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PREFACE

The International Working Group on Plant Viruses with Fungal Vectors was founded in 1988 during the Fifth International Congress of Plant Pathology under the chairmanship of C. Hiruki. It was decided that the group should join in a scientific symposium every three years in another country and that the first symposium should be held in Germany in 1990 before or after the VIIIth International Congress of Virology. This symposium was organized by the German Phytomedical Society in Braunschweig, August 21-24, 1990.

The Symposium was attended by more than 100 scientists from 18 countries in Europe, Asia, North America and Africa. Their contributions which have been compiled in the present volume reflect the broad range of activities which are undertaken with the aim of combating fungus-transmitted viruses. Several of the economically now most important diseases were almost unknown a few decades ago, e. g. sugarbeet rizomania and the different forms of barley yellow mosaic in Europe and East Asia. They were first detected in a few scattered spots only and have now spread to vast areas. No means have been found to fight the long-living resting spores of the plasmodiophorid vectors. Considerable success, however, has been achieved by the breeders which can supply now either tolerant, partially resistant or even immune cultivars. The example of barley yellow mosaic virus, nevertheless, demonstrates that this success may only be a temporary one. Recently, a new strain has been detected almost simultaneously in different countries which overcomes the immunity of the new cultivars. This strain is apparently readily spreading (W. Huth. p. 97 of this volume). New sources of resistance have to be found and this will also include genetechnical approaches which offer the potential of fighting the viruses with their own genes and which are tried now by several groups. A prerequisite for such attempts is a thorough knowledge of the molecular biology of the viruses and a considerable number of papers in this volume deal with this aspect. Much effort has also been spent on the development of new detection methods for the viruses, the fungi and the sources of resistance, on epidemiological studies and on the characterization of new viruses or virus-like agents and their vectors. General aspects, e. g. the risk assessment of transgenic plants, are dealt with in the last section of this volume.

The organization of the symposium was greatly facilitated by the financial support given by the institutions and firms listed on the following page. We are also greatly indebted to Mrs. Heidi Richter for her excellent typing work.

The Second Symposium of the International Working Group on Plant Viruses with Fungal Vectors will hopefully take place in Scotland (Dundee) in 1993.

Renate Koenig

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This support has greatly facilitated the organization of the symposium

CONTENTS

Section 1

Furoviruses of sugarbeets (beet necrotic yellow vein virus, beet soil-borne virus) and their vector Polymyxa betae

Section 2

Furovirus of potato (potato mop top virus)

Section 3

Bymo- and furoviruses of cereal crops and their vector Polymyxa graminis

Section 4

Viruses and virus-like agents not belonging to the furo- or bymovirus groups, including vector and transmission studies

Section 5

General subjects

A RAPID METHOD TO DETECT POLYMYXA-TRANSMITTED BEET VIRUSES FROM SOIL

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Summary

In the present method 50 g of the soil sample is put into an ordinary plastic cup for drinking and 50 ml pure sterilized water is added. After stirring the soil-water mixture and heating for 30 min at 40°C, two groups, each with five one-week-old beet seedlings, are transplanted into each cup. The cups are incubated in covered plastic boxes in order to reduce evaporation and the boxes are placed in a glasshouse at 20-30°C, or better, in a growth chamber at 25°C with a long photoperiod. After one week the roots are assayed for virus infection by ELISA and or transmission to Chenopodium quinoa.

If the inoculum level (viruliferous Polymyxa betae) is not too low a total baiting and incubation period of seven days usually seems to be enough for detecting occurrence of beet necrotic yellow vein virus (BNYVV) and other Polymyxa-transmitted beet viruses with ELISA. Advantages with the method and possibilities to increase its sensitivity are discussed.

Introduction

There has been some confusion about the occurrence of rhizomania in Sweden. Thus, in 1986 certain ELISA tests indicated that the rhizomania-virus, beet necrotic yellow vein virus (BNYVV), might be common. However, this has not later been possible to confirm. Instead, another somewhat similar but distinct Polymyxa-transmitted virus seems to be common. This virus is still not definitely characterized and identified and I prefer to use the isolate number, 86-109, until we know more. However, it is likely to belong to the beet soil-borne virus (BSBV)-group (Lindsten, 1989).

In order to learn more about rhizomania and Swedish soil-borne viruses in sugarbeets I have used rhizomania inoculum from infested German soils and inoculum (Polymyxa with virus) from Swedish beet soils in big cement tubes in a net-yard (wire netting enclosure) at Ultuna, Uppsala.

In addition to investigations of the sugarbeets sown in the tubes I have also tried to find a simple rapid method to detect and compare soil-borne viruses from the soil of the tubes with what can be found in arable fields.

The present method, which has been in use during 1989-90, is partly a modification of methods described earlier (Beemster and de Heij, 1989, Kloster et al., 1989). However, it is simpler, faster and less laborious without losing too much sensitivity.

Materials and Methods

The soil to be tested is carefully crumbled and mixed in order to get as representative samples as possible. 50 g of such soil is put in an ordinary plastic drinking cup (120 ml) and 50 ml of pure sterilized water is added. After careful stirring with a spoon the soil-water mixture is heated at 40°C for 30 min., as suggested by Beemster and de Heij (1987).

The bait plants used are one-week-old beet seedlings of the sensitive

cultivar Hilma, which is very densely sown in sterilized sand in clay pots (diameter 9 cm). Usually more than 100 seedlings are easily obtained from each pot. The seedlings can be easily rinsed from sand by running tap water and after additional stirring two groups of five seedlings in each are transplanted into each cup. The cups are incubated in partly covered plastic boxes with wetted paper towels at the bottom in order to reduce evaporation. Initially the boxes are placed in an ordinary glasshouse at 20-30°C. However, nowadays they are placed in a warm growing room (25°C) with high light intensity (18 000 Lux, Hg-lamps) and a photoperiod of 18 h.

After a baiting period of one week (6-8 days), the roots of 3-5 plantlets are washed and pressed in a Pollähne press. Three drops of sap are collected for ELISA in a plastic tube with 1 ml PBS-TP buffer. The rollers of the press are then first washed with 1 ml 0.01 M phosphate buffer which is collected for sap-transmission (if this is to take place) and then thoroughly washed with tap water before being used for the next sample.

ELISA is carried out in the usual way using our own antisera, one to BNYVV and one to a typical Swedish soil-borne beet virus isolate, 86-109, which most probably is an isolate of the BSBV-type. More details about the isolates used for antisera production and the set-up of isolates in the totally enclosed net-yard at Ultuna, Uppsala, are found in Lindsten (1989).

Results

In 1989 and 1988, but in contrast to 1987 (Lindsten, 1989), rather few sugarbeet fields were found from which clear positive ELISA for 86-109 were obtained directly from collected field plants. However, in 1989 we started to try to detect soil-borne viruses in soil by using the rapid method. From all 86-109 positive fields, and in addition from several of the negative ones, frequently high ELISA readings for 86-109 were obtained already after about one week for bait plants transplanted into small soil samples in water. In no case was any evidence found for occurrence of BNYVV. Thus, the ELISA readings after 60 min were generally < 0.05 and no transmission of BNYVV to Chenopodium spp was obtained.

On the other hand, in the enclosed net-yard at Ultuna, Uppsala, where also rhizomania was established already in 1984 in some cement tubes, clear positive ELISA was obtained both for BNYVV and 86-109. All tests according to the rapid method from five of the tubes in the net-yard during 1989 and so far in 1990 are summarized in Fig. 1 in order to give some idea of the usefulness of the method for detecting not only 86-109 but also BNYVV.

From a control tube without inoculum no evidence was obtained in ELISA either for 86-109 or for BNYVV. Neither was any indication of BNYVV found from the two tubes with Swedish inoculum (altogether 16+14 = 30 tests). However, all 16 tests from tube 1 gave positive results for 86-109 (lowest reading 0.27 and highest >2 but counted as 2 in the figure and giving an average of 1.08). Two negative readings (<0.1) but 12 clear positive were obtained in tube 2. From the tubes with German inoculum, on the other hand, clear positive readings were in general obtained for both BNYVV and 86-109. That the German inoculum was infested not only with BNYVV but also with virus of the 86-109 type has been known since 1988 (Lindsten, 1989).

Very similar results were obtained for all tubes when ELISA was made from sugerbeet plants sown in the tubes even if the infection rate varied with time of sowing and age of plants, which will be reported on elsewhere.

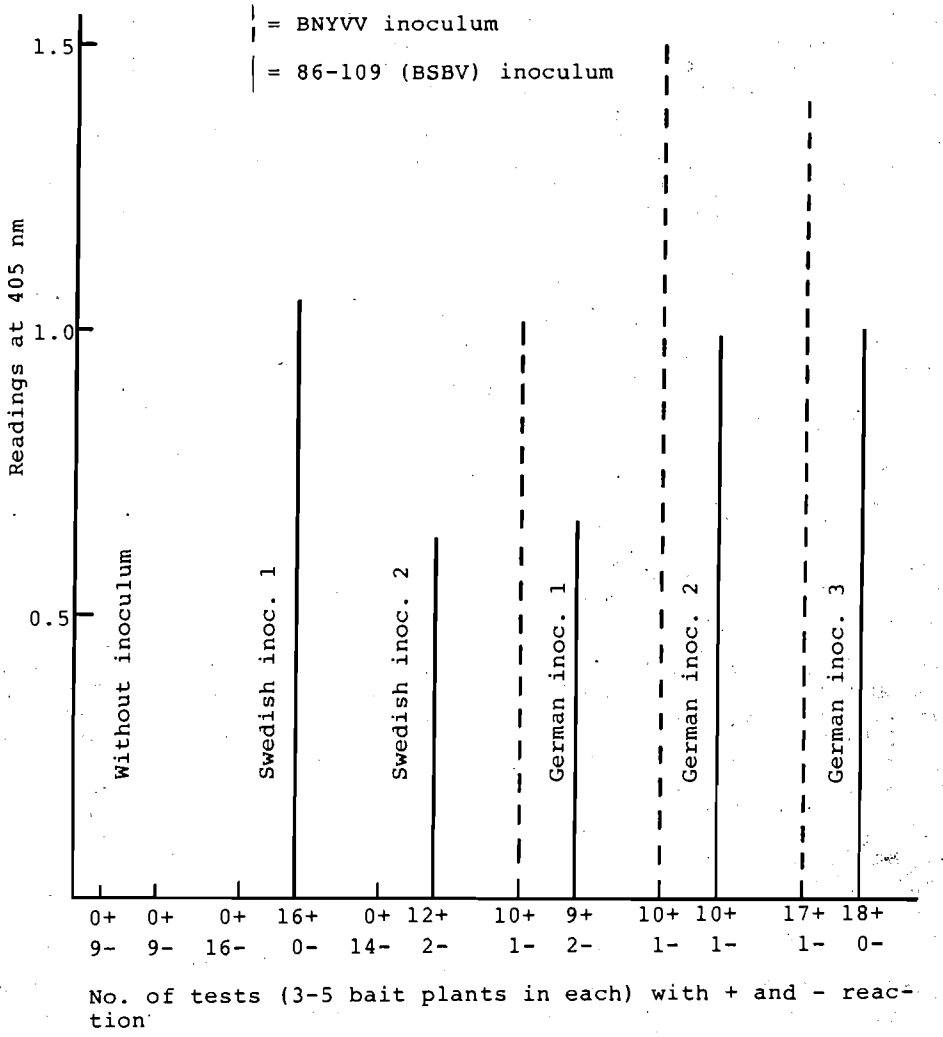

Fig. 1. Average of ELISA of bait plants in soil samples from inoculated net-yard at Ultuna during 1989-90.

Discussion

The simplest way to assay soil samples for occurrence of Polymyxa-transmitted viruses is to sow a sensitive beet cultivar in the sample and assay roots after the necessary baiting and incubation period. However, even when using the most optimal growing conditions for Polymyxa (ca 25°C and high moisture) it usually takes several and at least three weeks before a reliable testing of the bait plants can take place. Frequently in these growing conditions, Aphanomyces and other soil-borne patogens will cause dying, or in any case disturbance, of the bait plants. This is mainly overcome by the shorter time needed for the rapid method descrived. In addition, the riskts for unspecific reactions (high background) in the ELISA are less and much less growing space is needed.

Improvements of the bait plant methods have been made by Beemster and de Heij (1987) and Kloster et al. (1989). Probably, the sensitivity of the present method is less than for the above methods. However, it has so many advantages, including the simple procedure, that it may be worth trying at least as a preliminary screening test. According to experiments in progress, its sensitivity can also be increased very considerably by keeping the baiting-incubation temperature close to 25°C, having high light intensity and a long light period. Perhaps also an improvement of the heating procedure and a suitable nutrient solution instead of just pure sterilized water will become important, especially if the incubation period needs to be increased (cf. Peters and Godfrey-Veltman, 1989).

All the tests in Fig.1 apply to the first plant group tested and a baiting-incubation period of 6-8 days. However, we are routinely using two groups of bait plants with 5 plants in each to each cup with the soil-water mixture. If the growing conditions are good, most of these plants will survive some weeks and it is possible to make a second test after some additional incubation time, which usually increases the infection rate. This is, of course, of importance if the first test gives negative results and the viruliferous Polymyxa concentration is believed to be very low. Another possibility to check infection in the bait plants is by means of sap-transmissions to Chenopodium spp. However, in general the infection rate will then be lower than that given by ELISA.

We now have considerable experience of the rapid method for assaying viruses from soils of Swedish sugarbeet fields. Thus, we have found that viruses of the 86-109 type seem to be very common and in many fields they occur also in high frequency according to the ELISA tests, even if the concentration in the field beets may be low and difficult to detect (Lindsten, 1989). On the other hand, the ELISA plates for BNYVV which have always been included in the tests have always given negative results, with the exception of the holes of the positive controls from the net-yard. However, if this means that we have no BNYVV in Swedish sugarbeet fields, so far, or if the concentration in the samples tested is too low to be detected with this method, has to be further investigated.

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SURVEY OF SOIL-BORNE VIRUS DISEASES OF SUGAR BEET IN FINLAND AND ESTONIA

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Summary

The presence of beet necrotic yellow vein virus (BNYVV) could not been confirmed in Finland. A soil-borne sugar beet virus (isolate 86-109) could be detected by ELISA in sugar beet plants collected from fields and in bait plants. ISEM confirmed these results. No virus was detected in bait plants grown in soil samples from Estonia, USSR or in fodder beet plants from Estonia. *Polymyxa betae* was present in both Finnish and Estonian soils and beets.

Introduction

Rhizomania disease of sugar beet caused by beet necrotic yellow vein virus (BNYVV) is not known to occur in Finland. However, because of the monoculture of sugar beet, the presence of the vector of BNYVV (*Polymyxa betae* Keskin) in field soils (Vestberg, 1989) and the prevalent soil temperatures (Laurinen and Säkö, 1980), the occurrence of rhizomania in Finland is possible. This paper describes a field survey carried out to determine the incidence of *P. betae*, rhizomania and similar diseases of beets between 1988 and 1990.

Materials and methods

Sugar beet plants and soil samples were collected from regions of fields where sugar beet showed poor growth. Twenty plant and soil samples in 1988 and 47 plant and 44 soil samples in 1989 were collected from the main sugar beet growing areas in south-western Finland. In both years the crops were sown in the first week of May and sampled in August/September. In addition to this, five fodder beet and four soil samples collected from Estonia, USSR in 1989. The soil samples were air-dried for 4-7 weeks and tested using a modification (Kloster, Begtrup and Engsbro, 1989) of the bait plant method originally described by Beemster and de Heij (1987).

Roots of sugar beet plants from fields and bait plants were tested by enzyme-linked immunosorbent assay (ELISA) and the presence of viruses was confirmed by immunosorbent electron microscopy (ISEM) and sap inoculations to indicator plants. ELISA was performed by the direct method as described by Clark and Adams (1977) using antisera to BNYVV supplied by Boehringer and Sanofi and to a Swedish soil-borne sugar beet virus (isolate 86-109) kindly supplied by Prof K. Lindsten, Uppsala, Sweden. The threshold value used was the mean absorbance value plus three standard deviations of the healthy controls.

Results

In 1988 six sugar beet plants reacted positively to 86-109 antiserum and two reacted positively to the BNYVV antiserum (Table 1). One bait plant reacted positively to the 86-109 antiserum and one to the BNYVV antiserum.

Sap inoculations from the roots of these sugar beet and bait plants to *Chenopodium quinoa* and electron microscopy confirmed the presence of the virus isolate 86-109 in two samples, but the presence of BNYVV could not be confirmed by these methods. *P. betae* was seen in roots of 45% of the bait plants.

Table 1. ELISA-values for sugar beet plants from fields and bait plants giving positive reactions to the 86-109 and BNYVV (Boehringer) antisera in 1988 tests.

	Absorbance at 405 nm			
	86-109		BNYVV	
Sample	Bait plant	sugar beet	bait plant	sugar beet
Healthy control	0.025	0,121	0,005	0,016
1	*	0,633	*	0,373
2	*	0,877	0,184	0,337
3	*	4,443	*	*
6	*	1,126	*	*
10	*	0,378	*	*
11	*	0,944	*	*
18	0,225	*	*	*

* absorbance was below threshold value

In 1989 five bait plants and seven sugar beet plants reacted positively to the 86-109 antiserum; four of these bait plants corresponded to fields where infected sugar beet were also found (table 2). None of the sugar beet or bait plants reacted positively to the BNYVV antiserum in ELISA tests. The absorbance values for the bait plants was clearly negative, but those of for the sugar beets were close to the threshold value. *P. betae* was seen in roots of 39% of the bait plants and 63% of the sugar beet plants.

Yellow flecks appeared in *C. quinoa* leaves 6-8 days after inoculation with ground up roots of sugar beet and bait plants that reacted positively to the 96-109 antiserum. Symptoms of the sugar beet virus 86-109 were sometimes difficult to recognize due to tobacco necrosis (TNV) and tobacco mosaic (TMV) infections. Electron microscopical examination of dip preparations of these *C. quinoa* leaves and sugar beet roots revealed few rod-shaped particles. When the 86-109 antiserum was used to trap particles, about 120 particles from each of the three isolates could be measured. Particle length distributions varied between isolates, but those that were measured had common peaks at 90-150 nm and 200-260 nm. One

isolate had a third peak at 300-340 nm.

Table 2. ELISA values for sugar beet plants from fields and bait plants giving positive reactions to 86-109 antiserum in 1989 tests.

Sample	Absorbance at 405 nm 86-109 Bait plant	Sugar beet
Healthy control (for samples 24-53)	0,015	0,027
24	+	0,517
35	*	0,336
37	1,335	*
43	1,763	0,402
53	*	0,314
healthy control (for samples 54-70)	0,068	0,047
61	0,492	0,204
62	1,351	0,529
66	0,662	0,223

\+ no soil sample
* absorbance was below threshold value

No BNYVV or 86-109 was detected in bait plants grown in soil samples or in fodder beet plants collected from Estonia. *P. betae* was defected in one bait plant grown in an Estonian soil sample and in two fodder plants from Estonia.

Discussion

ELISA tests on sugar beet plants collected from fields often gave high absorbance values which indicated the presence of BNYVV or the virus isolate 86-109. ISEM and sap inoculations confirmed the presence of 86-109 in some of these samples, but failed to confirm the presence of BNYVV. Lindsten (1989) suggested that extracts from old sugar beet roots may cause unspecific reactions in ELISA and this may explain some of the high absorbance values observed. All the samples which gave positive or unclear results were tested two or four times by ELISA and sap inoculation tests. The high incidence of damping-off fungi in soil in 1988 may have weakened the physiological condition of sugar beet plants from the field and in bait tests. This may have caused the confusing ELISA results (cf. Gunn and Pares, 1988).

ELISA and ISEM tests on bait plants and sap inoculations from these plants to indicator plants confirmed the presence of the sugar beet virus isolate 86-109 in Finnish soils, but failed to

confirm the presence of BNYVV. The virus isolate 86-109 occured in several fields which were distant from each other indicating that it may have been present in field soils in Finland for a long time.

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CHARACTERIZATION OF A VIRUS ASSOCIATED WITH THE BEET NECROTIC YELLOW VEIN VIRUS: RECONSTITUTION WITH BNYVV-PROTEIN

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Summary

On the basis of symptoms produced on Chenopodium quinoa leaves by inocula prepared from field-grown sugar beet infected with the beet necrotic yellow vein virus (BNYVV) Hein (1987) demonstrated that BNYVV-infected plants sometimes contain tobacco necrosis virus and also an apparently unknown virus, which she called Gänsefuss-Nekrose-Virus (GNV). This virus produces large necrotic patches on C. quinoa leaves, which spread quickly along the veins. No virus-like particles could be found either in dip-preparations or in partially purified samples but phenol-extracts were highly infectious. The infective material can be reconstituted to rod-shaped particles using purified coat-protein of BNYVV.

Introduction

When a sap from sugar beet leaves infected with the beet nectrotic yellow vein virus was used to mechanically inoculate Chenopodium quinoa three different types of lesions developed: chlorotic lesions typical of BNYVV, tiny necrotic lesions, that could be identified as those of tobacco necrosis virus, and large necrotic patches that spead rapidly along the leaf veins. This "virus" was provisionally called "Gänsefuss-Nekrose-Virus" (GNV) by Hein (1987). GNV can be mechanically transmitted to C. quinoa, though with a poor efficiency. Here I report on our preliminary attempts toward a characterization of this new infectious agent.

Materials and Methods

The BNYVV infected sugar beet plants were collected from a grower's plot in Bavaria. Chenopodium quinoa plants, raised in an aphid-proof greenhouse, were used to purify the BNYVV from the other associated viruses. The isolate of BNYVV was increased in Spinach (Spinacia oleracea) and the virus was extracted by a butanol-chloroform procedure. Native coat protein of BNYVV was prepared by the acetic acid method of Fraenkel-Conrat (1957). The protein was dialysed exhaustively against double distilled water and dissolved in 0.05 M trisphosphate buffer of pH 8.0. BNYVV-RNA was extracted from the virus by a phenol-SDS method. Since no virus particle could be detected in GNV-infected plants, total nucleic acids were prepared from primary inoculated C. quinoa leaves, 6 or 7 days after inoculation according to a phenol-chloroform method.

For reconstitution of rod-shaped particles nucleic acid samples were mixed with BNYVV-protein in pyrophosphate buffer of pH 7.3 (Fraenkel-Conrat and Singer 1957). A typical reconstitution experiment was performed by mixing (in an ice bath) 2.0 ml of 0.2 M pyrophosphate buffer (pH 7.3) with 0.7 ml of double distilled water, followed by 0.74 ml BNYVV-protein (= 2.5 mg protein) and 1.56 ml of total Chenopodium nucleic acid (= about 0.125 mg total RNA) in a centrifuge tube. The mixture (5 ml) was gently shaken, incubated at room temp. for 1/2 hr and kept overnight at about 6° C. The opalescent solution was centrifuged at low speed (about 7000 x g) for 10 min at 5° C and the supernatant was centrifuged for one hour at 80.000 x g in an ultracentrifuge. The pellet was suspended in distilled water.

Results

Homogenates of infected C. quinoa leaves prepared in water or in HEPES buffer (pH 7,0) could be used for inoculation of C. quinoa but the infectivity was very low: only 1 or 2 lesions developed on each inoculated leaf. The homogenates lost infectivity in 24 hrs at room temp. and within 48 hrs when maintained at 6° C. Infected leaves frozen at -20° C retained infectivity for 10 days and freeze-dried leaves, kept over anhydrous $CaCl_2$, were infectious up to 4 weeks. Dip preparations (Brandes 1964) contained no rod-shaped or flexuous virus-like particles. Considering that the virus might consist of small isometric particles, a purification procedure was adoped, that had proved efficient for the isolation of cucumber mosaic virus (Lucas, C. 1985). No isometric virus-like particle was found by this method. However, the phenol-extracts were highly infectious.

Preparations containing only 5 ug of total RNA dissolved in tris-phosphate buffer (0.1 M, pH 8.8) produced more than 60 lesions per leaf of C. quinoa. Nucleic acid prepared from such leaves was used for reconstitution as mentioned under materials and methods. The particles obtained after reconstitution in vitro (and contrasted with 2% PTA, pH 6.1) were about 270 nm in length. Control experiments performed with total nucleic acids from healthy Chenopodium leaves did not yield any rod-shaped particle. Reconstituted particles of different lengths were obtained when RNA extracted from purified BNYVV particles was incubated with BNYVV-protein in pyrophosphate buffer. The E_{260}/E_{280}nm values of the particles reconstituted with BNYVV-protein using BNYVV-RNA and GNV-RNA were 1.2 and 1.1, respectively.

Discussion

The "new virus", presented here was obviously associated with BNYVV and could be isolated using the local lesion host Chenopodium quinoa. The symptom produced on C. quinoa is very similar to that caused by the beet soil borne virus (BSBV), reported by Henry, Jones and Coutts (1986). However, we could not find any particle of BSBV even after several attempts. The quick loss of infectivity in leaf sap speaks also against the presence of a virus particle in our samples. Moreover, the ability of BNYVV-protein to coat GNV-RNA in vitro indicates that we are not dealing with BSBV; assuming that BSBV-RNA most probably does not contain a nucleotide sequence for the initiation of assembly in vitro with BNYVV-protein. On the other hand, if a variant of one of the large RNAs of BNYVV is capable of inducing large necrotic lesions on C. quinoa, it could be reconstituted with BNYVV-protein in vitro; so that we may not be justified in giving a new name to our isolate. This question, as well as the possibility, that we have found the large RNA-component of a strain of tobacco rattle virus (a suggestion of Dr. B.D. Harrison, Scottish Crop Research Institute, Invergowrie, Dundee, U.K.) have yet to be examined.

Acknowledgement

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#1

A COMPARISON OF THREE EUROPEAN ISOLATES OF BEET SOIL-BORNE VIRUS WITH BEET NECROTIC YELLOW VEIN VIRUS

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Summary

The English isolate of beet soil-borne virus, a Swedish isolate (86-109), a Belgian isolate (1530) and a French isolate of beet necrotic yellow vein virus (BNYVV-Fr) were compared using immunoblotting and RNA-RNA blot hybridisation techniques. Immunoblots of extracts from plants infected with each virus were probed with antisera produced against the Swedish 86-109 isolate. The antisera reacted with a protein of Mr ca. 18,000 D found in extracts of plants inoculated with the isolates BSBV-N, 86-109 and 1530, this protein is thought to be the coat protein of BSBV. No serological relationship was detected between the 86-109 isolate and BNYVV in similar immunoblots. The dsRNA species of each isolate were probed reciprocally and significant cross-hybridisation was detected between the BSBV-N, 86-109 and 1530 isolates while there was no significant hybridisation between these isolates and BNYVV.

Introduction

Beet soil-borne virus (BSBV) was isolated in 1981 from the roots of sugar beet grown in a soil sample taken from a site at Norfolk, England (Ivanović and Macfarlane, 1982). This isolate of BSBV has been termed BSBV-N. The virus has tubular particles 19 nm in diameter with a central canal and a range of lengths, with a main peak at 150 nm and two smaller peaks at 65 nm and 300 nm (Ivanović and Macfarlane, 1982). BSBV-N resembles BNYVV in particle size and shape and both are transmitted by the plasmodiophoromycete fungus Polymyxa betae (Henry et al., 1986). Recently several tubular viruses similar in morphology to BSBV-N have been isolated from sugar beet in Sweden and West Germany (Lesemann et al., (1989) and Belgium (Verhoyen et al., 1987). Lesemann et al., 1989 have identified two serological groups within these isolates, group 1 which contains the German isolates Wiertha and Dornheim and group 2 which contains the German isolate Ahlum, the English isolate BSBV-N and the Swedish isolate, 86-109.

Materials and Methods

Virus culture. Isolates were cultured in Chenopodium quinoa. Plants were mechanically inoculated and maintained as described by Henry et al., (1986). Infected leaves were harvested 5 to 6 days after inoculation.

Immunoblotting. Extracts of infected or healthy leaf or root sap were centrifuged at 15,000g for 5 min and the supernatants and marker proteins denatured by heating to 100°C for 5 min in denaturation buffer (10% Glycerol, Bromophenol blue, Tris-HCl (62.5 mM, pH 6.8), 5% 2-mercaptoethanol, 0.1% SDS). The samples were electrophoresed through a 10% SDS-polyacrylamide gel and then immobilised on nitrocellulose by immunoblotting (Towbin et al., 1979). Blots were probed with a polyclonal antisera produced to the BSBV 86-109 isolate. Detection of bound antibodies with a Protein A-peroxidase conjugate by probing with the antisera followed by incubation with 4-chloro-1-napthol and hydrogen peroxide was carried out as described by Sherwood (1987).

DsRNA extraction and hybridisation. DsRNA was extracted from infected C. quinoa leaves by the CF-11 affinity column method (Morris and Dodds, 1979). Preparations were treated with DNase 1 to remove contaminating DNA and S1 nuclease to remove ssRNA. DsRNA preparations were electrophoresed through a 5% polyacrylamide gel for

16h at 60-80V. Nucleic acids were visualised by staining with ethidium bromide and photographed on a trans-illuminator. DsRNA was electroblotted from gels onto Zetaprobe membranes (Biorad) and probed using dsRNA probes produced by end labelling dsRNA preparations using [^{32}P]γ-ATP and T_4 polynucleotide kinase (Henry and Hutchinson, 1989).

Results

The serological relationship between two English isolates BSBV-N, BSBV-452N, the Swedish 86-109 and Belgian 1530 isolates were compared by immunoblotting. Blots were probed with a polyclonal antisera made against the 86-109 isolate. The antisera reacted with a protein with an Mr ca. 18 KD in both the 86-109 and

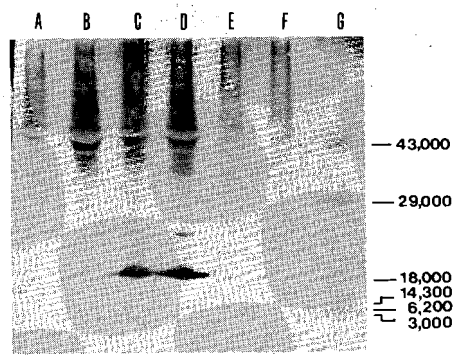

Fig. 1. Immunoblot analysis of proteins from virus-infected and healthy C. quinoa leaf extracts. A) BNYVV; B) Healthy C. quinoa; C) 1530; D) 86-109; E) BSBV-N; F) BSBV-491N; G) Prestained protein molecular weight standards; low range (3,000-43,000 D).

1530 isolates, this protein is thought to be the coat protein (Fig. 1). No serological reaction was detected when sap produced from C. quinoa leaves inoculated with the English BSBV isolate was tested, however when sap from roots of sugar beet (Beta vulgaris, cv. Monoire) plants infected with BSBV-N was tested a protein of similar Mr reacted positively (results not shown). The inability of the antisera to detect the coat protein of the English isolates in leaf extracts is probably due to the low virus titre present in leaves, as evidenced by an inability to routinely detect virions by electron microscopy of such extracts (results not shown). There was no cross reaction detected between the antisera and BNYVV. Double-stranded RNA preparations isolated from C. quinoa locally infected separately with BSBV-N, 86-109 and 1530 and a French isolate of BNYVV were compared following separation on non-denaturing polyacrylamide gels (Fig. 2). The dsRNA preparations from BSBV-N infected leaves contained 3 major, and occasionally 1 minor dsRNA species, the molecular weights of the 3 major dsRNA species being ca. 4.1, 1.95 and 1.68 x 10^{-6} (Henry and Hutchinson, 1989). DsRNA preparations from the 1530 and 86-109 infected leaves also contained 3 major dsRNA species which are of similar mobility to those of BSBV-N, however only the lower dsRNA species are shown in the result illustrated in Fig. 2.
Sequence homology between the virus isolates was investigated using RNA-RNA blot hybridisation. Blots were probed with [^{32}P] end-labelled BSBV-N dsRNA. Significant hybridisation was detected between the isolates BSBV-N, 86-109 and 1530, while

there was no significant hybridisation between BSBV-N and BNYVV (Fig. 3).

Discussion

Results of immunoblotting experiments suggest BSBV-N, 86-109 and 1530 are all

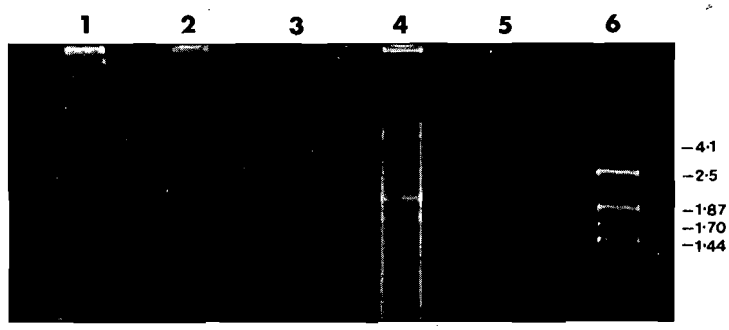

Fig. 2. Electrophoresis in a 5% polyacrylamide gel of dsRNAs isolated from leaves infected with the isolates: 1530 (lane 1), 86-109 (lane 2), BNYVV (lane 3), BSBV-N (lane 4). Healthy C. quinoa preparation (lane 5). Aspergillus foetidus viral dsRNA as standards (lane 6). Numbers to the side of the gel refer to the Mr ($\times 10^{-6}$) of the dsRNA markers.

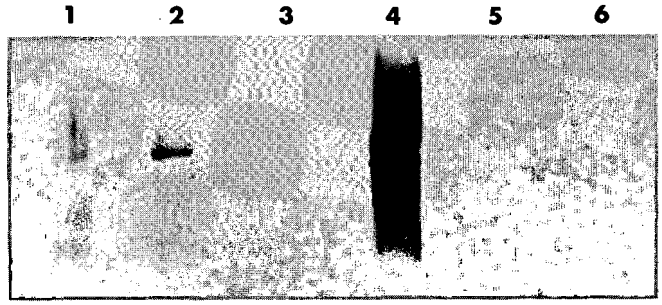

Fig. 3. Autoradiograph of RNA-RNA blot hybridisation. Nucleic acids were electroblotted on to Zetaprobe membrane and probed with [^{32}P] end labelled BSBV-N dsRNA. Lanes 1-6 as for Fig. 2.

serologically related to one another and that the Belgian isolate 1530 may be included in the BSBV serotype group 2 as described by Lesemann et al., (1989).

BSBV is apparently serologically distinct from BNYVV. The three BSBV isolates are apparently not only serologically related but have similar dsRNA profiles and composition which are also distinct from BNYVV.

Acknowledgements

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BEET SOIL-BORNE VIRUS: ELECTROPHORETIC PATTERNS OF ssRNAs AND dsRNAs AND PREPARATION OF cDNA CLONES

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Summary

The patterns of ss and dsRNAs associated with the Ahlum isolate of beet soil-borne virus were studied after agarose gel electrophoresis under denaturing (60% formamide) and undenaturing conditions, respectively. The RNAs were detected by means of ethidium bromide staining and by means of Northern blotting using cloned cDNAs. The cDNAs had been obtained by using denatured dsRNA from infected leaves as a template. By means of Northern blotting we detected virus-specific ssRNAs of 3.6 kb and 3.2 kb and dsRNAs of 3.6 kbp and 3.2 kbp, respectively, in extracts from infected leaves. The 3.2 kb ss-RNA or the 3.2 kbp dsRNA were apparently not derived from the 3.6 kb and the 3.6 kbp RNAs because many clones hybridized specifically with the 3.2 kb and the 3.2 kbp RNAs. Only RNA extracts from purified virus particles yielded in addition a 3.0 kb RNA which was apparently a degradation product of the 3.2 kb RNA. By means of ethidium bromide staining we detected sometimes a faint 5.8 kb band in ssRNA extracts from purified virus particles and a 5.6 kbp band in dsRNA extracts from infected leaves. None of our cDNA clones hybridized with this band and we do not know whether it represents a part of the viral genome.

Introduction

Beet soil-borne virus (BSBV) which was first described in England by Ivanović and McFarlane (1982), Ivanović et al., (1983) and Henry et al., (1986) resembles beet necrotic yellow vein virus (BNYVV), the causal agent of sugarbeet rizomania, morphologically and in its association with *Polymyxa betae*. However, serological relationships have not been found between the two viruses. BSBV is apparently widely spread in Europe (e.g. Verhoyen et al., 1987; Lesemann and Koenig, 1988; Lindsten, 1989) and several serotypes have been described (Lesemann et al., 1989; Henry and Hutchinson, 1989). The serological differentiation index (SDI) is 4-5 for the serotypes 'Ahlum' and 'Wierthe' from Germany. The Ahlum isolate is serologically closely related to the British BSBV isolate and the Swedish isolate 109-86 (Lesemann et al., 1989). Very little is known about the RNA composition of these viruses (Henry and Hutchinson, 1989). In this paper we describe the electrophoretic patterns of ssRNAs and dsRNAs associated with the Ahlum isolate of BSBV and the preparation of cloned cDNAs.

Materials and Methods

The Ahlum isolate of BSBV was propagated on *Chenopodium quinoa* and was purified as described for BNYVV by Putz and Kuszala (1978). The methods for extracting ssRNA either from infected leaves or from purified virus particles (Burgermeister et al., 1986) and dsRNA from infected leaves (Bar-Joseph et al., 1983) and the electrophoretic separation of RNAs in agarose gels under nondenaturing or denaturing (60% formamide) conditions (Burgermeister et al., 1986) have been described elsewhere. cDNAs were obtained by random-primed reverse transcription of denatured dsRNA from infected leaves and were cloned into the plasmid pT7T3 19U (Pharmacia).

Results

After electrophoresis in 60% formamide/1.25% agarose gels ssRNA from purified virus particles yielded three major bands corresponding to 3.6 kb, 3.2 kb and 3.0 kb, respectively, and occasionally a very faint band corresponding to 5.8 kb. The sizes of these RNAs differed clearly from those of the ssRNAs extracted from healthy *Chenopodium quinoa* or from ssRNAs of purified BNYVV (Fig.1). Extracts of dsRNA from infected, but not from healthy *Ch. quinoa* yielded 2 major bands corresponding to 3.6 kbp and 3.2 kbp in agarose gels under nondenaturing conditions; occasionally a minor band corresponding to 5.6 kbp was also seen (Fig. 2). There was no band corresponding to the 3.0 kb band in ssRNA from purified particles.

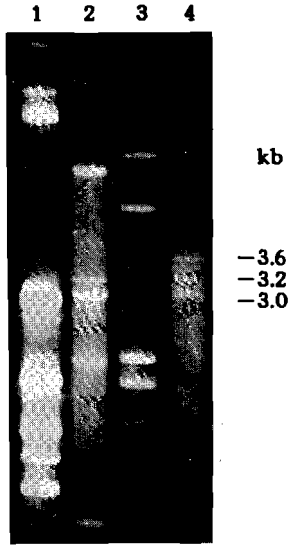

Fig. 1.

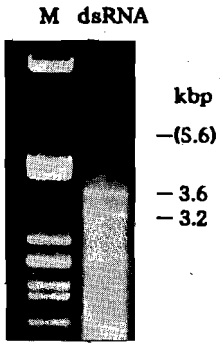

Fig. 2.

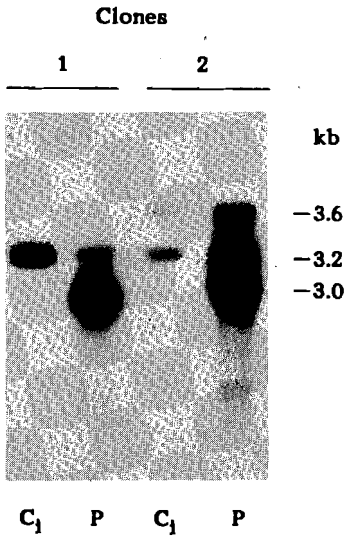

Fig. 3.

Fig. 1. Electrophoretic separation of ssRNAs in 60% formamide/1.25% agarose gels. The bands were visualized by staining with ethidium bromide (5 µg/ml). Lane 1 - ssRNA extract from healthy *Chenopodium quinoa*, lane 2 - size markers: RNA from tobacco mosaic virus (6395 b), RNA 1, 2, 3 and 4 from brome mosaic virus (3234 b, 2865 b, 2114 b and 876 b, respectively), lane 3 - beet necrotic yellow vein virus RNAs 1 to 4 (6746 b, 4612 b, 1774 b and 1467 b, respectively) and partially deleted form of the RNA 3 of unknown size, lane 4 - RNAs from purified particles of the Ahlum isolate of beet soil-borne virus.

Fig. 2. Electrophoretic separation of marker dsDNAs (11501 bp, 5080 bp, 4575 bp, 4505 bp, 2840 bp, 2454 bp, 2139 bp, 1986 bp and 1700 bp, respectively) on the left side and dsRNA from the *Ch. quinoa* leaves infected with the Ahlum isolate of beet soil-borne virus on the right side. The bands were visualized by staining with ethidium bromide (5 µg/ml).

Fig. 3. Northern blot of ssRNAs from *Ch. quinoa* leaves infected with the Ahlum isolate of beet soil-borne virus (C_1) and from purified virus particles (P) after electrophoresis in 60% formamide/ 1.25% agarose gels. Hybridization with clones 1 and 2, respectively (for explanation see text).

Seventeen virus-RNA-specific and 42 host-RNA-specific cDNA clones were obtained when denatured dsRNA was used as a template for reverse transcription. When ssRNA from purified virus preparations was used as a template only host-RNA-specific cDNA clones were obtained although the RNA from purified virus had yielded an electrophoretic pattern which was very different from that of host RNA (Fig. 1). This suggests that small amounts of host RNA which probably had contaminated the virus RNA were much more efficiently transcribed than the virus RNA.

The 17 virus-specific clones could be devided into two groups (Fig. 3). Clones 1 hybridized with the 3.2 kb and 3.0 kb ssRNA bands from purified virus, a 3.2 kb band in ssRNA extracts from infected leaves and (results not shown) a 3.2 kb band produced by denatured dsRNA. Clones 2 in addition detected the 3.6 kb band in ssRNA extracts from infected leaves or purified virus (Fig. 3) and (result not shown) in denatured dsRNA.

None of our clones hybridized with the 5.8 kb or the 5.6 kbp bands seen with ssRNA extracts from purified virus or dsRNA extracts from infected leaves, respectively.

Length measurements of the virus particles yielded a strong peak between 150 and 210 nm.

