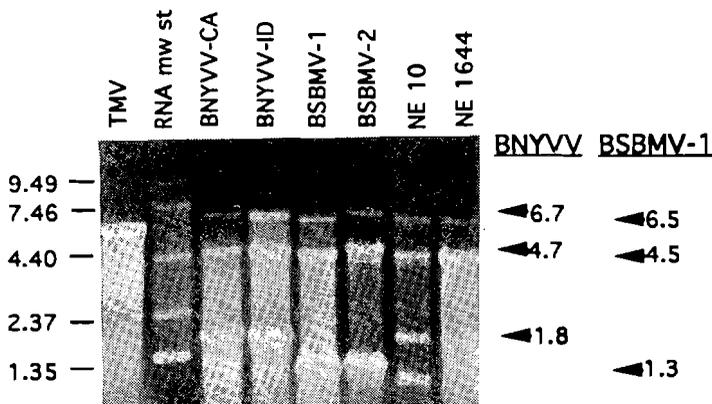
Fig. 1. Ethidium bromide stained 1% agarose gel of extracted and denatured sugarbeet furovirus RNAs. Electrophoresis was for two hr at 50 V. RNA ladder in kbp at left; arrows at right indicate relative sizes of BNYVV and BSBMV-1 RNAs.
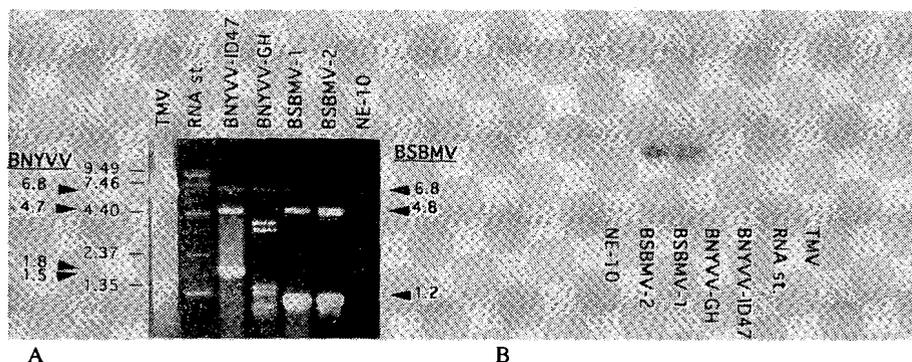
Fig. 2A; Ethidium bromide stained 1% agarose gel, with relative sizes of BNYVV and BSBMV-related isolates. B; Northern analysis of gel hybridized with a biotinylated oligo-dT probe, using streptavidin as the substrate.

The two BSBMV isolates from Texas had three RNAs, with RNA 3 being smaller than that reported for BNYVV RNA 3 or RNA 4 (Fig. 2 and Table 1). The two isolates that are serologically identical to BSBMV capsid protein, NE 10 and NE 1644, also had, different RNA patterns. NE 10 had an RNA pattern more like BNYVV, but with a small RNA 4 at 0.9 kb (Table 1 and Fig. 1). The isolate NE 1644 also showed an RNA pattern more like BNYVV than BSBMV. This isolate is especially interesting because it is the first sugarbeet furovirus isolate which infects *Nicotiana* species, *N. benthamiana* and *N. clevelandii.* Clones representing all four RNAs of BNYVV have been labeled with nonradioactive probes and used in Northern analysis of the isolates addressed in this study (data not shown). RNA 1, 2, and 3 of BNYVV are specific for the BNYVV isolates tested. The RNA 4 probe of BNYVV reacted with the RNA 3 of BSBMV-1 only (not with that of BSBMV-2) and with the RNA 3 of NE 10. This is the first definable difference seen between BSBMV-1 and -2, aside from a slight difference in host range.

Table 1	Approximate	Sizes of	RNAs from	Several Furoviruses	of Sugar	Beet
RNA component	BNYVV-CA	BNYVV-ID	BSBMV-1	BSBMV-2	NE 10	NE 1644
RNA-1	6.7[a]	6.7	6.5	6.7	6.7	6.7
RNA-2	4.7	4.7	4.5	4.7	4.7	4.7
RNA-3	1.8	1.8	1.3	1.4	1.8	1.8
RNA-4	nd[b]	n d	n d	n d	0.9	n d

[a] kilobases   [b] nd=not detected

Table 2. Nucleotide and amino acid similarities between BSBMV-2 & BNYVV		
RNA 1 (3'-1,800 nt)	RNA 2 (3'-1,000 nt)	RNA 3 (1,000 nt)
81% nt (RNA-1; BNYVV)	61% nt (RNA-2; BNYVV)	55% nt (RNA-5; BNYVV-Japan)
91% aa*	61, 76% aa	48% aa

* values represent amino acid similarities from individual open reading frames
nt = nucleotide   aa = amino acid

BSBMV-2 has been cloned, the RNAs 1, 2, and 3 have been partially sequenced, and their sequences have been compared. The 3'-1,000 bases of BSBMV-2 RNA 2 has a 81% similarity to the BNYVV RNA 1 (Table 2). This area represents a portion of the replicase gene. Although 81% is a relatively high similarity, it is still considered to be distinct with regard to individual viruses. The 3'-1,000 bases of RNA 2 shows a 60% sequence similarity with that of BNYVV. This area corresponds to the proposed cell-to-cell movement protein region of BNYVV. Interestingly, the BSBMV RNA 3 aligned with the RNA 5 of the Japanese isolate of BNYVV (Kiguchi et al., 1995), with a 55% nucleic acid similarity, and a 60.3% amino acid similarity.

Discussion

The molecular analyses of the BNYVV and BSBMV isolates in this study indicate, as the serological and host range analyses did, that the BNYVV isolates represent a homogeneous group, and that the BSBMV isolates represent a heterogeneous group. However, there are several interrelationships between and among these two groups. The molecular analyses confirm the serological analyses which indicate that the BSBMV-related isolates are distinct from BNYVV isolates in the U.S. The RNA analysis of the BNYVV isolates revealed only RNA 1, 2, and 3 (Fig. 2). This is not surprising, as according to the literature, RNA 4 can be in such a low concentration as to be lost by repeated mechanical transfers. Only one BNYVV isolate showed variation in the number and size of the RNAs and that was BNYVV-GH, an isolate which has been maintained for over 10 years by mechanical inoculation in the greenhouse. All furoviruses studied which infect sugarbeet in the U.S. belong to two groups , either a homogeneous BNYVV group or an heterogeneous group all of which are serologically identical to BSBMV. All have at least three RNAs, are polyadenylated, and are transmitted by *P. betae*.

References

1. Dolja, V. V., Karasev, A. V., and Koonin, E. V. 1994. Molecular biology and evolution of closteroviruses: Sophisticated build-up of large RNA genomes. Annu. Rev. Phytopathol. 32:261-285.
2. Kiguchi, T., Saito, M., and Tamada, T. 1996. Nucleotide sequence analysis of RNA-5 of five isolates of beet necrotic yellow vein virus and the identity of a deletion mutant. J. Gen Virol. 77:575-580.
3. Lecoq, H., and Pitrat, M. 1985. Specificity of the helper component-mediated aphid transmission of three potyviruses infecting muskmelon. Phytopathology 75:890-893.
4. McMaster, G. K., and Carmichael, G. C. 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels using glyoxyl and acridine orange. Proc. Natl. Acad. Sci. U.S.A. 74:4835-4838.
5. Wisler, G. C., Liu, H.-Y., Duffus, and J. E. Duffus. 1994. Beet necrotic yellow vein virus and its relationship to eight sugar beet furo-like viruses from the United States. Plant Disease 78:995-1001.

STUDIES ON POTATO MOP-TOP VIRUS REPLICATION

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Summary

Nucleotide sequence analysis of potato mop-top virus (PMTV) RNA 2 showed that it contains four open reading frames that encode three proteins with predicted molecular masses of 51 kDa, 13 kDa and 21 kDa respectively, and a cysteine-rich protein (8K) of predicted mass 8 kDa. This paper describes work to investigate the occurrence of putative subgenomic RNA for these proteins in infected plants and protoplasts. Also, *in vitro* translation studies were done to establish whether a protein can be translated from a plasmid containing the nucleotide sequence encoding the 8K protein.

Introduction

Potato mop-top virus (PMTV) has rigid rod-shaped particles and a plus-strand tripartite RNA genome of 6.5 kb (RNA 1), 3.0 kb (RNA 2), 2.5 kb (RNA 3) (Scott *et al.*, 1994). It is transmitted by the plasmodiophorid fungus *Spongospora subterranea* (Arif *et al.*, 1995). The complete nucleotide sequences of RNA 2 and RNA 3 of PMTV isolate T have been obtained. Sequence analysis indicated that RNA 2 encodes proteins of 51 kDa, 13 kDa, 21 kDa, and 8 kDa (Scott *et al*,1994). The sequence of RNA 3 contains an ORF for the coat protein (20 kDa) terminated by an amber codon, which can be suppressed to produce a read-through protein of 67 kDa (Kashiwazaki *et al.*, 1995). The 51 kDa, 13 kDa and 21 kDa proteins have amino acid sequence similarities to the triple gene block proteins (TGB) of other viruses, especially with those of beet soil borne virus (BSBV) RNA 3 (Koenig *et al.*, 1996). However, sequence analysis of RNA 2 shows that PMTV contains an additional gene to BSBV RNA 3 that encodes an 8 kDa cysteine-rich protein. Therefore, PMTV RNA 2 resembles the 3' portion of beet necrotic yellow vein virus (BNYVV) RNA 2 in which the ORFs encoding the TGB proteins are followed by an ORF for a 14 kDa cysteine-rich protein (Bouzoubaa. *et al.*,1986). The cysteine rich protein of BNYVV is found in the soluble (cytoplasmic) subcellular fraction of infected tissue and is expressed from a subgenomic RNA (Richards and Tamada, 1992). In this paper we investigate whether it is possible to identify subgenomic RNAs for the internal ORFs by Northern blot hybridization analysis of total RNA from infected plants and protoplasts. Also, the putative 8 kDa protein was cloned and expressed *in vitro* using the rabbit reticulocyte lysate system.

Materials and Methods

Virus isolates:
We used the Scottish isolates PMTV-T and PMTV-S (Arif *et al.*,1995) and a Danish isolate (PMTV-D) supplied by Dr S. L. Nielsen (Lyngby, Denmark).

58

Northern blot experiments:

PMTV particles were purified according to the method of Torrance *et al.* (1993) with the modification that 8 % polyethlene glycol (PEG) containing 0.1 M NaCl was added after chloroform extraction to precipitate the virus particles and then virus particles were further purified with high speed centrifugation as described in the orginal method.

Nicotiana tabacum "Xanthi nc" protoplasts were isolated and inoculated with purified virus particles using the poly-L-ornithine method (Kubo *et al.*, 1975b; Barker and Harrison, 1977a). RNAs were extracted from protoplasts and whole plants according to the methods of Robinson (1982) and Verwoerd *et al.* (1989), respectively. Synthesis of viral RNA was analysed by Northern blot hybridisation using ^{32}P-labelled cDNA probes, essentially as described by Sambrook *et al.* (1989).

Cloning and in vitro *translation of 8 K products:*

Polymerase chain reaction (PCR) was used to amplify the nucleotide sequence of RNA 2 ORF4. First, cDNA from viral templates was synthesized using "superscript reverse transcriptase" (Gibco, BRL) according to the manufacturer's instructions. PCR amplification was done in a reaction volume of 50μl using an upstream primer which had the nucleotide sequence of AGTAAGCAAGTACGCCCTGT, homologous to PMTV RNA 2 (nucleotides:2419-2438) and a downstream primer with the nucleotide sequence of CCGAATTCCTGTAAGCACTAACAC, complementary to PMTV RNA 2 (nucleotides:2678-2702). The reaction conditions were as follows:

94 °C for 1 min, then 25 cycles of 94 °C for 30 sec, 50 °C for 30 sec, 72°C for 1 min and a final 72 °C for 5 min. The PCR products were cloned into PT7Blue® (Novagen, USA).

The 8K protein products were translated *in vitro* using TNT® Coupled Reticulocyte Lysate system (Promega, USA). A circular plasmid carrying the fragment encoding the 8K protein (1μg) was used in a reaction volume of 25 μl containing 12.5 μl TNT® lysate, 1 μl TNT® reaction buffer, 0.5 μl TNT® RNA polymerase, 0.5 μl amino acid mixture minus methionine (1 mM) 2 μl ^{35}S-methionine (1000 Ci/mmol), 2μl RNasin® (40 U/μl) and incubated at 30 °C for 1-1.5 h. Translation products were analysed on 16% SDS-PAGE and visualised by autoradiography.

Results

In Northern blot hybridization experiments, a cDNA probe containing 1000 bp colinear with the 3'terminus of RNA 2 hybridised with bands of the expected size for genomic RNA 2 and three smaller bands (0.96 kb, 0.7 kb, 0.4 kb) equivalent to the size expected for subgenomic species (Fig. 1). However, on more detailed analysis with other cDNA probes specific for each ORF the same band patterns were obtained with each probe. This result indicates that the smaller bands obtained in Northern blots of protoplast and plant samples were viral-specific RNA bands but they were possibly associated with ribosomal RNAs.

The 8 K domains of PMTV-D and PMTV-S isolates were cloned and then were translated *in vitro* using the rabbit reticulocyte lysate system. It was possible to translate PMTV-S and PMTV-D isolates using both TNT® and conventional standard transcription/translation methods.
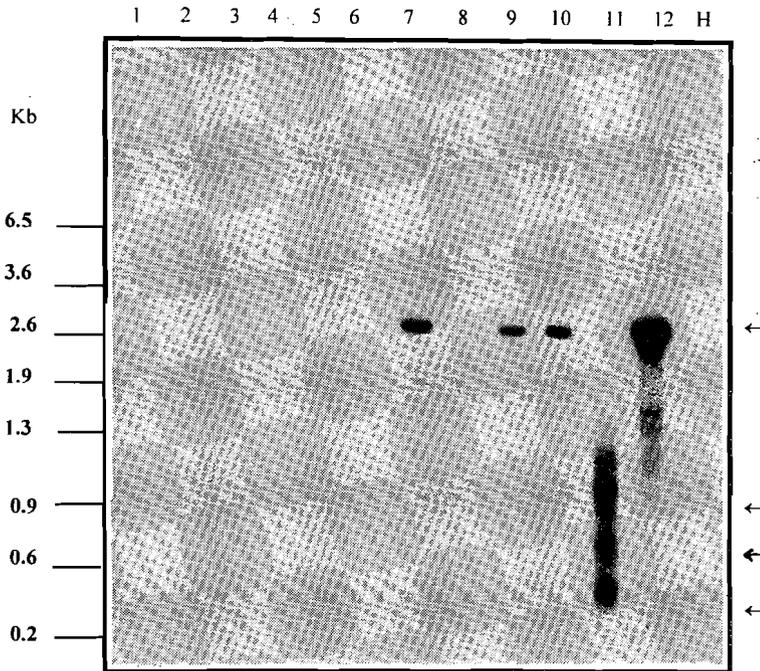
Fig. 1. Northern blot hybridisation of total RNA extracts from PMTV infected *N. benthamiana* plants. RNA was extracted 1-12 days after inoculation. Hybridisation was done using a cDNA probe complementary to the 3'terminal portion of RNA 2. Arrows indicate the sizes of genomic RNA 2 (3.0 kb) and the smaller bands (0.9 kb, 0.7 kb and 0.4 kb). Lanes 1-12 correspond to the samples taken between 1-12 days after inoculation. H: RNA from healthy plant.

Discussion

Many positive-strand RNA viruses produce subgenomic mRNAs to express their internal genes because usually only 5' proximal ORFs are translated from the genomic RNA (Miller *et al.,1985*). When full-length genomic RNA 2 of PMTV isolate T was translated *in vitro* in a rabbit reticulocyte lysate system, the 51 kDa protein was obtained as well as a protein of size expected for the 21 kDa, but it migrated close to other proteins endogenous to the system and may be an artefact. No evidence was obtained to indicate the expression of other proteins (B. Reavy, unpublished data). However, in our experiments we were unable to find evidence for subgenomic RNA for expression of the internal ORFs on RNA 2. The Northern hybridisation results suggest that the viral RNAs that were associated with polyribosomes and subgenomic RNA could not be distinguished. Similar associated ribosomal RNAs were obtained in studies on tobacco mosaic virus (Palukaitis *et al.*, 1983) and potato leafroll virus (Tacke *et al.*, 1990). Further work is being done to extract dsRNAs to investigate the occurrence of replicative forms (RF). We have shown that the 8 kDa protein can be translated *in vitro* and further work is being done to obtain evidence of production of the protein in infected plants.

References

Arif, M. Torrance, L. and Reavy, B. (1995). Acquisition and transmission of potato mop-top furovirus by a culture of *Spongospora subterranea* f.sp *subterranea* derived from a single cystosorus. Ann. Appl. Biol.126;493-503

Barker, H. and Harrison, B.D. (1977a). Infection of tobacco mesophyll protoplasts with rasberry ringspot virus alone and together with tobacco rattle virus. J.Gen Virol, 35:125-133

Bouzoubaa, S., Ziegler, V., Beck, D., Guilley, H., Richards, K. and Jonard, G. (1986). Nucleotide sequence of beet necrotic yellow vein virus RNA2. J.Gen.Virol. 67: 1689-1700

Kashiwazaki, S., Scott, KP., Reavy, B. and Harrison, B.D. (1995). Sequence analysis and gene content of potato mop-top virus RNA 3:Further evidence of heterogenity in the genome organisation of furoviruses. Virology, 206:701-706

Koenig, R., Beier, C., Commandeur,U., Luth, U., Kaufmann, A. and Luddecke,P. (1996) Beet soil-borne virus RNA 3: A further example of the heterogenity of the gene content of furovirus genomes and of triple gene block carrying RNAs. Virology, 216:202-207

Kubo, S., Harrison, B.D. and Barker, H. (1975b). Tobacco rattle virus in tobacco mesophyll protoplasts:infection and virus multiplication. J. Gen.Virol,27,293-304

Miller,W.A., Dreher, T.,and Hall,T. (1985). Synthesis of brome mosaic virus subgenomic RNA *in vitro* by internal initiation on (-) sense genomic RNA Nature, 313:68-70

Palukaitis, P., Garcia-Arenal, F., Sulzinski, M.A. and Zaitlin, M.(1983). Replication of tobacco mosaic virus.VII. Further characterisation of single and double-stranded virus related RNAs from TMV-infected plants. Virology, 131:533-545

Richards, K. E. and Tamada, T.(1992). Mapping functions on the multipartite genome of beet necrotic yellow vein virus. Annu.,Rev.Phytopathol, 30:291-313

Robinson, D. J. (1982). Apparent lack of nucleotide sequence homology between the satallite and genome RNA species of tomato black ring virus. J.Gen.Virol., 58:453-456

Sambrook, J., Fritsch, E. F.and Maniatis, T.(1989). Molecular cloning. A laboratory Manual, 2nd edn.Cold Spring Harbor Laboratory, New York

Scott, K. P., Kashiwazaki, S, Reavy, B. and Harrison, B. D.(1994). The nucleotide sequence of potato mop-top virus RNA 2:A novel type of genome organisation for a furovirus. J.Gen.Virol., 75:3561-3568

Tacke, E., Prufer., D., Salamini, F and Rohde, W.(1990). Characterisation of a potato leafroll luteovirus subgenomic RNA:differential expression by internal translation initiation and UAG supression. J.,Gen.,Virol., 71:2265-2272

Torrance, L., Cowan, G. and Pereira, L. G.(1993). Monoclonal antibodies specific for potato mop-top virus and some properties of the coat protein. Ann.Appl.. Biol, 122:311-322

Verwoerd, T. C., Dekker, B. M .M. and Hoekema, A.(1989). A small scale procedure for the rapid isolation of plant RNAs. Nucleic Acid Research 17, 2362

EVIDENCE THAT READTHROUGH OF THE POTATO MOP-TOP VIRUS COAT PROTEIN GENE OCCURS IN PLANTS AND THAT THE READTHROUGH DOMAIN IS PRESENT AT ONE EXTREMITY OF SOME PARTICLES.

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Summary

Potato mop-top furovirus (PMTV) RNA3 contains an open reading frame for the 20 K coat protein that terminates in an amber stop codon allowing translational readthrough to produce a larger readthrough protein of 67K. The nucleotide sequence encoding a 21 K C-terminal fragment of the potato mop-top virus (PMTV) readthrough domain was cloned and expressed in *Escherichia coli* fused to glutathione *S*-transferase, and the fusion protein was used to produce an antiserum. The antiserum was used in ELISA and immunogold labelling experiments to show that the readthrough protein was located near one extremity of some virus particles.

Introduction

Potato-mop top furovirus (PMTV) is transmitted by the soil-borne powdery scab fungus *Spongospora subterranea* (Arif *et al.*, 1995) and PMTV particles contain three species of single-stranded RNA (Scott et al., 1994). Nucleotide sequence analysis of the RNA 3 of PMTV isolate T shows that it contains an open reading frame encoding the coat protein (20 K) terminating in an amber stop codon followed in-phase by a second open reading frame encoding a readthrough protein of 47 K (Kashiwazaki *et al.*, 1995). This paper reports the production of a readthrough specific antiserum and detection of the readthrough domain in virus particles.

Materials and Methods

Cloning, expression and purification of readthrough domain:
A 575 bp *Bam*HI and *Eco*RI digested fragment from an RNA 3 clone (pPMTV-21) of PMTV-T (Kashiwazaki *et al.*, 1995) was ligated into the expression vector pGEX3X (Pharmacia). The resulting plasmid, pGEXRT, was used to transform *E. coli* strain TG1 cells to express a glutathione *S*-transferase protein C terminally fused to readthrough (GST-RT). An anti-GST-RT serum was prepared (code name anti-RT) which had a GST-RT specific titre of 1/2000 in plate trapped antigen ELISA tests, and partially purified immunoglobulin (Ig) fraction was obtained by ammonium sulphate precipitation.
ELISA:
An antibody trapped antigen (ATA) ELISA method (Torrance, 1992) was used, where virus was trapped from sap extracts by either the anti-RT serum or a polyclonal serum (anti-CP) specific for the PMTV coat protein (PMT-81; Torrance et al., 1993), followed by the coat protein specific monoclonal antibodies (MAbs) SCR68 and

SCR69 (Torrance *et al.*, 1993) as detecting antibodies. Briefly, microtitre plates were incubated with coating Ig preparations, then either PMTV- infected or virus-free *Nicotiana benthamiana* sap added (extracted 1g / 9 ml PBS containing 0.05 % Tween 20 and 0.1 % non-fat dried milk powder) and incubated overnight at 4 ^{0}C. Detecting antibodies were then added for 2 h at 37 ^{0}C followed by a species specific alkaline phosphatase conjugate (Sigma) diluted 1/1000 for 2 h at 37 ^{0}C. The substrate p-nitrophenyl phosphate (Sigma) was added and incubated at room temperature before recording absorbance values at 405 nm. PMTV isolates used were PMTV-T (Torrance et al., 1993); S (Arif et al., 1995) and M-18 (Mayo et al., 1996).

Immunosorbent electron microscopy and immunogold labelling: The methods described by Roberts (1986) and Periera et al. (1994) were used. The average number of particles per standard aperture at 10K magnification were recorded to compare numbers of particles trapped by the different antisera. Anti-RT Ig preparation (0.5 mg) was dialyzed against 2 mM borax buffer, pH 9.0 then conjugated to colloidal gold (Aurobeads G10 RPN476, Amersham) following the manufacturer's instructions.

Results and Discussion

A 551 bp C-terminal portion of the readthrough protein was cloned and the GST-RT fusion protein was expressed in *E.coli* strain TG1, the complete fusion protein was used as immunogen. Immunoblot results using the anti-RT serum showed that the antiserum contained antibodies specific for the GST-RT fusion protein (data not shown).

The anti-RT antiserum was used in ELISA to try to detect an association of readthrough protein with virus particles. Anti-RT or anti -CP Ig preparations were used to trap virus particles and MAbs SCR 68 and SCR 69 were used as detecting antibodies. Tests (Table 1) showed that the anti-RT serum trapped virus particles from infected sap (as judged by reactions with anti-CP MAbs), although the absorbance values obtained using anti-RT for virus capture were less than those obtained with anti-CP and unequivocal results were obtained only after prolonged substrate incubation.

The anti-RT serum trapped PMTV-S particles in ISEM experiments, but far fewer than the anti-CP serum, for example, the average number of particles trapped with the anti-RT or anti-CP sera were 37 and 323 respectively (a mean of 5 virus particles were counted on control grids coated with a potato virus Y antiserum).

Immunogold electron microscopy experiments were done to try to determine the location of the readthrough protein in the virus particles. In five experiments only 18 virus particles were found to be associated with gold, 15 particles were labelled at one extremity (Fig. 1) and three were labelled elsewhere along the particle. Of these three, only one particle of gold was observed per virus particle. There were virtually no gold particles present elsewhere on the grids.

Taken together, the ELISA, immunoblotting and electron microscopy data suggest that only a small proportion of particles contain full-length readthrough protein, and it is located at one extremity. Many fewer particles were labelled with gold in our experiments compared with the 4 - 20% labelled in experiments with BNYVV (Haeberle et al., 1994). Full details of our work will be published in Cowan et al., 1997.

Table 1. Absorbance values (A_{405nm}) obtained in ELISA to detect association of PMTV readthrough domain with virus particles

PMTV isolate*	Capture antibody+	Detecting monoclonal antibody	
		SCR 68	SCR 69
M18	RT	0.17 (2.59)**	0.11 (2.54)
	CP	1.43 (2.46)	0.63 (2.59)
S	RT	0.12 (2.05)	0.10 (1.97)
	CP	0.92 (2.28)	0.46 (2.38)
T	RT	0.09 (0.84)	0.08 (0.59)
	CP	0.63 (2.28)	0.25 (2.36)
Control sap	RT	0.09 (0.23)	0.08 (0.33)
	CP	0.09 (0.23)	0.07 (0.34)

* PMTV-infected (or control non-infected) sap extracts were used at 1/10 dilution.
+ RT = anti-readthrough serum; CP = anti-PMTV coat protein serum.
** A_{405nm} recorded after 1 h, and 16 h in parenthesis.

Fig. 1 Electron micrographs of PMTV particles labelled with anti-readthrough protein-immunogold conjugate. Bar represents 100 nm.

64

References

Arif, M., Torrance, L., and Reavy, B. (1995). Acquisition and transmission of potato mop-top furovirus by a culture of *Spongospora subterranea* f.sp. *subterranea* derived from a single cystosorus. *Annals of Applied Biology* **126**:493-503.

Cowan, G. H., Torrance, L. and Reavy, B. (1997). Detection of potato mop-top virus capsid readthrough protein in virus particles. Manuscript in preparation.

Haeberle, A., Stussi-garaud, C., Schmitt, C., Garaud, J-C., Richards, K. E., Guilley, H. and Jonard, G. (1994). Detection by immunogold labelling of P75 readthrough protein near an extremity of beet necrotic yellow vein virus particles. *Archives of Virology* **134**:195-203.

Kashiwazaki, S., Scott, K. P., Reavy, B., and Harrison, B.D. (1995). Sequence analysis and gene content of potato mop-top virus RNA 3: Further evidence of heterogeneity in the genome organization of furoviruses. *Virology* **206**:701-706.

Mayo, M.A., Torrance, L., Cowan, G., Jolly, C. A., Macintosh, S., Orrega, R., Barrera, C and Salazar, L F. (1996). Conservation of coat protein sequence among isolates of potato mop-top virus from Scotland and Peru. Archives of Virology **141**:1115 - 1121.

Pereira, L. G., Torrance, L., Roberts, I. M., and Harrison, B. D. (1994). Antigenic structure of the coat protein of potato mop-top furovirus. *Virology* **203**:277-285.

Roberts, I. M. (1986). Immunoelectron microscopy of extracts of virus-infected plants. In *Electron microscopy of proteins: 5. Viral Structure*. (J R Harris and R W Horne, Eds), pp 293-357. Academic Press, NY.

Scott, K. P., Kashiwazaki, S., Reavy, B., and Harrison, B. D. (1994). The nucleotide sequence of potato mop-top virus RNA 2: a novel type of genome organization for a furovirus. *Journal of General Virology* **75**:3561-3568.

Torrance, L. (1992). Serological methods to detect plant viruses: production and use of monoclonal antibodies. In *Techniques for the Rapid Detection of Plant Pathogen*. (J. M. Duncan and L Torrance, Eds.), pp 7-33. Blackwell Scientific Publications Ltd, Oxford.

Torrance, L., Cowan, G. H., and Pereira, L.G. (1993). Monoclonal antibodies specific for potato mop-top virus, and some properties of the coat protein. *Ann. appl. Biol.* **122**:311-322.

NUCLEOTIDE SEQUENCES OF BEET SOIL-BORNE VIRUS RNAs 2 AND 3

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Summary

Beet soil-borne virus (BSBV) has never been obtained in a highly purified form and previous attempts to obtain virus-specific cDNAs from partially purified virus preparations had failed. Short cDNA stretches were obtained, however, when denatured preparations of dsRNA were used as a template. RT-PCR techniques done with combinations of random and specific primers have enabled us now to amplify known and unknown parts of BSBV RNAs 2 and 3 from random primed cDNAs which were obtained using plus or minus strand RNAs from immunocaptured virus particles or denatured preparations of dsRNA, respectively, as templates. In its genetic organization BSBV RNA 3 resembles potato mop top (PMTV) RNA 2 and vice versa. There are, however, also striking differences between the RNAs of these two viruses. BSBV RNA 3 contains a triple gene block but in contrast to PMTV RNA 2 it lacks the coding region for a cysteine-rich protein. BSBV RNA 2 encodes the viral coat protein and a coat protein readthrough protein, but the readthrough portion of the latter is almost twice as big as that encoded on PMTV RNA 3. The 3'-ends of BSBV RNA 2 and 3 have the potential to fold into tRNA-like structures, in contrast to that of PMTV RNA 3. The deduced amino acid sequences for the proteins encoded on BSBV RNAs 2 and 3 show the highest amount of sequence identities with the corresponding proteins of PMTV, although the two viruses are unrelated serologically.

Introduction

Beet soil-borne furovirus (BSBV) is widely spread in sugarbeet growing areas (e.g. Ivanovic *et al.*, 1983; Henry *et al.*, 1986; Verhoyen *et al.*, 1987; Lesemann *et al.*, 1989; Lindsten, 1989). It has several properties in common with beet necrotic yellow vein virus (BNYVV), the causal agent of sugarbeet rizomania. Both viruses have tubular particles of several modal lengths, they are transmitted by *Polymyxa betae* and they have similar host ranges (Henry *et al.*, 1986). However, no relationships have been detected between the two viruses in serological and in cross hybridization tests and they differ in the number and size of their RNAs (Ivanovic *et al.*, 1983; Lesemann *et al.*, 1989; Hutchinson *et al.*, 1992; Kaufmann *et al.*, 1992). Also, the RNAs of BNYVV are polyadenylated, whereas those of BSBV and of other definitive furoviruses have non-polyadenylated RNAs (Kaufmann *et al.*, 1992). BSBV has never been obtained in a highly purified form. Studies with dsRNAs from infected plants suggest that its genome consists of three RNA species with sizes ranging in different strains between 6.1-6.4 kb, 3.0-3.6 kb and 2.6-3.3 kb, respectively (Hutchinson *et al.*, 1992; Kaufmann *et al.*, 1992). Our previous attempts to obtain cDNA clones for the Ahlum isolate of BSBV by using RNA from partially purified virus preparations as a template had failed; we had been able, however, to clone a number of short cDNA stretches (average size between 200 and 300 nucleotides) when denatured preparations of dsRNA isolated from infected *Chenopodium quinoa* leaves were used as a template (Kaufmann *et al.*, 1992). These clones have served us as starting material for determining the complete nucleotide sequences of BSBV RNAs 2 and 3 from cDNAs obtained from immunocaptured virus

particles and denatured preparations of dsRNA using RT-PCR techniques with random and specific primers for amplifying unknown parts of the sequence (Koenig et al., 1996; Koenig et al., 1997).

Materials and Methods

The Ahlum isolate of BSBV was originally obtained from a sugarbeet field near Braunschweig (Germany) and was propagated on leaves of C. quinoa where it causes local lesions only. IC-RT-PCR, the amplification of unknown parts of the nucleotide sequences and the sequencing techniques have been described in detail by Koenig et al. (1996 and 1997).

Results and Discussion

RNA 2 of the Ahlum isolate of BSBV consists of 3454 nucleotides and RNA 3 of 3006 nucleotides. In its genetic organization BSBV RNA 3 resembles potato mop top (PMTV) RNA 2 and vice versa. There are, however, also striking differences between the RNAs of these two viruses. BSBV RNA 3 (Fig. 1) is unique among the plant virus RNAs studied so far in containing apparently only the coding sequences of a triple gene block (TGB). In contrast, the TGB-carrying RNAs of PMTV (Scott et al., 1994) and peanut clump furovirus (PCV) (Herzog et al., 1994) contain, in addition, the coding regions for a cysteine-rich protein or for the coat protein and an additional protein with unknown function, respectively. BSBV thus represents another example for the heterogeneity of the genome organization of furoviruses. Additonal genes are also found on the TGB-containing RNAs of other viruses which are only possible furoviruses (BNYVV) or do not belong to that genus at all (hordeiviruses, potexviruses, carlaviruses) (Fig. 1).

BSBV RNA 2 resembles PMTV RNA 3 (Kashiwazaki et al., 1995) in containing only the coding regions for the coat protein and a coat protein readthrough protein whereas other furoviruses carry additional genes on their coat protein gene-containing RNAs (Fig. 2). There are, however, also considerable differences between RNA 2 of BSBV and RNA 3 of PMTV. BSBV RNA 2 is more than 1000 nucleotides longer than PMTV RNA 3. The calculated molecular weight of the readthrough portion of the BSBV readthrough protein is 85 kD, whereas that of the PMTV readthrough protein is only 47 kD. Also, the 3'-end of BSBV RNA 2, like those of BSBV RNA 3 and other definitive furoviruses, but unlike that of PMTV RNA 3, have the potential to fold into a tRNA-like structure (Fig. 2).

Analyses of the deduced amino acid sequences for the putative proteins encoded on BSBV RNA 2 (coat protein and coat protein readthrough protein) and BSBV RNA 3 (TGB proteins) also revealed that BSBV and PMTV are more closely related to each other than to any other furoviruses. In view of the sequence similarities in their coat proteins, it is surprising that the two viruses are unrelated serologically. This may be due to the fact that BSBV coat protein lacks in its N-terminal part 10 amino acids which in PMTV coat protein form the immunodominant epitope (Pereira et al., 1994). - The 3'-termini of BSBV RNA 2 and 3 also show a high level of nucleotide sequence identity with that of PMTV RNA 2, but not with that of PMTV RNA 3 which possibly has a defective 3'-end (Kashiwazaki et al., 1995).

Conclusions concerning the second closest relationships of BSBV depend on the part of the genome which is considered. With respect to amino acid sequence identities in the TGB-encoded proteins, BSBV has its second closest relations to PCV. With respect to similarities in the nucleotide sequences of the 3'-UTRs of its RNAs and the amino acid sequences of its coat protein and coat protein readthrough proteins, BSBV shows its second closest relationships to soil-borne wheat mosaic virus (SBWMV) (Shirako and Wilson, 1993).
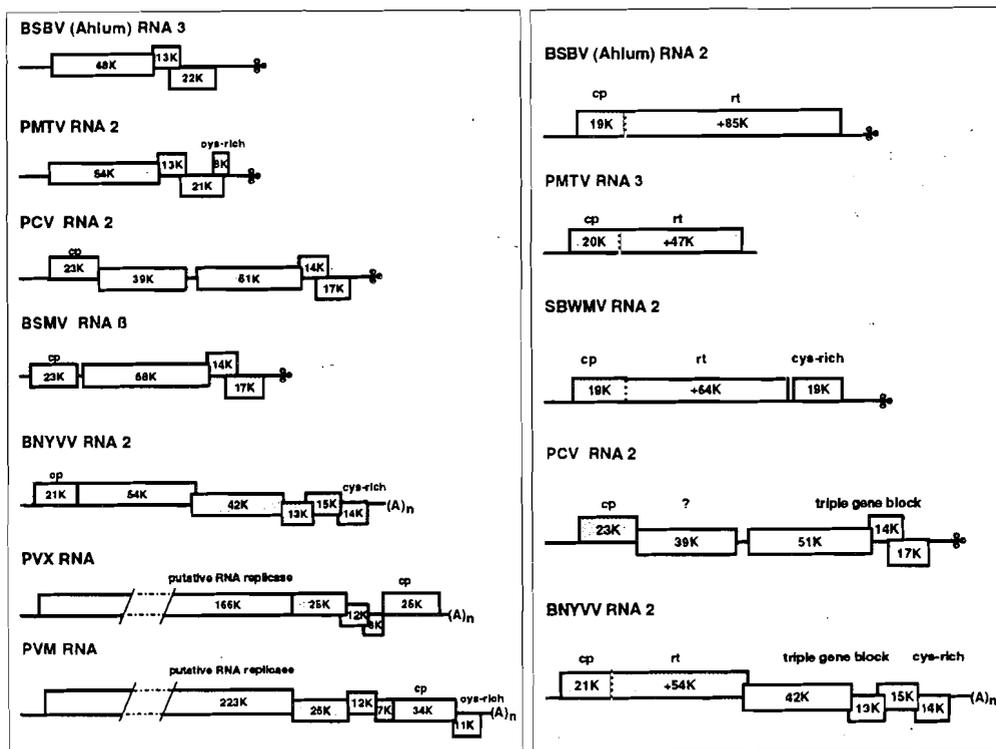
Fig. 1 (left). Gene content of BSBV RNA 3 and other triple gene block-containing RNAs. Triple gene blocks are shaded. ⚑ indicates the potential of the 3'-end of an RNA to fold into a tRNA-like structure (modified from Koenig et al., 1996).

Fig. 2 (right). Gene content of BSBV RNA 2 and of the coat protein gene-containing RNAs of other furoviruses. The coding regions for coat proteins and coat protein readthrough proteins are shaded. ⚑ indicates the potential of the 3'-end of an RNA to fold into a tRNA-like structure (modified from Koenig et al., 1997).

Detailed accounts of our studies on BSBV RNA 3 (Koenig et al., 1996) and RNA 3 (Koenig et al., 1997) are given elsewhere.

ACKNOWLEGDEMENTS

REFERENCES

BOUZOUBAA, S., QUILLET, L., GUILLEY, H., JONARD, G. AND RICHARDS, K. (1987). Nucleotide sequence of beet necrotic yellow vein virus RNA-1. *J. Gen. Virol.* **68**, 615-626.

GOELET, P., LOMONOSSOFF, G.P., BUTLER, P.J.G., AKAM, M.E., GAIT, M.J. AND KARN, J. (1982). Nucleotide sequence of tobacco mosaic virus RNA. *Proc. Natl. Acad. Sci. USA* **79**, 5818-5822.

GUSTAFSON, G. and ARMOUR, S.L. (1986). The complete nucleotide sequence of RNA b from the type strain of barley stripe mosaic virus. *Nucl Acids Res.* **14**, 3895-3909.

HENRY, C.M., JONES, R.A.C. and COUTTS, R.H.A. (1986). Occurence of a soil-borne virus of sugar beet in England. *Plant Pathol* **35**, 585-591.

HERZOG, E., GUILLEY, H., MANOHAR, S.K., DOLLET, M., RICHARDS, K., FRITSCH, C. and JONARD, G. (1994). Complete nucleotide sequence of peanut clump virus RNA 1 and relationships with other fungus-transmitted rod-shaped viruses. *J. Gen. Virol.* **75**, 3147-3155.

HUTCHINSON, P.J., HENRY, C.M. and COUTTS, R.H.A. (1992). A comparison, using dsRNA analysis, between beet soil-borne virus and some other tubular viruses isolated from sugar beet. *J. Gen. Virol.* **73**, 1317-1320.

IVANOVIC, M., MACFARLANE, I. and WOOD, R.D. (1983). Viruses of sugarbeet associated with *Polymyxa betae. Rothamsted Exp. Station* for 1982, 189-190.

KASHIWAZAKI, S., SCOTT, K.P., REAVY, B. and HARRISON, B.D. (1995). Sequence analysis and gene content of potato mop top virus RNA 3: further evidence of heterogeneity in the genome organization of furoviruses. *Virology* **206**, 701-706.

KAUFMANN, A., LI, Y., KOENIG, R., BREYEL, E., MAISS, E., LÜDDECKE, P. and COMMANDEUR, U. (1992). Single- and double-stranded RNAs associated with an isolate of beet soil-borne virus. *Intervirology* **33**, 97-102.

KOENIG, R., BEIER, C., COMMANDEUR, U., LÜTH, U., KAUFMANN, A. and LÜDDECKE, P. (1996). Beet soil-borne virus RNA 3 - a further example for the heterogeneity of the gene content of furovirus genomes and of triple gene block-carrying RNAs. *Virology* **216**, 202-207.

KOENIG, R., COMMANDEUR, U., LOSS, S., BEIER, C., KAUFMANN, A. and LESEMANN, D.-E. (1997). Beet soil-borne virus RNA 2: similarities and dissimilarities to the coat protein gene-carrying RNAs of other furoviruses. Manuscript submitted.

LESEMANN, D.-E., KOENIG, R., LINDSTEN, K. and HENRY, C.M. (1989). Serotypes of beet soil-borne furovirus from FRG and Sweden. *Bull OEPP/EPPO Bull* **19**, 539-540.

LINDSTEN, K. (1989). Investigations concerning soil-borne viruses in sugarbeet in Sweden. *Bull OEPP/EPPO Bull* **19**, 531-537.

PEREIRA, L.G., TORRANCE, L., ROBERTS, I.M. and HARRISON, B.D. (1994). Antigenic structure of the coat protein of potato mop-top virus. *Virology* **203**, 277-285.

SCOTT, K.P., KASHIWAZAKI, S., REAVY, B. and HARRISON, B.D. (1994). The nucleotide sequence of potato mop top virus RNA2: a novel type of genome organization of a furovirus. *J. Gen. Virol.* **75**, 3561-3568.

SHIRAKO, Y. and WILSON, T.M.A. (1993). Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. *Virology* **195**, 16-32.

VERHOYEN, M., VAN DEN BOSSCHE, M. and VAN STEYVOORT, L. (1987). Identification de nouveaux virus de la betterave en Belgique. *Rev. Agric.* **40**, 1463-1468.

THE GENOME ORGANIZATION OF BROAD BEAN NECROSIS VIRUS (BBNV) AND HETEROGENEITY OF THE GENUS *FUROVIRUS*

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Summary

The complete nucleotide sequence of broad bean necrosis virus, was determined from the cDNAs of BBNV-O genomic RNAs [RNA1 (6.0Kb), RNA2 (2.8Kb) and RNA3 (2.4Kb)]. The computer analysis indicates that methyltransferase, helicase and the RNA-dependent RNA polymerase (RdRp) motifs are present in RNA1. The viral capsid protein (CP) cistron is located on the 5' terminus of RNA2 and the *M*r of CP (19.4Kd) is close to that of PAGE analysis. An ochre codon (UAA) of the CP cistron is suggested to be partially suppressed to produce a large readthrough protein. RNA3 reveals to possess a typical motifs of triple-gene-block proteins, also reported in several other plant viruses. The genome organization and phylogenetic analysis using RdRp and CP amino acid sequences of furoviruses suggests that BBNV is closely related to potato mop-top virus but relatively remote from other furoviruses. The data also suggests that the genus Furovirus could be separated into several group (or genera): the genus *Furovirus*, PMTV group including BBNV, beet necrotic yellow vein virus group and peanut clump virus group.

Introduction

Furoviruses are a defined taxonomic group of fungal-transmitted, rod-shaped, single-stranded RNA viruses (Brunt *et al.*, 1986 & 1989; Fauquent *et al.*, 1988). Soil-borne wheat mosaic virus (SBWMV) is the type species of this genus (Brunt, 1991). Broad bean necrosis virus (BBNV) was first identified and described in Japan in 1952 (Fukano and Yokoyama, 1952) and BBNV had been considered to be a tentative member of the genus *Furovirus* (Fujikawa 1955 & 1957; Inoue *et al.*, 1967 & 1968).

Based on the results of recent studies and the results presented here, the genus *Furovirus* is thought to consist of more than one single group. The genome organization of BNYVV is quite different from that of SBWMV in presence of triple-gene-block which encodes proteins with roles in cell-to-cell movement of the virus, it was also found in PCV and PMTV (Bouzoubaa *et al.*, 1986; Gilmer *et al.*, 1992; Scott and Kashiwazaki, 1994). However, the putative movement protein of SBWMV is encoded by an ORF in RNA1 (Shirako and Wilson, 1993). The RNA2 of PCV has the same gene structure as that of BNYVV (Manohar *et al.*, 1993). Studies on PMTV show that its RNA2 similar to BNYVV RNA2 at the 3'-terminal. Among furoviruses, the CP genes of SBWMV, BNYVV and PMTV are followed by a leaky stop-codon and readthrough (RT) protein, except for PCV. Here, we determined the nucleotide sequence of RNA1, 2 and 3 and compared the sequence data of BBNV with the nucleotide sequences of other furoviruses. We also discussed the diversity of gene organization of furoviruses based on comparisons of the gene organizations among these furoviruses.

Materials and Methods

Virus purification:

BBNV particles were purified from infected stems. The infected stems were ground by mortar and pestle in liquid nitrogen, and then suspended by borate buffer. Being filtered with 2-layers cheese-cloth, the mixture was incubated at room temperature, and centrifuged to remove the impurities. The pellet was resuspended in boric acid buffer and filtered by celiteR. The filtrate was centrifuged and resuspended in boric acid buffer again. After the suspension was obliged to the cesium sulfate density gradient centrifugation, the virus fraction was collected by a needle and syringe. In order to remove salt, the virus fraction was diluted with borate acid buffer and centrifuged. The resultant pellet was resuspended in boric acid buffer and mixed with omnipaque, then the omnipaque density equilibrium gradient centrifugation was performed before and after collecting the virus band.

cDNA synthesis and cloning:

BBNV RNAs was obtained by suspending the virus pellet in TE buffer containing SDS and proteinase K. The mixture was incubated at 37℃, and obligated to phenol extraction and ethanol precipitation. Five μ g of RNAs was used for cDNA synthesis (Gubler and Hoffman 1983).The resultant ds-cDNA was ligated into pBluescript KS II (+). The ligation products were transformed into competent *E. coli* strain JM109.

Nucleotide sequence determination

The sequences of the plasmids containing inserts were determined in both directions by a Dye Primer Cycle Sequencing Kit using the DNA Sequencer. CLUSTAL V and DNASIS was used for sequence analysis.

Result

Particles of BBNV measures 130-150 and 250-270 nm in length and 18 nm in width (Fig. 1).Sequence analysis of the cDNAs of BBNV RNA1 show that there is two long ORFs in RNA1 genome. ORF1 starting at the first AUG codon and terminating with an opal stop codon potentially codes for a polypeptide of amino acids with an estimated Mr of 150000 Da. An in-phase coding sequence extends beyond this opal codon for an additional nucleotides before reaching a termination followed by a 3' untranslated region forms the ORF2. A potential RT (readthrough) protein has a Mr of 209000 Da (Fig.2).
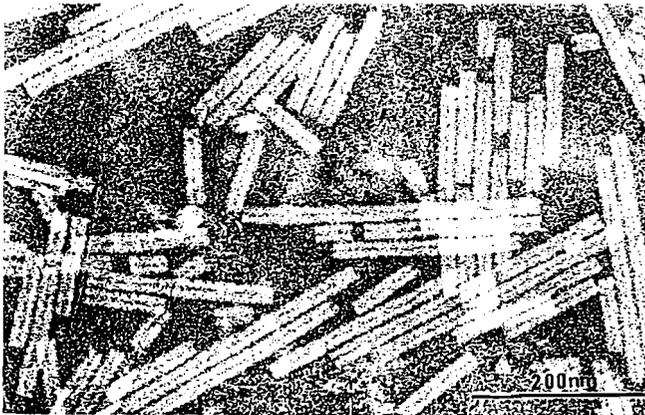
Fig. 1. Electron micrographs of purified BBNV
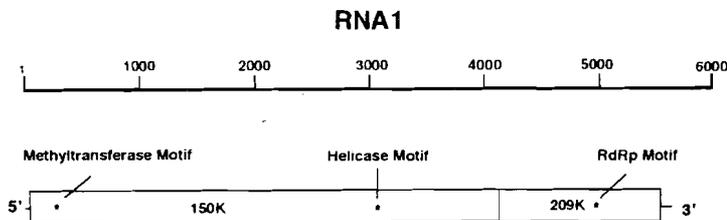
RNA1

Fig. 2. Arrangement of the ORFs of BBNV RNA1

The sequence of RNA2 contains only one possible ORF in RNA2 genome. This ORF starting from first AUG has a coding capacity of polypeptide of Mr of 194660 Da which is almost as same as the BBNV CP estimated by SDS-PAGE analysis. This ORF is interrupted by an UAA cotriplet for an additional nucleotides before reaching the stop codon. The RT protein beginning at the CP AUG codon, has a Mr of 730000 Da (Fig.3).

The sequence of RNA3 contains four long ORFs in RNA3, and the encoded proteins are 49K, 13K, 20K and 6K, respectively. The first three proteins resembles the triple-gene-block proteins found in species of several plant virus genera (Fig.4).
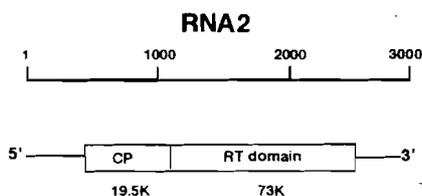
RNA2

RNA3

Fig. 4. Arrangement of the ORFs of BBNV RNA2
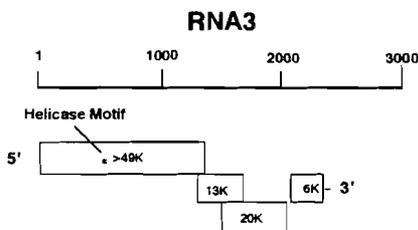
Fig. 3. Arrangement of the ORFs of BBNV RNA3

Discussion

The sequence data we analysised here includes the genes of the RdRp, helicase, methyltransferase, intergenic, CP and the triple-gene-block. These genes cover most of the genome. The results of these analysis are interpreted with respect to the classification of furoviruses. The genus *Furovirus* could be separated into several groups (or genera): the genus *Furovirus* which contains type species SBWMV, and several other species and tentative species; PMTV group including BBNV which close to the genus *Furovirus*; BNYVV group, PCV and IPCV consist of the fourth group which is close to the genus *Hordeivirus*.

Reference

Bouzoubaa, S., Ziegler, V., Beck, D., Guilly, H., Richards, K., Jonard, G. (1986) Nucleotide sequence of beet necrotic yellow vein virus RNA-2. *Journal of General Virology* **67**: 1689 -1700.

Brunt, A. A., Shikata, E. (1986) Fungus-transmitted and similar labile rod-shaped viruses. *The Plant Viruses*, Plenum Press, New York. **vol.2**, pp. 305-335.

72

Brunt, A. A., Richards, K. E. (1989) Biology and molecular biology of furoviruses. *Adv Virus Res* **36**: 1-32.

Brunt, A. A. (1991) Furovirus group In: Francki RIB. Fauquet CM, Knudson DL. Brown F (eds) Classification and Nomenclature of viruses. *Vth Report of the International Committee on Taxonomy of Viruses.* Springer, Wien New York, pp 377-379 (*Arch Virol* [Suppl 2])

Fauquet, C., Desbois, D., Fargette, D., Vidal, G. (1988) Classification of furoviruses based upon the amino acid composition of their coat proteins. Pages 19-36 in: *Development in Applied Biology 2, Viruses with Fungal Vectors.* J. I. Cooper and M. j. C. Asher, eds. University of St. Andrews, UK.

Fukano, H., Yokoyama, S . (1952). The necrosis disease of broad bean and pea. *Kyushu agricultural research.* **10**: 133-134.

Fujikawa, T. (1955) Study on broad bean necrosis disease. *Agricultural and Horticulture* **30**: 85 -86.