Table 1. Comparision of the size (kb) of the ssRNAs of some furoviruses

BSBV[1)]	(5.8 ?)	3.6	3.2	3.0[b)]
BNYVV[2)]	6.7	4.6	1.7[a)]	1.4[a)]
PMTV[3)]	6.5/6.4	3.2/3.0	2.5	
SBWMV[4)]	6.5-6.9	3.5[a)]		
PCV[5)]	5.9	4.3		

a) partially deleted forms may also occur
b) not present in leaves which had been frozen in liquid nitrogen and were extracted before they were unfrozen

1) this paper
2) Bouzoubaa et al., 1985; 1986; 1987
3) Scott et al., 1990; Kallender et al., 1990
4) Shirako et al., 1984
5) Reddy et al., 1985

Conclusions

Our results suggest that a 3.6 kb and a 3.2 kb ssRNA are part of the genome of the Ahlum isolate of BSBV. The fact that clones 2 hybridized with both RNAs indicates that the two RNAs have some common sequences as it has been found with the RNAs of many viruses with polypartite genomes.

The 3.2 kb RNA is apparently not a breakdown product or a subgenomic form of the 3.6 kb RNA, because clones 1 specifically hybridized with the 3.2 kb RNA. Only in purified virus we found another ssRNA of 3.0 kb which is apparently derived from the 3.2 kb RNA, because clones 1 which are specific for the 3.2 kb RNA also hybridized with the 3.0 kb RNA. We are presently checking in which stage of the purification procedure the conversion of the 3.2 kb RNA to the 3.0 kb RNA takes place. Possibly it occurs already in crude sap, because the ssRNAs in which the 3.0 kb band is missing are extracted from leaves which had been frozen in liquid nitrogen and were extracted with a buffer/phenol mixture before unfreezing had taken place.

Provided that the RNAs in BSBV are arranged in a similar way as in BNYVV the expected size of the particles with 3.0 to 3.6 kb RNAs would be 170 to 205 nm which agrees well with the actual measurement of 150 to 210 nm.

Since we did not obtain clones specific for the faint 5.6 to 5.8 kb band we do not know whether a RNA of this size also forms part of the viral genome. For all other furoviruses a large ssRNA of 5.9 to 6.9 kb has been reported to form part of the genome (Table 1). In case of the other furoviruses the large RNA forms a strongly staining band (see Fig. 1 for BNYVV). It is evident from Table 1 that the RNA pattern of the Ahlum isolate of BSBV differs from that of all other furoviruses listed. Preliminary experiments suggest that it also differs from that of the Wierthe serotype of BSBV. A purified preparation of this virus yielded only two RNA bands of approximately 3.0 and 2.8 kb.

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Heterogeneity of cytological effects produced by three viruses assigned to the furovirus group.

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Summary

The cytological effects induced by beet necrotic yellow vein (BNYVV), beet soil-borne (BSBV), peanut clump African source (PCV-A) and peanut clump Indian source (PCV-I) viruses were compared. The three viruses differed in the structure of their particle aggregates, the occurrence and structure of membrane accumulations and/or the vesiculation of peroxisomes. Individual isolates of a virus may differ in the cytological effects induced.

Introduction

The present study was undertaken to obtain comparative information on the cytological alterations induced by different viruses assigned as members or possible members to the furovirus group (Brown, 1989; Brunt and Richards, 1989; Lesemann et al.,1989) in order to define, whether there might be cytological effects in common to all members of the group and also whether effects specific for individual viruses exist. Four isolates of BNYVV (89/1, 89/2, 89/4, Wierthe), three isolates of BSBV (Ahlum, Wierthe and 86/109) , two isolates of PCV-A (Burkina Faso, Niger) and one isolate of PCV-I (Ludhiana) were studied in tissues from different host plants embedded in Epon at the time of optimal symptom expression either from local lesions or systemically infected leaves (BNYVV: *Beta macrocarpa, Chenopodium quinoa* and *Tetragonia expansa*; BSBV: *C. quinoa*; PCV-A: *Arachis hypogaea, C. amaranticolor, Nicotiana clevelandii, Triticum* sp.; PCV-I: *N. clevelandii*). The BNYVV isolates 89/1, 89/2 and 89/4 had not been passaged through *C. quinoa*, isolate Wierthe was studied in this host.

Results and Discussion

In those cases were individual isolates were studied in different host plants no qualitative differences of the cytological effects were detected although quantitative differences e.g. in amounts of virion aggregates or of membrane accumulations were observed. Since the embedded material was not selected for studying the development of cytological alterations, only qualitative aspects of the alterations are decribed here (Table 1).

1. Cells infected with BNYVV isolates contained virion aggregates of two different arrangements. The particles could either occur parallely aggregated in more or less dense masses like with many rod-shaped viruses (Francki et al.,1985) (Fig. 1) or they could form angle layer aggregates (Fig. 2) as has already been described from BNYVV-infected cells (Tamada, 1975; Putz and Vuittenez, 1980) and also from strains of tobacco mosaic virus (Francki et al.,1985). The amount of the different aggregation types differed with the isolates: with 89/1 only masses were found, with 89/2 both types, with 89/4 only angle layer structures and with Wierthe there was no aggregation of particles

found, but only scattered particles. Isolate 89/1 induced formation of cytoplasmic crystals, not bounded by a membrane (Fig. 1) and isolates 89/1 and Wierthe induced accumulations of endoplasmic reticulum (ER) (Table 1), similar as described by Russo et al.(1981).

2. Virions of BSBV isolates were characteristically aggregated in small, dense bundles very much different from the aggregates of BNYVV (Fig. 3). Only with isolate Wierthe some masses of parallely arranged particles were found additionally to the small dense bundles. All BSBV isolates induced conspicuous ER-accumulations. Isolates Ahlum and 86/109 induced also crystalline cytoplasmic inclusions and additionally isolate 86/109 induced formation of electron-dense, branched, rod-like cytoplasmic inclusions enclosed between proliferated membranes(Table 1).

3. The Ludhiana isolate of PCV-I exclusively induced massive aggregates of parallel particles(Fig. 4) whereas PCV-A Burkina Faso induced mainly aggregates with angle-layer-like arrangements(Fig.5),but also some massive aggregates. With isolate Niger, which contained only low particle concentrations in crude extracts, no aggregates have been detected in ultrathin sections. All three isolates induced the formation of conspicuous accumulations of ER which, especially with isolate Burkina Faso, attained a well defined rounded shape (Fig. 6), and which showed with all three isolates a tendency of being surrounded by a peripheral ER element. The isolates of PCV additionally induced the formation of small vesicles at the periphery of peroxisomes which contained fibrous material resembling ds-RNA. Similar vesicles have not been reported to be induced by any rod-shaped virus, but are known to be induced by tombusviruses in general and some other isometric viruses (for review see Lesemann, 1988).

Table 1. Cytological alterations observed with three furoviruses.

Virus isolates	VP-aggregates			Cytoplasmic inclusions		Membrane alterations	
	large masses	dense bundles	angle layer	crystalline	rodlike	ER-accumulation	Vesicles of peroxisomes
BNYVV 89/1	+	-	-	+	-	+	-
89/2	+	-	+	-	-	-	-
89/4	-	-	+	-	-	-	-
Wierthe	-	-	-	-	-	+	-
BSBV Ahlum	-	+	-	+	-	+	-
86-109	-	+	-	+	+	+	-
Wierthe	+	+	-	-	-	+	-
PCV-A							
Burkina Faso	+	-	+	+	-	+	+
Niger	-	-	-	-	-	+	+
PCV-I Ludhiana	+	-	-	-	-	+	+

In Table 1 the observations are summarized and it is clearly seen, that individual isolates of one virus may differ in the details of the cytological alterations induced. However, the principal alterations may be constant with individual isolates. Thus all BSBV isolates had in common the rather unique small dense bundles of particles, and all PCV-isolates induced the very specific vesiculation of peroxisomes. Within the group of furoviruses there is, on the other hand, much heterogeneity of cytological effects between the viruses studied here and also among the other members of the group (broad bean necrosis, hypochoeris mosaic, potato mop top, and soil-borne wheat mosaic viruses) which have been studied in this respect (citations see Brunt and Richards, 1989). The heterogeneity is much greater in the furovirus group than in many other virus groups like tombus-, tymo-, cucumo-, and potyviruses, which all exhibit at least the principal effects in common (Francki et al.,1985).

Acknowledgements: Isolates 86/109 of BSBV, Niger of PCV-A and Ludhiana of PCV-I were kindly provided by Drs. K.Lindsten, Uppsala, P. Reckhaus, Niamey and D.V.R. Reddy, Patancheru, respectively.

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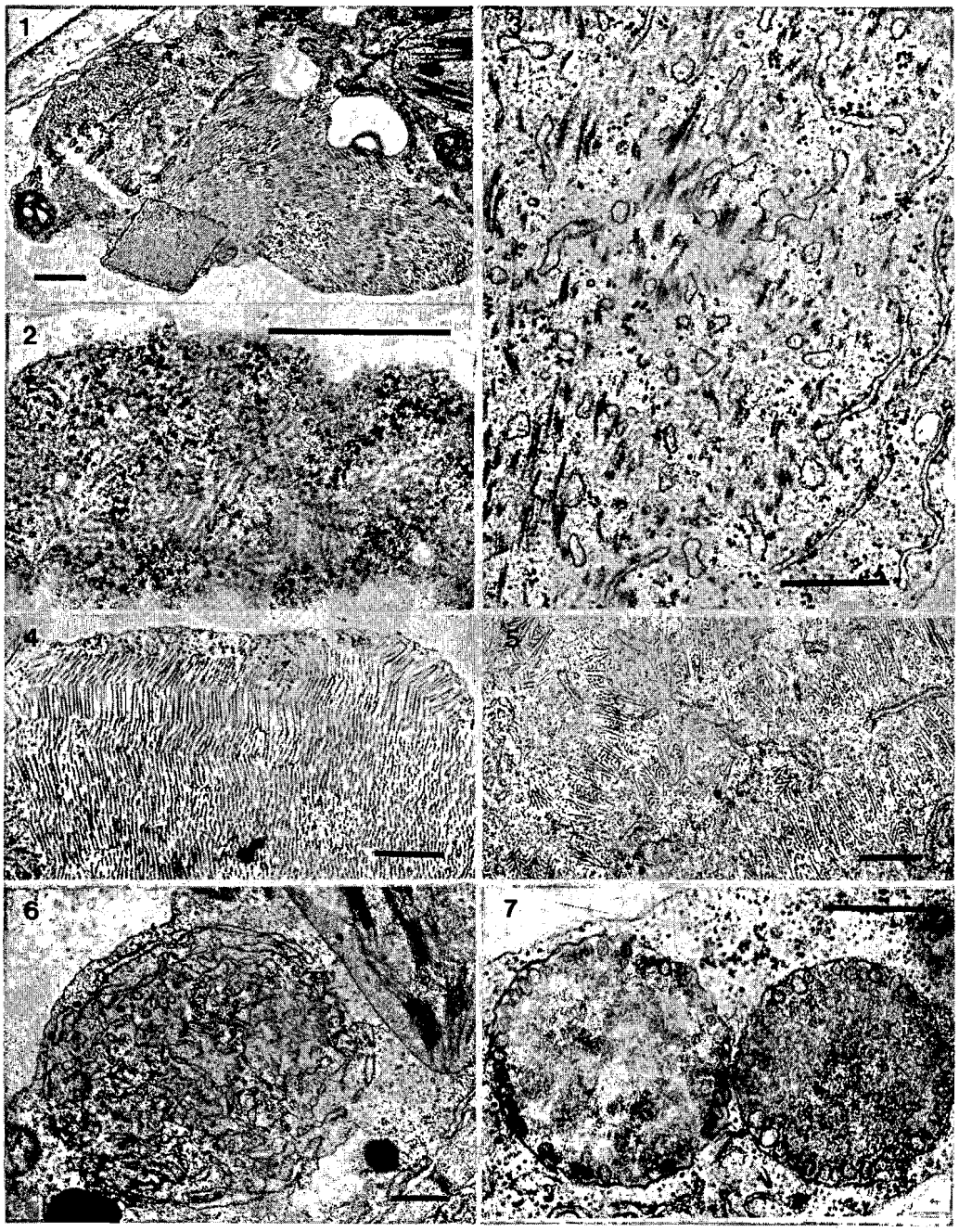

Figs. 1-7. Ultrathin sections of leaf parenchyma cells infected with furoviruses. 1. BNYVV isolate 89/1 in *Tetragonia expansa*, 2. BNYVV isolate 89/4 in *Beta macrocarpa*, 3. BSBV isolate Wierthe in *Chenopodium quinoa*, 4. PCV-I in *C. quinoa*, 5. PCV-A isolate Burkina Faso in *Triticum sp.*, 6. PCV-A isolate Burkina Faso in *Triticum* sp., 7. PCV-I in *Nicotiana clevelandii*. Bars = 500 nm.

MOLECULAR TOOLS FOR THE STUDY OF BEET NECROTIC YELLOW VEIN VIRUS: INFECTIOUS TRANSCRIPTS AND ANTIBODIES DIRECTED AGAINST VIRAL NON-STRUCTURAL PROTEINS

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Summary

Biologically active RNA molecules corresonding to each of the four beet necrotic yellow vein virus genome components have been synthesized *in vitro* from cloned full-length cDNA by run-off transcription with bacteriophage T7 RNA polymerase. The transcripts are being used to map functions on the genome by site-directed mutagenesis at the cDNA level. Sequence elements essential *in cis* for RNA 3 replication are situated within the 70 residues preceding the 3'poly(A) tail and within the 5'terminal 300 residues. The poly(A) tail is essential for productive replication. So far, deletions or frameshifts in open reading frames (ORFs) 2, 3, 5, and 6 of RNA 2 have all been found to abolish infectivity when the transcripts are inoculated to leaves along with RNA 1. ORF 4 has not been tested. Mutants carrying in-frame deletions in the coat protein cistron were viable. Using antisera raised against fusion proteins expressed in transformed bacteria or synthetic peptides, expression in leaves of all the ORFs on RNAs 2, 3 and 4 except ORF 5 of RNA 2 has been detected. Specific internal deletions can occur rapidly and spontaneously in transcript-derived RNA 3 and 4 during multiplication in leaves when coinoculated with RNA 1 and 2.

Introduction

The aim of this paper is to review recent findings concerning genome structure and function of beet necrotic yellow vein virus (BNYVV). BNYVV, agent of sugar beet rhizomania, is a plus-strand RNA virus with a quadripartite genome (Brunt and Richards, 1989). The virus is transmitted by the soil-borne fungus *Polymyxa betae* Keskin. All four viral RNAs appear to play a role in the natural infection process, i.e. fungus-mediated transmission to beet roots and proliferation of the virus in root tissue. Only RNA 1 and 2 are required for infection of mechanically inoculated *Chenopodium quinoa* or *Tetragonia expansa* leaves (Koenig *et al.*, 1986; Lemaire *et al.*, 1988; Tamada and Abe, 1989), although the presence of RNA 3 has dramatic effects on the symptoms (Kuszala *et al.* 1986; Tamada *et al.*, 1989).

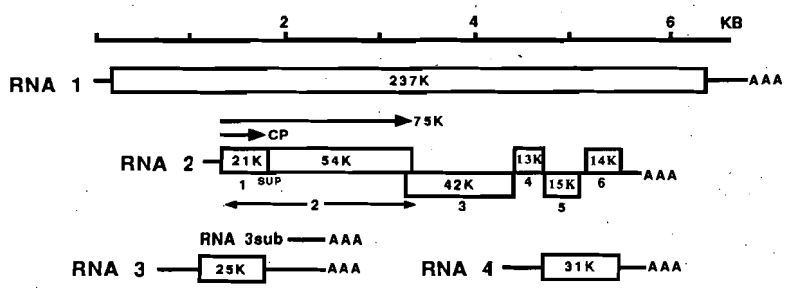

Figure 1. Genetic map of BNYVV RNA 1-4. Long ORFs are indicated by hollow rectangles. Arrows represent translation of the coat protein (CP) and the 75 kd readthrough translation product. The position of the suppressible amber termination codon is indicated by 'sup'.

The genetic map of BNYVV as determined by sequence analysis (Bouzoubaa *et al.*, 1985, 1986, 1987) is presented in Figure 1. RNA 1 has a single long ORF with consensus sequences characteristic of a viral replicase. RNA 2 has six ORFs. ORF 1 is the viral coat protein. The amber codon terminating this cistron can undergo translational readthrough both *in vitro* (Ziegler *et al.*, 1985) and *in vivo* (Niesbach-Klösgen *et al.*, 1990) to produce a 75 kilodalton (kd, apparent M_r 85 kd) species with the coat protein at its N-terminus. P42 (ORF 3 on RNA 2) carries a nucleotide triphosphate binding fold (Gorbalenya *et al.*, 1989) and P13 has two hydrophobic membrane-spanning domains (Morozov *et al.*, 1987). P42 and P13 have sequence homology with a pair of contiguous ORFs in barley stripe mosaic virus, potexviruses and carlaviruses (see Memelink *et al.*, 1990, and references therein). BNYVV RNAs 3 and 4 each contain one long ORF. A nonencapsidated subgenomic RNA, termed RNA 3sub, corresponding to the 3'terminal approx. 545 residues of RNA 3, has also been observed (Fig. 1).

Expression and subcellular localization of BNYVV proteins

Antiserum specific for BNYVV coat protein (raised against purified virions) is readily available. Immunoreagents specific for viral nonstructural proteins predicted from the sequence were raised against portions of ORFs 3, 5 and 6 of RNA 2 and of the 25 kd and 31 kd ORFs of RNA 3 and 4 fused in-frame to the N-terminal part of the bacteriophage λ CI protein in a bacterial plasmid expression vector (Niesbach-Klösgen *et al.*, 1990). For RNA 2 ORFs 2 and 4, synthetic peptides were used to raise antisera. By means of the antisera all of the above viral gene products were detected in extracts of infected *C. quinoa* leaves except for P15 (ORF 5) of RNA 2 (Niesbach-Klösgen *et al.*, 1990). P42 and P13 copurified predominantly with the 30,000g pellet (membrane fraction) while coat protein and P75 were found in all subcellular fractions (Fig. 2). The other species were predominantly in the 30,000g supernatant (cytosol). The presence of P13 in the membrane fraction is not surprising in view of its above mentioned hydrophobic character. This is not the case for P42, however, suggesting that its association with the membrane fraction may be indirect. Perhaps it is anchored to membranes by interaction with P13.

RNA	ORF	CELL COMPARTMENT			
		CELL WALL	1000g PELLET (NUC+CHLORO)	30,000g PELLET (MEMBRANES)	30,000g SUPER (CYTOSOL)
2	CP	←	—	+	→
	P75	←	—	+	→
	P42	±	−	+	−
	P13	±	−	+	−
	P15	?			
	P14	−	−	−	+
3	P25	−	−	−	+
4	P31	−	−	−	+

Figure 2. Subcellular localization of BNYVV proteins six days after infection of leaves of *C. quinoa*.

Infectious transcripts of BNYVV RNA

We have employed purified bacteriophage T7 RNA polymerase to drive *in vitro* run-off transcription of BNYVV RNAs from full-length cDNA cloned behind a synthetic T7 polymerase promoter (Ziegler-Graff *et al.*, 1988; Quillet *et al.*, 1989). Mixtures of these transcripts are infectious to leaves provided that the RNA 1 and 2 transcripts are both present (Quillet *et al.*, 1989). Using this system we have begun to map sequence signals important *in cis* for RNA 3 replication by introducing mutations at the cDNA level and monitoring the ability of transcripts carrying the mutations to be amplified when inoculated to leaves with RNA 1and 2 to provide replication functions *in trans*. Such analysis has shown that all but the 5'terminal approx. 300 nt. and the 3'terminal approx. 70 nt. can be deleted without abolishing the ability of RNA 3 to replicate (Jupin *et al.*, 1990a). The deleted region can be

replaced by a foreign sequence, the β-glucuronidase (GUS) gene, and GUS expression can be detected *in situ* in infected leaves. The 3'terminal *cis*-essential domain of RNA 3 is highly homologous with the 3' region of the other three BNYVV RNAs and the homologous domains can be folded into an identical secondary structure (Jupin *et al.*, 1990a). This region probably corresponds to the promoter for (-)-strand RNA synthesis. The 5'terminal sequences are not well conserved among the four BNYVV RNAs and all the elements essential for replication (i.e. sequences composing the (+)-strand promoter which are expected to map to this region) in the RNA 3 5'terminal *cis*-essential domain have not yet been located. Fine scale mapping of the 5'terminal region, however, indicates that essential sequence boxes are separated by nonessential spacers and there are indications that secondary structure may be important (authors' unpublished observations).

All four BNYVV RNAs carry a 3'poly(A) tail (Putz *et al.*,1983). Removal of the tail from the RNA 3 transcript greatly reduced but did not eliminate its biological activity (Jupin *et al.*, 1990b). Successful infection events were accompanied by reappearance of the poly(A) tail indicating that the tail is essential for replication or stability of RNA 3. The newly acquired tail is separated from the 3'heteropolymeric portion of RNA 3 by a novel U-rich tract (ranging from 1to 18 nt. in length in six clones examined), of unknown origin. In the case of transcripts containing U-rich tracts of 1 nt. and 6 nt. length the novel sequences were stably maintained in the descendence during two subsequent passages (Jupin *et al.*, 1990b).

Site-directed mutagenesis studies have also been carried out on RNA 2 with the aim of determining if all of the ORFs on this RNA are essential for the infection process. Deletions or frameshift mutations in ORFs 2, 3, 5, and 6 abolished infectivity on leaves when transcripts carrying the modifications were coinoculated with RNA 1 (unpublished observations). RNA 1 alone gave no detectable infection on leaves. RNA 2 molecules bearing in-frame deletions (unpublished observations) or a single amino acid substitution in the coat protein cistron (Quillet *et al.*, 1989) were viable but stable virions were not formed and long distance movement in a systemic host, spinach, was inhibited.

Internal deletions in RNA 3 and 4

When maintained by mechanical inoculation to leaves, RNA 3 and 4 of BNYVV may undergo internal deletions or disappear entirely from the inoculum (Bouzoubaa *et al.*, 1985; Kuszala *et al.* 1986; Burgermeister *et al.*, 1986). This behavior is presumably due to the absence of selective pressure for retention of the small RNAs when virus is propagated in this way. The deletion process is remarkably specific, generally giving rise to only one or two abundant species for a given isolate rather than a 'smear' of deleted forms. Thus there appear to be 'hot spots' in the sequence, distinct sites between which deletions take place, presumably by a copy-choice mechanism during replication.

Using full-length transcripts of BNYVV isolate F3 as the original source of inoculum, we have observed that deletions in RNA 3 and 4 arise rapidly, appearing after only one or two passages on leaves (unpublished observations). In RNA 4 the major deletion event takes place between 15 nt. direct repeats in the full-length prototype sequence in such a way that the left-hand repeat is eliminated in the deleted form. No such repeats were present at the boundaries of the two abundant deleted forms of RNA 3 but, instead, the deletions occurred in a region predicted to have extensive secondary structure. It is possible that this secondary structure slows down the replicase and favors its dissociation from the template. Furthermore, the 5' and 3' boundaries of each deletion are juxtaposed in the predicted secondary structure, suggesting that proximity in space may be important in determining the loci between which deletions take place. It will now be possible to test such ideas by designing various altered forms of full-length RNA 3 and 4 and studying the consequences of the alterations on the deletion process.

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EFFECT OF ISOLATES OF BEET NECROTIC YELLOW VEIN VIRUS WITH DIFFERENT RNA COMPONENTS ON THE DEVELOPMENT OF RHIZOMANIA SYMPTOMS

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SUMMARY

Japanese field isolates of beet necrotic yellow vein virus (BNYVV) contained RNA-1, RNA-2, RNA-3 and RNA-4, and some isolates, in addition, RNA-5. Field and laboratory isolates of BNYVV with different RNA components were tested by inoculation using virus-carrying Polymyxa betae. The efficiencies of transmission by P. betae were: isolate S (RNA-1+2+3+4+5) = S-34 (RNA-1+2+3+4) > S-45 (RNA-1+2+4+5) > S-4 (RNA-1+2+4) > S-35 (RNA-1+2+3+5) = S-3 (RNA-1+2+3) > S-5 (RNA-1+2+5) > S-0 (RNA-1+2). This indicates that presence of RNA-4 increases the frequency of fungus transmission. All the isolates with RNA-3 caused a massive proliferation of fine rootlets and typical foliage symptoms. In contrast, the other isolates without RNA-3 caused no apparent symptoms in shoots and roots of sugar beet. In glasshouse tests, a severe reduction in sugar content and root weights was caused by isolates with RNA-3, but not by those without RNA-3, although root weights decreased slightly. The virus content in taproots infected with isolates with RNA-3 was about 10 times greater than that with isolates without RNA-3. These results show that BNYVV RNA-3 not only increases the spread of virus in root tissue, but also is necessary for rhizomania symptoms to develop in roots and shoots of sugar beet, and that it affects greatly a reduction in sugar content.

INTRODUCTION

Rhizomania "root madness" 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 (Tamada & Baba, 1973; Tamada, 1975). The disease has now been distributed in many sugar beet growing countries and is economically very important, because it causes a severe loss in root yield and sugar content. In naturally-infected sugar-beet plants, BNYVV is usually confined to roots of the plants, and the symptoms of BNYVV infection are characterized by a massive proliferation of the lateral rootlets of taproots and the other abnormalities (Tamada, 1975).

BNYVV virions are rod-shaped and contain four or five RNA components, RNA-1 (7.1 kb), RNA-2 (4.8 kb), RNA-3 (1.85 kb) and RNA-4 (1.5 kb) (Richards et al., 1985) and, in some isolates, RNA-5 (1.4 kb) (Tamada et al., 1989). The nucleotide sequence of this new RNA-5 had different homology to that of RNA-3 or RNA-4, except for 3' terminal (Kiguchi et al., 1989). The two larger RNA species are needed for virus infection, whereas the smaller ones behave like satellites in mechanically inoculated leaves of host plants (Bouzoubaa et al., 1985; Koenig et al., 1986; Tamada et al., 1989). As to function of these smaller RNA species, it has been reported that RNA-3 confers an ability to intensify the symptoms (Kuszala et al., 1986; Tamada et al., 1989), whereas RNA-4 is essential for efficient transmission by P. betae (Tamada & Abe, 1989). However, it is unclear how these RNA species affect the symptom expression in roots of sugar beet. In this paper, we describe the effect of virus isolates containing distinct smaller RNA species on symptom development, virus content and sugar yield in roots of sugar beet, and present evidence that BNYVV RNA-3 determines the development of rhizomania symptoms in roots of sugar beet.

MATERIALS AND METHODS

Virus isolates: The following isolates of BNYVV containing different combinations of RNA species were used as laboratory isolates: isolate S (=field isolate, RNA-1+2+3+4+5), S-34 (RNA-1+2+3+4), S-35 (RNA-1+2+3+5), S-45 (RNA-1+2+4+5), S-3 (RNA-1+2+3), S-4 (RNA-1+2+4), S-5 (RNA-1+2+5) and S-0 (RNA-1+2). These isolates were originally obtained by single-lesion transfers in Tetragonia expansa leaves inoculated mechanically (Tamada et al., 1989). Eighteen samples, derived from rootlets of sugar beet or soils collected from various areas throughout Hokkaido, were also used as field isolates.

Fungus inoculation: Acquisition and inoculation of BNYVV isolates by P. betae were done as described by Tamada & Abe (1989). Dried rootlets containing resting spore clusters of P. betae were used as inocula. Inoculation was done by sowing sugar-beet seeds (cv. Monohill) on quartz sand in special test tubes, to which a dilution series of inoculum was added. The seedlings in the test tubes were supplied with nutrient solution every day and grown in a growth cabinet at 25 C in 16 hr light a day (Tamada et al., 1989).

Detection of BNYVV: BNYVV was detected by ELISA, when necessary, by inoculation to T. expansa leaves. The ELISA procedure was conducted as described by Tamada & Abe (1989). The virus content in root tissue was also estimated by the ELISA.

Detection of BNYVV RNA: BNYVV RNA were analysed by agarose gel electrophoresis as described by Tamada et al. (1989). RNA was extracted from the virus particles purified from inoculated leaves of T. expansa or rootlets of sugar-beet seedlings. Northern blot hybridization tests were made by using digoxigenin-labbeled DNA probes specific to each of five RNA species (Saito et al., 1990).

Glasshouse tests: After sugar-beet seedlings inoculated by P. betae were grown in the growth cabinet for one to two months, they were transplanted into clay pots containing sterilized soils and grown in a glasshouse, which kept at at a mean temperature of about 23 C°. T. expansa and Beta macrocarpa used as test plants were grown in the glasshouse.

RESULTS

RNA components and pathogenicity of field isolates of BNYVV: We tested the diversity of RNA components of 18 field isolates, which were collected from single fields in different areas in Hokkaido. The virus from each sample was multiplied in T. expansa plants by inoculation with sap from rootlets of sugar-beet seedlings, which had been previously inoculated with P. betae. RNA components contained in these field isolates were analysed by agarose gel electrophoresis and Northern blot hybridization using cDNA probes specific to each of RNA-3, RNA-4 and RNA-5. The results showed that, out of 18 field isolates tested, eight isolates contained four RNA species (RNA-1+2+3+4), and the other ten isolates contained five RNA species (RNA-1+2+3+4+5). There was no apparent difference between these field isolates in the size of RNA-1, RNA-2, RNA-3, RNA-4 and RNA-5. Inoculation tests with P. betae showed that all the isolates caused rhizomania symptoms in roots and shoots, although there was slight difference between these isolates in the severity of the symptom development.

Symptoms of BNYVV isolates in test plants: Table 1 shows symptom expression of virus isolates in the two local and systemic host plants, which were inoculated mechanically. Isolates with RNA-3 caused bright yellow lesions in inoculated leaves of T. expansa, whereas isolates without RNA-3 caused chlorotic lesions. However, isolate S-45 with both of RNA-4 and RNA-5 induced severe chlorotic (yellow) lesions, although S-4 (RNA-4) or S-5 (RNA-5) caused only chlorotic lesions, indicating that synergistic effect occurs between smaller RNA species (Tamada et al., 1989). In Beta macrocarpa, Isolates with RNA-3 caused systemic severe stunting and mosaic. Isolates with RNA-5 later produced systemic mild mottle in some plants, but S-4 or S-0 did not become systemic. This suggests that isolates with RNA-5 may be more virulent than those with RNA-4.

Table 1. Effect of BNYVV isolates on symptom expression and transmission efficiency of P. betae

Isolate	RNA component	Symptoms in inoculated leaves of T. expansa	Symptoms in systemically infected leaves of B. macrocarpa *	Transmission efficiency of P. betae	Proliferation in rootlets of sugar beet seedlings inoculated by P. betae
S	1+2+3+4+5	Yellow spots	Stunt, mosaic	+++++ **	+
S-34	1+2+3+4	Yellow spots	Stunt, mosaic	+++++	+
S-35	1+2+3+5	Yellow spots	Stunt, mosaic	++	+
S-3	1+2+3	Yellow spots	Stunt, mosaic	++	+
S-45	1+2+4+5	Yellow spots	(Mild mottle)	++++	-
S-4	1+2+4	Chlorotic spots	0	+++	-
S-5	1+2+5	Chlorotic spots	(Mild mottle)	+	-
S-0	1+2	Faint chlo. spots	0	+	-

* 0, not infected; parentheses indicate symptoms produced occasionally.
** +, less; +++++, much

Transmission efficiency of BNYVV isolates by P. betae: Tamada & Abe (1989) reported that S-4 was transmitted by P. betae about 100 times more efficiently than S-3 and about 1000 times more efficiently than S-0. Results of further transmission tests are shown in Table 1. These results indicate that presence of RNA-4 increases the frequency of the fungus transmission, and if RNA-3 or RNA-5 is present, virus transmission by the fungus is much more frequent.

Rootlet proliferation induced by BNYVV isolates: Forty days after inoculation, a massive proliferation of rootlets of sugar-beet seedlings was caused by S-34 and S-3, but not by S-4 and S-0 (Table 2). The virus concentration in rootlets to which S-3 was transmitted was almost similar to that in rootlets to which S-4 was transmitted (Table 2). Two months later, the amount of rootlets infected with S-3 was about 1.5 to 2.0 fold greater than that of rootlets with S-4 or with virus-free P. betae, which were similar. Virus-free P. betae caused a brown colour (slight necrosis) in rootlets of sugar-beet seedlings, as well as inoculated with S-4. In such a case, numerous resting spore clusters were found in most of epidermal cells of rootlets. Further tests using other isolates showed that RNA-5 is not associated with rootlet proliferation (Table 1). These results indicate that proliferation of rootlets, a typical symptom of rhizomania, is caused by isolates with RNA-3, but not by any isolates without RNA-3. Such an increase of fine rootlets is not always correlated with the virus concentration in root tissue.

Table 2. Effect of BNYVV isolates on the virus content and proliferation of rootlets of sugar-beet seedlings

Virus isolate (RNA component)	Transmission	Virus content in rootlets	Proliferation of rootlets
S-34 (1+2+3+4)	15/15 *	280 **	+
S-3 (1+2+3)	15/15	98	+
S-4 (1+2+4)	30/30	109	-
S-0 (1+2)	12/30	6	-
Virus-free P. betae	0/15	0	-

* Virus infection was checked by ELISA at 40 days after inoculation. Numerator is number of seedlings in which the virus was detected, denominator is number inoculated.

** Figure is expressed as μg virus/one g fresh root tissue.

Effect of BNYVV isolates on sugar yield: Table 3 shows the results of the glasshouse tests. Isolate S-3 caused typical foliage symptoms of rhizomania, such as yellowing, elongation of petioles, wilting and stunting. Isolate S-34 showed much more severe symptoms, followed in many plants by systemic yellow vein. In contrast, Isolate S-4 or S-0 did not cause any symptoms in shoots of sugar beet. Root weights were reduced greatly by infection of S-34 or S-3, and slightly by S-4 or S-0. However, it is interesting that infection of S-4 or S-0 had no effect on sugar content, whereas S-3 or S-34 reduced greatly the sugar content. The virus content in taproots infected with S-3 or S-34 was about 10 times greater than that infected with S-4 or S-0. Thus, BNYVV RNA-3 greatly affects sugar yield of sugar beet, and also the effect of isolate with both of RNA-3 and RNA-4, a kind of field isolates, is much greater.

Table 3. Effect of BNYVV isolates on symptoms, sugar yield and virus content in roots of sugar-beet plants grown in the glasshouse *

Virus isolate (RNA component)	Symptoms	Root weight	Sugar content	Virus content in parts of		
				rootlets	tip of taproots	centre of taproots
S-34 (1+2+3+4)	+	6.6 g	2.3 %	29.8 **	5.7	3.0
S-3 (1+2+3)	+	16.1	8.2	13.0	4.4	1.5
S-4 (1+2+4)	-	34.0	14.8	3.6	0.3	0.3
S-0 (1+2)	-	35.2	14.6	1.4	0.3	0.1
Virus-free P. betae	-	38.9	15.0	0	0	-
Not inoculated	-	42.1	14.9	0	0	-

* Forty days after grown in the growth cabinet, the sugar-beet seedlings were grown in the glasshouse for 3 months.

** Figure is expressed as μg virus/one g fresh root tissue.

CONCLUSIONS

In this paper, we have provided evidence that the presence of BNYVV RNA-3 is necessary for rhizomania symptoms to develop in roots of sugar beet, indicating that RNA-3 is strongly associated with abnormal proliferation of fine rootlets. Our results also showed that RNA-3 increases the spread of virus in root systems. However, the higher virus content in rootlets is not always correlated with such a proliferation, because although isolates with RNA-4 showed the high virus content in rootlets at the young stage of sugar-beet seedlings, they did not cause any proliferation of rootlets. It is of particular interest that isolates without RNA-3 had no effect on sugar content of sugar-beet roots. Similar results were also obtained from field experiments (T. Tamada and others, unpublished data), indicating that BNYVV RNA-3 affects greatly a reduction of sugar yield.

In a previous paper (Tamada & Abe, 1989), we have shown that RNA-4 is essential for efficient transmission by P. betae. This indicates that RNA-4, unlike RNA-3, plays other important roles in virus spread. In this paper, moreover, we suggest that RNA-5, which is commonly present in fields in Hokkaido, may assist the transmission efficiency together with RNA-4, or may assist the virus spread in root tissue, but this RNA-5 is not associated with rootlet proliferation.

Thus, we conclude that BNYVV RNA-3 contains the genetic determinant for development of rhizomania symptoms, whereas RNA-4 contains the main genetic determinant for efficiency of fungus transmission. Therefore, isolates containing a combination of RNA-3 and RNA-4, and probably RNA-5, are very stable to survive in nature (Koenig et al., 1986; Tamada et al., 1989).

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INFECTION OF PROTOPLASTS WITH BEET NECROTIC YELLOW VEIN VIRUS RNA

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Summary

Conditions for infection of *Chenopodium quinoa* mesophyll protoplasts with beet necrotic yellow vein virus RNA or synthetic transcripts have been established. Polyethylene glycol treatment was used to render the protoplasts competent for uptake of the inoculum. After a latent period during which excess input RNA was degraded, net accumulation of viral RNA could be detected by Northern hybridization 24 hr post-inoculation and continued until at least 48 hr. Newly synthesized viral coat protein could also be detected by Western blot analysis of extracted proteins. Beet necrotic yellow vein virus RNA 3, provided as a transcript, was also replicated if RNA 1 and 2 were included in the inoculum.

Introduction

Use of leaf mesophyll protoplasts as host in infection experiments with a virus such as beet necrotic yellow vein virus (BNYVV) provides a convenient means of obtaining synchrony of infection and of investigating the replication cycle at the single cell level without complicating secondary factors such as cell-to-cell movement of the virus, host defense reactions etc. With this in mind we have established conditions for reproducible infection of *Chenopodium quinoa* protoplasts with BNYVV. Since our ultimate intention is to infect such protoplasts with synthetic transcripts containing various engineered modifications of the genome and to study the consequences on the viral replication cycle we have concentrated on procedures employing RNA rather than virions as inoculum.

Materials and Methods

Plant material, protoplast preparation and inoculation

The sugarbeet cell suspension culture, a gift from J. Kallerhoff, Biosem, and the procedure for protoplast isolation from cultured cells was essentially as described elsewhere (Kallerhoff *et al.*, 1990) with minor modifications. Mesophyll protoplasts were isolated from lacerated leaves of 4 week old sugarbeets (*Beta vulgaris* var. Monosvalof) or *C. quinoa* by treatment with cellulase and macerozyme as will be described elsewhere (Bouzoubaa *et al.*, to be published).

BNYVV isolate F13 and the transcript-derived series of Stras BNYVV isolates have already been described (Ziegler *et al.*, 1985, Quillet *et al.*, 1989). Procedures for synthesis of bacteriophage T7 RNA polymerase run-off transcripts are given by Ziegler-Graff *et al.*, 1988, and Quillet *et al.*, 1989. Protoplasts were inoculated with BNYVV RNA or transcripts by the polyethylene glycol (PEG) method as described by Maule *et al.*, 1980, with minor modifications. Culture medium for infected protoplasts from the beet cell suspension culture were as in Kallerhoff *et al.*, 1990. The infected beet and *C. quinoa* protoplasts were maintained in 0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 5 mM $CaCl_2$, 1 µM KI, 10 nM $CuSO_4$, 0.6 M D-mannitol and 1% sucrose, pH 5.6, under continuous diffuse lighting at a temperature of 25°.

Detection of viral RNA and protein

Coat protein and P85 readthrough protein were detected on Western blots of total protoplast protein as described by Quillet *et al.*, 1989, using antisera (a gift of O. Lemaire, INRA, Colmar, France) raised against purified virions. RNA was extracted from protoplasts as described by Weiland and Dreher, 1989. Detection of viral RNA was by Northern hybridization using ^{32}P-labelled antisense viral RNA transcripts as probes (Lemaire *et al.*, 1988).

Results and Discussion

Choice of plant material

In preliminary experiments protoplasts from three sources were evaluated as to their suitability in infectivity studies: a beet cell suspension culture, beet leaves and *C. quinoa* leaves. The yield of protoplasts per gram starting material with the suspension culture was superior to that obtained from leaves but, with the protoplasts from the cultured cells, the percentage of infected protoplasts (using the PEG infection procedure described in Methods to inoculate the protoplasts and immunofluorescence (Otsuki and Takebe, 1969) to monitor infection) was only 30-40% while infections ranging from 60% to >80% were routinely achieved with protoplasts from leaves (data not shown). Furthermore, production of viral coat protein was readily detected in protein extracts of leaf protoplasts on Western blots but was much less abundant in extracts of the protoplasts from suspension cultures (data not shown). Consequently, experiments with the beet cell suspension culture were discontinued.

Newly synthesized viral RNA was detectable in infected protoplasts by Northern hybridization (see below) 24 hr. post-inoculation (pi) and accumulated in comparable amounts in the protoplasts from beet and *C. quinoa* leaves. The viral RNA from the beet protoplasts, however, was reproducibly more extensively degraded than that of the *C. quinoa* protoplasts (data not shown). It remains to be seen if this degradation takes place *in vivo* or during RNA extraction but its preferential occurrence with the beet protoplasts led us to concentrate our further efforts on the *C. quinoa* system.

Time course of infection

With *C. quinoa* protoplasts and the PEG infection protocol we have investigated the time course of appearance of viral RNAs during infection. In these experiments, the excess input RNA was generally degraded during the first approx. 5 hr pi; newly synthesized viral RNA was visible by 20 hr pi and was abundant by 40 hr (Fig. 1A, lanes 5 and 6). RNA synthesis undoubtedly continues beyond this point but protoplast death can become a significant problem. About the same kinetics were observed whether or not RNA 3 and 4 were present in the inoculum (data not shown).

Infection with synthetic transcripts

In the first experiments, protoplasts were coinoculated with natural RNA 1 and 2 and a 5'-capped run-off transcript corresponding to full-length RNA 3 (Ziegler-Graff *et al.*, 1988). The transcript, t35, contains a single supplementary G residue at its 5'terminus and 12 nonviral residues following the 3'poly(A) tail. When inoculated alone to protoplasts t35 was not detectably replicated and the input RNA was totally degraded 48 hr pi (Fig. 1B, lane 5). When RNAs 1 and 2 were included in the inoculum to provide *trans*-acting replication functions, however, multiplication of RNA 3 occurred readily (Fig. 1B, lane 6) indicating that, as in transcript-infected leaves (Ziegler-Graff *et al.*, 1988), the extra residues at the transcript extremities do not greatly interfere with its replication. We assume that the nonviral sequences are eliminated in the course of replication as has been shown to be the case when BNYVV transcripts are inoculated to leaves (Ziegler-Graff *et al.*, 1988; Jupin *et al.*, 1990).