Fujikawa, T. (1957) Study on soil transmission of broad bean necrosis disease. *Phytopathological society of Japan* **22(1)**: 19.

Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K. E., Jonard, G. (1992) Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA2. *Virology* **189**: 40-47.

Inoue, T., Asatani, M. (1967) Properties of broad bean necrosis . *Phytopathological society of Japan* **33**: 94-95.

Inoue, T., Asatani, M. (1968) Broad bean necrosis virus. *Phytopathological society of Japan.* **34**: 317-322.

Kuszala, M., Ziegler, V., Bouzoubaa, S., Richards, K., Putz, C., Guilley, H., Jonard, G. (1986) Beet necrotic yellow vein virus:different isolates are serologically similar but differ in RNA composition. *Ann.appl.Biol* **109**: 155-162.

Manohar, S. K., Guilley, H., Dollet, M., Richards, K., Jonard, G. (1993) Nucleotide sequence and genetic organization of peanut clump virus RNA 2 and partial characterization of deleted forms. *Virology* **195**: 33-41.

Shirako, Y., Brakke, M. K. (1984) Two purified RNAs of soil-borne wheat mosaic virus are needed for infection. *Journal of General Virology* **65**: 119-127.

Shirako, Y., Wilson, T. M. A. (1993) Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. *Virology* **195**: 16-32

Scott, K. P., Kashiwazaki, S., Reavy, B., Harrison, B. D. (1994) The nucleotide sequence of potato mop-top virus RNA 2: a novel type of genome organization for a furovirus. *Journal of general virology* **75**: 3561-3568.

Tamada, T., Shirako, Y., Abe, H., Saito, M., Kiguchi, T., Harada, T. (1989) Production and pathogenicity of isolates of beet necrotic yellow vein virus with different numbers of RNA compoments. *Journal of General Virology* **70**: 3399-3409.

THE NUCLEOTIDE SEQUENCE OF RNA-1 OF INDIAN PEANUT CLUMP VIRUS COMPLICATES ITS TAXONOMY BUT OFFERS BROAD SPECTRUM DIAGNOSTICS

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Summary

The nucleotide sequence of the 5841 nucleotides of RNA-1 of Indian peanut clump virus (IPCV) contains three open reading frames which encode a M_r 129687 protein containing a methyltransferase domain (ORF 1), a M_r 60188 protein which contains a polymerase domain (ORF 2), and a M_r 14281 protein (ORF 3). The nucleotide sequence of IPCV RNA-1 is similar to that of peanut clump virus (PCV). The translation products are 88% (ORF 1), 95% (ORF 2) and 75% (ORF 3) identical to those of PCV RNA and are also similar to those of soil-borne wheat mosaic virus and barley stripe mosaic virus (BSMV). These similarities and those with proteins of other furoviruses call into question the taxonomic status of the genera *Furovirus* and *Hordeivirus*. A cDNA probe derived from the 3' end of RNA-1 of IPCV-H hybridized with RNA of isolates of other serotypes of IPCV and of PCV. Also, primers based on sequences common to RNA-1 of IPCV and PCV, were effective in reverse transcription-PCR amplification of these RNA and that of L and T serotype isolates of IPCV.

Introduction

Indian peanut clump virus (IPCV) induces clump disease in groundnut crops in many parts of India, and is transmitted by *Polymyxa graminis* (Reddy *et al.*, 1988). IPCV resembles peanut clump virus (PCV), which induces clump disease of groundnut crops in West Africa (Thouvenal and Fauquet, 1981). There are three serotypes of IPCV (H, L or T) (Nolt *et al.*, 1988) and this serological diversity is a potential complication for disease diagnosis. IPCV has a bi-partite genome of positive-sense RNA molecules encapsidated in rod-shaped particles of different lengths (Reddy *et al.*, 1985); it is classified in the genus *Furovirus.*

Materials and methods

Virus isolates and propagation:
Hyderabad (H) and Ludhiana (L) isolates, which belong to the H and L serotypes (Nolt *et al.*, 1988), and the D isolate, collected by P. Delfosse in 1994 from Durgapura, Rajasthan (serotype T), were propagated in *Phaseolus vulgaris* and purified as described by Reddy *et al.* (1985).
Nucleotide sequencing:
RNA was extracted from purified virus particles and cDNA was synthesized using a commercial kit (Boehringer) and cloned in *Sma* I – digested pUC19. The 5'- and 3'-extremities of IPCV RNA-1 were cloned using the 5'Amplifinder RACE kit (Clontech).
Nucleotide sequences were determined by dideoxy chain termination using Sequenase (Amersham) and were compared using DIAGON (Staden, 1982) or CLUSTALV (Higgins *et al.*, 1992).
RT-PCR:
The locations of the primers chosen which matched the sequences of IPCV RNA-1 and PCV

RNA-1 (Herzog *et al.*, 1994) with minimum degeneracy are shown in Fig. 1. Template was 0.5 μg of virus RNA or 5 μg of RNA extracted from the leaf tissue of IPCV–infected *Nicotiana benthamiana*. Reverse transcription at 42°C for 1 hour was followed by 30 cycles of PCR at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min.

Results

A sequence of 5841 nucleotides was established. It contained 3 open reading frames
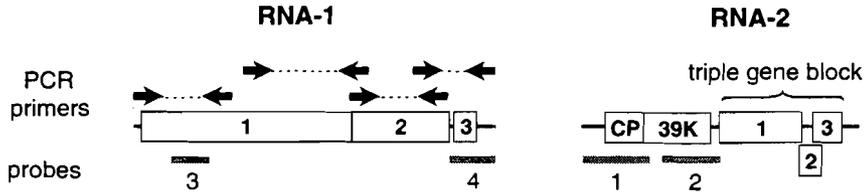
Fig. 1. Diagram of the genome of Indian peanut clump virus
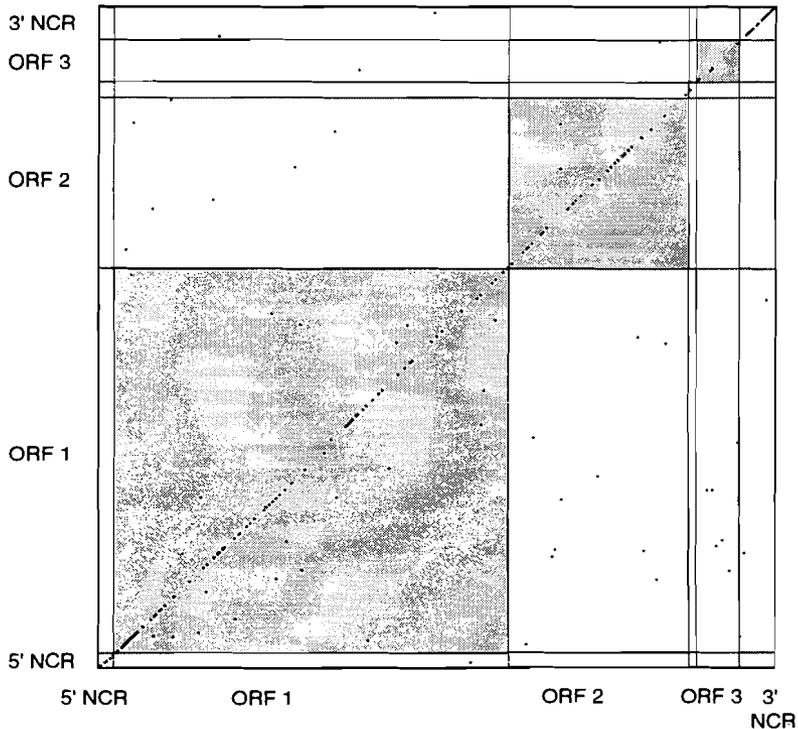
Fig. 2. DIAGON comparison of RNA-1 molecules of IPCV-H (horizontal) and PCV (vertical). Matches shown are for 11+/15 nucleotides identical.

(ORF). ORF 1 has domains suggestive of methyltransferase activity, ORF 2 has a polymerase domain but ORF 3 did not resemble proteins of known function. The genome organization (Fig.1) is very like that of PCV (Herzog *et al.*, 1994).

Comparison of the two sequences using DIAGON recording matching strings in which 11 of 15 nucleotides were identical (Fig. 2) showed a non-uniform distribution of similarity. The non-coding regions (NCR) were the most alike, there were patches of strong similarity in ORF1 and ORF2, and ORF3 were least alike.

Fig. 3 shows the result of comparisons, made using CLUSTALV, among the proteins
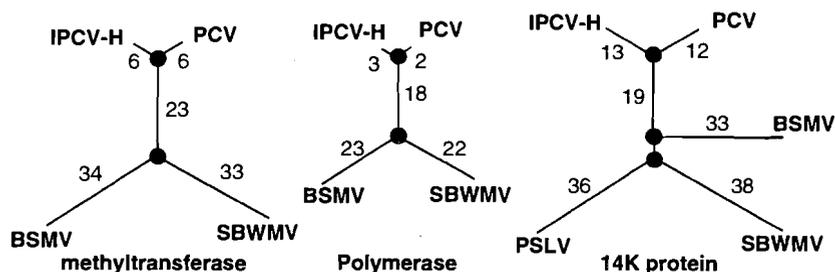
Fig. 3. Relatedness among corresponding proteins assessed by CLUSTALV

encoded by each of the ORF and the corresponding proteins of PCV and other viruses, when any perceptible similarities could be discerned.

Fragments of cDNA corresponding to different parts of the IPCV genome were tested in hybridization tests with RNA of each of the three IPCV serotypes as well of PCV RNA. The result (Table 1) was that probes from RNA-2 were relatively specific for the homologous H

	probe 1	probe 2	probe 3	probe 4
IPCV-H	++++	+++	+++	++++
IPCV-L	+/-	-	++	++++
IPCV-D	-	-	+++	+++
PCV	+/-	-	-	+++

Table 1. Reactions of hybridization probes with 0.1µg IPCV and PCV RNA

serotype, probe 3 from ORF1 of RNA-1 reacted with RNA from all 3 IPCV serotypes and probe 4, which included the 3'-non-coding region, hybridized well with all the RNA types.

RT/PCR tests yielded clear bands of cDNA equivalent in size (for IPCV-H) to the predicted products. Thus the sequences shared between IPCV-H RNA-1 and PCV RNA-1 were also shared completely or nearly completely with RNA-1 of IPCV-L and IPCV-D.

Discussion

We have obtained a broad specificity hybridization probe which is being used currently in disease surveys in India. IPCV infects many hosts and multiplies well in graminaceous hosts

such as wheat (Delfosse *et al.*, 1995). Groundnut came from South America and presumably encountered the ancestors of the viruses inducing clump on arrival in the Old World. It is possible that in the weed flora there are viruses related to IPCV and PCV which do not infect groundnuts. The new probe might be expected to detect such viruses. The results also suggest that with any new virus isolate it will be possible by RT/PCR to identify how closely such a new virus is related to existing viruses.

From the sequences of the putative polymerases of IPCV and PCV, it would be reasonable to classify the viruses as strains. But other proteins encoded by RNA-1 suggest less close relatedness and those encoded by RNA-2 of IPCV and PCV (Naidu *et al.*, this volume) are even less alike. Practical considerations suggest that IPCV and PCV be considered as different viruses. Sequence comparisons also show that, as with the coat proteins (Wesley *et al.*, 1994), BSMV is relatively closely related to IPCV, and more so than are other furoviruses. Taken together, these data strongly suggest that the general class of viruses with rod-shaped particles and multipartite genomes require taxonomic re-alignment.

References

Delfosse, P., Reddy, A. S., Devi, P., Murthy, A. K., Wesley, S. V., Naidu, R. A. and Reddy, D. V. R (1995) A disease of wheat caused by Indian peanut clump virus (IPCV). *Plant Disease* **79**:1074.

Herzog, E., Guilley, H., Manohar, S. K., Dollet, M., Richards, K., Fritsch, C. and Jonard, G. (1994). Complete nucleotide sequence of peanut clump virus RNA 1 and relationships with other fungus-transmitted rod-shaped viruses. *J. Gen. Virol.* **75**:3147-3155.

Higgins, D. G., Bleasby, A. J. and Fuchs, R. (1992). CLUSTALV: improved software for multiple sequence alignment. *CABIOS* **8**:189-191.

Nolt, B. L., Rajeshwari, R., Reddy, D. V. R., Bharatan, N. and Manohar, S. K. (1988). Indian peanut clump virus isolates : host range, symptomatology, serological relationships, and some physical properties. *Phytopathol.* **78**:310-313   .

Reddy, D. V. R., Robinson, D. J., Roberts, I. M. and Harrison, B. D. (1985). Genome properties and relationships of Indian peanut clump virus. *J. Gen. Virol.* **66**:2011-2016.

Reddy, D. V. R., Nolt, B. L., Hobbs, H. A., Reddy, A. S., Rajeshwari, R., Rao, A. S., Reddy, D. D. R. and McDonald, D. (1988). Clump disease in India: isolates, host range, transmission and management. In: Cooper, J. I. and Asher, M. J. C. (eds) Viruses with Fungal Vectors, pp239-246.

Staden, R. (1982). An interactive graphics program for comparing and aligning nucleic acid and amino acid sequences. *Nucleic Acids Res.* **10**: 951-2961.

Thouvenal, J. C., Fauquet, C. (1981) Further properties of peanut clump virus and studies on its natural transmission. *Ann. Appl. Biol.* **97**: 99-107

Wesley, S. V., Mayo, M. A., Jolly, C. A., Naidu, R. A., Reddy, D. V. R., Jana, M. K., and Parnaik, V. K. (1994). The coat protein of Indian peanut clump virus: relationships with other furoviruses and with barley stripe mosaic virus. *Arch. Virol.* **134**: 271-278.

THE NUCLEOTIDE SEQUENCE OF INDIAN PEANUT CLUMP VIRUS RNA 2

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Summary

The RNA-2 molecule of an isolate of the L serotype of Indian peanut clump virus (IPCV) is 4290 nucleotides in length and contains five open reading frames (ORF). The arrangement of the ORFs is similar to that in RNA-2 of peanut clump virus (PCV) from West Africa. Proteins encoded by IPCV-L RNA-2 are between 32% and 89% identical to those of PCV. The coat protein of IPCV-L is as similar to the corresponding coat proteins of isolates belonging to H and T serotypes of IPCV as they are to the coat protein of PCV. The results support the distinction of IPCV-L and PCV as separate virus species, although there are strong similarities among triple gene block proteins.

Introduction

Peanut clump is an economically important soil-borne virus disease of peanuts (*Arachis hypogaea* L) caused by Indian peanut clump virus (IPCV) in India (Reddy *et al.*, 1983) and peanut clump virus (PCV) in West Africa (Thouvenel and Fauquet, 1981). Particles of both viruses are rod-shaped, of two predominant lengths and contain two genomic RNAs; they have been classified in the genus *Furovirus*. The currently known isolates of IPCV fall into three serotypes, viz. IPCV-H, IPCV-L and IPCV-T (Nolt *et al.*, 1988) and each is serologically distinct from PCV.

The nucleotide sequence of the two genomic RNAs of an isolate of PCV is known (Manohar *et al.*, 1993; Herzog *et al.*, 1994). The nucleotide sequence of the coat protein gene of IPCV-H (Wesley *et al.*, 1994) showed the coat protein to be 61% identical in amino acid sequence to that of the PCV, which suggests that IPCV and PCV are different viruses. However, attempts to clone and sequence remainder of IPCV-H RNA-2 were not successful. In order to extend comparisons between IPCV and PCV, we have determined the nucleotide sequences of all of IPCV-L RNA-2 and of the coat protein gene of isolate D, which was collected by P. Delfosse in Durgapura, Rajasthan in 1994 and belongs to serotype T.

Materials and Methods

Purification of virus and extraction of RNA
The IPCV-L was purified as described by Reddy *et al.* (1985). RNA was extracted from purified virus particles and RNA 2 was separated from RNA 1 in low melting agarose gels.
Cloning and sequencing
RNA-2 was heat denatured at $65^{\circ}C$ for 5 min, annealed with random composition hexa-deoxynucleotides and used as a templete for cDNA synthesis. The ds cDNA was cloned in pUC119. Nucleotide sequencing was done by dideoxy chain termination using Sequenase (Amersham). The extremities of the RNA-2 were cloned using the 5'Amplifinder[TM] RACE Kit (Clontech). All the sequences were determined in both directions. Nucleotide sequences were compared by using programs DIAGON (Staden, 1982) and CLUSTALV (Higgins *et al.*, 1992).
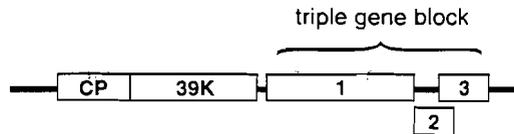
Fig. 1. Diagram of the genome organization of IPCV-L RNA-2

The IPCV–L RNA–2 is 4290 nucleotides (nt) in length and contains five open reading frames (Fig.1). The coat protein is the 5'-most gene and is followed by a $M_r 39000$ protein gene (39K) and three overlapping genes at the 3' end which form a triple gene block (TGB). The genome organization of IPCV-L RNA-2 is similar to that of PCV.

Fig. 2 shows a DIAGON comparison of RNA–2 sequences between IPCV–L and PCV. The results suggest that the sequences in the 3' half of the two molecules (which encode the TGB proteins) are more alike than sequences in the 5' half of the molecules (which encode
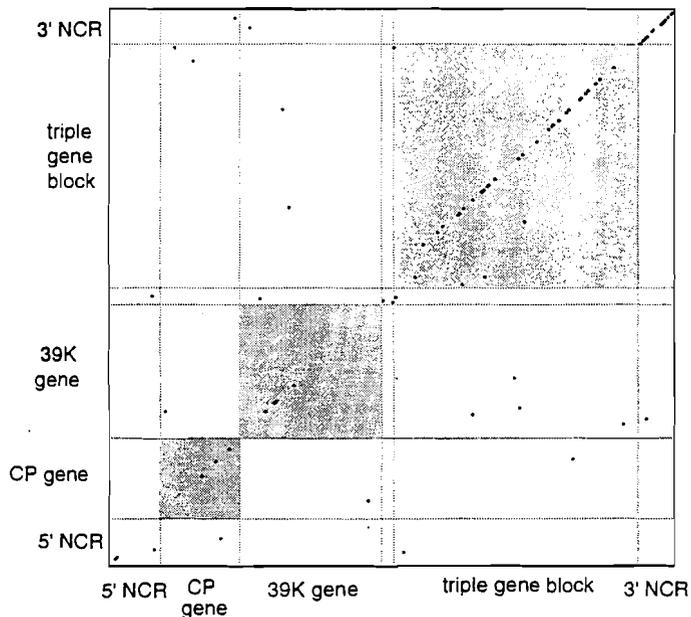
Fig. 2. DIAGON comparison of RNA-2 molecules of IPCV-L (horizontal)
and PCV (vertical). Matches shown are for 11+/15 identical

the coat protein and 39K). Different parts of RNA–2 molecule differ in the extent to which they vary among IPCV–L and PCV, the ORF2 of the TGB is highly conserved (89% identical) whereas the 39K is only 32% identical.

Analysis by using CLUSTALV of the coat protein sequences of IPCV and PCV suggested that the coat protein of PCV is 59% identical to that of IPCV–H, 67% identical to that of IPCV–L, and 62% identical to that of IPCV–D. The coat proteins of IPCV and PCV were also

compared with corresponding proteins of other viruses. Fig. 3 shows an unrooted tree illustrating the similarity distances between coat protein genes of IPCV serotypes, PCV, BSMV, potato mop-top (PMTV), soil-borne wheat mosaic (SBWMV), beet necrotic yellow vein (BNYVV) and Nicotiana velutina mosaic (NVMV) furoviruses. The only marked clustering detected (shaded in Fig. 3) was between IPCV serotypes and PCV. However, the coat proteins were more similar to that of BSMV than to coat proteins of any other furovirus. Thus, different furoviruses were as dissimilar from each other as they were from IPCV or PCV.

BSMV   **PMTV**   **SBWMV**
BNYVV
IPCV-L   **PCV**
IPCV-D
IPCV-H
33   33   36   39
18   17   16
17   **NVMV**
15   11   40

Coat Proteins

BSMV
PMTV   **BNYVV**
PCV   36   **NVMV**
IPCV-L   10   32   35
9   36
19
6

TGB 1

IPCV-L   **PCV**
3   4   **PMTV**   **BSMV**   **BNYVV**   **NVMV**
19   28
23   22   35
4   13

TGB 2

PMTV
BSMV   **NVMV**
PCV   38
IPCV-L   19   35
16   47
14   2

TGB 3
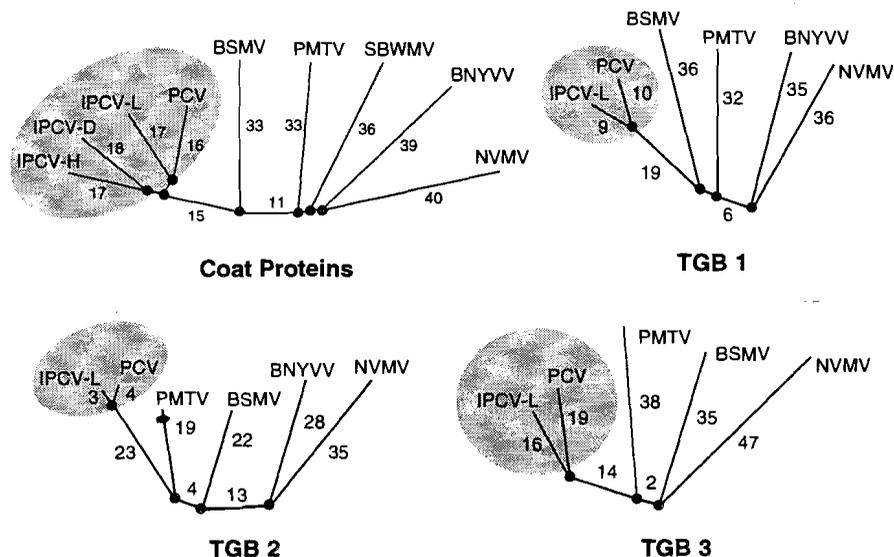
Fig. 3. Unrooted tree showing relationships among coat protein sequences and triple gene block proteins estimated by CLUSTALV

Fig. 3 also shows an analysis of relationships among the TGB proteins. The distances between IPCV-L and PCV are like those between the coat proteins for protein TGB 3 but the other TGB proteins of the two viruses are more alike. The striking difference between comparisons among coat proteins and TGB proteins is that whereas the coat protein of PMTV is distant from that of IPCV, the TGB proteins of PMTV are as close to those of IPCV and PCV as are the BSMV TGB proteins.

Discussion

The results show that the RNA-2 of IPCV-L and PCV are distinct, although their genome organizations are similar. The extent to which corresponding genes were similar depended on which genes were compared; the 5'-most genes were more different than those in the 3' half of the RNA-2 molecule. Comparisons among the sequences of the corresponding genes of RNA-1 of IPCV-H (Miller *et al.*, this volume) and PCV (Herzog *et al.*, 1994), and RNA-2 of IPCV-L and PCV (Manohar *et al.*, 1993; Herzog *et al.*, 1994) showed that RNA-2 gene products differed more than RNA-1 products. There is no strong similarity between RNA-1 and RNA-2 of IPCV and PCV except in the 3' non-coding region. The pattern of genome variation in IPCV and PCV is like that in tobacco rattle virus (TRV); RNA-1 molecules of all

TRV strains are relatively similar whereas RNA-2 molecules are distinct (Robinson, 1989).

The differences in coat protein sequence between IPCV serotypes and PCV suggest that they should be considered as separate viruses, but the stronger similarities among some TGB proteins might suggest that all are strains of the same virus. The apparent relatedness between IPCV and PMTV differs markedly according to which proteins are compared. It is possible that the TGB proteins have been acquired by an ancestor of one or other viruses by recombination. The similarities between IPCV and PCV proteins and those of BSMV reinforce the idea that different genome segments have been exchanged among these viruses. and thus the possibility of there being an evolutionary link between BSMV and furoviruses.

References

Herzog, E., Guilley, H., Manohar, S. K., Dollet, M., Richards, K., Fritsch, C., and Jonard, G. (1994). Complete nucleotide sequence of peanut clump virus RNA 1 and relationships with other fungus-transmitted rod-shaped viruses. *J. Gen. Virol.* **75**:3147-3155.

Higgins, D. G., Bleasby, A. J., and Fuchs, R. (1992). CLUSTALV: improved software for multiple sequence alignment. *CABIOS* **8**: 189-191.

Manohar, S. K., Guilley, H., Dollet, M., Richards, K., and Jonard, G. (1993). Nucleotide sequence and genetic organization of peanut clump virus RNA 2 and partial characterization of deleted forms. *Virology* **195**:33-41.

Nolt, B. L., Rajeshwari, R., Reddy, D. V .R., Bharatan, N., and Manohar, S. K. (1988). Indian peanut clump virus isolates : host range, symptomatology, serological relationships, and some physical properties. *Phytopathology* **78**: 310-313.

Reddy, D. V. R., Rajeshwari, R., Iizuka, W., Lesemann, D. E., Nolt B. L., and Goto, T. (1983). The occurrence of Indian peanut clump, a soil-borne virus disease of groundnuts (*Arachis hypogaea*) in India. *Ann. Appl. Biol.* **102**:305-310.

Reddy, D. V. R., Robinson, D. J., Roberts, I. M., and Harrison, B. D. (1985). Genome properties and relationships of Indian peanut clump virus. *J. Gen. Virol.* **66**:2011-2016.

Robinson, D. J. (1989). Tobacco rattle virus: variation among strains and detection by cDNA probes. *EPPO Bulletin* **19**:619-623.

Staden, R. (1982). An interactive graphics program for comparing and aligning nucleic acid and amino acid sequences. *Nucleic Acids Res.* **10**: 951-2961.

Thouvenal, J. C. and Fauquet, C. (1981). Further properties of peanut clump virus and studies on its natural transmission. *Ann. Appl. Biol.* **97**:99-107.

Wesley, S. V., Mayo, M. A., Jolly, C. A., Naidu, R. A., Reddy, D. V. R., Jana, M. K., and Parnaik, V. K. (1994). The coat protein of Indian peanut clump virus: relationships with other furoviruses and with barley stripe mosaic virus. *Arch. Virol.* **134**:271-278.

COMPLETE NUCLEOTIDE SEQUENCE OF WHEAT YELLOW MOSAIC BYMOVIRUS GENOMIC RNAS*.

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Summary

Complete nucleotide sequences of wheat yellow mosaic virus (WYMV) RNAs 1 and 2 were determined. WYMV RNA 1 is 7636 nucleotides, excluding the 3' poly(A) tail, and contains one large open reading frame (ORF), coding for a polyprotein of 2404 amino acids with an Mr of ca. 269 kDa. The putative ORF products contain the capsid protein(CP) at the C termini, and five putative non-structural proteins are arranged in the same manner as in RNA 1 of barley yellow mosaic virus and barley mild mosaic virus. The deduced CPs of WYMV and WSSMV (Southern France isolate) have only 77 % sequence identity of both complete region and core region of each CP. WYMV RNA2 is 3650 nucleotides excluding the 3' poly(A) tail, and contains one large ORF, coding for a polyprotein of 904 amino acids with an Mr of ca. 101 kDa. The data suggest that WYMV is unexpectedly different from WSSMV.

Introduction

Yellow mosaic of wheat was first recorded in Japan in 1927 (Sawada, 1927) and later its causal agent, wheat yellow mosaic virus (WYMV) was identified (Inouye, 1969). In Canada, the virus responsible for a similar yellow mosaic disease of wheat was designated as wheat spindle streak mosaic virus (WSSMV) (Slykhuis, 1970), which corresponds to the WSSMV-C in this paper. Usugi and Saito (1979) considered WYMV and WSSMV-C to be strains of the same virus because they have many similar features and only slight differences in their serological and pathological characteristics.

Particles of WYMV and WSSMV-C are slightly flexuous filaments with two modal lengths of ca. 550 and 275 nm, 13 nm in diameter (Usugi and Saito ,1979), and contain two 3'-polyadenylated single-stranded RNA species of M_r 2.6×10^6 (RNA I) and 1.4 to 1.5×10^6 (RNA2) (Usugi et al., 1989). They are soil-borne by a common vector fungus *Polymyxa graminis* (Slykhuis and Barr, 1978). Their natural host range is restricted to *Triticum* spp., but they both mechanically infect rye (Usugi, 1984). The virus responsible for the yellow mosaic of wheat has also been reported in the USA, India, France, Italy and China, where the virus has been called either WYMV or WSSMV. However, only little information is available on the comparative characteristics of WYMV or WSSMV isolates from different locations. WYMV and WSSMV, together with other *Polymyxa*-transmitted filamentous viruses are classified into the genus *Bymovirus* of the family *Potyviridae* (Barnett, 1991). Serologically, WYMV and WSSMV are closely related to barley yellow mosaic virus (BaYMV), a type species of the genus *Bymovirus*. But these viruses are distantly related or unrelated to other bymoviruses (Usugi, 1984). BaYMV is clearly different from WYMV or WSSMV in its host range which is restricted to *Hordeum* spp. (Usugi, 1984).

The complete nucleotide sequence has been determined for the BaYMV genome (Kashiwazaki et al., 1990, 1991). Nucleotide sequences have also been reported for the 3'-terminal half of RNA1 of WSSMV (a French isolate) (Sohn et al.,1994), and for the 3'-terminal half of RNA1 and the whole of RNA2 of barley mild mosaic virus (BaMMV), another bymovirus. The capsid proteins (CPs) of BaYMV and WSSMV-F have 74% amino acid sequence identity, but they have only 35 to 36% identity with the BaMMV-CP (Sohn et al.,1994).

In this study, we determined the complete nucleotide sequence of the genome of a Japanese isolate of WYMV (Usugi and Saito, 1976), and assessed its relationships to WSSMV-F, BaYMV, BaMMV, and potyviruses, based on comparisons of nucleotide and deduced amino acid sequences.

Materials and methods

Virus:
The WYMV isolate was propagated in wheat plants (*Triticum aestivum* L. cvs. Hatakeda-komugi or Norin 61) by mechanical inoculation. Virus particles were purified from systemically infected leaves 3-4 weeks after inoculation as described by Usugi and Saito (1976).

Double-stranded cDNA synthesis:
Approximately 5 µg of WYMV RNAs (a mixture of both RNA1 and RNA2), suspended in water, was denatured by heating at 60°C for 1 min and immediately chilled on ice. The RNA was used as the template for oligo(dT)-primed cDNA synthesis, followed by second strand synthesis, using a commercial kit following the manufacturer's protocol. The double-stranded cDNA (ds cDNA) was treated with 17 units of T4 DNA polymerase at 37°C for 10 min in the presence of 10 µg/ml RNase A, phenol/chloroform-extracted and ethanol-precipitated.

cDNA cloning and DNA sequencing:
The ds-cDNA was electrophoresed on an 0.7% agarose gel, and the gel was stained with ethidium bromide. Two cDNA bands corresponding to the full lengths of RNA1 (7.6 kb) and RNA2 (3.6 kb) were excised from the gel, respectively. Each cDNA fragment was electro-eluted from the gel slices and ligated into a dephospholylated, *Sma*l-cut pBluescriptKS(+). The cDNAs were used to transform *Escherichia coli* JM109 cells. Plasmid DNAs were isolated by a modified alkali method, and the sizes of the cDNA inserts were measured electrophoretically.

Two large clones (7.6 kbp and 3.6 kbp) were selected for sequence analysis. Their cDNA inserts were subcloned using restriction enzyme fragments or exonuclease III-generated nested deletions. The DNA sequences were determined by the dideoxynucleotide chain termination method with *Taq* DNA polymerase in an DNA sequencer. All parts of the cDNA were sequenced in both orientations.

Results

Computer analysis revealed a single long open reading frame (ORF) in one of the reading frames of the RNA1 sequence (Fig. 1). The predicted translation product of this ORF contains 2404 amino acids with a calculated Mr of 269167 (269K protein). The 269K protein encoded by RNA1 is thought to be a polyprotein that contains some functional proteins besides the CP, which was located in the C-terminal region. This large protein showed homology with BaYMV 270K protein. Significant homologies in the 432-880 amino acid alignment of the 269K protein with the cytoplasmic inclusion protein (CI) (71K protein) of potato virus Y (PVY) polyprotein and tobacco etch virus (TEV) polyprotein were found. The genome-linked protein (VPg), the two nuclear inclusion proteins (NIa and NIb), and the CP of TEV also showed homologies with 1146-2404 amino acid alignment of this large protein.

According to the sequencing data, CP consists of 293 amino acids and therefore has a calculated Mr of 32.0K. The estimated Mr of 33K has been reported after SDS-PAGE of virus particle proteins (Usugi *et al.*, 1989), which is in the same range.

Homology in the amino acid sequence of CP between WYMV and WSSMV-F (77%) is higher than that between WYMV and BaYMV (68%). WYMV-CP showed a homology with BaMMV-CP (35%) almost the same as that with potyvirus CPs (30%).

WYMV RNA2 was 3650 nts long, excluding the 3' poly(A) tail, of which 3639 nts were determined from cDNA clones and 11 nts were determined from RNA sequencing. The WYMV RNA2 genome is 65 nts longer than the BaYMV RNA2 genome (Kashiwazaki *et al.*, 1991). This sequence contains a single long ORF. The predicted translation product contains 904 amino
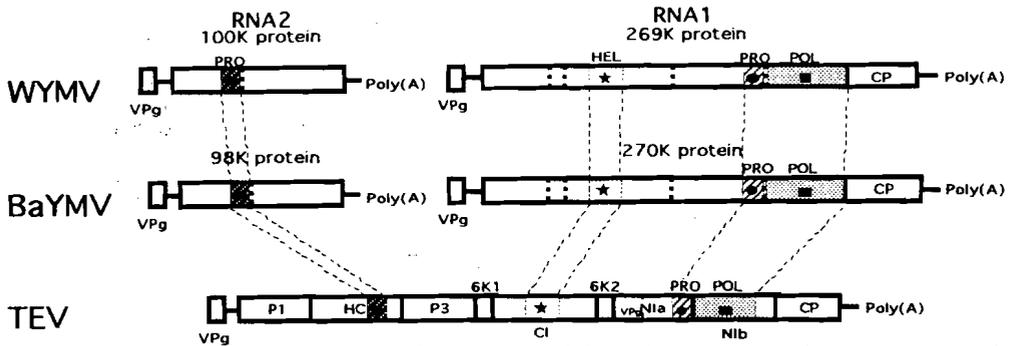
Fig. 1. Schematic representation of genomes of fungal-transmitted bymoviruses and aphid-transmitted potyviruses. Coding regions in the genomes are indicated as open bars; regions of amino acid sequence identity in the gene products are indicated by similar shading. The symbols are: CP, coat protein; HEL, helicase; PRO, proteinase; POL, polymerase; poly(A), poly(A) tail; Vpg is denoted by the small open square at the N-terminal end of each polyprotein.

acids with a calculated Mr of 100885 (101K).

A P2 protein of WYMV has sequence homologies with those of BaMMV and BaYMV and the CP-RT(readthrough) protein of two furoviruses, soil-borne wheat mosaic virus (SBWMV) and beet necrotic yellow vein virus (BNYVV) (Dessens *et al.*, 1995). These amino acid domains look like the "pseudocapsid protein domain". Other conserved sequence homologies, specific for bymo- and furoviruses, were found in the following region of the "pseudo-capsid protein domain" of bymoviruses and RT region of furoviruses as reported recently (Dessens *et al.*, 1995). This domain also contains a high number of hydrophobic residues, especially the major hydrophobic peaks in the RT proteins of furoviruses (Dessens *et al.*, 1995). These data suggest that th P2 protein of WYMV and other bymoviruses may be functionally and evolutionarily related to the furovirus capsid RT protein. Deletion of the 3'-terminal part of the bymovirus P2 protein and furovirus RT protein has been reported to be strongly associated with the loss of fungal transmission (Dessens *et al.*, 1995). This suggests the possibility of horizontal transfer of the capsid and RT protein genes from a furovirus to a bymovirus associated with a change in function.

The putative NIa proteinase may be responsible for the proteolytic processing of the polyprotein of WYMV-RNA1, as it is assumed for BaYMV (Kashiwazaki *et al.*, 1990), WSSMV-F (Sohn *et al.*,1994) and for potyviruses (Dougherty and Carrington, 1988).

The amino acid motif GYCY found in the WYMV 100K protein, which also occurs in the polyprotein encoded by the WYMV RNA2, and in the HC-Pro of aphid-transmissible potyviruses, was shown to contain a proteolytically active cysteine (Oh and Carrington, 1989). Furthermore, a histidine residue known as being essential for cleavage activity of HC-Pro of potyviruses has also been found in the polyprotein of WYMV RNA2. These data from the computer analysis suggest that these regions may be a proteinase domain and a cleavage site, respectively.

We compared the deduced amino acid sequence of the RNAdependent RNA polymerase (RdRp) and the coat protein sequences of several other species of the family Potyviridae to identify similarities and possible relationships. And we generated a cluster dendrogram illustrating the sequence relationships and the tentative phylogenetic relationships inside this large family of virion coat proteins. The resulting dendrogram shows that the genetic distance between WYMV and WSSMV-F on the CP-dendrogram is relatively larger than the others between strains of the same viruses.

84

Discussion

The results shown here strongly suggest that it is difficult to conclude that WYMV and WSSMV are different strains of the same species, which has long been suggested (Usugi, 1984). There may be a hypothetical common ancestor of both viruses which might have evolved and diverged to their stage just before different virus species appeared on the evolutionary pathways. However, since 77% is the lowest level in the second level of sequence identity, it may be wise to consider them as separate viruses.

Other questions remain before reaching a final conclusion about the relationship of these two viruses. There is the evidence of cross-protection between WYMV and WSSMV-Canadian isolate (Usugi and Saito, 1979). This strongly suggests that WYMV and WSSMV are strains of the same virus species (Hamilton *et al.*, 1981). Complete sequences of WYMV (present paper) and WSSMV-C need to be compared. However, the genomic organization of original isolate of WSSMV which was first reported in Canada (Slykhuis, 1970). If the sequence data support their being strains of the same virus species, WSSMV-French isolate may be identified as being another new bymovirus closely related to WYMV and WSSMV-C.

References

Barnett, O. W. (1991). Potyviridae, a proposed family of plant viruses. *Arch. Virol.* **118**: 139-141.

Dessens, J. T., Nguyen, M. and Meyer, M. (1995). Primary structure and sequence analysis of RNA2 of a mechanically transmitted barley mild mosaic virus isolate: an evolutionary relationship between bymo- and furovirus. *Arch. Virol.* **140**: 325-333.

Dougherty, W. G. and Carrington, J. C. (1988). Expression and function of potyviral gene products. *Annu. Rev. Phytopathol.* 26: 123-143

Hamilton, R. I., Edwardson, J. R., Francki, R. I. B., Hsu, H. T., Hull, R., Koenig, R. and Milne, R.G. (1981). Guidelines for the identification and characterisation of plant viruses. *J. Gen. Virol.* **54**: 223-241.

Inouye, T. (1969). Filamentous particles as the causal agent of yellow mosaic disease of wheat. *Nogaku Kenkyu* **53**: 61-68

Kashiwazaki, S., Minobe, Y. and Hibino, H. (1991). Nucleotide sequence of barley yellow mosaic virus RNA 2. *J. Gen. Virol.* **72**: 995-999

Kashiwazaki, S., Minobe, Y., Omura, T. and Hibino, H. (1990). Nucleotide sequence of barley yellow mosaic Virus RNA 1: a close evolutionary relationship with potyviruses. *J. Gen. Virol.* **71**: 2781-2790.

Oh, C. S. and Carrington, J. C. (1989). Identification of essential residues in potyvirus proteinase HC-Pro by site-directed mutagenesis. *Virology* **173**: 692-299.

Sawada, E. (1927). Control of wheat yellow mosaic virus. *J. Plant Protection* **14**: 444-449.

Slykhuis, J. T. (1976). Wheat spindle streak mosaic virus. C.M.I./A.A.B. "Description of Plant Viruses", no 167.

Slykhuis, J. T. and Barr, D. J. S. (1978). Confirmation of *Polymyxa graminis* as a vector of wheat spindle streak mosaic virus. *Phytopathology* **68**: 639-643.

Sohn, A., Schenk, P., Signoret, P. A., Schmitz, G., Schell, J., Steinbiß, H. H. (1994). Sequence analysis of the 3'-terminal half of RNA 1 of wheat spindle streak mosaic virus. *Arch. Virol.* **135**: 279-292.

Usugi, T. (1984). Studies on soil-borne filamentous viruses in gramineous plants. Doctoral Thesis.

Usugi, T., Kashiwasaki, S., Omura, T. and Tsuchizaki, T. (1989). Some properties of nucleic acids and coat proteins of soil-borne filamentous viruses. *Ann. Phytopathol. Soc. Japan* **55**: 26-31.

Usugi, T. and Saito, Y. (1976). Purification and serological properties of barley yellow mosaic virus and wheat yellow mosaic virus. *Ann. Phytopath. Soc. Japan* **42**: 12-20.

Usugi, T, and Saito, Y. (1979). Relationship between wheat yellow mosaic virus and wheat spindle streak mosaic virus. *Ann. Phytopathol. Soc. Japan.* **45**: 397-400.

FUNGAL VECTORS IN THE TOMBUSVIRIDAE.

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Summary

Some viruses in the Tombusviridae, especially those in the Carmovirus and Necrovirus genera, have fungal vectors, others have beetle vectors, and one virus has both types. Thus, these viruses are contrary to the principle that there is one type of vector within a group and the evidence for vectors should be re-examined, particularly with regard to the role of fungal vectors which can be overlooked because of their size, their host specificity, and their vector specificity. The possibility of contamination by fungal vector(s) in the beetle transmission trials is examined and can not be excluded. The nature of "soil transmission" of both carmo- and tombusviruses is also evaluated. Non-vectored soil transmission (NVST) has been demonstrated in experimental situations but its importance in natural soils remains unproven. The plurivorous strain(s) of O. bornovanus has (have) not been tested as vectors for tombus-, diantho-, or carmoviruses.

Introduction

The methods of transmission reported for the Tombusviridae; includes beetle vectors, fungal vectors, aphid vectors, non-vectored soil transmission (NVST), and water-borne. This array is difficult to reconcile with the current concepts of virus-vector relationships, especially the principles that there is a single type of vector within a given group and that plant viruses are not capable of infecting non-wounded plant cells. The objective of this paper is to evaluate transmission studies from the fungal vector position in order to determine if these viruses are the exception to the rule and to suggest methods for resolving conflicting reports.

Results and Discussion

1) Fungal vectors. Our recent trials have confirmed prior results or provided new evidence that six viruses are transmitted in the in vitro manner either by O. bornovanus or by O. brassicae (Campbell et al., 1991; 1995). The viruses are: cucumber necrosis tombusvirus (CNV), tobacco necrosis necrovirus (TNV), and the cucurbit-infecting carmoviruses: melon necrotic spot virus (MNSV), cucumber leaf spot virus (CLSV) including the fruit streak strain (CLSV-FS), cucumber soil borne virus (CSBV), and squash necrosis virus (SqNV). The viruses were diluted nearly to their dilution-end-point and were acquired by single sporangial cultures of the vector in conditions that eliminated the possibility of inadvertent beetle, aphid, or NVST contamination.

A high degree of host specificity exists among the cucurbit strains of O. bornovanus and, to some extent, in isolates of O. brassicae and can confound efforts to work with or identify them as vectors. Furthermore, host specificity, especially in O. bornovanus, is correlated with virus-vector ability which ranges from none, to inefficient, to efficient depending on the virus and strain of the vector. The host specificity and the vector ability of the plurivorus strain(s) of O. bornovanus (Lange and Insunza, 1977; Sahtiyanci, 1962) need to be studied especially for the diantho-, tombus-, and carmoviruses infecting legumes.

2. Beetle transmission. Beetle vectors have been reported for five carmoviruses: MNSV (Coudriet et al., 1979), cowpea mottle virus (CPMoV) (Shoyinka et al., 1978; Thouvenel et al., 1990), bean mild

mosaic virus (BMMV) (Hobbs, 1981; Waterworth et al., 1977), black gram mottle virus (BMoV) (Scott and Phatak, 1979), and turnip crinkle virus (TCV) (Martini, 1958). These reports must be re-examined to see if the results could have been due to fungal vectors, or, less likely, to NVST. An unrecognized fungal vector could affect results in two ways.

A) The fungal vector and the virus could be present as unrecognized contaminants in the soil in the greenhouse. Sooner or later systemic symptoms develop "inexplicably" from sporadic root infections. Either this scenario or NVST could explain the sporadic appearance of BMMV in a beetle-free greenhouse (Hampton and Hancock, 1981). This type of contamination could be excluded in beetle transmission trials by having control seedlings not exposed to the putative beetle vector. Such controls are conspicuously absent from all the beetle transmission experiments (Coudriet et al., 1979; Hobbs, 1981; Martini, 1958; Scott and Phatak, 1979; Shoyinka et al., 1978; Thouvenel et al., 1990; Waterworth et al., 1977). On the other hand, TCV was reported to be transmitted both by beetles and by aphids (Martini, 1958). If one accepts the principle that a beetle-transmitted virus is probably not transmitted by aphids, one could consider the plants exposed to aphids as the missing controls that were not exposed to beetles. Thus, their infection could indicate contamination of experimental plants by an unrecognized (fungal) vector. The work on TCV was done at Rothamsted at a time when the roots of many greenhouse plants were "spontaneously" infected by TNV (Kassanis and Macfarlane, 1964).

B) A fungal vector contaminating the soil could acquire virus transported by beetles. This virus could be regurgitated or deposited in feces and washed into the soil where it is acquired in the in vitro manner and transmitted by a fungal vector. This situation could be ruled out if the methods of culturing bait plants to avoid soil-borne fungal contaminants were described, eg. the use of pasteurized soil, soil-less benches in the greenhouse, or watering to avoid splash contamination. No such evidence is given in any of the cited references.

Beetle transmission of carmoviruses deserves further study using methods that provide assurance that fungal vectors are not involved. Another approach would be to demonstrate that carmoviruses infect in the presence of beetle regurgitant with the gross wounding technique (Gergerich and Scott, 1991). Until then, it is not possible to conclude that the carmoviruses are transmitted by beetles in addition to, or instead of, O. bornovanus.

3) **Soil and water transmission.** The term "soil transmission" has been applied to a variety of situations in which soil is involved and often without regard for the mechanism of transmission. At least 3 definitions of soil transmission can be recognized using carmoviruses as examples: A) Virus infection occurs when seeds are planted in infested soil in which no vector is recognized eg. CLSV (Weber et al., 1982), CSBV (Koenig et al., 1983), and MNSV (Avgelis, 1985). A fungal vector is now known for each of these viruses. B) Virus can be detected in a soil extract as has occurred with CLSV (Weber et al., 1982), MNSV (Bos et al., 1984), and Tephrosia symptomless virus (Bock et al., 1981). C) Virus can be added to soil and seedlings become infected eg. CLSV (Gallitelli et al., 1983), MNSV (Avgelis, 1985), and Galinsoga mosaic virus (Shukla et al., 1979). In a few cases, precautions have been taken to eliminate unrecognized vectors and non-vectored soil transmission (NVST) has been demonstrated (Avgelis, 1985; Shukla et al., 1979). The mechanism of NVST has not been studied but seems to be mechanical inoculation resulting from abrasion of roots. The abrasion can be caused by transplantation of seedlings into infested soil, or by movement of plants or pots. Occasionally, the virus has been said to be "acquired by" roots (Gallitelli et al., 1983; Martelli et al., 1988) but this term is inappropriate because it connotes a vector.

The in vitro type of acquisition and transmission of soil-borne viruses by zoospores is probably several times more efficient than is NVST. Fungal vectors could account for all of the other instances of "soil transmission" reported above, except for trials done in sterilized soil. Furthermore, it is virtually impossible to prove that NVST occurs in the absence of fungal vectors when tests are done in natural soils.

The paradigm of NVST is firmly established for the tombusviruses (Kegler et al., 1991; Murphy et al., 1995). The recognition of CNV as a tombusvirus (Rochon and Tremaine, 1988) with a fungal vector (Dias, 1970) and of fungal vectors for carmoviruses argues for further testing of this paradigm. It is

noteworthy that the fungi tested as vectors for the tombusviruses other than CNV (Campbell, 1968; Campbell et al., 1995; Teakle and Hiruki, 1964) have not included the plurivorus strain(s) of O. bornovanus.

Tombus-, carmo-, and necroviruses, among others, have been detected in rivers, streams, and agricultural water (Koenig, 1988; Li et al., 1992; Tomlinson and Faithfull, 1984; Tomlinson et al., 1983). Viruses in waters are unlikely to cause infections in agricultural crops unless the water is returned to the crops either by irrigation or by flooding and virions are deposited at sites and times of wounding of susceptible cells. This process requires an efficient mechanism considering the dilution of viruses in surface waters. NVST has been invoked but in vitro acquisition by a fungal vector offers a much more efficient method.

References

Avgelis A. (1985) Occurrence of melon necrotic spot virus in Crete (Greece). Phytopath. Z. 114:365-372.

Bock K. R., Gutherie E. J. and Figueiredo G. (1981) Purification and some properties of tephrosia symptomless virus. Ann. Appl. Biol. 97:277-283.

Bos L., Dorst H. J. M.-V., Huttinga H. and Maat D. Z. (1984) Further characterization of melon necrotic spot virus causing severe disease in glasshouse cucumbers in the Netherlands and its control. Neth. J. Pl. Path. 90:55-69.

Campbell R. N. (1968) Transmission of tomato bushy stunt virus unsuccessful with Olpidium. Pl. Dis. Rptr. 52:379-380.

Campbell R. N., Lecoq H., Wipf-Scheibel C. and Sim S. T. (1991) Transmission of cucumber leaf spot virus by Olpidium radicale. J. Gen. Vir. 72:3115-3119.

Campbell R. N., Sim S. T. and Lecoq H. (1995) Virus transmission by host-specific strains of Olpidium bornovanus and Olpidium brassicae. Eur. J. Pl. Path. 101:273-282.

Coudriet D. L., Kishaba A. N. and Carroll J. E. (1979) Transmission of muskmelon necrotic spot virus in muskmelons by cucumber beetles. J. Econ. Ent. 72:560-561.

Dias H. F. (1970) Transmission of cucumber necrosis virus by Olpidium cucurbitacearum Barr and Dias. Virology 40:828-839.

Gallitelli D., Vovlas C. and Avgelis A. (1983) Some properties of cucumber fruit streak virus. Phytopath. Z. 106:149-162.

Gergerich R. C. and Scott H. A. (1991) Determinants in the specificity of virus transmission by leaf-feeding beetles. (pp. 1-13). In: Harris K. F. (ed.) Advances in Disease Vector Research. Vol. 8, Springer-Verlag, New York.

Hampton R. O. and Hancock C. L. (1981) Soil-related greenhouse spread of bean mild mosaic virus. Phytopathology 71:223.

Hobbs H. A. (1981) Transmission of bean curly dwarf mosaic virus and bean mild mosaic virus by beetles in Costa Rica. Pl. Dis. 65:491-492.

Kassanis B. and Macfarlane I. (1964) Transmission of tobacco necrosis virus by zoospores of Olpidium brassicae. J. gen. Microbiol. 36:79-93.

Kegler H., Kontzog H. G. and Kleinhempel H. (1991) The behaviour of plant pathogenic viruses in soil (Review). Arch. Phytopath. Pflschutz 27:339-346.

Koenig R. (1988) Detection in surface waters of plant viruses with known and unknown natural hosts. (pp. 305-313). In: Cooper J. I. & Asher M. J. C. (eds.) Developments in Applied Biology II. Viruses with fungal vectors. Association of Applied Biologists, Wellesbourne, U.K.

Koenig R., Lesemann D.-E., Huth W. and Makkouk K. M. (1983) Comparison of a new soilborne virus from cucumber with Tombus-, Diantho-, and other similar viruses. Phytopathology 73:515-520.

Lange L. and Insunza V. (1977) Root-inhabiting Olpidium species: the O. radicale complex. Trans. Brit. Mycol. Soc. 69:377-384.

88

Li Y., Lesemann D. E., Koenig R., Rudel M. and Pfeilstetter E. (1992) Isometric plant viruses in ditches and streams in agricultural areas: recovery of previously found viruses and identification of hitherto unrecorded carmo- and tombusviruses including grapevine Algerian latent virus. J. Phytopath. 134:121-132.

Martelli G. P., Gallitelli D. and Russo M. (1988) Tombusviruses. In: Koenig R. (ed.) The Plant Viruses. Vol. 3 (pp. 13-72) Plenum Press, New York.

Martini C. (1958) The transmission of turnip viruses by biting insects and aphids. (pp. 106-113). In: Quak F., Dijkstra J., Beemster A. B. R. & van der Want J. P. H. (eds.) Proc. 3rd Conf. Potato Virus Diseases, 1957, Lisse-Wageningen. Vol. Veenman & Zonen, Wageningen.

Murphy F. A., Fauquet C. M., Bishop D. H. L., Ghabrial S. A., Jarvis A. W., Martelli G. P., Mayo M. A. and Summers M. D. (1995) Virus Taxonomy: classification and nomenclature of viruses. Arch. Virol. Suppl. 10, 586 pp.

Rochon D. M. and Tremaine J. H. (1988) Cucumber necrosis virus is a member of the tombusvirus group. J. Gen. Vir. 69:395-400.

Sahtiyanci S. (1962) Studien über einige wurzelparasitäre Olpidiaceen. Arch. Mikrobio. 41:187-228.

Scott H. A. and Phatak H. C. (1979) Properties of blackgram mottle virus. Phytopathology 69:346-348.

Shoyinka S. A., Bozarth R. F., Reese J. and Rossel H. W. (1978) Cowpea mottle virus: a seed-borne virus with distinctive properties infecting cowpeas in Nigeria. Phytopathology 68:693-699.

Shukla D. D., Shanks G. H., Teakle D. S. and Behncken G. M. (1979) Mechanical transmission of Galinsoga mosaic virus in soil. Aust. J. Biol. Sci. 32:267-276.

Teakle D. S. and Hiruki C. (1964) Vector specificity in Olpidium. Virology 24:539-544.

Thouvenel J. C., Tia E. and Fishpool L. D. C. (1990) Characterization of cowpea mottle virus on cowpea (Vigna unguiculata) in the Ivory Coast and the identification of a new vector. Tropical Agriculture 67:280-282.

Tomlinson J. A. and Faithfull E. M. (1984) Studies on the occurrence of tomato bushy stunt virus in English rivers. Ann. Appl. Biol. 104:485-495.

Tomlinson J. A., Faithful E. M., Webb M. J. W., Fraser R. S. S. and Seeley N. D. (1983) Chenopodium necrosis: a distinctive strain of tobacco necrosis virus isolated from river water. Ann. Appl. Biol. 102:135-147.

Waterworth H. E., Meiners J. P., Lawson R. H. and Smith F. F. (1977) Purification and properties of a virus from El Salvador that causes mild mosaic in bean cultivars. Phytopathology 67:169-173.

Weber I., Proll E., Ostermann W.-D., Leiser R.-M., Stanarius A. and Kegler H. (1982) Description of cucumber leaf spot virus, a virus unknown so far in greenhouse cucumber. Arch. Phytopath. Pflschutz. 18:137-154.

FACTORS AFFECTING THE RELEASE OF PRIMARY ZOOSPORES FROM CYSTOSORI OF *SPONGOSPORA SUBTERRANEA* ASSESSED USING MONOCLONAL ANTIBODY ELISA TEST.

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Summary

An ELISA technique was tested to quantify the release of zoospores from *Spongospora subterranea* sporeballs under laboratory conditions to study the factors affecting the process of germination. The ability of sporeballs to germinate under favourable conditions (15°C) was not affected by pre-treatment storage at temperatures ranging from -26 to 35°C. Rapid germination occurred at 15 and 25°C followed by a rapid decline in zoospores number whilst at 10°C zoospore number both increased and declined more slowly. The degree of dormancy within cystosori influenced the response of the spores to root exudates. Dormant spores were stimulated by root exudates of both the host and of non-hosts of *S.subterranea* whereas germination of non-dormant spores was regulated by a suppressive or feed back effect. ELISA enabled the detection of zoospores from *S.subterranea* sporeballs suspended in deionised water at a concentration as low as 10 per ml carbonate buffer although the technique was not reliable in root exudate experiments as the Monoclonal Antibodies reacted strongly with root leachates.

Introduction

Spongospora subterranea is the causal agent of powdery scab, an important and widespread disease of potatoes, and the vector of the Potato Mop Top Virus (PMTV) (Jones and Harrison, 1972). The importance of powdery scab has seriously increased in Scotland over the past 15 years and control of the disease has relied on an Integrated Control Strategy, based on numerous factors related to the characteristics of *S.subterranea*.

Epidemiological studies on *S.subterranea* have been very limited compared to the work on powdery scab under field conditions. Very little has been done on the resting spore germination and its role in the epidemiology of the pathogen (Kole,1954, Merz, 1989). Information on the factors affecting this process are of particular importance in the development of better control measures against both powdery scab and PMTV as zoospores seem to play an important role in both diseases.

In the present study, an ELISA technique, using Monoclonal Antibodies specific to *S.subterranea* zoospore, was evaluated, *in vitro*, to quantify the release of zoospores from resting spores. The effect of temperature and plant root exudates on *S.subterranea* sporeball germination was studied by using either this serological technique or a haemocytometer.

Materials and methods

ELISA technique:

Cystosori of *S.subterranea* were scraped from well-developed powdery scab lesions on washed tubers, passed through a 100 μm sieve and air-dried at room temperature for 24 hours. Zoospore suspensions were prepared by mixing 16 mg aliquots of cystosori with 80 ml deionised water followed by incubation at 15°C in darkness for 5 to 10 days to encourage release of zoospores. Six unpurified Monoclonal Antibodies (Mab's) to the zoospores of *S.subterranea* (Harrison et al., 1994) were purified using an HPLC technique. These Mab's were stored at -20°C and their sensitivity to zoospores evaluated using a PTA-ELISA method (Harrison et al., 1994) on a range of known concentrations of zoospores determined

by haemocytometer. This serological method was used in further experiments on factors affecting sporeball germination either in combination with the haemocytometer or as a substitute.

Effect of temperature on resting spore germination

Two sources of sporeball inoculum (infected Record and Erntestoltz potatoes) were used in the experiments as they gave the most consistent release of zoospores within 5-6 days at 15°C in the dark.

Temperature pre-treatment effect on resting spore viability: Air-dried sporeballs of infected Record potatoes were stored for 7 and 14 days at 6 temperatures (-26, -12, 3, 7, 15 and 35°C). Their subsequent ability to germinate was evaluated in deionised water at 15°C in the dark. Release of zoospores was assessed after 5 days using ELISA.

Temperature effect on zoospore release: Germination of sporeballs (16 mg/80 ml deionised water) from both sources was tested at four incubating temperatures (0, 10, 15, 25°C). The release of zoospores was quantified either after 5 and 10 days with ELISA or daily with a haemocytometer.

Effect of root exudates on zoospore release

Potato and wheat root exudates: Two plastic chambers separated with a mesh were used. In one chamber, sporeballs (16 mg) were suspended in 80 ml deionised water and the roots of a micropropagated Estima potato plant or of a wheat plant placed in the other chamber. The mesh was small enough (1μm) to prevent any movement of either sporeballs or zoospores from one chamber to the other . For the control, no plant was added. The concentration of zoospores was assessed after 5 days at 15°C in the dark by ELISA.

Tomato, oil seed rape and wheat root exudates: Tomato, oilseed rape and wheat seedlings were grown for a week in 1/20 Hoagland solution to promote the release of exudates. Sporeballs (16 mg) were suspended in 80 ml of these different solutions containing the root exudates and incubated at 15°C in the dark. The release of zoospores was assessed after 5 days by both ELISA and haemocytometer. This experiment was repeated 8 times.

Results

The six Mab's tested showed a good sensitivity to *S.subterranea* zoospores when these were suspended in deionised water with a detection limit of 10 zoospores per ml carbonate buffer.

Effect of temperature on resting spore germination

Temperature pre-treatment: Pre-treatment of the sporeballs from -26 to 35°C for either 7 or 14 days did not affect the ability of the spores to germinate. Zoospores were quantified after all the pre-treatments at levels ranging from 0.52 to 0.75×10^6 per g sporeballs.

Temperature effect on zoospore release: After 5 days, sporeballs from infected Record potatoes germinated at all the temperatures tested with an optimum at 15 and 25°C (Fig.1). There was, however, a significant increase in zoospores released at 0°C after 10 days. The apparent decline in zoospores after 10 days at 15 and 25°C may be due to zoospore death.

The effect of temperature on zoospore release was investigated further by looking in more detail at the germination process. The number of swimming zoospores was therefore recorded daily (3 to 11 days) for the Erntestoltz inoculum using a haemocytometer. Germination of spores was rapid at both 15 and 25°C compared to 10°C where zoospores were not recorded before the 5th day (Fig.2). The decline in zoospore number was also slowest at 10°C. No zoospores were recorded at 0°C when sporeballs from Erntestoltz were used.

Effect of root exudates on S.subterranea sporeball germination

Potato and wheat root exudates: The number of zoospores was highest in the presence of potato root exudates although not significantly different to the germination of sporeballs suspended in deionised water only (Table 1). Potato root exudates appeared, however, to have a regulating effect on sporeball germination as there was a significantly lower variance in zoospore release with the root host exudates. Germination of *S.subterranea* was significantly stimulated by wheat root exudates. The low variance indicated that the germination was consistent in both treatments.
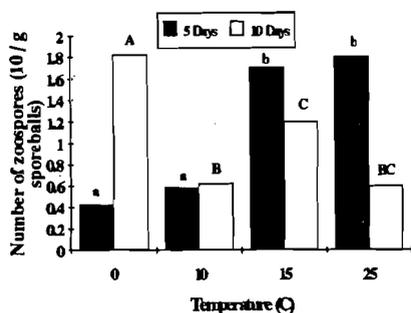
Fig1: Effect of temperature on sporeball germination (Record)
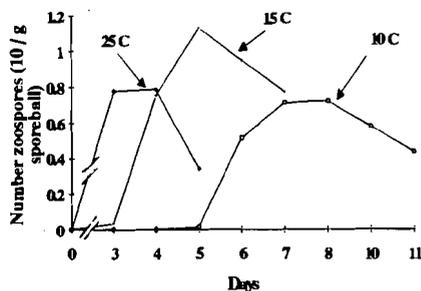
Fig2: Effect of temperature on sporeball germination(Erntestoltz)

Table 1: Effect of potato and wheat root exudates on *S.subterranea* sporeball germination

Number of zoospores(10^6/g sporeballs) after 5 days at 15°C (5 replicates)					
	Mean	Variance		Mean	Variance
Control	3.65	9.31	Control	2.54	0.281
Potato plant	4.30	1.28	Wheat plant	3.76	0.509

Tomato, oilseed rape and wheat root exudates: Two contrasting sets of results were obtained from the 8 repeat runs of this experiment. Results from two experiments typical of these contrasting results are presented in Table 2. In 5 of the repeat experiments, where the release of zoospores in the control was relatively low, there was a stimulating effect of root exudates with a significant increase in sporeball germination in presence of both tomato and oilseed rape root exudates (Group 1). However, in the 3 other experiments, where the germination was high in the control, root exudates appeared to have a suppressive or feed-back effect as there was a significant reduction in the number of zoospores for both tomato and oilseed rape exudates (Group 2). In this experiment, the concentration of the zoospores was assessed by both ELISA and haemocytometer. There was a high level of background reaction with ELISA for the tomato and oilseed rape leachates.

Table 2: Effect of root exudates on *S.subterranea* sporeball germination

	Group 1 (5 expts)		Group 2 (3 expts)	
	Number of zoospores (10^6/g sporeball) after 5 days at 15°C (Mean on 7 replicates)			
Control	0	a	2.266	a
Wheat	0.258	a	1.737	a
Tomato	1.305	b	0.577	b
OSR	1.698	b	0.884	b
LSD (p<0.05)		0.349		0.718

Discussion

Use of Monoclonal Antibodies in ELISA enabled the detection of zoospores from *S.subterranea* sporeballs at a concentration as low as 10 per ml carbonate buffer. This serological method appeared to be reliable in quantifying zoospores from sporeballs in deionised water. A high level of background reaction in the presence of root exudates may be

explained by cross-reaction to other microorganisms (Harrison *et al.*, 1994). The count of zoospores with a haemocytometer appeared to be more reliable in these cases.

S.subterranea can survive in the soil as dormant resting spores for many years and still be able to infect a potato crop. In the present study, temperatures ranging from -26 to 35°C did not affect the ability of the spores to germinate under optimal conditions (15°C). The resistance of the sporeballs to adverse conditions may be due to their thick structure, a 3 layered-wall (Lahert *et al.*1985). This survival structure of the pathogen makes it more difficult to control powdery scab.

Powdery scab is favoured by cool and wet conditions (Wale, 1987). More rapid germination was revealed here at both 15 and 25°C within 5 days but this followed by a rapid decline. In contrast, at the lower temperature 10°C, the release of zoospore was delayed but their number declined more slowly. Thus, the presence of active zoospores for longer at lower temperature may explain the greater infection at low temperature. The effect of 0°C was not clear as different results were obtained for the two inoculum used. Similar results on the effect of temperature were obtained for the crook root fungus on infected watercress roots (Claxton, 1995).

Contrasting responses of sporeballs to the presence of root exudates suggested that the stage of the spores influences their reponse as stipulated by MacFarlane (1959) for *Plasmodiophora brassicae*. Thus, exudates stimulated sporeball germination when little spontaneous germination occured, i.e. when spores were dormant. This stimulation was not host-specific as spore germination was stimulated by wheat and oilseed rape. In contrast, when spores were not dormant (high spontaneous germination), exudates had a suppressive or feed back effect on sporeball germination. Similar results were obtained by MacFarlane (1970) on *Plasmodiophora brassicae* who observed more rapid germination of spores from young galls in the presence of root exudates and a decrease in germination for spores from old galls. The high variation observed in the release of zoospores from the same source of inoculum of *Spongospora subterranea* may be explained by this balance between dormant and non-dormant spores within the cystosori.

References

Claxton, J.R., Arnold, D.L., Blakesley, D. and Clarkson, J.M. (1995). The effects of temperature on zoopores of the crook root fungus *Spongospora subterranea f.sp.nasturtii*. *Plant Pathology* 44: 765-7771.

Harrison, J.G., Lowe, R., Wallace, A. and Williams, N.A. (1994). Detection of *Spongospora subterranea* by ELISA using monoclonal antibodies. *In: Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification* pp.23-27. CAB International, Wallinford, Oxford.

Jones, R.A.C. and Harrison, B.D. (1972). Ecological studies on Potato Mop Top Virus in Scotland. *Ann.appl.Biol.*71:47-57.

Kole, A.P. (1954). A contribution to the knowledge of *Spongospora subterranea* (Wallr.) Lagerh., the cause of powdery scab of potatoes. *Tijdschrift over Plantenziekten* 60:1-65.

Lahert; H. and Kavanagh, J.A. (1985). The fine structure of the cystosorus of *Spongospora subterranea*, the cause of powdery scab of potato. *Canadian Journal of Botany* 63: 2278-82.

MacFarlane, I. (1959). Factors affecting the survival of *Plasmodiophora brassicae* in the soil and its assessment by a host test. *Ann.appl.Biol.*39: 239-256.

MacFarlane, I. (1970). Germination of resting spores of *Plasmodiophora brassicae*. *Transaction of the British Mycological Society* 55: 97-112.

Merz, U. (1989). Infectivity, inoculum density and germination of *Spongospora subterranea* resting spores: a solution culture test system. *European Plant Protection Organisation Bulletin* 19: 585-592.

Wale, S.J. (1987). Powdery scab-are there any easy solutions? *Potato World* 4(4): 8-9.

MOLECULAR STUDIES OF VARIATION AMONGST ISOLATES OF *POLYMYXA GRAMINIS* AND *POLYMYXA BETAE*.

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Summary

Several molecular methods have now been developed to simplify and improve the detection of *Polymyxa* and discriminate between species (*P. betae* and *P. graminis*) and isolates. We previously reported the use of RFLP analysis of PCR-amplified ribosomal DNA to discriminate between species and isolates of *Polymyxa* and we are now characterising these differences further by sequencing. We have sequenced a region of about 850bp in 10 isolates of *Polymyxa* and one isolate each of *Olpidium brassicae* and *Plasmodiophora brassicae*. This consists of about 330 bp at the 3' end of the 16S-like gene, the 5.8S DNA and the two internal transcribed spacers. These sequences are now being used to study phylogenetic relationships between these fungi and to devise primers for specific amplification of *P. graminis* DNA.

Introduction

The genus *Polymyxa*, members of which are responsible for transmission of at least 12 different plant viruses, has two recognised species *P. graminis* and *P. betae*. The two species have different host ranges (*P. graminis* occurs on cereals and grasses and *P. betae* on sugar beet and related plants) but they are morphologically indistinguishable. Because *Polymyxa* species are obligate parasites of plant roots they are difficult organisms to work with. Traditional methods of screening for *Polymyxa* infection involves skilled and tedious microscopic examination and molecular techniques should result in more reliable and quicker methods of detection. They also allow the possibility of distinguishing between isolates e.g. with different host plant or virus specificities.

Several molecular methods are now available which have improved identification and detection of *Polymyxa* species (Mutasa *et al.*, 1993, 1995, Ward *et al.*, 1993, 1994). We have already reported the use of RFLP analysis of PCR-amplified ribosomal DNA from *Polymyxa* and related species for identification and to study differences between isolates (Ward *et al.*, 1994). Results from more isolates are reported below. A region of rDNA was also sequenced to study the relationships further and to provide data to design of PCR primers specific for *Polymyxa* species.

Materials and Methods

Growth of Isolates and DNA Preparations:
Isolates were propagated and DNA extracted from zoospores, resting spores or infected plant roots as described in Ward *et al.*, 1994.
PCR and Digestion of Amplified DNAs:
This was done as described in Ward *et al.*, 1994. The primers used for PCR and sequencing, which recognise conserved regions of fungal ribosomal DNA, are described in Fig. 1 and White *et al.* (1990). For RFLP analysis the primers ITS4 and ITS5 were used to amplify

the DNA, which was then cut, separately, with enzymes *Dde*l and *Cfo*l (*Hha*l).

DNA Sequencing

For sequence analysis the primers NS7 and ITS4 were used to amplify a region of about 850bp (Fig. 1). This region was then sequenced using primers NS7, NS8, ITS4, ITS5, ITS3 and ITS2mod (our modified version of the published ITS2 primer: TTCGCTGCGTTCTTCATCG). Two methods of sequencing were used - direct sequencing of gel-purified PCR products and sequencing of cloned PCR products. Sequences were determined using ABI automated sequencing and analysed using programs in the Wisconsin (GCG) package (Genetics Computer Group, 1994).
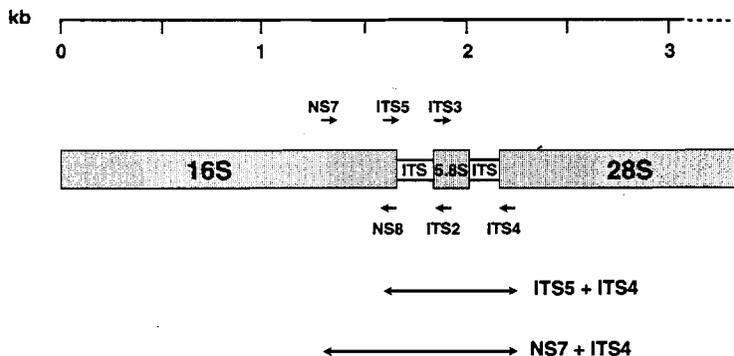
Fig. 1. Nuclear ribosomal DNA regions used for RFLP analysis and sequencing.

Results

RFLP analysis was done on 59 isolates of *P. betae* and *P. graminis* using ITS4 and ITS5 primers for the amplification and restriction enzymes *Dde*l and *Cfo*l. These were isolated from several plant hosts: barley, oats, *Poa* sp., sorghum, sugar beet and wheat, and were from a range of locations worldwide: UK (28 sites), Canada (2 sites), China (2 sites), France, Germany, Netherlands, India and the former Yugoslavia. All but one group of related isolates showed RFLP patterns identical to those already seen before (Ward, 1994). All the *Polymyxa betae* isolates showed pattern A for *Dde*l and pattern D for *Cfo*l (*Hha*l), and all the new *Polymyxa graminis* isolates showed pattern B for *Dde*l and pattern E for *Cfo*l. The only exceptions were 3 isolates which transmit Indian Peanut Clump Virus (see Legrève *et al.,* these Proceedings).

Almost complete sequence has been obtained for 4 *P.betae* isolates, 6 *P. graminis* isolates and one isolate each of *Plasmodiophora brassicae* and *Olpidium brassicae* . Over the NS7-ITS4 region all of the *Polymyxa* isolates showing identical rDNA RFLP patterns also had identical DNA sequences. The NS7-NS8 region of *P. betae* F62 was used to search the EMBL and Genbank databases (using FASTA [Pearson, 1990]) to find the most homologous

sequences from other organisms. The closest matches were to oomycetes (*Phytophthora megasperma* [75%], *Lagenidium giganteum* [75%], *Achlya bisexualis* [75%]) and heterokont algae (*Pseudo-nitzchia multiseries* [75%]). A thorough phylogenetic analysis will be undertaken when the sequencing is complete, but a provisional table of homologies has been produced using FASTA data (Table 1).

Olpidium brassicae was included in this study since it is also a fungal vector of viruses, rather than because any presumed closeness to the plasmodiophoromycetes. The rDNA analysis shows that it is not closely related to *Polymyxa* and *Plasmodiophora*, and the highest homologies were to the Zygomycete fungi *Basidiobolus ranarum* (91%) and *Endogone pisiformis* (90%). There was also high homology to other chytridiomycetes *Spizellomyces acuminatus* (89%) and *Chytridium confervae* (84%).

	Polymyxa graminis F40	*Polymyxa graminis I1-229*	*Polymyxa graminis F51*	*Polymyxa betae F62*	*Plasmodiophora brassicae*	*Phytophthora megasperma*	*Pseudo-nitzschia multiseries*	*Olpidium brassicae*	*Neurospora crassa*
Polymyxa graminis F40	100	98	95	93	84	74	74	72	71
Polymyxa graminis I1-229		100	95	93	85	74	74	72	72
Polymyxa graminis F51			100	94	85	76	76	74	73
Polymyxa betae F62				100	86	75	75	72	71
Plasmodiophora brassicae					100	75	71	70	71
Phytophthora megasperma						100	nd	75	nd
Pseudo-nitzschia multiseries							100	76	nd
Olpidium brassicae								100	82
Neurospora crassa									100

Table 1. Provisional nucleotide homologies (%) between fungal isolates in the NS7-NS8 region of rDNA. The table was constructed using a series of FASTA analyses. Sequence data is not complete on both strands for *Olpidium brassicae* and *Plasmodiophora brassicae*. Sequences used for comparison were obtained from Embl/Genbank - *Phytophthora megasperma* (X54265), *Pseudo-nitzschia multiseries* (U18241) and *Neurospora crassa* (X04971).

Discussion

The RFLP analysis of PCR-amplified ribosomal DNA did not reveal many polymorphisms within *Polymyxa* species. However analysis of this region generally only allows discrimination at the species or variety level, so this result is expected. All 16 *P.betae* isolates so far studied gave the same patterns for digestion with *Dde*I and *Cfo*I. However, only one of these isolates was from outside the UK. There were 3 RFLP groups within *P. graminis*. One of these groups (RFLP type B,E [Ward *et al.*, 1994]) is much more common than the rest, particularly among barley isolates. The second group (RFLP type C,F) of 4 isolates were from 3 hosts - barley, wheat and oats. The third group is probably specific for isolates transmitting Peanut Clump Virus (Legrève *et al.*, these Procedings). Biological studies to see whether these RFLP groups correlate with the ability to infect different host plants or the abilty to transmit virus have so far been inconclusive (Adams *et al.*, 1994). However, it may be significant that

all of the isolates of RFLP type C,F have so far been unable to transmit barley mild mosaic virus.

The sequence analysis indicated that the 3 RFLP groups within *P. graminis* are more closely related to one another than any of them are to *P. betae* (Table 1).

The NS7-ITS4 region in *Plasmodiophora brassicae* is larger than the corresponding region in *Polymyxa* and *Olpidium* (1330bp rather than 850bp). This is due to an insert of about 480bp in the middle of the region where the NS8 primer is situated. A FASTA search of EMBL and Genbank databases revealed that this insert sequence was homologous to type I introns found in several species, and typically in rDNA genes. The 2 closest matches were with the green alga *Mesotaerium caldorum* (homology 68%) and the yeast-like organism *Pneumocystis carinii* (65%). In both of these, the insert occurs in a similar postion to that in *Plasmodiophora* towards the 3' end of the 18S gene.

Acknowledgements

References

Adams, M.J., Ward, E. and Mutasa, E.S. (1994). Variabilité du genre *Polymyxa*: études biologiques et génomiques. *Journée Mosaïques. Blois 7.8 Avril 1994. Recueil de Communications.* pp27-28.

Genetics Computer Group (1994). *Program manual for the Wisconsin Package, version 8.* 575 Science Drive, Madison, Wisconsin, USA 53711.

Mutasa, E.M., Ward, E., Adams, M.J., Collier, C.R., Chwarzczynska, D.M. and Asher, M.J.C. (1993). A sensitive DNA probe for the detection of *Polymyxa betae* in sugar-beet roots. *Physiological and Molecular Plant Pathology* **43**, 379-390.

Mutasa, E.M., Chwarzczynska, D.M., Adams, M.J., Ward, E. and Asher, M.J.C. (1995). Development of PCR for the detection of *Polymyxa betae* in sugar beet roots and its application in field studies. *Physiological and Molecular Plant Pathology* **47**:303-313.

Pearson, W. R. (1990). Rapid and sensitive sequence comparison with FASTP and FASTA. *Methods in Enzymology* **183**:63-98.

Ward, E., Mutasa, E.S., Adams, M.J., Collier, C.R. and Asher, M.J.C. (1993). Development of molecular methods for the identification of *Polymyxa* species. Proceedings of the Second Symposium of the international working group on plant viruses with fungal vectors. C.Hiruki (ed.) American Society of Sugar beet technologists pp. 87-90.

Ward, E., Adams, M.J., Mutasa, E.S., Collier, C.R. and Asher, M.J.C. (1994). Characterization of *Polymyxa* species by restriction analysis of PCR-amplified ribosomal DNA. *Plant Pathology* **43**:872-877.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. (eds). *PCR Protocols. A Guide to Methods and Applications.* Academic Press, San Diego, USA. pp 315-322.

ULTRASTRUCTURAL STUDIES OF RESTING SPORE DEVELOPMENT IN *POLYMYXA GRAMINIS*

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Summary

Transmission electron microscopy was used to study the development and structure of resting spores and sporosori of *Polymyxa graminis.* Lipophilic bodies generally distributed in the cytoplasm moved to the periphery as spores matured. The spore wall consisted of four layers, two of which extended into spines linking adjacent spores. An unstained plug in the wall, facing towards the outside of the sporosorus, was probably associated with germination. Some spores germinated *in situ* by producing primary zoospores. Bundle-like structures in some spores may have been virus particles.

Introduction

The life cycle of *Polymyxa graminis* is not completely understood but has two phases. In the sporangial phase, the plasmodium formed after infection by a zoospore develops into a lobed zoosporangium, which releases more zoospores either to the outside, or to infect deeper layers of the root. Sporogenic plasmodia, initially indistinguishable from the sporangial ones, undergo what appears to be meiotic division and then develop into sporosori (often termed cystosori), each consisting of numerous, thick-walled resting spores which remain viable (together with any viruses they may be carrying) in soil for many years. Here we report transmission electron microscopy of different stages in sporosoral development .

Materials and Methods

Monofungal isolates of *P. graminis* were obtained by isolating sporosori from naturally-infected barley roots and inoculating barley roots in irrigated sand culture (Adams, Swaby & Macfarlane, 1986; Adams & Jacquier, 1994). Roots from inoculated plants heavily infested with sporosori were dried and used as inoculum for further cultures. Pieces of root for electron microscopy were selected under a light microscope at different times after inoculation, fixed in 2.5% glutaraldehyde, post-fixed with osmium tetroxide, dehydrated and embedded in L.R.White medium grade resin containing 1% (v/v) silicon fluid (Dow Corning 200) or in Agar 100 resin using standard techniques. Ultramicrotome sections were stained in uranyl acetate and lead citrate before examination using a JEM-1200EX transmission electron microscope at 60 kV.

Results and Discussion

At the ultrastructural level, the first distinct stage in resting spore development was the development of transitional plasmodia as described by Braselton (1984). This was followed by nuclear division and the division of the plasmodium into uninucleate cells by the formation of membranous sheets within the cytoplasm. A spore wall was then deposited between the cells

and, while the walls remain fused in places, progressively larger spaces developed between the spores. In immature resting spores, lipophilic bodies were generally distributed in the cytoplasm (Fig. 1) but these seemed to move to the periphery as the spores matured (Fig. 2) and sometimes appeared to be associated with a membrane system. Each spore wall had an unstained plug, extending up to about one third of the circumference of the spore and facing towards the outside. We assume that this plug is associated with germination, although it is not clear how it opens to release the primary zoospore.
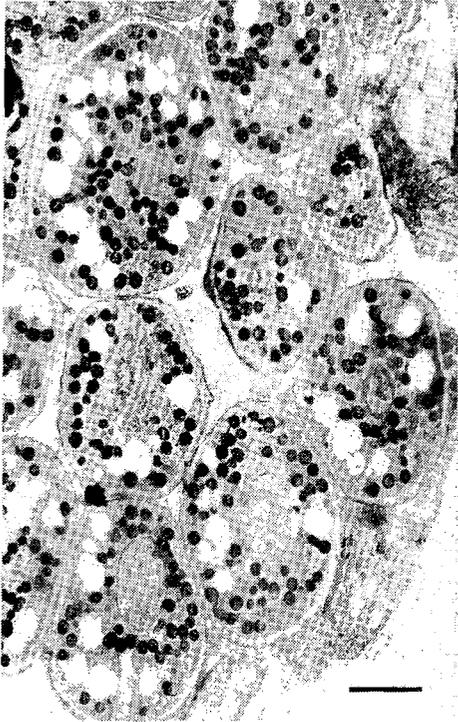
Fig. 1. Immature resting spores of *Polymyxa graminis* with lipohilic bodies generally distributed. Bar represents 1 µm
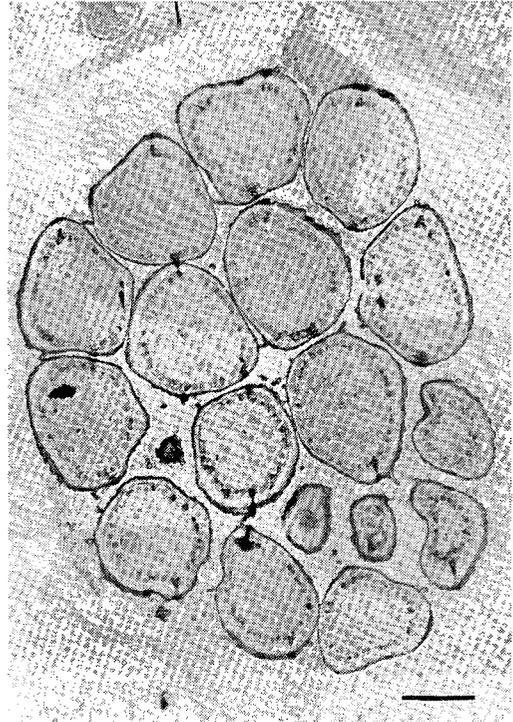
Fig. 2. Mature sporosorus of *Polymyxa graminis* with lipohilic bodies at periphery and germination plugs in the walls. Bar represents 2 µm

In a SEM study of the closely-related *Polymyxa betae*, Ciafardini & Marotta (1988) claimed that immature resting spore walls were folded with numerous empty spaces between them, while spores in older sporosori had an inflated appearance and adjacent spore walls were in contact without empty spaces. This seems to be the opposite of what is shown by TEM and we suggest that artefacts introduced during specimen preparation for SEM may be responsible for the apparent difference.

The mature spore wall consisted of four layers. The plug was within the fourth layer and adjacent spores had connecting links formed from the two outer layers (Figs 3-4). The outer

layer was thin and extremely electron-dense, while the second layer had the appearance of a matrix containing electron-dense granules. The third layer was the least osmiophilic and was microfibrillar in appearance, while the fourth (inner) layer more closely resembled the second in appearance but was very variable in thickness.
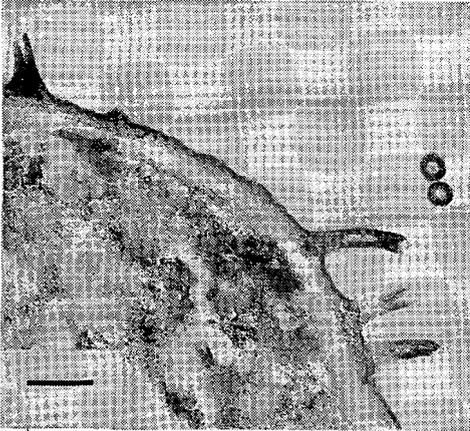
Fig. 3. Four-layered wall of mature spore. Bar represents 500 nm
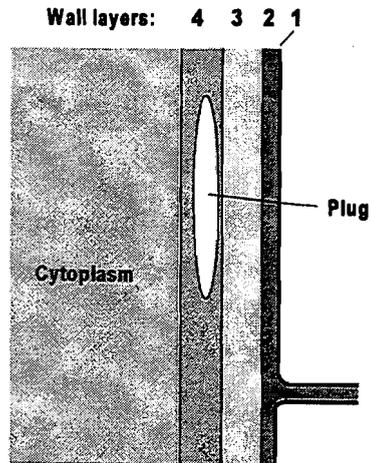
Fig. 4. Diagram illustrating spore wall structure

Fig. 5. Zoospore flagellum inside resting spore. Bar represents 1 μm
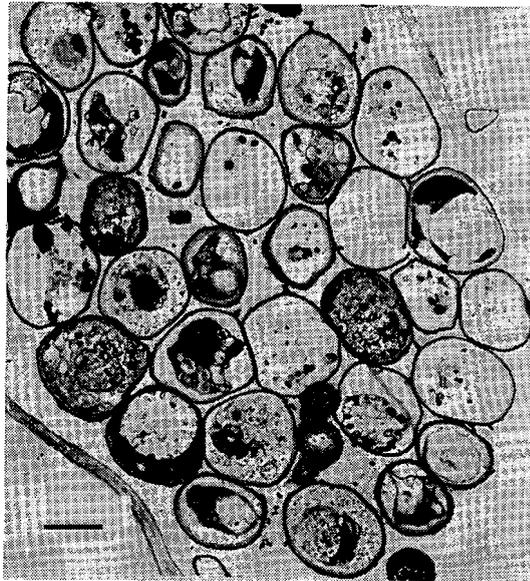
Fig. 6. Sporosorus containing spores at different stages of development into zoospores. Bar represents 2 μm

100
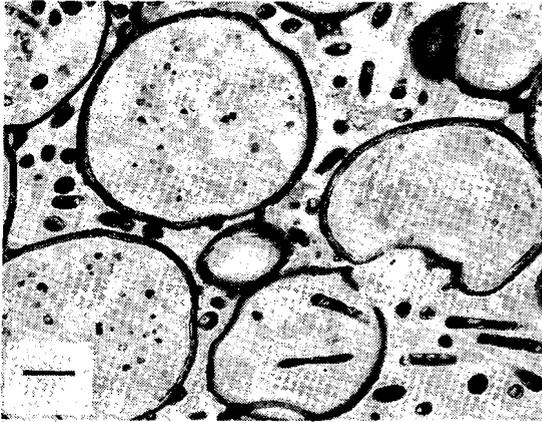
Fig. 7. Sporosorus with empty (germinated) spores occupied by bacteria. Bar represents 1 μm

Fig. 8. Possible virus particles inside resting spore. Bar represents 200 nm

In some spores, the cytoplasm contracted from the wall, the intervening space was filled with a granular matrix (perhaps from the peripheral osmiophilic vesicles), and primary zoospore differentiation began as shown by the occasional presence of flagella (Fig. 5). Frequently, sporosori had individual spores at different stages of development (Fig. 6) and empty spores, usually associated with many bacteria, sometimes occurred (Fig. 7), suggesting that germination had occurred *in situ*. The ability of *Polymyxa* spp. to persist in soil for many years suggests either that spores have a variable, inbuilt, dormancy, or that a host-specific stimulus is needed for their germination. Some filamentous virus-like bundles were observed inside a few resting spores (Fig. 8). Because they were only seen in the cytoplasm of an isolate transmitting BaYMV and appeared similar to virions of BaMMV identified by immunogold labelling in zoospores (Chen *et al.,* 1991), we think that these structures may consist of BaYMV particles.

References

Adams, M. J. & Jacquier, C. (1994). Infection of cereals and grasses by isolates of *Polymyxa graminis* (Plasmodiophorales). *Annals of Applied Biology* **125**, 53-60.
Adams, M. J., Swaby, A. G. & Macfarlane, I. (1986). The susceptibility of barley cultivars to barley yellow mosaic virus (BaYMV) and its fungal vector, *Polymyxa graminis. Annals of Applied Biology* **109**, 561-572.
Braselton, J. P. (1984). Karyotypic analysis of *Polymyxa graminis* (Plasmodiophoromycetes) based on serial thin sections of synaptonemal complexes. *Canadian Journal of Botany* **62**, 2414-2416.
Chen, J., Swaby, A. G., Adams, M. J. & Ruan, Y. (1991). Barley mild mosaic virus inside its fungal vector, *Polymyxa graminis. Annals of Applied Biology* **118**, 615-621.
Ciafardini, G. & Marotta, B. (1988). Scanning electron microscopy of the sporosorus of *Polymyxa betae* (Plasmodiophoromycetes). *Canadian Journal of Botany* **66**, 2518-2522.

WATERCRESS YELLOW SPOT VIRUS (WYSV): CYTOPATHIC ALTERATIONS SUGGEST IT IS A MEMBER OF THE *TOMBUSVIRIDAE*.

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Summary

Ultrastructural observations on sections of watercress tissue infected with watercress yellow spot virus were made using transmission electron microscopy (TEM). Multivesicular bodies, which were never seen in healthy tissues and the cytopathic effects on cell organelles are described. The occurrence and distribution of virus particles are also discussed. Results include features characteristic of members of the family *Tombusviridae* .

Introduction

Watercress yellow spot virus (WYSV) particles were first observed in the UK in 1983 (Walsh, *et al.*, 1989) from watercress (*Rorippa nasturtium-aquaticum*) plants displaying symptoms of watercress chlorotic leafspot disease. Previous attempts to isolate virus particles had been unsuccessful and the causative agent had been described as watercress chlorotic leafspot virus (Tomlinson & Hunt, 1987).

WYSV has isometric particles 37 - 38 nm in diameter and despite having some properties in common with the *Tombusvirus, Carmovirus and Dianthovirus* genera, other properties such as host range, instability of particles, difficulty of mechanical transmission and lack of serological relationship suggest only a distant relationship with these groups. This paper describes cytopathic changes in cells of watercress leaves infected with WYSV, which give strong indications as to which virus family WYSV could belong.

Materials and Methods

Plant material collection
Infected leaves showing the characteristic yellow spots were selected from plants growing in commercial watercress beds in Dorset, in January 1987 and May 1993.
Preparation for TEM
Small portions of leaf including a yellow spot were cut out, fixed in 2 % glutaraldehyde and post-fixed with 1 % osmium tetroxide, each made up in 0.1 M sodium cacodylate or 0.1 M sodium phosphate buffer pH 7.2, dehydrated in a graded ethanol series and embedded in LR White resin. Healthy control leaves were treated identically. 'Silver' ultrathin sections (*ca* 70 nm) were cut with a Diatome diamond knife using a Reichert Ultracut ultramicrotome. Sections were stained with uranium acetate and lead citrate using an LKB Ultrostainer and subsequently viewed and photographed with a JEOL 100 CX II TEM operated at 80 kV.

102

Results

The most striking feature seen in infected tissues was the presence of multivesicular bodies (mvbs), which were never seen in healthy controls and thought to be derived from peroxisomes (Figs 1, 3). The peripheral vesicles of these structures frequently contained fibrils (Fig. 1).

Nuclei were the least affected organelle, even in otherwise severely disrupted cells. Chloroplasts were frequently seen in very close contact with mvbs, but appeared relatively unaffected. Some mitochondria appeared altered in affected cells being dilated and showing reduction in the number of cristae and changes in electron opacity. Some affected cells showed a general increase in vesiculation. Cell walls did not appear to be modified or suberised. Some vacuoles contained a granular substance.

Accumulations of virus particles were seen in very few spongy mesophyll cells (Fig. 2). No crystalline inclusions of virus particles were seen, nor were particles seen in the vacuoles, nuclei, chloroplasts, mitochondria or mvbs. Presumed virus particles were seen in bubble-like protrusions of the tonoplast.

To date no mvbs or virus particles have been seen during examination of ultrathin sections from roots of watercress or in *Spongospora subterranea* f. sp. *nasturtii*, the vector of WYSV (Clay & Walsh, 1990).

Discussion

The cytopathic effects described (induction of mvbs derived from peroxisomes) suggest that WYSV is a member of the family *Tombusviridae* (Martelli & Russo, 1994). The former *Tombusvirus* (Martelli *et al.*, 1988) and *Carmovirus* (Morris & Carrington, 1988) groups have been brought together as two genera of the family *Tombusviridae* (Martelli & Russo, 1994).

It has been suggested that mvbs associated with members of this family may be involved in virus replication (Francki *et al.*, 1985). Fibrils similar to the ones we show in peripheral vesicles of mvbs have been interpreted in studies on tomato bushy stunt virus as nucleic acid suggesting that the mvbs could be the sites of viral RNA synthesis (Francki *et al.*, 1985).

Although virus particles have been observed in spongy mesophyll cells of leaves, the absence of virus particles in other tissues shows that WYSV differs from other reported members of the family, which typically induce large accumulations of virions throughout all types of tissues. This may explain the difficulty of visualising WYSV initially by the quick leaf dip method.

Analysis of the size and structural organisation of the genome of WYSV may clarify its taxonomic position within the family.
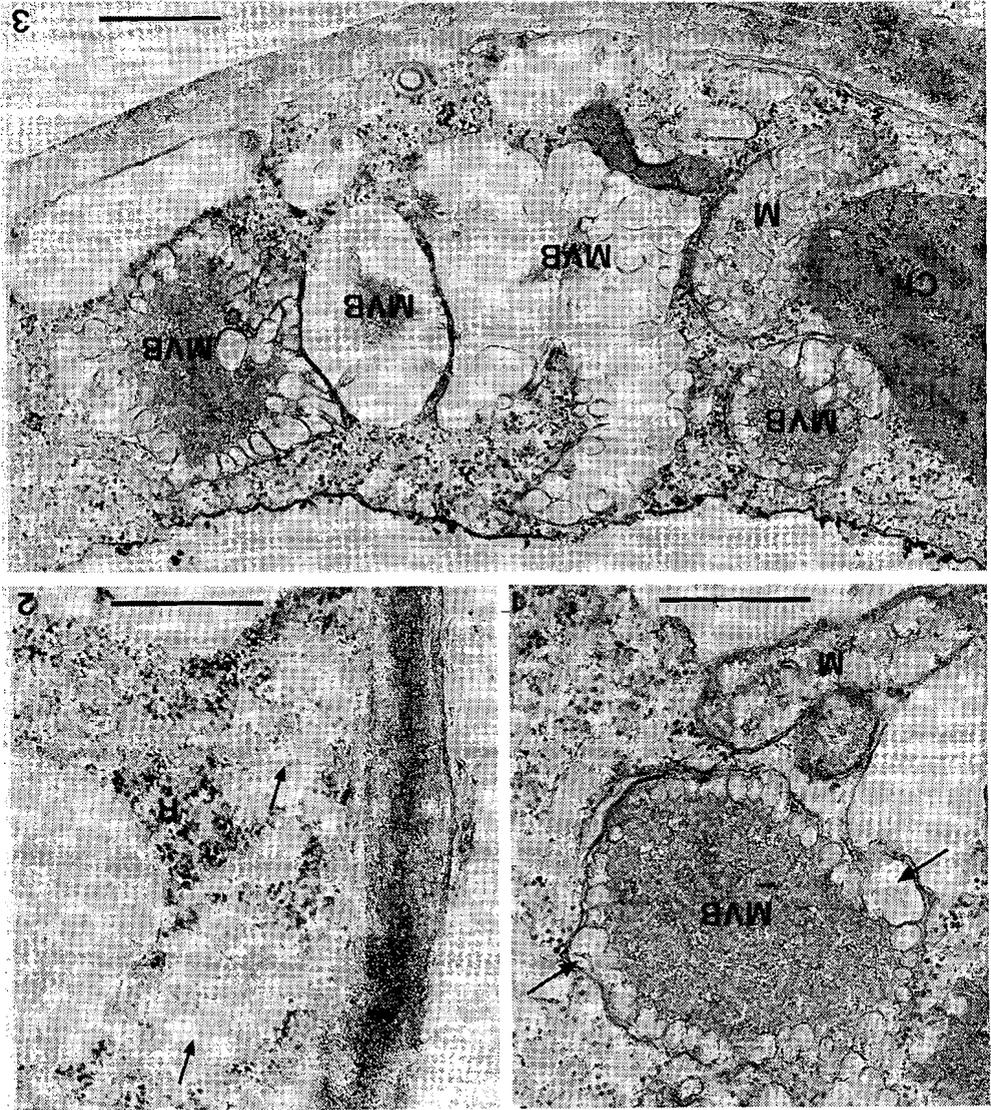
Fig. 1 Thin section showing a mitochondrion (M) and a multivesicular body (MVB) with moderate vesiculation. The vesicles contain densely stained fibrils (arrows).

Fig. 2 Thin section showing WYSV particles (arrows) in the cytoplasm, clearly distinguishable from cytoplasmic ribosomes (R).

Fig. 3 Thin section showing parts of four mvbs (MVB) with moderate to severe vesiculation. The mvbs to the left and right consist largely of the granular matrix of the peroxisomes from which they were derived plus vesicles, whereas the two central mvbs show severe vesiculation. Parts of a chloroplast (Ch), a mitochondrion (M) and the cell wall can be seen.

Figs 1 - 3 Scale bar = 500 nm.

104

References

Clay, C.M. and Walsh, J.A. (1990). Ultrastructure of sporangial development in *Spongospora subterranea* f. sp. *nasturtii* infecting watercress. *Mycological Research* **94**: 462-471.

Francki, R.I.B., Milne, R.G. and Hatta, T. (1985). Tombusvirus group. In: *Atlas of Plant Viruses* (eds. Francki, R.I.B., Milne, R.G. and Hatta, T.), Vol. I, pp 181-197. CRC Press: Florida, USA.

Martelli, G.P., Gallitelli, D. and Russo, M. (1988). Tombusviruses. In: *The Plant Viruses, Volume 3, Polyhedral Virions with Monopartite RNA Genomes* (ed. R. Koenig), pp 13-72. Plenum Press: London, UK.

Martelli, G.P. and Russo, M. (1994). Family *Tombusviridae*. In: *Classification and Nomenclature of Viruses. Sixth Report of the International Commitee on Taxonomy of Viruses* (eds. C.M. Fauquet, F. Murphy, D.H.L. Bishop, M.D. Summers, G.P. Martelli, S.A. Ghabrial and A.W. Jarvis), *Archives of Virology*, Suppl. 10, pp 392-397. Springer-Verlag: Vienna, Austria.

Morris, T.J. and Carrington, J.C. (1988). Carnation Mottle Virus and viruses with similar properties. In: *The Plant Viruses, Volume 3, Polyhedral Virions with Monopartite RNA Genomes* (ed. R. Koenig), pp 73-112. Plenum Press: London, UK.

Tomlinson, J.A. and Hunt, J. (1987). Studies on watercress chlorotic leafspot virus and on the control of the fungus vector (*Spongospora subterranea*). *Annals of Applied Biology* **110**: 75-88.

Walsh, J.A., Clay, C.M. and Miller, A. (1989). A new virus disease of watercress in England. *EPPO Bulletin* **19**: 463-470.

MICROSCOPICAL OBSERVATIONS ON RELEASE AND MORPHOLOGY OF AND HOST INFECTION BY PRIMARY ZOOSPORES OF *SPONGOSPORA SUBTERRANEA* F.SP. *SUBTERRANEA*

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Summary

Zoospores are of particular interest in the life cycle of the plasmodiophorids as they spread infection in the field, carry viruses, their morphology is fundamental in taxonomy and they are the main target of direct control measures. A solution culture test system with tomato bait plants was used to create a pulse of primary zoospore production and subsequent host root infection. Light/fluorescence microscope, TEM and SEM photographs were made. Zoospore formation was found to take less than one hour. Many spores with developing exit pores did not show any changes in cytoplasmic content. Several types of exit pores were observed with an average diameter, for the opening, of 1 μm. The pores were often encircled by a ringlike fusion of the outer wall layers. Primary and secondary zoospores were similar in morphology. Internal 'Rohr'-like structures were observed in encysted zoospores on tomato bait plant roots and zoospores attached to root hairs showed a swelling resembling the 'adhesorium', as described for *Plasmodiophora brassicae*. Papillae and uninucleate plasmodia were observed as post-infection stages.

Introduction

All members of the plasmodiophorids are zoosporic parasites. The zoospore stage is of particular interest because the zoospores spread infection in soil, carry virus and are the main target of control measures. Several authors have made microscopical studies on different aspects of primary or secondary zoospores of *Spongospora subterranea* f.sp. *subterranea* (Ledingham, 1935; Kole, 1954; Diriwächter, 1981). Merz (1992) documented the emergence of secondary zoospores from zoosporangia, their swimming behaviour and their morphology. Nobody has yet investigated the corresponding aspects of primary zoospores. The mechanism of host infection has been studied in detail only for other members of the plasmodiophorids, *Polymyxa betae* (Keskin und Fuchs, 1969) and *Plasmodiophora brassicae* (Aist and Williams, 1971). In this paper, the use of a bioassay to induce release and enable microscopical observation of primary zoospores and spore balls is described and the results of the observations are presented.

Material and Methods

Primary zoospore production:
Spore balls were suspended in nutrient solution and incubated for 10 days as described by Merz (1989). To create a pulse of primary zoospores, the inoculum was then separated from the solution by filtration (10μm), resuspended in fresh nutrient solution and the tomato plants were added. After 5 hours the zoospores were collected by filtration (filtered through 7μm and collected on 0.2μm) and centrifugation and fixed and prepared on glass discs as described in Merz (1992).

Primary zoospore observation:

Before centrifugation small samples were taken for observation of zoospore movement under a light microscope.

After the five hour baiting period the tomato plant roots were washed and immersed in a staining solution of di-ethanol-stilben, a fluorescent dye (500ppm; Gysi, 1975) for 30 min. Root pieces of 1-2 cm were then arranged on slides with a drop of glycerine-PBS-DABCO mounting medium, covered with a coverslip and observed under an epi-fluorescence microscope (with UV excitation).

Resting spore preparation:

Before and one, two or five hours after the tomato plants have been added, the inoculum was again separated by filtration (collected on 7μm), fixed in 3% glutaraldehyde in 0,1 M cacodylate buffer (pH 7,4) for 12 hours, dehydrated in an ethanol series (30/50/70/90/99%, v/v; each for 5 min) followed by 100% acetone. For TEM observation, the spore material was embedded in Epon-Araldit, sectioned and stained with uranyl acetate and lead citrate. For SEM observation, the dehydrated spore material was further 'critical point'-dried from CO_2 and coated with gold palladium (3-30nm).

Results

All inoculum preparations contained numerous resting spores with a developing exit pore showing a ring-like thickening of the innermost wall layer; the part of the layer thus enclosed was organised differently from the remainder. No relationship between these changes in wall structure and zoospore formation could be observed. The number of empty spores increased with increasing baiting period but mature zoospores were never seen inside resting spores. Also non-baited sporosori contained empty spores.