In contrast to the situation with the RNA 3 transcript, infection of protoplasts with transcripts of RNA 1 and 2 has proven to be unexpectedly difficult. This appears to be due to the fact that the specific infectivity of the RNA 1/2 transcript mix is about tenfold lower than that of the corresponding natural

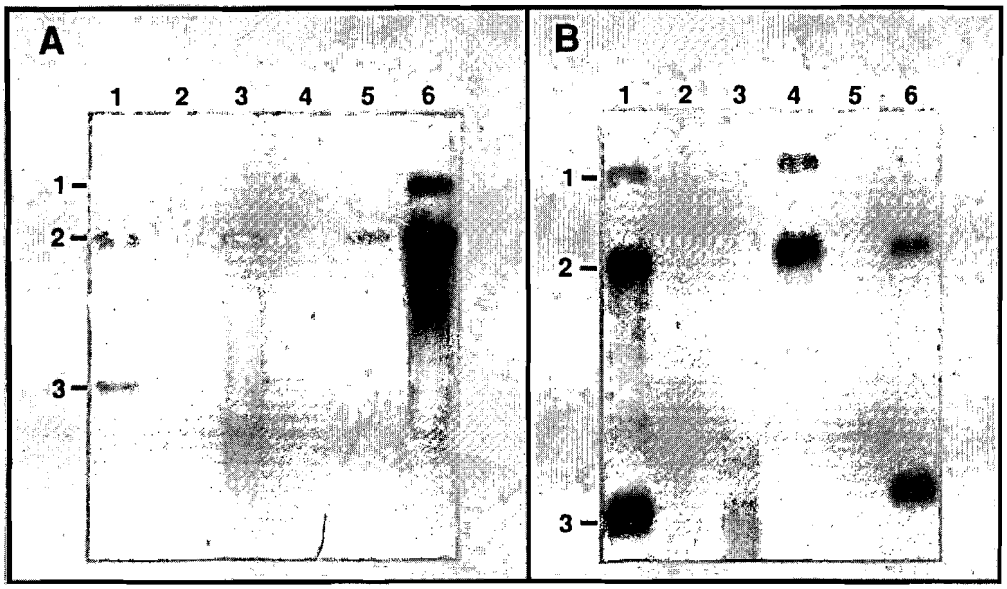

Figure 1. Detection by Northern hybridization of BNYVV RNAs in *C. quinoa* mesophyll protoplasts inoculated with viral RNA or viral RNA plus synthetic transcript. (A) Time course of infection with RNA of BNYVV isolate Stras 12 (contains only RNA 1 and 2; Quillet *et al.*, 1989). The samples consisted of uninfected protoplasts (lane 2) or infected protoplasts collected 1 hr (lane 3), 5hr (lane 4), 20 hr (lane 5) or 40 hr (lane 6) pi. RNA extracted from 20,000 protoplasts was loaded in each lane. Lane 1 contains viral RNA (BNYVV isolate F13) to provide length standards. (B) Inoculation with the RNA 3 transcript t35. Samples were uninfected protoplasts (lane 2), protoplasts infected with RNA of BNYVV isolate Stras 12 collected 1 hr (lane 3) and 48 hr (lane 4) pi, protoplasts inoculated with t35 alone (lane 5) or with Stras 12 RNA plus t35 (lane 6) collected 48 hr pi. Lane 1 as in lane 1 of Fig. 1A.

RNAs (authors' unpublished observations), presumably because of incomplete capping and/or the aforesaid nonviral sequences at the transcript extremities (Quillet *et al.*, 1989). Apparently, the biological activity of the RNA 1 and 2 transcripts is more sensitive to such factors than is RNA 3. A similar situation holds for brome mosaic virus where the biological activities of the RNA 1 and 2 transcripts, both of which encode proteins involved in replication, are particularly sensitive to terminal modifications (Ahlquist *et al.*, 1987). By increasing the amount of BNYVV RNA 1 and 2 transcripts in the inoculum we hope to be able to overcome this concentration effect and obtain infection.

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INFECTIOUS IN VIVO TRANSCRIPTS OF BEET NECROTIC YELLOW VEIN VIRUS cDNA CLONES CONTAINING THE 35S PROMOTOR

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Summary

Plasmids containing the full length cDNA sequences of beet necrotic yellow vein virus (BNYVV) RNA 3 and 4 under the control of the cauliflower mosaic virus 35S promotor and polyadenylation signal were rubbed on the leaves of Chenopodium quinoa and Tetragonia expansa together with a BNYVV isolate (Rg 1) which contained only RNA 1 and 2. The appearance of RNA 3 and 4 in the progeny virus suggests that the cDNA sequences were transcribed in the plants and that the viral replicase which is provided by the Rg 1 isolate of BNYVV led to the production of large amounts of RNA 3 or 4, respectively. RNA 4 was replicated from transcripts which contained 40 additional nonviral nucleotides on the 5′ end. With RNA 3, however, replication was observed only when the number of nonviral 5′ nucleotides was reduced from 40 to 25. The original transcripts must have contained several hundred 3′ nonviral nucleotides plus an extra poly(A) tail which were apparently lost during replication, because the sizes of the progeny RNA 3 or 4 resembled those of natural RNA 3 or 4.

Introduction

Infectious in vitro transcripts of viral cDNA clones have proved to be indispensible tools for the functional analysis of viral genomes and for the generation of gene expression vectors from viruses (e.g. Ahlquist et al., 1987). For the four RNAs of beet necrotic yellow vein virus (BNYVV), the causal agent of sugarbeet rizomania, infectious in vitro transcripts have been obtained by Ziegler-Graff et al. (1988), Quillet et al. (1989) and Jupin et al. (1990). We have investigated in this study whether viral cDNA sequences can be transcribed directly in plants when they are inserted in plasmids which contain the cauliflower mosaic virus 35S promotor and polyadenylation signal and whether the transcripts can serve as templates for further replication. The cDNA sequences used were full length copies of BNYVV RNA 3 or 4. Because RNA 3 and 4 alone are not infectious in plants, the plasmids were applied to the plants together with a BNYVV isolate (Rg 1) containing only RNA 1 and 2 which are essential for viral replication. For comparison we have also prepared in vitro transcripts of BNYVV RNA 3 or 4. They were readily infectious although the transcript for RNA 4 contained more nonviral nucleotide sequences behind the poly(A) tail than those described by Ziegler-Graff et al.(1988).

Materials and Methods

For in vitro transcription the full length cDNA sequences of BNYVV RNA 3 and 4 were subcloned into plasmid pGEM3ZF(+) (Promega) where they are under the control of the bacteriophage SP6 promotor. The resulting plasmids were named pGB533 and pGB543 for BNYVV RNA 3 and 4, respectively. In vitro transcripts were capped as described by Ziegler-Graff et al. (1988).

For in vivo transcription the full length cDNA sequences were subcloned into plasmid pRT103 which contains the cauliflower mosaic virus 35S promotor and polyadenylation signal (Toepfer et al., 1987). The resulting plasmids were named pTB533 and pTBX533 for RNA 3 and pTB543 for RNA 4.

The in vitro and in vivo transcripts contain various numbers of additional nucleotides on the 5′ and 3′ ends of the virus RNA sequences which are outlined in Fig. 1.

In vitro transcripts (1-2 µg/leaf) were rubbed on leaves of Chenopodium quinoa or Tetragonia expansa together with total RNA extracts from leaves infected with the Regensburg 1 (Rg 1) isolate of BNYVV which was first

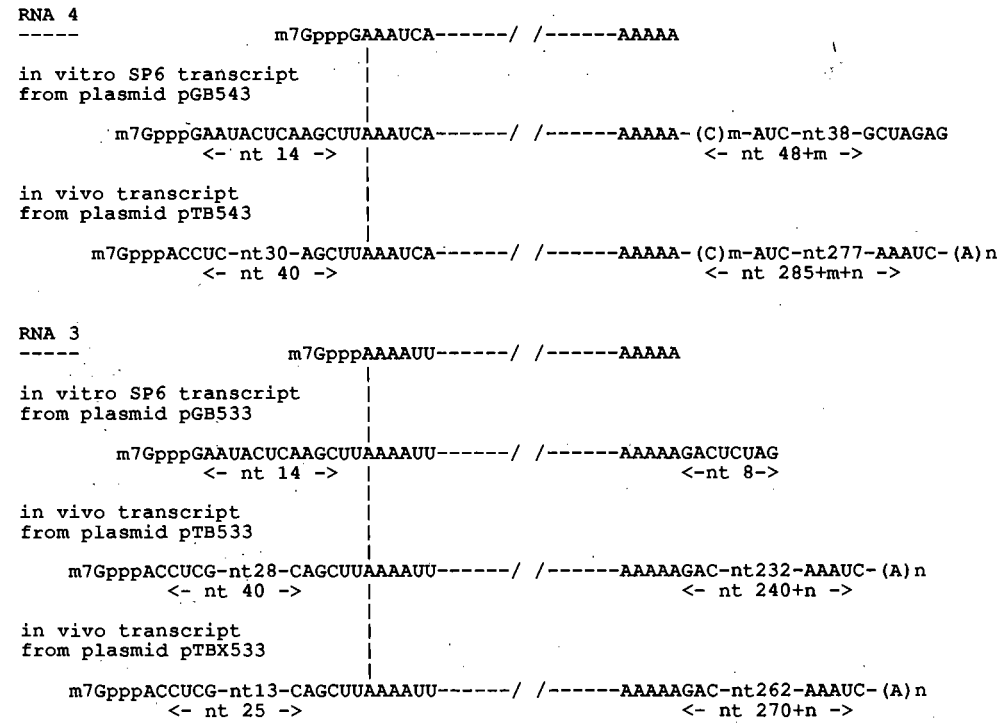

Fig. 1. Structure at the 5′ and 3′ termini of natural BNYVV RNAs 3 and 4 and of the in vitro and in vivo transcripts.

described by Burgermeister et al. (1986). This isolate lacked RNA 3 and 4 which have not reappeared during 5 years of continuous passages on C. quinoa or T. expansa. Plasmids (2.5 µg/leaf) were rubbed on the leaves of test plants together with intact virions of the Rg 1 isolate. The inoculation medium consisted of 0.05 M phosphate buffer pH 7.2 containing 1 to 5% bentonite. One to two weeks after inoculation the leaves were harvested and the total RNA was extracted either directly or the sap of the leaves was rubbed on fresh leaves of either C. quinoa or T. expansa and RNA extractions were made after the second passage. The last procedure was usually preferred because we anticipated that in the first passage transcripts may have been available to the virus only in a few lesions. However, in the second passage progeny RNA 3 and 4 should be more generally available and propagated to high concentrations in all lesions.

RNA extractions and Northern blot hybridizations with cDNA probes specific for RNA 3 and 4 were done as described by Burgermeister et al. (1986).

Results

The results of our experiments are summarized in Table 1. No infectivity was observed with the plasmids of the pGB series which do not contain a plant specific promotor and polyadenylation signal. The RNA transcripts obtained from these plasmids, however, were infectious. This was already suggested by the different color of the local lesions which was especially spectacular with RNA 3 transcripts and by the appearance of a high proportion of smaller virus particles in electron micrographs (results

Table 1

Infectivity tests with the transcripts and plasmids described in Fig. 1 and BNYVV isolate Rg 1* which contains only RNA 1 and 2.

RNA 4 Composition of inoculum		Color of local lesions	Detection of RNA 4 in Northern blot hybridizations
Rg 1	transcript or plasmid		
+	-	pale green	no
+	pGB543	pale green	no
+	transcript of pGB543	greenish/yellowish	yes
+	pTB543	greenish/yellowish	yes
-	pTB543	-	no#
+	pRT103	pale green	no#

RNA 3 Composition of inoculum		Color of local lesions	Detection of RNA 3 in Northern blot hybridizations
Rg 1	transcript or plasmid		
+	-	pale green	no
+	pGB533	pale green	no
+	transcript of pGB533	bright yellow	yes
+	pTB533	pale green	no
+	pTBX533	bright yellow	yes
+	pRT103	pale green	no#

* whole virus in experiments with plasmids, RNA extracts in experiments with transcripts
\# tested after first passage, all others were tested after second passage
Experiments with individual plasmids or transcripts were repeated up to four times on either Chenopodium quinoa or Tetragonia expansa with identical results.

not shown). The final proof that RNA 3 or 4 were present in the progeny virus was obtained in Northern blot hybridizations with cDNA probes specific for RNA 3 or 4.

Plasmid pTB543 which in addition to the RNA 4 cDNA sequence contains the cauliflower mosaic virus 35S promotor and polyadenylation signal was obviously transcribed in vivo and the transcript had served as a template for the production of large amounts of RNA 4 in the progeny virus (Table 1). This was observed in all four experiments with C. quinoa or T. expansa. The progeny RNA 4 had a similar size as natural RNA 4 (Fig. 2) although the original transcript must have had a large number of additional nonviral nucleotides especially at the 3′ end (Fig. 1).

Plasmid pTB533 which is basicly similar to pTB543 except that it contains the cDNA sequence of RNA 3 rather than RNA 4 was not infectious in our experiments. Since it is known that the infectivity of in vitro transcripts may decrease with increasing numbers of nonviral 5′ nucleotides (Ahlquist et al., 1987; Ziegler-Graff et al., 1988), we have prepared another vector construct pTBX533 which would yield a transcript with only 25 rather than 40 nonviral 5′ nucleotides (Fig. 1). This plasmid, indeed, readily gave rise to RNA 3 formation. As in the case of RNA 4 the progeny RNA 3 had about the same size as natural RNA 3 and apparently lacked the large number of additional nonviral nucleotides on the 3′ end.

Discussion

Our results confirm earlier observations by Ahlquist et al. (1987) and Ziegler-Graff et al. (1988) that virus cDNA-containing plasmids which do not possess a plant specific promotor do not give rise to infections in plants. An exceptional case has been reported by Dore and Pinck (1988).

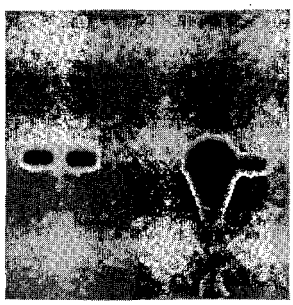

Fig. 2. Northern blot analysis of RNA extracts from leaves which had been infected with the progeny virus obtained from plants to which various combinations of virus, transcripts or plasmids had been applied. A cDNA clone specific for RNA 4 was used as a probe.

1 and 2 - BNYVV isolate Rg 1 + pTB543, results of two different experiments
3 - BNYVV isolate Rg 1 + pGB543
4 - BNYVV isolate Rg 1
5 - BNYVV isolate with full length RNA 3 and 4
6 - BNYVV isolate Rg 1 + transcripts of pGB543

However, when we placed the virus cDNA sequences under the control of the cauliflower mosaic virus 35S promotor and polyadenylation signal which are present in pRT103 (Toepfer et al., 1987) infections were readily obtained provided that the 5′ nonviral nucleotide sequences in the transcripts which are produced in the plants are not too long. The number of 5′ nonviral nucleotides which can be tolerated apparently differs with different RNAs. With RNA 4 40 nonviral 5′ nucleotides were tolerated, but with RNA 3 their number had to be reduced to 25. The number of nonviral nucleotides which can be tolerated on the 3′ end is much larger and can amount to several hundred. These nonviral 3′ nucleotides, however, are apparently at least in part lost during replication, because the RNAs 3 and 4 produced in the progeny virus had the sizes of natural RNAs 3 and 4 and not those which would be expected when the whole transcripts were replicated. Similar observations were reported by Dore and Pinck (1988).

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IMPROVED TECHNIQUE FOR PRODUCING MONOCLONAL ANTIBODIES AGAINST BEET NECROTIC YELLOW VEIN VIRUS (BNYVV) AND MABS APPLICATIONS

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Summary

The somatic cell hybridization technique is discussed and some modifications are suggested to improve the efficiency of monoclonal antibody production. MAbs against Beet necrotic yellow vein virus were obtained and evaluated in ELISA as second antibodies or conjugates. The situation of rizomania in Italy was investigated using polyclonal and monoclonal antibodies.

Introduction

Since 1975, when Kohler and Milstein first developed it, the monoclonal antibody (MAb) production technique has undergone continuous improvement. The working stages carried out to produce MAbs can be summed up as follows: antigen preparation, animal immunization, fusion technique, hybridoma selection, cloning method, production of purified antibodies, conjugation and testing of MAbs.
We report some comments on these stages and indicate further solutions to improve the production of MAbs. Some applications of MAbs are reported.

Production and selection of monoclonal antibodies

The animals normally used to produce monoclonal antibodies are Balb/c mice, although using animals of different strains it was possible to obtain antibodies with increased variability.
The myeloma X63 Ag 8.653 line proved to be less sensitive than the PAI or Sp2/0 line to the problems of in vitro growth and more effective in the fusions. Other lines with further improved myeloma have recently been developed: among these, NP2 demonstrates excellent characteristics.
The antigen preparation and preservation stage already has a certain effect on its integrity and antigenicity. The antigen can also be treated so as to reduce or eliminate the effect of contaminating substances. To do this, it is possible to act directly on the antigen to be injected or on the animal which will be immunized. In the first case, the normal plant components can be absorbed in an affinity column by means of antisera or with a mixture of specific monoclonal antibodies against normal plant components. In the second case, it is possible to use the mechanism of immunological tolerance induced in the animals to be injected (Hsu et al., 1990).
Particular substances, commonly called adjuvants, can be added to the antigen, or mixtures of several antigens can be used (Halk et al., 1984). In an experience concerning the production of monoclonal antibodies against Potato leaf roll virus, we mixed PLRV with alfalfa mosaic virus. In this trial, the total number of specific clones versus PLRV was notably higher than in the previous fusions, in which PLRV was used alone.
Another system which results in a greater stimulus in the animal, consists of inserting in the peritoneum pieces of nitrocellulose to which the antigen is bound. A granuloma forms around the nitrocellulose, consisting of an accumulation of cells which are immunocompetent against the antigen bound to the support. If this

tissue is used for the fusion, there will be a greater probability of obtaining monoclonal antibodies directed against the antigen used.
The immunization protocol is very important for a good result of the cellular fusion. In our trials we chose long-term immunization, using the traditional Freund Adjuvant.
The terminal stage of immunization is certainly the most decisive since it is the last injections which give the animal the stimulus to produce that particular population of lymphocytes which, once fused with the myeloma cells, will give rise to secreting hybridomas. We normally administer the last injections of antigen 4 days and 1 day before the fusion, without using adjuvants and preferably intravenously.
The fusion method we used is Fazekas de St Groth and Sheidegger's (1980). A other sophisticated fusion system consists of binding, at the time of fusion biotin to myeloma cells and avidin to the antigen. The two bound substances have a high affinity and favour the binding of sensitized antigen to treated myeloma and also the activated spleen cells interact with the antigen.
The good result of a cellular fusion also depends on the treatment of the plates on which the cells are distributed. We compared fusion plates with and without a feeder layer of mouse macrophages or with growth factors in the medium. The number of antibody-secreting clones obtained was highest when 10^4 macrophages were used in each well of a 24-well plate.
The antigen can also be bound to the plates in order to sensitize the lymphocytes in vitro.
Normally, single colonies of hybridomas can be detected in the plate wells 10 days after fusion. In order to limit the possibility of losing particularly interesting clones, we adopted the system of isolating the cells directly from the fusion plates. With a micromanipulator obtained from a pasteur pipette drawn out in the flame, it is possible to isolate a few cells from individual clusters. In this way, right from the initial stages it is possible to work with more or less monoclonal lines and also to test a good number of clones representing the variability of the hybridomas produced. Moreover, by avoiding the limit dilution, lines which survive with difficulty or grow slowly when the cells are highly diluted are no longer lost. In a single fusion of BNYVV, in which five 24-well plates were used, 292 hybridomas were isolated and tested (Grassi et al., 1988).
In the first stages of selection it is essential to have a rapid and efficient test to identify the secreting clones. We used the triple antibody sandwich version of ELISA where the antigen was bound by a polyclonal antibody (Al Moudallal et al., 1984). The secreting hybridomas were cloned twice and characterized according to class and subclass. The production of reasonable quantities of antibodies is obtained by multiplying the cells in the peritoneum.
To purify the anti BNYVV monoclonal antibodies from ascitic fluid, we use the method of affinity purification with Sepharose-Protein A.

Monoclonal antibody applications.

The purified IgGs of two monoclonal antibodies were used in ELISA both as a second antibody and as a coating. In both cases they made it possible to improve the sensitivity of the test. When they were used as a coating at a concentration of 0.5 - 1 µg/ml, they improved the accuracy of the estimation of viral concentration in the infected plant juice as compared with polyclonal antibodies. This permitted us to establish new methods of selecting sugar beet for resistance to BNYVV (Grassi et al., 1989).
The IgGs of MAb 47 (IgG_{2b}) were conjugated with the alkaline phosphatase enzyme and with biotin. The reactivity of the two conjugates was evaluated in ELISA using two different polyclonal

antibodies and one monoclonal antibody (MAb 41) as a coating. We found that the MAbs used in TAS-ELISA made the test more sensitive than DAS-ELISA in which the monoclonal antibody conjugated with alkaline phosphatase was used (fig. 1).

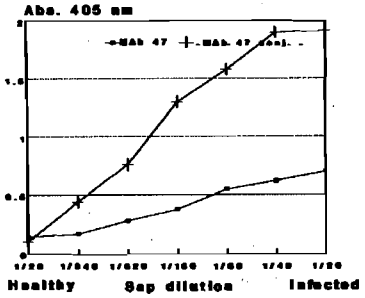

Fig. 1: Different sensitivity of DAS-ELISA and TAS-ELISA performed with Mabs

Fig. 2: Survey on Rizomania incidence in Italy: dots represent areas where BNYVV was detected

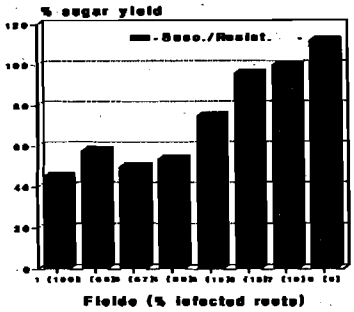

Fig. 3: Sugar yield reduction in relation to different degrees of Rizomania detected in 8 fields

MAb 47 conjugated with biotin, in preliminary trials, showed some problems of unspecific enzyme binding. The conjugation problems of MAbs have already been reported by other authors (Martin, 1987); some suggest adopting other methods of conjugation or using bifunctional binding reagents. In our opinion the loss of reactivity due to the conjugation with enzymes may also be linked to the IgG subclass. In three cases, where the conjugated IgGs belonged to IgG_{2b} or IgG_{2a} subclasses, a marked loss of reactivity was noted while a MAb of conjugated IgG_1 subclass remained highly reactive.

Monoclonal antibodies were used in research during a five-year project financed by the Ministry of Agriculture. Every year, in some 40 different places in Italy, some sugar beet varieties have been evaluated. A preliminary evaluation was carried out to verify the presence of BNYVV in the fields through ELISA analysis of bait plants grown in soil samples. On the basis of the analysis results, soils were chosen for the trails. At harvest time sugar beet tip samples of the susceptible (Kawegigamono) and the resistant variety (Rizor) were analyzed in turn . In the course of these years, approximately 300 samples from various regions were checked for the presence of BNYVV using polyclonal and monoclonal antibodies in ELISA.
Most of the soil samples were also checked for the presence of the fungal vector of BNYVV, Polymyxa betae. This fungus and the virus was widespread in almost all the beet-growing areas, including some parts of Sardinia and southern Italy (fig. 2). The percentage of infected fields was quite variable over the years (about 15-30%). Our samplings were not based on random collection but, they depended on fields which each researcher had available. Sometimes soils with some previous signs of the disease were also chosen.
The results on the degree of infection of the individual fields and the yield losses are interesting. The number of beet roots of the susceptible cv. affected by BNYVV rarely reached high percentages. It was observed that there was a significant difference in sugar yield between susceptible and resistant cultivars only when the number of infected roots exceeded the value of about 15% (fig. 3).

Conclusions

The MAbs production technique is in continuous evolution and up until

now there have been many methods of obtaining MAbs against plant viruses. However the results of this work are stronger conditioned by many variable factors such as the response of the animal immunized, the condition of cells at the moment of the fusion and the manipulation of cells. Probably other factors play an important role in the production of MAbs and all these variables may determine differences between MAbs obtained in different laboratories.
The MAbs against BNYVV permitted us to study more precisely the reaction of sugar beet to BNYVV infection and to improve the methods of selection for resistance. At the same time MAbs increased the sensitivity of serological assays which make it possible to perform a reliable survey of BNYVV in Italy. We can now conclude that rizomania is present in almost all the Italian areas where sugar beet is grown. However the percentage of infected fields and the level of infection are not very high: in fact the productivity of resistant sugar beet varieties has been satisfactory in almost all the Italian regions (Biancardi et al., 1990).

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ANTIGENIC ANALYSIS OF BEET NECROTIC YELLOW VEIN VIRUS BY MEANS OF MONOCLONAL ANTIBODIES

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Summary

Five epitopes were identified on the particles of beet necrotic yellow vein virus by means of monoclonal antibodies. Epitope 1 is located on one extremity of the particles and epitopes 2 and 3 on the opposite one. Epitopes 4 and 5 occur along the entire length of the particles. Only epitopes 3 and 4 are denaturation-resistant (SDS-stable). Epitope 4 occurs on the exposed C-terminus of the protein chain (aa 183-188) which is removed when the particles are treated with trypsin. Epitope 3 occurs on the N-terminal part of the protein chain (aa 1-103), but probably not on the N-terminus itself. It is not removed when the particles are treated with trypsin.

Introduction

Torrance et al. (1988) described the preparation of four monoclonal antibodies (MAbs) which readily detected beet necrotic yellow vein virus (BNYVV) in sap from infected Chenopodium quinoa in an indirect ELISA on plates which had been coated with polyclonal antibodies. In the immunoelectron microscopical decoration test, however, only two of these MAbs (MAFF 6 and 7) formed a dense coat around the particles, whereas the other two (MAFF 8 and 9) apparently did not react. Further studies using the immunogold technique revealed that the latter two MAbs were also bound to the particles, but not along the entire length, but only at one extremity (Lesemann et al., 1990). This prompted us to do a more comprehensive study on the antibody binding sites on BNYVV particles with MAbs from different laboratories.

Materials and Methods

MAbs were prepared by Torrance et al. (1988) in England, Boonekamp et al. (1988) in Holland, Grassi et al. (1988) in Italy and M. Alric and J. Kallerhoff (unpublished) in France. Details of the treatment of BNYVV particles with trypsin (Koenig et al., 1990) or SDS-containing buffer (Laemmli and Favre, 1973), of the preparation of cro-lacI-lacZ fusion proteins (Kocken et al., 1988; Koenig et al., 1990), of the dot blot and Western blotting techniques (Burgermeister and Koenig, 1984) and the immunogold technique (Lesemann et al., 1990) were described elsewhere.

Results and Discussion

MAbs which decorated the particles only on one extremity were obtained in England (MAFF 8 and MAFF 9), Holland (6D8) and Italy (41, 47). Mixtures of the Dutch and the Italian MAbs or of the two English MAbs also yielded particles which were labelled only at one extremity. However, when the Dutch or the Italian MAbs were mixed with the English MAbs

many particles were found which carried the gold lable on both extremities. This suggests that the epitope(s) for which the English MAbs MAFF 8 or 9 are specific (epitope 1 in Fig. 1) and the epitopes for which the Dutch MAb 6D8 (epitope 2 in Fig. 1) or the Italian MAbs 41 and 47 (epitope 3 in Fig. 1) are specific occur on the opposite extremities of the virus particles.

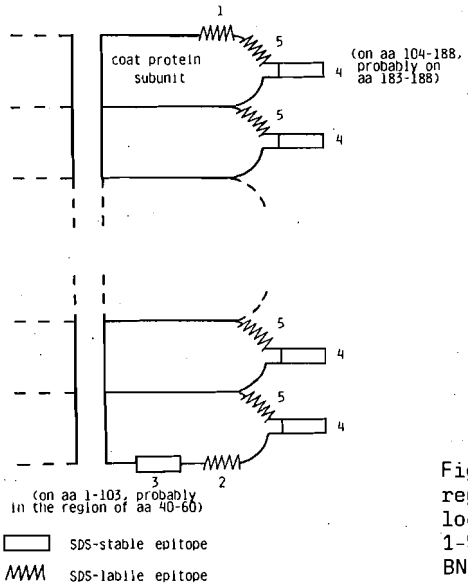

Fig. 1. Schematic representation of the location of epitopes 1-5 on a particle of BNYVV

Epitopes 1 and 2 were destroyed when the particles were either treated with trypsin or were boiled in SDS-containing buffer. Epitope 3, however, was resistant to these treatments. It was readily detected by means of Western blotting on the coat protein isolated from BNYVV particles (Fig. 3) or on fusion proteins containing amino acid (aa) 1-103 of BNYVV coat protein (Fig. 2).

At least two epitopes (epitopes 4 and 5 in Fig. 1) were detected along the entire length of the particles. Epitope 4 was SDS-stable, epitope 5 SDS-labile. Epitope 4 is apparently strongly immunogenic, because MAbs specific for this epitope were obtained in England, Holland and France. In Western blotting epitope 4 was readily detected on the fusion protein containing aa 104-188 of BNYVV coat protein (Fig. 2).

After treatment with trypsin BNYVV particles were still visible in the electron microscope, but binding of antibodies specific for epitope 4 was no longer observed. In Western blots, untreated BNYVV preparations yielded only one coat protein, whereas preparations treated with trypsin yielded two slightly smaller proteins (Fig. 3). It was estimated that these two smaller proteins lacked about 5 and 10-15 aa, respectively. Polyclonal antisera and MAbs specific for epitope 3 readily detected the undegraded as well as the partially degraded proteins. MAbs specific for epitope 4, however, reacted only with the undegraded protein suggesting that trypsin treatment had removed epitope 4 from the very C-terminus of the coat protein chain. From the data of Bouzoubaa et al. (1986) it is known that there is a trypsin cleavage site on the carboxyl side of the arginine at position 182. Thus, epitope 4 is apparently located on the exposed C-terminus comprising aa 183-188 (Fig. 1).

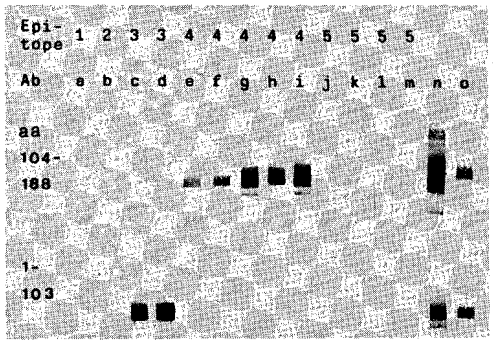

Fig. 2 Reactivity or lack of reactivity of various monoclonal or polyclonal antibodies (Ab) on Western blots with fusion proteins containing either aa 1-103 or 104-188 of BNYVV coat protein. a) MAFF 8, b) 6D8, c) 41, d) 47, e) MAFF 6, f) MAFF 7, g) 3H12, h) 4F11, i) 8B6, j) 17G2, k) 15, l) 379, m) 400, n) polyclonal Ab to BNYVV, o) polyclonal Ab to ß-galactosidase.

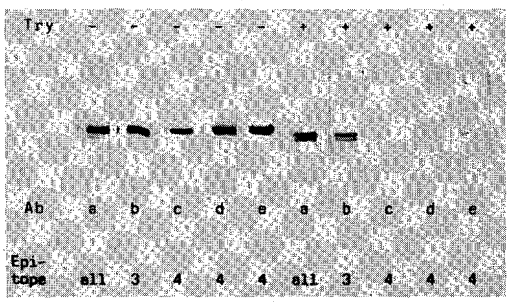

Fig. 3 Influence of BNYVV treatment with trypsin on the size and reactivity of the coat protein with polyclonal Ab (a), MAb 41 which is specific for epitope 3 (b) and MAbs MAFF 6 (c), 8B6 (d) and 3H12 (e) which are specific for epitope 4. Lanes Try + particles treated with trypsin, lanes Try - untreated controls

An exposed C terminus has also been reported for other elongated plant viruses such as tobacco mosaic virus (TMV) (Bloomer et al., 1978), potato virus X (Koenig et al., 1978; Sawyer et al., 1987) and potyviruses (Allison et al., 1985; Shukla et al., 1988). These viruses also have an exposed N terminus which in the case of PVX (Koenig and Torrance, 1986; Sober et al., 1988) and potyviruses (Allison et al., 1985; Shukla et al., 1988) is strongly immunogenic. So far we have obtained no evidence for an exposed and immunogenic N terminus on the protein subunits of BNYVV. Epitope 3 which is exposed only on one extremity of the particles and which is not removed when the particles are treated with trypsin has now been located around the region of aa 40 to 60 (Commandeur et al., 1990, p. of this volume). We cannot exclude, however, the possibility that the SDS-labile epitope 5 which is sensitive to treatment with trypsin and is exposed along the entire length of the particles, involves the N terminus of the coat protein chain.

The susceptibility of the exposed epitope 4 to trypsin and also to proteolytic enzymes in plant sap (Koenig et al., 1990) may be the reason for the observed failure of MAbs MAFF 6 and 7 to detect BNYVV in low concentrations in extracts from sugarbeet roots (Torrance et al., 1988).

A detailed description of the studies presented here has been given by Lesemann et al. (1990) and Koenig et al. (1990).

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EPITOPE MAPPING ON *E.COLI* EXPRESSED FRAGMENTS OF BEET NECROTIC YELLOW VEIN VIRUS COAT PROTEIN

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Summary

In order to map the positions of the antigenic determinants on the amino acid (aa) sequence of the beet necrotic yellow vein virus (BNYVV) coat protein (cp), deleted forms of the cp gene were expressed as ß-galactosidase (ß-gal) fusion proteins (fp) in *E.coli*. With a panel of monoclonal antibodies (MAbs) and polyclonal antibodies (PAbs) we were able to determine the approximate position of three SDS-stable epitopes. One epitope (previously named epitope 3) was located between (aa) 37-59, another one (previously named epitope 4) between aa 176-188 and a third one (now named epitope 6) between aa 116-140. All epitopes reacted with PAbs. However, MAbs were obtained so far only for epitopes 3 and 4. We found only a very limited correlation between the antigenic and nonantigenic regions defined in this study and the computer-predicted antigenicity of BNYVV cp regions.

Introduction

In previous studies (for review see preceeding paper by Koenig et al., 1990) we were able to define five different epitopes on the particles of BNYVV using the electron microscopical immunogold technique, ELISA and Western blotting. Epitopes 1,2 and 5 were found to be SDS-labile, whereas epitope 3 and 4 were SDS-stable. By means of Western blotting epitopes 3 and 4 were located on *E.coli* expressed fps containing aa 1-103 or aa 104-188 of BNYVV cp, respectively.
In this paper we describe the results of a more refined mapping for epitopes 3 and 4 using fps with shorter BNYVV cp sequences, and the detection of a third SDS-stable epitope (epitope 6) for which so far no MAbs are available.

Materials and Methods

MAbs were prepared by the authors listed under 2-5 in the heading of this paper. A set of deletion clones was prepared from the cp gene of BNYVV (Fig. 1). The truncated forms of the cp were expressed as fps with N-terminal parts of different length of ß-gal using the plasmid pEX and its derivatives (Kocken et al., 1988). Plasmids with the initials pEV contain a ß-gal fragment producing a 45K fusion part, whereas pEC plasmids yield a 37K fusion part. Recombinants were screened on the basis of the size of the inserts and of the expressed fps and were checked by restriction analysis. Culture of the bacteria, induction of expression and fp purification was essentially done as described by Kocken *et al.* (1988). Western blotting was done as described by Burgermeister and Koenig (1984).

Results

The portion of the BNYVV cp aa sequence contained in individual fps and the respective regions on the BNYVV cp gene cDNA are outlined in Fig. 1. As expected all fps reacted with PAbs to ß-gal, however, considerable differences were found in their reactivity with PAbs to BNYVV particles (Fig. 1 and 2). The fp containing aa 1-43 of the BNYVV cp (derived from pEVN-ΔSty) failed to react with PAbs to BNYVV particles, whereas the fps containing aa 1-59 and 1-103 (derived from pEV-NΔAva and pEV-N) strongly

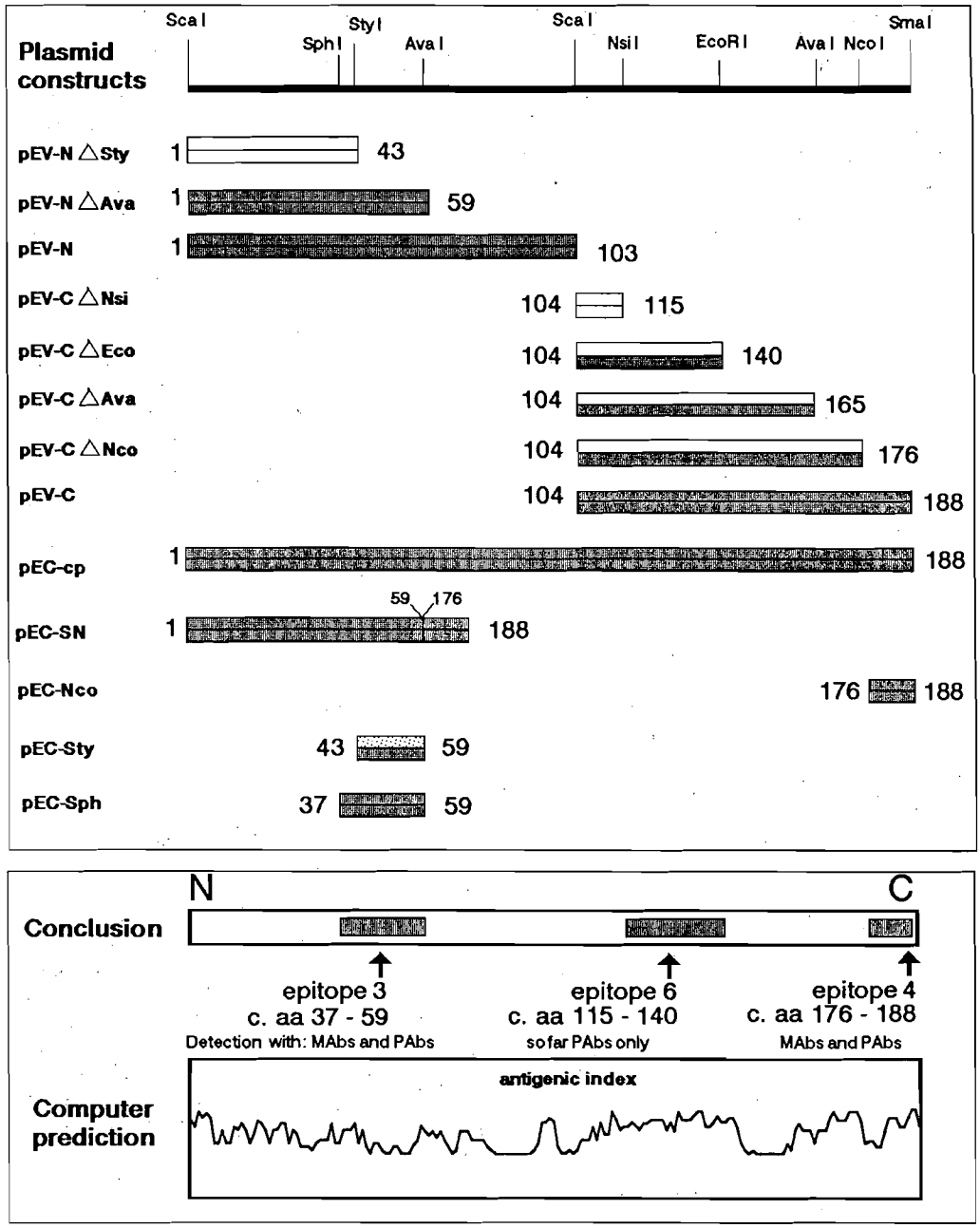

Fig. 1. Strategy and results. The first bar shows the locations of the restriction enzyme recognition sites that were used to generate the respective subclones. The boxes represent the BNYVV cp regions present in individual fps. The numbers indicate the aa at the deletion junctions. Hatching of the boxes indicates that the fps had reacted on Western blots with PAbs (lower part of the box) or MAbs (upper part of the box). MAbs reacting with the N-terminal (aa 1-103) or C-terminal (aa 104-188) portion of the cp were those specific for epitopes 3 and 4, respectively. Trypsin digestion experiments with BNYVV particles had previously suggested the location of epitope 4 on aa 183-188.

reacted with these PAbs (Fig. 1 and 2). The latter two fps also strongly reacted with MAbs specific for epitope 3, but the fp containing aa 1-43 failed to do so.

The fp containing aa 104-115 (derived from pEV-CΔNsi) also failed to react with PAbs to BNYVV particles, but the fps containing aa 104-140, 104-165, 104-188 (derived from pEV-CΔEco, -ΔAva, -ΔNco and pEV-C, respectively) strongly reacted with BNYVV PAbs (Fig. 1 and 2). A reactivity with MAbs specific for epitope 4 was seen, however, only with fps containing aa 104-188 (Fig. 1). These results suggested that at least three SDS-stable epitopes occur on the BNYVV cp aa sequence, i.e. one on aa 43-59 which by means of the respective MAbs was identified as epitope 3, a second one on aa 115-140 for which so far no MAbs are available and a third one on aa 176-188 which was identified by means of the respective MAbs to be epitope 4.

As a next step we prepared a fp which contained aa 1-59 and aa 176-188, i.e. the putative epitopes 3 and 4 and the region preceeding epitope 3 on the N-terminal side. This fp reacted well with PAbs to BNYVV and MAbs specific for epitopes 3 and 4 (Fig. 1). We have also prepared fps containing only the putative aa sequences for epitopes 3 (aa 43-59) and epitope 4 (aa 176-188) (Fig. 1). The latter reacted well with MAbs specific for epitope 4, but surprisingly the former showed only a very weak reaction with MAbs specific for epitope 3 (Fig. 1 and 3). Since the fps containing either aa 1-57 or 1-57 + 176-188 had reacted well with MAbs specific for epitope 3 we investigated the possibility whether a few additional aa on the N-terminal side of aa 43-59 which in the fp containing aa 1-43 were not reactive could nevertheless form part of epitope 3. This seemed to be indeed the case, because a fp containing aa 37-59 strongly reacted with MAbs specific for epitope 3 (Fig. 3).