Several types of exit pores were observed with an average diameter of 1μm for the opening. The pores of empty spores were often encircled by a ringlike fusion of the outer

Fig. 1 Resting spores of a sporosorus of *Spongospora subterranea*, prepared after a 5 hour-baiting period for SEM. Most had liberated their zoospores. The exit pores show a ringlike fusion of the outer wall layers and residues of an inner layer (1). Some spores still had an intact inner layer (2) (Bar=1μm).

wall layers, whereas residues of an inner layer could be observed at the edge of the opening (Fig. 1/1). Some exit pores of resting spores which had not yet released their zoospores still showed an intact inner layer (Fig. 1/2).

Swimming primary zoospores showed a straight movement with sudden changes in direction. The biflagellated zoospores had a long flagellum with a tapering end-piece, which trailed behind, and, in the opposite direction, an active short flagellum with an abrupt transition from thick to

thin (Fig. 2/1). The flagellae were inserted laterally at an angle of about 180^0 to each other with a bulging ring at the points of insertion (Fig. 2/2).

When the tomato plant roots - after a 5h baiting period - were stained with an optical brightener and observed under an epi-fluorescence microscope, encysted primary zoospores were seen on the root surface with a pear-shaped, tubular structure inside, with one end bound to the wall. Zoospores which were attached to root hairs showed an adhesorium-like swelling at the place of attachment. In a few cases a bright fluorescing spot could be observed inside root hairs. The first post-infection stage seen under the light microscope was an uninucleate plasmodium.
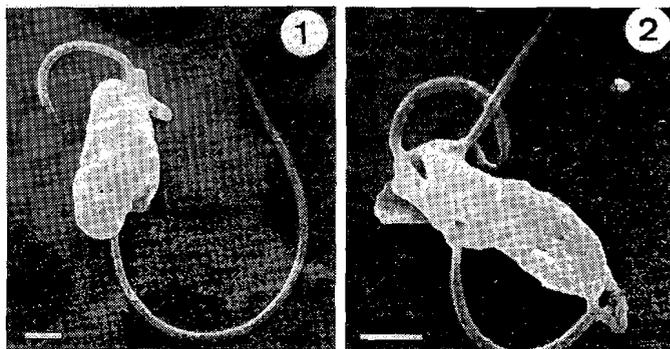
Fig. 2 SEM micrographs of primary zoospores of *Spongospora subterranea*, released from sporosori within a 5 hour-baiting period. A zoospore showing the long flagellum with a gradually tapering end-piece and the short flagellum with an abrupt transition from thick to thin (1). The flagellae are inserted laterally at an angle of about 180^0 to each other showing bulging basal rings at the insertion positions (2). (Bar=1μm).

Discussion

Most of the resting spores released their zoospores within the 5h baiting period. The changes in the wall structure forming the exit pore were also observed in TEM for *S. subterranea* by Lahert and Kavanagh (1985) and for *Plasmodiophora. brassicae* by Yukawa and Tanaka (1979). The postulation of the authors that these changes occur prior to zoospore formation could not be confirmed by the findings reported here. Other work (not reported here) has shown that these changes occur independently of spore ball treatment. As developing zoospores were never seen in the different spore ball samples, it is concluded that zoospore formation may happen in the first part of the baiting period and take place within less than one hour. This confirms findings that bait plant roots can be infected within a baiting period shorter than one hour (not presented here).

The TEM observations on the exit pore structure are similar to those observed in SEM. It is suggested that the ring-like thickening of wall W_3 seen inside intact spores corresponds to the ringlike fusion forming the exit pore of empty spores observed in SEM. Yukawa and Tanaka indicate (1979) the same size of exit pore for *P. brassicae*. As the zoospores have a diameter greater than 1μm they have to force themselves - like the secondary zoospores (Merz, 1992) - or are forced passively through the opening.

So far, emergence of primary zoospores has never been observed. It is therefore difficult to be sure about the correct identity of the swimming zoospores, a subject which has been discussed in more detail by Merz (1992). Apart from this difficulty, both swimming pattern and morphology were similar to those of secondary zoospores.

The elongated cavities observed in encysted zoospores under the fluorescence microscope look strikingly similar to those reported by Keskin and Fuchs (1969) for *Polymyxa betae* and Aist and Williams (1971) for *P. brassicae* zoospores. Keskin and Fuchs called

these 'Rohr' and found that each contained a 'Stachel'. The 'adhesorium', a term introduced by Aist and Williams, located at the end of the 'Rohr', fixes the zoospore body to the host wall. This structure enables the fungus to penetrate the host mechanically. The findings reported here confirm the statement by Braselton (1995) that this infection process is unique and an important feature of the plasmodiophorids.

The optical brightener used as a fluorescent dye in this work is easy to use and helps to reveal more structural details compared to light microscopy. Gisi (1975) used it as a vital stain for growth-studies with *Phytophthora*, giving better results than Calcofluor White.

The further post-infection development seems to follow roughly the description by Aist and Williams (1971) for *B. brassicae*: the bright fluorescing spot inside the root hair is supposed to be a papilla and the uninucleate plasmodia appear similar to one of their micrographs of the same stage.

We know now more of the biology of *S. subterranea* f.sp *subterranea* but there remains still an important gap: little is known about the factors controlling dormancy and zoospore formation and release of the resting spores.

Acknowledgement

References

Aist, J.R. and Williams, P.H. (1971). The cytology and kinetics of cabbage root hair penetration by *Plasmodiophora brassicae. Can. J. Bot.* **49**: 2023-2034.

Braselton, J.P. (1995). Current status of the plasmodiophorids. *Critical Reviews in Microbiology* **21**: 263-275.

Diriwächter, G. (1981). *Spongospora subterranea*, Erreger des Pulverschorfes der Kartoffel: Entwicklung in Kulturmedien und Versuche zur Bekämpfung. *Diss. ETH Zürich*, Nr. 6868

Gisi, U. (1975). Eine neue Methode zur quantitativen Direktbeobachtung der Sporangien von *Phytophthora cactorum* (Leb. et Cohn) Schroet. im Boden. *Z. PflKrankh. PflSchutz* **82**: 30-47.

Keskin, B. and Fuchs, W.H. (1969). Der Infektionsvorgang bei *Polymyxa betae. Arch. Mikrobiol.* **68**: 218-226.

Kole, A.P. (1954). A contribution to the knowledge of *Spongospora subterranea* (Wallr.) Lagerh., the cause of powdery scab of potatoes. *Tijdschr. Pl. Ziekt.* **60**: 1-65.

Lahert, H. and Kavanagh, J.A. (1985). The fine structure of cystosorus of *Spongospora subterranea*, the cause of powdery scab of potatoes. *Can. J. Bot.* **63**: 2278-2282.

Ledingham, G.A. (1935). Occurrence of zoosporangia in *Spongospora subterranea* (Wallroth.) Lagerheim. *Nature* **135**: 394.

Merz, U. (1989). Infectivity, inoculum density and germination of *Spongospora subterranea* resting spores: a solution-culture test system. *EPPO Bulletin* **19**: 585-592.

Merz, U. (1992). Observations on swimming pattern and morphology of secondary zoospores of *Spongospora subterranea. Plant Pathology* **41**: 490-494

Yukawa, Y and Tanaka, S. (1979). Scanning electron microscope observations on resting sporangia of *Plasmodiophora brassicae* in clubroot tissues after alcohol cracking. *Can. J. Bot.* **57**: 2528-2532.

THE p37 OF SOILBORNE WHEAT MOSAIC VIRUS (SBWMV) MOVES FROM CELL TO CELL IN WHEAT VIA PLASMODESMATA

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Summary

Several plant virus movement proteins move from cell to cell when intracellularly injected. The p37 is the putative movement protein of SBWMV. To determine whether p37 can move from cell to cell, the gene encoding p37 from RNA1 of SBWMV was cloned into the protein expression vector pET11-b and the expressed protein was labeled with fluorescein isothiocyanate (FITC). The FITC-labeled p37 was injected into root epidermal cells or cortical cells of wheat seedlings three days after their emergence. Movement of FITC-labeled p37 was followed by fluorescence microscopy. The labeled protein moved from cell to cell rapidly while the fluorescein-labeled dextran of molecular weight 40 kD remained in the injected cells. Hence, p37 moves from cell to cell in the host plant as do movement proteins of other plant viruses.

Introduction

Soilborne wheat mosaic furovirus (SBWMV) is transmitted by *Polymyxa graminis* Ledingham (Brakke, 1987) and causes mosaic leaves and stunting in wheat and barley (Brakke, 1971). The viral genome is divided into two RNA species that are individually encapsidated. One particle is 280-300 x 20 nm and the second is 140-160 x 20 nm. Both RNA species are required for virus replication. RNA1 is 7099 nucleotides (nt) and encodes the RNA replicase in its 5' region (Shirako, 1993). The 37 kD protein is encoded in the 3' end and has sequence similarity with the cell to cell movement protein of red clover necrotic mosaic dianthovirus (RCNMV) (Shirako, 1993). RNA2 is 3593 nt and encodes the 19 kD coat protein at the 5' end. A cysteine-rich 19 kD protein is encoded at its 3' end. Isolates with deletions in the coat protein readthrough region have been characterized (Chen, 1994).

Several plant virus movement proteins (MP) have been microinjected into a single plant cell to follow the movement of the MP, complexes of MP and single-stranded RNA, or coinjected fluorescent dextrans (Heinlein, 1995). These experiments have demonstrated that most plant viral MPs move in host cells of different types. Most experiments used *Nicotiana tabacum* as an experimental host. Although *N. tabacum* has been reported to be a host for some isolates of SBWMV, it is not infected by the isolate found in Oklahoma. The p37 is the putative MP of SBWMV. It has single-stranded RNA binding ability as do of other plant virus MPs (Zhu, 1995). Insertion of the gene encoding the p37 into RCNMV and tobacco mosaic virus resulted in clones that were not infectious on their respective hosts (Zhu, unpublished; Zhu, 1995). To further characterize the p37 of SBWMV, the p37 was

expressed in *Escherichia coli*, the purified protein labeled with fluorescein isothiocyanate, and the movement of the labeled protein injected into wheat root cells was observed by fluorescent microscopy.

Materials and Methods

Expression of p37 in E. coli:

The ORF encoding p37 of SBWMV was amplified by reverse transcription-polymerase chain reaction (RT-PCR) that introduced a *Nde* I site and a *Bam*H I site flanking the 5' end of the initiation codon and the 3' end stop codon, respectively. The PCR fragment was digested and directionally inserted into the expression vector pET11-b. The ligated DNA was transformed into *E. coli* BL21 (DE3) and the p37 expressed following the manufacturer's direction (Novagen, Madison, WI)

Protein purification and labeling:

Expressed protein was dissolved in 0.1 M NaHCO$_3$ with 0.1% SDS to a concentration of 2-5 mg. FITC was added to the protein solution at the ratio of 1 FITC to 10 protein molecules and held in the dark for 1 hour with occasional agitation. The solution was then passed through a 1 ml Sephadex G-25 column and the eluant dialyzed extensively against 10 mM Tris, 1 mM EDTA, 100 mM NaCl, 10% glycerol, 1 mM DTT, and 1 mM PMSF.

Microinjection:

Seedlings of wheat (*Triticum aestivum* L. cv. Vona) were grown for three days on wet filter paper with occasional white light. Roots 3 to 8 cm long, either maintained on or detached from the seedling, were incubated in 0.2 M mannitol for 30 min prior to injection. Roots were kept in the same solution through the experiment for up to 1 hr (Cleland, 1994). Cells in the root hair zone were used for injection.

The movement of the labeled protein was monitored by fluorescence microscope (model No. Optiphot-2; Nikon, Japan) with a blue (BP 390 to 490) excitation filter. Pressure microinjection was performed via a pneumatic PicoPump (model PV820; World Precision Instruments, Sarasota, FL). Images were processed and enhanced by a video-intensified microscopy system (model No. Argus-20; Hamamatsu Photonics K.K., Hamamatsu City, Japan). Images were videotaped by VCR (model No. BR-S378U; Victor Company, Japan) in real time. Processed false color images were displayed on a Sony Trinitron monitor (model No. PVM-1353MD) for direct Photography to a 35-mm color slide film.

Results

The FITC labeled protein moved out of the injected cells to the neighboring cell within 5 minutes (Table I.), (Fig 1A). In contrast, the 40 kD F-dextran remained in the injected cells after 30 minutes (Fig 1B). For each root injected, it was injected three consecutive times in close distance starting from the edge of the root tip. In most cases, the injected p37 moved out of the injected cells as did the 453 D Lucifer yellow. The 40 kD F-dextran never moved out of the injected cells. The 20 kD F-dextran injected also remained in the injected cells.

Table I. Movement of p37 of SBWMV in wheat root[1]		
Treatment	Excised root	Intact root
Labeled p37	10(12)	8(10)
40 kD F-dextran	0(6)	0(5)
20 kD F-dextran	1(5)	0(4)

[1]Number of experiments where movement of fluorescent dye was observed (number of experiments)
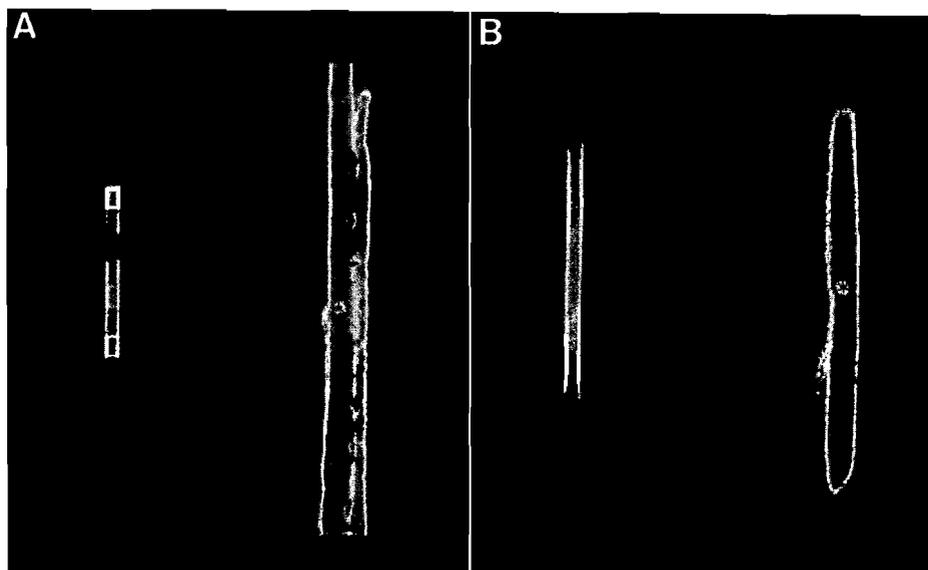
Figure 1.   Cell-to-cell movement of fluorescein isothiocyanate (FITC) labeled p37 of SBWMV in wheat root cells.   The intensities of fluorescence are presented as false-color images as shown by the bars.
A. FITC labeled p37 moved from injected cell to the neighboring cell. White represents the highest intensity.
B. The 40 kD F-dextran remained in the injected cell. Red represents the highest intensity.
* Star indicates the injected cell.

Discussion

Most plant viruses encode a MP to facilitate cell to cell movement.   The MP is thought to sequester the viral RNA to form regular-shaped protein-single stranded RNA

112

complexes and locate to the plasmodesmata (Citovsky, 1990). A domain in the MP may interact with a host factor(s) so that the MP-viral RNA complexes pass through the plasmodesmata. In this process, the size exclusion limit (SEL) of plasmodesmata is increased. Waigmann et al. (1994) reported that injection of TMV MP into tobacco mesophyll cell increased the SEL of plasmodesmata from 1 kD to 40 kD.

In our previous experiments, we determined that the p37 of SBWMV is a ss RNA binding protein. Here, we report that SBWMV p37 moved from cell to cell in wheat root cells. Cleland et al. (1994) reported that the SEL of the wheat root cortical plasmodesmata was 1 kD. The movement of the p37 from cell to cell in the wheat root demonstrated that p37 increase the plasmodesmata SEL to 37 kD. In other studies, it was demonstrated that the fluorescein labeled BL1 MP of bean dwarf mosaic virus (Noueiry, 1994) and RCNMV MP also moved from cell to cell (Fujiwara, 1993). Collectively, it seems a common characteristic for plant viral MPs to move from cell to cell, although the mechanism of cell to cell movement of plant virus is yet to be clarified.

Reference

Brakke, M. K. (1971) C.M.I./A.A.B. Descriptions of plant viruses. No. 77

Brakke, M. K. (1987) Chapter 8 "Wheat Disease" in: Wheat and wheat improvement, 2nd edition, E.G. Heyne, ed., Agronomy Monograph 13. American society of Agronomy, Crop Science of America, and Soil Science Society of America, Madison, WI.

Citovsky, V., Knorr, D., Schuster, G., Zymbryski, P. (1990) Nucleic acid binding by p30 of TMV. Cell 60:637-647.

Cleland, R. E., Fujiwara, T., and Lucus, W. J. (1994) Plasmodesmal-mediated cell-to-cell transport in wheat roots is modulated by anaerobic stress. Protoplasma 178:81-85.

Ding, B., Haudenshield, J.S., Hull, R.J., Wolf, S., Beachy, R.N. (1992a). Secondary plasmodesmata are specific sites of localization of the tobacco mosaic virus movement protein in transgenic tobacco plants. The Plant cell 4:915-982.

Fujiwara T, Glesman-Cokmeyer D., Ding, B., Lommel, S. A., and Lucus W. J. (1993) Cell to cell trafficking of macromolecules through plasmodesmata potentiated by the red clover mosaic necrotic virus movement protein. The Plant Cell 5:1783-1794.

Heinlein M., Epel, B.L., Padgett, H.S., and Beachy, R.N. (1995) Interaction of tobamovirus movement protein with the pant cytoskeleton. Science 270:1983-1985.

Noueiry, A. O., Lucas, W. J., and Gilbertson, R. L. (1994) Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. Cell 76:925-932.

Shirako, Y., and Wilson, T.M.A. (1993) Complete nucleotide sequence and organization of the bipartite RNA genome of soil-borne wheat mosaic virus. Virology 195:16-32.

Zhu X., and Sherwood, J. L. (1995) Characterization of p37 of wheat soil-borne mosaic furovirus (WSBMV). Phytopathology 85:1165.

EFFECTS OF CULTIVAR AND SOWING DATE ON THE INCIDENCE OF BARLEY MOSAIC VIRUSES AND ON YIELD

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Summary

Eight winter barley cultivars were grown at two sites, one heavily infested with barley yellow mosaic virus (BaYMV) and the other with barley mild mosaic virus (BaMMV) for 3 years. There was a single sowing date at the BaYMV site, but three or four at the BaMMV site. Disease incidence and yield data showed that the malting cultivars Pipkin, Halcyon and Puffin were much more susceptible to BaMMV than to BaYMV, whereas the feeding cv. Fighter was equally susceptible to both viruses. At the BaMMV site, disease incidence on the susceptible cultivars decreased as sowing was delayed. Yields of resistant cultivars were usually greatest from the first sowing date, declining steadily as sowing was delayed, whereas susceptible cultivars yielded better after later sowing, although they never reached those of the resistant cultivars sown early.

Introduction

Barley yellow mosaic (BaYMV) and barley mild mosaic (BaMMV) viruses were first detected in the UK in 1980 but were almost certainly present, but unrecognised, for several years before that. Since then, they have been reported from all the areas of the UK where winter barley is grown intensively. BaYMV and BaMMV cause similar symptoms, may occur singly or together within plants, and can only be distinguished by serological tests (Adams, 1991). However, there seem to be important differences between cultivars in their response to the two viruses, which have implications for disease control. There have been reports, mostly of an anecdotal nature, that virus incidence is more severe the earlier crops are sown in the autumn. However, the only experimental evidence is a brief report from Japan (Watanabe, Toshima, Ueda & Ogawa, 1989), where environmental conditions and cultivars are very different to those in Europe. Control of the disease therefore relies upon attempts to minimise the spread of contaminated soil (e.g. on farm machinery) and on resistant (immune) barley cultivars.

Laboratory and field trials have identified a number of UK cultivars which are resistant to both viruses and which yield better on infested sites than susceptible cultivars. The disadvantages of using resistant cultivars have been that on uninfested land yields have often been less than those of the best susceptible cultivars and that no resistant cultivar suitable for malting has been available. Amongst the susceptible cultivars, there appear to be some differences in response to the viruses, but they have not been well characterised. Surveys of virus incidence on samples from different parts of the UK have suggested that malting cultivars are especially prone to BaMMV whereas feeding cultivars are more usually infected with BaYMV (e.g. Adams, 1991; 1993). The principal purpose of this study was to examine the effects of sowing date on the performance of malting barley cultivars on land infested with BaMMV. Delayed sowing would normally be expected to depress yields, but it was thought that this might be offset, at least to some extent, if the crops suffered less from virus. Susceptible and resistant feeding cultivars were also included so that the effects of delayed sowing in the absence of virus could also be assessed. A second objective was to get comparative disease

and yield data for the same group of cultivars grown in the presence of BaYMV, especially because the survey data had suggested that malting cultivars were rarely affected by BaYMV.

Materials and Methods

Eight winter barley cultivars were grown at two sites, one heavily infested with BaYMV and the other with BaMMV in each of 3 years. The sites were on similar soil types and within 4km of each other in Gloucestershire. Both had grown continuous winter barley for more than 10 years and the viruses present had been monitored extensively in previous seasons. There was a single sowing date at the BaYMV site, but three or four at the BaMMV site with plots (16 x 1.25 m) arranged in a three-replicate randomised block design. Sites were visited at monthly intervals during the winter (December to May) and virus incidence assessed by estimating the approximate percentage of plants infected from inspection of several positions in each plot. In each year, samples of leaves with symptoms were collected from plots at both sites (usually 5 plants per plot) and tested by ELISA (Adams, 1991) to determine which virus was present. Grain yields were recorded at harvest.

Results

Virus incidence and yield on selected cultivars from two years of the experiments and at both sites are shown in Table 1. Yields of all cultivars were disappointing, especially in 1994/5, owing to a combination of soil conditions and low rainfall. No disease developed on the resistant cv. Epic at either site, whereas cv. Fighter was highly susceptible to both viruses. In the absence of disease, Fighter would have been expected to yield at least as much as Epic, suggesting yield losses of up to about 30% as a result of infection. The malting cvs Halcyon and Pipkin were highly susceptible at the BaMMV site, but had much less virus at the BaYMV sites, where their yields were sometimes as great as that of the resistant cultivar. Extensive sampling and testing by ELISA demonstrated that the sites had virtually only the one virus present.

Table 1. Virus incidence (% plants infected) and yield (t/ha) of four barley cultivars in two years on sites infested with either BaYMV or BaMMV

	BaYMV site				BaMMV site							
Year ...	1993/4		1994/5		1993/4				1994/5			
Sown ...	7 Oct		22 Sept		22 Sept		16 Oct		23 Sept		13 Oct	
	%	t/ha	%	t/ha	%	t/ha	%	t/ha	%	t/ha	%	t/ha
Epic	0	5.04	0	3.14	0	5.85	0	5.28	0	4.79	0	4.40
Fighter	57	4.75	60	2.93	97	4.50	84	4.76	100	3.25	100	3.69
Halcyon	14	4.86	27	3.52	87	4.51	77	4.85	100	2.95	100	3.65
Pipkin	2	5.02	12	3.55	75	4.02	62	4.44	77	2.37	93	2.73
LSD		0.57		0.53		0.73		0.60		0.71		0.63

The effects of sowing date on virus incidence and yield at the BaMMV site in 1993/4 are summarised in Fig. 1. Yields of resistant cultivars were usually greatest from the first sowing date, declining steadily as sowing was delayed. Amongst the fully susceptible cultivars, virus incidence was greatest at the earliest sowing and declined as sowing was delayed. Yields of these cultivars at the first sowing date were the lowest of any of the dates and the highest yields were at the third (Nov 3) sowing, although they never reached those of the resistant cultivars at the earliest sowing. Delayed sowing also delayed the onset of symptoms (Fig. 2) and the cv. Sprite, which is claimed to have some disease tolerance, escaped symptoms when sown later. In 1994/5, the trends were similar but the effects were usually less than in the previous year, and in 1992/3 when the first sowing was delayed by wet weather, there was less disease and results were not so clear.

Fig. 1. Mean virus incidence (% plants infected) and yield of resistant or susceptible cultivars sown on four dates in 1993 at a site infested with BaMMV
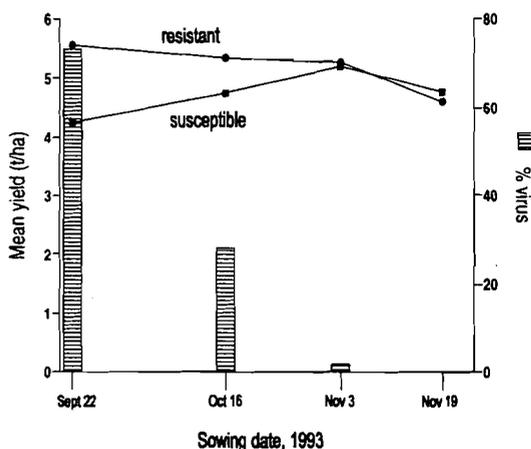
Fig. 2. Symptom development on three barley cultivars sown on 2 dates in 1993 at a site infested with BaMMV

Discussion

The experiments have given a conclusive demonstration, and for the first time under European conditions, that disease (at least if caused by BaMMV) is less if sowing is delayed. The decrease in disease resulting from later sowing was also reflected in the yield data, and it was clear that a susceptible cultivar sown on an infested site would yield better if sown in late October than in late September. It is, however, unlikely that these cultivars could ever be made to yield as well as the resistant ones sown early in the autumn.

The effects of delayed sowing on the disease are probably related to soil temperatures during the first few weeks of crop growth. The higher the temperature, the greater is likely to be the infection by the fungus vector, *Polymyxa graminis*, and multiplication of virus in the root cells. This in turn will lead to more symptoms in winter

and spring when virus moves from the roots to the shoots. This also explains why the effect of delayed sowing on disease expression was less in 1994/5, when the autumn was exceptionally mild, than in the two previous seasons. An effect of autumn temperature on the response to delayed sowing was also noted in Japan (Watanabe, Toshima, Ueda & Ogawa, 1989). In 1994/5 it was possible to compare disease levels on early-sown (3rd week of September) cultivars on the two sites, and this showed that the malting cultivars (Pipkin, Halcyon, Puffin) were much more susceptible to BaMMV than to BaYMV. Indeed, on BaYMV sites, their yields were sometimes comparable to those of the resistant cultivars. This confirms what has long been suspected from analysis of samples sent from commercial crops (Adams, 1991; 1993). By contrast, and rather unexpectedly, the feeding cv. Fighter proved to be equally susceptible to both viruses. Although cv. Sprite is not immune to the viruses, and sometimes a large proportion of plants became infected by BaMMV, symptom development was slower than on the fully susceptible malting cultivars and it appeared to have suffered much less yield penalty from the disease. It can therefore be regarded as partially resistant to BaMMV (as demonstrated in laboratory tests: Adams, 1994) and, like the other malting cultivars, does not seem very prone to BaYMV. There was no evidence in these experiments that BaYMV symptoms appeared earlier in the winter than those of BaMMV as reported by Stanarius, Proeseler & Kühne (1988).

References

Adams, M.J. (1991). The distribution of barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV) in UK winter barley samples, 1987-1990. *Pl. Path.* **40**, 53-58.

Adams, M.J. (1993). Fungally-transmitted mosaic viruses of barley. In: *U.K. Cereal Pathogen Virulence Survey, 1992 Annual Report*, pp. 50-51. Cambridge: UKCPVS Committee.

Adams, M.J. (1994). Partial resistance to barley mild mosaic virus in barley cv. Sprite. *Tests of Agrochemicals and Cultivars* No. 15, (*Ann. Appl. Biol.* **124** Supp.), pp. 78-79.

Stanarius, A., Proeseler, G. & Kühne, T. (1988). Veränderungen im Auftreten von Stämmen des Gerstentgelbmosaik-Virus (barley yellow mosaic virus) in Wintergestenpflanzen eines Befallstandortes. *Arch. Phytopath. Pfl., Berlin* **24**, 437-440.

Watanabe, K., Toshima, I., Ueda, Y. & Ogawa, K. (1989). Effects of the seeding time of two-rowed barley on the yield loss caused by infection with barley yellow mosaic virus. *Proc. Kanto-Tosan Pl. Prot. Soc.* **36**, 30-32.

THE PRESENCE OF *SPONGOSPORA SUBTERRANEA* F.SP. *SUBTERRANEA* IN THE NORTHERN AREAS OF PAKISTAN CONFIRMED BY MICROSCOPY, SEROLOGY AND BIOASSAY

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Summary

Reports in the mid 1980's mentioned the occurrence of powdery scab-like lesions on potatoes in Pakistan, mainly in the plains of the province Punjab. The cause was reported to be *S. subterranea*. There were however, doubts as to the true identity of the causal agent and this led to a collaborative project between CDRI and SFIT, initiated in 1992. Further investigations have so far failed to confirm the presence of *S. subterranea* in this region. During a survey in 1994 in the Skardu region of Northern Areas, potato plants were found in Astak valley with root galls and tuber lesions similar to those caused by powdery scab and these were analysed further. Three plants were replanted in pots containing the original soil. After 4 months one plant again showed root galls on new roots and 'cauliflower' stage lesions on young tubers, typical of powdery scab. Light microscopic preparations of root galls showed the presence of the characteristic spongy-like spore balls with the honeycomb-like spore wall structure. The soil gave a high root infection score for the presence of zoosporangia in a bioassay test using tomato as bait plants. It also reacted very strongly in ELISA with an antisera produced against the sporosori of *S. subterranea*. Preparations from root galls and the `cauliflower' stage lesions also gave high reactions in ELISA.

Introduction

Powdery scab was reported for the first time in Pakistan during a survey of bacterial and fungal diseases of potaoes in 1986 by Turkensteen (1987). He found tubers with what he called 'deep-pitted' lesions mainly in some fields in the plains of Punjab province and confirmed the presence of spore balls of *S. subterranea* by microscopic observation of lesions. Surveys of potato crops in the subsequent years in different regions showed that this type of lesion was much more common than previously expected (Turkensteen, 1988; Ahmad *et al.*, 1991; Iftikhar *et al.*, 1993; Ahmad *et al.*, 1993; Soomro *et al.*, 1994; Munir *et al.*, 1994). The surveys also revealed that common scab, caused by *Streptomyces scabies*, was a widespread disease in many of these areas.

Since powdery scab-like symptoms were also found on numerous tubers in 1991 in a large consignment of imported seed, it was decided to pay more attention to this disease. First contacts were initiated between CDRI, Islamabad, and SFIT. There were increasing doubts as to the true identity of the causal agent of the powdery scab-like lesions. This led to a collaborative project, which was then started in 1992. So far, further investigations (Iftikhar *et al.*, 1995) gave no indication of the presence of *S. subterranea* in the plains.

During a survey of potato diseases in the Skardu region of Northern Areas of Pakistan in 1994, potato plants were found in a field in Astak valley showing root galls and tuber

symptoms typical for powdery scab. The plants together with soil probes from root zones were taken to CDRI, replanted and investigated using visual methods, bioassay and serology.

Material and Methods

Cultivation of plants:
Three plants were replanted in the original soil in pots and cultivated in a growth room at 20 °C with a 16 hour light (10,000 lux) / 8 hour dark regime. The plants were watered when the soil started to dry out. After four month the plants were harvested and the roots and tubers washed for further examination.
A portion of original roots with their galls was dried and stored.

Microscopy:
Transverse sections of fresh galls (from new roots) were made, stained with cotton blue and examined under the light microscope at 40fold magnification.
Bioassay:
The original soil together with two other Pakistani soils (from fields where tubers with 'deep-pitted' lesions were found) and two Swiss soils (highly infested with *S. subterranea* / uncontaminated) were tested using a modified bioassay (Merz 1989). The air-dried soils (100g) were mixed with 0.5l of nutrient solution (NS) and incubated for 10 days after which the tomato bait plants and another 0.5l of NS were added. After baiting for one day the tomato roots were washed and the plants transferred to containers with fresh NS and cultivated for another 7 days. Roots were then stained and examined under a stereo microscope for the presence of zooporangia. Root infection was scored on a scale of 0 (no infection) to 4 (severe infection) as described by Merz (1989).
ELISA:
A polyclonal antiserum to the spore ball stage of *S subterranea* was used in plate-trapped antigen (PTA)-ELISA in all experiments (Merz and Walsh; these proceedings). Soil samples and spore ball preparations were ground in a pestle and mortar in 0.05M sodium carbonate buffer (pH 9.6). Sample homogenates were pipetted into wells of microtitre plates (Nunc-Immuno Plate Maxi Sorp F96) and incubated overnight (16h) at 6°C. Subsequently the plates were incubated for 2h at room temperature with the γ-globulin fraction of the AS. This was followed by goat anti-rabbit γ-globulin conjugated to alkaline phosphatase for 3h at room temperature. Finally the plates were incubated with substrate (2-nitrophenyl in 10% diethanolamine adjusted to pH 9.8 with HC1) at room temperature and the optical absorbance at 405 nm (A_{405}) was measured.
The same soil samples tested in the bioassay were also tested by ELISA. Samples from fresh and dried root galls and tuber lesion scrapings (of the original plant) were prepared and compared with scrapings of 'deep-pitted' lesions and Swiss spore ball material (scrapings of fresh lesion and dried spore ball preparations).

Results

After a cultivation period of 4 months one plant had new roots with galls and lesions in the early 'cauliflower' stage on young tubers showing white, wartlike outgrowths of infected host cells. Parts of the galls were still white whereas others had already turned to a dark-brown colour. When the galls were examined under the light microscope at 40fold magnification, spore balls typical for *S. subterranea* were seen. Their size was in the normal range (40-

80µm). Each consisted of numerous single spores forming the characteristic honeycomb-like structure with hollows.
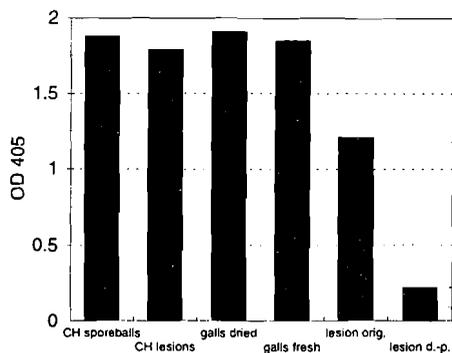
Fig. 1 A_{405} values of *S. subterranea* spore ball prepa-rations and lesion scrapings from Switzerland, samples of galls and lesion scrapings from a Pakistani potato plant with powdery scab symptoms and scrapings of 'deep-pitted' lesions from Pakistani tubers in PTA-ELISA.
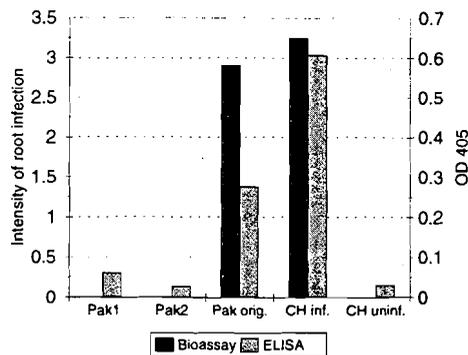
Fig. 2 Comparision between bioassay scores and A_{405} in PTA-ELISA of two Swiss soils (infested with *S. subterranea*/healthy), one Pakistani soil where potato plants with powdery scab symptoms have been found and two other Pakistani soils where tubers with 'deep pitted' lesions were found.

Both dried and fresh galls gave the same A_{405} values as Swiss lesion scrapings and spore ball preparations in PTA-ELISA (Fig. 1). The scrapings of the 'cauliflower' stage lesions produced a weaker but still much higher reaction compared to scrapings of the 'deep-pitted' lesions. When the original Astak soil was baited with tomato plants a root infection score similar to that of a highly infested Swiss soil was obtained. No infection was found in the bait plant roots of the other soils. In PTA-ELISA the A_{405} of the Astak soil was about half of the Swiss infested soil whereas the other soils gave only a weak reaction (Fig. 2).

Discussion

A combination of tests (bioassay/ELISA) and microscopical observation was used to confirm the presence of *S. subterranea* in a soil located in Astak valley, Pakistan. This combination is uniquely characteristic for the identification of the fungus.

Known spore ball material and soil samples from Switzerland were used as controls. The structure and shape of the spore balls from root galls of the plants found in Astak were identical to those originating from Swiss potatoes. Zoosporangia in bait plant roots grown in Astak and Swiss soil were identical.

The antiserum was produced against mature spore balls. The lesions on young tubers were in an early stage of development therefore not many mature spore balls might have been present. This could explain the weaker reaction in ELISA.

The difference in bioassay scores and A_{405} of Astak soil compared to Swiss infested soil shows the different abilities of the two test methods: Highly infested natural soils like the Swiss soil, originating from a potato production area, give both high A_{405} values in ELISA and

root infection rates in bioassay whereas medium infested soils may still cause high infection rates but only moderate reaction in ELISA (Merz and Walsh; these proceedings).

Both bioassay scores and A_{405} values of deep-pitted lesions and soils where tubers with this type of lesion have been found did not gave any indication for the presence of *S. subterranea*.

Based on our observations and tests, we confirmed that the disease found in Astak was powdery scab caused by *Spongospora subterranea* f.sp. *subterranea*. This is the first substantiated report of *S. subterranea* in Pakistan. Further surveys for the presence of powdery scab will be made in the Northern Areas to assess the potential risk for Pakistani potato production through seed trade. In India increasing importance of the disease in the hills was observed in 1985 (Bhattacharyya *et al.*, 1985). However it was found later that the disease could not establish in the plains because of unfavourable environmental conditions (Bhattacharyya, pers. comm.).

Further investigation is also needed identitify of the organism reported to cause powdery scab-like or 'deep-pitted' lesions on tubers.

References

Ahmad, I., Soomro, M.H., Iftikhar, S. and Aslam, M. (1991). Investigations on powdery scab of potato in Pakistan. Pakistan Agricultural Research Council, Crop Diseases Research Institute, Pakistan Swiss Potato Development Project, pp 50.

Ahmad I., Iftikhar, S., Soomro, M.H. and Aslam, M. (1993). Distribution of powdery scab of potato in the irrigated plains of Central Punjab, Pakistan. Proccedings of the Fourth National Conference of Plant Scientists, February 16-18, 1993, AARI, Faisalabad, Pakistan, pp 26.

Bhattacharyya, S.K., Raj, S. and Dwivedi, R. (1985). Sources of resistance to powdery scab in potatoes. *Indian Phytopathology* **38**: 174-175.

Iftikhar, S., Ahmad, I., Soomro, M.H. and Aslam, M. (1993). Powdery and common scab in spring potato crop in irrigated plains of Central Punjab (zone2) and Pabbi area of NWFP, Pakistan. CDRI-PSPDP, Islamabad, Pakistan, pp 15.

Iftikhar, S., Ahmad, I. and Merz, U. (1995). Powdery scab of potato in Pakistan: Identity of the pathogen. Proceedings of the National Seminar on Research and Development of Potato Production in Pakistan. PSPDP, PARC, Islamabad, Pakistan, 156-161.

Merz, U. (1989). Infectivity, inoculum density and germination of *Spongospora subterranea* resting spores: a solution-culture test system. *EPPO Bulletin* **19**: 585-592.

Munir, A., Iftikhar, S., Ahmad, I., Soomro, M.H. and Aslam, M. (1994). Tuber diseases of potato in autumn 1992-93 crop in Pak-Swiss potato Development Project Pilot Areas of irrigated plains of Central Punjab (zone2), Pakistan. CDRI-PSPDP, PARC, Islamabad, Pakistan, pp 14.

Soomro, M.H., Ahmad, I., Iftikhar, S. and Aslam, M. (1994). Powdery and common scab of potato in the Galis, Hunza-Gojal (NA) and Kaghan Valley, Pakistan. CDRI-PSPDP, PARC, Islamabad, Pakistan, pp 17.

Turkensteen, L.J. (1987). Survey on bacterial and fungal diseases of potato in Punjab Province and Pappi area of NWFP, Dec., 1986, PSPDP, PARC, Islamabad, Pakistan, pp 24.

Turkensteen, L.J. (1988). Survey on bacterial and fungal diseases of potato in Balochistan Province of Pakistan, Aug., 1987, PSPDP, PARC, Islamabad, Pakistan, pp 11.

SURVEY OF SOIL-BORNE VIRUS DISEASES OF SUGAR-BEET IN ITALY

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Summary
Most Italian sugar beet-growing areas were surveyed to determine the incidence of beet necrotic yellow vein virus (BNYVV), beet soil-borne virus (BSBV), and beet soil-borne mosaic virus (BSBMV also called Tx7). During 1994 and 1995, 170 soil samples were collected from 126 sites in 27 provinces. Sugar beet seed was planted in the soil samples, and root tissue was later harvested and tested by ELISA: 29% were positive for BNYVV alone, 5% for BSBV alone, and 35% BSBV and BNYVV in mixed infection. Samples positive for BSBMV with ELISA were never confirmed by Western blot analysis or immuno sorbent electron microscopy (ISEM): BSBMV presence in the soil samples tested was therefore excluded.

Introduction

Rhizomania, is a very damaging disease of sugar beet which can cause yield losses up to 45-50% in taproot weight, and up to 60-79% in sugar content (Casarini Camangi, 1987). The disease was first described in Italy in the mid 1950's (Canova, 1959). An epidemiological study carried out during the early sixties showed that infection was widespread in the Po Valley sugar-beet growing areas (Bongiovanni, 1964) . Etiological and fungal transmission studies provided the basis for a different approach to "Rizomania" diagnosis (Canova, 1966; Faccioli and Giunchedi, 1974). BNYVV was then 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). Studies carried out in Italy over the last 15 years mainly concentrated on genetic improvement of sugar beet varieties for resistance to BNYVV and on its localization in the taproots tissues (Giunchedi and Poggi Pollini, 1987; Giunchedi *et al.*, 1987) To date, a few soil samples in the Po Valley area tested positive for beet soil borne virus (BSBV) (Giunchedi, personal com.; Lindsten, 1993).
Recently, a partial molecular characterization of two Italian BNYVV isolates from the Po Valley classified them in the strain group A (Kruse *et al.*, 1994; Koenig *et al.*, 1995).
Our report highlights the prevalence and importance of other furoviruses in the main Italian sugar beet production areas. Some preliminary results are given of a future study on biological and molecular diversity of a wide collection of Italian BNYVV and BSBV isolates.

Materials and methods.
Soil sample collection.
Soil samples were collected during autumn and winter of 1994, and 1995 from 170 fields previously planted with sugar beet in 126 towns distributed in the most productive regions in Italy. Five subsamples were randomly collected in each field and bulked. Soil from each bulked sample was mixed with sterile sand (1/4, soil/sand) and placed in 13 cm diameter pots and planted with sugar beet seed cv Duro. Two replications for each sample were planted.

Serology on sugar beet bait plants

Sugar beet, roots grown for 6-8 wks, were assayed for the presence of BNYVV, BSBV and BSBMV with various serological methods. Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) was carried out according to Clark and Adams (1977). Rootlets samples were ground in a mortar in 1:10 (w:v) phosphate buffered saline (PBS) containing 0.05% Tween-20 and 2% PVP (m.w. 25,000). BNYVV antiserum was purchased from Bioreba Inc., Chapel Hill, NC. BSBV antiserum, provided by K. Lindsten, was diluted 1/200 for the coating step, and 1/100 for the coniugated antibody step. For BSBMV (Tx7), primary antibodies and biotinilated secondary antibodies were kindly provided by C. Rush. Two different ELISAs were processed: a DAS-ELISA was carried out as above than, the biotinylated secondary antibody was probed with avidin-conjugated alkaline phosphatase (Sigma), diluted 1/3000 in PBS. A protein A sandwich ELISA (PAS-ELISA) was carried out as described by Rubies-Autonell and Turina (1995): Tx7 IgG were used at 1/1000 dilution for both steps. Positive and negative controls were included for each test. Values at 405 nm were read with a microplate reader. Absorbance values of at least three times the average of the healthy controls were considered positive.

SDS-PAGE and Western blot analysis were carried out on the samples positive for BSBMV in DAS-ELISA according to the standard protocols (Towbin *et al.*, 1979).

Immuno-sorbent electron microscopy (ISEM) and gold labelled antibody decoration (GLAD) were carried out on ELISA positive samples for BSBV and BSBMV according to the protocols described previously (Rubies-Autonell and Turina, 1995). Tissue print immunoblotting (TiPIB) was carried out according to the protocol described in detail by Resca *et al.* (1992).

Mechanical inoculation

Mechanical inoculation of *C. quinoa* and *C. amaranticolor* was carried out using infected beet rootlets sap diluted 1/4 (w/v) in 0.1 M Na-K phosphate buffer with 0.2% sodium sulfite. *Chenopodium spp.* were grown in the greenhouse at 27°± 1°C with natural illumination supplemented in winter with 3000 lux, and a 16 h photoperiod (tube Fluora Osram 77).

Double stranded RNA analysis

Double stranded RNA (dsRNA) analysis was carried out on five BNYVV isolates mechanically transmitted to *C. quinoa* using the LiCl separation method (Morris and Dodds, 1979).

Results

Out of 170 soil samples, 49 were positive for BNYVV alone, 8 for BSBV, and 59 for both viruses. Both viruses are widespread in the beet growing areas, and virtually no province was free of the Rhizomania complex disease (Figure 1). Western blot analysis and GLAD confirmed the presence of BSBV coat protein and particles respectively in the positive DAS-ELISA samples. TiPIB on sugar beet infected by BSBV gave a further confirmation of the presence of the virus. Some samples had mean values higher than the Tx7 control in Avidin-Biotin DAS-ELISA, not confirmed by PAS-ELISA, Western blot analysis and mechanical inoculation on test plants.

After transmitting 5 BNYVV isolates to *C. quinoa* and *C. amaranticolor* leaves, 3 different patterns of symptom expression were observed. Nevertheless, DsRNA analysis from *C. quinoa* leaves infected with the 5 BNYVV showed the same pattern for RNA1, RNA2 and RNA3 size.

Figure 1. Distribution of BNYVV (A) and BSBV (B) infected soil samples.

Discussion
The results of our survey confirm the presence of viruliferous *Polymyxa betae* resting spores throughout all Italian sugar beet growing areas. A map of BSBV incidence in Italy was prepared for the first time, and it indicastes that this virus is endemic. Further studies on its importance for the damage caused to the Italian sugar-beet crops are being carried out. Although several positive samples were obtained for BSBMV (Tx7) with the Avidin Biotin DAS-ELISA, they were never confirmed with other methods of diagnosis and isolation: therefore its presence in the soil samples tested must be excluded, even if we can not explain the positive ELISA. The result of our dsRNA analysis did not link the symptomatic differences observed on *C. quinoa* with a difference in the RNA1, RNA2 and RNA3 pattern: a different method of extraction (Hutchinson *et al*, 1992) could lead to a better separation from contaminant ribosomal RNA which prevented screening for the presence and size of the smaller dsRNAs which are likely to be involved in symptom expression on *C. quinoa*. Furthermore, many symptomatic differences have already been noted for BNYVV isolates on *C.quinoa*, but they did not correspond to differences in the original BNYVV isolates present in the sugar beet taproots (Koenig *et al*, 1986). Future studies on the molecular aspects of the Italian BNYVV isolates will take in account the techniques already used and will provide new data for the maps of the pathotype distribution in Italy.

124

References

Bongiovanni, G.C. (1964). La diffusione della rizomania in Italia. *Inf. Fitopat.*, **14**:263-265.

Canova, A. (1959). Appunti di patologia della barbabietola. *Inf. Fitopat.*, **9**:390-396.

Canova, A. (1966). Si studia la rizomania della bietola. *Inf. Fitopat.*, **16**:235-239.

Casarini Camangi, P. (1987). Incidenza della rizomania sulla produzione quali-quantitativa della barbabietola da zucchero. *L'Inf. Agr.*, **43**:149-152.

Di Franco A. e D. Gallitelli. (1980). La Rizomania della bietola da coste. *Inf. Fitop.* **3**:9-11.

Faccioli, G., and L. Giunchedi (1974). On the viruses involved on rizomania disease of sugarbeet in Italy. *Phytopath. Medit.*, **13**:10-16.

Giunchedi, L., and C. Poggi Pollini. (1988). Immunogold-silver localization of BNYVV antigen in susceptible and moderately resistant sugar-beets. *Phytopath. Medit.* **27**:1-6.

Giunchedi, L., M. De Biaggi, and C. Poggi Pollini (1987). Correlations between tolerance and Beet Necrotic Yellow Vein Virus in sugar beet genotipes. *Phytopath. medit.*, **26**:23-28.

Hutchinson, P.J., Henry, C.M. and Coutts, R.H.A. (1992). A comparison, using dsRNA analysis, between beet soil-borne virus and some other tubular viruses isolated from sugar beet. *J. gen. Virol*, **73**:1317-1320.

Koenig, R., Burgermeister, W. Weich, H., Sebald, W., and Kothe, C. (1986). Uniform RNA patterns of beet necrotic yellow vein virus in sugarbeet roots, but not in leaves from several plant species. *J. gen. Virol*, **67**:2043-2046.

Koenig, R., P. Luddecke, and A.M. Haeberlè. (1995). Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphism of immunocapture RT-PCR products. *J. of Gen. Virol.* **76**:2051-2055.

Kruse, M. R. Koenig, A. Hoffmann, A. Kaufmann, U. Commandeur, A.G. Solovyev, I. Savenkov, and W. Burgermeister. (1994). Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. *J. of Gen. Virol.* **75**:1835-1842.

Lindsten, K. (1993). Rhizomania- are both Beet necrotic yellow vein virus (BNYVV) and Beet soil-borne virus (BSBV) involved? Proceedings of the 2nd Symposium of the international Working Group on Plant viruses with Fungal vectors. Montreal, Canada, July 25-27, 1993., pp.67-70

Rana, G.L., A. Di Franco, and M. Russo. (1978). La rizomania della barbabietola in Italia meridionale. *Inf. Fitopat.* **2**:5-7.

Resca, R., Giunchedi, L., and Poggi Pollini, C. (1992). Localizzazione immunologica del virus agente della rizomania nell'impronta su membrana di radici di barbabietola. *Sementi elette*, **38**: 21-24.

Rubies Autonell, C. and M. Turina. (1995). Characterization and cytopathology of Hibiscus latent ringspot virus isolated from kenaf (Hibiscus cannabinus L.) in Italy. *J. Phytopath.*, **143**:211-215

Rubies Autonell C., M. Turina, and M.G. Fantino. (1995). Infezioni da virus in alcune coltivazioni di spinacio in Italia. *Inf. Fitopat.* **1**:43-47.

CONTROL OF AUGUSTA DISEASE CAUSED BY TOBACCO NECROSIS VIRUS IN TULIP AFFECTED BY CULTURE CONDITIONS AND SOIL DISINFESTATION

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Summary
The control of Augusta disease caused by the fungus *Olpidium brassicae* and tobacco necrosis virus (TNV) in tulip was studied. In early October-planted bulbs in the field high infection rates were obtained contrary to those planted at Occtober 28 or later. High rates were obtained in boxes under favourable moisture conditions kept in a store room for 14 days at 5, 9, 13 and 17°C in November and December. The mostly variable rates during forcing were considerably decreased in two treatments after late planting. Very substantial differences between cultivars were found in the recurrence of symptoms in replanted bulbs which were not evident in the primary infection. The spread of infectivity on to a field by soil remains and tunics and roots appeared in the second year after application. The soil disinfestation by cyprofuran, dazomet, etridiazool, and metalaxyl proved ineffective, or even was stimulatory on the infection. Tolclofosmethyl was considerably effective. The impact of the various factors involved in the control was discussed.

Introduction
The incidence of Augusta disease was at high rate in the late 1980s. Especially in one region on heavy soil types, whereas in the 1990s the occasional occurrence was prevalent (Asjes, 1993). The soil infectivity in its persistence and spread of the fungus/virus complex was described elsewhere (Asjes and Blom-Barnhoorn, 1997). In this paper data about control by the effect of culture conditions and soil disinfestation will be presented.

Material and methods
Trial fields. Fields previously grown with severely primarily infected tulips were used, viz., 1990-1995 on heavy soil (16.5-25.5 % lutum; indicated as 'loam'), 1990-1992: on sand (3 % lutum; 'sand'), and a second on sand in 1992-1993. The plots (1x2 m; 100-120 bulbs (10-11 cm) in 10 rows) in triplicate were flanked by paths (0.5 m).The test cultivars in loam and sand were in 1990: Don Quichotte, in 1991: Angelique, in 1992 on sand: Leen van de Mark , and in 1993 and 1994 on loam: Apeldoorn. A thermograph registered temperatures in loam and sand at 15 and 35 cm depth in 1990.
Soil infestation. In 1993 an amount of moist soil remains from severely infected forced tulips was mixed in loam at 0-15 cm and 0-30 cm depth before planting. Previously the plots were practically free from Augusta disease (0-1 %) in 1991 and 1992.
Soil disinfestation. The chemicals were 1. cyprofuran in Tubosan (20% powder) at normal dosis of 30 g/m^2; 2. dazomet in Basamid (100% powder; normal dosis 20 g/m^2); 3. etridia-zool in AAterra (liquid; 700 g/l; normal dosis 4 ml/m^2), 4. metalaxyl in Ridomil (5G; powder; normal dosis 20 g/m^2), and 5. tolcolfosmethyl in Rizolex (powder 50%; normal dosis 10 g/m^2). The amounts were either spread with sand, or with water (2 l) and mixed through the soil at 0-15 and 0-30 cm depth before planting.

Box trials. PVC-like boxes described elsewhere (Asjes and Blom-Barnhoorn, 1997) were filled with infested field sand stored before at 5°C in plastic bags and planted with bulbs (2 x 20), furtherly kept at, 5, 9, 13 and 17°C for 14 days with sprinkling and then brought to the field. Forcing trials were also done in these boxes.

Results
Planting date in the field
In 1990 tulips planted on different dates in loam and sand were infected, respectively, in cv. Angelique (Oct. 7): 35 and 68%, (Oct.31): 0.3 and 3 %, and (Nov.21): 0 and 0%; in cv. Christmas Marvel (Oct. 7): 42 and 46%, (Oct.31): 0.7 and 0.5% and (Nov.21): 0 and 0 %; and in cv. Don Quichotte (Oct.7): 0.3 and 4%, (Oct.31): 0.7 and 0.5% and (Nov.21): 1.3 and 0%. Symptoms mostly in dwarfed plants were most severe after the October 7 planting. The data indicated that planting dates strongly affected the infection rates in cvs. Angelique and Christmas Marvel. Soil temperatures with daily fluctuations were in October ca. 15°C at 15 cm and ca. 13°C at 35 cm depth. In November and December temperatures went down to ca. 9-7°C and 8-1°C at 15 cm, and 10-8°C and 8-3°C at 35 cm, respectively.

Planting in boxes in storage rooms
In 1990 trials with cv. Angelique started on October 24 rated 85, 100, 100, and 100% infection, if the boxes were kept at 5, 9, 13, and 17°C, respectively. In cv. Christmas Marvel started on November 20 these rates were 85, 95, 95, and 90%. In 1991 in cv. Angelique in a trial at 5, 9, and 17°C started on December 15 the rates were on soil 1: 11, 16, and 54%, on soil 2: 0, 25, and 11%, and on soil 3: 17, 12, and 93%, respectively. The data indicate that tulips were infected under favourable moisture conditions at different temperatures late in November and December.

Forcing to flower in the greenhouse in winter
In 1990 81 secondarily infected lots of 23 cultivars showed symptoms rather more affected by the sensitivity of cultivars than by the treatments subjected to bulbs to induce flowering in late January and March (Asjes 1993). In 1991 47 and 14% infection was observed after the forcing (20°C till planting in boxes followed by 17 weeks at 9°C) of secondarily infected cv. Angelique planted on October 7 and November 4, respectively. In the growers' forcing primary infection more often occur in early October-plantings than later on. Severe infection may occur in some series of forced tulips and not in others, notwithstanding the planting in similarly infested soil (Asjes and Blom-Barnhoorn, 1997).

Primary susceptibility and recurrence of secondary symptoms
In box trials four of 35 cultivars, viz., Arma, Lustige Witwe, Prinses Irene and Roccoco, did not become infected. In samples from field lots (50-90% infection) nine cultivars, e.g., Angelique, Apricot Beauty, Arie Alkemade's Memory, etc., proved most sensitive during the forcing and culture on sand by the recurrence of symptoms up to one third of the primary rates, whereas on loam this was near to the previous field rate. In little sensitive cultivars these rates were 0-5% and 10-25%, respectively. The sensitive cultivars rated in between. The data indicate that primary high rates were not similar to those found in the forcing and culture in sand, and in loam more proportionate in the most sensitive cultivars.

Spread of infectivity by soil and tunics and roots debris

The infectivity of soil from the rinsing of bulbs after lifting, especially from heavy soil, and of debris of soil, tunics and roots from the cleaning of bulbs, and of soil remains from the forcing of bulbs was earlier indicated (Asjes and Blom-Barnhoorn, 1997).

In 1994 humid soil remains was spread over the soil surface and mixed with the loam at different depths. In 1995 in cv. Apeldoorn no symptoms of infection were observed. In box trials with soil sampled from these three plots per treatment in July 1995 rates of infection obtained in 1996 in cv. Apeldoorn were: 0-3 cm: 25% (12-40%), 0-15 cm: 71% (52-82%), and 0-30 cm: 48% (25-78%). In 16 samples from plots with high infection rates in 1991 and 1992 the average rate in 1996 was 9% (0-38%). So the effect of soil infestation in 1994 was indicated in 1996, while the original infestivity of soil observed in 1991 and 1992 had been decreased considerably.

Soil disinfestation

Soil disinfestation was done in loam with cyprofuran, dazomet, etridiazool and tolclofosmethyl with the following results: 1990 (October 7); 1. dazomet; 0-15 cm depth: 0 g/m^2: 14; 10 g/m^2: 45, 20 g/m^2: 18, and 30 g/m^2: 1%; 0-30 cm: 10 g/m^2: 53, 20 g/m^2: 52, and 30 g/m^2: 1%; 2. cyprofuran; 0-15 cm depth; 30 g/m^2: 35%; 3. metalaxyl; 0-15 cm depth; 10 g/m^2: 17%. The data indicate that dazomet gave control in two and the infection was increased in three treatments. Cyprofuran gave an increased rate of infection.

1991 (October 7); dazomet; 0-15 and 0-30 cm depth; 0 g/m^2: 20 and 15%; 20 g/m^2: 36 and 31%; 40 g/m^2: 46 and 28%; 60 g/m^2: 22 and 31%, and 80 g/m^2: 36 and 9%. The October 28-treatments rated 1% (0-2%). The October 7-treatments indicated the increased incidence after the use of dazomet.

1991 (October 7); etridiazool; 0-15 and 0-30 depth; 0 ml/m^2: 6 and 25%; 4 ml/m^2: 16 and 1%; 8 ml/m^2: 11 and 4%; 16 ml/m^2: 6 and 11%. The data indicate variable control.

1991 (October 7); tolcofosmethyl; 0-15 and 0-30 cm depth; 0 g/m^2: 15 and 13%; 10 g/m^2: 5 and 1%; 20 g/m^2: 2 and 5%; 40 g/m^2: 5 and 2%. The data indicate considerable control. The October 28-treatments of both compounds rated 0 and 0% (0-1%).

In sandy soil treatments were applied in 1992 as follows:

1. etridiazool; October 7; 0-15 and 0-30 cm depth; 4 ml/m^2: 9 and 12%; 8 ml/m^2: 6 and 11%, and 16 ml/m^2: 1 and 3%. The treatments with four times the normal dosis of 4 ml/m^2 was effective and comparable with other non-treated parts of the field. October 28-treatments gave average infection of 0 and 0.3%;

2. metalaxyl; October 7; 0-15 and 0-30 cm depth; 10 g/m^2: 8 and 28%; and 20 g/m^2: 18 and 13%; October 28: average 1.1% (0-2%). The data indicate increase of infection if applied on October 7;

3. tolclofosmethyl; October 7; 0-15 and 0-30 cm depth; 10 g/m^2: 1 and 4%; and 20 g/m^2: 0 and 1%; October 28: 0, 2, 1 and 0%. The data indicate very substantial control of Augusta disease.

The data of all years indicate that the control by tolclofosmethyl was effective, whereas dazomet, cyprofuran, etridiazool, and metalaxyl mostly were not, or even stimulated the infection. The planting at October 28 proved the most effective in the control.

Discussion

The very low rate of infection after late planting at the end of October confirmed earlier data (Van Slogteren, 1963). This may be due to the prevalent soil temperature near to 15°C in October at which the optimal release of fungal zoospores of *Olpidium brassicae* occurs (Nahata et al., 1988). However, the increase of inoculum potential from infested soil by favourable moisture conditions largely overcame the control by late planting. Symptomless plants, possibly because of underdeveloped infection throughout the whole plants, especially after late planting, may show up in the next season in replanted progeny bulbs in heavy soil, particularly if the most sensitve cultivars are grown (Asjes, 1993; Nahata et al., 1988).

The spread of infectivity in remnants with the disease complex obtained from water mixed with hightly infestive loam or debris of soil, tunics and roots, which was stuck to healthy bulbs of four susceptible cultivars failed to infect any bulb grown in loam afterwards. The soil infectivity apparently decreased in 1993 when the trial field laid fallow. The infectivity introduced into the soil did not become evident in the following season, which may be due to the incomprehensible build-up of inoculum potential. Activities of loosening the soil, the depth of planting except shallow (5-10 cm), the relative compaction, the excessive wetting of soil, or the prevention of water influx on top of the soil for weeks, were not in any way indicative in this process (Asjes, unpublished results).

Soil disinfestation treatments may be subject to the more or less prominent patchy occurrence of variable infestive soil. Nevertheless most chemicals showed no control, or rather stimulation of infection, which is contrary to earlier reports on substantial control by dazomet (Van Slogteren, 1970) and metalaxyl (Nahata et al., 1988). So far the most effective control in the field will be by late planting, while tolclofosmethyl is applicable if it is necessary to plant early, and in pot soil used for forcing, especially if the most sensitive cultivars are grown.

References

Asjes, C.J., 1993. Occurrence and recurrence of symptoms of Augusta disease caused by tobacco necrosis virus in tulip. Proceedings Second Symposium International Working Group on Plant Viruses with Fungal Vectors, Montreal, Canada, 141-144.

Asjes, C.J., and Blom-Barnhoorn, G.J., 1997. Soil infectivity and occurrence of Augusta disease caused by tobacco necrosis virus in tulip. Proceedings Third Symposium International Working Group on Plant Viruses with Fungal Vectors, Dundee, Scotland, submitted.

Nahata, K., Kusaba, T., and Mukobata, H., 1988. Studies on the ecology and control of tulip virus diseases. Bulletin Toyama Agricultural Research Centre 2: 1-132.

Slogteren, D.H.M. van, 1963. Infectieproeven met gebroeide tulpen op grond besmet met tabaksnecrosevirus ('Augustaziek'). Jaarverslag Laboratorium voor Bloembollenonderzoek: 50-51.

Slogteren, D.H.M. van, 1970. Augustaziek in tulpen (tabaksnecrosevirus). Jaarverslag Laboratorium voor Bloembollenonderzoek: 34.

SOIL INFECTIVITY AND OCCURRENCE OF AUGUSTA DISEASE CAUSED BY TOBACCO NECROSIS VIRUS IN TULIP

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Summary

The incidence and maintenance of the fungus/virus complex *Olpidium brassicae*/tobacco necrosis virus (TNV) was studied. The infectivity in the soil profile occurred at depths down to 60 cm. Infested sandy soil was infective up till 1:40 dilution. The complex in peaty pot soil and loam kept under desiccating conditions at 5°C after storage for 3 - 8 years caused symptoms in tulips planted in boxes in the field. The debris of soil, tunics and roots stored under fairly humid conditions at 5°C infested non-polluted soil effectively. The infectivity of severely infected lots of tulips was transferred to fields planted in the following season. Healthy roots up till 30 cm down in the profile were infected. To some extent horizontal spread of infectivity occurred in the soil. The impact over the years of the infectivity of soil, cleaning debris of bulbs and migration of highly infected Augusta-diseased lots under different culture conditions is discussed.

Introduction

Tobacco necrosis virus (TNV) as cause of the 'Augusta disease' named by its occurrence in cv. Queen Augusta in 1928 (De Bruyn Ouboter and Van Slogteren, 1949) occurs worldwide in tulips, e.g., Britain (Mowat, 1970), Denmark (Lange, 1976) and Japan (Nahata et al., 1988). In the Netherlands in the late eighties high virus rates were fairly frequently observed in cultivars mainly grown in heavy soil types in some regions (Asjes, 1993). In the 1990 - 1996 period the incidence was only occasional. In this paper data on the incidence and maintenance of soil infectivity over the years will be presented.

Material and methods

Storage of material. Soil of fields, or from the rinsing of lifted bulbs, debris of soil, tunics and roots from the cleaning of bulbs, and soil remains plus tunics and roots from tulips forced to flower in boxes in the greenhouse in winter were stored in plastic bags at 5°C to preserve fairly humid conditions over the years.

Trials in boxes. PVC-like boxes (37 x 55 cm; 10 cm in height plus 5 cm at two sides) with a multi-open frame in the bottom and sides to accomodate equivalent moisture regimes with soil in the open field were used. The bottom covered with fairly wide-meshed nets prevented leakage of soil. The boxes in duplicate planted with 20 bulbs (circumference 10 - 11 cm) of different cultivars (Ang. = Angelique; Ap. = Apeldoorn; CM = Christmas Marvel; DQ = Don Quichotte; LM = Leen van de Mark) on top (10 - 12 cm) of infested soil or other debris was covered with disease-free sand (ca. 10 cm) in the field.

Results

Infectivity in soil profile

In primary infestations in cv. Prominence sandy soil of the profile was collected in 1990. In 1991 cv. Ang. planted in boxes was infected at rates of 5% (0 - 10 cm), 40% (10 - 20

cm), 30% (20 - 30 cm), and 50% (30 - 40 cm).
Similar samples from loam with cv. DQ caused infection in cv. Ang.: 0 - 10 cm: 80%, 10 - 20 cm: 70%, 20 - 30 cm: 80%, 30 - 40 cm: 55%, 40 - 50 cm: 15%, and 50 - 60 cm: 15%. So the infectivity was detected throughout the profile.

Soil dilutuion
The sand and loam diluted with sand and heavy loam, respectively, gave infection of 60% in cv. Ang. in sand at 1:2, 1:4, and 1:8, and 55% in cv. DQ with 1:16, and 10% with dilutions of 1:20, 1:30, and 1:40. Loam mixtures of 1:4 and 1:8 gave 65% infection in cv. Ang.. So the soil was infective at high dilutions.

Retention of infectivity in dry soil
In 1988 primarily infested heavy loam planted with cv. Ang. was collected and afterwards continuously stored at 5°C without any protection against desiccation. Samples of this gave infection rates of 53% in cv. Ang. and 11% in cv. Ap. in 1992, 29% in cv. LM and 70% in cv. Ap. in 1994, 0% in cv. Ap. in 1995, and 60% in cv. Ap. in 1996.
In early 1993 remains of peaty pot soil with roots and tunic debris of primarily infected forced tulips cv. CM was collected and stored at 5°C without plastic enclosure. In 1994 in box trials the infection rate in cv. Ap. was 50%, and in 1996 in cv. Ap. 85%. A sample similarly kept at 20°C gave 10% in 1995. So the infectivity survived several years under conditions of desiccation.

Retention of infectivity in humid soil
In 1990 sand was collected from primarily infected tulips cv. Ang. and stored at 5°C in plastic bags. In 1996 in cv. Ap. 65% infection was observed. Loam collected in 1992 gave 75% infection in cv. Ap. in 1996. Peaty soil from forcing cv. Monte Carlo in open soil under glass collected in early 1992 gave a rate of 50% in cv. Ang. in 1993, and 40% in cv. Ap. in 1996. Peaty pot soil from boxes with forced tulips cv. Monte Carlo collected in early 1993 gave infection rates of 10% in 1994, 80% in 1995, and 100% in 1996. So the infectivity was well maintained for years during the storage at 5°C in plastic enclosure.

Infectivity of soil rinsed from lifted bulbs
Soil rinsed with water from bulbs lifted out of heavier soil types than sand was collected in 1991 and tested on the retained infectivity in 1992: Ap.: 0%, Ap.: 5%, LM: 5%, and LM: 15%.

Infectivity of debris of tunics and roots
In 1993 debris of tunics and roots free from soil was collected from secondarily infected tulip cv. Ang.. This debris mixed in coarse sand in boxes gave 15% infection in cv. Ap.. The debris of primarily infected cv. DQ gave 70% infection in 1994.

Infectivity of debris of soil, tunics and roots
Debris of soil, tunics and roots from cv. Ang. mixed in coarse sand gave infection rates in cv. Ap. of 35% in 1991, 100% in 1992, 80% in 1994, and 15% in 1996. Similar results were obtained from the samples stored outside in the open. So the infectivity was well preserved over the years.

Infectivity of soil after replanting infected bulbs
In 1991 soil was collected in fields with replanted lots of previously severely infected lots (70 - 90%) of cvs. Ang., CM, and Monte Carlo from different locations. These gave the following infection rates, viz., location 1 (sand): Ap.: 0%, LM: 0%, and Ang.: 20%; location 2 (sand): Ap.: 80, 60, 55, 10, and 0%; location 3 (sand): Ap.: 75%, and LM: 20%; and location 4 (loam): Ap.: 80 and 70%, LM: 15 and 25%. So the newly planted fields originally free from the Augusta disease complex appeared infestive.
These rates were compared with tests of soil from fields replanted with severely infected lots of cvs. Ang., Apricot Beauty, CM, and LM in the regions where high infection rates prevailed in the late 1980s. These rates were: location 1 (sand): Ang.: 85%; location 2 (loam): Ap.: 0% and LM: 35%, and location 3 (loam): Ap.: 20% and LM: 70%. The infectivity of these samples was comparable with that reflected in the data of the newly planted fields. The soil (loam) from the severely infested fields with cv. Ang. in 1988 and grown with grass till 1991 gave rates in cv. Ap.: 0, 0, and 0%, and LM: 5%. Similar samples from sand severely infested in 1990 with cv. Ang. and grown with hyacinths gave no infection from the soil collected in 1991. So the high level of infectivity originally detected was not found later on.

Depth of inoculum in soil infective to roots
Infestive sand or loam in boxes to infect roots of cv. DQ directly (layer 1 = 1-1; depth of root in contact with soil: 0 cm), or at a level below 1-1 either for 10 cm (half-layer 2 = hl-2; 15 cm), or up to the bottom of 1-1 (full-layer 2 = fl-2; 10 cm) was used. The interspace between hl-2 and 1-1, was filled with non-infested sand. The results were: 1. Infested sand in 1-1: 25 and 75%; in fl-2: 25 and 45%; and hl-2: 25 and 15%; 2. Infested loam in 1-1: 55 and 75%, in fl-2: 10 and 15%, and hl-2: 0 and 25%; 3. Infested sand in fl-2 and non-infested loam in l-1: fl-2: 45 and 10%, and hl-2: 90 and 60%; 4. Infested loam in 1-2 and non-infested loam in 1-1: hl-2: 20 and 25%, and fl-2: 5 and 25%; 5. Infested sand or loam at layer-3 (l-3; 30 cm) did not cause symptoms of infection. If infested sand in l-2 (15 cm) and l-3 was tested with coverage of sand free from boxes, disease rates of 20 and 15% were obtained, respectively. Infested loam at 1-3 did not cause symptoms. The data indicate that roots growing up to 30 cm in depth may be infected by the fungus-virus inoculum.

Horizontal spread of infectivity in sand
Debris of soil (loam), tunics and roots of cv. Ang. was mixed with non-infested sand in boxes. The dilution 1:1 rated in cv. Ang.: 80% and in cv. DQ: 75% infection. The dilution 1:10 rated in cv. Ang.: 45% and in cv. DQ: 45%, both uniformly over the area of the boxes. The dilution 1:1 at 5 cm from one side of the box gave 15% in cv. Ang. and cv. DQ, over or nearest the infested soil debris. If the bottom was covered with plastic the rates were 35% in cv. Ang. and 20% in cv. DQ. A similar experiment with soilless debris of tunics and roots gave the following rates: 1:10 in cv. Ang.: 30 and cv. DQ: 10%; 5 cm-debris: 0% in cv. Ang. and cv. DQ; and 5 cm-debris with plastic on the bottom: cv. Ang.: 30% and cv. DQ: 40% nearest to the infested area. The data indicate that the infectivity remained fairly confined to the area with the infested material and it was enhanced by favourable moisture conditions.

132

Discussion

The soil infectivity of primarily TNV-infected tulips was detectable deep down the soil profile. The persistent infectivity in soil from forced tulips variably induced Augusta disease at low rate in some forced sets of tulips (Asjes, unpublished results). The incidence of Augusta disease not necessarily linked to the high level of soil infectivity was also observed in a trail field used from 1990 till 1995 (Asjes, unpublished results). The spread of the fungus/virus complex to other fields was indicated by soil rinsed from bulbs after the lifting from heavier soil types than sand, especially in rainy lifting seasons, or by debris of soil, tunics and roots obtained after the cleaning of bulbs, or by the transfer of Augusta-infested lots. The latter is often done to sandy soil to decrease the rates of symptom development most strongly, if the tulips were grown in loam or clay in the previous season (Asjes, 1993). The extent of infestation of new fields may differ considerably as the recurrence of Augusta disease greatly differed per cultivar (Asjes, 1993). The build-up of infestive soil to crops such as tulips as affected by this incoming material, or in the field as such is not comprehensible because of a diversity of factors involved, e.g., increase and release of fungal zoospores of *Olpidium brassicae* (Nahata et al., 1988), infestive occurrence in heavy and light soil, soil moisture, effect of weeds and crops (Lange, 1976; Nahata et al., 1988), and the different reflection of varying weather conditions per season over the years (Asjes, 1993). The retention of the fungus/virus infectivity in the soil remainder of forced tulips, and in field soil (loam) stored under desiccating conditions for years is surprising as the virus is known to be non-persistently transmissible (Adams, 1991; Campbell, 1993), and it is not to be maintained in rest sporangia (Kassanis, 1970), while it may likely be on soil components which is apt for further investigation.

References

Adams, M.J., 1991. Transmission of plant viruses by fungi. Annals of Applied Biology 118: 479-492.

Asjes, C.J., 1993. Occurrence and recurrence of symptoms of Augusta disease caused by tobacco necrosis virus in tulip. Proceedings Second Symposium International Working Group of Plant Viruses with Fungal Vectors, Montreal, Canada, 141-144.

Campbell, R.N., 1993. Persistence: a vector relationship not applicable to fungal vectors. Phytopathology 83: 363-364.

De Bruyn Ouboter, M.P., and Van Slogteren, E., 1949. Augustaziek der tulpen, een virusziekte van het tabaksnecrosetype. Tijdschrift Plantziekten 55: 262-267.

Kassanis, B., 1979. Tobacco necrosis virus. CMI/AAB Descriptions of Plants Viruses, 14

Lange, L., 1976. Augusta disease in tulips. Field experiments concerning tobacco necrosis virus and its vector Olpidium brassicae. Tidskrift Planteavl 80: 153-169.

Mowat, W.P., 1970. Augusta disease in tulip - a reassessment. Annals of Applied Biology 66: 17-28.

Nahata, K., Kusaba, T., and Mukobata, H., 1988. Studies on the ecology and control of tulip virus diseases. Bulletin of the Toyama Asgricultural Research Centre 2: 1-132.

TRANSGENIC RESISTANCE TO POTATO MOP-TOP FUROVIRUS

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Summary

In previous work (Reavy et al. 1995) we found that transgenic *Nicotiana benthamiana* plants, transformed with a sequence encoding the coat protein (CP) gene of a Scottish isolate of potato mop-top furovirus (PMTV), were highly resistant to inoculation with PMTV. Transgenic seedlings (T_1 generation) from additional lines have now been tested for resistance to PMTV; some lines were immune, i.e. no plants supported viral replication, whereas other lines were only partially resistant and a proportion of transgenic plants became infected. Various characteristics of the CP transgene, such as gene copy number, level of RNA transcript and coat protein accumulation were assessed in pooled plant samples from different lines. However, although these characteristics varied between lines, the observed variation could not be correlated with differences in resistance to inoculation. Transgenic plants were also highly resistant to inoculation with four Scandinavian isolates of PMTV.

Introduction

Potato mop-top furovirus (PMTV) is responsible for economic losses in potato crops grown in areas with cool climates and has been identified in Northern Europe, Canada, China, Japan and the Andean region of South America. PMTV is transmitted by the motile zoospores of the plasmodiophoromycete fungus *Spongospora subterranea* (Arif *et al.*, 1995). Infection with the virus can cause some yield loss but more importantly, qualitative damage, known as 'spraing', which can occur as brown arcs and circles in the flesh of tubers of susceptible cultivars (Harrison and Jones, 1971). Some potato cultivars are particularly sensitive and PMTV-infected plants produce tubers with severe spraing symptoms. For example, cv. Saturna, which is widely used in the Scandinavian potato processing industry, is a particularly sensitive cultivar. Effective and environmentally acceptable chemical control of the fungal vector is not commercially available, and there are no sources of resistance or tolerance to PMTV that have been deliberately used in breeding programmes.

The advantages of transgenic resistance to plant viruses using pathogen-derived transgene sequences, are well established and there are many examples where this approach has been successfully applied against potato viruses. The most frequently-used pathogen-derived transgene sequences are those encoding viral coat proteins, and from which coat protein-mediated resistance (CP-MR) is derived. Reavy *et al.* (1995) demonstrated that some lines of *Nicotiana benthamiana* transformed to express the coat protein gene from a Scottish isolate of PMTV, were immune to infection with two Scottish isolates following manual, graft or fungal inoculation. We now report tests to examine whether the CP transgene derived from the Scottish isolate provides resistance against Scandinavian PMTV isolates. We also describe preliminary work in which we have attempted to identify some features of the behaviour of the CP transgene which can be correlated with its effectiveness in different independent transformed lines of *N. benthamiana*.

Materials and Methods

Transgenic plants:
Transgenic lines of *N. benthamiana* expressing the CP gene were obtained as described by Reavy *et al.* (1995). The transgene contained cDNA encoding the CP gene of a Scottish isolate of PMTV (T isolate) under the transcriptional regulation of a cauliflower mosaic virus 35S promoter. All experiments were made with plants grown from seed (T_1 generation) arising from self-fertilisation of the original transformants (Reavy *et al.*, 1995). Seedlings of transgenic test plants were grown without phenotypic selection and transgenic plants were identified by PCR using oligonucleotide primers which amplified the CP transgene.

Virus isolates and inoculation:
Scandinavian and Scottish isolates of PMTV were maintained in *N. benthamiana*. Virus was transmitted by manual inoculation in which extracts of freshly macerated leaves from infected *N. benthamiana* plants (1 g of leaf per 5 ml of water) were rubbed onto Carborundum-dusted leaves of test plants.

ELISA:
Coat protein production in non-inoculated transgenic plants and accumulation of PMTV antigen in inoculated plants was assessed by triple antibody sandwich ELISA using monoclonal antibody SCR69 (Torrance *et al.*, 1993).

RNA transcript analysis:
Total RNA extracts from leaves of non-inoculated plants were subjected to Northern blotting, essentially as described by Barker *et al.* (1992), using a DIG-labelled cDNA probe prepared using sequence homologous to the CP gene of PMTV.

Virus resistance tests:
Test plants of transgenic lines and of a control non-transgenic line (wild type) were manually inoculated. Extracts of these plants were tested by ELISA and bioassay as described by Reavy *et al.* (1995) approximately 18 and 27 days post inoculation. Bioassay was performed by inoculating indicator plants of *N. benthamiana* with macerated leaf tissue obtained from test plants. Infection of indicator plants was assessed by symptomatology and ELISA 3 to 4 wks after inoculation.

Results

Assessment of transgenic resistance to Scandinavian PMTV isolates conferred by the Scottish CP transgene:
Approximately 15 plants from the T_1 generation of three lines selected for resistance tests were challenged by manual inoculation with four Scandinavian PMTV isolates. Plants were

Table 1 Manual inoculation with four Scandinavian PMTV isolates of plants from three transgenic lines of *N. benthamiana* expressing the PMTV-T CP gene

Line	PMTV-174	PMTV-DK	PMTV-31	PMTV-47
W5	NT [*]	1/13	0/13	1/14
W15	0/16 [†]	NT	NT	NT
W16	2/15	NT	0/14	3/13
Wild type (control)	16/17	15/15	11/15	11/14

[*] NT = not tested.
[†] Data given are no. plants infected/no. plants tested

assayed by ELISA and sensitive bioassay. Most transgenic plants were resistant and infectious virus was not detected in the majority of inoculated plants, although virtually all non-transgenic control plants became infected (Table 1).

Characteristics of the CP transgene in relation to resistance expressed to PMTV:

A more extensive series of resistance tests was made and over 100 T_1 plants of several lines were challenge inoculated. Although all transgenic plants of some lines were immune to inoculation with PMTV (e.g. W5 and W16), others were not and a small proportion of transgenic plants became infected (Table 2). However, plants of those lines which did not become infected after inoculation remained virus-free throughout their life, as did those of lines where all transgenic plants were resistant. The level of resistance in individual lines does not seem to be related to: (i) CP gene copy number, (ii) the level of coat protein expression in non-inoculated plants determined by ELISA, or (iii) the amount of CP gene transcript detected by Northern blotting (Table 2).

Table 2 Expression of resistance and some characteristics conferred by the PMTV CP gene in *N. benthamiana*

Line	% resistance*	CP gene copy no. **	CP expression (ELISA A405 values) †	RNA transcript (arbitrary units) ‡
W1	81	2	1.89	16.9
W2	65	1	1.31	13.4
W5	99	2	0.27	0.6
W8	86	2-3	0.4	6.7
W9	87	2-3	0.4	9.6
W16	99	1	0.73	13.2
W20	82	>3	0.3	4.1
Wild type (control)	0	-	0.25	-

* Data given are % of plants that were virus-free after inoculation with four Scandinavian isolates of PMTV. Approx. 100 plants were tested from each line.
** CP gene copy number estimated by PCR of a segregating seedling population.
† Coat protein expression in pooled samples from batches of non-inoculated plants.
‡ Estimated from Northern blots of RNA extracts from pooled samples from batches of non-inoculated plants.

Correlation between coat protein accumulation and level of CP gene transcript:

Our preliminary results suggest that the level of endogenous coat protein accumulation in non-inoculated transgenic plants is correlated with the amount of RNA transcript that can be detected (Table 2), and generally those lines in which high levels of transcript accumulated were those in which most coat protein was found. In further tests, individual plants from T_1 progenies of some lines were examined for coat protein accumulation and RNA transcript production. We found that the individual plants differed greatly in the amount of RNA transcript and coat protein accumulation, (Table 3). Most importantly, we found a strong correlation between RNA transcript and coat protein accumulation (Table 3).

Discussion

A very strong form of CP-MR to Scottish isolates T and S of PMTV is conferred by

transgenic expression of the PMTV (isolate T) CP gene in *N. benthamiana* (Reavy *et al.*, 1995). This resistance has been shown to be effective against inoculation by mechanical means, grafting or by the fungal vector. We have now shown that such CP-MR is effective against four Scandinavian PMTV isolates whose coat protein genes have a high degree of sequence conservation compared with the Scottish isolates (unpublished results).

Table 3 Correlation of coat protein accumulation with accumulation of CP gene transcript in *N. benthamiana*

Plant	CP expression*	RNA transcript accumulation†
W8-24	0.12	Low
W8-27	1.15	High
W8-29	0.13	Low
W8-30	0.7	High
W8-43	0.48	High
W8-44	1.05	High
W8-53	0.79	High
W8-56	0.12	Low
Wild type (control)	0.1	-

* Data given are A_{405} values from ELISA.

† Estimated from Northern blots. Accumulation levels in 'high' plants were approximately 30-fold greater than those in 'low' plants.

A high degree of virus resistance is conferred by the PMTV CP transgene. In some lines this resistance is expressed in all transgenic plants, whereas in other lines it is partial and some transgenic plants become infected. To date, our assessment of some characteristics of the CP transgene in different lines have not pointed to any correlation with the level of resistance expressed. The identification of factors which affect the expression of resistance will be important in determining how to select and utilise this transgene in a potato breeding programme.

References

Arif, M., Torrance, L. and Reavy, B. (1995). Acquisition and transmission of potato mop-top furovirus by a culture of *Spongospora subterranea* f.sp. *subterranea* derived from a single cystosorus. Ann. Appl. Biol. **126**:493-503

Barker, H., Reavy, B., Kumar, A., Webster, K.D. and Mayo M.A. (1992). Restricted virus multiplication in potatoes transformed with the coat protein gene of potato leafroll luteovirus: similarities with a type of host gene-mediated resistance. Ann. Appl. Biol. **120**: 55-64.

Harrison, B.D. and Jones, R.A.C. (1971). Factors affecting the development of spraing in potato tubers infected with potato mop-top virus. Ann. Appl. Biol. **68**:281-289.

Reavy, B., Arif, M., Kashiwazaki, S. Webster, K.D. and Barker, H. (1995). Coat protein-mediated immunity to potato mop-top virus in *Nicotiana benthamiana* is effective against fungal inoculation Mol. Plant -Microbe Interact. **8**:286-291.

Torrance, L., Cowan, G.H. and Pereira, L.G. (1993). Monoclonal antibodies specific for potato mop-top virus and some properties of the coat protein. Ann. Appl. Biol. **122**:311-322.

INDUCTION OF RESISTANCE OF SUGAR BEET PLANTS TO *POLYMYXA BETAE* TRANSMITTED BEET NECROTIC YELLOW VEIN VIRUS (BNYVV) BY SALICYLIC ACID

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Summary

The aim of the study was to determine a possible role for salicylic acid in the induction of resistance to rizomania. The influence of SA on the course of infection was evaluated on the basis of the number of infected plants by ELISA. No significant differences in the number of infected plants were found between control and 500 μM SA-treated plants of any cultivar. Pretreatment of plants with both 1mM and 3mM SA resulted in a significant decrease in the number of BNYVV in susceptible cultivars 'Monosvalof' and partly in 'Domona'. In relatively resistant 'Steffi' and 'Gala' no decrease in the number of BNYVV infected plants was found. These results indicate that the effect of SA induction is markedly dependent on the particular cultivars, with no observed induced resistance in cultivars where low numbers of plants become infected. Intercellular fluids from leaves of SA treated plants of cvs.Monosvalof were analyzed for the presence of newly formed proteis and their enzyme activities. Three bands corresponding to m.w.17000,38000,40000 which were not present in untreated control appeared on SDS-PAGE gel . Electrophoresis of acidic proteins under non-denaturing conditions showed 4 newly formed protein bands. One of them displayed β-1,3-glucanase activity that was not observed for control plants. One band corresponding to a chitinase activity appeared in untreated control plants and two such bands in SA-treated plants.

Introduction

The phenomenon of 'induced ' or 'acquired' resistance in plants has been extensively studied since 1929 when McKinney demonstrated the protective effect of 'preimmunization' using different strains of tobacco mosaic virus. This type of resistance can be induced by inoculation with pathogens or chemical treatment and it is thought that both types of inducer stimulate the same signal transduction pathways. Resistance may be induced at the site of treatment / inoculation (local acquired resistance-LAR) or in distant parts of a plant (systemic acquired infection - SAR), and it is usually accompanied by synthesis of pathogenesis-related proteins (PR-proteins). It has been shown that some PR-proteins have 1,3-β-glucanase (PR-2 group) or chitinase (PR-3 group) activity at least *in vitro* (Broekaert et al., 1988, Jacobsen et al. 1990). It is suggested that these hydrolases have roles in the defence ability of the plant, e.g. they are capable of degrading fungal cell wall polysaccharides and so could inhibit fungal growth (Roulin and Buchala, 1995), and could also act to break down bacterial cell walls (Bernasconi et al. 1987).

A number of exogenously applied chemicals, including salicylic acid, polyacrylic acid, isonicotinic acid, have been shown to induce resistance. The best studied are salicylic acid (SA) and 2,6-dichloroisonicotinic acid (INA). SA is known to induce both PR-proteins accumulation and

resistance (White et al. 1986, Malamy and Klessig, 1992) and has been implicated as a component of signaling pathways that lead to a state of systemic resistance following pathogen infection.
In sugar beet, SA was found to induce synthesis of PR-proteins (Fleming et al. 1991) but only INA possessed the ability to induce resistance against *Cercospora beticola* (Nielsen et al. 1994). The role of SA in infection of sugar beet with the plasmodiophoromycete fungus *Polymyxa betae* Keskin and with beet necrotic yellow vein virus (BNYVV), for which *P.betae* acts as vector, has not yet been studied .

Materials and Methods

Plant material, fungus and SA induction

Seedlings of *Beta vulgaris* cvs.Domona, Steffi, Gala and Monosvalof were grown in sand and watered with half -strength Steiner solution. After three weeks, plantlets were removed from the sand, roots were rinsed with water and plants were placed into bottles of half-strength Steiner solution containing 500μM , 1mM or 3mM sodium salicylate (SA). Control plants were treated in the same manner, but omitting sodium salicylate. Plants were transferred to infested soil after 72 hours and intercellular fluid from leaves of 'Monosvalof'was isolated.
Soil infested with *P. betae* containing BNYVV was collected from Erstein region in Alsatia (France).
ELISA. Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was as described by Clark and Adams (1977). Rabbit IgG and IgG coupled to alkaline phosphatase were purchased from Boehringer Manheim. *Extractions of intercellular fluid* were made (Pierpoint et al. 1987), lyophilised, dissolved in sample buffer and centrifuged at 10 000g for 40min.
Electrophoresis. SDS-PAGE was done according to Laemmli using 4% stacking and 10% resolving 1mm thick gels. *Non-denaturing PAGE* according to Davis using 1mm thick slab gels with 4% stacking and 10% resolving gels was used to separate acidic proteins . All separations were at constant current of 25mA. *Gels were stained* using Coomasie brilliant blue R 250 (SDS gels) or silver stained (non-denaturing PAGE).
Chitinases were detected in owerlaying gel containing 1% glycolchitin (Trudel and Asselin 1989) stained with Calcofluor white.
β-1,3-glucanase activity was detected directly in the gel by the method of Shimoni (1994) using 2% laminarine solution as a substrate and 2,3,5-triphenyltetrazolium chloride for visualisation.
Statistic evaluation. The data were tested by Duncans test.

Results

Test of cultivars. Roots of plants which had grown for 3 weeks in infested soil were analyzed by ELISA .Results presented in Fig.1 indicate that in glasshouse-grown sugar beet, cultivars differ in their susceptibility to BNYVV. Average numbers of BNYVV infected plants in susceptible cultivars 'Domona' and 'Monosvalof' were significantly higher than in partly resistant 'Gala' and 'Steffi'. No symptoms were observed on leaves of any cultivar.
Induction of resistance. Plants were treated with 500 μM, 1mM or 3mM sodium salicylate and their roots were analyzed by ELISA after cultivation for 3 weeks in infested soil.
No significant differences in the nuber of infected plants were found between control and 500 μM SA-treated plants of any cultivar. Pretreatment of plants with both 1mM and 3mM SA resulted in a significant decrease in the number of BNYVV infected plants of susceptible cultivars 'Monosvalof', and to a lesser extend in cv. 'Domona'. In moderaly resistant cultivars 'Gala' and 'Steffi', in which the number of infected plants is lower, the effect of SA pretreatment was not pronounced. No difference was observed in the effect of the different concentrations of SA (1mM and 3mM).

Intercellular fluid analysis. Intercellular fluid isolated from leaves of SA-treated and untreated plants was analyzed for the presence of newly synthesized proteins and their enzyme activities. At least three bands corresponding to m.w 17000,38000,40000 which were not present in untreated control appeared on SDS-PAGE gel . Electrophoresis of acidic proteins under non-denaturing conditions (Davis system) showed 4 newly formed protein bands. One of them displayed β-1,3-glucanase activity that was not observed for control plants. One band corresponding to a chitinase activity appeared in untreated control plants and two such bands in SA-treated plants.

Fig.1. The percentage of plants infected with BNYVV
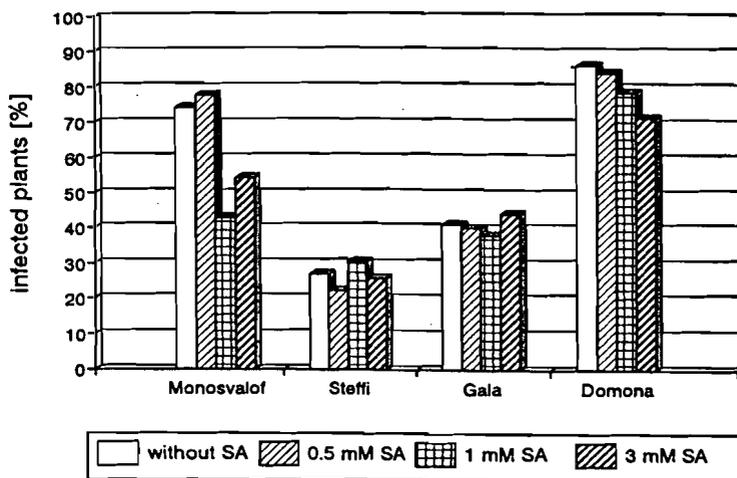
Discussion

SA has been shown to induce resistance to different pathogens in many plant species (White et *al.* 1986, Malamy and Klessig, 1992). The aim of this study was to determine a possible role for salicylic acid in the induction of resistance to rizomania. The influence of SA on the course of infection, evaluated on the basis of the number of infected plants, was only evident in susceptible cultivars 'Monosvalof' and 'Domona'. In partly resistant 'Steffi' and 'Gala' no decrease in the number of BNYVV infected plants was found. These results indicate that the effect of SA induction is markedly dependent on the particular cultivars, with no observed induced resistance in cultivars where low numbers of plants become infected.

When IFs were analyzed, the presence of newly formed proteis showing β-1,3-glucanase and chitinase was in agreement with findings of Fleming et *al.* (1991) and Nielsen et *al.* (1994). In contrast to our results indicating induced resistance to BNYVV in the susceptible cultivar, Nielsen et *al.* (1994) reported that SA treatment induced PR-proteins with β-1,3-glucanase and chitinase activities but did not induce resistance to the *Cercospora beticola.* Probably, different resistance mechanisms are involved.

The presence of β-1,3-glucanase and chitinase activities in roots could influence the course of *Polymyxa betae* infection, affecting zoosporangium or plasmodium formation .

References

Bernasconi, P., Locher, R., Pilet, P.E., Jolles, J., Jolles, P. (1987), Purification and N-terminal amino-acid sequence of a basic lysozyme from *Partenecissus quinquifolia* cultured *in vitro*. *Biochimica Biophysica Acta* **915**: 254-260.

Broekaert, W.F., Parijs, J.V., Allen, A.K., Peumans, W.J. (1988). Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. *Physiol.Mol. Plant Pathol.***33**:319-331.

Fleming, T.M., McCarthy, D.A., White, R.F., Antoniw, J.F., Mikkelsen, J.D. (1991). Induction and characterization of some of the pathogenesis-related proteins in sugar beet.*Physiol.Mol.Plant Pathol.***39**:147-160.

Jacobsen, S., Mikkelsen, J.D., Hejgaard, J. (1990). Characterization of two antifungal endochitinases from barley grain. *Physiologia Plantarum* **79**:554-562.

Malamy, J., Klessig, D.F. (1992). Salicylic acid and plant disease resistance. *The Plant J.* **2**:643-654.

Nielsen, K.K., Bojsen, K., Collinge, D.B., Mikkelsen, J.D.(1994). Induced resistance in sugar beet against *Cercospora beticola*: induction by dichloroisonicotinic acid is independent of chitinase and β-1,3-glucanase tanscript accumulation. *Physiol.Mol.Plant Pathol.* **45**:89-99.

Pierpoint, W.S., Tatham, A.S., Pappin, D.J.C. (1987). Identification of virus induced protein of tobacco leaves that resemble the sweet-tasting protein thaumatin. *Physiol.Mol. Plant Pathol.***31**:291-298.

Roulin, S., Buchala, A.J. (1995). The induction of 1,3-β-glucanases and other enzymes in groundnut leaves infected with *Cercospora arachidicola*. *Physiol.Mol.Plant Pathol.* **46**:471-489.

Shimoni,M. (1994). A method for activitz staining of peroxidase and β-1,3-glucanase isozymes in polyacrylamide electrophoresis gels. *Anal.Biochem.***220**:36-38.

Trudel, J., Asselin, A. (1989). Detection of chitinase activity after polyacrylamide gel electrophoresois.*Anal.Biochem.* **178**:362-366.

Van Loon, L.C., and Antoniw, J.F. (1982). Comparison of the the effects of salicylic acid and ethephon with virus-induced hypersensitivity and acquired resistance.*Neth.J.Pl.Pathol.* **88**: 237-256.

White, R.F., Dumas, E., Shaw, P., Antoniw, J.F. (1986). The chemical induction of PR(b) proteins and resistance to TMV infection in tobacco.*Antivir.Res.***6**:177-185.

EPIDEMIOLOGY OF INDIAN PEANUT CLUMP VIRUS TRANSMITTED BY *POLYMYXA* sp.

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Summary

The peanut clump virus disease, caused by Indian peanut clump virus (IPCV) is widespread in the Indian subcontinent. It occurs in sandy soils. Epidemiology of the Hyderabad isolate of IPCV has been studied in rainy and postrainy seasons in India. The virus produces severe symptoms not only on groundnut but also on pigeon pea, wheat and barley under field conditions. Sorghum, pearl millet, finger millet, foxtail millet, maize, rice, cowpea, chickpea, soya bean, mung bean and rapeseed were infected by the virus without showing any overt symptoms. Sunflower was not infected by the virus. Various monocotyledonous weeds naturally infected by IPCV were *Cynodon dactylon*, *Dactyloctenium aegyptium*, *Digitaria ciliaris*, *Eragrostis ciliaris* and *E. uniloides*. The dicotyledonous ones were *Celosia argentea*, *Macroptilium atropurpureum* and *Oldenlandia corymbosa*. The virus is seed-transmitted in groundnut, pearl millet, finger millet, foxtail millet and wheat. *Polymyxa* sp. is often detected in the roots of monocotyledonous hosts. The fungus has only been detected once in groundnut roots and not in the roots of other dicotyledonous plants collected from IPCV infested patches. The infection of roots by *Polymyxa* sp. in India is influenced by the climatic conditions, especially those of rainfall distribution and temperatures.

Introduction

Peanut clump is a seedborne (Reddy, 1991 and Konaté and Barro, 1993) and soilborne virus causing severe damage to the crop in semi-arid areas of West-Africa (PCV) (Thouvenel *et al.*, 1988). In the Indian subcontinent, where it is referred to as Indian peanut clump virus (IPCV), at least three distinct serotypes are known (Nolt *et al.*, 1988) and none of the IPCV isolates cross react with those reported from West Africa. This high variability complicates detection of the virus by serology (Miller *et al.*, this volume). IPCV is known to infect many monocotyledonous and dicotyledonous plants but the fungus, *Polymyxa* sp., which was shown to be the vector of IPCV, is known to prefer monocotyledonous plants to complete its life cycle (Ratna *et al.*, 1991). However, those hosts which play a crucial role in perpetuation of the fungus and transmission of the virus with severe incidence are yet to be identified. IPCV has been shown to be seed-transmitted in groundnut and several monocotyledonous hosts, however it is not known if seedborne inoculum can initiate the disease in areas where non-viruliferous populations of *Polymyxa* sp. occur. Resistance to IPCV has so far not been located in cultivated or wild *Arachis* sp. Control may have to rely on cultural practices and information generated from epidemiological studies is likely to guide disease management.

Geographical Distribution and Cropping Systems

The Durgapura isolate (D-IPCV) is a member of the most widespread serotype of IPCV. It occurs in the Indian States of Gujarat (Talod), Rajasthan (Boraj, Dausa, Durgapura, Kallavas and Ranoli), Andhra Pradesh (Bapatla, Chinnaganjam, Ganapavaram, Pallipalem, Ramapuram and Tsundupalle), Tamil Nadu (Pondicherry) and in the Punjab Province of Pakistan in Attock, Chakwal and Rawalpindi districts (Delfosse *et al.*, 1995a). A second serotype, which occurs in Punjab in India (Jalandhar, Ludhiana and Sangrur), is

referred to as Ludhiana isolate (L-IPCV) and is also present in Pakistan in Attock district. In Hyderabad, Andhra Pradesh (A.P.), a third serotype (H-IPCV) occurs. The disease in India and Pakistan is very severe in the areas where groundnut is rotated with cereals and particularly with wheat. In Punjab the disease caused severe damage to groundnut until the early eighties when the wheat-groundnut rotation was replaced by a wheat-rice system.

Natural Host Range

Various naturally occurring weeds were tested at ICRISAT Asia Center (IAC) for the presence of H-IPCV and *Polymyxa* sp. Results for monocotyledonous weeds (Table 1) show that highly pernicious weeds can act as carry-over hosts for the virus and its vector. Moreover all vegetatively reproducing rhizomes of *C. dactylon* arising from an infected plant contained the virus and recent experiments indicate that rhizomes carrying the virus but not the vector contributed to the establishment of the disease in soil harbouring nonviruliferous *Polymyxa* sp. For *Cyperus rotundus*, roots were rarely found to be infected by the virus and tubers and leaves were never found to be positive in ELISA and ISEM. Various dicotyledonous weeds (*Celosia argentea, Desmodium sp., Eclipta alba, Euphorbia hirta, Flaveria australasica, Indigofera sp., Macroptilium atropurpureum, Merriemia gangetica, Oldenlandia corymbosa, Phyllanthus niruri, Tridax procumbens, and Vernonia cinerea*) collected in H-IPCV infested areas did not show the presence of the vector. *Celosia argentea, Macroptilium atropurpureum* and *Oldenlandia corymbosa* were symptomless hosts for the virus.

Table 1. Results of monitoring of *Polymyxa* sp. in the roots of various monocotyledonous weeds collected from a clump infested field at ICRISAT Asia Center and, HIPCV in roots and leaves, by ELISA.

	Number of plants infected (n)/Number of plants tested (N)			
	Polymyxa sp.		H-IPCV	
Species	n/N	Degree of infection	Leaves	Roots
Cynodon dactylon	6/26	+	11/26	12/26
Cyperus rotundus	19/28	+ to +++	0/28	2/28
Cyperus diffusus	1/2	+++	0/2	0/2
Dactyloctenium aegyptium	13/22	+ to +++	5/22	6/22
Digitaria ciliaris	3/22	+ to +++	1/22	2/22±
Eragrostis ciliaris	3/13	+ to ++	11/13	11/13
Eragrostis uniloides	2/22	++ to +++	10/22	11/22

(+ : low infection, ++ : medium infection, +++ : high infection).

The virus incidence in roots and leaves of various monocotyledonous crops and groundnut from an H-IPCV infested field was monitored during the 1995 rainy season. Twelve days after sowing, plants containing the viral antigen in their roots were found in all the crops when tested by ELISA. The virus was also detected in the leaves of barley (13%), maize (6%) and wheat (31%). Wheat showed the highest virus incidence in the roots (94%) followed by maize (50%). Two months after germination it was possible to detect the virus in wheat and barley leaves but no longer in roots. For others species the virus incidence stayed higher in roots than leaves. Over the entire season, the incidence of the virus in roots and in leaves was: 62% and %56 in barley, 28% and 21% in finger millet, 21% and 8% in groundnut, 38% and 4% in maize, 3% and 0% in pearl millet, 3% and 1% in rice, 26% and 6% in sorghum and 85% and 76% in wheat. The same samples were also analysed for the presence of *Polymyxa* sp. Maize, pearl millet and sorghum were seldom found to be infected by the fungus but those plants with infection showed a very high number of resting spores in their roots. Though the other crops were infected by the virus, *Polymyxa* sp. could not be found in the roots. However evidence has been obtained to show (Ratna *et al.*, 1991) that both the vector and the virus can infect most of these crops so there is a high risk that rotation with these crops would lead to an

increase in the disease incidence. When barley was grown under irrigation during the postrainy season, H-IPCV infected plants showed symptoms very similar to those of wheat plants (stunting and dark green leaves with yellow stripes turning necrotic as the plants aged, Delfosse *et al.*, 1995b).