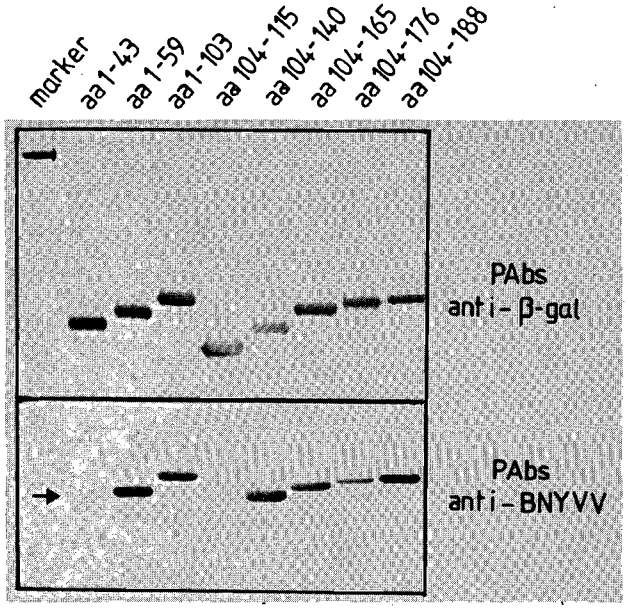

Fig. 2. Reactivity of various fps with PAbs to ß-gal and to BNYVV particles. The weak line marked by an arrow→ is the product of a non-specific reaction of BNYVV PAbs with a contaminating bacterial antigen. The locations of the non-reacting fps containing aa 1-43 and 104-115 in the lower part of the figure can be deduced from the positions of the corresponding fps in the upper part of the figure.

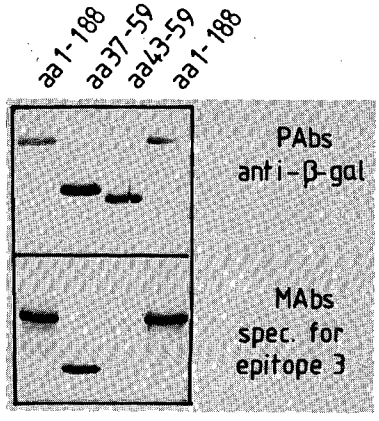

Fig. 3. Reactivity of PAbs to ß-gal and MAbs specific for epitope 3 with fps containing BNYVV cp aa sequences 37-59 and 42-59, respectively.

Conclusion

The results of this paper confirm our earlier conclusion derived from work with trypsin-treated particles that epitope 4 occurs on the C-terminus of BNYVV cp. From the results of the present paper it may be concluded that epitope 4 is located on aa 176-188, our earlier work had indicated its occurrence on an even narrower region between aa 182-188 (for review see preceeding paper by Koenig *et al.*, 1990). The present work also confirms our earlier conclusion that the N-terminus does not carry an SDS-stable epitope. Epitope 3 which in the particles is exposed only on one extremity (Koenig *et al.* 1990) was located in a region between aa 37-59. Interestingly, epitope 3 is part of a putative RNA-binding site (E.V. Koonin, pers. communication). A third epitope for which we so far have no MAbs was located in the region of aa 115-140 (Fig. 1).
We have compared our results on the location of epitopes 3, 4 and 6 with the computer-predicted antigenicity of BNYVV cp regions (Fig. 1) (Jamson and Wolf, 1988). Epitope 4 and 6 occur in regions of predicted high antigenicity (Fig. 1), but in many other regions of a predicted high antigenicity we could not detect SDS-stable epitopes, e.g. on the N-terminus (Figs. 1 and 2). Most of the aa sequence of epitope 3, on the other hand, lies in a region of predicted low antigenicity (Fig. 1).

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THE HOST RANGE OF *POLYMYXA BETAE* AND RESISTANCE IN *BETA* SPECIES

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Summary

Examination of 24 British arable weed species grown in soils naturally infested with *Polymyxa betae* showed that only *Chenopodium album, Atriplex patula* and *Silene alba* were infected significantly. However, cross-infection studies, using zoospores from purified cultures of *P. betae*, demonstrated that isolates derived from sugar beet were unable to infect any of these three weed species. Nevertheless, isolates from *C. album* were able to infect sugar beet and this common weed must therefore be considered a potential alternative host in the epidemiology of rhizomania in Europe.

Amongst *Beta* species, *B. patellaris* and *B. procumbens* are known to be completely resistant whereas *B. vulgaris* and *B. maritima* are, in general, susceptible to *P. betae*. However, resistant populations of *B. maritima* have been found and their potential use in sugar-beet breeding programmes, as a means of enhancing resistance to rhizomania disease, is discussed.

Host Range of *Polymyxa* species

The host range of *Polymyxa betae* in Britain, particularly in relation to the major arable weeds, was investigated, as has been done in a number of other countries, e.g. Germany (Keskin, 1964), Italy (D'Ambra, 1967), Canada (Barr, 1979), Japan (Abe & Ui, 1986), USA (Gerik & Duffus, 1987), Yugoslavia (Ivanovic, 1988) and Belgium (Goffart *et al.*, 1989). A total of 24 species from 16 families were tested by growing plants in naturally infested soils, collected from different sugar-beet growing areas of Britain, in the glasshouse at *ca* 22°C. The plants were sampled after 2-3 months and examined for the presence of cystosori in the roots. Most of the plants were found to be uninfected by *Polymyxa*. These were *Avena fatua, Daucus carota, Galium aparine, Matricaria perforata, Myosotis arvensis, Polygonum aviculare, P. lapathifolium, P. persicaria, Portulaca oleracea, Raphanus raphanistrum, Sinapsis arvensis, Solanum nigrum, Spergula arvensis, Veronica hederifolia, V. persica* and *Viola arvensis*. A trace of infection was found in *Papaver rhoeas, Stellaria media* and *Urticaria urens*. *Amaranthus retroflexus* and *Poa annua* both contained low levels of infection. These levels are not considered to be significant in increasing the amount of fungal inoculum in the soil.

By contrast, a few of the weeds tested were found to contain *Polymyxa* species at the same high levels that were found in sugar beet. Significant levels of infection were found in *Atriplex patula* and *Chenopodium album* of the Chenopodiaceae and in *Silene alba* of the Caryophyllaceae. The latter is a new host species for *Polymyxa*. In order to determine whether these weeds could act as alternative hosts to the sugar-beet strain of *Polymyxa*, purified cultures of the fungus were developed by maintaining isolates on each of the host species separately. Zoospores derived from each host species were then used in cross-infection studies to examine their host range (Table 1). Isolates from *B. vulgaris* were unable to infect either *C. album* or *S. alba* and therefore it appears likely that these species have their own species or strain of *Polymyxa*. The strain of the fungus from *C. album*, however, was able to infect *B. vulgaris*. This suggests that *C. album* could act as a potential alternative host of *P. betae* and thus be

important in the epidemiology of rhizomania. Further work is being carried out to determine the host specificity of the other two heavily infected species, *A. patula* and *S. alba,* so as to ascertain their role, if any, in the disease.

Table 1: Host range of *Polymyxa betae* - zoospore inoculum.

	Test plant		
Donor species	*B. vulgaris*	*C. album*	*S. alba*
Beta vulgaris	●	○	○
Chenopodium album	●	●	○

● able to cross-infect ○ unable to cross-infect

Resistance in *Beta* species

The host range of *P. betae* amongst *Beta* species was first examined by Fujisawa & Sugimoto (1979). *B. patellaris* and *B. procumbens* were found to be completely resistant whereas *B. vulgaris* was susceptible and *B. maritima* populations, though generally susceptible, appeared to have some partially resistant plants (Table 2). The resistance in *B. patellaris* appears to act at an early stage in the infection process since, in our time-course studies, no post-penetration structures have been observed in roots exposed to inoculum. However, the potential durability of such resistance, if it was introduced widely into sugar beet, might be questioned, since it may be under relatively simple genetic control. In addition, crosses between these two resistant wild species and *B. vulgaris* are difficult, often leading to sterility or loss of vigour.

Table 2: Host range of *Polymyxa betae* among *Beta* species

Beta patellaris	Resistant
Beta procumbens	Resistant
Beta vulgaris	Susceptible
Beta maritima	Susceptible

In contrast, *B. maritima* is fully compatible with *B. vulgaris* and has already been exploited in sugar-beet breeding programmes for its resistance to BNYVV (Lewellen *et al.,* 1987). We have recently screened a number of populations of *B. maritima* from an international germplasm collection for their resistance to *P. betae*. About 20 plants from each population were grown in naturally infested soil in a glasshouse at *ca* 22°C for a minimum of 8 weeks and then assessed microscopically for intensity of infection with cystosori of *P. betae*. The disease scores of individuals in the populations usually followed a normal distribution pattern (Fig. 1) but one population was skewed towards low levels of infection and a number of highly resistant

individual plants were identified for use in a sugar-beet breeding programme. This resistance appears to be quantitative in character and work is in progress to determine whether it is inherited polygenically.

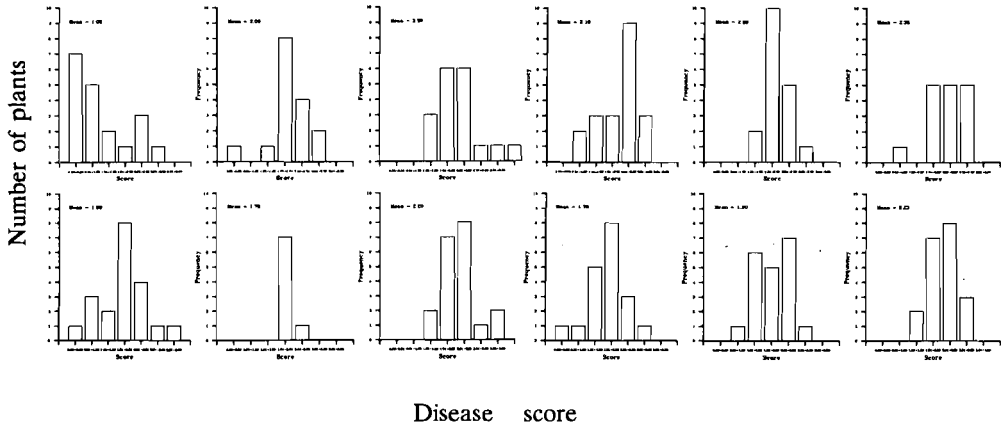

Figure 1: Frequency distribution of susceptibility to *Polymyxa betae* in 12 populations of *Beta maritima*. Note abnormal frequency of resistant plants in population at top left.

Variation in levels of susceptibility to *P. betae* also appears to be present in *B. vulgaris*. In our study of 18 rhizomania 'resistant' selections of sugar beet that are being trialled in The Netherlands this year some differences between lines were observed (Fig. 2). The variation was much less than that found in *B. maritima* populations and no high levels of resistance to *P. betae*, that might have contributed to their reputed rhizomania resistance, were detected.

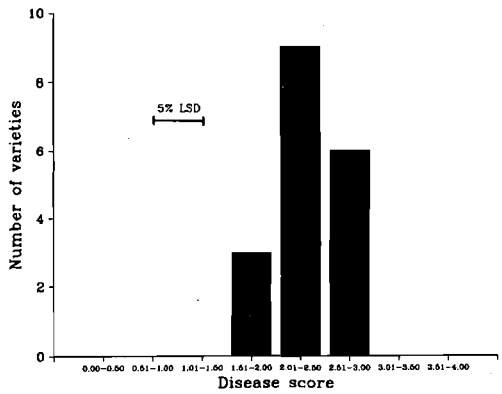

Figure 2: Frequency distribution of susceptibility to *Polymyxa betae* in 18 rhizomania 'resistant' varieties of sugar beet.

In the longer term, if resistance to the fungal vector is to be exploited as a means of controlling rhizomania disease, in addition to the virus resistance currently being developed, it must be (a) at a high enough level to prevent significant entry and multiplication of the virus in the root and (b) sufficiently complex to prevent the development of fungal races able to overcome it. Experience with other Plasmodiophoromycetes (Crute *et al.*, 1980) suggests that resistance that is simply inherited is unlikely to be durable.

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INOCULUM CHARACTERISTICS OF ZOOSPORE SUSPENSIONS OF *POLYMYXA BETAE* INFECTED WITH BEET NECROTIC YELLOW VEIN VIRUS

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Summary

Polymyxa betae zoospore suspensions produced by beet roots infected with beet necrotic yellow vein virus proved to be highly infectious inocula. Suspensions of 50 ml in which an infected seedling had been placed for five days, could be diluted for at least 250 times to infect half of the plants exposed. Although the spores are evenly distributed through the inoculum the amount of virus in the infected roots varied considerably as has to be concluded from the large standard deviations of the ELISA readings found between the plants in each experiment.

Introduction

Beet necrotic yellow vein virus (BNYVV), a furovirus, is a soil-borne virus that causes a severe disease in sugar beet known as rhizomania. The virus is transmitted by the plasmodiophoraceous fungus Polymyxa betae (Abe & Tamada, 1986; Barr, 1988).
The infected plant responds by a proliferation of lateral roots, a yellowish or a brownish discoloration of the vascular rings and a sudden size reduction of the tap roots in their lower parts. Virus can be detected in the crown of the beet when symptoms develop in the leaves, which rarely happens. Virus can readily be detected in the tap roots, especially in the developing parts such as the tip of the tap root and the youngest lateral roots. The distribution of the virus in the plants indicates that transport of virus is often restricted to parts around the infection site. Restriction of BNYVV transport through plants has been shown by Hillman (1984). That part of the root system of young plants which grew in BNYVV-infected soil became infected whereas the one in uninfected soil remained virus-free. Highly variable amounts of virus have also been found in root systems of plants inoculated in breeding programs with soil conducive to the development of rhizomania (Bürcky & Büttner, 1985; Müller, 1986). These authors explained their results by differences in the rate of virus replication due to the genetic variability of the plants and by the moment at which the plants were infected during the period of exposure to inocula. However, their results and also those obtained by Hillman may indicate that roots have to be re-inoculated several times by zoospores to obtain a more thorough infection of the root system.
The use of highly infectious inocula may result in a more uniform development of the infection and a more rapid and thorough infection of the root system.
Resting spores of P. betae germinate at a low rate. Treatments to accelerate germination, except heating of soil at 40°C (Beemster & de Heij, 1987), have not been developed yet. Cultures of zoospores produced by plants in nutrient solutions may be better inocula yielding more constant infection levels. Rössner & Grösz (1987) showed that identical results were obtained with the use of zoospore suspensions and that of infected plants. Peters & Godfrey-Veltman (1989) demonstrated that zoospore suspensions are highly infectious. Some properties of these zoospore suspensions will be described in this report.

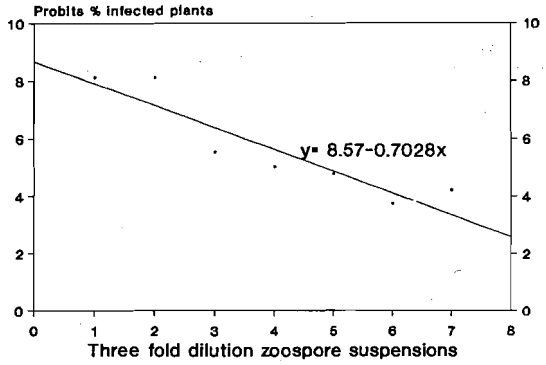

Fig. 1. Probit analysis of the percentage of beet plants infected by BNYVV after exposure to a three-fold dilution series of a suspension of Polymyxa betae zoospores released by BNYVV-infected plants in a nutrient solution.

Materials and Methods

The general procedure to produce zoospore suspensions infected with BNYVV, and the techniques used to infect and to culture the seedlings in nutrient solutions were as described by Peters & Godfrey-Veltman (1989). Seedlings of which the roots were assayed for the virus content were incubated in 50 ml of a Steiner (1966) based nutrient solution after inoculaton. The composition of this nutrient solution can be obtained from the present authors.

Results

Peters and Godfrey-Veltman (1989) showed that half of the seedlings placed in zoospore suspensions were infected with BNYVV in 3 to 10 min and that after removal of the infected plants, the suspensions remained infectious for at least 8 h. These results indicate that the zoospore suspensions produced were highly infectious. This conclusion could be confirmed by dilution experiments (Fig. 1). A zoospore solution was produced by transferring seedlings infected in soil, to the nutrient solution for four days. Fresh seedlings were exposed to zoospores for 1 h in a three-fold dilution series of this zoospore suspension using 1:10 nutrient solution as diluent. Infectivity could be demonstrated in roots of seedlings inoculated in all zoospore supension dilutions made. Probit analysis of the results showed that the dilution at which 50 percent of the plants were infected, was estimated to be 1:262 (Fig. 1).
The infectivity of the diluted zoospore suspensions depends on the diluent used. Peters & Godfrey-Veltman, 1989) observed that a dilution of 1:4 of the zoospore suspension using tap water resulted in a complete loss of infectivity. Detrimental effects of tap water could also be demonstrated in an experiment in which the seedlings after being inoculated in a zoospore suspension were placed for 7 days in tap water. The amount of virus in seedlings placed after inoculation in undiluted, 1:10 and 1:50 diluted suspensions showed a rapid increase over the first five days after inoculation (Fig. 2). Only limited amounts of virus were found in the seedlings placed in tap water after being inoculated in the undiluted zoospore suspension. These results may indicate that zoospores released during the incubation in tap water do not re-infect the roots leading to new foci of infection.
Besides the fact that tap water negatively affects the development of virus in the roots it may also affect the establishment of infection in the roots. Plants were placed in a highly infectious suspension and then, after dividing them into

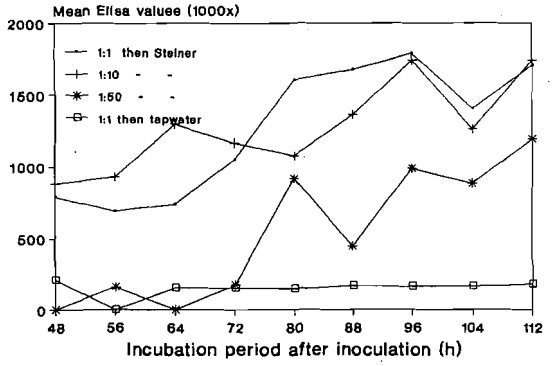

Fig. 2. Virus content of seedling roots either inoculated in undiluted and diluted zoospore suspensions and then placed in nutrient solution, or inoculated in an undiluted zoospore suspesion and then placed in tap water.

groups, incubated first for different periods in tap water and then in 1:10 nutrient solution for a total period of ten days. The results obtained show that the length of the period in which the roots are incubated in tap water decreases the number of plants infected and the amount of virus detected (Table 1). Low amounts of virus in roots have been considered to be indicative for resistance in beets. We tested the development of the virus in the susceptible cultivar Arigimono, the partially resistant cvs Rima and Univers and three populations of partially resistant hybrids (R 602, R660 and R650). Roots were assayed 3, 5 and 7 days after inoculation. The results indicate that all plants develop high levels of virus (Fig. 3). The virus reaches its maximum concentration more rapidly in 'Arigomono' than in the other lines.

Discussion

Zoospore suspensions produced by P. betae and BNYVV-infected beet seedling cultures in a nutrient solution are highly infectious. This can be concluded from the short infection periods and the dilutions of the suspensions at which 50 % of the plants become infected. At the moment of dilution the suspension contained so many viable spores that the original suspension (50 ml) produced by one plant could be diluted 260 times to infect 50% of the plants exposed. In contrast to this high infection potential it is surprising that the virus level in the roots varies considerably between the infected plants (Table 1). This has to be explained by other factors than by the inoculation process itself, as the zoospores are evenly distributed in the suspension so that each unit of root has the same chance to become infected. Other factors may be either

Table 1. Development of virus in roots of 'Arigomono' beet seedlings which after exposure to highly infectious suspensions were grown for a number of days in tap water and then in nutrient solution.

Incubation of roots first in tap water and then in nutient solution (days)		% infected plants	Mean Elisa values	Std
0	10	90	1.434	0.534
2	8	80	1.589	0.396
4	6	80	1.535	0.429
6	4	60	0.981	0.539
8	2	50	1.065	0.595
10	0	25	0.551	0.141

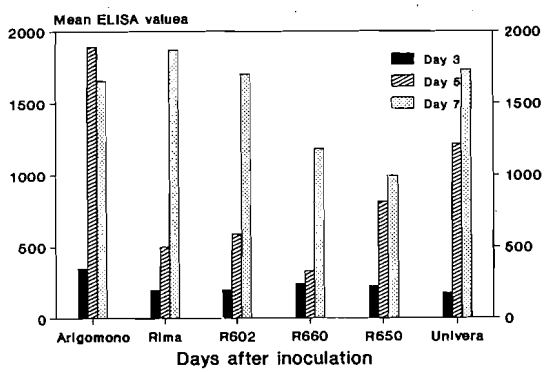

Fig. 3. The virus titer in roots of susceptible and partial resistant beet cultivars 3, 5 and 7 days after inoculation in a zoospore suspension and placing the roots in 1:10 nutrient solution.

a large difference in the infection potential of these spores or the susceptibility of the roots to the virus due to the genetic variability of the spores and plants. Genetic variablity may determine the ratio of zoospore and resting spores produced, the number of zoospores released, the number of viruliferous zoospores, the number of virus particles per viruliferous spore and the replication rate of the virus and transport through the plant.

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DEVELOPMENT OF RHIZOMANIA AT DIFFERENT INITIAL INOCULUM LEVELS OF THE SOIL

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Summary

Five inoculum levels of rhizomania were created by application of different amounts of BNYVV-infested soil to a non-infested field. Sugar-beets were grown during two consecutive years (1988 and 1989).

Bioassays on soil samples collected at the end of the first growing season revealed an increase in BNYVV infestation at all inoculum levels.

Root symptoms could not be observed in 1988, but BNYVV-infected plants were detected by ELISA in low numbers at all inoculum levels.

After late drilling in 1989 high numbers of infected plants were already detected in June, especially at the highest inoculum level. Disease incidence at the lower inoculum levels increased during the season, but differences between levels remained present. Root symptoms were observed from the end of June, and yellowing of the leaves started at about mid July.

Effects of rhizomania on sugar content and yield were already observed in the first year. At the highest inoculum level sugar yield was reduced by 9% and in 1989 by 65%.

SOIL MATRIC POTENTIAL EFFECTS ON INFECTION BY POLYMYXA BETAE AND BNYVV

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Summary

Infection by Polymyxa betae and beet necrotic yellow vein virus was studied in four soils of varying textures at soil matric potentials of -400 mbars and higher. Infection was found to occur at matric potentials ≥ -400 mbars. Infection in soil at higher matric potentials appeared more frequent in the course texture soils.

Introduction

Beet necrotic yellow vein virus (BNYVV), the causal agent of rhizomania, is transmitted to roots by Polymyxa betae (Fujisawa & Sugimoto, 1976; Giunchedi & Langenberg, 1982; Tamada et al., 1974). Many important environmental factors favoring activity by P. betae and development of rhizomania have been reported (Abe, 1987; Asher & Blunt, 1987; Horak & Schlösser, 1980), but information on the effect of soil matric potential has remained undocumented.

The effect of soil matric potential on zoosporic soil fungi is well recognized (Duniway, 1975a, b, 1976, 1979; MacDonald & Duniway, 1978a, b; Smith et al., 1990; Westerlund et al., 1978). As soil matric potential decreases, formation of zoosporangia is limited (Duniway, 1975a, b), zoospore discharge is reduced (MacDonald & Duniway, 1978b, c; Pfender et al., 1977), and zoospore motility is decreased (Duniway, 1976; MacDonald & Duniway, 1978a; Westerlund et al., 1978). Soil matric potential effects have been studied also on infection from resting spores of other plasmodiophoromycetes (Dobson et al., 1982).

In California rhizomania is a serious problem for sugarbeet production, especially near the city of Los Banos. In this area sugarbeets are planted in late spring, when soil temperatures are > 20°C, for harvest the following spring. Sugarbeets are sown into dry soil, then extensively watered until they emerge. This practice is conducive to early infection of the elongating taproot, which can result in extensive yield loss. An alternative method of sugarbeet culture would be to plant into pre-irrigated soils which have drained to a point were soil moisture conditions were no longer conducive for infection by P. betae and BNYVV.

This study reports preliminary results concerning the soil matric potential requirements at planting for infection by P. betae and BNYVV.

Materials and Methods

Soils, varying in texture, were collected from four fields known to be infested with rhizomania. In an effort to overload the soils with inoculum, they were placed in 15 cm diameter plastic pots and planted with sugarbeet hybrid 'USH11'. The plants were grown in a greenhouse maintained between 20° - 25°C for 6 weeks, then the tops of the plants were removed and the soils were air dried. This procedure was repeated once more.

The dry soils were passed through a 1 mm sieve and added to steel cylinders (4 cm tall X 5 cm diameter, opened on both ends) and planted with seed of `USH11'. The cylinders were placed in a tray and water was added so that the soil would become saturated. The cylinders were

then immediately transferred to a ceramic plate soil moisture extractor where the matric potential of the soils was adjusted to various levels. The soils were allow to adjust for at least 18 hours after which the cylinders with the soil were carefully transferred individually to 250 ml plastic beakers which were sealed with a plastic lid to prevent drying. The beakers were incubated at 24°C for 14 days, after which the plant roots were removed and assayed for infection by ELISA.

Results

The soil texture classes and particle size distributions are listed in Table 1. Initial experiments were performed with the sandy loam soil. The matric potential of this soil was maintained between -60 and -180 mbars with the use of Büchner funnels with fritted glass plates (Duniway, 1976). Sugarbeets were grown in these funnels at constant matric potentials for 4 weeks, then assayed by ELISA. After 4 weeks the plants at all matric potentials were infected by BNYVV. Attempts to use the Büchner funnels at matric potentials < -180 mbars failed due to the inability of the fritted glass plated to hold tensions of this magnitude. Because of this limitation, the procedure using the moisture extractor was adopted.

Table 1. Texture and particle size distribution of soils.

Soil Class	Sand	Silt	Clay
Clay	26.0%[a]	12.5%	61.5%
Clay Loam	31.5%	29.5%	39.0%
Sandy Loam	76.0%	6.5%	17.5%
Loamy Sand	74.0%	12.0%	14.0%

[a]Values are the mean of 2 analyses.

The results of the experiment conducted with the four soils are presented in Table 2. In general, infection was greater in the lighter texture soils at the higher matric potentials. Infection at the lowest matric potential appeared to be reduced, but still occurred to some extent.

Table 2. Effect of soil matric potential at planting on infection by Polymyxa betae and BNYVV.

Soil Class	Number of Infected Plants out of 4[a]				
	Matric Potential (-mbars)				
	200	250	300	350	400
Clay	1	2	0	2	1
Clay Loam	4	4	1	0	0
Sandy Loam	4	3	4	2	1
Loamy Sand	4	4	4	1	2

[a]Infection determined by ELISA test 14 days after planting.

Discussion

The results of the experiment are somewhat inconclusive because soil matric potentials were not tested at levels sufficiently low to limit all infection. The data do point out that infection can take place at relatively dry soil moisture conditions. Studies with Phytophthora spp. suggest little or no infection from zoospores at soil matric potentials ≤ -50 mbars (Duniway, 1976, 1979; MacDonald & Duniway, 1978a, b; Pfender et al., 1977). Studies with Olpidium brassicae indicate that infection from resting spores will occur at soil matric

potentials ≥ -60 mbars, but do not occur at matric potentials ≤ -100 mbars (Westerlund et al., 1978). Our data indicate that infection resulting from resting spores of P. betae can occur at relatively dry soil conditions. Similar data have been reported for Plasmodiophora brassicae (Dobson et al., 1982) where researchers have described root-hair infection to occur at soil matric potentials as low as -800 mbars

The data indicate that soil texture may interact with the matric potential requirements for infection, especially in wetter soils. Frequency of infected plants appeared to be greater in the coarser texture soils compared to the clay soil; however, the inoculum densities of the different soils were not standardized and this observation could be erroneous. But previous researchers have indicated increased zoospore activity in coarser compared to finer soils (Duniway, 1976; MacDonald & Duniway, 1978a).

The method used in this experiment to adjust soil matric potential did not provide constant soil moisture conditions during the entire experiment, but only provided defined conditions at the time of planting. Experiments utilizing more exacting techniques should be performed to obtain better data concerning the effect soil matric potential has on infection. Field experiments will be required to determine if planting in pre-irrigated soils will provide for control of early infection by P. betae and BNYVV.

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GENETIC CONTROL OF RHIZOMANIA IN SUGAR BEET (BETA VULGARIS)

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Summary

In various accessions of Beta vulgaris and B. maritima a reduced level of beet necrotic yellow vein virus has been found. However, no immunity to the virus has been detected yet.
No resistance to Polymyxa betae has been found in B. vulgaris, while partial resistance was observed in B. maritima. Preliminary results with the latter resistance suggest that high levels of vector resistance are necessary to give effective protection against rhizomania. Complete resistance to P. betae was reported in wild species of the sections Corollinae and Patellares. The vector resistance found in B. procumbens is likely to be under relatively simple genetic control.
A more intensive screening for resistance to the virus and the vector in wild Beta species is recommended.

Introduction

Breeding for resistance to rhizomania in sugar beet is thought to be the best way for controlling the disease. Both resistance to beet necrotic yellow vein virus (BNYVV) (Tamada, 1975) and to its vector Polymyxa betae Keskin might be used in breeding programmes.
In this paper a short review is given of the perspectives for using different Beta species in breeding rhizomania resistant sugar beet varieties.

Resistance to beet necrotic yellow vein virus (BNYVV)

Intensive screening among Beta vulgaris germplasm, under BNYVV-infested conditions, revealed great differences between accessions in resistance to rhizomania (Bolz & Koch, 1983; Johansson, 1985; Lewellen et al., 1987). Lewellen & Biancardi (1990) reported on both quantitative inherited and monogenic resistance in B. vulgaris. Breeding activities have resulted in the development of several varieties that give good performance on infested fields. However, no immunity to the virus has been reported yet and under severely infested conditions considerable losses still occur even with these new varieties.
Among the accessions of B. vulgaris kept at CPO, which were tested in the greenhouse, some interesting sources of resistance were found. In accession R39, earlier selected for tolerance to virus yellows, the concentration of BNYVV was only 20% of that of the susceptible cv. Regina (Paul et al., 1990). Within these populations and within rhizomania resistant varieties, however, plants with high and low concentrations of the virus were found. Further selection might improve the level of resistance of the resistant

material. In the roots of all accesions and varieties of B. vulgaris, both susceptible and resistant, cystosori of P. betae occurred abundantly. After inoculation of the susceptible cv. Regina and the partial resistant cvs Nymphe and Rima with a virulifurous and a non-virulifurous isolate of P. betae, no significant differences in numbers of cystosori were detected, whereas virus concentration between the varieties differed significantly (P=0.05). It is therefore assumed, that resistance to rhizomania in B. vulgaris material is based on resistance to the virus.
Simply and dominantly inherited resistance to the virus has also been reported to be present in B. maritima (Whitney, 1989).
Fujisawa & Sugimoto (1979) mechanically inoculated four accessions of B. maritima, together with B. vulgaris, B. perennis and B. orientalis of the section Beta, B. patellaris ,B. procumbens and B. webbiana of the section Patellares and B. trygina, B. corolliflora and B. intermedia of the section Corollinae. The occurrence of lesions on all these species indicate that these species are, at least to some extent, susceptible to the virus.

Resistance to Polymyxa betae

Habibi (1969) observed differences in numbers of cystosori in the roots of various accessions of Beta vulgaris. However, Bolz & Koch (1983) tested numerous varieties and breeding lines, and could not detect significant differences in infection by P. betae.
Fujisawa & Sugimoto (1979) tested the accessions that were mechanically inoculated with BNYVV (see above) also for resistance to P. betae.
B. vulgaris, B. perennis and B. orientalis were classified as susceptible to P. betae. In three of the four accessions of B. maritima (SP581103-0, SP581105-0 and WB37) low grade infection by P. betae was observed. No P. betae could be detected in the roots of the species of the sections Corollinae and Patellares.
Also at CPO, the accessions SP581103-0 SP581105-0 and WB37 were tested for resistance to the vector and to the virus. In all three accessions less cystosori were detected than in the susceptible cv. Regina. Numbers of cystosori were found to be lowest in SP581103-0, 61% less than in cv. Regina. Virus concentrations, however, in the three accessions were more than twofold that of cv. Regina.
The studies with species of the section Patellares were repeated and extended. No cystosori of P. betae were detected in the roots of either Patellares species nor in hybrids of B. procumbens and B. patellaris with B. vulgaris. Virus concentrations in the Patellares species and in the hybrids were very low (Paul et al., 1990). Subsequently, tests were carried out with the complete series of nine monosomic additions of B. procumbens in B. vulgaris, made by Lange et al. (1988). The additions Type 4 and Type 8 had only little cystosori of P. betae. Virus concentrations in the addition plants of these two types were much lower than in sib-plants, but were higher than in the wild species and the hybrids. The seven other additions were found to be susceptible to both P. betae and the virus (Paul et al., in preparation). These results suggest that the resistance to P. betae found in B. procumbens, is controlled by genes or gene complexes on only two of the nine chromosomes, and therefore is likely to be under relatively simple genetic control.

Perspectives for breeding

In Table 1 a summary is given of present knowlegde about resistance to BNYVV and P. betae in Beta species. Also the perspectives of the use of the species in breeding sugar beet with resistance to rhizomania are indicated.

Table 1. Resistance to beet necrotic yellow vein virus (BNYVV) and Polymyxa betae in Beta species and perspectives for their use in breeding sugar beet with resistance to rhizomania (for references, see text).

Beta species	Virus resistance	Perspectives	Vector resistance	Perspectives
Some accessions of Beta vulgaris	partial	+	no	-
Some accessions of B. maritima	partial	+	partial	±
Patellares spp.	no	-	complete	±
Corollinae spp.	no	-	complete	±

+: good perspectives; ±: currently investigated; -: no perspectives.

Within Beta vulgaris several sources of resistance to BNYVV have been found and several rhizomania resistant varieties have been developed. Improvement of the level of resistance might be obtained by further selection and combination of different sources of resistance (Lewellen & Biancardi, 1990). The resistances present in B. maritima can also be used, since this species can be easily crossed with B. vulgaris.
Within B. vulgaris no interesting sources of resistance to P. betae have been detected yet. Some partial resistance to the vector has been found in B.maritima accessions. However, results of a preliminairy experiment with this partial resistant material indicate that levels of resistance to P.betae must be very high to be effective. Complete resistance to the vector has been found in wild species of the section Corollinae and Patellares. Although the use of Patellares species has resulted in nematode resistant sugar beet material (Lange et al., 1990), the transfer of genes from these species to cultivated beet appeared to be extremely difficult. The possibilities to transfer vector resistance from Patellares species to cultivated beet, using both classical and biotechnological approaches, are currently investigated at CPO.
Although resistance to the virus is present in B. vulgaris accessions, additional sources of resistance, either to the virus or the vector, are needed to give sufficient control. Therefore a intensive screening of wild species for resistance to the virus and the vector is recommended.

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CONTENT AND DISTRIBUTION OF BEET NECROTIC YELLOW VEIN VIRUS (BNYVV) IN SUGAR BEET VARIETIES WITH DIFFERENT DEGREES OF SUSCEPTIBILITY TO RHIZOMANIA

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Summary

The concentration of beet necrotic yellow vein virus (BNYVV) in the lateral rootlets, tap roots and hypocotyls of young plants of sugar beet varieties known to differ in susceptibility to rhizomania was examined. Independent of the degree of susceptibility of the variety, the concentration of BNYVV was always higher in lateral rootlets than in the tap root or in the hypocotyl, but ELISA-readings of extracts from lateral rootlets as well as from tap roots were higher in plants of the susceptible varieties. Higher temperatures and permanent illumination led to an increase in virus content in these tissues. Generally, the virus content of tap roots and/or hypocotyls was a better indicator of varietal differences in susceptibility than that of lateral rootlets. Possible reasons for the differences in virus content and distribution as well as the use of such differences in the selection of genotypes more resistant to rhizomania are discussed.

Introduction

In order to achieve fast and effective breeding of sugar beet varieties with a high degree of resistance to rhizomania, an understanding of the characteristics of such resistance is essential for selection. In a previous investigation (Bürcky and Büttner, 1985) differences in the content of beet necrotic yellow vein virus (BNYVV) in young plants of different varieties of sugar beet grown in rhizomania-infested soil were found. In the early stages of growth a considerably lower virus content in the lateral rootlets of varieties first of all selected for tolerance against rhizomania in the field was observed. Consequently these varieties could be termed resistent. Subsequently, the difference in virus content between these resistant and susceptible varieties declined until it became almost undetectable. In contrast, in fully developed plants the virus content of tap root slices and/or brei prepared from mature roots differs clearly between resistant and susceptible varieties (Bürcky and Büttner, 1989 a).

Further investigations were therefore undertaken to examine the following:

- the development of BNYVV in roots of sugar beet plantlets differing in susceptibility to rhizomania
- the relative distribution of virus in lateral rootlets, the tap root and hypocotyl
- the extent to which growing conditions influence the amount and distribution of BNYVV in roots.

Material and Methods

Sugar beet seedlings of three susceptible and three resistant varieties were grown in disease-free Blähton-hydroponic culture for two weeks and then transplanted into a rhizomania-infested soil mixture. After growing in infested soil for 3 weeks, each plant was separated into lateral rootlets,

tap root and hypocotyl . BNYVV determinations were done by means of ELISA (Büttner and Bürcky, 1987).

In order to achieve a comparable estimate of the amount of antigen (virus) in the various plant tissue homogenates, a dilution series of a highly purified virus preparation was included in all tests and a standard calibration curve prepared. Tissue samples were assigned a relative virus content according to their ELISA-readings by reference to this standard curve (Bürcky and Büttner, in press).

Results

The highest virus content was found in the lateral rootlets (Table 1),

Tab. 1: BNYVV-concentration in the lateral roots, tap root and hypocotyl of young plants of susceptible and resistant varieties of sugar beet. n = 36 plants/variety

Variety	Variety against BNYVV	BNYVV concentration (relative) lateral roots	tap roots	hypocotyl
Kawemono	susceptible	1691	336	18
Novadima	susceptible	1735	357	34
Hilma	susceptible	1799	443	35
Rizor	resistant	781	83	1
Donna	resistant	901	162	3
Nymphe	resistant	807	83	5

the tap roots and the hypocotyls containing progressively less virus. This gradation was found to be independent of the level of resistance of the variety. Overall, resistant varieties contained less virus in their roots. This difference was particularly pronounced in tap roots and even more so in hypocotyls. Whereas BNYVV could be detected in the lateral rootlets of all plants, including those of resistant varieties, there were quite a number of resistant plants in which no virus could be found in the tap root and hypocotyl.

Tab. 2: Mean concentration and distribution of BNYVV in young plants of three susceptible and three resistant varieties of sugar beet grown in infested soil at different temperatures for 21 days

Temperature during growth	Varieties	BNYVV concentration (relative) lat.roots	tap roots	hypocotyl	BNYVV concn.susc.varieties / BNYVV concn.resist.varieties lat.roots	tap roots	hypocotyl
12°C/18°C 14h light	suscept.	226	47	- *	2.0	2.8	-
	resistant	114	17	-			
18°C/22°C 14h light	suscept.	1742	379	29	2.1	3.5	9.3
	resistant	830	109	3			
25 °C permanent light	suscept.	3877	494	89	1.4	2.9	3.6
	resistant	2758	170	25			

* not examined

Higher temperatures and, as a consequence, an acceleration of the metabolic processes in the host plant, led to an increase in virus content (Table 2). This occurred regardless of the degree of susceptibility of the plant and was particularly noticeable in the tap root and hypocotyl. The ratio of the values derived from the susceptible and resistant plants show that rhizomania resistant varieties can readily be differentiated on the basis of the virus content of these tissues.

Discussion

The results of the present study indicate that, in addition to known differences in total virus content, differences also exist in the relative distribution of BNYVV within plants differing in their degree of susceptibility to rhizomania. These different distribution patterns may result from different rates of translocation of the virus.

At this stage, we can only speculate as to the cause of the apparent differences in virus translocation from the primary sites of infection, the lateral rootlets, into the tap root and further into the hypocotyl. According to the "source and sink model" of transport physiology, differences in concentration are the driving forces in transport processes. However, whether the differences found in virus content of lateral rootlets are the result of different translocation rate, cannot be established from the results reported here.

Several authors have postulated a relationship between the molecular organization of the virus, specifically the number and type of subunits, and its spread in plants (e.g. Koenig and Burgermeister, 1989). The possibility has also been suggested that the virus vector, Polymyxa betae, contributes to the spread of the virus in the plant, in that damage to host tissue caused by the fungus would facilitate virus movement (Gerik and Duffus, 1988). Finally, anatomical-morphological barriers, which would interfere with the translocation of BNYVV from lateral rootlets into the upper parts of the plant, have been proposed (Hillman and Schlösser, 1984). However, the nature and location of these barriers have yet to be defined.

Preliminary experiments, carried out in cooperation with sugar beet breeders, have yielded good results with regard to the usefulness of the young plant test (Bürcky and Büttner, 1989 b). Information on differences in the distribution pattern of the virus in roots, as an additional criterion, could improve the value of such greenhouse tests for selecting breeding lines with enhanced resistance to rhizomania.

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DISTRIBUTION OF BEET NECROTIC YELLOW VEIN VIRUS IN MECHANICALLY INOCULATED SUGARBEET PLANTLETS OF CULTIVARS WITH DIFFERENT DEGREES OF RIZOMANIA RESISTANCE

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Summary

Mechanical inoculation of sugarbeet seedlings 6 to 9 days old with beet necrotic yellow vein virus (BNYVV) by means of vortexing led not only to root infections, but also to a limited systemic spread of the virus in the upper parts of the plants. Cotyledons as well as the first leaf pairs and their petioles all had a high virus content independent of the sugarbeet cultivar. In the later leaf pairs, however, and especially in their petioles the virus content was higher in highly susceptible than in partially resistant cultivars. This could be the basis for a preselection test for virus resistance, because petiols are very easy to handle, do not require time-consuming washings as roots do and the plants can be saved with a minimum of injury for further breeding. Seasonal differences in the results of our tests suggest that susceptibility to rizomania is not the only parameter which influences the spread of the virus in a plant. Temperature and perhaps light may also play an important role. A careful investigation and standardization of these parameters is necessary before the test can be passed on to the breeders.