When a selected number of dicotyledonous crop plants was raised in an infested field during the 1995 rainy season, the proportion of infected plants was, chickpea (4%), cowpea (6%), groundnut (28%), mung bean (1%), pigeon pea (26%), rapeseed (1%) and soya bean (4%). Interestingly sunflower was the only crop not infected, although it could be manually infected with H-IPCV in the laboratory. Infected pigeon pea plants were severely stunted compared to healthy plants and showed mosaic symptoms on young leaves. Presence of H-IPCV particles in leaves of stunted pigeon pea plants was confirmed by ISEM.

Factors influencing virus transmission and disease occurrence

The temporal and climatic factors influencing H-IPCV transmission by *Polymyxa* sp. to groundnut, sorghum, pearl millet and finger millet, were studied during the 1994 rainy season. The groundnut crop was scored at 2 weeks intervals for virus incidence. Most infection occurred in July with the onset of monsoon rains (80% of the total infected plants during the season). Cereals were sampled at weekly intervals and analysed for the presence of the fungus in their roots and the virus in their leaves. Infection by *Polymyxa* sp. varied greatly during the season and was correlated with the cumulative weekly rainfall (CWR) recorded 15 days before sampling, in agreement with the time needed (10 to 12 days) to form cystosori. This correlation was supported by another experiment where seedlings were raised in a glasshouse for one week, then exposed for one week in a virus infested field and finally transplanted into sterile sand. They were subsequently maintained in a glasshouse to allow the fungus to complete its life cycle. Results of this experiment showed that the incidence of *Polymyxa* sp. in the roots of sorghum and pearl millet was correlated with the CWR recorded during the week of exposure in the field. In both cases, a CWR of 14 mm was sufficient to induce *Polymyxa* sp. infection. The incidence of the fungus recorded during the entire season was higher in pearl millet (21%) and sorghum (18%) compared to finger millet (5%). However the virus incidence was higher in the millets (6%) than in sorghum (3%). The fungus was present in plants grown both in virus infested and virus free areas. During the rainy season the daily mean soil temperature ranged from 24 to 29°C but generally remained higher than 25°C, a conducive condition for infection by *Polymyxa* (Legrève *et al.*, this volume).

The incidence of IPCV in groundnut is influenced by the date of sowing. April sown crops in Pakistan showed low incidence while those sown in July with the onset of the monsoon rains were severely affected. However in India, crops sown after initial heavy rains, until 15 days after the onset of the monsoon, were weakly affected by the disease. In India, the soil temperature in summer reaches 40 to 50°C and this dry heat induces the breakdown of fungal spore dormancy (Legrève *et al.*, unpublished data). Heavy rains at the onset of monsoon, are assumed to induce the release of many primary zoospores, which infect even nonpreferred hosts such as groundnut. Barley, wheat and sorghum grown in the postrainy season under irrigation in A.P. were infected by the virus while groundnut was not. In this case, the germination of resting spores is likely to be induced by the root exudates from the cereal hosts. In Rajasthan where the air temperature in postrainy season is much lower than in A.P., no virus infection was found in thousands of wheat and barley samples, roots and leaves, either at early stage or close to maturity. The virus can multiply at temperature close to 15°C (Reddy et al., 1988) but such low temperature would not have been conducive to activation of *Polymyxa* sp. (Legrève et al., this volume) so transmission of the virus did not occur.

Perspectives and Management

Resistance in nearly 9000 *A. hypogaea* germplasm lines could not be identified for IPCV, and biocides, though effective in reducing disease incidence are hazardous and not economical. Therefore it is imperative to identify cultural methods for the control of IPCV. Although H-IPCV was detected in several

dicotyledonous plants, resting spores of *Polymyxa* sp. were rarely detected in such plants (Ratna et al., 1991). They were few in number so they probably contribute little to increase the incidence of H-IPCV. Additionally roots of naturally infected groundnut plants failed to induce incidence when incorporated into sterile sandy soils. Preliminary observations at IAC have shown that it is possible to reduce disease incidence by growing a crop which does not support *Polymyxa* sp. multiplication in the postrainy season prior to a rainy season groundnut crop. The disease incidence in plots where the postrainy season crop was groundnut was much lower than in plots where sorghum was grown. On the other hand, in the rainy season, a bait crop (pearl millet) grown for 15 days and then ploughed into the soil prior to sowing groundnut reduced disease incidence from 22-36% to 4-8%. The presence of a preferred host such as pearl millet could have induced the germination of *Polymyxa* sp. cystosori and before the fungus completed its life cycle the host was killed by ploughing it into the soil. Clean cultivation is recommended since monocotyledonous weeds, such as *C. dactylon* can contribute to disease establishment. The virus was found to be seed transmitted in finger millet, foxtail millet, groundnut, pearl millet and wheat. The use of virus free seed is essential to prevent the spread of the disease. In preliminary experiments infected groundnut seed failed to provide the inoculum for *Polymyxa* sp. in uninfested soils but this aspect needs further investigation. Currently influence of cereal seed, in contributing to disease establishment, is being tested.

References

Delfosse P, Bashir M, Malik SN and Reddy AS (1995a). Survey of groundnut virus diseases in Pakistan. *International Arachis Newsletter* **15**: 51-52.

Delfosse P, Reddy AS, Devi PS, Murthy AK, Wesley SV, Naidu RA and Reddy DVR (1995b). A disease of wheat caused by the Indian peanut clump virus (IPCV). *Plant Disease* **79**: 1074.

Konaté, G and Barro, N (1993). Dissemination and detection of peanut clump virus in groundnut seed. *Annals of Applied Biology* **123**: 623-627

Nolt, BL, Rajeshwari, R, Reddy, DVR, Bharatan, N and Manohar, SK (1988). Indian peanut clump virus isolates: host range, symptomatology, serological relationships, and some physical properties. *Phytopathology* **78**: 310-313.

Ratna AS, Rao AS, Reddy AS, Nolt BL, Reddy DVR, Vijayalakshmi M and McDonald D (1991). Studies on the transmission of Indian peanut clump virus disease by *P. graminis*. *Annals of Applied Biology* **118**: 71-78.

Reddy, DVR, Nolt BL, Hobbs HA, Reddy AS, Rajeshwari R, Rao AS, Reddy DDR and McDonald D (1988). Clump virus in India: isolates, host range, transmission and management In: *Viruses with fungal vectors*. Cooper, JI and Asher MJC (ed.). AAB, Wel., Warwick, UK. pp. 239-246.

Reddy, DVR. (1991). Groundnut viruses and virus diseases: distribution, identification and control. *Review of Plant Pathology* **70**: 665-678.

Thouvenel J-C, Fauquet C, Fargette D and Fishpool DC. (1988). Peanut clump virus in West Africa. In: *Viruses with fungal vectors*. Cooper, JI and Asher MJC (ed.). AAB, Wel., Warwick, UK. pp. 247-254.

OBSERVATIONS ON THE EPIDEMIOLOGY OF SOIL-BORNE WHEAT INFECTING VIRUSES IN GERMANY

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Summary

Soil-borne wheat mosaic (SBWMV) and wheat yellow mosaic virus (WYMV) have been detected in several regions in Germany. In each of these regions which preferentially are used for rye production only rye and Triticale but very rarely (>0.1%) wheat became infected. One possible reason for the low infection rate of wheat might be that in the growing areas with light soils a biotype of *Polymyxa graminis* occurs which might be specialised to rye. In contrast to the 100% infection of barley growing on fields severely infested by barley yellow mosaic and/or barley mild mosaic viruses, only relatively small proportions of rye plants on infested fields were infected by SBWMV and/or WYMV. The low number of infected plants (rye: 30-50%; Triticale: 5-20%) may be due to a reduced vector content of the light soil. The low content of these soils in organic or inorganic adsorbing materials might be the reason for the elution of resting spores and zoospores of the vector. It is supposed that the late sowing date of winter wheat in autumn in Germany may be one reason for the failure of transmission of SBWMV and WYMV to wheat by wheat-specialised biotypes of *Polymyxa graminis*.

Introduction

Barley yellow mosaic (BaYMV) and barley mild mosaic bymoviruses (BaMMV) are common soil-borne pathogens of winter barley in Germany. First detected in 1977 both spread rapidly into most of the regions in Germany where winter barley is cultivated and occur now also in most of the other European countries (Huth, 1988). Additionally to these barley infecting soil-borne viruses Huth and Lesemann reported in 1990 on the occurrence of soil-borne wheat mosaic furovirus (SBWMV) in a region of Lower Saxony in Germany and detected some years later also wheat yellow mosaic bymovirus (WYMV) as the fourth cereal-infecting soil-borne virus (Huth and Lesemann, 1996). Besides these viruses which all are transmitted by *Polymyxa graminis* a fifth cereal-infecting soil-borne virus, the nematode-transmitted tobacco rattle virus (TRV) has been recorded in one location in Northern Germany (Huth and Lesemann, 1984). In the following paper the occurrence of WYMV and SBWMV, which differ in their epidemiological behaviour from the barley infecting soil-borne viruses will be reported.

Distribution of WSBMV and WYMV in Germany

In Germany SBWMV and WYMV occur in several distant regions. In each of these areas only few fields are known to be infested. From small initial patches inside fields the infested areas enlarged by machinery from year to year. Although a spread to other fields has not yet been noticed, from the distribution inside the fields it can be concluded that both viruses have been present there for long time. Most of these fields are already totally infested.

In contrast to BaYMV/BaMMV which predominantly occur in fields with heavy soils the regions in Germany where WYMV and SBWMV are common are characterised by very light soils. In some regions the area of light soil is rather limited and only few fields there are virus infested.

Most of the fields known to be infested contain both SBWMV and WYMV. Plants of rye or of Triticale growing on these fields can be either singly infected by one of the two viruses only or, can be double infected by both. Up to now only one field is known where probably SBWMV only occurs.

Symptoms of SBWMV and WYMV infected plants of rye

Although wheat plants are the main natural hosts of both SBWMV and WYMV in Italy and France as well as in the USA (Fouchard et al. 1995; Rubies-Autonell and Vallega 1990; Elliot and Lommel 1991) in Germany up till now wheat plants were never found to be infected by either virus, except on experimental sites. Because regions of light soils are used preferentially for rye (*Secale cereale*) production not wheat but plants of rye are the main hosts of SBWMV and WYMV in Germany. Besides of rye also plants of Triticale were found to be infected by both viruses.

Symptoms on infected plants of rye and Triticale caused by both viruses are similar to those produced on wheat plants or caused on barley plants by BaYMV/BaMMV. Because of their pale green colour rye plants infected by SBWMV and/or WYMV are much less conspicuous than barley plants infected by BaYMV/BaMMV and therefore the recognition of fields of rye even when they are totally infected by SBWMV and/or WYMV is sometimes difficult.

Depending on climatic conditions, first symptoms appear on autumn sown rye plants when temperatures are rising in early spring, e. g. in March/April. Pale or light green streaks or short stripes irregularly distributed on the leaf surface indicate the infection. In agreement to cereal plants infected by other soil-borne viruses the symptoms already appear on the youngest still rolled leaves. During the leaf growth also size and number of streaks become larger and especially old leaves sometimes show chlorotic to whitish stripes between the veins from the leaf tip to the ground. The tillers of infected plants are often shortened to more than half of the length of symptomless plants.

Infectivity studies

Mechanical inoculations: Using crude sap from plants of rye naturally infected either by SBWMV or by WYMV, both viruses were transmitted mechanically to plants of several cultivars of rye as well as of wheat. After inoculation plants grow in growth chambers at 16/8 hours light/darkness and at constant temperatures of c. 18°C. Of the two viruses SBWMV was much more easily transmitted mechanically than WYMV. After inoculation of the furovirus SBWMV nearly all plants became infected but using plant sap containing the bymovirus WYMV for inoculations in general 20 to 30% of the plants became infected, occasionally also 40 to 60%. No remarkable differences in the response of plants of different cultivars to virus inoculations were observed. All cultivars of wheat as well as of rye used for this study were susceptible to both viruses.

Natural infections: In the contra-distinction to the response of plants mechanically infected by both viruses, the average number of naturally infected plants is much smaller and differs from year to year. Plants of 15 cultivars of rye and 5 cultivars of Triticale were cultivated on two experimental sites close Glentorf, Lower Saxony, and close Zerbst, Saxony-Anhalt. Depending from the cultivars and probably from environmental conditions 30 to 50%, occasionally also 70% of rye plants and 5 to 20% plants of Triticale became infected. Most of the infected plants of rye were doubly infected, but when infected only by one of the two viruses predominantly SBWMV was detected serologically. Results from Zerbst differ from those of Glentorf. On the field in Zerbst most plants of rye and Triticale were singly infected by WYMV (Table 1). On both experimental sites plants of Triticale were much more often singly infected by either SBWMV (>90%; e. g. cv 'Alamo' and cv 'Modus') or by WYMV (±90%; cv 'Prego'), but very rarely doubly infected by both viruses.

Table 1: Proportions of plants of rye (*Secale cereale*) and Triticale infected by SBWMV and/or WYMV. Symptom-bearing plants of several cultivars were collected from fields close to Glentorf (Lower Saxony) and Zerbst (Saxony-Anhalt) and were tested serologically. Data represent the average values of 15 cultivars of rye and 5 cultivars of Triticale.

Experimental sites	No. infected plants tested	plants infected by		
		SBWMV	WYMV	SBWMV +WYMV
Glentorf				
Secale cereale (1992 - 1994)	700	300 (43%)	5 (0,7%)	395 (56%)
Triticale (1993 and 1994)	95	51 (54%)	37 (39%)	7 (7,3%)
Zerbst				
Secale cereale (1992 and 1994)	288	80 (28%)	156 (54%)	56 (19%)
Triticale (1994)	20	4 (20%)	16 (80%)	0 (0%)

Wheat plants grown from 1992 to 1996 at the same plots of 1 x 12 m on the experimental site close Glentorf became much more rarely infected than plants of rye or Triticale. In 1993, only 8 plants, and only 1 plant in 1995 out of c. 36 000 wheat plants cv 'Florida' sown each year were recognised to be infected by visual assessment. No infected plants were found in 1994 and 1996 and even plants of French cultivars, cv 'Hardi' and cv 'Soissons', known to be highly susceptible to SBWMV and WYMV in France, which were first grown in 1996 at the experimental site close Glentorf, remained symptomless. Serological tests revealed that the few symptom-bearing plants of wheat were exclusively infected by SBWMV detected (Table 2).

Table 2: Proportions of wheat plants which became infected by SBWMV when cultivated for 4 years in succession at the identical places on the experimental field close Glentorf (Lower Saxony).

cultivars of *Trit. aestivum*	1992/93			1993/94			1994/95			1995/96		
	No plants	inf.	%	No plants	inf.	%	No plants	inf.	%	No plants	inf.	%
c. v. 'Florida'	c 36000	8	0,02	c 36000	0	0	c 36000	1	0,003	c 12000	0	0
c. v. 'Hardi'	-	-	-	-	-	-	-	-	-	c 12000	0	0
c. v. 'Soissons'	-	-	-	-	-	-	-	-	-	c 12000	0	0

- not tested

Discussion

Of the fungus-transmitted soil-borne viruses, BaYMV and BaMMV are the most widely spread pathogens of small grains in Germany whereas the occurrence of SBWMV and WYMV is limited to a comparably small number of fields at distantly separated regions. Comparing the occurrences of these viruses the barley infecting viruses occur predominantly in regions with heavy soils. There is in general all susceptible barley plants become infected by BaYMV and/or BaMMV. This might be correlated with the high content of zoospores and also resting spores of the vector, the fungus *Polymyxa graminis*, in heavy soils. The high humidity of these soils as well as the high content of organic and inorganic adsorbing materials as humic acids and clay are probably necessary conditions for an optimum propagation of the vector.

In contrast to BaYMV/BaMMV in Germany both SBWMV and WYMV occur only on very light, sandy soils and of the plants cultivated there only occasionally more than 50% became infected. No indications have been found for resistances of the used cultivars at least to one of both viruses. Reasons for the low

number of infected plants may be the in general low amounts of adsorbing materials on light soils which probably are the reason for an easy elution and reduction of zoospores as well as resting spores by rain water. The variability of the infection rates in different years apparently indicates an influence of variable soil humidity or an inhomogeneous distribution of both viruses.

In Germany, but also in Poland (Jezewska, 1995), not wheat but mainly rye becomes naturally infected by SBWMV and WYMV. Although found to be susceptible to both viruses isolated from rye after mechanical inoculations only very few wheat plants became infected when cultivated on light soil. One possible reason for the very low infection rate might be that in the growing areas with light soils, where not wheat but preferentially rye is grown, a biotype of *Polymyxa graminis* occurs which might be specialised to rye. The cultivation of wheat which was sown during 4 years in succession at the same plots on the experimental fields and at the same time as rye (middle of September) did not result in an increasing number of infected plants. That means that *Polymyxa graminis* did not became adapted to wheat during this time. Triticale as intergeneric hybrid has an intermediate position between rye and wheat. Correspondingly, the proportion of infected Triticale plants was lower than that of rye but higher than that of wheat.

From the results it might be concluded that the comparably low rates of infections of rye and Triticale on soils totally infested by SBWMV and WYMV do not result from resistances of these cultivars against both viruses. Environmental conditions in regions where both viruses in Germany occur, as light soils and low soil humidity, appear to be the main factors which reduce the number of vectors. Especially for wheat, which is susceptible to both virus isolates from rye probably a resistance to a presumed rye specific biotype of *Polymyxa graminis* seems to be a further factor which is responsible for the failure of infections. The in general late sowing date of wheat in autumn may be one reason for the lack of transmission of soil-borne viruses to wheat by wheat-specialised biotypes of *Polymyxa graminis* in Germany.

Literature

Elliot, L. G. and Lommel, S.A. (1991). Identification and partial characterisation of oat mosaic and oat golden stripe virus co-infecting in the South-eastern United States. In: Koenig, R. (ed.): Proc. First Symposium Intern. Working Group on Plant Viruses with Fungal Vectors, Schriftenr. Dt. Phytomed. Ges. Bd. 1, pp. 131-134

Fouchard, M., Hariri, D., Prud'homme, H., Leburn, H. I., Koenig, J. and Lapierre, H. (1996). Resistance of old French cultivars of bread wheat to soil-borne wheat mosaic virus (SBWMV) and wheat yellow mosaic virus (WYMV). Agronomie 15, 502-503.

Huth, W. (1988). Ein Jahrzehnt barley yellow mosaic virus in der Bundesrepublik Deutschland. NachrBl. Dt. PflSchutzdienst **40**, 49-55.

Huth, W. und Lesemann, D.-E. (1984) Vorkommen von Tobacco rattle virus in Roggen (*Secale cereale*) in der Bundesrepublik Deutschland. Phytopath. Z. **111**, 1-4.

Huth, W. and Lesemann, D.-E. (1990). Soil-borne wheat mosaic virus isolated from rye in Germany. In: Koenig, R. (ed.): Proc. First Intern. Symposium Working Group on Plant Viruses with Fungal Vectors. Schriftenr. Dt. Phytomed. Ges. Bd. 1, pp 139-141.

Huth, W. and Lesemann, D.-E. (1996). Fungus-transmitted soil-borne viruses on rye in Germany. Z. Pflanzenkrankh. und Pflanzenschutz **103**, 120-124.

Jezewska, M. (1995). Detection of Polish isolate of wheat soil-borne mosaic virus in cereal seeds. Phytopathol. Polonia **10**, 61-67.

Rubies-Autonell, C. and Vallega, V. (1990). Soil-borne wheat mosaic virus and wheat spindle streak mosaic virus in Italy. In: Koenig, R. (ed.): Proc. First Symposium of the Intern. Working Group on Plant Viruses with Fungal Vectors, Schriftenr. Dt. Phytomed. Ges., Bd 1, pp 135-138.

MOLECULAR ANALYSIS OF BARLEY MILD MOSAIC VIRUS IN RELATION TO HOST RESISTANCE

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Summary

Two Japanese strains of barley mild mosaic virus, BaMMV–Ka1 and BaMMV–Na1, differ in pathogenicity and symptomatology on barley cultivars. Mechanical inoculation of barley cultivars with a mixture of BaMMV–Ka1 and BaMMV–Na1, followed by RT–PCR to detect RNA components in infected plants, revealed that the RNAs of the two strains were exchangeable *in vivo* to generate all nine possible combinations containing at least one RNA1 and one RNA2. Infected plants with reassorted RNAs were selected and used as inocula for further analysis of cultivar reactions. The results demonstrate that the pathogenicity and symptomatology are determined solely by RNA1. During repeated trials of single inoculation of BaMMV–Ka1 onto barley cultivars having the resistance gene *Ym*, only two plants became infected, and the virus progenies from these plants efficiently infected cultivars with *Ym*. Sequence analysis of the capsid protein genes of these possible resistance–breaking mutants and the original BaMMV–Ka1 showed no consistent differences.

Introduction

Barley mild mosaic bymovirus (BaMMV), often in co–infection with barley yellow mosaic bymovirus, causes serious damage to winter barley crops in East Asia and Europe. BaMMV has flexuous rod–shaped particles with two modal lengths, containing two species of ssRNAs of 7.6 kb (RNA1) and 3.5 kb (RNA2) (Huth *et al.*, 1984; Kashiwazaki *et al.*, 1989). In Japan, two BaMMV strains, BaMMV–Ka1 and BaMMV–Na1, differing in pathogenicity towards barley cultivars and serologically, have been reported (Kashiwazaki *et al.*, 1992; Nomura *et al.*, 1996). Complete nucleotide sequence determined for BaMMV–Na1 (Kashiwazaki *et al.*, 1992, 1996) shows that RNA1 and RNA2 are translated into single polyproteins which undergo proteolytic cleavage to give functional proteins. The capsid protein is located at the C terminus of the RNA1–encoded polyprotein.

In order to examine relationships between the RNA components and pathogenicity, mechanical inoculation of barley cultivars with a mixture of BaMMV–Ka1 and BaMMV–Na1, followed by RT–PCR analysis to detect RNA components in infected plants, has been conducted. Further inoculation experiments have also been made using plants with reassorted RNAs resulted from the co–inoculation (Kashiwazaki & Hibino, 1996). In addition, possible resistance–breaking mutants derived from BaMMV–Ka1 were analyzed.

Materials and Methods

BaMMV–Ka1 and BaMMV–Na1 were maintained in barley (cv. Ishukushirazu, unless otherwise stated) by mechanical inoculation. Barley seedlings were inoculated at the one and half leaf stage with infected plant sap, and grown at 13–15 °C as described by Kashiwazaki *et al.* (1989). For co–inoculation with two strains, equal volumes of sap were mixed and inoculated to barley seedlings.

Oligonucleotide primers for RT–PCR were designed on the nucleotide sequences reported for

the 3' halves of RNA1 of BaMMV-Kal and BaMMV-Nal (Kashiwazaki *et al.*, 1992) and newly determined for the 3' non-coding regions in RNA2 of both strains. Forward primers S1 (5' AAGGATCCATGGCAGGACACGAAGAACCA 3') or S7 (5'AAGGATCCATGTCAGGAAAAG-ATGATCCA 3') were used in combination with a reverse primer S3 (5' AAAGGCCTCTACCGG-AGGAGCGCCTCGTG 3') to amplify the capsid protein coding region of BaMMV-Kal or BaMMV-Nal, respectively. These three primers contain additional nucleotides (underlined) for a restriction site or an initiation codon (not used in this study). Forward primers S20 (5' TCATCTT-AATAGGTAGGCATCAG 3') or S21 (5' GACATCGTCTCAATAGATGATGA 3') and a reverse primer S25 (5' TTACGAGCTGATAGTAATCAGCG 3') were used to amplify the 3' non-coding region in BaMMV-Kal or BaMMV-Nal RNA2, respectively.

Total nucleic acid was extracted from approx. 10 mg of leaf tissue with a Sepa Gene nucleic acid extraction kit (Sanko Junyaku, Japan), and dissolved in 50 µl of sterile distilled water. One microliter of the nucleic acid was used for RT reaction at a 5 µl scale with 250 ng of a reverse primer. Whole of the RT mixture was used for PCR at a 25 µl scale with 250 ng of a forward primer in a 30 cycle program of 1 min at 94 °C, 2 min at 65 °C and 3 min at 72 °C. An aliquot (5 µl) of the PCR product was electrophoresed on a 1% agarose gel. RT-PCR with any of the four primer pairs were able to detect specifically the target RNA components in 1 µl of a 1/5000 dilution (about 10 pg) of the total nucleic acid samples.

For sequence analysis, the whole of the capsid protein gene was amplified by RT-PCR using a forward S19 (5' CCATCGTGGAGGAAACAT 3') and reverse S17 (5' CATTGCTTAAAAGGA-AAT 3') primer, cloned into pT7Blue-T (Novagen), and sequenced from both ends using a Perkin-Elmer 377A DNA seqeuncer.

Results

In an initial attempt for co-inoculation, 25 seedlings of Ishukushirazu were inoculated with a mixture of BaMMV-Kal and BaMMV-Nal. Three weeks later, mosaic symptoms began to appear on the third leaves, and sixteen of the inoculated plants eventually developed systemic mosaic. RT-PCR detected RNA1 and RNA2, from either or both strains, from the 3rd and 5th leaves of all 16 plants. Each plant had the same combination of RNA components in the 3rd and 5th leaves. The nine remaining plants, with no symptoms, gave no RT-PCR product. Therefore, in subsequent experiments, the 3rd leaves, showing symptoms, were used for RT-PCR.

Seedlings of five barley cultivars were inoculated with a mixture of BaMMV-Kal and BaMMV-Nal, or with each strain separately. After separate strain inoculations, plants (16-32%) of New Golden and Ishukushirazu were infected with each strain, but no plants of cv. Misato Golden were infected with BaMMV-Kal, and none of cvs. Shiromugi 6 or Tosan Kawa 73 infected with BaMMV-Nal.

After the co-inoculation, plants (16-62%) of all five cultivars were infected, and all infected plants were examined by RT-PCR (Table 1). Although Ishukushirazu and New Golden are susceptible to both strains, only a few infected plants of Ishukushirazu had two pairs of RNA1 and RNA2 from both strains ($K_1N_1K_2N_2$). A number of infected plants of the two cultivars contained only one pair of RNAs from either BaMMV-Kal (K_1K_2) or BaMMV-Nal (N_1N_2). Other infected plants had heterologous combinations of two RNA1 and one RNA2 molecules ($K_1N_1K_2$ or $K_1N_1N_2$), or one RNA1 and two RNA2 ($K_1K_2N_2$ or $N_1K_2N_2$), or one RNA1 and one RNA2, but from different sources (i.e. pseudorecombinants; K_1N_2 or N_1K_2). In Misato golden, which is susceptible to BaMMV-Nal but resistant to BaMMV-Kal, all infected plants contained RNA1 only from BaMMV-Nal. Conversely, in Shiromugi 6 and Tosan Kawa 73, both of which are susceptible to BaMMV-Kal but resistant to BaMMV-Nal, all infected plants, except two Shiromugi 6 plants, had RNA1 only from BaMMV-Kal. The occurrence of two Shiromugi 6 plants with RNA1 from both strains could be explained by a complementation between the two

RNA molecules.

Table 1. Proportion of infected plants of five barley cultivars and their RNA combinations after co-inoculation with BaMMV-Ka1 and BaMMV-Na1[*]

Barley cultivar	Number of plants infected/total	Number of plants with each RNA combination								
		$K_1N_1K_2N_2$	$K_1N_1K_2$	$K_1K_2N_2$	$N_1K_2N_2$	$K_1N_1N_2$	K_1N_2	N_1K_2	K_1K_2	N_1N_2
Ishukushirazu	25/58	3	2	3		1	3	2	8	3
New Golden	18/29			1	1	1	2		10	3
Misato Golden	7/32					1		3		3
Shiromugi 6	9/46	1				1	4	3		
Tosan Kawa 73	5/44			1				4		

[*] A mixture of sap of plants infected singly with each strain was used.

Ishukushirazu plants with either pseudorecombinants K_1N_2 or N_1K_2, obtained by the co-inoculation, were used as inoculum to seedlings of the five barley cultivars. Ishukushirazu and New Golden were infected with either pseudorecombinant. Misato Golden was infected only with N_1K_2, whereas Shiromugi 6 and Tosan Kawa 73 were infected only with K_1N_2. The results consolidate the above described indication that RNA1 determines the pathogenicity. Furthermore, the symptoms on these cultivars induced by K_1N_2 and N_1K_2 were very similar to those induced by the parental strains, BaMMV–Ka1 and BaMMV–Na1, respectively (Nomura *et al.*, 1996). Thus, RNA1 also determines the symptoms on these cultivars.
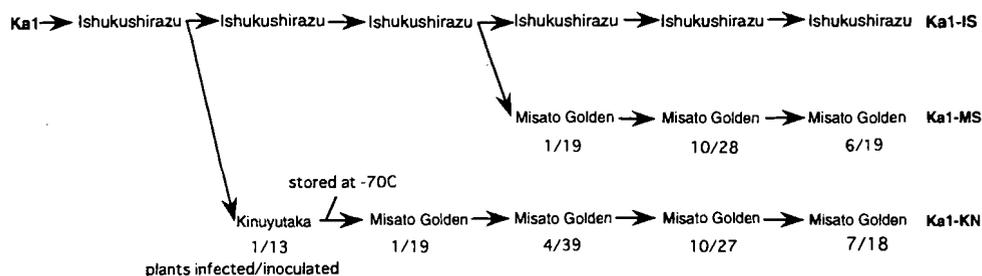

Fig. 1. Separation of possible resistance-breaking mutants from BaMMV-Ka1 by mechanical passages through resistant barley cultivars. Cvs. Misato Golden and Kinuyutaka have the resistance gene *Ym*.

Barley cultivars with the *Ym* gene are resistant to BaMMV–Ka1 (Nomura *et al.*, 1996). However, during repeated inoculation experiments of BaMMV–Ka1 to cultivars with *Ym*, only one plant of Kinuyutaka and one of Misato Golden, among more than 100 inoculated plants of each cultivar, became infected. The progeny viruses from the infected plants of Kinuyutaka (Ka1–KN) and Misato Golden (Ka1–MS), after mechanical passages through Misato Golden, efficiently infected Misato Golden (Fig. 1) and other cultivars with *Ym*, but the virus passaged only through Ishukushirazu (Ka1–IS) did not infect any of cultivars with *Ym*.

As the progenies Ka1–KN and Ka1–MS were thought to be mutants derived from BaMMV–Ka1, nucleotide sequences of their capsid protein (CP) genes were analyzed and compared with the sequence reported for BaMMV–Ka1 (Kashiwazaki *et al.*, 1992). The entire CP genes of Ka1–

KN and Ka1–MS were amplified by RT–PCR and cloned. Four independent cDNA clones from Ka1–KN and two from Ka1–MS were sequenced. All clones, except one from Ka1–KN, had a nucleotide sequence completely identical to that of BaMMV–Ka1. One clone from Ka1–KN had only a single base change (A to G at position 82 from the 5' terminus of the CP gene), which is probably unrelated to pathogenicity as other clones had no such change.

Discussion

The results from co–inoculation with BaMMV–Ka1 and BaMMV–Na1 revealed that RNA components of the two strains were sufficiently compatible to generate all nine possible combinations, containing at least one RNA1 and one RNA2, in infected barley plants. The infection rate was generally low after co–inoculation with the two strains, as well as after separate strain inoculations. The low infection rate would be one reason why, after co–inoculation, the mixed population of RNAs from the inoculum segregated into different combinations. It is also likely that there are competition or affinity between RNAs *in vivo*.

The reactions of barley cultivars after co–inoculation with BaMMV–Ka1 and BaMMV–Na1 and inoculation with sap containing their pseudorecombinants demonstrate that RNA 1 has determinants for the pathogenicity and symptomatology. These two strains were obtained from field samples collected at different locations and sequence analysis of the 3'–terminal halves of their RNA1 shows approx. 10% nucleotide differences distributed throughout the sequenced parts (Kashiwazaki *et al.*, 1992). Hence, any relationship between sequence differences between the two strains and their differences in pathogenicity or symptomatology cannot easily be elucidated.

The progeny viruses from cultivars with *Ym* infected after single inoculation with BaMMV–Ka1 are thought to have mutation(s) to overcome the host resistance. Sequence analysis the CP genes of these possible mutants showed no consistent differences from BaMMV–Ka1, suggesting that the CP gene is unrelated to resistance–breaking. Thus, to identify gene(s) involved in pathogenicity towards cultivars with *Ym*, other parts of their genomes need to be analyzed.

References

HUTH, W., LESEMANN, D. –E. & PAUL, H. –L. (1984). Barley yellow mosaic virus: purification, electron microscopy, serology, and other properties of two types of the virus. *Phytopathol. Z.* 111, 37–54.

KASHIWAZAKI, S., OGAWA, K., USUGI, T., OMURA, T. & TSUCHIZAKI, T.(1989). Characterization of several strains of barley yellow mosaic virus. *Ann. Phytopathol. Soc. Jpn.* 55, 16–25.

KASHIWAZAKI, S., NOMURA, K., KURODA, H., ITO, K. & HIBINO, H. (1992). Sequence analysis of the 3'–terminal halves of RNA1 of two strains of barley mild mosaic virus. *J. Gen. Virol.* 73, 2173–2181.

KASHIWAZAKI, S. & HIBINO, H. (1996). Genomic reassortment of barley mild mosaic virus: evidence for the involvement of RNA1 in pathogenicity. *J. Gen. Virol.* 77, 581–585.

KASHIWAZAKI, S. (1996). The complete nucleotide sequence and genome organization of barley mild mosaic virus (Na1 strain). *Arch. Virol.* (in press).

NOMURA, K., KASHIWAZAKI, S., HIBINO, H., INOUE, T., NAKATA, E., TSUZAKI, Y. & OKUYAMA, S. (1996). Biological and serological properties of strains of barley mild mosaic virus. *J. Phytopathol.* 144, 103–107.

DETECTION OF BEET NECROTIC YELLOW VEIN VIRUS STRAINS BY MEANS OF RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) AND SINGLE STRAND CONFORMATION POLYMORPHISM (SSCP) ANALYSES OF IMMUNOCAPTURE RT-PCR PRODUCTS

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Summary

Reverse transcription (RT)-PCR, especially when it is carried out in its immunocapture version, not only allows the very sensitive detection of plant viruses, but in combination with restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP) analyses, it also enables the highly sensitive differentiation of virus strains which may not be distinguishable by serological means. By means of these two methods we were able to demonstrate the existence of three strain groups of beet necrotic yellow vein virus in Europe. In most countries the A type is prevalent, but in Germany and France mainly the B type was found. The P type was detected in a small area around the French town of Pithiviers where rizomania is known to be especially severe.

Introduction

Sugarbeet rizomania which is caused by beet necrotic yellow vein virus (BNYVV) has been recorded in the past three decades in an increasing number of countries and has now become a major problem in most sugarbeet growing areas in the world. In some areas the symptoms seem to be more severe than in others, therefore the question was raised whether there may be different strains of BNYVV. Earlier attempts to detect major serological differences between isolates by means of serology had failed, but by means of restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP) analyses of RT-PCR products which were obtained from various parts of the BNYVV genome we have been able to demonstrate the existence of various strain groups of BNYVV (Kruse *et al.*, 1994; Koenig *et al.*, 1995).

Materials and Methods

BNYVV-infected sugarbeet samples were received from all major sugarbeet growing areas in Europe and also from some other parts of the world (e.g. China, Japan and USA). Details of RFLP and a non-radioisotopic variant of SSCP analyses of RT-PCR products have been described by Kruse *et al.* (1994) and Koenig *et al.* (1995). The principle of SSCP analysis is outlined in Fig. 1.

Results and Discussion

In our first studies we used restriction fragment length polymorphism (RFLP) analyses in attempts to detect differences between the RT-PCR products obtained from various sources of BNYVV. By means of this method we could clearly demonstrate the existence of two major strain groups of BNYVV in Europe which were named type A and type B (Kruse *et*
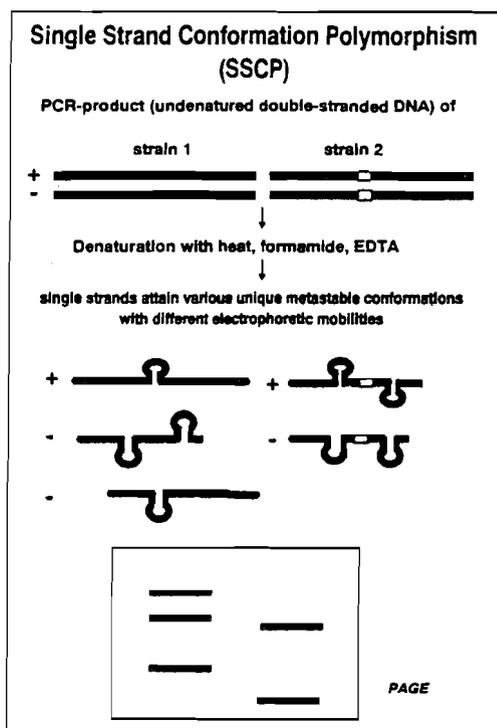
154

Fig. 1. Schematic representation of the principle of SSCP analyses

al.,1994). The restriction patterns obtained for the B type corresponded to those which were to be expected from the nucleotide (nt) sequences published by Bouzoubaa et al. (1985, 1986 and 1987) for the French BNYVV isolate F2/F13. The restriction patterns obtained for the A type, however, were in agreement with those to be expected from the nt sequences of RNAs of various isolates from other parts of Europe (for review see Kruse *et al.*, 1994). The few amino acid exchanges occurring in the coat protein regions of the A and B types and the C type described below lie outside the four antigenic regions which we have identified previously (Commandeur *et al.*, 1994). This might explain the lack of serological differences between the various BNYVV types (Kruse *et al.*, 1994).

For analyzing large numbers of samples, RFLP analyses were found to be inconvenient, because they are expensive and labour-intensive. In later studies we have, therefore, adopted a non-radioisotopic variant (Oto *et al.*, 1993) of SSCP analysis (Orita *et al.*, 1989). The results of such tests with 18 different sugarbeet samples for which RT-PCR products for four different regions of BNYVV RNAs 1 to 4 were checked are shown in Fig. 2. A and B type as well as mixed infections (Fig. 2, lanes 10) and minor variants of BNYVV (Fig. 2b, lanes 17 and 18,) were readily distinguished. Deviating patterns for all four RNA species (Fig. 2, lanes 2) were detected with all 12 beet samples which were obtained from various fields around the French town of Pithiviers where rizomania is known to be especially severe. This suggests that a different strain (P type) is present in this area. Another deviating BNYVV type (C type) was identified in beets received from China (Fig. 2, lanes 1).
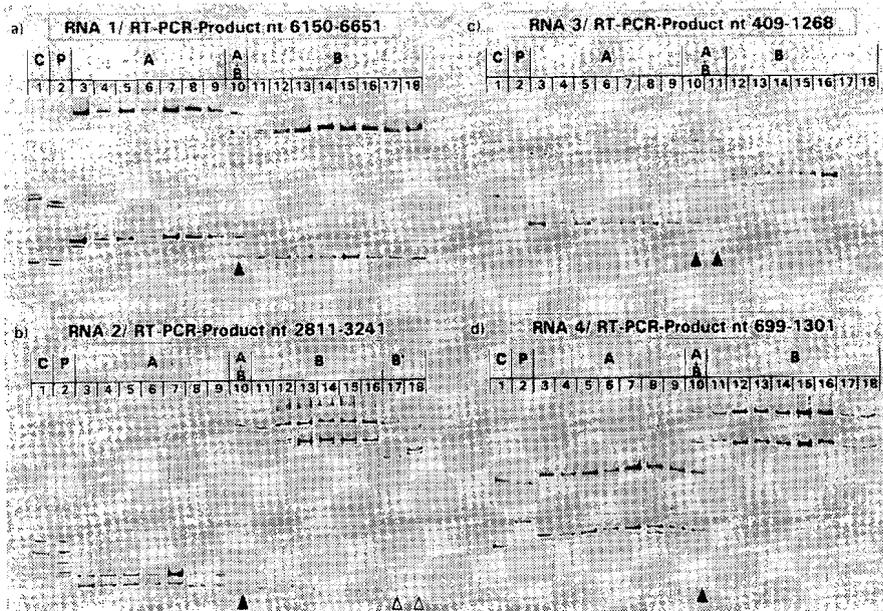
Fig. 2. SSCP patterns produced by the RT-PCR products obtained for various regions on BNYVV RNAs 1 to 4. Origin of samples: 1 - China (Inner Mongolia), 2 - France (Pithiviers), 3 - USA (Texas), 4 - Turkey, 5 - Spain, 6 - Italy, 7 - Austria, 8 - Netherlands, 9 - England (Thetford), 10 - Germany (Lower Saxony), 11 - England (West Stow), 12, 13, 14, 18 - France (Puy de Dôme, Oise, Aube and Marne et Ardennes), 15, 16, 17 - Germany (Lower Saxony, upper Rhine valley and Bavaria). ▲ - mixed infections, △ - mutants.

The results shown in Fig. 2 were obtained with PCR products which in preliminary tests had allowed an optimal differentiation of the A and the B type of BNYVV. Not all parts of the BNYVV genome yielded PCR products which were equally suitable for distinguishing the various virus types despite the fact that they may have contained the same number of nt exchanges as other genome regions which yielded well-differentiating PCR products. This confirms observations made by others that the effect of nt changes on the electrophoretic mobility is difficult to predict and that some sequence changes may not appreciably affect the mobility (e.g. Orita *et al.*, 1989; Takahashi-Fujii *et al.*, 1993).

Fig. 3 shows the geographical distribution of the A, B and P types of BNYVV in various parts of Europe. In most countries the A type is prevalent, but in Germany and France we found mainly the B type. In England where rizomania has been introduced only recently, the A type and the B type as well as mixed infections were found suggesting that the disease has been introduced to Britain several times from various parts of Europe.

From our results it is evident that SSCP analyses which are less expensive and time-consuming than RLFP analyses are a convenient tool for assigning large numbers of BNYVV sources to a certain strain group and for detecting mixed infections, minor variants or new strain groups of the virus. It is suggested that this method should be of general use for large scale comparative studies on the genomes of other viruses and especially for the differentiation of serologically undistinguishable strains.

156
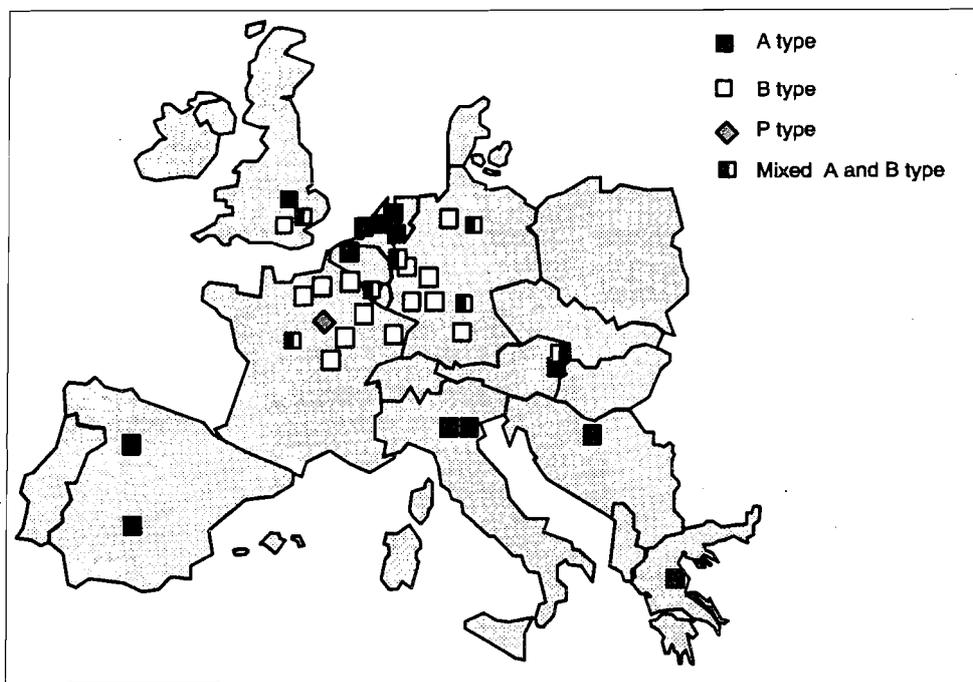
Fig. 3. Identification of various types of BNYVV in Europe

ACKNOWLEGDEMENTS

REFERENCES

KOENIG, R., LÜDDECKE, P. and HAEBERLÉ, A.M. (1995). Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. *J. Gen. Virol.* **76**, 2051-2055.

KRUSE, M., KOENIG, R., HOFFMANN, A., KAUFMANN, A., COMMANDEUR, U., SOLOVYEV, A.G., SAVENKOV, I. and BURGERMEISTER, W. (1994). RFLP analysis of RT-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. *J. Gen. Virol* **75**, 1835-1842.

These papers also contain further references and more detailed informations.

CHARACTERIZATION OF *POLYMYXA* SP. ASSOCIATED WITH THE TRANSMISSION OF INDIAN PEANUT CLUMP VIRUS.

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Summary

Temperature and soil moisture requirements as well as host range of *Polymyxa* strains associated in India with Indian peanut clump virus transmission (IPCV-*Polymyxa*) were compared to those of *P. betae* and *P. graminis* from temperate areas. IPCV-*Polymyxa* has a narrow optimum temperature range close to 30°C compared to 15-20°C for *P. graminis* and 20-25°C for *P. betae*. Sorghum planted on heavily infested Indian soil became infected by IPCV-*Polymyxa* even at very low humidity (suction > 80 millibars, 8% w/w). The preferred hosts for IPCV-*Polymyxa* appeared to be sorghum and pearl millet, compared to barley and wheat for *P. graminis* and Chenopodiaceae for *P. betae*. RFLP-analysis using PCR-amplified rDNA gave for IPCV-*Polymyxa* a pattern different from those obtained for *P. graminis* and *P. betae*. This suggests that IPCV-*Polymyxa* is taxonomically distinct.

Introduction

Peanut clump is a soil- and seed-borne virus disease widely distributed in India (Reddy *et al.*, 1988) and in West Africa (Thouvenel *et al.*, 1988). *Polymyxa*, a plasmodiophoromycete fungus, was reported to be the vector of Indian peanut clump virus (IPCV) and was referred to as *Polymyxa graminis* (Ratna *et al.*, 1991). This intracellular obligate root parasite is widespread and has been associated with the transmission of 10 other viruses on cereals (Maraite, 1991). IPCV has a wide host range and is transmitted on dicotyledonous species in addition to monocotyledonous plants. The work of Ratna *et al.* (1991) suggested that the host range of *P. graminis* from IPCV infested areas is different and larger compared to the one of isolates involved in the transmission of virus on cereals in temperate areas. *Polymyxa* cystosori were detected in the roots of monocotyledonous plants, such as *Eleusine coracana*, *Pennisetum glaucum*, *Sorghum bicolor*, *S. sudanense*, *Triticum aestivum*, *Zea mays*, and dicotyledonous plants from several families, such as *Arachis hypogea*, *Beta vulgaris*, *Lactuca sativa*, *Spinacea oleracea*. The taxonomy of *Polymyxa* spp. being delineated by the host range, *Polymyxa* involved in the IPCV transmission is here referred to as IPCV-*Polymyxa*.

The ability of IPCV-*Polymyxa* to grow in conditions different from those generally described for zoosporic fungal vectors of viruses, the transmission of IPCV to dicotyledonous species, the wide geographical distribution and the large host range of both IPCV and IPCV-*Polymyxa* increase the interest for further characterization of the fungus. Single cystosorus strains of *Polymyxa* from various origins were produced. This paper reports the first results concerning the ecological requirements, the host range and the DNA polymorphism of IPCV-*Polymyxa* strains compared to *Polymyxa* spp. from temperate areas.

Materials and Methods

Inoculum:

Single cystosorus strains were produced for *Polymyxa* isolates from various origins : on *S. bicolor* for IPCV-*Polymyxa* from Patancheru, India (strains I1-1, I1-20 and I1-229), on *Hordeum vulgare* for *P. graminis* from Loupoigne, Belgium (strain B1), Ottawa, Canada (strain C1), and Carcassonne, France (strain F11), and on *B. vulgaris* for *P. betae* from Opprebais, Belgium (strain

A26/41) and Turkey (strain T17). Roots infected with each strain were air-dried and stored at room temperature in the darkness.

Host range:

Cystosori suspensions of the strains B1 from Belgium, C1 from Canada, and I1-20 from India, were prepared in sterile distilled water from heavily infected dried roots and a volume containing 1000 cystosori was distributed into glass culture tubes (15 cm height; 2,4 cm diameter). Two-day-old seedlings of *B. vulgaris* cultivar (cv.) Cadyx, *Dactyloctenium aegyptium*, *Digitaria ciliaris*, *E. coracana*, *H. vulgare* cv. Corona, *P. glaucum* cv. ICMV 8790 and CIVT M1, *S. bicolor* cv. IRAT 204, and *T. aestivum* cv. Capitaine and HD 2329 were transplanted in culture tubes. Each strain was inoculated to 10 seedlings for each species. The plants were grown for 12 weeks in controlled cabinets, with 12 hours light at 15-20°C (night temperature-day temperature) for *P. graminis* strains B1 and C1, or at 25-30°C for IPCV-*Polymyxa* strain I1-20. The infection was assessed by observation of roots under the macro- and microscope following staining in cotton blue lactophenol.

Temperature requirement:

The effect of temperature on the development of IPCV-*Polymyxa* strains was studied by transplanting 2-day-old seedlings of *S. bicolor* in individual glass culture tube filled with sterile sand and inoculated with about 3000 cystosori (200 tubes/strain). The plants were grown at 15-18°C, 19-22°C, 23-26°C and 27-30°C. The infection was analysed on the roots of 6 to 10 plants harvested between 15 and 56 days incubation for each strain and for each tested temperature.

Water requirement:

Influence of soil water potential on the sorghum roots infection by IPCV-*Polymyxa* was studied in controlled conditions following the method described by Duniway (1976) where various suctions were applied to IPCV infested soil in which sorghum was grown. The soil was allowed to equilibrate for 2 days at a given suction. Six 2-day old pregerminated seeds of *S. bicolor* were then transplanted per funnel and allowed to grow for 3 weeks in controlled cabinets at 25-30°C at a given suction (6 plants/repetition, 3 repetitions/suction). The plants were then removed from the soil, thoroughly rinsed with water and transplanted in glass tubes on sterile sand to be cultivated for 3 weeks in conditions favouring the multiplication of the fungus. The roots were then stained to assess *Polymyxa* infection. The soil tested was the soil from which IPCV-*Polymyxa* strains were isolated. The inoculum potential of the soil was increased by addition of 1400 cystosori of *Polymyxa* strain I1-229 per gramme of dried soil.

DNA polymorphism:

DNA of 8 single cystosorus strains of *Polymyxa* was extracted from cystosori and zoospores and amplified (Ward *et al.,* 1994), with the primers ITS4 and ITS5 in a Perkin Elmer thermal cycler for 25 cycles of 94°C for 30 sec, 42°C for 2 min, and 72°C for 2 min. Amplified DNA was digested with restriction enzyme (Ddel) overnight at 37°C. Products were separated on gels containing 3% agarose NA (Pharmacia Biotech). The DNA was stained with ethidium bromide.

Results

Most *S. bicolor* and *P. glaucum* roots were infected by I1-20 (Table 1). The infection intensity with plasmodia, zoosporangia and cystosori was higher on sorghum than on pearl millet. On wheat cv. HD2329 and sugar beet, only a few cystosori were spotted in one of the plants observed. No infection by I1-20 was detected on the other plants tested. A large proportion of barley and wheat cv. Capitaine were infected by the strains B1 and C1, with infection levels being always much higher on barley than on wheat (Table 1). Strain B1 produced a few cystosori on wheat cv. HD 2329 as well as on sorghum. No infection was detected with B1 and C1 on the other plants tested; *Dactyloctenium aegyptium* and *Digitaria ciliaris* were poorly germinating at 15-20°C.

All three tested IPCV-*Polymyxa* strains showed the fastest and highest infection rate at 27-30°C with cystosori being already detected 15 days after inoculation and representing almost the only fungal structure detected after 46 days (Table 2). At 23-26°C, both infection rate and evolution of the zoosporangial to the resting spore stage is clearly delayed compared to 27-30°C. No infection was detected in the roots of the plants grown at 15-18°C nor 19-22°C except in one

single plant harvested 56 days after inoculation at the latter temperature. Surprisingly the roots of this plant showed abundant infections by cystosori.

Infection of sorghum roots by *Polymyxa* on the sandy IPCV infested soil occurred at the suctions of 30, 50 and 80 millibars (mb), the proportion of infected roots being even slightly higher at the last suction given a water content of 8% (Table 3).

The RFLP pattern of the 3 tested IPCV-*Polymyxa* strains are different from those of *P. graminis* strains from Belgium, Canada and France, or *P. betae* strains from Belgium and Turkey (Fig. 1).

Discussion

The host range of the single cystosorus strain I1-20 of IPCV-*Polymyxa* appeared more restricted than the one reported by Ratna *et al.* (1991) with unpurified inoculum. This discrepancy may be due to : i) differences in cultivar susceptibility of the used host species as observed f. i. for wheat; ii) heterogeneity of the *Polymyxa* populations with regards to the host range, or iii) differences in the definition of a possible host. Our data with a defined inoculum show indeed a ranking in the degree of compatibility from "preferred" hosts, on which primary infections and multiplication are high, f. i. sorghum, or moderate, f. i. pearl millet, and can be easily detected, to "fortuitous" hosts, on which some primary infections evolve into resting spores apparently without going through the zoosporangial stage f. i. beet, wheat or peanut (Legrève *et al.*, unpublished data). Probability of detection will strongly depend on the inoculum potential and evolution of primary infections into cystosori. A fortuitous host can become infected by IPCV vectored by this *Polymyxa* without supporting extensive multiplication of the latter. One outstanding characteristic of the IPCV-*Polymyxa* is its wide range of potential hosts extending from Gramineae to various dycotyledons, while *P.*

Table 1. Host range of IPCV-*Polymyxa* strain I1-20 extracted from sorghum roots, *P. graminis* strains B1 and C1, both extracted from barley roots. Proportion of infected plants 12 weeks after inoculation with 1000 cystosori per plant. The plants were maintained at 15-20°C for the strains B1 and C1, at 25-30°C for the strain I1-20.

	Origins of the strains		
	I1-20	B1	C1
Species and cultivar	India	Belgium	Canada
B. vulgaris (Cadyx)	1/10+	0/10	0/10
Dactyloctenium aegyptium	0/6	0/1	0/1
Digitaria ciliaris	0/6	-	0/1
E. coracana	0/8	0/6	0/10
H. vulgare	0/7	9/10+++	9/10+++
P. glaucum (ICMV 8790)	4/7++	0/5	0/7
P. glaucum (CIVT M1)	5/6++	0/4	0/5
S. bicolor (IRAT 204)	5/6+++	3/8+	0/9
T. aestivum (Capitaine)	0/9	8/10+	9/10+
T. aestivum (HD 2329)	1/6+	3/9+	0/10

trace (+), moderate (++) or high (+++) infection.

Table 2. Effect of temperature on development of IPCV-*Polymyxa* in roots of sorghum plants grown on sand inoculated with 3000 cystosori/ treatment.

Strains	Sampling day	Percentage of infected plants and degree of infection at (°C) (night-day)			
		23-26		27-30	
I1-1	15	0		90	++PZ(C)
	25	67	++(+)PZ	67	++PZC
	35	33	++PZC	100	++PZC
	46	70	++PZC	100	++C
	56	83	++(+)ZC	100	++(+)C
I1-20	15	20	+PZ	80	++PZ
	25	10	+PZ	70	++(+)PZC
	35	60	++(+)PZC	100	+++PZC
	46	100	++(+)PZC	100	++(+)C
	56	100	+++C	100	++C
I1-229	15	10	++PZ	67	++PZ(C)
	25	40	+(+)PZC	88	++(+)PZC
	35	40	++PZC	78	+++PZC
	46	83	+++C	100	++(+)C
	56	67	+++PZC	100	++C

+ : trace of infection; ++ : moderate infection; +++ : high infection by P : Plasmodia; Z : Zoosporangia or C : Cystosori. (+) some plants with higher infection.

Table 3. Percentage of sorghum roots infected by *Polymyxa* when grown on Indian inoculated soil (I) and water content (W) at various suctions (mb).

Suction	W (%) ± st. dev.	I (%) ± st. dev.
0	16,9±1,2	0
30	16,4±0,7	46,6±30,5
50	12,5±1,9	61,1±19,2
80	8,0±1,3	77,5±38,5

Fig. 1. RFLP analysis (using DdeI) of *Polymyxa* strains using PCR-amplified rDNAs. M, size marker φX174*Hae*III; 1, 2, 3, negative control for the cystosori DNA preparation from barley, sorghum and sugar beet; 4. *P. graminis* C1 (cystosori DNA); 5, *P. graminis* B1 (cystosori DNA); 6, *P. graminis* F11 (zoospores DNA); 7, *P. betae* T17 (cystosori DNA); 8, *P. betae* A26/41 (cystosori DNA); 9, 10, 11, IPCV-*Polymyxa* I1-1, I1-20, I1-229 (cystosori DNA).
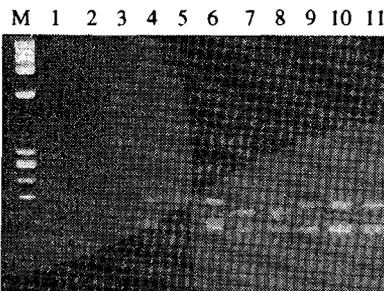
graminis appeared restricted to some Gramineae and *P. betae* to Chenopodiaceae (Barr, 1979). Further studies with mixed zoospore inoculum will analyse the eventual gene flow among these populations and the possible adaptation from a fortuitous to a preferred host. The IPCV-*Polymyxa* strains show several properties that make them different from *P. graminis* and *P. betae*; they have a wide host range, they develop at high temperatures, they can infect at low water potential and they can be distinguished from the other *Polymyxa* species at the molecular level. These differences, which reflect adaptation to conditions prevailing in India, might therefore merit a separate taxonomic designation for IPCV-*Polymyxa*.

Acknowledgements

References

Barr, D.J.S. (1979). Morphology and host range of *Polymyxa graminis, Polymyxa betae* and *Ligniera pilorum* from Ontario and some other areas. *Canadian Journal of Plant Pathology* 1: 85-94.

Duniway, J.M. (1976). Movements of zoospores of *Phytophthora cryptogea* in soils of various textures and matric potentials. *Phytopathology* 66:877-882.

Maraite, H. (1991). Transmission of viruses by soil fungi. In *Developments in agricultural and managed-forest ecology 23 : Biotic interactions and soil-borne diseases, proceedings of the first conference of the European Foundation for Plant Pathology*, pp. 67-82. Eds A.B.R. Beemster, G.J. Bollen, M. Gerlagh, M.A. Ruissen, B. Schippers & A. Tempel, Elsevier, Amsterdam.

Ratna, A.S., Rao, A.S., Reddy, A.S., Nolt, B.L., Reddy, D.V.R., Vijayalakshmi, M., and McDonald, D. (1991). Studies on transmission of Indian peanut clump virus disease by *Polymyxa graminis*. *Annals of Applied Biology* 118:71-78.

Reddy, D.V.R., Nolt, B.L., Hobbs, H.A., Reddy, A.S., Rajeshwari, R., Rao, A.S., Reddy, D.D.R., and McDonald, D. (1988). Clump virus in India : isolates, host range, transmission and management. In *Developments in Applied Biology II. Virus with fungal vectors*, pp. 239-246. Eds J.I. Cooper and M.J.C. Asher. Wellesbourne, UK : Association of Applied Biologists.

Thouvenel, J.C., Fauquet, C., Fargette, D., and Fishpool, L.D.C. (1988). Peanut clump virus in West Africa. In *Developments in Applied Biology II. Virus with fungal vectors*, pp. 247-254. Eds J.I. Cooper and M.J.C. Asher. Wellesbourne, UK : Association of Applied Biologists.

Ward, E., Adams, M.J., Mutasa, E.S., Collier, C.R., and Asher, M.J.C. (1994). Characterization of *Polymyxa* species by restriction analysis of PCR-amplified ribosomal DNA. *Plant Pathology* 43:872-877.

ETIOLOGY OF VASCULAR NECROSIS SYNDROME OF SUGARBEET

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Summary

A vascular necrosis syndrome (VNS) of sugarbeet has been observed in the Imperial Valley of California. The incidence of VNS can be up to 80% in some fields. No evidence of fungi, bacteria or mycoplasma-like organisms has thus far been implicated in the syndrome. Three soil-borne viruses, tomato bushy stunt virus (TBSV), tobacco necrosis virus (TNV), and tobacco mosaic virus (TMV) have been isolated from sugarbeet roots with VNS. The infectious agents have been purified and antisera produced. An inoculation procedure developed for TBSV resulted in beets that showed yellowing and stunting symptoms. However, no symptoms of VNS have been produced to date. Attempts to inoculate TNV to beets have failed. At this point the causal agent or agents of VNS are not known.

Introduction

Since about 1981, a vascular necrosis syndrome (VNS) of sugarbeet (Fig. 1) has been observed in the Imperial Valley of California. The incidence of VNS can be up to 80% in some fields. Early observations suggested the possibility that beet curly top virus (BCV) and lettuce infectious yellows virus (LIYV) might be involved. This early hypothesis was soon proved to be erroneous. In a few instances similar symptoms have been found in the San Joaquin Valley. No evidence of fungi or bacteria have thus far been implicated in the syndrome. The etiology of VNS has not been determined, therefore, we have undertaken the identification and characterization of the viruses associated with vascular necrosis syndrome.
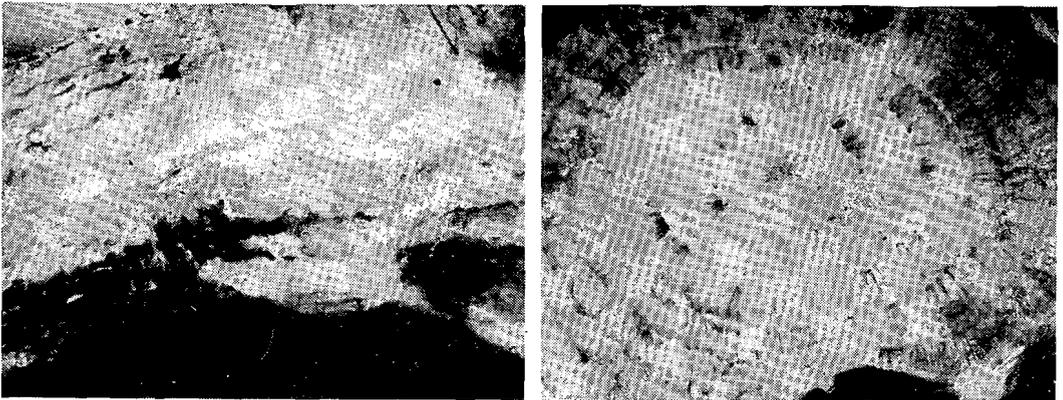

Fig. 1: Sugarbeet roots found in Imperial Valley of California with vascular necrosis syndrome.

Materials and Methods

Field survey:
    Fifty fields, selected at random, were sampled in the Imperial Valley during 1994-1995. Ten beets were selected from the lower end of each field. Each beet root was cut in cross sections starting at the tip and continuing about half-way to the crown. Vascular discoloration was graded on a scale from 0 to 5. Each root was tested by double antibody sandwich ELISA (Clark and Adams, 1977) for beet necrotic yellow vein virus (BNYVV), tobacco mosaic virus (TMV), tobacco necrosis virus (TNV), and tomato bushy stunt virus (TBSV). All roots sampled were mechanically innoculated to indicator plants including *Beta marcocarpa* Guss., *B. vulgaris* L., *Chenopodium album*, *C. amaranticolor*, *C. capitatum* (L.) Asch., *C. murale* L., *C. quinoa* Willd., *Datura stramonium* L., *Gomphrena globosa* L., *Nicotiana clevelandii* Gray, *N. glutinosa* L., *N. tabacum* L., *Spinacia oleracea* L., and *Tetragonia expansa* Murr.

Virus characterization:
    Virus isolates were maintained in the greenhouse in *C. quinoa* after three single-lesion passages of the original isolations from beet root with VNS. Viruses were purified by extraction in 0.1 M phosphate buffer, pH 7.2, and then clarified by chloroform/butanol (1:1). The virus preparations were precipitated by PEG and high-speed centrifugation as concentrating steps. The partially purified viruses were layered onto 10-35% (w/v) sucrose density gradients and centrifuged for 2 hr at 82,700 g and light-scattering virus zone were collected. Purified virus in aliquots of 0.5 ml (100 µg/ml) was prepared for six intramuscular injections into a rabbit for antiserum production as previously described (Liu and Duffus, 1990).
    Serological relationships were determined by agar double diffusion tests. Immunodiffusion media consisted of 0.8% Ionagar and 0.1% sodium azide. Electrophoretic comparison of the isolates was made on 1.0% agarose gels in 6.0 mM Tris, 200mM glycine, pH 8.2 (modified from Hillman, B. et al., 1982, and Koenig, R. and Gibbs, A., 1986). The gels were stained with ethidium bromide (1 µg/ml) and photographed with UV illumination.

Pathogenicity test:
    Beet isolates of tomato bushy stunt virus or tobacco necrosis virus infected *C. quinoa* were homogenized in a mortar and pestle and were incorporated into pasteurized soil in plastic pots. Sugar beet (*B. vulgaris*) seedlings were transplanted into the pots. Water draining from each pot was collected and added back to the same pot. Innoculated plants were maintained under greenhouse conditions at 26-32 C. Three months later the plants were evaluated for disease symptoms.

Results

    Eighty percent of the fields we surveyed in the Imperial Valley, California in 1994 had VNS, ranging in incidence from 10-100%. Viruses were recovered from

68% of the symptomatic beets. Of the isolated viruses 6% were TNV, 21% were TMV, 64% were TBSV, and 9% were not identified.

Two soil-borne viruses, TBSV and TNV, have been isolated from sugarbeet roots with VNS as determined by agar double diffusion tests (Figs. 2 and 3) and electron microscopy (Figs. 4). Different isolates of TBSV virions could be readily differentiated by agarose gel electrophoresis (Fig. 5).
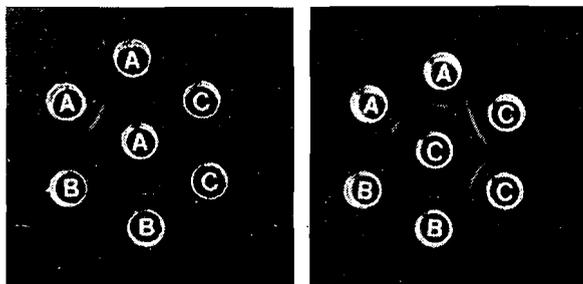
Fig. 2. Agar double diffusion of tomato bushy stunt virus isolate. Outside wells are purified virus and inside wells are antisera. A=tomato isolate, B=beet isolate, C=cherry isolate. The production of antiserum to beet isolate is under way.
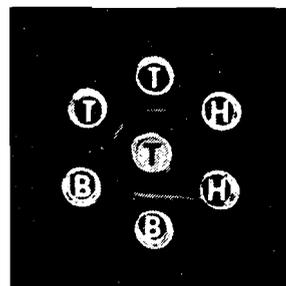
Fig. 3. Agar double diffusion test. Outside wells are plant sap and inside well is antiserum. T=Tobacco necrosis virus, B= beet isolate, H=healthy C. *quinoa*
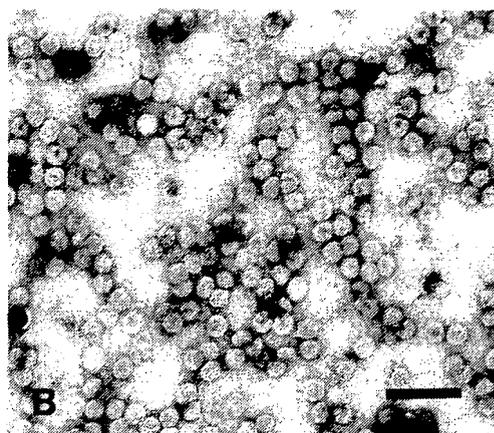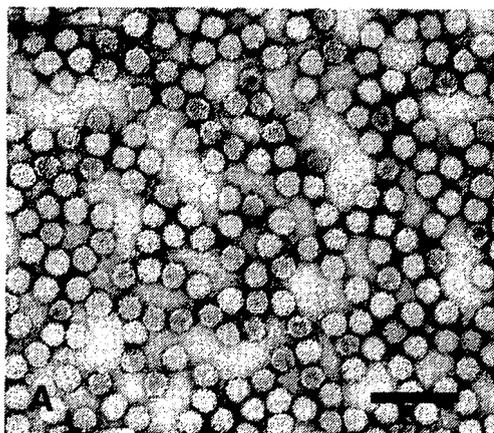
Fig. 4. Electron micrograph of purified (A) beet isolate of tomato bushy stunt virus and (B) beet isolate of tobacco necrosis virus particles stained in 2% uranyl acetate. Bars represent 100 nm.

A B C

Fig. 5. Agarose gel electrophoresis of whole virions of tomato bushy stunt virus isolates. Lane A contains cherry isolate, lane B contains tomato isolate, and lane C contains beet isolate. Although the beet and tomato isolates could not be distinguished in serological analyses, the virions could be readily differentiated by characteristic mobility differences in agarose gel electrophoresis.

In the pathogenicity tests, when sugarbeet seedlings were innoculated with TBSV the inoculated beets showed yellowing and stunting symptoms. The infected beet roots also showed positive reaction in ELISA tests. However, no vascular necrosis syndrome has been produced. Attempts to infect beets with TNV by mechanical inoculation or by soil inoculation have failed.

Discussion

Three identified viruses (TBSV, TNV, and TMV) and two unidentified viruses have been isolated from sugarbeet roots with VNS. At present time, we can inoculate and infect TBSV into sugarbeet successfully. However, no vascular necrosis syndrome has been produced from these inoculations. It is not clear at this point what the causal agent or agents of VNS are. In the 1995 field survey in the Imperial Valley, California, the VNS incidence was dramatically decreased to only 2% of the fields showing symptoms. The possible roles that varietal differences, environmental factors and/or possible vectors play in this vascular necrosis syndrome need further study.

References

Clark, M. F. and Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34: 475-483.

Hillman, B., Morris, T. J., Kellen, W. R., Hoffman, D., and Schlegel, D. E. (1982). An invertebrate calici-like virus: evidence for partial virion disintegration in host excreta. J. Gen. Virol. 60: 115-123.

Koenig, R. and Gibbs, A., (1986). Serological relationships among tombusviruses. J. Gen. Virol. 67: 75-82.

Liu, H. Y. and Duffus J. E. (1990). Beet pseudo-yellows virus: Purification and serology. Phytopathology 80: 866-869.

PRODUCTION OF POLYCLONAL ANTISERA TO THE COAT PROTEIN OF WHEAT SPINDLE STREAK MOSAIC VIRUS EXPRESSED IN *Escherichia coli*

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Summary

The CP coding region of Wheat spindle streak mosaic virus (WSSMV) has been cloned into the expression vector pET-3A. WSSMV CP overexpression in *Escherichia coli* and purification from polyacrylamid gels allows the immunization of a rabbit and recovery of a good-titered polyclonal serum. Due to its mode of obtention this serum gives no reaction with healthy plant sap and circumvents difficult bymovirus purification.

Introduction

Slykhuis (1960,1970) identified a soil-borne virus on winter wheat in Ontatrio, Canada which was designated as Wheat Spindle Streak Mosaic Virus (WSSMV). The cause of the disease was identified as a filamentous virus inducing cylindrical inclusions and transmitted by the soil-borne fungus *Polymyxa graminis*. It is now classified in the family *Potyviridae* in the genus *Bymovirus* which type member is the BaYMV (Barley Yellow Mosaic Virus). WSSMV has also been reported in the USA (Williams et al., 1975, Lommel et al., 1986 ;), India (Ahalawat et al., 1974); France (Signoret et al., 1974), Italy (Rubiess et al 1987) and China (Chen, 1993). In Japan the causal agent of a yellow mosaic virus of winter wheat has been called Wheat Yellow Mosaic Virus (WYMV) (Inouye, 1969). cDNA complementary to the 3' -terminal half of RNA1 of WSSMV from Southern France has been cloned and sequenced (Sohn et al., 1994). The clone has been used to amplify and clone the CP coding sequence into the vector pET-3a for expression in *E.coli*. The overexpressed CP has been used to prepare a polyclonal serum. We present here briefly the characterization of this polyclonal antiserum.

Material and methods

Construction of the prokaryotic expression vector
Amplification of the CP-region was done with oligo B (5' *GAC ATT GAA* **Cat atg** *GCT GCG GAC AC* 3') corresponding to the end of the NIb and the start of the CP-coding region of RNA1 (nt 3517-3542 ; Sohn et al., 1994) and with oligo A (5' *CCA CAC* **GgG Atc** *CGC GAT GTA C* 3') corresponding to the nt 4456-4477 (Sohn et al., 1994). (Nucleotides in bold correspond to restriction sites used for cloning, nucleotides in small letters do not correspond to viral sequences). Because the CP-gene is part of a sequence encoding a polyprotein it was necessary to design a start codon in oligo A. Furtheron, oligo A includes the restriction site NdeI and oligo B the restriction site BamHI which enable the cloning of the amplified fragment in the polylinker of the prokaryotic expression vetctor pET-3a.
The PCR-reaction containing 50 pmoles of each primer, 20 ng of vector pGEMMoI (Sohn et al., 1994), 2.5 U Taq polymerase (Boehringer), 10 mM dNTPs in a total volume of 20 µl was performed under the following conditions : 2 min 95°C,30 cycles 2 min 92°C, 2 min 72°C on a Biometra Trio Thermoblock.

After checking an aliquot on an agarose gel the PCR reaction was directly used for the digestion with the restriction enzymes NdeI and BamHI. The expected fragment of 960 bp was excised from a 1% agarose gel and eluted using an extraction kit (Quiagen). The fragment was then ligated into the expression vector pET-3a cut with the same enzymes resulting in the expression vector pET-CPW. All molecular cloning techniques were performed as described by Sambrook et al., 1989.

Protein purification and antibody production

One colony of BL21 (DE3) containing pET-3a (as negative control) and three colonies of BL21 bearing the recombinant plasmid pET-CPW were grown overnight at 37 °C with vigourous shaking in 3 ml of Luria Broth containing 100μg/ml of ampicillin.

30ml of LB supplemented with ampicillin were inoculated with each culture and incubated at 37°C until OD_{600} reaches 0.5. Expression of the CP was induced by adding IPTG to a final concentration of 0.4 mM and induction was kept at 37°C, shaking during three hours.

Bacteria were harvested at 5000 rpm in the JA20 (Beckman J21) and resuspended in 1.2 ml of TE (Tris /EDTA : 10/1).

Bacteria lysis was achieved by three cycles of congelation/decongelation (-80°C), a benzonase treatement (12u/ml) of thirty minutes at 37°C, and ultra sonication / 3 min of efficient treatment.

A following centrifugation of 10 min at 12000 g separates a supernatant containing the soluble proteins and a pellet containing insoluble proteins as inclusion bodies. Pellets were resuspended in 1.2 ml of TE and washed with the following procedure : after a 10 min centrifugation at 12000 g pellets were resuspended in three volumes of TKET buffer (Tris-Cl 50 mM pH 7.8; KCl 0.1 M ; EDTA 10 mM and Triton X-100 0.5 %) and incubated 10 min at room temperature; this step was repeated and followed by a wash with TKE (TKET without Triton) and finally pellets were resuspended in TE.

Proteins were separated through a 10% polyacrylamid gel containing SDS. Proteins were visualized with cold KCl (0.2M) during 5 min, the CP band was excised, and conserved at - 20°C overnight. Protein was recoverd by grinding the band in elution buffer (Tris-Cl 50mM pH 8 ; EDTA 0,1 mM; NaCl 150 mM) and incubating it two hours at 37°C.

A final centrifugation separates acrylamide from the eluted protein.

Rabbits were injected intradermally with 100-200 μg of antigen supplemented with an equal volume of complete (first injection) or incomplete Freund's adjuvant. The two first injections were done at 15 days interval and the third one month after the second. Bleeedings were collected from the third injection and then regularly during three months.

Checking the serum

Serum was checked by ACP-ELISA and by western blot analysis using conventional protocols. For western blot analysis samples conserved at -20°C were used and ground in PBS-Tween (0.05%).

Results and discussion

For the overexpression in bacterial cells the CP-encoding region of RNA1 of WSSMV was modified and amplified by PCR using oligos A and B. The oligos were designed creating an NdeI restriction site at the 5' end including the ATG start codon and at the 3' end a BamHI restriction site was inserted in the 3' non translated region. This enables the direct cloning of the amplified fragment into the expression vector pET-3a. The resulting vector pET3CPW allows the transcription and translationn of the complete CP gene under the control of the T7 promotor without any fusion with other amino acids.

Fig 1 shows that a protein of expected molecular weight was obtained only in colonies bearing pET-CPW plasmid and not in the negative control. The protein was found mainly in the pellets.
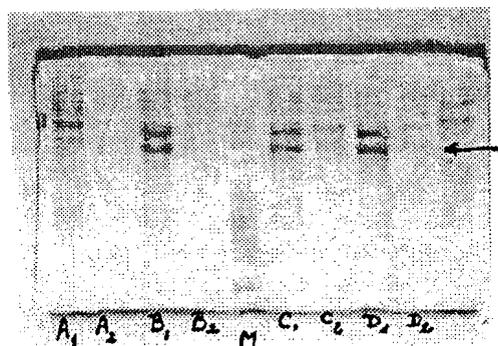
Fig 1: 10% polyacrylamid SDS gel colored with Coomassie brillant blue. All samples have been prepared as described above.

A: BL21(DE3) bearing pET-3a

    1: pellet ; 2: supernatant

B,C,D, BL21(DE3) bearing pET-CPW (three independent colonies)

    1: pellet ; 2: supernatant

overexpressed CP is observed in B1,C1,D1.

A single induction gave enough material to immunize one rabbit.

Serum was checked by ACP Elisa and has a titer of about 1/100000.
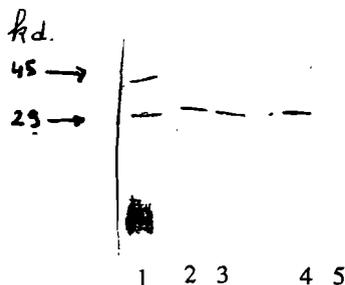
Fig 2 : shows the reactivity of the serum in western blot analysis.

1: molecular weights ; 2, 3, 4 : protein extracts of WSSMV-infected wheat leaves from Southern France, 5: healthy wheat leaves.

The serum react specifically with a protein of 30-32 kD of WSSMV-infected leaf cells. This is consistent with the molecular weight expected from the sequence and reported from previous experiments. No signal is detected using protein extracts from healthy wheat leaves.

The produced antisera was also tested in ACP-ELISA with WSSMV-infected and BaYMV-infected leaf-extracts. A cross reaction of the polyclonal antisera was observed to the BaYMV-infected extract but the determined OD values were 5 times lower compared to those of WSSMV-infected extracts. The cross reaction can be explained by the high homology of the amino acid sequence of the coat proteins of WSSMV and BaYMV (74 %). Only some monoclonal antibodies can distinguish between these to related bymoviruses (Hariri et *al.*, 1996). The antisera described here can be easily used in ELISA and Western blot analysis to detect WSSMV in plant extracts.

168

References

Ahalawat, Y.S., Majumdar, A. and Chenulu, V.V. (1974). First record of wheat spindle streak mosaic in India. *Plant Dis Rep* **60** : 782-783.

Chen, J. (1993). Occurrence of fungally transmitted wheat mosaic viruses in China. *Ann Appl Biol* **123** : 55-61.

Hariri, D., Delaunay, T., Gomes, L., Filleur, S., Plovie, C. and Lapierre, H. (1996).Comparison and differentiation of Wheat Yellow Mosaic Virus (WYMV), Wheat Spindle Streak Mosaic Virus (WSSMV) and Barley Yellow Mosaic Virus (BaYMV) using WYMV monoclonal antibodies. *European Journal of Plant Path.* **102** :283-292.

Inouye, T. (1969). Filamentous particles as the causal agent of yellow mosaic disease of wheat. *Nogaku Kenkyu* **53** : 61-68.

Lommel, S.A., Willis, W.G. and Kendall, T.L.(1986). Identification of wheat spindle streak mosaic virus and its role in a disease of winter wheat in Kansas. *Plant Dis* **70** :964-968.

Rubiess-Autonell, C. and Vallega, V. (1987). Observations on mixed soil-borne wheat mosaic virus and wheat spindle streak mosaic virus infection in durum wheat . *J Phytopathol* **119** : 111-121.

Sambrook, J., Fritsch, E.F. and Maniatis , T. (1989). Molecular cloning : a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, New York.

Signoret, P.A., Alliot, B. and Poinso, B. (1974). Présence en France du wheat spindle streak mosaic virus. *Ann Phytopathol* **9** : 377-379.

Slykhuis, J.T. (1960). Evidence of a soil-borne mosaic of wheat in Ontario. *Can Plant Dis Surv* **40** :43.

Slykhuis, J.T. (1970). Factors determining the development of wheat spindle streak mosaic virus caused by a soil-borne virus in Ontario. *Phytopathology* **60** : 319-331.

Sohn, A., Schenk, P., Signoret, P.A., Schmitz, G. , Schell, J. and Steinbiβ, H.H. (1994). Sequence analysis of the 3'-terminal half of RNA1 of wheat spindle streak mosaic virus. *Arch. Virol.* **135** :279-292.

Usugi, T. and Saito, Y. (1979). Relationship between wheat yellow mosaic virus and spindle streak mosaic virus. *Ann Phytopathol Soc Jap* **45** : 397-400.

Usugu, T. , Kashiwasaki, S., Omura, T. and Tsucizaki, T. (1989). Some properties of nucleic acids and coat prtoeins of soil-borne filamentous viruses. *Ann Phytopathol SOC Jap* **55** : 26-31.

Williams, A.S., Pirone, T.P., Slykhuis, J.T. and Tutt, C.R.(1975). Wheat spindle streak mosaic virus in Kentucky. *Plant Dis Rep* **59** : 888-889.

SEROLOGICAL DETECTION OF *SPONGOSPORA SUBTERRANEA* F.SP. *SUBTERRANEA*

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Summary

A polyclonal antiserum to the spore ball stage of *Spongospora subterranea* f.sp. *subterranea*, the causal agent of powdery scab of potato and vector of mop-top furovirus (PMTV) was produced to evaluate the potential of serology for detection. It detected as little as 0.02 spore balls in enzyme-linked immunosorbent assay (ELISA). Spore balls of different geographical and cultivar origin were detected equally well. Spore balls were detected in spiked soil down to 100 spore balls/g soil and also in naturally infested field soils. Comparison between ELISA and a bioassay utilising tomato bait plants for the detection of spore balls showed that discrimination of spore ball levels improved at concentrations above 2000/g soil in ELISA, whereas the opposite was the case for the bioassay.

Introduction

Powdery scab of potatoes is caused by infection of tubers, roots and stolons by the obligate zoosporic parasite *Spongospora subterranea* f.sp. *subterranea*. However, the scab-like lesions on tubers are the most damaging as they reduce the proportion of marketable crop and carry infection to subsequent crops. *S. subterranea* is also important as the vector of potato mop-top furovirus (PMTV). These diseases have become more important in parts of Europe over the last decade including the U.K. (Wale, 1987), Switzerland (Merz, 1993), Nordic countries (Rydén *et al.*, 1989; Kurppa, 1989), Holland (Turkensteen, pers. comm.) and also other parts of the world including Turkey (Eraslan and Turhan, 1989), USA (Mohan, pers comm), Australia (de Boer, 1991), New Zealand (Braithwaite *et al.*, 1994) and Pakistan (Ahmad *et al.*, 1991). This increase in importance is thought to be due to the intensification of potato production, shorter rotations, increased irrigation of crops and spread of resting spores on seed stocks.

Control of the disease is difficult as the resting spores are able to survive in a dormant state in soils for a number of years and are difficult to kill. Disease avoidance through planting clean seed into uncontaminated land would be the only reliable form of control. However, even though seed tubers with scabs may be identified by visual inspection, symptomless infections have been reported (de Boer *et al.*, 1982; Diriwächter and Parbery, 1991). Determination of the presence and contamination levels of resting spores in field soils is laborious. Sensitive and rapid serological detection of resting spores on tubers and in field soils would be a great aid to the development of IPM strategies and certification schemes. This paper describes the development of serological techniques for the detection the spore ball stage of *S. subterranea*.

Material and Methods

Production of antiserum:
Spore balls from potato tubers cv. 'Bintje' were used as immunogens. A New Zealand White rabbit was injected subcutaneously with 1 ml of spore ball preparation emulsified with 1 ml Freund's complete adjuvant. One and two weeks later further injections were made intramuscularly with 0.5 ml of spore ball preparation emulsified with 0.5 ml of Freund's incomplete adjuvant. A booster injection intramuscularly with 0.5 ml of spore ball preparation emulsified with 0.5 ml Freund's incomplete adjuvant was made seven months later. A final intramuscular booster injection was given after a further three months. Final blood serum samples were then collected and tested by ELISA. The γ-globulin containing fraction of the serum was prepared as described by Clark and Adams (1977) and some samples of raw serum were cross-absorbed with uninfested Swiss field soil.
ELISA:
Plate trapped antigen (PTA) ELISA was used in all experiments. Prepared spore balls were ground with pestles and mortars in carbonate buffer. Sample homogenates were pipetted into wells of microtitre plates and incubated overnight (16h) at $6^{o}C$. Subsequently the plates were incubated for 2h at room temperature with raw serum. This was followed by goat anti-rabbit γ-globulin conjugated to alkaline phosphatase for 3h at room temperature. Finally the plates were incubated with substrate at room temperature and the optical absorbence at 405 nm (A_{405}) was measured.
Sensitivity and specificity of the antiserum:
A sample of spore balls from potato cv. 'Bintje' was weighed and quantified by counting in a haemocytometer slide. Dilutions containing 4000, 1000, 50, 12.5, 2.5, 0.5 and 0.1 spore balls/ml carbonate buffer were then prepared. Samples of spore balls were collected from cv 'Bintje', 'Indira' and 'Ukama' and 30 mg of each was suspended in 2 ml of buffer and ground or left unground. Spore ball preparations from Switzerland, Scotland and USA were quantified and further diluted in carbonate buffer to give concentrations of 50 spore balls/ml. These were all tested by PTA-ELISA as described above.
Detection of resting spore balls in soil:
A dilution series containing approximately 8000, 6000, 4000, 2000, 1000, 500, 100 and 0 spore balls per gram of soil was prepared from uninfested field soil and spore balls from potato cv. 'Bintje'. A sample of each dilution (1g) was taken and ground in 2 ml carbonate buffer and tested by ELISA. The spore dilutions were also tested by bioassay (Merz, 1989). A range of seven infested soils with known bioassay scores and an uncontaminated one from Swiss potato fields were ground in carbonate buffer (1g/2 ml) and tested by ELISA.

Results

The prepared and quantified spore balls reacted strongly in PTA-ELISA with the raw serum and A_{405} values increased consistently with increased spore ball concentration (Fig. 1). The sample containing 0.02 spore balls (200μl of 0.1 spore balls/ml) gave an A_{405} of 0.04 whereas blank wells gave a value of 0.02. In contrast equivalent weights of scraping from uncontaminated potatoes gave low absorbence values (Fig. 1).
A comparison of the A_{405} for equal quantities of ground and unground scrapings from infected potatoes showed that grinding almost doubled the values (A_{405} = 0.18 not ground; 0.34 ground). In tests on equal weights of resting spore ball preparations from three different
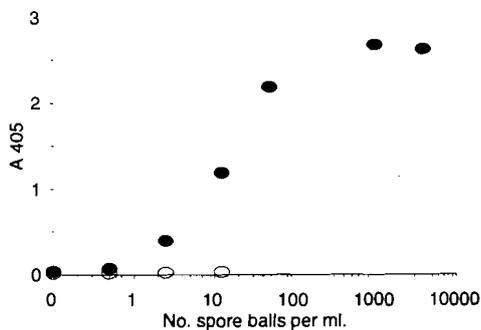
Fig. 1 The relationship of *Spongospora subterranea* spore ball concentration and equivalent concentration of healthy potato scrapings in PTA-ELISA. (●) spores; (○) healthy potato
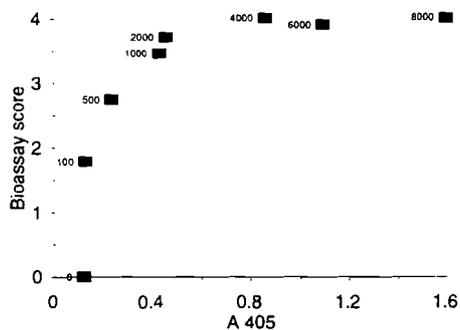
Fig. 2 The relationship between bioassay scores for soils spiked with spore balls of *Spongospora subterranea* (spore balls/g soil) and A $_{405}$ in PTA-ELISA.

potato cultivars - 'Bintje', 'Indira', 'Ukama' - there were remarkably no differences in A_{405} values.

Tests on equal quantities of spore balls (50/ml) of different geographical origin demonstrated the ability of raw serum to detect these in PTA-ELISA. The A_{405} values obtained showed differences between spores of different origins: Swiss = 0.93, Scottish = 1.11, USA = 1.18.

The raw serum detected spore balls mixed with uninfested field soil. The relationship between A_{405} values and spore ball concentration/g soil appeared to be linear. The antiserum detected 100 spore balls/g soil but discrimination of spore ball levels was better for concentrations greater than 2000/g soil. In contrast the bioassay discriminated spore ball levels better in soils containing less than 2000/g soil (Fig. 2).

The raw serum discriminated two of three Swiss field soils with high bioassay scores (>3) from those with lower scores in PTA-ELISA, however soils with bioassay scores ranging from zero to three were not differentiated from each other. When tested with the cross-absorbed serum or the purified γ -globulin fraction the differentiation of the contaminated soils from the uncontaminated ones was much improved.

Discussion

Further to the earlier description of serological detection of *S. subterranea* (Harrison *et al.*, 1993) this paper describes improvements in the sensitivity of serological detection of spore balls from tubers and soils. There was a quantifiable relationship between the concentration of spore balls and the A_{405} values. The assay detected 0.02 spore balls and was slightly more sensitive than the test described by Harrison *et al.* (1993), perhaps due to differences in antisera titres or assay methods.

The ability to detect spore balls of different potato cultivar and geographical origin suggests that the antiserum has wide application. In addition to the detection of spore balls from Switzerland, Scotland, and USA the antiserum has also been shown to detect spore balls from Pakistan (Ahmad *et al.*, these proceedings) and Australia (Wilson, pers. comm.). From

the results presented it can be recommended that all samples should be ground prior to testing.

The reduced ability of the antiserum to discriminate spore ball concentrations of less than 2000/g soil in spiked soil may have been due in part to detection of non-target antigens in the soil itself, masking spore ball antigen detection or cross reaction of substrate with alkaline phosphatase in soil extracts. With soils with <2000 spore balls/g soil the bioassay may be more sensitive because the pathogen has the opportunity to multiply on the host during the 8 days of the assay.

Soils that are considered to be heavily infested are thought to contain >500 spore balls/g soil and such soils have given bioassay scores of >3 (Merz, 1993). However, many soils that gave high bioassay scores, did not give rise to diseased tubers (Merz, 1993). It would be easy to conclude that in soils where high inoculum levels were detected and no tuber symptoms were observed, the environmental conditions (e.g. soil moisture) were unfavourable for infection. However, ELISA tests on some such soils suggested that they contained fewer spore balls, therefore bioassay scores may have been anomalously high due to the "amplification" effect of the bioassay. Further detailed comparative studies are needed.

The comparison of the bioassay and ELISA data presented here suggests that the most sensitive system for soil detection may be a bioassay involving serological quantification of plasmodia or zoosporangia in the roots of bait plants.

The results of this research are described in greater detail by Walsh et al. (1996).

References ·

Ahmad, I., Soomro, M.H., Iftikhar, S. and Aslam, M. (1991). Investigations on powdery scab of potato in Pakistan. Pakistan Agricultural Research Council, Crop Diseases Research Institute, Pakistan Swiss Potato Development Project, pp 50.

de Boer, R.F. (1991). Evaluation of potato cultivars in the greenhouse and field for resistance to powdery scab. *Australian Journal of Experimental Agriculture* 31: 699-703.

de Boer, R.F., Flett, S.P. and Taylor, P.A. (1982). A survey of Victorian foundation seed potatoes for spore balls of *Spongospora subterranea*. *Australasian Plant Pathology* 11: 6-7.

Braithwaite, M., Falloon, R.E., Genet, R.A., Wallace, A.R., Fletcher, J.D. and Braam, W.F. (1994). Control of powdery scab of potatoes with chemical seed tuber treatments. *New Zealand Journal of Crop and Horticultural Science* 22: 121-128.

Clark, M.F. and Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34: 475-483.

Dirwächter, G. and Parbery, D.G. (1991). Infection of potato by *Spongospora subterranea*. *Mycological Research* 95: 762-764.

Eraslan, F. and Turhan, G. (1989). Investigations of mycological characteristics of *Spongospora subterranea* (Wallr.) Lagerh. in the Aegean region and reactions of some potato cultivars and clones. *Ege-Universitesi-Ziraat-Fakultesi-Dergisi* 26: 313-331.

Harrison, J.G., Rees, E.A., Barker, H. and Lowe, R. (1993). Detection of spore balls of *Spongospora subterranea* on potato tubers by enzyme-linked immunosorbent assay. *Plant Pathology* 42: 181-186.

Kurppa, A. (1989). The distribution and incidence of potato mop-top virus in Finland as determined in 1987 and on the variation of disease symptoms in infected tubers. Annales Agriculturae Fenniae 28: 285-295.

Merz, U. (1989). Infectivity, inoculum density and germination of *Spongospora subterranea* resting spores: a solution-culture test system. *EPPO Bulletin* 19: 585-592.

Merz, U. (1993). Epidemiological aspects of powdery scab of potatoes caused by *Spongospora subterranea*. Proceedings of the Second Symposium of the International Working Group on Plant Viruses with Fungal Vectors, Montreal, Canada, July 25-27: 103-106.

Rydén, K., Lövgren, L. and Sandgren, M. (1989). Investigation on potato mop-top furovirus in Sweden. *EPPO Bulletin* 19: 579-583.

Wale, S. (1987). Powdery scab - are there any easy solutions? Potato World 4: 8-9.

Walsh, J.A., Merz, U. and Harrison, J.G. (1996). Serological detection of spore balls of *Spongospora subterranea* and quantification in soil. *Plant Pathology* 45 (in press).

DEVELOPMENT AND APPLICATION OF MOLECULAR METHODS FOR THE STUDY OF *POLYMYXA BETAE*

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Summary

A simple method for cloning *Polymyxa betae* DNA from infected sugar-beet roots has been developed and used to isolate a multi-copy genomic DNA fragment, pPbKES-1, located on at least four different *P. betae* chromosomes. This fragment proved to be a good probe for detecting the fungus by Southern/dot blot hybridisation, and a good source of PCR primer sequences for the specific amplification of *P.betae* DNA. PCR is now used routinely for detecting *P.betae* in sugar-beet roots and in experiments investigating the epidemiology of the fungus. Single copy DNA probes have also been isolated and are beginning to provide useful information on variability at the molecular level.

Introduction

Polymyxa betae transmits and spreads beet necrotic yellow vein virus (BNYVV), the causal agent of rhizomania, a disease of great economic importance to the sugar-beet industry. The obligate parasitic nature of *P. betae* has until recently made it difficult to carry out studies at the molecular level. Here we describe molecular biological techniques that allow the sensitive and specific detection of *P. betae* in sugar-beet roots, as well as more detailed molecular studies.

Materials & Methods

Fungal isolates. Polymyxa betae was grown in sugar-beet roots, and *Polymyxa graminis* in barley roots as described by Adams *et al.* (1988). *Olpidium brassicae, Pythium ultimum* and *Aphanomyces cochlioides* were cultured as previously described (Mutasa *et al.* 1993).

Nucleic acid extraction, and cloning of P. betae DNA. Genomic DNA from infected roots was extracted using a high salt CTAB buffer, and shotgun cloned into pBluescript KS+ (Stratagene) to make a random plasmid library in *E coli* DH5α, which was then screened for repetitive *P. betae* sequences (Mutasa *et al.* 1993). DNA probes generated from the cloned fragments were tested for specificity and sensitivity by southern blot hybridisation. Alternatively, RNA was extracted in guanidinium thiocyanate buffer (Chomcynski *et al.* 1987), mRNA isolated and converted into cDNA which was then cloned in the Uni-ZAP XR lambda vector (Stratagene). *P. betae* clones were isolated by plus/minus differential screening, (Mutasa-Göttgens, Chwarszczynska & Asher: *manuscript in preparation*).

Sequencing, PCR primers and amplification conditions. *P. betae* DNA was sequenced by the chain termination method (Sanger *et al.* 1977) with T7 DNA polymerase (Pharmacia kit). Sequence data was then used to design primers suitable for PCR, which was carried out on not more than 100ng of genomic DNA, with primer pairs Pb-3a/Pb-3b and Pb-N3a2/Pb-N3b2 as described in Mutasa *et al.* (1995), or in a single-tube nested reaction with the four primers Pb-5a, Pb-5b, Pb-6a and Pb-4b as described in Mutasa *et al.* (1996).

Effect of soil temperature on the growth of P. betae. Sugar-beet seeds were sown in experimental field plots with different temperatures generated using polythene covers and soil-warming cables, such that the uncovered (bare) soil remained at ambient, covered soil was just above ambient, and covered soil with warming cables at 5 cm depth was maintained at *ca.* 5°C above ambient. Ambient soil temperature at 5cm was *ca.* 12°C at the start of the experiment,

rising to c.a. 25°C at the end. Plants were sampled at 37 and 65 days after sowing and their roots examined for *P. betae* infection by microscopy and PCR.

RFLP analysis. *P.betae* cDNA clones isolated from the lambda-Zap library were used to probe different digests of genomic DNA (20µg) from sugar-beet roots infected with different *P.betae* isolates by southern hybridisation, under stringent conditions. DNA from non-sterile sugar-beet roots free of *P. betae* infection was used as the negative control.

Results & Discussion

The nucleic acid sequence of the *P.betae* DNA fragment pPbKES-1 is shown in Fig. 1. Although there is as yet no evidence to suggest that this is a coding sequence, the fragment has been shown to be specific to *P.betae* by southern hybridisation analysis.

```
   1   GAATTCTTTA AGGACAACAA CCTAAGAAGA TGAATCAGAG GTGCAAGATA   Primer code:
  51   ACTGAGGAAA CTGATCCCGA GTATGATGAG ACGCGCGATC GAATTGAGGA
 101   AGTCGATGAA GACGTTGAGG GCAAGCCTGT AACGCAGTGT ACCAATGAGG
 151   AACTGGTCCG CAAACAGCGA GCCGGCAAGC GTGTGGCTTC AGGACATGTT
 201   ACGTTACTGG AGACCAACAA GGCACCGGAA TTGAGGACTG CAGATCCGAA
 251   GATATTCTTC GTTTCCAGCA GAAGCGAGAA AAGTACGAGC GCGTACATTT
 301   CGAATCAGGG GCAGACGGAT CGCAGTTACG ATCATTGATA TCAATGATTG   Pb-5a
 351   AGCCTGTTTT TCTGTTCGCC ATCTGCAGAT ATCAACTGCA GAAGGCAATT
 401   GAGGATGTGA CGGATGAGGA TCTTGAGCAC TTGACCTTAG GAGTTCTCCG
 451   CGATGATCGA TGATCGACCG TGATGTTCTC TGATCAAGTG TCACATTGAA
 501   GATGAGGATG TCAGTCAGGT CGCCAGTGCC TTGGGTGTTG CATTAGCAGC   Pb-6a
 551   ,CGATTCACGC CTTGAAAGTT ACGATGGACG ACTATTGAGG GGCGAGGGTA   Pb-3a
 601   AAACGCACGT AAATCTACTT AATGCTGTCC ATCCACCGGT CTAAAGATAT
 651   GATGACAGCG CGTGGGCGCG CGAGACACAG CTGTCAAAGC CCGGACGCAT
 701   TTACGGAATG CTTGCAGGAG GCCGCGGTCT ATTATCAAGA GCCTCTATAT
 751   ATCAACGACG TGAGGCAAAG ATGAATCCGC GTAATGAGGC AACGTCCGGG
 801   CGCAGGTAAC AAACGCCTGA AATCATCTAA CTCAACTTCG TTTAAGGTCC
 851   GAATTGGTAC CAAAGAGGAA AAGCCTGAGG GGTCCGCCGA ATCTATTAAT
 901   GGAGGAAAGG GACAGGGTAA GCGCGAATAT CCCAATTTGC GTTGCTATGA   Pb-N3a/2
 951   GTGTAAGGAA TTGGGCCATC CTATCTTTAA ATGCCCTAAG AATTTATCAA
1001   AACAACATGT ATATGAGGTT TTGAAAAAGA ATGCTAATAA ATCAAAGCAA
1051   TCTTCCAACT ACActtgggt ttgccacata gTAGCAAGTG AGGACAGTTA   Pb-4b
1101   AGAAGTTTTA GTGAGGGTAA ATAATAGTCT TTACTTTCCT GCGATCTTGG
1151   ATAGTGGCGC TAGAGGGGTA TCTCTAATAC CACGGAGGTT AGCTAAAGAG
1201   GCCATGAGGA ATGATAAAAG TATTACGTTA CAGAAGCTAT CAGAGCCAGT
1251   ACGACTAAAA TTGGGCGATA ATAGCTCAAT TGTAGAAGCC ACGGACTTTG
1301   TTTGCGTAAC ATTGAGGCTG CGCACAAAGC AGGTGATCTG ATCACAAGAA
1351   AGAGGCAGTG CTGATATGGG ATGTGCCCAG TGATGAGATC ATACTGGGGA
1401   GCGACTTATT AGAGGAGCTC GGAATAGAgc caagaactgc gctcgacgCA   Pb-5b
1451   GTTATCCTGA GGAAGAGACA AAAGATGACT CAAGAAGAGA GTCATGAGGA
1501   TTTCAAAACT AAGATATGAT AAggcaactg acaagtTTCA AGTTGCTAGA   Pb-N3b/2
1551   ATCACCGTAA TGAGGAATAA CTGCGGTTTA AAGGAAATGA GTTGAATAAG
1601   TGAAGGTCGT AAAGAAATAG CCAAACAGTG GTGTCTGAGG TTAGAACAAC
1651   TGCTGCAAGA GCATACAGAT GTATGGCGAA TCAACATGGG GCCAGATGGA
1701   GGAGTTAAAG TCAcgccatt tgtgactagg ctgcTGCCGA ACGCGAGGCC   Pb-3b
1751   GTTCAGATGT CCAAATAGGA GATACAGCCC TGAGGATAGC GAATTC
```

Fig 1   Sequence of fragment pPbKES-1 with *P.betae* primer sequences in bold print. For all sequences underlined in lower case, the correct primer orientation is given by the reverse complement of that shown. (EMBL accession No. X83745)

Probes generated from pPbKES-1 detected *P.betae* down to 10ng of total genomic DNA, but only from artificially inoculated roots with high levels of infection. This level of sensitivity was considered insufficient for detecting the low levels of fungus that might occur in roots naturally infected in the field. PCR primers (see Fig 1) and protocols were therefore designed to address this problem, and were successfully used in both laboratory and field experiments (Mutasa *et al.* 1995 & 1996). In the soil temperature experiment, it was clearly demonstrated by both PCR and microscopy that increased temperatures resulted in higher levels of infection (Fig 2). PCR was more sensitive than microscrocopy, being able to detect the much lower levels of infection occurring at 37 days after sowing. With microscopy, *P. betae* was detected under the highest temperature regime only, where the fungus had already formed c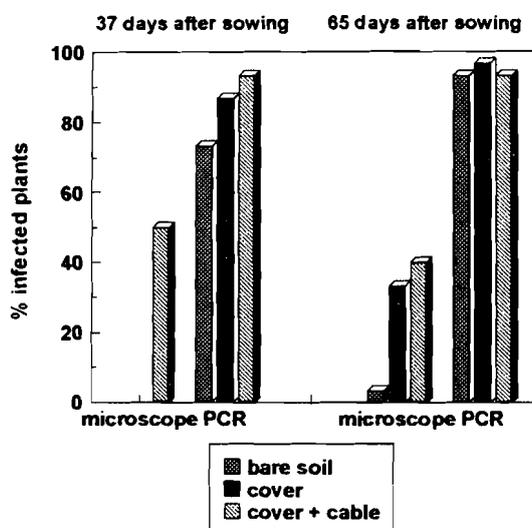ystosori in roots; this is the only stage in the life cycle that can be reliably distinguished from all other root-infecting fungi under the microscope. After 65 days it was just possible to detect *P.betae* by microscopy in all three treatments whereas PCR revealed nearly 100 per cent infection. PCR can therefore detect the fungus at very low levels and at much earlier stages in its life cycle than are possible by microscopy. The degree of specificity conferred by the use of nested PCR and stringent annealing temperatures allow reliable identification of the fungus at any stage in its life cycle. In addition, PCR may offer a fast and efficient method of screening plants for *P. betae* resistance, as a means of combatting rhizomania and for use in epidemiological studies in the field.