Introduction

Under natural conditions beet necrotic yellow vein virus (BNYVV) is transmitted from sugarbeet roots to sugarbeet roots by means of the vector Polymyxa betae (Tamada, 1975). For scientific as well as for some practical purposes the mechanical inoculation of sugarbeet roots with BNYVV offers a number of advantages. The behaviour of isolates with different RNA compositions and of mutants which may not be transmissible by the vector can easily be studied. Also, the time of inoculation is exactly defined and there is only one inoculation event which is especially important in studies on the translocation of the virus. For breeding purposes it is important that the virus resistance can be clearly distinguished from vector resistance. In previous studies using mechanical inoculation we found that isolates which contain intact RNA 3 and 4 are spreading more readily in the root system than isolates with partially deleted RNA 3 and 4 or isolates which lack these small RNAs altogether (Koenig and Burgermeister, 1989; Koenig and Ehlers, 1989). We also found that the spread of the virus is more efficient in highly susceptible than in partially resistant varieties (Koenig and Ehlers, 1989). In such studies we also often observed systemic symptoms in the first two or three leaf pairs, especially in highly susceptible cultivars (Koenig and Stein, unpublished). Leaves which were formed later rarely showed systemic symptoms. We have checked these phenomena in more detail now with the aims of getting more information on the mechanism of resistance and of possibly developping a preselection test for partially resistant sugarbeet cultivars.

Materials and Methods

Seeds of sugarbeet cultivars with different degrees of rizomania susceptibility were kindly provided by German breeders (KWS, Einbeck; Dieckmann/Strube Nienstädt, Schöningen). Several means of introducing the virus into seedling roots were tried, e. g. rubbing,

pricking with glass wool, vortexing etc. The best results were obtained when young seedlings (15 at a time) were placed in a glass centrifuge tube, diameter c. 2.3 cm, together with 3 ml inoculation mix containing 0.09 g carborundum and were vortexed for 1 minute (Vortex Genie 2TM, speed setting 6 to 7). The inoculation mix consisted of 1 volume of sap from leaves of Tetragonia expansa which were heavily infected with an isolate of BNYVV having intact RNAs 1, 2, 3 and 4 and of 3 volumes of 0.05 M sodium phosphate buffer pH 7.2. After vortexing the plants were left for 5 minutes in the inoculation mix and were then planted into a 2:1 mixture of soil (TKS 1) and sand. ELISA was done as described by Clark and Adams (1977).

Results

The optimal age for seedling inoculation was 6 to 9 days. At that time the seedlings had fully developped cotyledons but no true leaves. Seedlings older than 12 days never became infected.

In attempts to determine the minimum time necessary for a preselection test we checked the virus concentrations in different parts of the plants at various times after inoculation. Cotyledons which were tested 2 weeks after inoculation (Fig. 1a) had a high virus content in susceptible as well as partially resistant cultivars. Young leaves and petioles tested 3 weeks after inoculation had a somewhat lower virus content in the partially resistant than in the highly susceptible cultivars, but the differences were not very pronounced (Fig. 1b). Two months after inoculation we observed pronounced differences in the virus concentration in highly susceptible and partially resistant cultivars especially in tap and side roots and in the petioles of the youngest fully developped leaves. Less pronounced differences were observed in the virus concentrations in leaves and hypocotyls (Fig. 1c). In another experiment we compared 6 weeks after inoculation the virus concentrations in petioles of the first, second and third leaf pair of plantlets belonging to 4 cultivars with different degrees of resistance to BNYVV. A clear correlation between virus content and susceptibility was found with the petioles of the third, but not of the first or second leaf pair (Fig. 1d). Such a correlation was still detected in petioles of young leaves 3 months after inoculation, but at that time virus concentrations on the whole had become very low (Fig. 1e).

Conclusions

It is evident from our experiments that the mechanical inoculation of seedlings by means of the vortexing method results not only in root infections (Koenig and Burgermeister, 1989; Koenig and Ehlers, 1989) but also in a limited spread of the virus in the upper parts of the plants which at the time of inoculation had not yet been developped. This spread is apparently more efficient in highly susceptible than in partially resistant cultivars. A correlation between the efficiency of virus spread and rizomania susceptibility had previously also been observed in roots which were either inoculated mechanically (Koenig and Ehlers, 1989) or by means of the vector Polymyxa betae (e.g. Bürky and Büttner, 1988; Casarini Camangi and Canova, 1988; Poggi Pollini and Guinchedi, 1989) and in local lesions on sugarbeet leaves (Grassi et al., 1988).

The results of our experiments suggest that measurements of the virus concentration in the petiols of the third or possibly a later leaf pair of mechanically inoculated plantlets could form the basis for a preselection test for rizomania resistance. As compared with roots

petiols offer a number of advantages: they are easier to handle than roots, they do not require time-consuming washings and interesting plants can be saved with a minimum of injury for further breeding.

The susceptibility of a cultivar to rizomania, however, is not the only parameter which influences the spread of the virus in the plant. In addition to the RNA composition of the virus isolate used for inoculation (Koenig and Ehlers, 1989) apparently also other parameters such as temperature and perhaps light play an important role in the spread of the virus. The experiments described in this paper were done in the winter months when the greenhouse temperatures were around 22° C. In a recent experiment which was started in summer when the temperatures in the greenhouse were between 25 and 30° C the plants showed unusually strong leaf symptoms and many of them were severely stunted even in the most resistant cultivar 1. The virus content in all plants was very high and seven weeks after inoculation the differentiation between cultivars on the basis of virus content in the petiols was much less clear than in Figs. 1c, 1d and 1e. Thus, before a preselection test can be offered to the breeders further research will be necessary in order to define the optimal growing conditions for the plants after mechanical inoculation.

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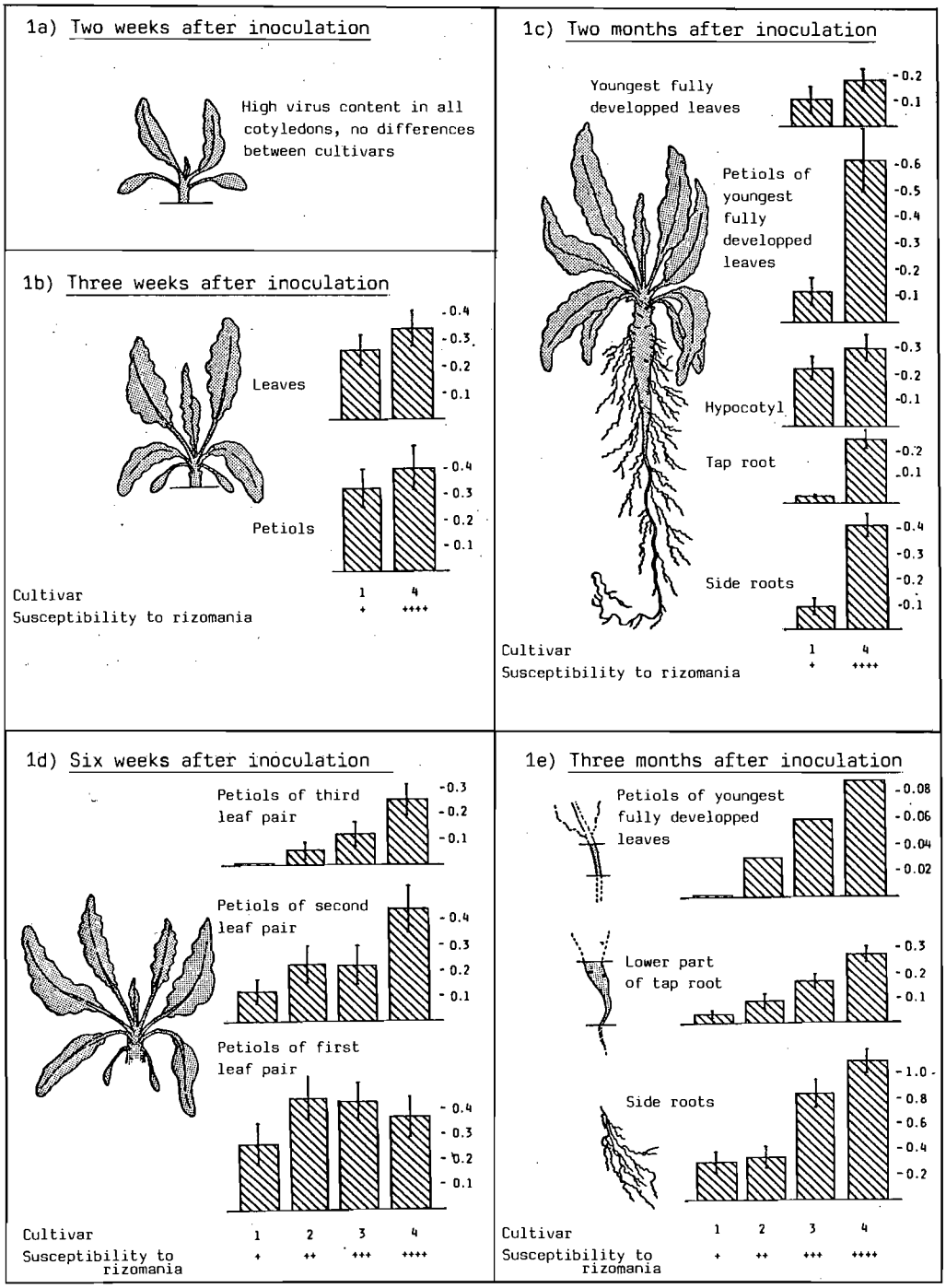

Fig. 1 BNYVV ELISA readings (per unit weight) in various parts of sugar beet plants of cultivars with increasing degrees of susceptibility (+ to ++++) to rizomania

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Sugar-beet transformation for rhizomania resistance

Coat protein-mediated protection has thus far been proven to successfully protect against virus infection in the case of 8 virus-host combinations. As a first step towards the obtention of sugar-beet resistant to rhizomania, we have investigated whether engineered BNYVV coat protein protection could be extended to sugar-beet using protoplasts as a model system.

Transformed sugar-beet suspension cultures were obtained after co-cultivation of sugar-beet cells with *Agrobacterium tumefaciens* harbouring a binary vector containing the coat protein gene of BNYVV inserted between the kanamycin resistance gene and a ß-glucuronidase reporter gene. Protoplasts were isolated from untransformed and transformed cells expressing the viral coat protein and both were infected with BNYVV. Comparison of the levels of infectivity shows that the expression of the coat protein gene in sugar-beet protoplasts mediates high levels of protection against infection by BNYVV.

In parallel, transformation methods for sugar-beet have been worked out.

Transformed sugar-beet,expressing the BNYVV coat-protein gene specifically in the roots,have been obtained. Whether the coat protein mediated protection observed in protoplasts will also be effective in the plants,will now be assessed.

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POTATO MOP-TOP VIRUS, DETECTION AND DIAGNOSIS

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Summary

An investigation of incidence of potato mop-top virus in Sweden was made during 1986-1989. Methods to detect the virus in field soil and soil adhered to the potato tubers was examined.

Introduction

Spraing symptoms in potato tubers caused by potato mop-top virus (PMTV) has been known in Sweden since 1985 (Rydén et al, 1986). This is a serious disease for the production of table- and seed potatoes. The virus is transmitted by the powdery scab fungus *(Spongospora subterranea)*, whose cystosori are able to survive for more than a decade in the field (Jones & Harrison, 1969, Jones & Harrison, 1972). As the virus is carried inside the fungal spore it also survives there and when the disease once is established it has to be a risk whenever a sensitive host is grown later in the same field.

Materials and results

An investigation of the occurrence of PMTV in soil samples from potato fields, mainly from the south of Sweden, was carried out during 1986-1989 (Rydén et al, 1989a). The results of the survey showed PMTV to be fairly common in areas of contract cultivations of potato. Of 376 soil samples, representing 104 farms, PMTV was found in 72 samples (19%), originating from 45% of the farms. The survey was done with the bait plant method.

PMTV was detected in 20 of 48 (42%) soil samples collected during potato grading (Rydén et al, 1989b). This "grading soil" method seems to be of value in the diagnostic work. Compared to the ordinary soil sampling with a probe in the field, collecting soil adhering to the tubers have advantages and a larger part of the field will thus be represented. Probably a more reliable detection of the incidence of PMTV will be achieved by the "grading soil", but it has to be tested by samles from the same field, taken at the same time with each method. Powdery scab spores that might contain virus will fall of infected tubers during grading in a riddle and the "grading soil" contain no clumps and are nearly dry and therefore easy to handle. We continue our work in 1990, testing the important seed producing areas in the north of Sweden with this method. Preliminary, 40 tested samples have been negative with respect to PMTV.

We have also detected PMTV in the "washing water" from potatoes by growing small bait plants in it. There is, however, a risk that these plants become infected by other pathogens and therefore evaporation of the "washing water" in advance and/or antibiotics often are necessary.

Further investigations

Ultrathin sections of PMTV-infected tissue from *Chenopodium quinoa* and *C. amaranticolor* and systemically infected bait plant leaves of *Nicotiana benthamiana* and *N. debneyi* are now being studied in the electron microscope. We will continue the ultrastructural studies with tubers and roots of potato and also tobacco using the immunogold technique.

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PRELIMINARY ANALYSIS OF THE RNA OF POTATO MOP-TOP VIRUS

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SUMMARY

The rod-shaped particles of the T strain of potato mop-top virus have commonest lengths of 250-300 nm and 100-150 nm, and contain either RNA-1 (6.4 kb), RNA-2 (3.0 kb) or RNA-3 (2.5 kb). RNA-2 of strain R was about 0.3 kb smaller than that of strain T. Hybridization tests with cDNA probes showed that RNA-2 is not derived from RNA-1 and that RNA-3 is not derived from RNA-2. The main product of in vitro translation of unfractionated PMTV RNA in a reticulocyte lysate system was a polypeptide of Mr c.20,000, similar to that of the virus coat protein. RNA-2 contains the coat protein gene.

INTRODUCTION

About twenty years have elapsed since the particles and properties of potato mop-top virus (PMTV) were described (Harrison & Jones, 1970), its dependence on fluctuating temperatures for the induction of brown arcs in the flesh of tubers of sensitive potato cultivars was reported (Harrison & Jones, 1971), and evidence was obtained of its transmission by the plasmodiophoromycete fungus, Spongospora subterranea (Jones & Harrison, 1969). Further characterisation of the virus has been handicapped by the low concentration and fragility of PMTV particles in tissue extracts from even the best experimental hosts. However, the particles were found to contain infective RNA (Harrison & Jones, 1970) and a protein of Mr c.20,000 (Randles et al., 1976). In addition, tests with polyclonal antisera detected a distant relationship to tobacco mosaic virus (Kassanis et al., 1972) and a closer one to soil-borne wheat mosaic virus (Randles et al., 1976). Recently, PMTV has attracted renewed interest because of the increasing damage it has caused in crops of a few potato cultivars that are widely grown for processing in Northern Europe (Ryden et al., 1989). Initial results from new studies on the virus are described below and by Torrance (this volume).

RESULTS AND DISCUSSION

Properties of PMTV RNA

Particles of strain T of PMTV were purified from extracts of leaves of Nicotiana benthamiana and N. debneyi by a modification (L. Torrance, personal communication). of the method of Randles et al. (1976). The preparations contained rod-shaped particles of a range of lengths but the RNA extracted from these particles by a phenol-cresol procedure was found to contain three principal components by electrophoresis in 1.2% agarose gel. The sizes of these molecules were estimated, by reference to tobacco mosaic virus RNA and Escherichia coli ribosomal RNA, to be 6.4 kb (RNA-1), 3.0 kb (RNA-2) and 2.5 kb (RNA-3). These figures parallel those reported by Kallender et al. (1990). RNA of PMTV strain R, examined by the same method, had a smaller RNA-2 (2.7 kb) but RNA-1 and RNA-3 of sizes similar to those of strain T.

When unfractionated PMTV RNA was translated in a rabbit reticulocyte lysate system supplied with ^{35}S-methionine, a range of virus-specific

products were made, the largest of Mr >100,000. The main product was of Mr c.20,000, similar to that of PMTV coat protein (Randles et al., 1976).

PMTV cDNA

A random-primed PMTV cDNA library was made in lambda ZAPII phage. One of the pBluescript plasmids released by in vivo excision contained an 80 bp insert which, when used to probe PMTV RNA, reacted with RNA-2 but not RNA-1 or RNA-3. This insert was also used to screen the cDNA library and thereby to select a further set of clones, the largest of which (Tb2) had an insert of 2.3 kb. This insert reacted in Northern blots with PMTV RNA-2 but not with the other two RNA species, showing that RNA-2 is not derived from RNA-1 and that RNA-3 is not derived from RNA-2.

In further tests the lambda expression library was screened, for fusion proteins containing epitopes from PMTV coat protein, by exposing plaque blots to a mixture of four monoclonal antibodies prepared to PMTV particles (Torrance, this volume). Antibody binding was detected by exposure to goat anti-mouse globulin/alkaline phosphatase conjugate and then to NBT/BCIP (p-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoylphosphate p-toluidine salt) substrate. Obvious purple spots were obtained and several of the corresponding phage clones were plaque-purified. One clone (C21) contained a sequence of 500 bp which also occurred in RNA-2, showing that the PMTV coat protein gene is in this genome segment. The results of Northern blotting, restriction endonuclease mapping and nucleotide sequencing indicated that the C21 sequence is contained in Tb2, towards its centre. Further sequencing is in progress to determine the structure of RNA-2 and the arrangement of open reading frames within it.

The results obtained to date show that PMTV differs from tobamoviruses and resembles furoviruses in having a multipartite RNA genome, with the coat protein gene being in the second largest RNA segment, as well as in having a fungal vector. These findings, taken together with the strong serological relationship between PMTV and soil-borne wheat mosaic furovirus, reinforce the evidence that PMTV should be considered a definitive furovirus.

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A PRELIMINARY ANALYSIS OF THE PROTEIN AND NUCLEIC ACID COMPONENTS OF POTATO MOP-TOP FUROVIRUS

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Summary

Analyses by polyacrylamide gel electrophoresis indicated that potato mop-top virus particles contain a single polypeptide with an estimated molecular weight of 19.1 Kd and three ss-RNAs of 6.5, 3.2 and 2.5 Kb, respectively. Infected, but not healthy, leaves of *Nicotiana benthamiana* and *N. debneyi* contained a ds-RNA of 3.2 Kbp and, in lower concentrations, two ds-RNAs of 6.5 and 2.4 Kbp. The M_r of the capsid polypeptide and the number and sizes of the viral ss-RNAs provide additional evidence for the inclusion of PMTV in the furovirus group.

Introduction

When the furovirus group was recognised by the International Committee on the Taxonomy of Viruses as a separate taxon (Brown, 1989), potato mop-top virus (PMTV) was included as a definite member mainly on the basis of its transmission by the plasmodiophorid fungus *Spongospora subterranea* and having rod-shaped particles *c.* 20 nm wide and of two modal lengths of *c.* 125 and 290 nm (Kassanis, Woods and White, 1972; Roberts and Harrison, 1979). Virus-infection significantly decreases the yield and quality of susceptible potato cultivars in Northern and Central Europe, the Andean region of South America, Africa, Israel, Japan and possibly also in other countries in which *S. subterranea* occurs (Harrison and Jones, 1970; Jones, 1988). There is much recent information on the molecular organisation and replication strategies of other furoviruses such as wheat soil-borne mosaic (Shirako and Brakke, 1984a, b; Hsu and Brakke, 1985a, b; Shirako and Ehara, 1986), peanut clump (Reddy *et al.*, 1985; Mayo and Reddy, 1985) and sorghum chlorotic spot (Kendall, Langenberg and Lommel, 1989), and of possible furoviruses including beet necrotic yellow vein (Steven *et al.*, 1981; Bouzoubaa *et al.*, 1985, 1986 and 1987; Ziegler *et al.*, 1985, 1989) and Nicotiana velutina mosaic (Randles and Rohde, 1990). Despite its similarities to these viruses and its considerable economic importance, the particle components of PMTV have long remained uncharacterised. We report here that PMTV particles contain a single capsid polypeptide and three ss-RNAs which correspond to three ds-RNAs extracted from infected plants, results which provide additional support for the inclusion of the virus in the furovirus group.

Materials and Methods

All plants were grown in glasshouses maintained at 16-22°C, with natural light being supplemented as required to an 18 hr photoperiod with mercury vapour lamps. Isolate T of PMTV, kindly supplied by the late P. Massalski (Scottish Crop Research Institute, Invergowrie, Dundee, Scotland) was usually propagated in *Nicotiana benthamiana* seedlings in which it induced chlorosis and distortion of systemically infected leaves; it was, however, occasionally propagated in *N. debneyi* plants in which, under our conditions, it sporadically induced only mild symptoms.

PMTV was purified from *N. benthamiana* (Kallender, Buck and Brunt, 1990) by slight modification

of the procedure used for the purification of beet necrotic yellow vein virus (Putz and Kuszala, 1978).

The molecular weight of the capsid protein was determined by discontinuous polyacrylamide gel electrophoresis as described by Laemmli (1970). Nucleic acid released from particles (Kallender, Buck and Brunt, 1990) was analysed under denaturing conditions in 1% agarose gels containing 10 mM methymercuric hydroxide as described by Bailey and Davidson (1976), using tobacco mosaic virus RNA (6.4 Kb) and red clover necrotic mosaic virus RNA-1 (4.0 Kb) and RNA-2 (1.4 Kb) as size standards.

Double-stranded RNA was obtained by extracting total RNA from systemically infected *N. benthamiana* plants (Taylor and Powell, 1982) and removing ss-RNA by precipitation with 2M LiCl (Baltimore, 1966). The ds-RNA was then precipitated from the solution by incubation at -70°C for 1 hr after the addition of 0.1 vol. of 3M sodium acetate, 0.1 vol. isopropanol and 10 μg/ml of tRNA, collected by centrifugation, dried under vacuum and resuspended in sterile distilled water. It was analysed in 5% polyacrylamide gels (Bozarth and Harley, 1976) by co-electrophoresis with ds-RNA size standards from *Penicillium stoloniferum* and *Aspergillus foetidus* viruses (Bozarth and Harley, 1976; Buck and Ratti, 1977).

Results

Components of Virus Particles

i. Capsid protein. Only a single polypeptide was detected in virus preparations by PAGE. Its size, estimated from three determinations, was found to be 19,100 daltons.

ii. Nucleic acid. Nucleic acid extracted from particles was completely digested by incubating preparations for 30 min at 37°C with ribonuclease A (1 μ/ml) in 10 mM tris-HCl at pH 8.0, results indicating that the particles contain ss-RNA.

Analysis by PAGE showed that the RNA preparations contained three ss-RNAs in near equimolar amounts of 6.5, 3.2 and 2.5 Kb.

Double-Stranded RNA from Infected Plants

Three double-stranded RNAs were extracted from infected, but not healthy, *N. benthamiana* and *N. debneyi* plants and had sizes of 6.5, 3.2 and 2.4 Kbp respectively; the 3.2 kbp ds-RNA was always present in the highest concentration. The nucleic acids were shown to be double-stranded RNAs by their resistance to DNase 1, and their resistance to ribonuclease A in high salt but susceptibility in low molar saline sodium citrate buffer (Buck *et al.*, 1971).

Discussion

Members of the furovirus group characteristically have bipartite ss-RNA genomes (Brown, 1989; Brunt and Richards, 1989). RNA-1 and RNA-2 of wheat soil-borne mosaic virus (WSBMV), the type member of the group, are 6.5-6.9 Kb and 3.5 Kb (Shirako and Brakke, 1984a; Shirako and Ehara, 1986) although deleted forms (2.1-2.4 Kb) of RNA-2 also occur in cultures of the virus maintained in plants at 15-17°C (Shirako and Brakke, 1984a, b; Hsu and Brakke, 1985a, b; Shirako and Ehara, 1986). The sizes of the two larger RNAs of PMTV (6.5 and 3.2 Kb) are very similar to those of WSBMV, and the smallest (2.5 Kb) is comparable to deleted forms of its RNA-2. The genomic RNAs of PMTV are of similar sizes also to those of other definite furoviruses such as

peanut clump (Reddy *et al.*, 1985) and sorghum chlorotic spot (Kendall *et al.*, 1989).

The estimated size of the single capsid polypeptide of PMTV is similar to that reported previously (Kassanis *et al.*, 1972; Randles *et al.*, 1976), and comparable with values reported for other definite and possible furoviruses (Brunt and Richards, 1989).

Our preliminary results thus provide additional evidence for the inclusion of PMTV in the furovirus group. However, the genomic components of the virus now need to be further characterised, and their sequence, organisation and expression determined.

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INVESTIGATION OF POTATO MOP-TOP VIRUS WITH MONOCLONAL ANTIBODIES

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Summary

Monoclonal antibodies (MAbs) specific for potato mop-top furovirus (PMTV) have been produced. They detected nine PMTV isolates from five countries in enzyme-linked immunosorbent assay (ELISA). The antibodies were labelled with biotin, and an ELISA incorporating biotin-labelled and unlabelled antibodies was developed for routine virus testing.
Preparations of PMTV coat protein produced one band of approximate Mr 22,500 after electrophoresis in polyacrylamide gels and staining with Coomassie blue. However, after silver staining some additional bands were seen (approx. Mr 21,000 & 25,000). All of the MAbs reacted with the 22.5K band (the putative coat protein), and most of the MAbs also reacted with the other bands in immunoblotting tests. The nature of the extra bands is uncertain but one is probably a degraded form of the coat protein.
Electron microscope serology with MAbs SCR 68 and SCR 69 showed that SCR 69 coated the full length of the virus particles whereas SCR 68 did not seem to coat the particles. Immunogold labelling revealed that SCR 68 was specific for an epitope at one extremity of the particles.

Potato mop top furovirus (PMTV) is transmitted by Spongospora subterranea. It causes brown arcs and rings in the flesh of tubers of sensitive potato cultivars and these can be confused with the symptoms of tobacco rattle virus (Jones, 1988). PMTV occurs in the Andean region of South America, Northern and Western Europe and Japan. In the last few years there have been serious outbreaks of PMTV in potato crops in Scandinavia and Finland (Kurppa, 1990; Ryden et al., 1989). Supplies of specific antisera to PMTV are scarce and for want of a more reliable procedure most diagnosis has been based on visual symptoms in the tubers. However, satisfactory results have been obtained recently using ELISA to test extracts of tuber flesh and sprouts (Kurppa, 1990).

A Scottish isolate of PMTV (PMTV-T) was purified by a modification of the method of Randles et al. (1976) and both polyclonal and monoclonal (MAb) antibodies were produced (L. Torrance, unpublished results). Four out of the ten MAbs investigated reacted in indirect ELISA where virus antigen was trapped on microtitre plates by polyclonal antibodies. All four readily detected nine PMTV isolates from Scotland, Northern Ireland, Sweden, Finland and Denmark. An assay for PMTV was devised utilising MAb SCR 68 to coat microtitre plates, biotin-labelled MAb SCR 69 as detecting antibody and a streptavidin-alkaline phosphatase conjugate to reveal the bound biotin-labelled MAb. This assay reliably detected PMTV in leaf sap of both potato and Nicotiana benthamiana.

Usually only one band of approximate Mr 22,500 was seen after PMTV-T coat protein preparations were subjected to electrophoresis in polyacrylamide gels (PAGE) and stained with Coomassie blue. However, when the gels were silver stained some additional bands were seen (approximate Mr 21,000 and 25,000). When the bands were transferred electrophoretically to nitrocellulose membrane and then reacted with the MAbs (immunoblots), all ten MAbs reacted with the 22.5K band and most of the MAbs also reacted with the other two. The 25K and 22K bands were excised from the gel and treated with cyanogen bromide then separated in 16.5% PAGE (Nikodem & Fresco, 1979). The products of CNBr treatment suggested some homology between the two bands.

The nature of the different bands is uncertain at present. The 21K protein is probably a degradation product of the virus coat protein as treatment of the particles of another PMTV isolate (R) with trypsin causes conversion of the 22.5K to the 21K form (L.G. Pereira & L. Torrance, unpublished results). The 25K band may result from a read-through of the coat protein termination codon as occurs with soil-borne wheat mosaic virus (Brakke & Langenberg, 1987). Alternatively, both the 22.5K and 21K bands may be degradation products of the 25K band.

In electron microscope serology tests, PMTV particles were trapped on electron microscope grids which had been previously coated with either MAb SCR 68 or 69. In addition, SCR 69 coated the full length of the particles thickly and evenly whereas SCR 68 and SCR 70 did not seem to coat the particles. After labelling SCR 68 with 10 nm gold particles (I.M. Roberts & L. Torrance, unpublished results), tests showed that SCR 68 reacted with an epitope which was present at only one extremity of the particles.

Therefore the results of the different tests indicate that MAbs SCR 68, 69 and 70 react with three distinct epitopes on PMTV particles. Further work is in progress to determine the nature of the three bands found in the coat protein preparations and to investigate the reactivities of the other MAbs.

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BARLEY YELLOW MOSAIC VIRUS AND BARLEY MILD MOSAIC VIRUS: STRAINS AND HOST RESISTANCE

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Summary

Comparative studies of twenty-three isolates of barley yellow mosaic virus (BaYMV) collected at various localities in Japan revealed presence of six strains with characteristic pathogenicities to differential barley cultivars. The incidence of the BaYMV strains showed regional variations, and appeared to be related to the cultivation of different types of barley. The six BaYMV strains were serologically indistinguishable and unrelated to barley mild mosaic virus from West Germany (BaMMV-M). Two BaMMV isolates newly collected at different localities in Japan were different from the six BaYMV strains but similar to BaMMV-M in their pathogenicities to cvs. Ishukushirazu and Haganemugi. They differed from each other in their pathogenicities to cv. Misato Golden and in their serological relation to BaMMV-M, suggesting that they are different strains of BaMMV. Comparative sequence analysis of the BaYMV strains is in progress.

Characterization of Japanese strains of barley yellow mosaic virus

Barley yellow mosaic virus (BaYMV), transmitted by the fungus Polymyxa graminis, is the most widespread and economically important virus of winter barley in Japan. It causes particularly serious damage to two-rowed barley crops. In the absence of suitable chemical control of the virus or its vector, use of cultivar resistance is the only practical means to avoid damage caused by the virus. In the last decade, several two-rowed barley cultivars resistant to BaYMV have been developed. Misato Golden, Mikamo Golden, Nishino Gold and Kinu Yutaka inherit a resistance gene Ym1 from a Chinese six-rowed cultivar, Mokusekko 3. Ishukushirazu inherits a gene ym3 from a Japanese six-rowed cultivar, Haganemugi. Their success was dramatic in eradicating BaYMV from the crops in some barley-growing areas. However, the occurrence of BaYMV in Misato Golden was observed in 1984 at one site and thereafter at three other sites. Characterization of BaYMV strains with different pathogenicities is thus required.

The sap inoculation tests of twenty-three BaYMV isolates collected at various localities in Japan revealed significant differences in their pathogenicities to barley cultivars (Table 1) (Kashiwazaki et al., 1989a). On the basis of their pathogenicities to selected two-rowed barley cultivars, they are grouped into three types, I, II and III. Type I is separated into three subtypes, I-1, I-2 and I-3, based on their pathogenicities to two six-rowed barley cultivars, Kashimamugi (hulled) and Joshushiro Hadaka (naked). Type II is also divided into

two subtypes, II-1 and II-2, based on their pathogenicities to Kashimamugi. The data suggest presence of six strains (pathotypes) of BaYMV in Japan. Strain III is remarkable for its pathogenicity to Misato Golden. Barley mild mosaic virus from West Germany (BaMMV-M) (Huth et al., 1984) differs from the six BaYMV strains in its pathogenicity to Ishukushirazu and Haganemugi which are resistant to all the six BaYMV strains.

Table 1. Differential reactions of barley cultivars to BaYMV and BaMMV isolates by sap inoculation (Kashiwazaki et al., 1989a)

Barley cultivar	BaYMV I-1	I-2	I-3	II-1	II-2	III	BaMMV M
Two-rowed							
New Golden	+[a)]	+	+	+	+	+	+
Haruna Nijo	+	+	+	-	-	+	+
Misato Golden (Ym1)	-	-	-	-	-	+	+
Ishukushirazu (ym3)	-	-	-	-	-	-	+
Six-rowed							
Tosan Kawa 73	+	+	+	+	+	+	+
Kashimamugi	-	+	+	-	+	+	-
Joshushiro Hadaka	-	-	+	-	-	-	+
Mokusekko 3 (Ym1)	-	-	-	-	-	-	-
Haganemugi (ym3)	-	-	-	-	-	-	+

a) Letters indicate infected (+) or not infected (-).

Table 2. Comparison of the properties of BaYMV and BaMMV isolates (Kashiwazaki et al., 1989a)

	BaYMV I-1	I-2	I-3	II-1	II-2	III	BaMMV M
Capsid protein Mr($x10^3$)	33	33	33	33	33	32	31
RNA-1 Mr($x10^6$)	2.57	2.57	2.57	2.57	2.57	2.57	2.57
RNA-2	1.40	1.40	1.40	1.45	1.40	1.40	1.35
Serological reaction							
Ant-II-1	1280[a)]	1280	1280	1280	1280	1280	<10
Ant-III	1280	1280	1280	1280	1280	1280	<10
Ant-M	<10	<10	<10	<10	<10	<10	640

a) Figures are reciprocals of dilution end points of respective antisera with positive reaction in complement-fixation tests.

Some properties of the six BaYMV strains and BaMMV-M are presented in Table 2. Strain III is somewhat different from five other strains in the Mr of its capsid protein. Strain II-1 also slightly differs from others in the Mr of RNA-2. The six BaYMV strains are serologically indistinguishable and unrelated to BaMMV-M. In the absence of specific antisera to the BaYMV strains, the use of differential barley cultivars is the only means available for their diagnosis.

The incidence of the BaYMV strains showed regional variations, and appeared to be related to the cultivation of different types of barley. Strain I-1, for example, occurred in the areas where the planting of two-rowed barley is common. It did not infect most six-rowed barley cultivars tested by sap inoculation. Likewise, strains I-2 and I-3 showed clear correlation to the planting of six-rowed hulled and naked barley, respectively.

According to the latest field survey at various localities in Japan, Kashimamugi had BaYMV infection only in the areas where six-rowed hulled barley, especially Kashimamugi, has long been planted. In contrast, Joshushiro Hadaka had BaYMV infection only in the areas where six-rowed naked barley has long been planted. Neither Kashimamugi nor Joshushiro Hadaka had BaYMV infection in the areas where two-rowed barley has long been grown. Therefore, the distribution of three major strains, I-1, I-2 and I-3, in barley-growing areas in Japan was confirmed based on the cultivar reactions in the fields.

In the fields where Misato Golden had BaYMV infection, the virus occurred in this cultivar at the first planting. This suggests that the virus which infects Misato Golden had been present in the fields before this cultivar was introduced. Fortunately, the incidence of BaYMV in Misato Golden has so far been limited in the four sites. Moreover, in Misato Golden, the symptoms caused by BaYMV were mild and the yield reduction due to the virus was less than 10%.

Occurrence of barley mild mosaic virus in Japan

BaMMV has been identified in several European countries, but there are no reports of similar viruses outside Europe (Huth & Adams, 1990). In 1987, the occurrence of yellow mosaic symptoms in Ishukushirazu was first observed at two sites in Japan, from where BaMMV was obtained. The two BaMMV isolates, one (Ka1) from Ishukushirazu and the other (Na1) from Kinu Yutaka, have been partially characterized (manuscript in preparation).

The two isolates infected Ishukushirazu and Haganemugi by sap inoculation as did BaMMV-M. Na1 infected Misato Golden, whereas Ka1 did not. In agar gel diffusion tests, Ka1, Na1 and BaMMV-M reacted with antisera against Ka1 and BaMMV-M, but did not with antisera against BaYMV strains II-1 or III. With antisera against Ka1 and BaMMV-M, both Ka1 and BaMMV-M spurred over Na1, but no spur was observed between Ka1 and BaMMV-M. The data suggest that Ka1 and Na1 are different strains of BaMMV.

In the field from where Na1 was obtained, all the BaYMV-resistant two-

rowed barley cultivars which inherit Ym1 or ym3 had BaMMV infection. BaYMV occurred in susceptible barley cultivars in this field. The barley plants naturally infected with BaMMV developed very mild symptoms in spring later than the appearance of symptoms in the plants infected with BaYMV.

The occurrence of BaMMV has so far been confirmed only at the two sites, although a great number of barley samples collected at various localities have been examined by ELISA. It seems therefore likely that the incidence of BaMMV is at present limited in Japan.

Comparative sequence analysis of BaYMV strains

The BaYMV genome consists of two single-stranded RNA species, RNA-1 and RNA-2, which contain poly (A) tails at the 3' termini (Usugi et al., 1989; Kashiwazaki et al., 1989b). It seems possible to differentiate the BaYMV strains by hybridization with specific cDNA or oligonucleotide probes.

A cDNA library has been constructed for the genome of BaYMV strain II-1 (Kashiwazaki et al., 1989b). The complete sequences of RNA-1 and RNA-2 of this strain have been determined. RNA-1 encodes a single 270K polyprotein in which the capsid protein is mapped at the carboxy terminus by protein sequencing (Kashiwazaki et al., 1989b). This 270K protein contains three other regions which share significant amino acid homologies with the NIb polymerase, NIa proteinase and cytoplasmic inclusion proteins of potyviruses. RNA-1 may therefore play a major role in replication of the virus. RNA-2 also encodes a single 98K protein which contain a region corresponding to the helper component proteinase of potyviruses.

The cDNA libraries of strains I-1 and III have been constructed, and their sequence analysis and hybridization experiments are in progress.

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Resistance to barley yellow mosaic virus and to barley mild mosaic virus in barley.

Barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV) cause severe diseases in winter barley in western Europe.In France,high-yielding cvs resistant to the common strain of BaYMV ($BaYMV_1$) and to BaMMV have been developed in the last ten years. These resistant cvs,however,do not have sufficient brewery qualities. More recently,the existence of other strains or pathotypes,named collectively $BaYMV_2$,which infect cvs resistant to $BaYMV_1$,is a major threat to barley crops. The aim of this paper is to specify which are the areas where the $BaYMV_1$-BaMMV have been detected,and to indicate how the $BaYMV_2$-contaminated surface areas have progressed in the last few years in France. In addition,results on the behaviour of barley towards the BaYMV-BaMMV complex and the vector of these two viruses are reported.

Materials and methods

BaMMV was ELISA-detected.Two antisera were used:one kindly provided by Dr. HUTH (Braunschweig RFA) and our own antiserum which has been more recently prepared. Plant extracts were diluted 10 times in a citrate buffer pH 7 + 0,5 urea. A French isolate of BaMMV (from cv Magie,Reims) was maintained in a growth chamber at 17°C. BaMMV inoculum was prepared as follows: young leaves with clear mosaic symptoms were grinded in a phosphate buffer 0.04 M pH 7 at 4°C. Leaf extracts were immediately inoculated to 24 plants per cv. After being inoculated twice by finger,the plants were kept for 20 days,and the presence of the virus detected by ELISA.BaYMV(pathotypes1and2) was only ELISA-detected with an antiserum (supplied also by Dr. HUTH) under the same conditions as for BaMMV. A BaYMV isolate was considered as $BaYMV_2$,when it had infected barley cvs resistant to $BaYMV_1$. Susceptibility to *Polymyxa graminis* was measured through the quantity of cystosori produced on barley roots. The quantity of cystosori from plants grown in a growth chamber was estimated by the surface of contaminated roots. Roots of field plants were obtained by gently washing soil samples of approximately 20 X 20 X 20 cm. Cystosori were counted after their partial purification.The10µ filter residue was resuspended in one ml of sterile water.The cystosori in two samples of one µl were counted under a dissecting microscope.The concentration of cystosori from a sample of about 10 plants was calculated for one plant.

Results

1.Distribution of $BaYMV1$ and BaMMV in France.

The $BaYMV_1$-BaMMV complex was present in most of the French departments where winter barley is grown. In the areas reclaimed from the Ocean (Poitou-Charentes region),the complex was observed less than ten years after the land was first sown with barley. However,the viral complex was not detected in all of France,especially in Brittany whose soil and cultivation conditions,at least locally,promote the expression of the two viruses.$BaYMV_2$ was detected in all barley-growing areas,although few fields have been found contaminated until now. However,the number of infected fields is rapidly increasing (Tabl.1).$BaYMV_2$ was frequently observed as from the first cropping of a $BaYMV_1$-resistant cv,which implies the simultaneous presence of $BaYMV_1$.

2. BaMMV presence in fields already contaminated by $BaYMV_2$.

BaYMV has been detected in two fields which had previously been infected by $BaYMV_2$.$BaYMV_1$-suceptible and -resistant barley cultivars were sampled at three differents stages of development on one of the two fields located near Reims (Tabl.2, I to II).In all of the cultivars sampled,BaMMV was never detected in the absence of $BaYMV_2$. The presence of BaMMV was observed for the three sampling dates in most of the $BaYMV_1$-suceptible cultivars,with a large number of plants being infected. BaMMV was absent or detected less frequently in $BaYMV_1$-resistant cultivars.

3. Barley susceptibility to mechanical inoculation with BaMMV.

Numerous barley cultivars which,in the field were susceptible to BaYMV-BaMMV complex were susceptible to mechanical inoculation of BaMMV,whereas most cultivars resistant to the complex in the field were also resistant to mechanical inoculation of BaMMV. A few local French cultivars have the latter type of behaviour (Ile de Ré,Hâtif de Grignon,Marne). It is also the case for cvs for which the genetic basis of resistance is not known (cv Bison). However,several barley cvs which are resistant to the BaYMV-BaMMV complex in the field,have been found susceptible to mechanical inoculation of BaMMV : Comanche,Marianne,Margot,Friberga,L5 351,J6 180.3.

For all these cvs the rate of mechanical transmission was high, although less than one (Tabl.3.I). Lastly,some cultivars which were susceptible in the field,appeared to be either very difficult to inoculate with BaMMV (Vixen) or resistant (Acton,Flamenco, Huron) (Tabl.3.II).