Fig 2. A comparison of infected plants detected by microscopy vs. PCR at 37 and 65 days after sowing in soils maintained at different temperatures.

The cDNA library proved to be a rich source of *P. betae* single copy sequences, with almost 40 per cent of all clones identified in the first round of screening being of fungal origin. These sequences provide more informative probes for RFLP analysis than those from multi-copy probes. We have now identified 9 probes that not only show differences and similarities between isolates, but also provide interesting clues about isolate purity and allelic differences that may be linked to biotype. For example, we tested four different isolates, F41 from Norfolk UK, F62 and F65 from Broom's Barn UK, and F67 from the former Yugoslavia. Of the three UK isolates, the two from Broom's Barn are known to be from distinct biotypes, one (F65) which is able to infect *Silene alba* (Barr and Asher 1992) and the other (F62) which cannot. When *Pst* I digested DNA from these isolates was hybridised with one of our probes (pPcD-3), two RFLP patterns (A and B) were observed (Fig 3). The two Broom's Barn isolates had the same pattern (B) as the Yugoslav isolate, whereas the Norfolk isolate had its own distinct pattern (A). However, a closer inspection of the autoradiograph revealed a difference in the intensity of bands in pattern B. The probe hybridised more strongly to groups 2 and 3 bands in isolates F65 and F67, compared to F62. For the Broom's Barn isolates, this difference may be linked to observed differences in host

range, and it would be interesting to determine whether F67, like F65, is able to infect *S. alba*. Another possible explanation may be that the isolates are mixtures of different fungal strains, such that F65 and F67 have equal amounts of each strain whereas, in F62 one strain, represented by the darker bands (group 1), predominates. Since the group 3 band is shared by F41, this too suggests an additional strain. Thus a total of at least three different fungal strains, each with a different allele at this locus, may have been identified with this single probe. The question of whether or not the fungus has a mating system has yet to be resolved.

The advantages of single copy probes are self evident, and their availability should now open up new areas of study, particularly in relation to parasite variability and genetics.
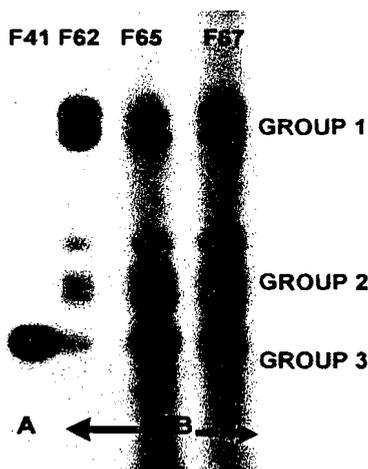
Fig 3. RFLPs identified by probe pPcD-3 on *Pst*I digested DNA of sugar-beet roots infected by four different *P.betae* isolates, showing two distinct hybridisation patterns A & B. Different levels of band intensity can also be seen in pattern B.

References

Adams MJ, Swaby AG, Macfarlane I. 1988. The susceptibility of barley cultivars to barley yellow mosaic virus (BaYMV) and its fungal vector *Polymyxa graminis*. Annals of Applied Biology **109**: 561-572.

Barr KJ, Asher MJC. 1992. The host range of *Polymyxa betae* in Britain. Plant Pathology **41** 64-68.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical biochemistry **162** 156-159.

Mutasa ES, Ward E, Adams MJ, Collier CR, Chwarszczynska DM, Asher MJC. 1993. A sensitive DNA probe for the detection of *Polymyxa betae* in sugar beet roots. Physiological and Molecular Plant Pathology **43** 379-390.

Mutasa ES, Chwarszczynska DM, Adams MJ, Ward E, Asher MJC. 1995. Development of PCR for the detection of *Polymyxa betae* in sugar beet roots and its application in field studies. Physiological and Molecular Plant Pathology **47** 303-313.

Mutasa ES, Chwarszczynska DM, Asher MJC. 1996. Single-tube nested PCR for the diagnosis of *Polymyxa betae* infection in sugar beet roots and colorimetric analysis of amplified products. Phytopathology **86**(5) 493-497.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. **47**(12) 5463-5467.

SPREAD OF BEET NECROTIC YELLOW VEIN VIRUS (BNYVV) AND *POLYMYXA BETAE* IN RHIZOMANIA-RESISTANT AND -SUSCEPTIBLE SUGARBEET.

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Summary

The spread of BNYVV in *P. betae*/BNYVV-infected sugarbeet was directly monitored by ELISA and tissue print immuno blot detection, whilst that of *P. betae* was indirectly monitored using the zoospore antagonist *Bacillus subtilis*. These studies revealed a fungus-related component of rhizomania resistance in one German sugarbeet variety. Nucleic acid based *P. betae* detection methods have also been developed for direct detection of fungus development. It was shown that quantitative development and distribution of *P. betae* in roots could be followed by dot blot hybridization and tissue print hybridization blotting respectively. BNYVV detection methods can now be complemented by *P. betae* detection methods, making it possible to differentiate between virus- and fungus-related rhizomania resistance mechanisms in sugarbeet varieties.

Introduction

Rhizomania disease of sugarbeet is characterized by severe stunting of the tap root, proliferation of lateral roots and reduction in sugar yield. The causal agent of rhizomania disease is beet necrotic yellow vein virus (BNYVV) which is transmitted to sugarbeet roots by the soil-borne plasmodiophoraceous fungus *Polymyxa betae* (Tamada, 1975).

Breeding for resistance to rhizomania disease has become an important task for sugarbeet breeders. Both resistance to BNYVV and its vector *P. betae* might be useful for control of rhizomania disease. The two different types of resistance cannot be distinguished in conventional screening of sugarbeet varieties with reduced virus levels.

Spread of virus in sugarbeet roots may be caused by fungus-independent transport within the root tissue and by means of *P. betae* zoospores. Transmission of BNYVV by zoospores that are secondarily released during the fungal development cycle may play an important role for the efficiency of virus spread in sugarbeet roots (Peters and Godfrey-Veltman, 1990).

In this paper we describe infection studies using a combination of direct and indirect detection methods for BNYVV and its vector *P. betae*. We have used the biological *P. betae* zoospore antagonist *Bacillus subtilis* to investigate the role of secondarily released zoospores for virus spread within roots in order to estimate the relative importance of fungus- and virus-related rhizomania resistance in different sugarbeet varieties.

Materials and Methods

For inhibition of BNYVV transmission by P. betae zoospores Bacillus subtilis strain A1/3 was grown on YDC agar for 48 hours at 28°C, bacterial cells were collected with 10% Steiner solution and the suspension was adjusted to 10^7 cfu/ml.

For infection studies with sugarbeet seedlings three week old plants were inoculated for three days with *P. betae* zoospore-releasing BNYVV-infected plants and subsequently incubated in 10% Steiner solution in the presence or absence of *B. subtilis*. Every day ten roots were tested for BNYVV levels by DAS-ELISA (Clark and Adams, 1977) and examined for BNYVV distribution by tissue print immuno blotting (Kaufmann *et al.*, 1992). In some experiments, roots were also examined for *P. betae* levels by dot blot hybridization of DNA extracts. In these experiments roots were dried for 2 hours at 50°C, homogenized with 0.5 ml PBS/mg root and aliquots of the suspension used for ELISA and for nucleic acid isolation followed by dot blot hybridization.

For cloning of P. betae-specific DNA probes a random genomic DNA plasmid library was prepared from *Eco*RI-fragmented DNA of *P. betae*-infected sugarbeet roots and screened for *P. betae* repetitive sequences according to Mutasa *et al.* (1993). Two *P. betae*-specific clones pPbetaeBS1 (1.5 kb) and pPbetaeBS2 (1.8 kb) were obtained.

For quantitave P. betae detection by dot blot hybridization DNA was extracted from roots using the QIAamp extraction procedure for plant material (QIAGEN). DNA (200 ng) of each heat denatured sample was applied in 16x SSC solution to a nylon membrane using a vacuum dot blot apparatus. Hybridization and detection was carried out using 20 ng/ml digoxigenin-labeled *P. betae*-specific DNA fragment PBetaeBS2 and the DIG Detection System (Boehringer Mannheim).

For double tissue printing with nucleic acid and immuno detection of P. betae and BNYVV roots were printed between two nylon membranes presoaked in 20x SSC by means of a roller applying uniform pressure. One membrane was used to determine the distribution of BNYVV by colorimetric visualization with alkaline phosphatase-conjugated BNYVV coat protein-specific antibodies according to Kaufmann *et al.* (1992). The other membrane was used to determine the distribution of fungus structures in the same roots by hybridization with 100 ng/ml *P. betae*-specific digoxigenin-labeled antisense RNA of cDNA pPcD-3 (Mutasa, unpublished) according to Más and Pallás (1995).

Results

In infection studies with ELISA and tissue print immuno blotting analysis the transmission of BNYVV by *P. betae* from infected sugarbeet seedlings to healthy seedlings was completly inhibited by co-cultivation with *Bacillus subtilis* in liquid culture medium (Kastirr and Griesbach, 1990). Microscopic observations indicate a direct inhibitory effect of *B. subtilis* on zoospores of *P. betae* in the liquid culture medium but no influence on the development of *P. betae* or BNYVV inside the root tissue (Kastirr, unpublished).

The inhibitory effect of *B. subtilis* on *P. betae* zoospores can be used in infection studies to inhibit BNYVV transmission by reinfection of the roots with newly released secondary zoospores. Virus levels and virus distribution in the roots were detected by ELISA and tissue print immuno blotting respectively, but no direct detection methods for *P. betae* were available so far. For detection of fungus-dependent virus spread in the roots we compared virus spread under conditions of reinfection and inhibited reinfection respectively. Virus development in the susceptible cv. Hilma and the partially rhizomania-resistant cv. Patricia is shown in Fig. 1. All plants had initially been inoculated for three days with zoospore-releasing BNYVV-infected plants.

mean ELISA values from ten roots

Days after inoculation

A —■— cv. Hilma, with reinfection
—□— cv. Hilma, reinfection inhibited

Days after inoculation

B —■— cv. Patricia, with reinfection
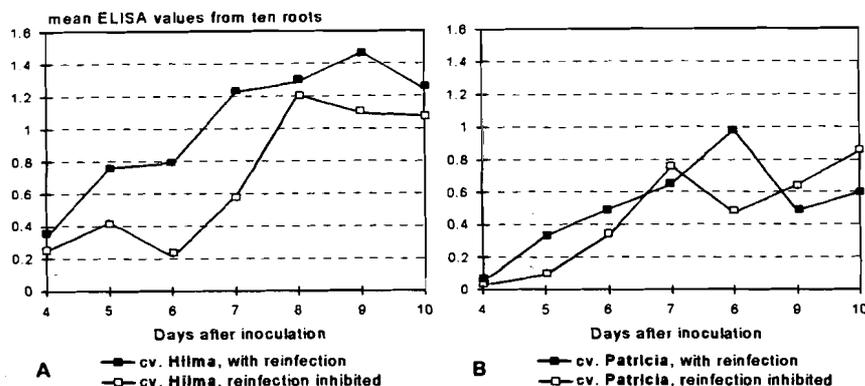—□— cv. Patricia, reinfection inhibited

Fig. 1: BNYVV development in sugarbeet roots of the rhizomania-susceptible cv. Hilma (A) and the partially rhizomania-resistant cv. Patricia (B) with and without reinfection by BNYVV-transmitting *P. betae* zoospores.

The increase of virus levels in roots of both cultivars under conditions of inhibited reinfection was slightly delayed compared to conditions of reinfection. After three days of inoculation tissue print immuno blotting revealed several local restricted infection sites in the root tissue. The severity of virus development in the root tissue was similar under conditions of reinfection and inhibited reinfection but the number of infection sites did not increase if reinfection was inhibited. Virus levels in roots of cv. Patricia were significantly lower after three days of inoculation compared to roots of cv. Hilma. Tissue print immuno blotting of roots showed that numbers of infection sites in roots of cv. Hilma were higher than in cv. Patricia.

For infection studies with direct parallel detection of virus and fungus in individual sugarbeet roots, nucleic acid based *P. betae*-specific detection methods have been developed. We have obtained a sensitive *P. betae*-specific cloned DNA probe and used it in a dot blot hybridization procedure for quantitative detection of *P. betae* in DNA extracts of sugarbeet roots. Hybridization signals were visualized by colorimetric substrate development on the membrane. No signal was obtained with DNA from healthy sugarbeet roots. Extinction values of a calibration series were linear from 1 to 200 pg of *P. betae*-specific DNA fragment. As little as 1 pg of the homologous *P. betae*-specific sequence in 200 ng root DNA could be detected, which corresponds to a weak *P. betae*-infection of roots after six hours of inoculation. Parallel measurements of BNYVV by ELISA and *P. betae* by dot blot hybridization in the same roots was possible by using one part of homogenized root material for nucleic acid isolation and the other part for ELISA.

P. betae-specific nucleic acid probes were also used in double tissue print experiments to detect fungus distribution in sugarbeet roots. One membrane was used for hybridization with antisense transcripts from the *P. betae*-specific cDNA pPcD-3 while the other was used for immuno detection of virus distribution. Intensity of tissue print hybridization signals was weaker than intensity of tissue print immuno signals. Hybridization signals were only obtained with *P. betae*-infected roots but not with healthy sugarbeet roots. Distribution of fungus-specific signals corresponded in most cases with distribution of virus-specific signals in the same roots. However, roots with a high proportion of *P. betae* resting spores showed high virus-specific signals but barely any fungus-specific signals.

180

Discussion

The inhibitory effect of *Bacillus subtilis* on zoospores of the fungus *Polymyxa betae* was used to inhibit BNYVV transmission to sugarbeet roots. In infection studies with and without secondary zoospore inhibition it was possible to distinguish between fungus-dependent and fungus-independent virus spread in the roots.

It was shown that high total virus levels can also be reached under conditions of inhibited secondary zoospore activity, but the virus remained restricted to lower root areas under these conditions. This suggests, that fungus independent virus spread within the root tissue is not very effective and virus transmission by secondary zoospores seems to be necessary for infection of the whole root tissue. Our observations of reduced virus levels after three days of inoculation confirmed the partial rhizomania resistance of the sugarbeet cv. Patricia. In spite of the reduced number of infection sites in roots of cv. Patricia there was a similar time-dependent increase of virus levels in roots of both rhizomania-susceptible cv. Hilma and partially rhizomania-resistant cv. Patricia. This suggests that the rhizomania resistance mechanism of cv. Patricia is fungus-related and not virus-related.

These conclusions were based on the indirect monitoring of fungal participation in virus spread in roots. To confirm these findings by direct detection of the fungus we have developed nucleic acid based *P. betae*-specific detection methods which can be used together with immuno based BNYVV detection methods for parallel analysis of the agents involved in rhizomania.

Dot blot hybridization of DNA extracts from roots proved to be a very sensitive and simple method for quantitative *P. betae* detection. Virus and *P. betae* levels in extracts of the same roots can thus be compared using ELISA and dot blot hybridization, respectively. Tissue print hybridization with *P. betae*-specific antisense RNA can be used to analyze the distribution of fungal structures in roots. This method detects fungal mRNA and perhaps also DNA from plasmodia. Failure to detect nucleic acids from resting spores may be due to the resistance of resting spores to disruption.

Dot blot hybridization and ELISA can be used to measure virus and fungus concentration while tissue print techniques can be used to visualize virus and fungus distribution in the root tissue. We are now applying these combined methods of *P. betae* and BNYVV detection in infection studies to differentiate between fungus-related and virus-related rhizomania resistance mechanisms in sugarbeet cultivars and lines.

References

Clark, M. F. and Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34: 475-483.

Kastirr, U. and Griesbach, E. (1990). *Institut für Phytopathologie Aschersleben, Jahresbericht*, 15.

Kaufmann, A., Koenig, R. and Lesemann, D.-E. (1992). Tissue print-immunoblotting reveals an uneven distribution of beet necrotic yellow vein and beet soil-borne viruses in sugarbeets. *Archives of Virology* 126: 329-335.

Más, P. and Pallás, V. (1995). Non-isotopic tissue-printing hybridization: a new technique to study long-distance plant virus movement. *Journal of Virological Methods* 52: 317-326.

Mutasa, E. S.; Ward, E.; Adams, M. J.; Collier, C. R.; Chwarszczynska, D. M.; Asher, M. J. C. (1993). A sensitive DNA probe for the detection of *Polymyxa betae* in sugar beet roots. *Pysiological and Molecular Plant Pathology* 43: 379-390.

Peters, D. and Godfrey-Veltman, A. (1990). Inoculum characteristics of zoospore suspensions of *Polymyxa betae* infected with beet necrotic yellow vein virus. In *Proceedings of the First Symposium of the International Working Group on Plant Viruses with Fungal Vectors*, pp. 69-72. Edited by R. Koenig, Stuttgart: Eugen Ulmer.

Tamada, T. (1975): Beet necrotic yellow vein virus. *CMI/AAB Description of Plant Viruses*, no. 144.

STRAND-SPECIFIC RT-PCR DETECTS REPLICATION OF BaYMV AND BaMMV IN LEAVES AND ROOTS

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Summary

Barley yellow mosaic (BaYMV) and barley mild mosaic (BaMMV) viruses have single-stranded plus (sense) strand RNA genomes. Roots and leaves of plants containing one or both of the viruses were tested by ELISA and RT-PCR. The reverse transcription step used primers designed to recognise and transcribe only the minus or the plus RNA strands. RNA was then destroyed by RNase digestion before PCR amplification and detection by gel electrophoresis. Both plus and minus strand RNAs were detected in roots and leaves of infected plants, demonstrating virus replication in both parts of the plant. RT-PCR was more sensitive than ELISA, but the two methods were in general agreement. This is the first report of strand-specific RT-PCR to study replication of plant viruses.

Introduction

Barley mild mosaic virus (BaMMV) and barley yellow mosaic virus (BaYMV) are filamentous, bipartite viruses belonging to the proposed bymovirus subgroup of the Potyviridae, with BaYMV the type member. Although both viruses cause systemic mosaic symptoms in susceptible barley cultivars and are transmitted by the same plasmodiophoraceous fungus Polymyxa graminis Led., they are serologically distinct and nucleotide homologies between them are only 35-43% (Kashiwazaki et al, 1992; Peerenboom et al, 1996). Their genomes consist of two plus strand RNA molecules of about 7.5 and 3.5 knt, each encoding a single polyprotein.

BaMMV and BaYMV can be detected in infected plants using ELISA, but there have been no studies of virus replication. The replication of plus strand RNA viruses is catalysed by an RNA-dependent RNA-polymerase, first by using the encapsidated plus strand RNA as a template to synthesize a minus strand RNA, and then using this product as the template for the synthesis of the plus strand virus RNA. The detection of minus strand RNA would therefore indicate the first stage in virus replication. In this paper we describe the development of RT-PCR protocols to detect BaYMV and BaMMV plus and minus strand RNAs and their use to demonstrate replication in roots and leaves of infected plants.

Experimental and Discussion

Plants were collected from two field sites in Germany (Köln, with BaYMV only; Bellnhausen, Hessen, with both BaMMV and BaYMV). Plants containing only BaMMV were glasshouse-grown plants which had been inoculated with viruliferous zoospores. Duplicate samples were taken from both leaves and roots of three plants from each source. One sample was analysed by ELISA, while RNA was extracted from the other for detection of plus and minus strand virus RNA by RT-PCR. Healthy glasshouse-grown barley was used as a negative control.

Antiserum prepared to a UK isolate of BaMMV was used in an indirect F(ab´)$_2$ method. Samples with absorbances (A_{405nm}) more than twice that of the relevant healthy control were considered to be infected.

Source	Plant no.	BaYMV		BaMMV	
		Leaves	Roots	Leaves	Roots
-ve control	1	0.123	0.071	0.103	0.067
Köln	2	0.331	0.361	0.067	0.075
(BaYMV)	3	0.430	0.229	0.079	0.078
	4	0.436	0.331	0.087	0.080
Bellnhausen	5	1.136	0.129	0.093	0.084
(BaYMV +	6	0.466	0.199	0.091	0.082
BaMMV)	7	0.213	0.093	1.342	0.218
Glasshouse	8	0.152	0.095	1.339	0.123
(BaMMV)	9	0.149	0.091	1.373	0.277
	10	0.158	0.097	1.227	0.277

Table 1. Absorbance values in ELISA of leaf and root samples from plants from different sources, assayed for BaYMV or BaMMV. (A_{405nm}; means of 2 replicate wells at 10 min)

RNA was extracted from 0.2 g samples of leaves and roots. Primers for reverse transcription polymerase chain reaction (RT-PCR) of BaMMV RNA1 were designed to regions of homology in the published sequences of RNA1 of the UK, German and Japanese isolates of BaMMV using the MS-DOS programs COMPARE and MATCH (Antoniw, 1995). The PCR primers M3 and M4 amplified an 899 bp DNA fragment from the 3' end of RNA1. For BaYMV, the primers were designed using the German RNA1 sequence (Peerenboom et al, 1992). The PCR primers 4800EP and 4799 EP amplified a fragment of 1018 nt. Reverse transcription of the RNA used the forward primers BaMMV M3 or BaYMV 4800EP for detection of the virus minus strands and the reverse primers BaMMV M4 or BaYMV 4799EP primers for the detection of the plus strands. The reverse transcription reaction used 5 μl RNA and 200 U Superscript II enzyme (BRL) according to the manufacturer's instructions in a total reaction volume of 20 μl. To eliminate the possibility that the Taq DNA polymerase might use an RNA template for cDNA synthesis, all the RNA was then destroyed by digestion with RNase A. PCR was then carried out with 1 μl reverse transcription product and 0.2 μg of each primer in a total volume of 100 μl, using the wax-mediated hot start procedure. The PCR products were analysed by electrophoresis in 1% agarose gels.

Preliminary experiments were done on the reliability and effectiveness of the strand-specific RT-PCR method to determine whether, under our PCR conditions, Taq polymerase could directly transcribe RNA templates as reported by Tse and Forget (1990). If this were to occur during PCR, when both forward and reverse primers were present, it would be impossible to distinguish between plus and minus RNA strands. Using RNA isolated from infected leaves in PCR assays for BaYMV, without prior cDNA synthesis, no PCR products could be detected, even if reverse transcriptase was included in the PCR reaction. This suggests that under our PCR conditions, the synthesis of DNA directly from RNA does not occur to a detectable extent, and that any reverse transcriptase carried over from the strand-specific cDNA synthesis step would be inactivated. This agrees with the manufacturer's specification that Superscript II RT is inactivated at 90° C. However, when cDNA, synthesized using either the forward or reverse primers was used in PCR, a product of the expected size was amplified. Although the presence of RNA in the PCR did not seem to be a problem, we decided to destroy the RNA after the strand-specific cDNA synthesis by RNase digestion. Incubation with RNase A for 35 min at 37° C before PCR did not interfere with the PCR.

ELISA analyses (Table 1) confirmed that all three samples taken from the field in Köln (plants 2-4) were infected only with BaYMV and that glasshouse grown plants (plants 8-10) contained only BaMMV. Of the three plants from Bellnhausen, two (plants 5 and 6) had only BaYMV and the other had only BaMMV. Leaf and root samples were in agreement. RNA extracted from the root and leaf samples of plants 1-7 were then assayed for plus and minus strand BaYMV RNA. Specific bands were detected in both the plus and minus strand virus RNA assays of both roots and leaves of plants 2-4 from Köln and 5-6 from Bellnhausen but not in the uninfected control. In plant 7, BaYMV was undetectable in leaves, but the root sample had a band for the plus but not the minus strand

virus RNA. In the BaMMV assay, using plants 1 and 5-10, both plus and minus strand BaMMV RNAs were detected in the roots of all test plants (5-10) and in the leaves of plants 7-10, but not in the controls.

The results indicate that BaMMV and BaYMV replicate in both leaves and roots. The RT-PCR signal for the plus strand RNA was much stronger than that for the minus strand, presumably reflecting differences in their relative abundance. During a natural infection of the crop, the virus first enters the roots following infection by the vector and probably first replicates in the roots before spreading to the rest of the plant. This is supported by the assays of plants 5 and 6, in which both plus and minus strand BaMMV RNAs were detected in roots but no virus or RNA was detected in leaves. The results from ELISA and RT-PCR were usually in good agreement, but RT-PCR appeared to be more sensitive. Both ELISA and RT-PCR indicate that the roots contain much less virus than the leaves.

The use of PCR to detect anti-sense RNA transcripts was first demonstrated using herpes simplex virus type 1 (Cantin *et al*, 1991). However, as far as we are aware, this is the first report of its use to investigate replication of plant viruses.

References

Antoniw, J.F. (1995) A new method for designing PCR primers specific for groups of sequences and its application to plant viruses. Mol. Biotech. 4, 111-119.

Cantin, E.M., Lange, W. and Openshaw, H. (1991) Application of polymerase chain reaction assays to studies of herpes simplex virus latency. Intervirol. 32, 93-100.

Kashiwazaki, S., Minobe, Y., Omura, T. and Hibino, H. (1990) Nucleotide sequence of barley yellow mosaic virus RNA1: a close evolutionary relationship with potyviruses. J. Gen. Virol. 71, 2781-2790.

Peerenboom, E., Jacobi, V., Antoniw, J.F., Schlichter, U.H.A., Cartwright, E.J., Steinbiss, H.-H. and Adams, M.J. (1996) The complete nucleotide sequence of RNA-2 of a fungally-transmitted UK isolate of barley mild mosaic bymovirus (BaMMV) and identification of amino acid combinations possibly involved in fungus transmission. Virus Res. 39.

Peerenboom, E., Pröls, M., Schell, J., Steinbiss, H.-H. and Davidson, A.D. (1992) The complete nucleotide sequence of RNA 1 of a German isolate of barley yellow mosaic virus and its comparison with a Japanese isolate. J. Gen. Virol. 73, 1303-1308.

Pröls, M., Davidson, A., Schell, J. and Steinbiss, H.-H. (1990) In vitro translation studies with cDNA clones corresponding to the RNA's of barley yellow mosaic and barley mild mosaic viruses. J. Phytopathol. 130, 249-259.

Tse, W.T. and Forget, B.G. (1990) Reverse transcription and direct amplification of cellular RNA transcripts by *Taq* polymerase. Gene 88, 293-296.

SURVEY OF SOIL-BORNE VIRUS DISEASES OF SUGAR-BEET IN ITALY

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Summary

Most Italian sugar beet-growing areas were surveyed to determine the incidence of beet necrotic yellow vein virus (BNYVV), beet soil-borne virus (BSBV), and beet soil-borne mosaic virus (BSBMV also called Tx7). During 1994 and 1995, 170 soil samples were collected from 126 sites in 27 provinces. Sugar beet seed was planted in the soil samples, and root tissue was later harvested and tested by ELISA: 29% were positive for BNYVV alone, 5% for BSBV alone, and 35% BSBV and BNYVV in mixed infection. Samples positive for BSBMV with ELISA were never confirmed by Western blot analysis or immuno sorbent electron microscopy (ISEM): BSBMV presence in the soil samples tested was therefore excluded.

Introduction

Rhizomania, is a very damaging disease of sugar beet which can cause yield losses up to 45-50% in taproot weight, and up to 60-79% in sugar content (Casarini Camangi, 1987). The disease was first described in Italy in the mid 1950's (Canova, 1959). An epidemiological study carried out during the early sixties showed that infection was widespread in the Po Valley sugar-beet growing areas (Bongiovanni, 1964) . Etiological and fungal transmission studies provided the basis for a different approach to "Rizomania" diagnosis (Canova, 1966; Faccioli and Giunchedi, 1974). BNYVV was then 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). Studies carried out in Italy over the last 15 years mainly concentrated on genetic improvement of sugar beet varieties for resistance to BNYVV and on its localization in the taproots tissues (Giunchedi and Poggi Pollini, 1987; Giunchedi *et al.*, 1987) To date, a few soil samples in the Po Valley area tested positive for beet soil borne virus (BSBV) (Giunchedi, personal com.; Lindsten, 1993).
Recently, a partial molecular characterization of two Italian BNYVV isolates from the Po Valley classified them in the strain group A (Kruse *et al.*, 1994; Koenig *et al.*, 1995).
Our report highlights the prevalence and importance of other furoviruses in the main Italian sugar beet production areas. Some preliminary results are given of a future study on biological and molecular diversity of a wide collection of Italian BNYVV and BSBV isolates.

Materials and methods.
Soil sample collection.

Soil samples were collected during autumn and winter of 1994, and 1995 from 170 fields previously planted with sugar beet in 123 towns distributed in the most productive regions in Italy. Five subsamples were randomly collected in each field and bulked. Soil from each bulked sample was mixed with sterile sand (1/4 soil/sand) and placed in 13 cm diameter pots and planted with sugar beet seed cv Duro. Two replications for each sample were planted.

Serology on sugar beet bait plants

Sugar beet, roots grown for 6-8 wks, were assayed for the presence of BNYVV, BSBV and BSBMV with various serological methods: double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) was carried out according to Clark and Adams (1977). Rootlets samples were ground in a mortar in 1:10 (w:v) phosphate buffered saline (PBS) containing 0.05% Tween-20 and 2% PVP (m.w. 25,000). BNYVV antiserum was purchased from Bioreba Inc., Chapel Hill, NC. BSBV antiserum, provided by K. Lindsten, was diluted 1/200 for the coating step, and 1/100 for the coniugated antibody step. For BSBMV (Tx7), primary antibodies and biotinilated secondary antibodies were kindly provided by C. Rush. Two different ELISAs were processed: a DAS-ELISA was carried out as above than, the biotinylated secondary antibody was probed with avidin-conjugated alkaline phosphatase (Sigma), diluted 1/3000 in PBS. A protein A sandwich ELISA (PAS-ELISA) was carried out as described by Rubies-Autonell and Turina (1995): Tx7 IgG were used at 1/1000 dilution for both steps. Positive and negative controls were included for each test. Values at 405 nm were read with a microplate reader. Absorbance values of at least three times the average of the healthy controls were considered positive.

SDS-PAGE and Western blot analysis were carried out on the samples positive for BSBMV in DAS-ELISA according to the standard protocols (Towbin *et al.*, 1979).

Immuno-sorbent electron microscopy (ISEM) and gold labelled antibody decoration (GLAD) were carried out on ELISA positive samples for BSBV and BSBMV according to the protocols described previously (Rubies-Autonell and Turina, 1995). Tissue print immunoblotting (TiPIB) was carried out according to the protocol described in detail by Resca *et al.* (1992).

Mechanical inoculation

Mechanical inoculation of *C. quinoa* and *C. amaranticolor* was carried out using infected beet rootlets sap diluted 1/4 (w/v) in 0.1 M Na-K phosphate buffer with 0.2% sodium sulfite. *Chenopodium spp.* were grown in the greenhouse at $27° \pm 1°C$ with natural illumination supplemented in winter with 3000 lux, and a 16 h photoperiod (tube Fluora Osram 77).

Double stranded RNA analysis

Double stranded RNA (dsRNA) analysis was carried out on five BNYVV isolates mechanically transmitted to *C. quinoa* using the LiCl separation method (Morris and Dodds, 1979).

Results

Out of 170 soil samples, 49 were positive for BNYVV alone, 8 for BSBV, and 59 for both viruses. Both viruses are widespread in the beet growing areas, and virtually no province was free of the Rhizomania complex disease (Figure 1). Western blot analysis and GLAD confirmed the presence of BSBV coat protein and particles respectively in the positive DAS-ELISA samples. TiPIB on sugar beet infected by BSBV gave a further confirmation of the presence of the virus. Some samples had mean values higher than the Tx7 control in Avidin-Biotin DAS-ELISA, not confirmed by PAS-ELISA, Western blot analysis and mechanical inoculation on test plants.

After transmitting 5 BNYVV isolates to *C. quinoa* and *C. amaranticolor* leaves, 3 different patterns of symptom expression were observed. Nevertheless, DsRNA analysis from *C. quinoa* leaves infected with the 5 BNYVV showed the same pattern for RNA1, RNA2 and RNA3 size.
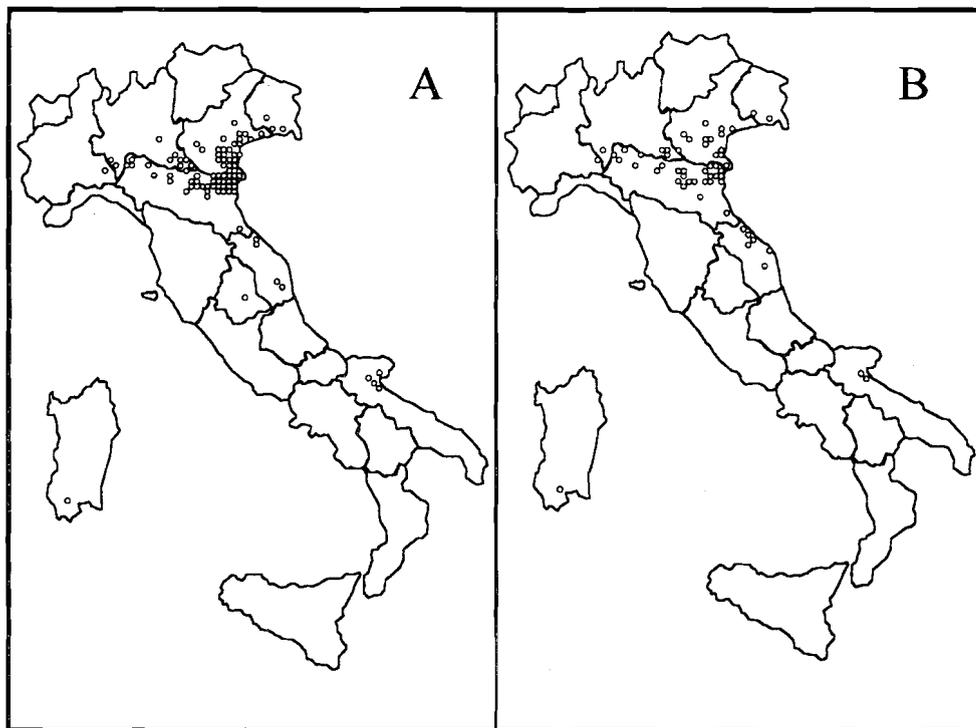
Figure 1. Distribution of BNYVV (A) and BSBV (B) infected soil samples.

Discussion

The results of our survey confirm the presence of viruliferous *Polymyxa betae* resting spores throughout all Italian sugar beet growing areas. A map of BSBV incidence in Italy was prepared for the first time, and it indicastes that this virus is endemic. Further studies on its importance for the damage caused to the Italian sugar-beet crops are being carried out. Although several positive samples were obtained for BSBMV (Tx7) with the Avidin Biotin DAS-ELISA, they were never confirmed with other methods of diagnosis and isolation: therefore its presence in the soil samples tested must be excluded, even if we can not explain the positive ELISA. The result of our dsRNA analysis did not link the symptomatic differences observed on *C. quinoa* with a difference in the RNA1, RNA2 and RNA3 pattern: a different method of extraction (Hutchinson *et al*, 1992) could lead to a better separation from contaminant ribosomal RNA which prevented screening for the presence and size of the smaller dsRNAs which are likely to be involved in symptom expression on *C. quinoa*. Furthermore, many symptomatic differences have already been noted for BNYVV isolates on *C.quinoa*, but they did not correspond to differences in the original BNYVV isolates present in the sugar beet taproots (Koenig *et al*, 1986). Future studies on the molecular aspects of the Italian BNYVV isolates will take in account the techniques already used and will provide new data for the maps of the pathtype distribution in Italy.

188

References

Bongiovanni, G.C. (1964). La diffusione della rizomania in Italia. *Inf. Fitopat.*, **14**:263-265.

Canova, A. (1959). Appunti di patologia della barbabietola. *Inf. Fitopat.*, **9**:390-396.

Canova, A. (1966). Si studia la rizomania della bietola. *Inf. Fitopat.*, **16**:235-239.

Casarini Camangi, P. (1987). Incidenza della rizomania sulla produzione quali-quantitativa della barbabietola da zucchero. *L'Inf. Agr.*, **43**:149-152.

Di Franco A. e D. Gallitelli. 1980. La Rizomania della bietola da coste. *Inf. Fitop.* **3**:9-11.

Faccioli, G., and L. Giunchedi. (1974). On the viruses involved on rizomania disease of sugarbeet in Italy. *Phytopath. Medit.*, **13**:10-16.

Giunchedi, L., and C. Poggi Pollini. (1988). Immunogold-silver localization of BNYVV antigen in susceptible and moderately resistant sugar-beets. *Phytopath. Medit.* **27**:1-6.

Giunchedi, L., M. De Biaggi, and C. Poggi Pollini (1987). Correlations between tolerance and Beet Necrotic Yellow Vein Virus in sugar beet genotipes. *Phytopath. medit.*, **26**:23-28.

Hutchinson, P.J., Henry, C.M. and Coutts, R.H.A. (1992). A comparison, using dsRNA analysis, between beet soil-borne virus and some other tubular viruses isolated from sugar beet. *J. gen. Virol*, **73**:1317-1320.

Koenig, R., Burgermeister, W. Weich, H., Sebald, W., and Kothe, C. (1986). Uniform RNA patterns of beet necrotic yellow vein virus in sugarbeet roots, but not in leaves from several plant species. *J. gen. Virol*, **67**:2043-2046.

Koenig, R., P. Luddecke, and A.M. Haeberlè. 1995. Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphism of immunocapture RT-PCR products. *J. of Gen. Virol.* **76**:2051-2055.

Kruse, M. R. Koenig, A. Hoffmann, A. Kaufmann, U. Commandeur, A.G. Solovyev, I. Savenkov, and W. Burgermeister. 1994. Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of beet necrotic yellow vein virus. *J. of Gen. Virol.* **75**:1835-1842.

Lindsten, K. 1993. Rhizomania- are both Beet necrotic yellow vein virus (BNYVV) and Beet soil-borne virus (BSBV) involved? Proceedings of the 2nd Symposium of the international Working Group on Plant viruses with Fungal vectors. Montreal, Canada, July 25-27, 1993., pp.67-70

Rana, G.L., A. Di Franco, and M. Russo. 1978 - La rizomania della barbabietola in Italia meridionale. *Inf. Fitopat.* **2**:5-7.

Resca, R., Giunchedi, L., and Poggi Pollini, C. (1992). Localizzazione immunologica del virus agente della rizomania nell'impronta su membrana di radici di barbabietola. *Sementi elette*, **38**: 21-24.

Rubies Autonell, C. and M. Turina. (1995). Characterization and cytopathology of Hibiscus latent ringspot virus isolated from kenaf (Hibiscus cannabinus L.) in Italy. *J. Phytopath.*, **143**:211-215

Rubies Autonell C., M. Turina, and M.G. Fantino. 1995. Infezioni da virus in alcune coltivazioni di spinacio in Italia. *Inf. Fitopat.* **1**:43-47.

A PLANT VIRUS NOTEBOOK FOR IBM-COMPATIBLE COMPUTERS

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Summary

A new computer program (Plant Virus Notebook) which displays and interprets plant virus genomes is described. The program is a Windows application for IBM-compatible PCs. It displays the classification of plant virus genera and families as well as genome structure and organisation. The features of virus sequences can be displayed in different ways, graphically or textually, so that the positions and relationships of the features can be examined more easily. A complete plant virus classification is included, together with sample genome maps of the type members of each genus (where available). Other, more advanced, features are demonstrated using bymovirus sequences as examples but should be applicable to any virus genus. The program is designed to be updated by down-loading files from a World Wide Web site.

Introduction

The new computer program (Plant Virus Notebook), is a Windows application for IBM-compatible PCs that has been written to display information on the classification, genome structure and organisation of plant viruses. The program, which is working but still under development, consists of a series of tabbed pages which are discussed briefly below.

Index

The opening page of the program (Fig. 1) consists of three columns: (a) plant virus classification according to the 6th report of the International Committee on Taxonomy of Viruses (Murphy *et al.,* 1995) displayed as an expandable tree (b) alphabetic list of plant virus genera and (c) list of species in any genus (or genera in any family) selected from columns (a) or (b). The species list can be searched for keywords or abbreviations and is designed as a series of html files (one per genus).

Genome Map

When a genus has been selected in the Index page, a diagram illustrating the genome organisation (where known) of the type member is displayed in an upper window of this page. The diagram shows the number and size of genome components as lines (or circles if appropriate) and distinguishes DNA, RNA, single-stranded, and double-stranded nucleic acids. The position, size and orientation of open reading frames and the processing of polyprotein products are also indicated (Fig. 2). In a lower text window, further details of the exact positions of the key features and database accession numbers are given.

Sequences

Selecting this loads a searchable list of all the plant virus sequences in the EMBL and
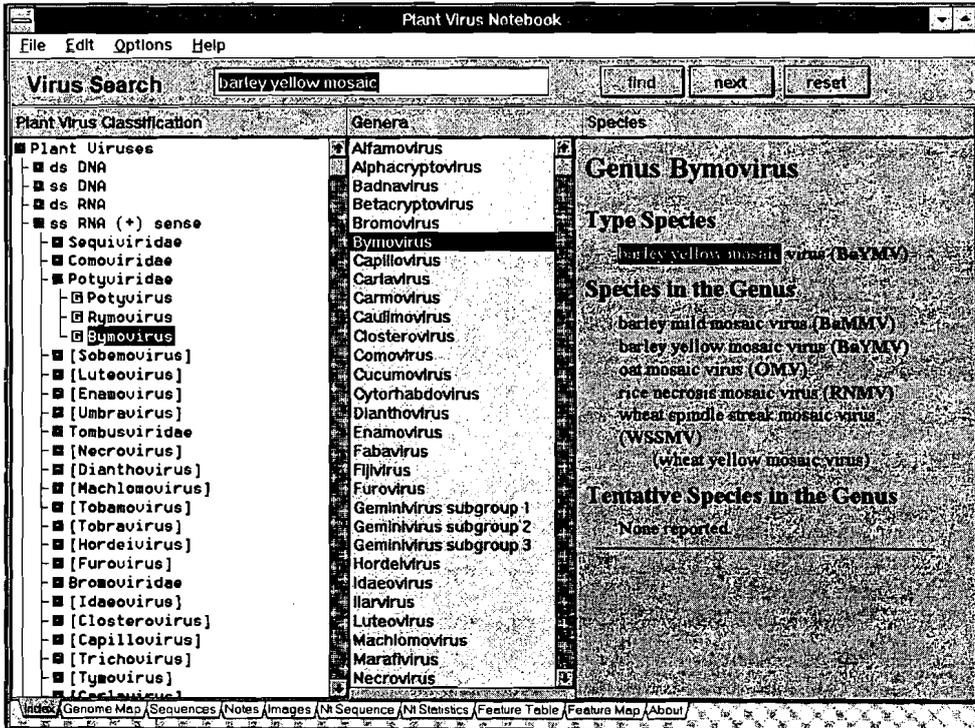
Fig. 1. Index page of Plant Virus Notebook, showing Plant Virus Classification tree (left), the list of Genera (centre) and the Bymovirus species list (right). The position of barley yellow mosaic bymovirus is highlighted
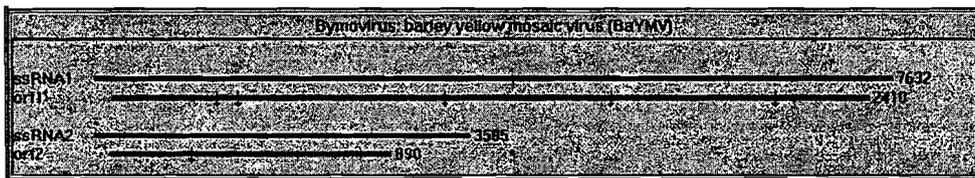
Fig. 2. Upper part of the Genome Map page of Plant Virus Notebook, displaying the map for barley yellow mosaic virus, the Bymovirus type member

Genbank databases together with their brief description and accession numbers. This greatly simplifies the task of locating available sequences for down-loading from these databases. The source text file is a separate component which can therefore readily be replaced with updated versions as necessary.

Notes and Images

When a plant virus genus is selected on the Index page, a related html file will be loaded on the Notes page describing more details of the genus and with key references. As yet, this feature is only working for the bymo- and furo-viruses. By following standard html links, images (e.g. electron micrographs, detailed genome maps) can be loaded onto the Images page and genome sequence files loaded. It is hoped that experts on different virus genera will contribute pages to complete this part of the project.

Nt Sequence, Nt Statistics and Feature Table

From the Sequence page, standard EMBL and GenBank nucleotide sequence files (in GCG format) can be loaded and their composition summarised in the Statistics page. Where available, a separate file containing an enhanced feature table will be automatically loaded on the Feature Table page, indicating the positions of open reading frames and other major features. This table, which is a simple text file, is used to construct the Feature Map (see below).

Feature Map

This page consists of three separate windows, each of which can be re-sized or hidden (Fig. 3). The lower (principal) window contains a coloured genome map of the virus (or genome segment) which has been loaded on the Sequence and Feature Table pages. This feature map shows, in different colours and drawn to scale, the nucleic acid, the different open reading frames and (where appropriate, as in the bymoviruses) protein products produced by processing a polyprotein. Other features, such as the VPg or polyA tail are also displayed. Although bymoviruses are used as a model system for this program, this format can be used to display single genome segments of any plant virus with a linear genome. As the mouse is moved within the window, the position on the genome (in nts from the 5'-end) is indicated and hint boxes appear as labels for each of the features when the mouse is pointed to them. By dragging the mouse it is possible to zoom in to a selected region. By pointing and dragging the mouse, different features, which initially overlay one another, can be dragged out vertically into one of nine different positions. A single button converts the whole map into exploded, or back to overlay, format. The top right window is an editable table based on the Feature Table (see above), and this can also be used to hide different features or to change their widths, colours or vertical positions. The top left window is used to display nucleotide or amino acid sequences. By selecting and dragging a region of the feature map into this box, DNA, RNA or amino acid sequences are displayed depending on the coloured box through which the mouse is dragged. The sequences derived can be copied to the clipboard for pasting to other Windows programs in the normal way.

Future development

The program is still being developed and is not yet available for general release. It is designed to use data files that can be down-loaded from the IACR-Rothamsted World Wide Web (WWW) pages and so can be easily updated or extended to include new virus genera. Future versions of the program will connect directly to the Internet integrating both WWW browser and analytical functions.
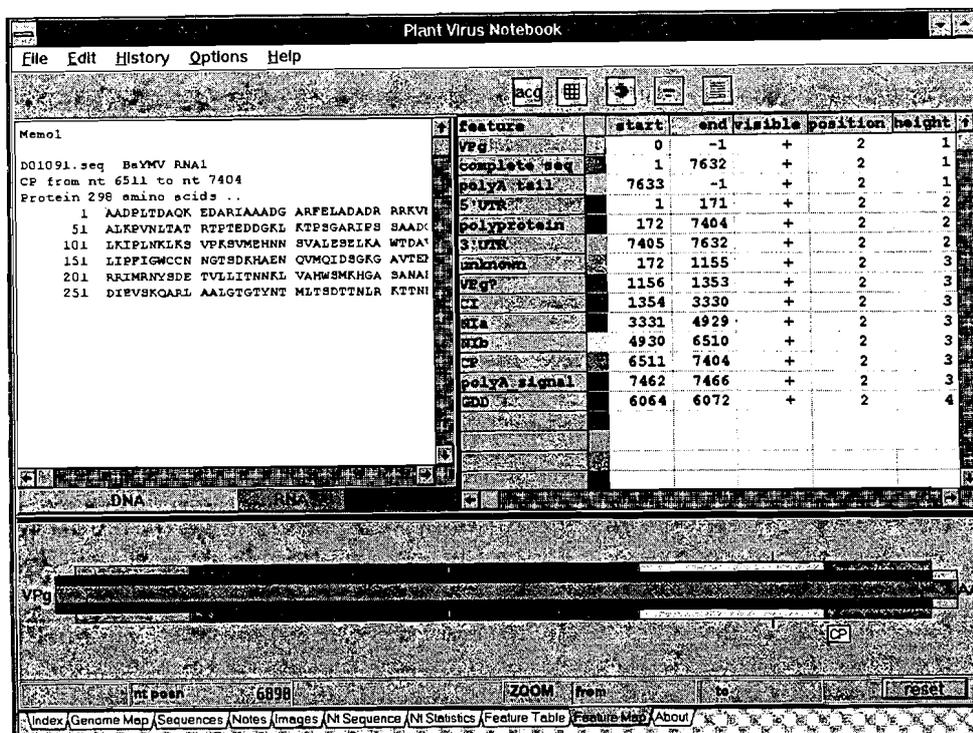
Plant Virus Notebook

File Edit History Options Help

Memo1

D01091.seq BaYMV RNA1
CP from nt 6511 to nt 7404
Protein 298 amino acids ..
     1 AADPLTDAQK EDARIAAADG ARFELADADR RRKVI
    51 ALKPVNLTAT RTPTEDDGKL KTPSGARIPS SAAD(
   101 LKIPLNKLKS VPKSVMEHNN SVALESELKA WTDA
   151 LIPPIGWCCN NGTSDKHAEN QVMQIDSGKG AVTE
   201 RRIMRNYSDE TVLLITNNKL VAHWSMKHGA SANAI
   251 DIEVSKQARL AALGTGTYNT MLTSDTTNLR KTTNI

feature	start	end	visible	position	height
Vpg	0	-1	+	2	1
complete seq	1	7632	+	2	1
polyA tail	7633	-1	+	2	1
5'UTR	1	171	+	2	2
polyprotein	172	7404	+	2	2
3'UTR	7405	7632	+	2	2
unknown	172	1155	+	2	3
VPg?	1156	1353	+	2	3
CI	1354	3330	+	2	3
NIa	3331	4929	+	2	3
NIb	4930	6510	+	2	3
CP	6511	7404	+	2	3
polyA signal	7462	7466	+	2	3
GDD	6064	6072	+	2	4

DNA RNA

VPg

CP

nt posn 6898 ZOOM from to reset

Index Genome Map Sequences Notes Images Nt Sequence Nt Statistics Feature Table Feature Map About

Fig. 3. The Feature Map page of Plant Virus Notebook, displaying RNA-1 of barley yellow mosaic virus. The map (bottom) is shown in overlay format with the complete RNA sequence centred within the single ORF and the 6 putative protein products. The coat protein (CP in hint box) has been dragged through the protein box to the sequence window (top left), where the amino acid sequence of this feature is displayed. The upper right window is the feature table on which the map is based and which can be used to change the map display format.

Reference

Murphy, F.A., Fauquet, C.M., Mayo, M.A., Jarvis, A.W., Ghabrial, S.A., Summers. M.D., Martelli, G.P. and Bishop, D.H.L. (1995). Sixth Report of the International Committee on Taxonomy of Viruses. Springer Verlag, Wien, New York.