4. Measurement of production of *P.graminis* cystosori by various barley cvs.

a.Plants grown under controlled conditions.Among 37 barley germ-plasm none was found to be totally resistant to *P.graminis* . The production of cystosori was , however, clearly different between cvs in three independent experiments. In cv Plana, J1-487,Margot and WS IA very few cystosori were found compared with the high numbers found in four barley lines from FRG (Giessen),a line from Japan (J5.1723) and two cvs (Viola,Express). Barley cvs for which production of cystosori was the highest were field resistant to the viral complex,whereas the cv Plana was susceptible to both viruses.

b. Field infected plants.Few lines or cvs with distinct susceptibility to P. graminis in growth chamber were tested for their cystosori content in field infected plants. The less susceptible germplasms (cv Plana,J1-487) showed a good correlation with their susceptibility in growth chamber. But for most cystosori productive germplasms (Mo 26,J4-1248) discrepant results were obtained(Tabl.4).

Conclusion

$BaYMV_1$ and BaMMV are widely distributed in France and contrary to the observations made in GB and FRG,both viruses were present simultaneously in almost all the fields surveyed. However,plants carrying only one virus species could be found in the different fields infested by the viral complex. It remains unclear why the BaYMV and BaMMV are absent in the western France whereas it is present in other french regions with smaller acreages of winter barley.The number of fields carrying $BaYMV_2$ have rapidly been increased in France since 1989 as in FRG. This lead to total inversion of $BaYMV_1$ and $BaYMV_2$ inoculums in soil if no $BaYMV_2$-resistant cvs are released in the next few years. For 2 out of 16 fields surveyed,the association of BaMMV with $BaYMV_2$,not exclusively in cvs susceptible to $BaYMV_1$,may be explained either by the presence of another strain of BaMMV or by a synergism between particular strains of BaMMV and BaYMV. Resistance to mechanical inoculation of BaMMV is considered as a good marker of the "Ragusa" type of field resistance to the $BaYMV_1$-BaMMV complex. As it is shown by Adams & al., 1988 and Huth & Adams 1990,we found that the field susceptibility to the viral complex is not always associated with mechanical susceptibility to BaMMV. But specific susceptibility of these cvs to soil infection by BaMMV remains to be demonstrated. On the other hand, those response to mechanical inoculation of BaMMV need to be more studied for this virus.Field resistant cvs which are susceptible to mechanical inoculation of BaMMV probably carry another gene than those of the "Ragusa" type,and may be compared to French winter wheat which are field resistant to wheat soil born mosaic virus(WSBMV). The roots of these wheat cvs are infected by WSBMV,but there is no transportation of this virus to the leaves (Hariri & al., 1987). No resistance to the production of *P. graminis* cystosori was found in 24 barley cvs resistant to $BaYMV_1$ and BaMMV. The same result has been obtained in Japan (Kashiwasaki et al., 1989),the resistance to *P. graminis* is probably very rare or absent in the species *Hordeum sativum* . For the cv Plana,only a few cystosori is sufficient to ensure that infection by the viral complex occurs. The high production of cystosori by cvs resistant to BaMMV and $BaYMV_1$,which are widely developped,is favourable to the dissemination of the less frequent strains of BaYMV.

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	Number of fields			
	1987	1988	1989	1990
ENGLAND	?	+	+	+
GERMANY	?	+	+	> 50 (a)
FRANCE	1	1	4	> 26
BELGIUM	0	0	0	1 (b)

Tabl.1 **Fields where a strain of BaYMV (BaYMV 2) was detected in barley cvs resistant to BaYMV 1.**
(a)From Dr.HUTH (personal communication)
(b)From Dr. MAROQUIN (personal communication)

		Detection of									Intensity of mosaic symptoms (February)
		BaYMV			BaYMV + BaMMV			BaMMV			
	cvs/Date	Feb.	Mar.	Apr.	Feb.	Mar.	Apr.	Feb.	Mar.	Apr.	
I	ASORBIA	10(a)	7	-	0	3	-	0	0	0 -	+ +
	ENERGY	10	8	7	0	1	0	0	0	0	+
	GAULOIS	10	8	2	0	2	0	0	0	0	+ +
	MARIANNE	10	6	6	0	4	3	0	0	0	+
	MELUSINE	10	9	10	0	1	0	0	0	0	+ +
	MOSAR	4	5	3	6	3	0	0	0	0	+ +
	REBELLE	10	5	4	0	4	4	0	0	0	+ +
	TALASSA	9	7	-	0	1	-	0	0	-	+ +
	TORRENT	8	8	-	2	2	-	0	0	-	+ +
	ALTAIR	10	10	7	0	0	0	0	0	0	+ +
	COMANCHE	7	2	0	0	0	0	0	0	0	(+)
	ELARA	10	7	1	0	0	0	0	0	0	+
	EXPRESS	10	10	2	0	0	0	0	0	0	+ +
	GIBSY	10	3	0	0	0	0	0	0	0	+
	MOHICAN	10	0	0	0	0	0	0	0	0	+
II	CATANIA	9	5	6	1	5	4	0	0	0	+ + +
	CELTIC	1	5	5	9	5	5	0	0	0	+ + +
	PLAISANT	7	5	5	2	4	3	0	0	0	+ +
	SONJA	7	5	4	3	5	6	0	0	-	+
	TARGET	5	4	-	0	3	-	0	0	-	+
	CLARINE	10	2	-	0	8	-	0	0	-	+

Tabl.2 **Presence of BaMMV in field plants infected with BaYMV 2.**
I. Barley cultivars resistant to BaYMV 1 (Samples of 10 plants per cv)
II. Barley cultivars susceptible to BaYMV 1
(a) number of infected plants - not tested

Cultivars	Field susceptibility FRANCE (BaYMV+BaMMV)	JAPON	RFA	% of BaMMV infection (Mechanical inoculation)
COMANCHE	R			70
FRIBERGA	R		R	30
MARIANNE	R			62
MARGOT	R			25
J6 1803	S	R		32
L5 35	aR		R	50
PLAISANT	S			100
ACTON	S			0
FLAMENCO	S			0
HURON	S			0
VIXEN	S			<5

Tabl.3 **Susceptibility of different barley cvs to mechanical inoculation of BaMMV.**

Lines or cvs	Field-cultivated plants number of cystosori per plant (1)	Growth chamber cultivated plant visual estimation of cystosori
Plana	80	1
J1 487	130	1
Ile de Ré	140	2
Barberousse	150	2
Classica	180	3
Vixen	180	2
Sentinel	180	1
MD 10	280	2
Magie	290	2
Acton	320	2
Marne	490	2
L 550	670	2
Birgit	730	3
1/2 Hatif Tourettes	2060	2
JS 1723	2240	4
Bison	2380	2
J4 1248	4560	2
Express	14790	4
Mo 26	16860	2

Tab.4 **COMPARAISON OF CYSTOSORI PRODUCTION IN BARLEY CULTIVATED IN THE FIELD OR IN A GROWTH CHAMBER**
(1) Counted from 10 µl filter residue

THE YELLOW MOSAIC INDUCING VIRUSES OF BARLEY IN GERMANY

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Barley infecting soil-borne viruses have been isolated in 1978 for the first time in Europe (Huth und Lesemann, 1978). The isolated viruses were called barley yellow mosaic virus (BaYMV) because at least some particles in crude plant sap as well as in purified virus samples reacted with an antiserum of a BaYMV isolate from Japan. Later it became evident that two different barley-infecting soil-borne viruses occur in Europe (Huth, Lesemann und Paul, 1983) which differ in some of their biological, serological and physical properties (Huth, 1989). The virus which did not react with the BaYMV antiserum has now been namend barley mild mosaic virus (BaMMV) (Huth and Adams, 1990).

Distribution of BaYMV and BaMMV in Germany

BaYMV and BaMMV are common in several European countries (e.g. Adams et al., 1987 , Proeseler et al. 1984). At least in Germany both belong to be the most important pathogens of winter barley. Since their first discovery at three different places they have been spread to most regions in Germany (Huth, 1991). Usually not only single fields but large areas are already totally infested by these viruses. The majority of the infested areas are found in the regions of Lower Saxony, Northrhine-Westfalia and Hessia.
Plants may be infected either by one of the viruses separately or by both simultaneously. The rate of infections by BaYMV and BaMMV has been compared during the past five years (Table 1). Only the results of plant material is listed which has been collected by members of the advisory service stations and was sent for examinations to the Biological Research Station in Braunschweig. From both viruses BaYMV is obviously

Table 1
Frequency of BaYMV and BaMMV in the former Federal Republic of Germany

	number tested plants	BaYMV		BaMMV		mixed infect. by BaYMV + BaMMV	
		no.	%	no.	%	no.	%
1986	115	84	73	18	16	13	11
1987	41	19	46	8	20	14	34
1988	39	25	64	1	3	13	33
1989	155	114	74	3	2	38	25
1990	124	81	65	0	0	43	35

more widely spread than BaMMV. On the average more than 50% of the tested plants were infected by BaYMV alone and nearly one third of all tested plants were in addition infected by BaMMV. On the contrary the percentage of plants infected only by BaMMV decreased and especially during the past three years only few plants were found to be infected by BaMMV alone. Obviously, BaYMV spread more effectively and also those fields became infested which earlier have been infested by BaMMV only.

Appearance of a second strain of BaYMV in Germany

In order to avoid yield losses by BaYMV/BaMMV infections, farmers have prefered for several years now to grow barley cultivars which do not become infected. These cultivars may be called to be immune according to the definition of Cooper and Jones (1983). On those fields where immune cultivars have been grown we have isolated a

new type of BaYMV, BaYMV-2 (Huth, 1989). This virus isolate can not be distinguished from the common European and Japanese BaYMV isolates on the basis of morphology (Lesemann, pers. communic.) nor by means of polyclonal antibodies nor by all those monoclonal antibodies prepared so far (Vetten, unpubl. data). It is distinguishable only by the range of cultivars which are or are not infected. Like BaYMV also BaYMV-2 is serologically unrelated to BaMMV.
BaYMV-2 was first identified in 1988 in three different regions in Germany and also in a barley sample from England (Huth, 1989). Since then in Germany it has been found already on more than 50 fields. Some fields became already totally infested by this virus, but in most fields only few plots sometimes smaller than one squaremeter indicate its occurrence. It seems likely that BaYMV-2 like the other barley infecting soil-borne viruses will continiously spread in the future and will like BaYMV/BaMMV become a threat to the European agriculture .
BaYMV-2 has been detected only on fields which are known to be infested by the common BaYMV/BaMMV and on which exclusively barley cultivars resistant to them have been planted for several years to prevent yield losses. From the primary occurrence of BaYMV-2 on small plots in such fields it may be concluded that the BaYMV/BaMMV susceptible cultivars grown before are rather inefficient propagation hosts of the strain; in those BaYMV/BaMMV susceptible cultivars, especially when simultaniously infected by BaYMV or BaMMV, the propagation of BaYMV-2 may be suppressed. BaYMV-2 is a very instable virus which obviously is transmissible mechanically with difficulty only. Using the method of Friedt (1983) less than 1% of the inoculated barley plants became infected.

Resistance to BaYMV-2

The occurrence of strains of BaYMV is not unusually. Hariri et al. (1990) report in this volume on the occurrence of isolates in France and Belgium with properties which obviously are comparable with them of the German isolate. In Japan, already six different strains of BaYMV have been described (Kashiwazaki et al., 1989). Like the European BaYMV isolates also the Japanese strains are differentiated by their host cultivars only.
In Europe the deviating BaYMV strain appeared spontaneously in several countries at nearly the same time. Even if there are presently no indications, the existence of further strains of BaYMV in Europe can not be excluded. Comparative investigations need to be started.
Future barley breeding programms should be aimed to protect this crop against BaYMV-2. The identification of suitable sources of resistance to BaYMV-2 will be one of the most important tasks. None of the Europeaen commercial barley cultivars is resistant to BaYMV-2.
More than 200 lines and cultivars of barley from several gene banks have been screened for their behaviour on BaYMV-2 infested fields. All these samples are originated from East-Asia and were previously selected for their resistance to both BaYMV and BaMMV (Huth, 1985). Seeds of these samples were planted in fields of two distinct regions south of Braunschweig, Lower Saxony, and close to Paderborn, Northrhine-Wetsfalia, respectively, which are homogeneously infested by all BaYMV, BaMMV and BaYMV-2. The results were the same in both regions; alltogether 116 lines/cultivars remained virus-free when the screening was done visually and by ELISA using BaYMV antiserum (Huth, 1991). These selected lines/cultivars obviously are no hosts of this virus and the quality of their resistance may be comparable with that to BaYMV/BaMMV; they appeare to be immune to all three viruses. One of these cultivars is Mokusekko 3 which is resistant to all of the six Japanese BaYMV strains too (Kashiwazaki et al, 1989).
All of the European cultivars with immunity to BaYMV and BaMMV used in the test became infected by BaYMV-2. Also Ragusa which has been found to be immune to BaYMV and BaMMV was susceptible to BaYMV-2. Because Ragusa probably was the donor of the BaYMV/BaMMV resistance gene (Huth, 1985), the lack of resistance to BaYMV-2 in the European cultivars to BaYMV-2 can be explained.
Differences in the genetics of the resistances of European cultivars and Mokusekko-3, respectively, have been repeatedly reported (e.g. Friedt, 1989). For the first one a recessive gene has been described to be responsible, for the second one a dominant

gene. Both are probably located on different chromosomes.
Different genetic systems in plants are obviously necessary to establish the resistance to the different mosaic-inducing barley viruses as well as their strains, BaYMV, BaMMV and BaYMV-2. They should be combined in future European commercial barley cultivars. Results of the above mentioned screening (Huth, 1991) showed that the resistance to BaYMV-2 is present in a large number of cultivars. Even if most of them are not adapted to the environmental conditions in Europe Mokusekko-3 seems to be one of the possible donors of these resistances because in it the resistance to all of the known virus strains are obviously combined.

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GENETICS OF RESISTANCE TO THE "BARLEY YELLOW MOSAIC VIRUS COMPLEX" AND STATUS OF BREEDING

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Summary

Barley yellow mosaic disease is present in all European countries now. In Germany where extensive yield losses are observed frequently the disease is caused by a complex of three viruses, i.e. BaYMV, BaMMV and a "new virus strain". Resistance of German winter barley cultivars, which is not effective against the "new virus strain", is inherited by a single recessive gene located on chromosome 3. Foreign varieties carrying different resistance genes were identified; some of them are even effective against the "new virus strain". Because of inferior agronomic performance of these exotic resistant germplasms, efficient breeding procedures are needed. In particular, the application of androgenetic doubled haploids in combination with marker based selection schemes is considered to be a very useful tool for the rapid incorporation of these alien resistance genes into adapted varieties.

Introduction

Barley Yellow Mosaic Virus (BaYMV) was first discovered in Japan in 1940 (INOUYE & SAITO, 1975) and is considered to be one of the most severe diseases of Japanese two-rowed malting barley (USUGI, 1988).
In West Germany barley yellow mosaic disease - now recognized to be caused by BaMMV, BaYMV-So (HUTH & ADAMS, 1990) and a "new virus strain" (HUTH, 1989) - was first reported by HUTH & LESEMANN (1978). Whereas the virus occurrence was first limited to some small areas in the northern part of Germany, the disease is now spread over the whole country. Because of this wide distribution and the severe yield losses frequently observed in susceptible barley crops (FRIEDT & GÖTZ, 1987; Table 1) barley yellow mosaic is now one of the most important diseases of winter barley in Germany and other central European countries.

Table 1. Grain yield of BaYMV-resistant as compared to susceptible barley cultivars and breeding lines in Northern Hesse, 1990

Cultivar/Line	BaYMV-reaction	Grain Yield * t/ha	Grain Yield * relative
Asorbia (6-row)	resistant	5.33	100
Corona (6-row)	susceptible	3.53	65
St. 367 (6-row)#	resistant	5.73	107
Romanze (2-row)	resistant	4.20	100
Marinka (2-row)	susceptible	2.38	57
St. 372 (2-row)§	resistant	4.28	102

* Gipper's Farm, Bellnhausen/Gilserberg: 10 m^2 plots, 3 replications; LSD 5% = 0.25t/ha; yield in absolute dry matter. # St. 367 = 'Dea' x 'Resistant Ym No.1', § St. 372 = 'Sonate' x 'Resistant Ym No.1'.

On account of transmission by the soil-borne fungus *Polymyxa graminis* chemical measures against barley yellow mosaic disease are either inefficent or uneconomic. Therefore, yield losses can only be prevented by cultivating resistant varieties. In Germany cvs. 'Asorbia', 'Banjo', 'Brunhild', 'Frances', 'Franka' (six-rowed), 'Diana', 'Romanze' and 'Sonate' (two-rowed) are available. These cultivars are resistant to both, BaMMV and BaYMV-So, but not against the "new virus strain".

For broadening the basis of resistance numerous germplasms were screened for BaMMV reaction by mechanical spraygun inoculation in the greenhouse (FRIEDT, 1983; FRIEDT *et al.* 1988) as well as by natural inoculation in the field (BaYMV-So, "new virus strain"). Several barley stocks, mainly originating in East Asia, were shown to be resistant, both in the greenhouse and in the field.

Genetic analysis of resistance to Barley Yellow Mosaic Virus

Chromosomal localization of the 'German resistance gene'

Analysis of crosses between resistant German cultivars lead to the conclusion that resistance of these varieties is due to one identical recessive gene, because these crosses do not segregate susceptible plants in F_2. This German gene, which was proposed to be called *ym4* (FRIEDT *et al.*, 1990) probably derives from a common parent, the Dalmatian spring barley land-race 'Ragusa' (FRIEDT, 1984).
To determine the definite genetic location of gene *ym4*, trisomic analysis was carried out with a complete trisomic set of the spring barley cv. 'Shin Ebisu 16' kindly provided by Dr. T. Tsuchiya, Colorado State University, Fort Collins (USA). Trisomic plants for each barley chromosome were crossed as females to German resistant cvs. like 'Sonate' and 'Ogra'. Trisomic F_1 plants were identified cytologically and F_2 plants of these crosses were infected mechanically with BaMMV. In the trisomic fractions unexpected segregations with an excess of resistant plants were observed (KAISER & FRIEDT, 1989) which may be explained by the weak growth habit of trisomics and the deleterious effects of the severe inoculation procedure. However, very clear results were obtained in the disomic fraction. All disomic F_2 populations of 'Ogra' crosses as well as of 'Sonate' crosses except the one with 'Pale' (trisomic for chromosome 3) showed a good fit to the uncritical segregation (3:1), while in the disomic F_2 derived from the cross to 'Pale' a good fit to the critical segregation (8:1) was observed. From these results it is concluded that the gene *ym4* is located on chromosome 3 (KAISER & FRIEDT, 1989).

Genetic diversity of resistance to Barley Yellow Mosaic Virus

Three different resistance genes have earlier been described in Japan: *Ym1* deriving from the Chinese landrace 'Mokusekko 3', *Ym2* from 'Mihori Hadaka 3' (TAKAHASHI *et al*, 1973) and *ym3* which was detected in 'Ea 52', an induced mutant of 'Chikurin Ibaraki 1' (UKAI, 1984). It is interesting to note that 'Ea 52' which is resistant in Japan is susceptible against BaMMV in Europe while the parent 'Chikurin Ibaraki 1' reacts resistant here and susceptible in Japan.

Table 2. Segregation of F_2 plants derived from crosses of foreign resistant varieties to resistant German cultivars after mechanical inoculation with BaMMV (GÖTZ, 1990).

Variety	Reaction observed resistant : susceptible	Genetic relationship to 'German resistance'
Asahi 9	16 : 0	allelic or tightly linked
France 7	16 : 0	allelic or tightly linked
Kanto Nijo 19	16 : 0	allelic or tightly linked
Yukishirazu C	16 : 0	allelic or tightly linked
Anson Barley	7 : 9	different
Chikurin Ibaraki 1	7 : 9	different
Ou 1	7 : 9	different

By means of marker analysis evidence for linkages between gene *Ym1* and the marker gene *K* (hooded lemma) as well as between gene *Ym2* and themarker gene *n* (naked kernel) was identified by TAKAHASHI *et al.* (1973), leading to the conclusion that *Ym1* is located on chromosome 4 and *Ym2* on chromosome 1. Furthermore, KONISHI & MATSUURA (1987) and KONISHI *et al.* (1989) observed, that resistant F_2 plants derived from crosses of 'Mokusekko 3' to susceptible varieties always carried the esterase isozyme pattern of 'Mokusekko 3' indicating that an additional resistance gene of 'Mokusekko 3' may be linked to an esterase isozyme gene block at the

terminal end of the long arm of chromosome 3. These results fit well to the observation that the F_1 plants of crosses of resistant German cultivars to 'Mokusekko 3' are all resistant and no susceptible plants segregate in F_2 (FRIEDT & FOROUGHI-WEHR, 1987). Therefore, these resistance genes must be either allelic or very tightly linked.
In order to obtain a more complete view of genetic diversity of resistance against Barley Yellow Mosaic Virus resistant German varieties were crossed to a large number of foreign donors of resistance (GÖTZ, 1990). Some of these crosses segregated in F_2 indicating that the parents carry different resistance genes, while in other crosses no segregation could be detected leading to the conclusion that the resistance genes are allelic or very closely linked (Table 2).
Even in crosses between exotic resistant varieties segregation could be observed (ORDON, 1990) raising hope that there are different resistance genes - some of them even effective against the "new virus strain" (Table 3), which may be used for broadening the basis of resistance of winter barley to barley yellow mosaic disease.

Table 3. Reaction of foreign barley germplasms against BaMMV, BaYMV and the "new virus strain" (GÖTZ, 1990).

Variety	BaMMV	BaYMV	"new strain"
Chikurin Ibaraki 1	resistant	resistant	resistant
Mihori Hadaka 3	resistant	resistant	resistant
Mokusekko 3 *	resistant	resistant	resistant
Muju covered 2	resistant	resistant	resistant
Russia 57	resistant	resistant	resistant
NIR 55 MN-28	resistant	resistant	susceptible
Russia 32	resistant	resistant	susceptible
Anson Barley	resistant	susceptible	?
Krasnodar 1920	resistant	susceptible	?

* in 'Mokusekko 3' plants resistant against the "new virus strain" as well as susceptible plants were identified.

Exploitation of foreign resistance genes by breeding

Because of inferior agronomic performance of exotic resistant varieties (Table 4) long lasting backcross-programmes are needed for incorporating their resistance genes in high yielding adapted cultivars (FRIEDT et al., 1990).

Table 4. Grain yield of foreign resistant varieties in comparison to adapted German resistant varieties at Giessen (Hesse), 1990 (ORDON, 1990).

Cultivar	Origin	Grain Yield *	
Franka (6-row)	Germany	6.89 t/ha	100 %
Asorbia (6-row)	Germany	6.83	99
Russia 32 (6-row)	USSR	4.52	66
Muju covered 2 (6-row)	Korea	3.94	57
Mokusekko 3 (6-row)	China	3.71	54
Chikurin Ibaraki 1 (6-row)	Japan	3.21	47
Mihori Hadaka 3 (6-row)	Japan	2.33	34
Romanze (2-row)	Germany	5.32	100
Russia 57 (2-row)	USSR	2.63	49
Hakei I-41 (2-row)	Japan	2.02	38

* 4.5 m^2 plots, 2 replications; LSD 5% = 0.41 t/ha; yield in absolute dry matter.

To abridge the process of combining foreign resistance genes with high agronomic performance the use of androgenetic doubled haploids in a breeding programme has been demonstrated to be

a very useful tool (FOROUGHI-WEHR & WENZEL, 1988; FRIEDT *et al.*, 1989). Field trials indicate that it is possible to select doubled haploid lines, derived from crosses including an exotic parent, e.g. 'Igri x 'Resistant Ym No.1' (jap. spring barley), which are nearly as high yielding as the adapted cv. 'Igri'.
Furthermore, identification of resistant progenies may be facilitated by marker based selection using either isozyme- and protein-electrophoresis or the RFLP-technique. The benefit of these methods is mainly due to the possibility of selecting resistant individuals in a very early stage of a breeding programme and without time and labour consuming natural or mechanical virus inoculation.

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HOST RANGE AND TRANSMISSION OF BARLEY VIRUSES BY ISOLATES OF POLYMYXA GRAMINIS

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Summary

Nineteen unifungal isolates of Polymyxa graminis from Britain, Germany, China and Canada, mostly obtained from barley were tested for their ability to infect barley, wheat, oats, rye and Lolium perenne. All isolates grew well on barley, but only a few grew on wheat and/or rye. No isolate grew on L.perenne and there were only traces of infection by six isolates on oats. A range of grass species were not infected by two isolates tested in more detail. The ability of some isolates to transmit barley mild mosaic (BaMMV) or barley yellow mosaic (BaYMV) viruses was tested by growing them on rooted tillers of virus-infected barley and inoculating zoospores from these roots to test seedlings. Five isolates transmitted BaMMV, apparently with similar efficiencies. Three isolates have transmitted BaYMV but simultaneous transmission of both viruses has not yet been achieved. One isolate of P.graminis (from Canada) appeared to be unable to acquire and/or transmit BaMMV.

Introduction

Polymyxa graminis Led., is the vector of important viruses of barley, oats, rice, wheat and other crops. The fungus is geographically widespread and has been reported to occur on the roots of many species of the family Gramineae and has occasionally been reported from other families. Most of the records have been made from plants growing in field soils but in inoculation experiments using sand culture, Barr (1979) demonstrated some differences in host range amongst six unifungal isolates from wheat Bromus or Agropyron. All isolates infected wheat and barley, three infected Agropyron and only one caused a trace of infection on oats. Bastin, Boute and Maraite (1989) tested the host range of five barley isolates. None was pathogenic on wheat but two infected oats and two rye. Several grass species, including Agropyron, were uninfected. To understand the epidemiology of the viruses transmitted by P.graminis, it is necessary to have more information on the host range of isolates and on their ability to transmit viruses. This paper reports results on the host range of 19 isolates and on the ability of some of them to transmit barley mild mosaic (BaMMV) and barley yellow mosaic (BaYMV) viruses.

Materials and Methods

Unifungal isolates were obtained in sand culture by selecting individual mature resting spore clusters from roots into a drop of water on a glass coverslip. When 20 or more clusters had been selected, the coverslip was placed on the surface of sterile silver sand in a disposable plastic pot, 3 pre-germinated seedlings of barley (usually cv. Maris Otter) placed on top and covered with more sand. Pots were watered with dilute nutrient solution, either by hand or using the automatic irrigation system described by Adams, Swaby and Macfarlane (1986), and maintained at 15-20°C. The purity of the isolates was checked microscopically and, when abundant resting spore clusters were formed, the roots were dried, powdered and kept in the laboratory as a source of inoculum. Infected root powder was dusted onto roots of seedlings to establish new cultures and for host range experiments in sand culture.

Results and Discussion

Host range experiments

Nineteen isolates of P.graminis were tested in detail by inoculating seedlings of barley (Maris Otter), wheat (Armada or Galahad), oats (Peniarth), rye (Dominant) and Lolium perenne (S.22) with resting spore powder and growing them in irrigated sand culture. Most of the isolates were from barley growing in British soils but one was isolated from Poa annua and two were from Germany, two from China and one was a Canadian isolate from wheat, kindly supplied by Dr D.J.S.Barr. All isolates caused heavy infection on barley and none infected L.perenne. Four isolates grew well on rye and two of these also caused a little infection on wheat. A further isolate infected wheat slightly but not rye. On oats, six of the isolates caused a trace of infection and this was not correlated with infection of the other crop species. Two British isolates were also tested on a range of grass species but infected none (Table 1).

Table 1. Non-hosts of two isolates of Polymyxa graminis

Agropyron repens[1]	Cynosurus cristatus	Holcus lanatus
Agrostis tenuis[2]	Dactylis glomerata	Phleum pratense
Alopecurus pratensis	Festuca pratensis	Poa annua
Anthoxanthum odoratum	Festuca rubra	

[1] now Elymus repens [2] now Agrostis capillaris

Some preliminary information indicates that wheat cultivars may differ in their susceptibility. In comparative experiments using several isolates, more infection was seen on cv Avalon than on Armada or Galahad, although the intensity of infection was never as great as on barley. This is similar to the results reported by Bastin, Boute and Maraite (1989). Nine UK winter and spring oat cultivars were also tested with two isolates which had caused a trace of infection in earlier experiments. Only slight amounts of infection occurred on all cultivars.

The results confirm experience from looking at roots of plants from the field, that oats and wheat are generally less infected by P.graminis than barley. It seems that some host specialisation occurs within isolates of the fungus but this was not simply related to the plant species from which the isolation was first made.

Virus transmission experiments

Because virus inoculated mechanically can loose its fungal transmissibility, the ability of isolates to transmit virus was tested by growing the fungus on freshly-rooted tillers of plants containing virus originally inoculated by the vector (Adams, Swaby and Jones, 1988). In one experiment, rooted tillers of plants with and without BaMMV were inoculated with resting spore powder of five British isolates of P.graminis believed to be virus-free. On two occasions, 6 and 8 weeks after inoculation, zoospore suspensions were obtained by soaking roots of inoculated plants in dilute nutrient solution and these were used to inoculate seedlings of Maris Otter barley as described by Adams, Swaby and Macfarlane (1986). Plants were maintained at about 20°C and symptoms of BaMMV were recorded 4-6 weeks later. All isolates transmitted virus and since the efficiency of transmission was closely correlated with the concentrations of zoospores, it was concluded that there were no differences between the isolates in their ability to transmit BaMMV (Table 2).

Table 2. Transmission of BaMMV from rooted tillers by isolates of Polymyxa graminis

Isolate no	First inoculation zoospores nos/ml	% plants	Second inoculation zoospores nos/ml	% plants
1	1.5×10^5	67.5	1.7×10^5	47.4
2	1.1×10^5	48.8	1.3×10^6	67.9
4	5.3×10^4	32.3	1.2×10^5	55.1
5	3.6×10^4	5.3	2.1×10^5	36.2
6	1.7×10^4	5.3	2.2×10^4	3.7
S.E.D. (16 D.F.)		6.59		5.85

In subsequent experiments, BaMMV has been transmitted by a further fungal isolate and three isolates have acquired BaYMV in experiments that were similar but done at lower temperatures (10-12°C). Simultaneous transmission of both viruses has not yet been achieved. One isolate of P.graminis (that from Canada, originally isolated from wheat), has not acquired and/or transmitted BaMMV despite multiplying well in barley roots infected with this virus.

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DETERMINATION OF THE PRIMARY STRUCTURE OF RNA 1 AND RNA 2 OF BARLEY YELLOW MOSAIC VIRUS

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Barley Yellow Mosaic Virus (BaYMV) has a bipartite positive sense ssRNA genome with RNA sizes of approximately 8 and 3.7 kb. We have prepared cDNA clones corresponding to the RNA genome of BaYMV. DNA and protein sequence analysis is being performed using the cDNA clones and their corresponding in vitro translation products to determine the molecular organization of the BaYMV genome.

The near full length cDNA clone psY35 corresponding to RNA2 has been fully sequenced and found to be 3570 nucleotides in length excluding a 3' terminal poly(A) tail. Primer extension analysis revealed that the 5' terminal region of the cDNA clone is complete with the exeption of 20 nucleotides. The first AUG codon at position 137-139 is the putative translation initiation codon as it is contained in the sequence ACCAUGU which is similar to the consensus sequence for eucaryotic translation initiation and is followed by a single large open reading frame. The AUG codon is preceded by a 5' untranslated region of 136 nucleotides which is adenosine rich and has similarities to other RNA virus untranslated regions. The open reading frame continues to position 2807 where it is terminated by a UAG codon. Analysis of the DNA sequence reveals no other open reading frames coding for proteins greater then 6500 daltons. The protein encoded by the open reading frame has a predicted mol. wt. of 110 kD. The sequence UAUGU which is thought to be a polyadenylation signal for some potyviruses was found 110 nucleotides upstream of the poly(A) tail.

In vitro translation of the RNA corresponding to psY35 results in the synthesis of four major translation products whose combined molecular weights are approximately that of the protein encoded by psY35. This indicates that the RNA encodes a polyprotein which is subsequently processed. Investigations are being performed to map these proteins to the nucleotide sequence by amino acid sequencing of the amino terminals of the proteins. DNA sequencing studies are now being performed on RNA1 so that all proteins encoded by BaYMV may be identified and positioned on the RNA genome and the translation mechanisms of this virus elucidated.

MOLECULAR CHARACTERIZATION OF A UK ISOLATE OF BARLEY MILD MOSAIC VIRUS

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Summary

The single-stranded (ss) genomic RNA of the Streatley isolate of barley mild mosaic virus (BaMMV) was isolated. The RNA consists of two separately -encapsidated species of 6.9 and 3.9 kb. Both are polyadenylated and act as messengers in a rabbit reticulocyte lysate *in vitro* translation system. It is possible that subgenomic forms of one or both of these RNAs exist in infected plants. Virus-associated double-stranded (ds) RNA was isolated from infected plants and three discrete species, of molecular weights 5.1, 2.4 and 2.1 $x10^6$ were present. The viral coat protein consists of one subunit of 38 kD, which degrades on purification, giving breakdown products of 29 to 37 kD. DNA complementary to the viral RNA has ben synthesised and a number of clones produced.

Introduction

Barley yellow mosaic virus (BaYMV) is transmitted in the soil by *Polymyxa graminis* (1) and is responsible for yield losses of up to 50% in susceptibls cultivars of winter barley (2). Two distinct serotypes of BaYMV, which differ in particle stability and mechanical transmissibility, have been identified as occurring in Europe (3,4,5). It has recently been accepted that these two serotypes are in fact distinct viruses (3,6) and they have been termed BaYMV (which includes the non-mechanically-transmissible types) and BaMMV (formerly the mechanically-transmissible type).

Previous work on BaYMV and BaMMV has established that their particles are slightly flexuous and rod-shaped (7,3). They are 13 nm in diameter, with a bimodal length distribution of 270-300 nm (7,3) due to the separate encapsidation of the two genomic RNA species - RNA-1 (≈8 kb) and RNA-2 (≈4 kb) (7,3). It has been shown (8,9) that there is little sequence homology between RNA-1 and RNA-2 in both viruses and Koenig and Huth (9) confirmed that bothspecies are needed for the infection of plants. Ehlers and Paul (10) investigated the coat protein subunits of BaYMV and BaMMV and found them to have the same molecular weight (≈35 kD) but to differ in their tendency to degrade and form breakdown products of 29.5-33 kD.

The work described here continues with the molecular characterisation of BaMMV, in order to provide some clues as to the infection and replication strategies of this economically-important pathogen.

Materials and Methods

Origin of the virus and its propagation. The BaMMV isolate used in this work originated from a site in Streatley, Bedfordshire (11) and was maintained by mechanical inoculation onto young *Hordeum vulgare* cv. Maris Otter plants using the method of Friedt (12).

Virus purification. A modified version of the method of Macadam (13) was used to obtain a partially-pure preparation of BaMMV. Leaf tissue was ground in liquid nitrogen, homogenised in 2 vols. 20 mM HEPES (pH 7.4), 10 mM DIECA, 5 mM EDTA, 1 mM PMSF, 0,1% v/v 2-mercaptoethanol and the breis filtered. The supernatent from a low-speed centrifugation of the filtrate was re-filtered and clarified by stirring with 0.5% v/v Triton X-100 for 30 min. Virus particles were concentrated by centrifugation through a 6ml, 30% w/w sucrose cushion, for 2.5 h at 27 k, using a Beckman Type 30 rotor. The pellets were drained, resuspended in homogenisation buffer overnight and usually subjected to a further round of differential centrifugation by pooling and centrifuging through a 12ml, 30% w/v sucrose cushion for 2 h at 24 k in a Beckman sw27 rotor. The pellet was drained and resuspended in homogenisation buffer, prior to extraction of RNA.

Extraction of viral RNA and further purification. Genomic RNA was extracted from a 'crude' virus preparation by incubation with 200µg/ml proteinase K in the presence of 0.1% w/v SDS and 20 mM Tris-Cl (pH 9.0), followed by phenol-chloroform extraction and precipitation with 2M lithium chloride.

The RNA was separated into its two components by electrophoresis through an agarose -TBE (134 mM Tris (pH 8.8), 44 mM boric acid, 2.5 mM EDTA) gel, excision of the two bands and electroelution of the RNA for 30 min. at 75 mA into dialysis tubes.

Extraction of virus-associated ds RNA. Ds RNA was purified directly from total nucleic acids from infected plants using CF-11 cellulose, as described by Morris and Dodds (14). Traces of DNA and ss RNA were removed by DNAse 1 digestion and lithium chloride fractionation.

Total RNA extraction from plants. Total RNA was extracted from plants using the method of Parish and Kirby (15) as modified by R. Townsend (pers. comm.). After DNAse 1 digestion, the single-stranded RNA was precipitated in 2M lithium chloride.

Polyadenylated RNAs were isolated from total, single-stranded plant RNA by oligo (dT) cellulose chromatography, essentially as described by Maniatis *et al* (16). The poly(A)+ and poly(A)- fractions were both collected and stored.

cDNA synthesis and cloning. cDNA to the viral RNA was synthesised using an Amersham kit, following manufacturer's instructions. First-strand synthesis was primed with either random primers, oligo(dT), or with synthetic oligonucleotides.

This DNA was digested with the restriction enzymes EcoR1 and Sma1 and was cloned into the corresponding sites within the multiple cloning site of the Bluescript™ (Stratagene) vector.

Gel electrophoresis of the virus and immunoblotting. Virus samples were disrupted by boiling for 5 min. in 62.5 mM Tris-Cl (pH 6.8), 2% w/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 0.001% w/v bromophenol blue and were then electrophoresed through a 12.5% w/v mini polyacrylamide (30% acrylamide, 0.6% bisacrylamide)/SDS gel.

After electrophoresis, gels were either stained with Coomassie Brilliant Blue or were used for immunoblot analysis. Soluble proteins were transferred to nitrocellulose by electroblotting using a Biometra fast blot system, with transfer buffer (25 mM Tris, 150 mM glycine, 10% v/v methanol). Filters were then incubated in PBS-Tween (10 mM sodium phosphate (pH 7.2), 0.085% w/v sodium chloride. 0,1% v/v Tween 20), 5% w/v Marvel, containing primary antibody at a dilution of 1:200, at 4°C overnight. After washing in PBS-Tween/Marvel, filters were incubated with a 1:500 dilution of goat anti-rabbit secondary antibody conjugated to alkaline phophatase for 2 h at 2room temperature. After washing and rinsing in 0.15M veronal acetate (pH9.6), the blots were incubated in 0.15M veronal acetate containing 1 mg/ml nitroblue tetrazolium, 0.5 mg/ml bromochloroindolyl phosphate.

***In vitro* translation.** BaMMV RNA was translated in rabbit reticulocyte lysate. Reaction conditions were 20 mM creatine phosphate, 0.5 mM magnesium chloride, 0.1M potassium chloride, 1 mM each amino acid and 10 mM DTT. RNA was added to 50 µg/ml and [^{35}S]methionine to 500 µCi/ml. The mixture was incubated for 90 min(unless stated otherwise) at 30°C and the reaction stopped by making the mix 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 2.3% w/v SDS, 62.5 mM Tris-Cl (pH8.0) and 0.025% bromophenol blue and heating to 90°C. Products were electrophoresed through 12.5% polyacrylamide/SDS gels, which were then fixed, dried and exposed to X-ray film.

Results

BaMMV genomic RNA. On a typical gel of RNA extracted from a partially-pure preparation of virus, the two bands corresponding to RNA-1 and RNA-2 can clearly be seen. The estimated sizes of these species are 6.9 and 3.9 kb.

When the products of first- and second-strand cDNA synthesis from viral RNA were electrophoresed, two distinct bands, corresponding in size to RNAs-1 and -2, were seen in both samples. The efficiency of priming by oligo(dT) (lanes 1 and 2) and the production of apparently full-length species would suggest that both BaMMV RNA-1 and

-2 are polyadenylated at or near their 3' termini. The presence of a poly(A) tract has been confirmed by sequence analysis of clones made using this cDNA.

A similar result to the above was obtained when the first-strand reaction was primed with a synthetic oligonucleotide made to an area within the 3' non-coding region of BaYMV RNA-1 (17). The sequence of this oligonucleotide is 5' ACGGCTGATCAAACGCTGTC 3'. The efficiency of priming from both RNA species suggests that they share sequence homology with this oligonucleotide. The production of nearly full-length species would suggest that this homology lies towards the termini of the genomic RNAs.

Virus-associated ds RNA. A resistance to digestion by RNAse A in 2xSSC, but a susceptibility in 0.1xSSC confirmed the ds RNA nature of samples of BaMMV-associated ds RNA derived from infected plants. Three species of ds RNA are typically present in such a sample, the larger two being of estimated molecular weights 5.1 and 2.4 $x10^6$, which equate closely with the expected sizes of replicative forms of RNA-1 ($4.6x10^6$) and RNA-2 ($2.6x10^6$). A third band is also present, of estimated molecular weght $2.1x10^6$. Its origin is unclear, although it could represent an artefact of preparation, a replicative intermediate, or a species derived from a co-infection. It is possible that it could represent the ds form of a subgenomic RBNA, but at present, there is no evidence to substantiate this.

Viral coat protein. Immunoblots of samples of 'crude' preparations of BaMMV were probed with antisera raised to whole virus particles. The pattern produced, of a major band and three, slightly smaller bands was very similar to that obtained by Ehlers and Paul (10).By analogy with their work, the major band of 38 kD represents the intact coat protein and the 37, 31 and 29 kD species are breakdown products resulting from purification and storage of the virus preparation.

***In vitro* translation.** The typical translation products from a sample of viral genomic RNA are major products of 23, 46, 56, 62, 71, 76, 84, 93 and 106 kD and minor products of 31, 37, 53, 59, 79 and 98 kD. Despite the presence of so many species, there is no major band that corresponds with the size of the coat protein. The appearance of so many translation products may be explained by the premature termination of protein synthesis, non-specific internal initiation, fragmentation of the RNA template and translation of those fragments, or by partial proteolytic processing.

To investigate the occurrence of either specific proteolytic processing or non-specific proteolysis, several time course experiments were performed, whereby samples of the translation mixture were removed at various times for electrophoresis. The results of such an experiment appear to be fairly complex Although the majority of the protein bands remain constant, others increase and decrease in intensity at different time points. For example, the 22 kD band is not apparent until after a 1 h. incubation and the 30 kD band is only visible after 2h. This suggests that the synthesis of these two proteins might be controlled by proteins produced earlier in the cycle. Alternatively, degradation of the RNA template might lead to an alteration in its secondary structure and an opening of new ribosome binding/initiation sites. The intensity of the 30, 33, 49 and 69 kD bands appears to decrease after an overnight incubation, indicating that some form of proteolysis might be occurring. However, there is no concurrent appearance of smaller products, which might be expected.

When the two viral RNAs were translated separately, very few products were visible after electrophoresis. However, a band of a 112 kD protein was present in the RNA-1 specific lane but not in the RNA-2 specific lane. It is possible that BaMMV RNA-1 directs the synthesis of only one large protein, as is probably the case with soil-borne wheat mosaic virus (SBWMV) (18) and beet necrotic yellow vein virus (BNYVV) (19).

Translation of the poly(A)+ and the poly(A)- fractions of total, ss RNA derived from infected plants also produced an interesting result. Two bands, of 20 kD and 30 kD, were present in the poly(A)+ track, but not in the poly(A)- track, which, if virus-specific, are further evidence for the polyadenylation of BaMMV RNA. The absence of these bands amongst the products of the size-separated genomic RNAs suggests that they may be translated from one or more subgenomic RNAs. The 20 kD and 30 kD products are present amongst the bands produced from 'mixed' virion RNA, so such subgenomic species would probably be encapsidated and thus co-purified with the full-length virus particles.

Dicussion

The results described here represent preliminary data on the molecular characterisation of BaMMV. The viral RNA is polyadenylated, a feature it shares with BaYMV (17) and BNYVV (20), but not with SBWMV (18). The presence of sequence homology at the termini of BaMMV RNAs -1 and -2 to an area in the 3' non-coding region of BaYMV RNA-1 suggests a similarity in the control of translation and/or replication between these two viruses.

The possibility that the replication strategy of BaMMV might involve the production of one or more subgenomic RNAs is raised by the presence of 3 bands of virus-specifc ds RNA in infected plants and by the results of *in vitro* translation experiments. Obviously, more work is neede to clarify the situation. It is also possible that polyprotein processing might play a role in the viral replication cycle. In this respect, BaMMV would be similar to BaYMV, the coat protein of which is thought to be produced from a polyprotein precursor (17).

Interest in BaYMV and BaMMV is growing, especially as they may require classification in a new group of viruses, the bymoviruses (3,6). Much further work is needed to elucidate the structure and the replication strategies of these two viruses, of which the work described here represents a start.

Acknowledgements

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[Due to circumstances beyond my control, copies of the figures were not available in time for publication. Should anyone wish to see them, please contact me directly.]

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IDENTIFICATION AND PARTIAL CHARACTERIZATION OF OAT MOSAIC AND OAT GOLDEN STRIPE VIRUSES CO-INFECTING OATS IN THE SOUTHEASTERN UNITED STATES.

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Summary

A soil-borne mosaic disease of oats has been recognized on winter oats in the southeast United States for over 50 years. Leaves of infected oats take on a chlorotic, mottled appearance in early spring. As temperatures rise in late March and April mottling gives way to bright golden yellow stripes running longitudinally on both young and old leaves. Symptoms are often very apparent on flag leaves during tillering and grain fill thus, severe infections may result in significant yield losses. The etiology of the disease has not been fully determined , therefore, we have undertaken the identification and characterization of the viruses associated with typical disease symptoms. Observations made with the aid of an electron microscope of partially purified virus preparations from symptomatic oat tissue indicated the presence of both long, flexuous, rod-shaped particles and short, stiff, rod-shaped particles. Based on previously published information, EM observations and SDS-PAGE analysis, we have identified the long flexuous particles as oat mosaic virus (OMV), a member of the potyvirus group, and the short stiff particles as oat golden stripe virus (OGSV), a member of the furovirus group. We have shown the genome of OGSV to be composed of 2 distinct RNA molecules of approximately 6.2 and 3.5 kilobases in size and are in the process of synthesizing cDNA to these molecules. There have been no published reports of OGSV in the United States, however, it has been reported in Europe. The relatedness of OGSV isolated in North Carolina (OGSV-NC) and that from the United Kingdom (OGSV-UK) has not yet been determined. Polyclonal antisera produced in rabbits to the capsid protein (CP) of OGSV-NC does show activity against OGSV-UK. Specificity of this antisera to viral CP has been confirmed by both enzyme linked immunosorbant assays and western blots. We are in the process of producing polyclonal antisera to the CP of OMV. We are also attempting to synthesize cDNA from the genome of OMV. Serological assays and cDNA probes will be used to screen tissue from field plots planted during the fall of 1990 in order to identify oat cultivars with levels of resistance useful in future breeding programs.

Introduction

Soil-borne oat mosaic virus (OMV) was first observed in the U.S. in 1943 (McKinney, 1946). It was observed in North Carolina in 1945 (Atkinson, 1945), and has since been found in fields throughout the southeast, Washington state, England, Wales and possibly New Zealand (McKinney, 1946, Bruehl and Damsteegt, 1961, MacFarlane, 1968 and Slykhuis, 1962). Symptoms vary with disease severity and may differ between virus isolates. McKinney (1946) proposed the existence of two different isolates. The first, *Marmor terrestre* var typicum causes apical mosaic symptoms. Chlorotic lesions are light green to yellow and may remain as narrow streaks or leaves may be mottled or blotched. Symptoms from this strain tend to be limited to the top 1 to 3 leaves of infected plants. The second, less common, strain (*M. terrestre* var oculatum) causes eyespot mosaic symptoms. Spindle shaped spots are bordered by ash gray margins and remain green at their center until the entire leaf becomes chlorotic. (More recent

publications refer to the virus simply as oat mosaic virus or OMV) Other general symptoms caused by both isolates include stunting or dwarfing, rosetting and failure to head in severe infections. Yield losses can range from 50 to 100% and seem to be greatest in genetically pure cultivars (Atkinson, 1945). The virus host range is strictly limited to *Avena* spp. (Toler and Hebert, 1963) and no cultivated *Avena* spp. is immune to infection although a range of tolerance has been reported (Coffman *et al.*, 1963). OMV has been tentatively classified as a furovirus due to its similarity to WSBMV, the type member of this group.

A thorough characterization of OMV has never been reported. Due to this lack of information, an accurate determination of OMV distribution and concentration throughout the Carolinas has not been possible in the past. Previous studies have also been hindered due to the lack of an accurate probe for OMV detection.

OMV has been known to exist in North Carolina oat fields since 1945. Despite its widespread distribution and its potential to cause large scale yield losses, very little information about the nature of this virus is available. It has been concluded that the host range of OMV is limited to *Avena* spp. and it is vectored by the soil-borne fungus *Polymyxa graminis*. Very little else is known about this virus except that it resembles WSBMV and has been tentatively classified as a furovirus. It has been suggested (but not yet proven) that oat mosaic in the Carolinas is actually caused by a complex of 2 virus particles; one, a short rod and the other a long flexuous rod.

Materials and Methods

Virus purifications:

OGSV Purification: Symptomatic oat tissue was collected during March and April 1990 and stored at -20°C. Frozen tissue was ground in liquid nitrogen in a mortar and pestle. Approximately 2 volumes of 0.5 M sodium borate, pH 9 buffer was added to the tissue prior to expression through cheesecloth. Clarified sap was centrifuged at 10,000 rpm in a Sorvall SS-34 rotor. Triton X-100 was added to 2% final volume and the supernatant was mixed for 30 min. at 4°C. Virus was collected by centrifugation through a 20% sucrose cushion at 28,000 rpm for 2 hrs. Pellets were gently resuspended in 50 mM Na borate, pH 8. The virus preparation was clarified by centrifugation at 10,000 rpm for 20 min prior to re-pelleting at 28,000 rpm for 2 hr. Final virus pellet was resuspended as above.

OMV purification: OMV was purified as described for OGSV except that it was extracted in 100 mM ammonium citrate, pH 6.5.

Antibody production and serological assays:

Purified virus was dissociated in Laemmli dissociation buffer and capsid protein separated by 12.5% SDS-PAGE. Protein band was visualized by soaking gel in cold 250 mM KCl. Protein was excised and eluted from the polyacrylamide and used as an immunogen for antibody production in rabbits as described by Kendall *et al.*, (1988). Western Blots and ELISA were performed as described by Kendall *et al.*, (1988).

Genomic RNA analysis

Purified OGSV virions dissociated with SDS and the virion RNAs were fractionated in sucrose density gradients. UV absorbing fractions were collected, pooled and assayed on 1% agarose gels.

Results

Oats expressing mosaic symptoms from North Carolina are infected with two distinct viruses as determined by electron microscopy. Long flexous rod viruses (600-700 x 13 nm) typical of members of the potyvirus group were observed. In addition, shorter stiff rod like viruses with two length classes were also observed. The rods averaged 150 nm and 300 nm in length (Fig. 1).

The morphology of the bimodal virus is indicative of the furoviruses and specifically OGSV (Adams *et al.*, 1988).

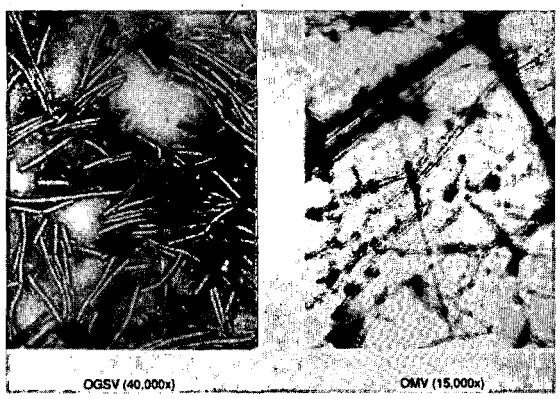

Fig. 1 Electron micrographs of purified OGSV OMV.

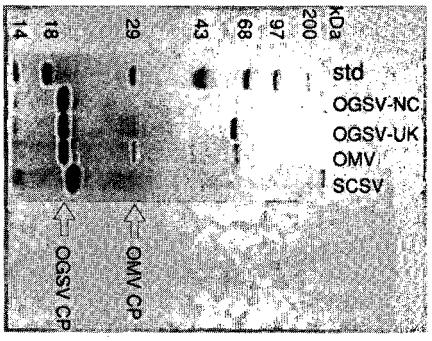

Fig. 2 12.5% SDS-PAGE analysis of OGSV and OMV capsid proteins.

Purification procedures were designed for both viruses. The high pH borate purification was selective for the purification of the furo-like virus. Ammonium citrate extraction of the mosaic symptomatic oat tissue enriched for the poty-like virus, however, significant amounts of furo-like virus co-purified (fig. 2).

The furo-like virus is composed of a single 19 kDa capsid protein (Fig. 2) and two single stranded RNA species of 6.2 kb and 3.5 kb (fig. 3). The poty-like virus possess a single capsid protein component of 29 kDa. A satisfactory estimation of the genomic RNA has yet to be determined.

An antibody has been raised to the furo-like virus. Western blot analysis using the antibody as well as wheat soil borne mosaic virus (WSBMV), sorghum chlorotic spot virus (SCSV), and oat golden stripe virus (OGSV-UK, kindly provided by M. J. Adams) antibodies were performed. OGSV-UK antibodies reacted strongly to OGSV-UK and OGSV-NC capsid proteins (Fig. 4). Weaker reactions were detected between OGSV-UK and OGSV-NC with the other furovirus antibodies. None of the antibodies detected OMV capsid protein.

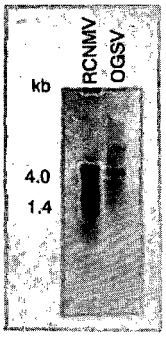

Fig. 3. Agarose gel electrophoresis of OGSV genomic RNA.

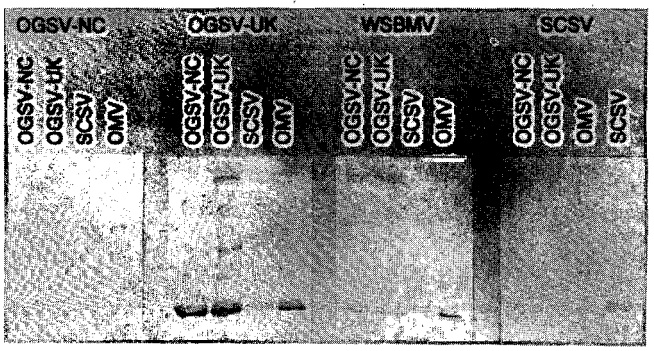

Fig. 4 Western blot analysis of OGSV-NC, OGSV-UK, SCSV and OMV capsid proteins with, WSBMV, OGSV-NC, OGSV-UK, and SCSV antibodies.

Discussion

From the physicochemical characterization of the furo-like virus from oats in North Carolina, we conclude it to be OGSV. We have yet to determine whether it is identical to or a serological strain of OGSV-UK reported by Adams *et al.*, (1988). From previous reports (Atkinson, 1945: Usugi and Saito, 1981) we conclude that the long flexous rod is OMV, a member of the fungus transmitted potyviruses.

Symptomatic oat tissue was collected from commercial fields in Georgia and North Carolina during the spring, 1990. These samples will be screened for the presence of both OGSV and OMV, using ELISA and cloned cDNA probes, in order to determine the distribution of these viruses in the Southeast United States. Experimental field plots are planned in order to determine the correlation between symptom expression and virus titer, virus titer and yield loss and to identify oat cultivars possessing levels of resistance useful for future breeding programs. In addition to completing the molecular characterization of OMV, we will determine the relatedness between OGSV-NC and OGSV-UK. Western blot analysis has shown that wheat soil-borne virus (the type member of the furovirus group) antisera does react with OGSV CP indicating that there is some degree of homology between the two viruses and thus provides further evidence that OGSV has been properly classified in the furovirus group. Further comparisons between OGSV and other furoviruses will be performed in the future.

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SOIL-BORNE WHEAT MOSAIC VIRUS AND WHEAT SPINDLE STREAK MOSAIC VIRUS IN ITALY

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Summary

In Italy, the most widely grown bread wheat cvs. are resistant to SBWMV. On the contrary, many of the high-yielding durums released during the past two decades are extremely susceptible to this disease. SBWMV is widespread, especially in North and Central Italy, but WSSMV appears only a minor menace to wheat crops. Various Italian durum wheat cvs. showed to be resistant to mixed SBWMV and WSSMV infection.

Introduction

In Italy, Soil-borne Wheat Mosaic Virus (SBWMV) was first reported in 1960, on wheat cvs. grown in the Northern regions of the country (Canova and Quaglia, 1960). More recent surveys have shown that this virus is widespread also in several Central provinces as well as in the traditional durum wheat growing areas of the South (Rubies-Autonell and Vallega, 1985; Vallega and Rubies-Autonell, 1987; 1989; Rana and Lafortezza, 1988). Wheat Spindle Streak Mosaic Virus (WSSMV), on the contrary has been reported only in a few fields and, so far, always in conjunction with SBWMV (Rubies-Autonell and Vallega, 1987, 1990). Mixed SBWMV and WSSMV infections are cause of concern in that the concomitant presence of these viruses is claimed to render SBWMV resistance genes ineffective (Lommel, Willis and Kendall, 1986). In this paper we summarizes the results of a series of varietal resistance trials and field surveys, as well of our studies on the interaction of SBWMV and WSSMV durum wheat cvs. infected by both pathogens.

Materials and Methods

Fifty-eight durum wheat cvs., 24 common wheat cvs., and 24 barley cvs. were evaluated for SBWMV resistance in a uniformly SBWMV-infested field near Rome. The trials, performed in different years, were layed out according to randomized block designs with three replicates (10 sqm each). SBWMV symptom severity was rated according to a 1-4 scale (see Vallega and Rubies-Autonell, 1985).

The presence of SBWMV and WSSMV in Central and Southern Italy was surveyed on randomly chosen durum wheat and common wheat fields. Plant extracts were processed by Isem.

The development of mixed SBWMV and WSSMV infections was studied on both root and shoot samples collected at various growth stages from eight durum wheat cvs. posessing different levels of SBWMV resistance. Investigations were carried out for three consecutive years in replicated trials grown in field naturally infested by both SBWMV and WSSMV. Viral particle counts were made using an amplified Isem procedure ('Isem-r', Stobbs 1984)in that previous experiments had shown that the use of this technique allows the detection of SBWMV and WSSMV even in resistant cvs. or during the earliest and latest phases of infection (Rubies-Autonell, 1989).

Results

As can be seen in Table 1, about two thirds of the durum wheat cvs., tested

demonstrated to be resistant or moderately resistant to SBWMV. However, both root and shoot tissues collected from these cvs. contained at least a few SBWMV particles when analysed by Isem-r. Many of the high-yielding semidwarf cvs. released during the past two decades showed to be extremely susceptible to SBWMV. Yield losses attributable to SBWMV as high as 70% were estimated for some of these cvs. (Vallega and Rubies-Autonell, 1985).

The results of our field experiments with bread wheats showed that most of the cvs. presently grown in Italy are either resistant or moderately resistant to SBWMV. As in the case of durums, particles of SBWMV were detected in root and shoot tissues of resistant cvs.

None of the 24 barley cvs. tested showed clear foliar symptoms of SBWMV, but the virus was detected in both root and shoot tissues of cvs. Jeff and Jaidor. SBWMV particles were observed also in root tissues of apparently symptomless sorghum plants grown in an adjacent field.

Fig. 1 illustrates the results of a series of surveys made by different researchers on the presence of SBWMV and WSSMV in Italy (Canova and Guaglia, 1960; Corino and Grancini, 1975; Giunchedi and Credi, 1977; Rubies-Autonell and Vallega, 1985; Vallega and Rubies-Autonell, 1987, 1989; Rana and Lafortezza, 1988). It whould be noted that, so far, WSSMV has been found only in conjunction with SBWMV.

Positive significant correlations between SBWMV and WSSMV particle counts, as well between SBWMV particle counts and SBWMV foliar symptom severity evaluations, were found, amongst eight durum wheat cvs. coinfected by SBWMV and WSSMV (Table 2). As illustrated in Fig. 2., particle numbers in the leaves tended to peak - for both

Fig. 1 - Distribution of SBWMV and WSSMV in Italy. The provinces were SBWMV is widespread are marked their initials; circles and asterisks represent, respectively, other sites where SBWMV and WSSMV have been detected.

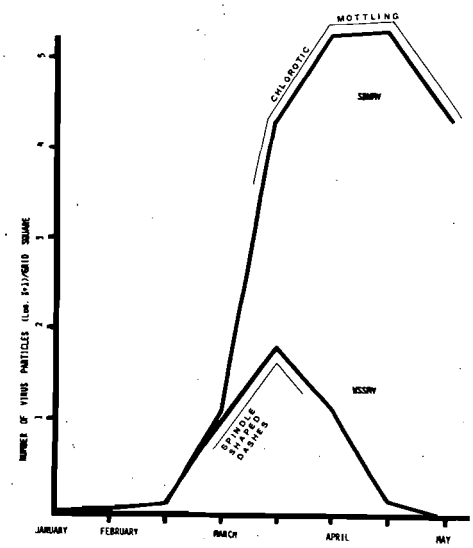

Fig. 2 - SBWMV and WSSMV particle numbers in leaves of eight durum wheats from January to May 1988.

viruses - at the time when foliar symptoms reached their maximum expression. In the case of WSSMV, the maximum no. of particles was reached about 20 days earlier than for SBWMV, and the same was observed for the WSSMV-characteristic spindle-shaped dashes, which appeared about 30 days earlier than the chlorotic mottling attributable to SBWMV.

Discussion

SBWMV is widespread in Italy, and may become a most serious limiting factor for wheat production if new cultivars are not carefully selected for resistance. Many of the high-yielding durums released during the past two decades are extremely susceptible to SBWMV. On such cvs., yield losses caused by SBWMV can be as high as 70%, that is, of the same magnitude as those reported some 25 years ago on some bread wheats grown in Northern Italy (Toderi, 1969). Presently, most bread wheat cvs. are either resistant or moderately resistant to SBWMV.However, since SBWMV particles are present even in resistant wheats, it is evident that these cvs. too contribute to the survival of SBWMV in infested fields. Indead, a similar role is probably played also by barley and sorghum crops.

So far, WSSMV has been detected only in a few fields, and therefore it appears to be -at present- only a minor menace to wheat crops in Italy. The simultaneous occurrence of WSSMV and SBWMV on wheat has been claimed to cause a breakdown of resistance to SBWMV (Lommel Willis and Kendall, 1986) but our results do not confirm these findings. In fact, various Italian durum wheat cvs. showed to be resistant to both SBWMV and WSSMV when coinfected by these viruses.

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Table 1 - Reactions of italian Triticum durum cvs. to SBWMV

Cultivars	Disease ratings	Cultivars	Disease ratings	Cultivars	Disease ratings
Adamello	MS	Grazia	MS	Rodeo	MS
Aldura	R	Iapigia	MR	Salapia	MS
Ambral	MS	Isa I	MR	San Alberto	MR
Amedeo	MR	Karel	MR	Simeto	R
Appio	MR	Latino	R	Solitario	MR
Appulo	MR	Lauria	MR	Tibula	R/MR
Arcangelo	MR/MS	Lira	S	Trinakria	MR
Athena	MR	Makit	MR	Ulisse	MR
Berillo	MS	Messapia	R	Valfiora	MR
Bravo	MS	Mito	R/MR	Valforte	MS/S
Brunda	MS	Murgia	R	Valgerardo	MS/S
Capeiti	R	Nepal	MS	Valitalico	MR
Cappelli	R	Nora	MS	Valnova	S
Castello	MR	Norba	R	Valriccardo	MS
Creso	MR	Orsini	MR/MS	Valselva	MS
Duilio	R	Piceno	R	Vespro	MS
Endura	R	Plinio	MR	Vezio	MS
Filippo	S	Procace	R	Vitron	R
Gabbiano	R	Produra	MR/MS		
Giano	MR	Quadruro	R		

* Disease severity scale: R= resistant (slight or no symptoms); MR= mildy resistant (mild mottling and stunting); MS= mildly susceptible (mottling and stunting); S= susceptible (severe mottling and stunting, dieback).

Table 2 - Field reactions and no. of SBWMV and WSSMV particles in root and leaf tissues of eight durum wheat cvs (means of eight collection dates, January-May 1988)

Cultivars	Field reaction	Average No. of viral particles/g.s.			
		SBWMV		WSSMV	
		leaves	roots	leaves	roots
Latino	R	0.5	0.5	0.1	0.1
Quadruro	R	3.7	2.6	0.1	0.2
Appio	MR	0.4	0.3	0.1	0.3
Creso	MR	1.0	3.5	trace	0.1
Grazia	MS	131.2	15.1	0.1	1.1
Vespro	MS	61.5	18.3	1.0	0.3
Lira	S	348.4	11.5	3.2	1.3
Valnova	S	606.9	22.5	7.7	5.5

WHEAT SOIL-BORNE MOSAIC VIRUS ISOLATED FROM RYE IN GERMANY

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The occurrence of wheat soil-borne mosaic virus (WSBMV) in Germany has been first described in 1982 (Proeseler et al., 1982). Near Haldensleben (earlier GDR) a few rye plants only were found in mixed infection with wheat yellow mosaic virus (WYMV). In 1990 Proeseler (pers. comm.) identified WSBMV also in rye plants collected near Zerbst in the earlier GDR, again in mixed infection. We report here WSBMV from a third field close Braunschweig in the former FRG. In this case an area of about two ha appears to be totally infested by WSBMV in the following designated WSBMV-G1. The uniform distribution of WSBMV in this field suggests that the virus occurs there already since several years. No indication was found in this case for the presence of WYMV in mixed infections with WSBMV.

Symptoms of infected plants

In March/April plants on infested areas in the field appeared lighter green than healthy plants. This impression was due to the light green streaks of different length irregularly distributed on the leaf surfaces. In general infected plants remained smaller than the healthy ones and symptom bearing leaves became necrotic and died earlier than leaves from healthy plants. On leaves developed in May at raising temperatures only a few virus induced streaks were produced.

Transmission studies

Crude sap of infected rye plants from the field mixed with Carborundum were used for mechanical inoculations. Infected leaves were homogenized in 0.1M K_2HPO_4 (pH 9,1) as well as in 0.1M phosphate buffer, pH 7.0. No differences in infection rates were detected for the two extraction media. Inoculated plants were cultivated in growth chambers at 12 - 15° C, since it had been observed that symptom expression in general was enhanced by relative low temperatures (below 15° C). Results revealed that all cereal species used in this study were hosts

Table 1
Infection of cereal species by WSBMV-G1 and OGSV after mechanical inoculation

		Test Plants				
Virus	Source of Inoculum	Oat	Barley	Wheat	Rye	N. bentham.
WSBMV-G1	rye	-*	4/60**	16/312	30/600	5/11
OGSV	oat	2/40	-	-	-	24/24

* not tested
** number of infected / number of inoculated plants

of the virus but, like with oat golden stripe virus (OGSV) inoculated plants became infected (Table 1). Nicotiana benthamiana was much more susceptible to both, WSBMV-G1 and OGSV, than the cereals; all of the plants inoculated with OGSV and nearly the half of the plants inoculated with WSBMV-G1 became systemically infected.

Electron microscopical studies

Particles of WSBMV-G1 were easily visualized electron microscopically in negative stained crude sap preparations in infected plants or preparations of purified viruses. For purification the method described by Adams et al. (1988) for OGSV was used. Length measurements of 1172 virus particles from crude sap preparations revealed two maxima length distributions (Fig. 1) similar with other furoviruses (Brunt and Richards, 1989). Seventy percent of the particles formed a maximum of shorter particles with a normal length of 171 nm, 14 % formed a second maximum at 327 nm. The sligthly higher length values obtained here compared to values reported in literature for WSBMV (Brunt and Richards, 1989) may be due to the use of uranyl acetate by us instead of phosphotungstate by Japanese authors (e.g. Hibino et al., 1981).

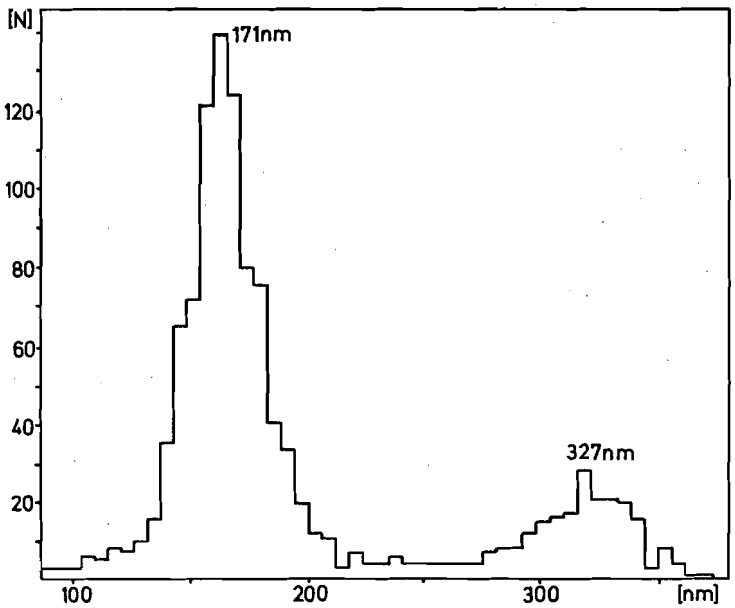

Fig. 1.
Length distribution of particles of WSBMV-G1 in crude sap preparations negatively stained with 1 % uranyl acetate.

Serological identification

WSBMV-G1 has been serologically compared in immunoelectron microscopical decoration tests with a WSBMV from the USA and OGSV from England. The decoration titers given in Table 2 revealed that OGSV and WSBMV-G1 reacted much more stronger with their homologous antisera than with the heterologous antisera. The results demonstrated furthermore that WSBMV-G1 is more closely related to WSBMV from the USA than to OGSV. We, therefore, classify the rye isolate as WSBMV.

Table 2

Serological relationship between WSBMV-G1 and OGSV revealed by decoration tests (figures are reciprocal decoration titers)

Antiserum	Antigen OGSV* from oat	Antigen WSBMV from rye
OGSV 230-4*	400	40
WSBMV**	200	800
WSBMV-rye	400	3200

virus and antiserum were kindly provided

* by M.J. Adams, Rothamsted Experimental Station, UK, and
** by M.K. Brakke, Lincoln, Nebraska, USA

Economical importance

The todays importance of WSBMV-G1 as a pathogen for cereals is limited to small regions in Germany only and it is not predictable whether it will, like the yellow mosaic inducing viruses of barley BaYMV and BaMMV (Huth, 1988) become more distributed during the coming years. In contrast to the latter viruses, WSBMV-G1 occurs on an area with very light soil. May be this is one of the reasons for the limited spreading of this virus. Similarly tobacco rattle-virus infections in rye grown on comparable soils in the northern German Lüneburger Heide (Huth and Lesemann, 1984) remained locally limited and did not spread in order to become an economically important pathogen of cereals.

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NUCLEOTIDE SEQUENCE AND GENOME ORGANIZATION OF SOIL-BORNE WHEAT MOSAIC VIRUS RNA II

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Soil-borne wheat mosaic virus (SBWMV), type member of the furovirus group, has a unique characteristic among plant viruses in that portions of wild-type (WT) RNA II frequently delete spontaneously in infected wheat plants at about 17°C. Deletion mutants cause more severe symptoms on wheat plants than WT virus. Infection and replication of both WT and mutant viruses are temperature-sensitive above 20°C. To investigate the mechanism of deletion and the determinants of increased virulence, as well as reasons for temperature sensitivity, and to identify fungus transmission factor(s), cDNA of WT RNAs I and II and deletion mutant RNA II were prepared to construct full-length cDNA clones from which biologically active RNA can be transcribed. WT virus was isolated from a field in Nebraska in 1988. A deletion mutant (Lab.1 isolate) was produced from WT virus collected in the same field in 1975 by successive mechanical transfers in wheat plants. Nucleotide sequencing of both WT and mutant RNA II species indicated that: (1) in WT RNA II (3592 nt), next to the 5' untranslated region of 333 nt, there is an open reading frame (ORF) for a 19.3 K polypeptide (capsid protein) terminated at an opal codon, followed by an in-frame coding region for an additional 64.4 K stopped by an ochre codon; (2) toward the 3' end, there is another ORF for a 18.8 K polypeptide, followed by the 3' untranslated region of 403 nt; (3) mutant RNA II (2424 nt) has two single nt deletions in the 5' untranslated region and two internal deletions in the opal read-through region; 108 nt immediately after the opal codon and 1058 nt before the ochre codon; and (4) in addition to the two deletions, there are two, one and two amino acid differences in the capsid protein, read-through polypeptide and 18.8 K polypeptide regions, respectively. *In vitro* transcription/ translation analyses indicate that the 3' proximal, 18.8 K polypeptide could be expressed *in vivo* from a subgenomic mRNA.

GENOME STRUCTURE OF TOBACCO NECROSIS VIRUS STRAIN A

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Summary

The genome organization of tobacco necrosis virus (TNV) strain A is very similar to that of the carmoviruses. Comparison of the amino acid sequences of the encoded proteins with other viral proteins revealed relationships with different small icosahedral plant viruses. The 82-kDa protein of TNV shows sequence similarity with the putative RNA polymerases of turnip crinkle virus, carnation mottle virus, cucumber necrosis virus, tomato bushy stunt virus, maize chlorotic mottle virus, red clover necrotic mosaic virus, and barley yellow dwarf virus, while TNV coat protein is related to the structural protein of southern bean mosaic virus. Based on these relationships, we suggest that TNV can still be classified in a monotypic virusgroup.

Introduction

Tobacco necrosis virus (TNV) is a small icosahedral, fungus-transmitted virus, which is often associated with satellite viruses (Kassanis, 1970; Fraenkel-Conrat, 1988). The single viral genomic RNA is 3.8-4.0 kb long (Condit and Fraenkel-Conrat, 1979, Bishop et al., 1967). It has no cap structure nor a VPg, the 5'-terminal sequence being ppApGpUp (Lesnaw and Reichmann, 1970). There is no poly(A) tail at the 3'terminus (Condit and Fraenkel-Conrat, 1979). Two double-stranded RNAs of less than full-length were isolated from TNV-infected tobacco (Condit and Fraenkel-Conrat, 1979). These correspond to two 3'-terminal subgenomic RNAs of resp. 1,6 and 1,3 kb (Meulewaeter et al., 1990a).

The almost complete sequence of the genomic RNA has recently been determined (Meulewaeter et al., 1990a). This shows that TNV is closely related to the carmoviruses with respect to its overall genomic organization. However, both the putative RNA polymerase and the coat protein of TNV show extensive sequence similarity with the corresponding proteins of several other small icosahedral viruses. The significance of these findings for the classification of TNV is discussed (Meulewaeter et al. 1990b).

Results and Discussion

Genome organization

The sequence of the first 3660 nucleotides from the 5'end of the RNA genome of TNV has been determined (Meulewaeter et al., 1990a; Genbank/EMBL Data Libraries Accession No.M33002). This shows the presence of three large open reading frames (Fig. 1). ORF 1 (nt 60-668) codes for a 23-kDa protein and read-through of its amber codon gives a 82-kDa protein (ORF2). The third large ORF (ORF 5 : nt 2613-3443) encodes TNV coat protein (30 kDa). Two small open reading frames are located between ORF 2 and 5. ORF 3, which extends from nt 2218 to 2436, encodes a 7.9-kDa protein while ORF 4 (nt 2440-2610) encodes a 6.2-kDa protein. A third small ORF (ORF 6 : nt 3467-3646) with a coding capacity for a 6.7-kDa protein was found downstream from the coat protein cistron.

The 23 and 82-kDa proteins are probably expressed from the genomic RNA. The hybridization pattern of the subgenomic RNAs with different probes suggests that the largest subgenomic RNA codes for the 7.9-kDa protein while the smallest one is the mRNA for the coat protein (Meulewaeter et al., 1990a). The significance of the other ORFs remains unclear. This gives a genome organization very similar to that of carnation mottle virus (CarMV; Guilley et al., 1985) and turnip crinkle virus (TCV; Carrington et al., 1989) (Fig.1).

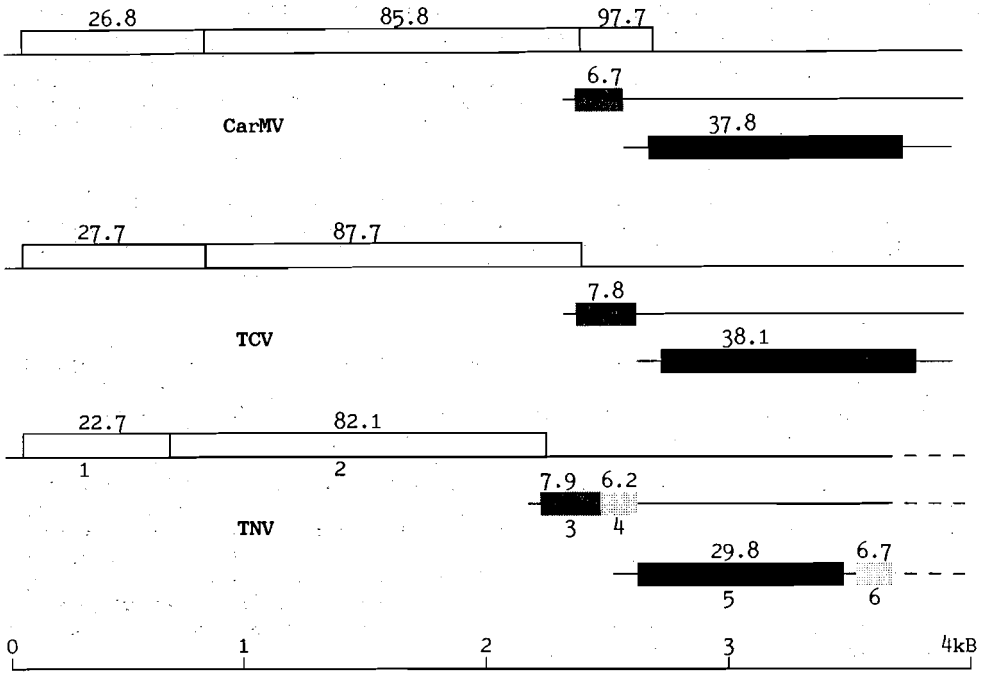

Fig.1 Comparison of the genome structures of CarMV, TCV, and TNV. Corresponding ORFs are drawn in the same way (white, black or gray blocks) and on the subgenomic RNAs which are thought to be responsible for their expression. The stippled boxes represent ORFs of TNV for which there are apparently no subgenomic RNAs. Numbers above the ORFs indicate the molecular weight (in kilodaltons) of the proteins encoded by these ORFs. For TNV the numbers of the ORFs as used in the text are given below the ORFs. The dashed lines represent the 3'end of TNV RNA which has not been sequenced.

Sequence similarity with other viruses

The read-through part of the 82-kDa protein contains the GXXXTXXXNX$_{18-50}$GDD sequence motif typical for RNA polymerases (Kamer and Argos, 1984, Haseloff et al., 1984). This protein also shows extensive sequence similarity with the putative RNA polymerases of CarMV (Guilley et al., 1985), TCV (Carrington et al., 1989), cucumber necrosis virus (CNV; Rochon and Tremaine, 1989), tomato bushy stunt virus (TBSV; Hearne et al., 1990), red clover necrotic mosaic virus (RCNMV; Xiong and Lommel, 1989), maize chlorotic mottle virus (MCMV; Nutter et al., 1989) and barley yellow dwarf virus (BYDV; Miller et al., 1988). The percentage of identical amino acid residues for the read-through part varies between 44% (CarMV and MCMV) and 33% (BYDV).

Moreover, the coat protein of TNV also shows sequence similarity with the structural proteins of several other small icosahedral plant viruses, like southern bean mosaic virus (SBMV; Hermodson et al., 1982), TBSV (Hopper et al., 1984), TCV (Carrington et al., 1987), CNV, CarMV, RCNMV and melon necrotic spot virus (MNSV; Riviere et al., 1989). The three-dimensional structure of the coat protein subunits of some of these viruses has been determined at high resolution (Olson et al., 1983, Hogle et al., 1986, Abad-Zapatero et al., 1980) and appeared to be similar. Essentially, the subunits are organized into four distinct domains: R (random, N-terminal), a (arm, connecting R and S), S (shell) and P (projecting, C-terminal). The highest degree of sequence similarity was found in the higly structured S domain. For TNV coat protein the highest number of identical amino acid residues in the S domain was found with SBMV coat protein (63

i.e. 34% of paired residues) whereas with the others there was only between 22% and 27% similarity (Meulewaeter et al., 1990b). Moreover, the total number of residues in the S domain of TNV (186) is closer to that of SBMV (191) than to that of all the others (between 162 and 167). Only a few small gaps are needed for the alignment of TNV and SBMV coat protein. So, TNV coat protein could be aligned against the α helices C and D of SBMV which are lacking in TCV and TBSV. TNV and SBMV coat protein are also more similar at their Ca^{2+} binding sites and both lack the P domain.
The 7.9-kDa protein shows, besides an identical C-terminal sequence (FNF), only limited sequence similarity with the small proteins of CarMV (p7), TCV (p8) and MCMV (p9). The translation products of the two other small ORFs do not show sequence similarity with any other known viral protein.

Relationship with other viruses

Based upon its overall genomic organization, tobacco necrosis virus is closely related with CarMV and TCV. However, the different proteins encoded by TNV show sequence similarity with those of various other small icosahedral viruses. First, the putative RNA polymerase of TNV is also homologous to those of CNV, TBSV, BYDV, MCMV and RCNMV. Second, the coat protein sequence is similar to that of SBMV, although the RNA polymerase of the latter (Wu et al., 1987) shows only limited sequence similarity with that of TNV (around the GDD motif). Less sequence similarity was found with the capsid proteins of other icosahedral viruses, like TCV, CarMV, CNV, RCNMV, TBSV and MNSV. All these relationships, together with those previously described (Mayo et al., 1989, Veidt et al., 1988), support the concept of modular evolution (Zimmern, D., 1987) by which genes or parts of genes can be exchanged between viruses, permitting a more or less independent evolution of different genes.

As suggested earlier (Morris and Carrington, 1988), TNV is clearly related to the carmoviruses, but there are also some major differences, as the absence of a cap structure at the 5'end of TNV RNA. Furthermore, the coat protein of TNV is more related to the structural protein of SBMV. The presence of two additional ORFs is also a potential difference. These arguments support the classification of TNV in a monotypic virus group.

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FUNGUS A HOST OF MAIZE INFECTING TOBACCO NECROSIS VIRUS

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Summary

A new severe disease of maize grown for seed production was found to be caused by tobacco necrosis virus (TNV). Infected plants exhibited leaf scorch, yellowing and premature death leading to severe crop loss in the 1988 season. Water samples taken from several irrigation sources in 1989, in three different regions, were found to contain infectious TNV. Water from the same sources had been used to irrigate maize in the 1988 season. Soil samples taken in 1989 from fields on which diseased maize had grown during the 1988 season also contained infectious TNV. The presence of a fungus appeared to be a common contaminant of seed, plants, water and soil samples analysed. Infectious TNV was isolated from fungal cultures grown on culture media. The presence of virus inside fungal hyphae was demonstrated by immuno-fluorescent technique. Electron microscopy confirmed the presence of hexagonally shaped virus particles in seed, water, soil and fungal preparations. The TNV isolated from maize reacted strong positive with anti-TNV-A and less strong with anti-TNV-D sera received from Professor R. Koenig, BBA, Braunschweig. A coat protein M_r of 30 KDa and ssRNA of M_r 1.3 Mda (3.9 kb) was determined for the maize infecting TNV-isolate. The isolate from the fungus after propagation in plants could not be differentiated from the maize-isolate.

Introduction

A new disease of maize was noticed for the first time in the 1988 winter season in the Eastern Transvaal Lowveld. The magnitude and destructiveness of the disease justified a more detailed investigation. Initial studies had indicated that a complex of viruses was present in natural infections including maize streak virus, maize dwarf mosaic virus (strain A & B), cucumber mosaic virus and another virus which was later identified as tobacco necrosis virus (TNV). The presence of a particular fungus in all materials investigated, focussed our attention on its possible role in causing the disease of maize. In this paper we provide evidence that this fungus is a host of TNV.

Materials and Methods

Material brought to the laboratory from the maize producing regions for analysis included diseased plant material, original and progeny seed, soil, irrigation water, grasses including *Panicum maximum* and *Sorghum halepense*. Plant material was ground in 0.01M phosphate buffer pH7 clarified by low speed centrifugation and concentrated by ultracentrifugation for two hours. Soil (300 ml) soaked in distilled water and water samples (300 ml) were concentrated by ultracentrifugation. The fungus was cultured on potato dextrose agar (PDA) or liquid potato dextrose. Fungal hyphae were fragmented in a mortar and pestle with celite or with an Ultraturrax homogeniser before concentration. Culture media in which hyphae had grown, were also concentrated. A variety of hosts were used for infectivity assays including *Nicotiana tabacum* cv. Soulouk, Xanthi and White Burley, *N. glutinosa*, *N. benthamiana*, *Chenopodium quinoa*, *C. amaranticolor* and *Phaseolus vulgaris* cv. Bonus. Assays were done on detached leaves on moist filter paper in large Petri dishes. Inoculated leaves were kept in the dark for 24 hours before placing them under cold fluorescent light. Antisera were prepared to concentrated preparations of naturally infected plants and after propagating virus isolates in laboratory plants. For comparative purposes antisera to TNV strains A and D were received from Dr. R. Koenig, BBA, Braunschweig and anti-TNV-A from Dr R.O. Hampton, Oregon State University. Immunological assays included the following: Ouchterlony double diffusion in agar gel, immuno-fluorescent technique (6), immunoelectroblotting (7) and immunosorbent electron microscopy (ISEM). Virus coat protein was sized on PAGE according to the method of Laemmli (5). ssRNA isolation was performed (2). Sizing of ssRNA was done on denaturing formaldehyde agarose gels with an RNA ladder (BRL) and tobacco mosaic virus RNA as reference. A digoxygenin-labelled probe was made by using the Boehringer protocol and a ssRNA end-labelled probe (1).

Results

Extracts prepared from cultured fungus yielded infectious TNV and typical hexagonal particles were viewed in the electron microscope (Fig. 4A). Examination with fluorescent-labelled antibodies revealed strongly fluorescing hyphae and apparent virus crystals inside hyphae (Fig. 1A). Sporangia with spore content intact fluoresced strongly (Fig. 1B). Single spore progeny of the positively infected cultures continued to be infected after several cycles of subculturing (results not shown). Free-living nematodes which came into the laboratory with field samples appeared to feed on the same fungus. When treated with fluorescent-labelled antibodies, such nematodes appeared to harbour fungal clusters in the esophageal and anterior intestinal regions (Fig. 1C) (9). Maize plants

infected with a mixture containing fungus, virus and nematodes became infected and fungal masses were seen in maize leaf cells when treated with fluorescent labelled antibodies (Fig. 1D).

Immunoelectroblots of concentrated specimens before or after passage through plants, with various antisera, showed a band in the 30 KDa position. The virus isolated from fungus growing in water (TNV-WF) was postively identified as a TNV-A type strain with anti-TNV-A serum. The virus isolate was subsequently used as marker to identify further new isolates. Fig. 2A & B illustrates a typical blot where samples prepared from water concentrates after one passage through plants were blotted with (A)anti-TNV-maize and (B)anti-TNV-WF. The TNV-isolates were further characterised by sizing the RNA. A size of 3.9 kb was determined for the RNA of TNV-WF, TNV from river water (Lo), and irrigation water (Br) (Fig. 3A). The same samples used in Fig. 3A were run on an agarose gel, blotted onto Hybond-N and hybridised with a digoxygenin-labeled TNV-WF-RNA probe (Fig. 3B). In PAGE the virus coat protein was sized at 30 KDa using standard M.W. markers (Fig. 3C). Both values correspond well with published data (Fraenkel-Conrat 1985). In ISEM studies antisera to strain-A and -D were utilised to determine the strain relationship of TNV-WF. Fig. 4 illustrates the strong positive trapping and decorating achieved with anti-TNV-A serum (b); similarly strong trapping and decorating with the anti-TNV-WF homologous serum (c) and positive trapping with anti-TNV-A but little or no decoration with anti-TNV-D (d). End-labelled ssRNA of TNV-WF was used to probe total nucleic acid extracts of several plant species suspected to be infected with TNV (Fig. 5A). The hybridisation blot further provides evidence that TNV replicates in fungal hyphae (row e, lane 3a and 4a) and that the virus product is either exported into the liquid media or released when hyphae age (row e, lane 5a, 6 & 6a). Virus particles and virus protein were repeatedly identified in liquid culture media.

Discussion

Initial biological and epidemiological studies had identified certain factors which apparently all contributed to the scorching disease in maize (10). Further in depth studies provides evidence for the presence of TNV inside fungal hyphae. Previous work provided evidence for the association of TNV and the zoospores of the fungus *Olpidium brassicae* (3). Our results provide evidence that TNV appears to have a firm association with a fungus belonging to the Zygomycetes. Evidence obtained with dot blot hybridisation confirmed the replication of TNV in fungal hyphae (Fig. 5A & B). This finding is supported by viewing virus crystals in hyphae (Fig 1A) and the isolation of fair quantities of infectious virions from fungal cultures and that the fungus did not lose the ability to support virus replication through various generations of subculturing. To our knowledge this is the first report of a fungus being host to TNV. It is also the first report of an alleged plant virus having a fungal host. In an investigation stretching over two seasons, including three geographic regions, the presence of the fungus and the virus appeared to be interlinked. The fact that TNV-maize was found to be seedborne (10) explains the gradual build-up of the disease over several generations, resulting finally in total crop loss of one hybrid during the 1988 season (10). The disease situation was further compounded by the presence of virus, fungus and free-living nematodes in water used for overhead irrigation. Maize irrigated with subterranean water did not become diseased.

TNV had previously been isolated from surface water (8) and from ditches and drainage canals (4). We now report a fungus being host to TNV found in fast flowing river water and in storage water of a large irrigation scheme. The fungus and virus was also isolated from soil irrigated with contaminated water. The finding that TNV was detected in several systematically diseased plants (Fig. 5A) indicates that its occurrence is fairly common in South Africa.

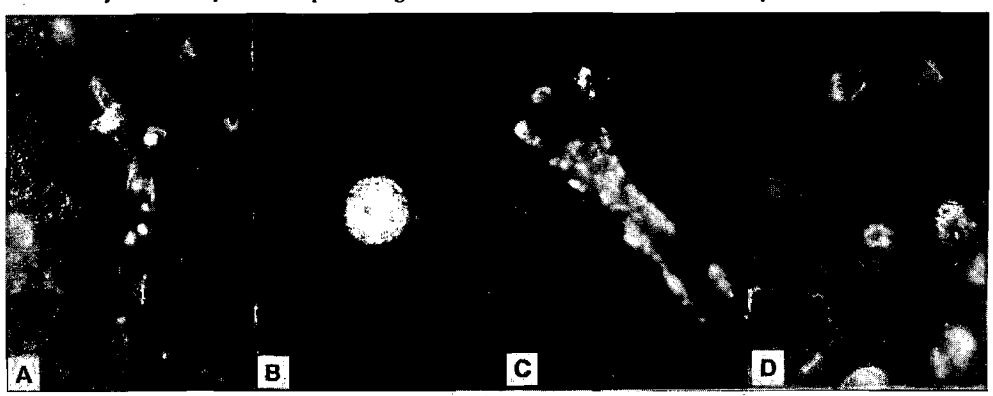

Fig. 1. (a) Fungal hyphae treated with fluorescent antibody showing virus crystals (b) a sporangium with fluorescing spores photographed with a Neofluar lense (40x); (c) nematode esophageal and anterior intestinal region with fluorescing fungal clusters and (d) maize leaf from plant infected with nematodes, fungus and virus illustrating fluorescing fungal masses in leaf cells.

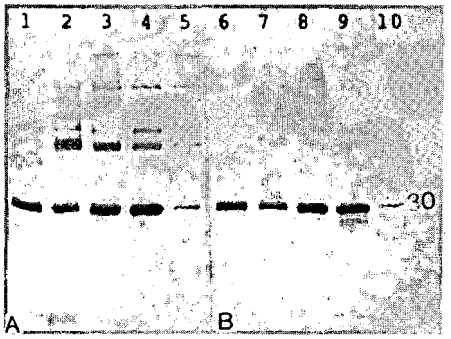

Fig. 2. Immunoelectroblot of viral isolates from plants inoculated with concentrated preparations from water collected from two sources. Blots were probed with anti-TNV-maize and anti-TNV-WF; 1 and 6, TNV-WF; 2,3 and 4, TNV isolate from river water (Lo) propagated on bean and two different tobacco cvs; 5 and 10, TNV isolate from irrigation water (Br) propagated on tobacco.

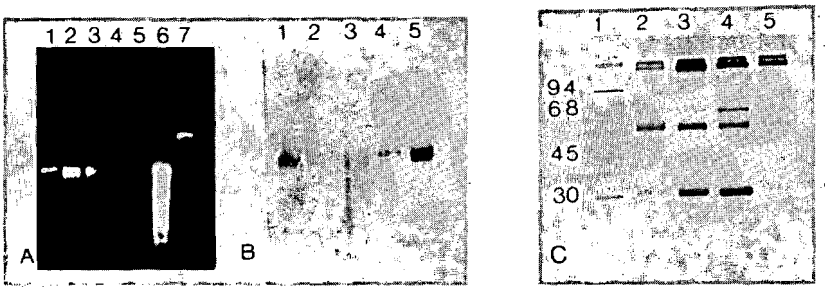

Fig. 3. A. Agarose gel of RNA. 1, TNV-WF; 2, TNV-irrigation water (Lo); 3, TNV-irrigation water (Br). 4, TMV; 5, tobamo; 6, TNV-WF; 7, TMV. B. Digoxygenin-labelled blot of RNA. 1, TNV-WF; 2, TNV-D; 3, TNV-A; 4, TNV-irrigation water (Br); 5, TNV-river water (Lo). Water-isolates of TNV were passaged once through plants for RNA isolation. C. PAGE of virus coat protein. 1, M.W. markers; 2-4, water concentrates (Lo) propagated on different plants; 5, water concentrate (Br) propagated on tobacco.

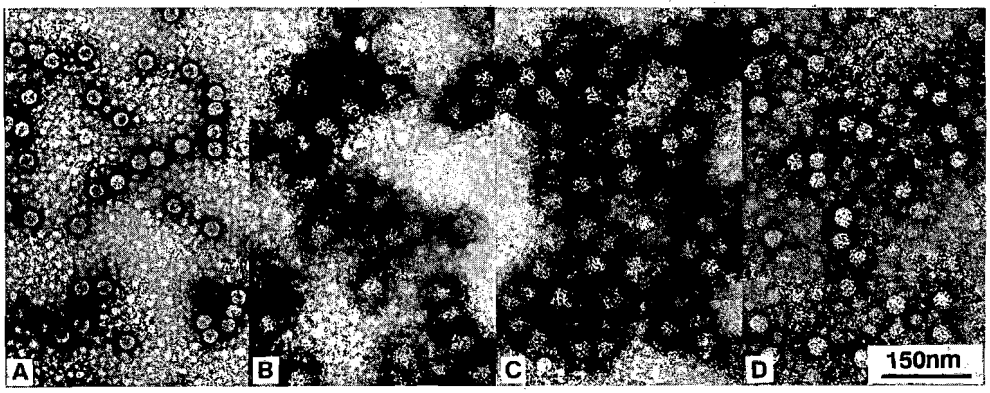

Fig. 4. Electron micrographs of TNV-WF (a) Negative stain, (b) and (c) positive trapping and decorating with anti-TNV-A and anti-TNV-WF and respectively and (d) trapping with TNV-A and decoration with anti-TNV-D serum.

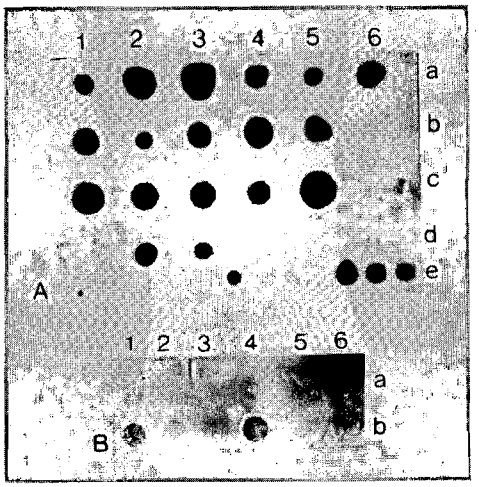

Fig. 5. A. Total nucleic acid extracts of several species of plants, one fungus isolate and its culture fluid, probed with end-labelled ssTNV-WF-RNA. (a) 1-6, *Passiflora* specimens; (b) 1-6, *Gramineae* specimens; (c) 1, TNV-WF; 2, TNV-soil isolate; 3, *Lachenalia*; 4 *Piper nigrum*; 5, commercial tobacco; (d) 1, TNV-D; 2, TNV-WF; 4 and 5, clean tobacco leaf; (e), 3a & 4a, Water-fungus hyphae grown on agar; 5a,6,6a, residual culture fluid after removal of water-fungus hyphae from liquid media. B. Digoxygenin-labelled probe of TNV-WF-RNA; (a) 1, TNV-A ssRNA; 6, TNV-WF ssRNA isolated from fungus; (b) 1-6, dilutions of TNV-WF-ssRNA.

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SOIL-BORNE VIRUSES OF MELONS AND CUCUMBERS IN FRANCE.

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Summary

Several viruses that produced necrotic lesions on most host range plants were isolated from systemically infected cucurbits in French glasshouses or plastic tunnels between 1979 and 1989. Single-lesion transfers were done for each of nine isolates. The isolates were identified serologically with the protein A-sandwich enzyme-linked immunosorbent assay (PAS-ELISA). Five of the isolates from cucumber and melon were melon necrotic spot virus (MNSV); two isolates from cucumber were A serotypes of tobacco necrosis virus (TNV); one isolate from cucumber was a D serotype of TNV; and one isolate from cucumber was petunia asteroid mosaic virus (PAMV). The MNSV isolates were restricted to cucurbit hosts and could be divided into strains originating from cucumber or those from melons by the differential reaction of Marketer cucumber. The cucumber strains caused 2-4 mm necrotic lesions on cucumber cv Marketer in 4-5 days whereas the melon strains produced pinpoint necrotic lesions. The present paper is the first report of a D serotype of TNV on cucumbers in Europe or of natural infection of cucurbit hosts by PAMV.

Introduction

Several soil-borne viruses have been described on cucurbits in recent years: cucumber soil-borne virus (CSBV), cucumber leaf spot virus (CLSV), cucumber fruit streak virus (CFSV), and melon necrotic spot virus (MNSV). These viruses are distinct from viruses reported earlier on cucurbit hosts: cucumber necrosis virus (CNV), and tobacco necrosis virus (TNV); or others such as tomato bushy stunt virus (TBSV) from non-cucurbit hosts. Suspected soil borne viruses were isolated at Montfavet between 1979 and 1989 from melons and cucumbers with systemic necrotic symptoms. The objective of this paper is the serological identification of nine viruses from this collection.

Materials and Methods

The viruses were stored in infected plant tissue dried over calcium chloride. The dry cultures were recovered by sap inoculation to host range plants and maintained in the cucurbit host from which the virus had been isolated originally. Single local lesion isolates were prepared for each unknown virus. Serological tests were done by the protein A sandwich-enzyme linked immunosorbent assay (PAS-ELISA) (Edwards & Cooper, 1985) using protein A-alkaline phosphatase conjugate from Sigma. The antisera were from collections of the authors plus CLSV from Dr. Weber, CFSV from Dr. Gallitelli, CSBV from Dr. Koenig, and MNSV from Dr. Bos. Homologous viruses were used as positive controls. The degree of serological relatedness of some isolates was visualized in gel double diffusion tests.

Results and Discussion

Melon necrotic spot virus (MNSV) which has been found in many countries (*Hibi* & Furuki, 1985) was the most commonly detected virus. Five of the isolates were identified as MNSV because they reacted with an antiserum to MNSV provided by L. Bos but not with antisera to CNV, CLSV, CFSV or TBSV. The host range of MNSV was restricted to cucurbits and we did not get necrotic local lesions on Chenopodium spp. as reported for the strain of this virus isolated from melons with the systemic disease, criblure (Marrou and Risser, 1967). Isolates of MNSV were grouped into 2 subgroups that were correlated with the host of origin (melon vs. cucumber) by the differential reaction of Marketer cucumber. Melon isolates caused pinpoint necrotic lesions in 5-7 days whereas cucumber isolates caused 2-4 mm necrotic lesions in 4-5 days. The cucumber and melon isolates caused similar local necrotic lesions on melons and pin-point necrotic lesions on cucumber cv K1700 in 5-7 days.

Three isolates, each obtained from cucumber in a different greenhouse near Orleans in June of 1987, were identified as tobacco necrosis virus strains (TNV). Two isolates reacted in PAS-ELISA with antiserum to Kassanis' A strain of TNV (KA TNV, A serotype) but not with antiserum to New Zealand strain of TNV (NZ TNV, D serotype). In gel double diffusion plates, no hooks formed between these two isolates and KA TNV when tested against KA TNV antiserum. The third isolate reacted with antiserum to NZ TNV but not with antiserum to KA TNV in PAS-ELISA. No hooks formed between this isolate and NZ TNV when they were tested against NZ TNV antiserum in gel double diffusion. An A serotype of TNV, originally named cucumber necrosis virus van Koot and van Dorst (1955), has been reported on cucumbers in Europe (Babos & Kassanis, 1963) and a D serotype of TNV has been reported from New Zealand (Thomas & Fry, 1972). All three French isolates of TNV were acquired in the in vitro manner and transmitted by a single sporangium isolate of O. brassicae from California. The present report is the first of a D type of TNV on cucurbits in Europe.

One virus isolate (E60) collected from cucumber in southern France in April 1980 was identified as PAMV. When it was increased in cucumber cotyledons, it reacted in PAS-ELISA with antisera of TBSV-type, TBSV-BS, and PAMV but not with antisera to CNV, CSBV, CLSV, or NZ TNV. The results with PAS-ELISA agreed with the serogrouping of tombusviruses (Koenig & Gibbs, 1986) and with lack of serological relationship between TBSV and CNV which has been placed in the tombus group (Rochon & Tremaine, 1988). In gel diffusion tests using antisera to PAMV and to E60, there were no hooks between either virus with either antiserum. Although PAMV and E60 are serologically identical, they are probably not biologically identical. The homologous optical density values obtained in PAS-ELISA for PAMV and TBSV that had been increased in melon or cucumber cotyledons were at background levels in one test and weakly positive in another test whereas these viruses were readily detected from N. clevelandii. PAMV was detected in cucurbit bait plants in greenhouse tests (Lovisolo et al. 1965) but has not been found previously in naturally infected cucurbits.

Diverse viruses have been found in necrotic syndromes of cucumbers and melons in France. Their incidence and economic impact are probably greater than previously recognized and warrant further study.

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ECOLOGICAL OBSERVATIONS ON PETUNIA ASTEROID MOSAIC VIRUS (PeAMV)

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Summary

PeAMV is a tombusvirus first found in petunia in 1954, but never isolated again from that plant. It has been reported mainly from woody hosts (cherries, plums, grapes, privet and dogwood), from hops, spinach, and from the roots of Chenopodium album, Cucumis melo, Plantago major and Stellaria media.
The virus has some properties that make it soil-borne: high concentration in roots, natural leaching of virus particles from roots, rather long persistance in soil, easy mechanical transmissibility.
Available data suggest that PeAMV is common in the roots of many plants, and is spread naturally mainly by surface water.
PeAMV, in the first environment where it was found (petunia and privet), may have been transmitted through a fungus, but such a vector has not yet been found. Further work using Olpidium radicale as a possible vector would be interesting.

PeAMV is a tombusvirus first found in petunia in 1954 (Lovisolo, 1957), but never isolated again from that plant. During the following ten years nothing was published on the biology and ecology of this virus. The second report of the presence of PeAMV in nature was that of Lovisolo et al. (1965) referring to the isolation of the virus in 1964 from roots mainly of privet (Ligustrum vulgare) but also of Chenopodium album, Cucumis melo, Plantago major and Stellaria media. This second isolation was from the same environment as 1954: 21 big concrete pots (each containing about 80 cubic dm of soil) in which privet plants were grown, but where occasionally petunia and volunteer plants, mainly weeds, were present.

Starting from 1976 PeAMV was reported from other plants and countries. First in Ontario (Canada) where the virus (then called tomato bushy stunt-P) was isolated from fruit pitting of sweet cherry (Allen and Davidson, 1967; Davidson and Allen, 1976). In the same year PeAMV was found in grapevines in West Germany (Bercks, 1967), and a few years later Brunt and Stace-Smith (1972) found a virus closely related to PeAMV, fairly common in dogwood and privet in Sussex, England (A. Brunt, personal communication).

PeAMV was reported more frequently starting from 1975. Albrechtova et al. (1975) found that the disease known since 1956 in Central Europe as detrimental canker of sweet cherry was associated with a virus similar to Allen and Davidson's TBSV-P and to Bercks' grapevine isolate. In the following years isolates or strains of PeAMV were found in Czechoslovakia, East and West Germany and Italy in cultivated cherries, plums and grapes, as summarized by Koenig and Kunze (1982) and Martelli et al. (1988). In addition PeAMV was also isolated from hops with yellow mottling and malformation in Czechoslovakia (Novak & Lanzova,

1976), and from spinach with stunting and leaf symptoms in Yugoslavia (Eric et al., 1986).

Important in understanding the ecology of PeAMV is the finding of the virus in water of ditches coming from an area of vineyards, orchards and asparagus fields in the Palatinate, West Germany (Koenig et al., 1989).

A review of the reports shows that the main hosts of PeAMV are woody plants, especially grapevine or cherry, and that particularly the root systems are invaded, rather than whole plants, at least in species like privet. It may be that hedge or wild plants present near the vineyard and the orchards from where infected water was coming are the main source of the virus. It is now clear that the name "petunia asteroid mosaic" is misleading because petunia is not the main host and because some strains of PeAMV do not infect petunia or cause only chlorotic or necrotic lesions (Koenig and Kunze, 1982).

PeAMV has a wide experimental host range covering about 60 species in 19 families (Lovisolo, 1957; Lovisolo et al., 1965; Allen & Davidson, 1967). It is serologically closely related to artichoke mottled crinkle virus, intermediately related to pelargonium leaf curl virus and to tomato bushy stunt virus-BS3, and distantly related to eggplant mottled crinkle virus (Koenig and Gibbs, 1986). Relationships between tombusviruses is still not completely clear: there are important serological variabilities at the strain level (Wetter and Luisoni, 1969; Hollings and Stone, 1975); serological relatedness is not correlated with the estimates of genome homologies (Gallitelli et al., 1985; Koenig and Gibbs, 1986).

PeAMV has some properties that make it soil borne: high concentration in roots, natural leaching of virus particles from roots, easy mechanical transmissibility, and long persistence in soil, especially in the presence of clays, silicates, or organic material.

PeAMV, in the first environment where it was found (petunia and privet), may have been transmitted through a fungus, but such a vector was not found (Lovisolo, 1966; Campbell et al., 1975). Further work would be interesting, using Olpidium radicale as possible vector. Until now transmission tests have been done with O. brassicae and Lagena radicicola (Campbell et al., 1975). Macfarlane (personal communication) suggested tests with O. radicale, being aware that in England this fungus becomes inactive in summer (Macfarlane, 1982). Furthermore O. radicale is the vector of cucumber necrosis virus (CNV) and of melon necrotic spot virus (MNSV). PeAMV and CNV are tombusviruses, and MNSV is a carmovirus, but between these two groups there are many affinities (Martelli et al., 1989).

PeAMV is probably of temperate origin, being present mainly in temperate Europe, and under natural conditions infecting plants of temperate origin. The virus is also present in Canada in sweet cherry, and probably in grapes (Davidson and Allen, 1976), where it could have been introduced with infected material.

If we consider the whole range of viruses which are fungus-transmitted, we see that this transmission is particularly important for viruses which are unstable outside the plants

and/or have low concentration in the roots, such as tobacco stunt and lettuce big vein viruses. For viruses such as the tombusviruses, which are very stable in the soil and reach high concentrations in the roots, possible fungus transmission is not so important for their epidemiology and ecology, as spread is helped by human activity. With PeAMV propagation of infected material (both infected seed and cutting/root-stock material) may be important, as it may be planting susceptible plants in infected soil.

However for the ecology and epidemiology of any virus it is important the existence of a vector even if the vector is not important from the agricultural point of view. Plant pathogens can not be maintained without horizontal transmission under purely natural conditions, as discussed by Purcell (1982). Furthermore fungus transmission may help the virus to infect certain hosts that possess resistance to infection by virus particles present in the soil. Information on the existence of a vector may also be interesting for evolutionary implications, because it may help to draw phylogenetic affinities between members of tombusviruses and carmoviruses, two groups which include fungus-transmitted viruses.

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NON-VECTORED TRANSMISSION OF PLANT VIRUSES

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Summary

After detection of petunia asteroid mosaic virus (PAMV) in pome and stone fruit trees its natural distribution without vectors was investigated. It was established that PAMV retains its infectivity in naturally virus contaminated soil up to 14 weeks. The virus was isolated from artificially contaminated soils after autoclaving 2 hours at 121 °C and 11.8 x 10^4Pa. PAMV was more stable in moistured soil and sand samples than in dry samples. After repeated freezing (-20 °C) and thawing the samples infectivity of PAMV decreased to 1/10th of the initial level. The virus adsorption was more strong in quartz-sand than in soil. Furthermore, it was demonstrated that PAMV could be released from roots of infected plants of *Celosia argentea* into the surrounding medium 20 d p.i. or 10 d after appearance of systemic symptoms. Virus-free plants of *C. argentea* growing under sterile conditions can be infected by PAMV via the roots from a virus contaminated medium. Thus, the transmission of PAMV from infected plants to healthy plants without virus vectors could be demonstrated. Analogous results were obtained with tobacco rattle virus and in some respect with cucumber mosaic virus but not with barley yellow mosaic virus.

Introduction

Outset of our investigations was the detection of a tombusvirus in pome and stone fruit trees (Kleinhempel et al., 1971; Richter et al.; 1977) which was originally described as tomato bushy stunt virus, later identified as petunia asteroid mosaic virus (PAMV) (Grüntzig et al., 1989). The detection of PAMV in fruit trees raises the question of its origin and natural spread. Lovisolo et al. (1965) described the occurence of this virus in soil but no vector was known.

Materials and Methods

Natural soils (loam with organic compounds) from one apple and one cherry orchard were suspended in phophate buffer and fractionated by centrifugation including ultracentrifugation. Sediments were used for mechanical transmission on carborundum dusted *Celosia argentea* L. leaves. The serological identification was done by agargel-double-diffusion tests (Kegler et al. 1980). Either soil samples close to infected trees or more distantly from these trees contained the virus. Because the isolation was successful repeatedly in several seasons, the stability of this virus in soil should be investigated.

Results and Discussion

Virus stability in soil

These investigations were carried out with naturally and artificially virus-contaminated soil. For artificial contamination we used infectious extracts from *C. argentea*-leaves. As substrates we applied steam-sterilized soil, sterilized quartzsand and bentonite. The elution of the virus was done as described by Kegler and Kegler (1981). The presence of viruses in the eluates was assayed by mechanical transmission on *Chenopodium quinoa* Willd. and counting the local lesions. Statistical analysis was done by one-way analysis of variance.

There was a clear evidence that PAMV could retain its infectivity in naturally contaminated soil, even after repeated drying and wetting up to 14 weeks. From artificially contaminated soils the elution of undestroyed particles could be demonstrated by electron microscopy . Moreover, infectious PAMV was isolated from naturally and artificially contaminated soils after autoclaving the soils 2 hours at 121 °C and 11.8 x 10^4 Pa. The virus could not retain its infectivity in plant sap treated in the same way. The virus in soil samples lost its infectivity after 2h at 137 °C and 14.7 x 10^4 Pa.
These relatively high stability could be explained by the protecting influence of organic and/or anorganic colloids. The influence of soil moisture on infectivity of the virus particles in the soil was demonstrated by further experiments.
At a constant temperature of 25 °C in moistured soil and sand samples PAMV was infective for 50 and 43 days, respectively. During this time the infectivity of the eluates decreased to nearly 50 % of the initial level . After drying the samples the infectivity decreased more quickly (Fig. 1).

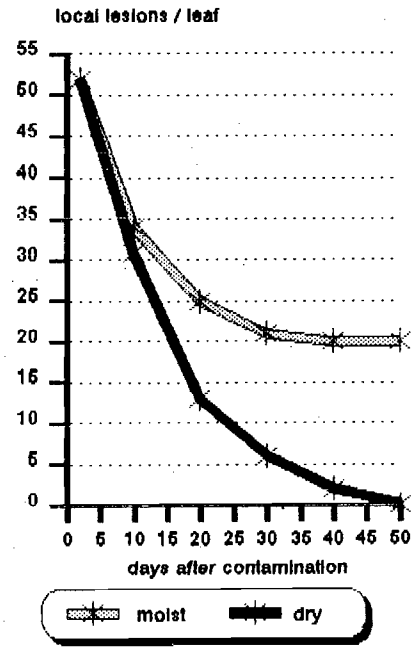

Fig. 1: Concentration of PAMV in soil

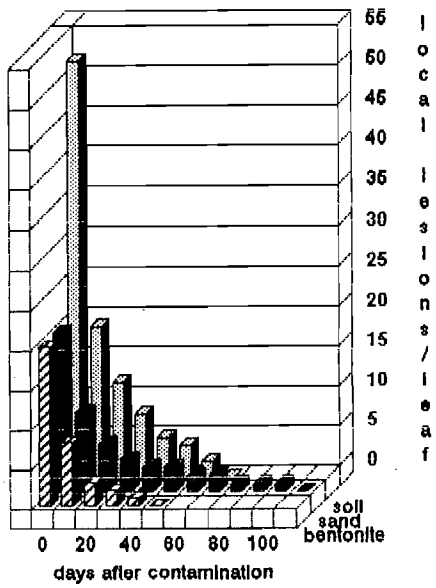

Fig. 2: Concentration of PAMV buffer after substrate elution

Storing the samples at 37 °C the infectivity decreased as quick as in dried samples. At 20 °C below zero the infectivity was stable for 6 weeks, after thawing the infectivity decreased to 1/10th of the initial value.
For comparative investigations on virus adsorption of different substrates the samples were eluted in equidistant intervals with 0.03 M phosphate buffer. The infectivity was evaluated by counting the

local lesions after mechanical transmission. It was shown that the adsorption was more strong in quartz-sand than in soil (Fig. 2).After 60 d (i.e. 7 cycles of elution) neither in eluates from bentonite nor from soil infectious virus was present, whereas PAMV could be isolated from quartz-sand even after 100 d (i.e. 10 cycles of elution).
To demonstrate the adsorption of PAMV according to the depth of soil, columns of different height were filled with noncontaminated soil. 5 ml of virus-containing leaf sap was placed on the top of each colum and then eluted with boiled rainwater (pH 6.9). The infectivity of the eluates decreased with increasing height of the soil in the columns (Tab. 1).

Tab. 1: Adsorption of PAMV by soil particles

Height of the soil column [cm]	Infectivity of the extracts [%]
0	100
5	48.6
10	4.9
20	0.8
40	0

All infectivity was lost by using columns of 40 cm height. In naturally contaminated soils there was also no virus detectable in the depth of 40 cm.

Release of viruses from roots of infected plants

The detection of "free" virus particles in soil raised the question of its sources. Releasing of virus particles from the roots of infected plants was the most suitable assumption. To proof this hypothesis test plants were grown in nutrient solution under sterile conditions and inoculated with a virus suspension passed through a bacterium filter. After inoculation each 1 ml of nutrient solution was removed in equidistant intervals and assayed for the presence of viruses by mechanical transmission on *C. quinoa* . Under these conditions PAMV could be detected in the nutrient solution 20 days after inoculation or 10 d after occurence of systemic symptoms on *C. argentea*. The concentration of viruses in the solution was nearly constant for 60 d. Thus, it was demonstrated that PAMV could be released from roots of infected plants into the surrounding medium.

Infection of healthy plants via the roots

Investigating the infection of healthy plants via the roots *C. argentea* was grown under sterile conditions in glass tubes on filter paper, using nutrient solution, agar or quartz-sand as substrates . These substrates were artificially contaminated with purified virus preparations which were sucked through bacterial filters before. After 5 weeks leafs of the test plants grown under sterile conditions were removed and mechanically assayed on virus infection. It was shown, that PAMV is able to infect the plants systemically via the roots, but there was only symptomless infection. The number of infected plants grown in quartz-sand was as twice as much compared with plants grown in nutrient solution or agar (Tab. 2).

Tab. 2: Infection of healthy plants of *C. argentea* by PAMV in sterile condotions

Nutrient solution	10.6 % infected plants
Nutrient agar	9.3 % infected plants
Quartz sand with nutrient solution	24.1 % infected plants

There is some evidence that micro wounding during the growth of the plants in quartz-sand favored infection by PAMV.
Summarizing it could be demonstrated that PAMV was released from the roots of systemically infected plants into the surrounding soil. In the soil the virus could survive extremly unfavourable conditions. Without any vector PAMV could infect healthy plants via the roots systemically. Thus, the non vectored transmission of PAMV was demonstrated.
Comparable results were obtained by Barchend (1983) in the case of tobacco rattle virus (TRV). TRV could be isolated from soil samples 100 cm below soil level. Non-vectored transmission could also be demonstrated with cucumber mosaic virus (CMV) by Pares and Gunn (1989) and Kontzog (1989). Both viruses have known vectors.
PAMV occurs naturally mostly in loamy soils rich on colloids whereas TRV is mostly restricted on sandy soils poor on colloids.
Experiments with non-vectored transmission of barley yellow mosaic failed. The particles of this virus lost it infectivity after 1 or 2 days in the soil.
Non-vectored transmission of other stable viruses, f.e. tobamoviruses seems to occur.

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THE ULTRASTRUCTURE OF THE RESTING SPORE FORMATION AND GERMINATION OF OLPIDIUM BRASSICAE

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Summary

The resting spores formed in root cells of tobacco plants, observed with a scanning electron microscope after freeze-fracturing, were smooth spheres initially covered with mucous material. Regular ridges later developed on the surface of the resting spores to form pentamer and hexamer facets. The number of facets was relative to the size of the resting spore. The wall structure, observed in thin sections with a transmission electron microscope, consisted of three layers: outer, inner, and intermediate. The cytoplasm was dense and contained many lipid-like bodies at the periphery. After individual zoospores were developed fully, an exit tube was formed by the protrusion of a membrane through the multilayered wall.

Introduction

Olpidium brassicae is one of the common root-infecting soil-borne fungi and is known as the vector of certain plant viruses. The fungus is endobiotic and holocarpic. The life-cycle of the fungus is simple and has three stages, i.e. zoospore, zoosporangium and resting spore. Zoosporangia and resting spores develop in the epidermal and cortical cells of plant roots following infection of the cells by zoospores. While the ultrastructure of zoospores and zoosporangia of O. brassicae has been studied in detail (Temmink and Campbell, 1968, 1969a, 1969b; Lesemann and Fuchs, 1970a, 1970b; Temmink, 1971), information concerning the resting spore formation and germination at the ultrastructural level has been limited (Hiruki and Alderson, 1976; Hiruki, 1987). This paper reports the results of observations using a tobacco strain of O. brassicae by scanning and transmission electron microscopy during the course of infection of tobacco plants.

Materials and Methods

Culture of O. brassicae. The tobacco strain of the fungus used in this study was originally obtained from a single isolate from tobacco after repeated culture on the roots of cowpea (Hiruki, 1965). In some experiments O. brassicae in tobacco seedlings fully infected with tobacco stunt virus (TSV) was used. The O. brassicae isolates were cultured on roots of tobacco seedlings grown in sterilized quartz sand, and watered with a half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Plants in microincubators (Hiruki, 1969) were maintained in growth cabinets at 17^{0}C with a 16 h light period. The roots were examined microscopically at 24 h intervals for the development of resting spores.

Scanning electron microscopy (SEM). Olpidium resting spores in root tissue were fixed for 1 h at 22^{0}C in 0.1 M phosphate buffer pH 7.0 containing 2% glutaraldehyde and 2% formaldehyde, washed with distilled water, postfixed in 2% osmium tetroxide for 30 min, washed again and then freeze-dried in liquid nitrogen. Some roots containing resting spores were freeze-fractured after fixation. Fixed roots were frozen rapidly in Freon 13 and fractured with a pre-cooled scalpel. The fractured roots were transferred into liquid nitrogen, freeze-dried and examined with a Cambridge Stereoscan S4.

Transmission electron microscopy (TEM). Olpidium resting spores in root tissue, 2-3 mm long, were fixed overnight at 4^{0}C in 0.1 M phosphate buffer pH 7.0, containing 2% glutaraldehyde and 2% formaldehyde. After being rinsed in the same buffer solution for 30 min, and then in distilled water, the samples were post-fixed with 2% osmium tetroxide for 4 h. They were embedded in Araldite after dehydration. Thin sections were stained with uranyl acetate and lead citrate. Micrographs were taken with a Philips Model 200 electron microscope operated at 60 kv.

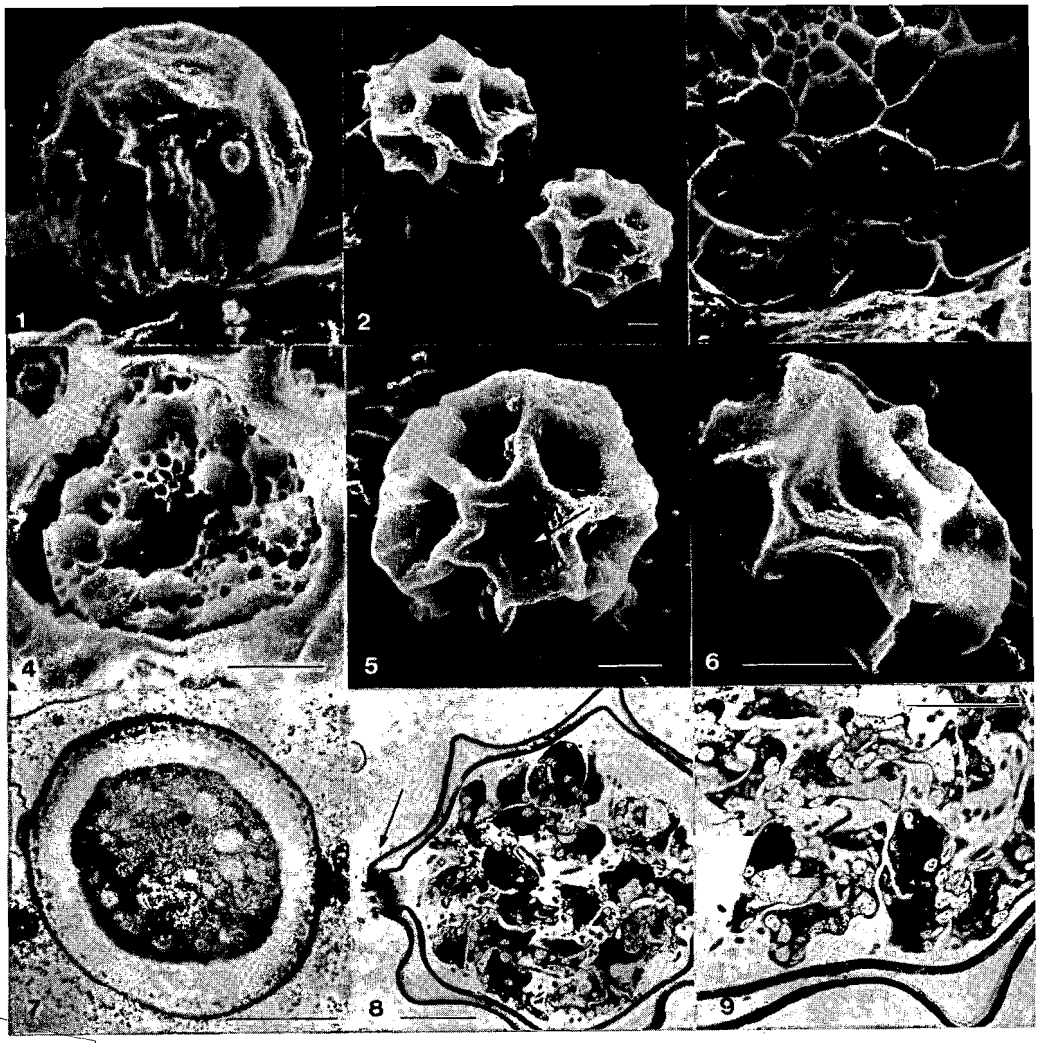

Fig. 1. Olpidium brassicae resting spore at 48 h after infection.
Fig. 2. O. brassicae resting spore showing pronounced ridges at 120 h.
Fig. 3. O. brassicae resting spores in root epidermal and cortical cells of a tobacco seedling.
Fig. 4. Fractured resting spore. Note the thick wall enclosing the fungal cytoplasm with numerous zoospores.
Fig. 5. Exit tubes protruding from the wall of a mature resting spore (arrow).
Fig. 6. Ovoid-shaped resting spores.
Fig. 7. Young resting spore with thick wall. Note the dense cytoplasm.
Fig. 8. Resting spore containing fully developed zoospores. Note the presence of an exit tube (arrow).
Fig. 9. Fully developed zoospores in a resting spore.
Bars, 3μm.

Results

In a time course study of resting spore formation, the initial stage of resting spore formation was difficult to distinguish from that of zoosporangia due to the absence of ridges at the spore surface. However, as the ridges were formed on the wall surface after 48 h, the characteristic morphology of Olpidium resting spore emerged (Fig. 1). At 96 h, resting spores, each with their characteristic thick undulating wall, were clearly recognizable. The ridges were more pronounced at 120 h, often showing a regular combination of pentamers and hexamers (Fig. 2).

Resting spores were present in the cells of the epidermis and cortex of freeze-fractured roots up to 4 weeks after root inoculation with zoospores (Fig. 3). No resting spores were observed in xylem or phloem elements. A freeze-fractured resting spore at 4 weeks showed the presence of numerous zoospores being enclosed in the thick walls (Fig. 4). Exit tubes were found protruding from the walls of mature resting spores (Fig. 5). In some cases, ovoid-shaped resting spores were also observed (Fig. 6).

In the TEM, developing resting sporangia at 72 h had dense cytoplasm containing mitochondria-like organelles enclosed in smooth but thick walls which were apparently composed of different layers (Fig. 7). The inner layer was electron transparent and contained fibrous material. In a mature spore at 4 weeks, the wall was composed of at least three distinct layers, and enclosed fully developed zoospores. (Figs 8, 9). When individual zoospores were developed fully, an exit tube was formed by the protrusion of an inner membrane through the multilayered wall (Fig. 8). The presence of plug material at the very short exit tube indicated that the release of the zoospores was imminent (Fig. 8).

Discussion

The development and differentiation of thalli into zoosporangia or resting spores were apparent approximately 48 h after infection. Unlike zoosporangia, the cytoplasm of thalli that were destined to develop into resting spores did not become multinucleate. Cellular organelles appeared to degrade as lipid bodies started to increase at the periphery of the cytoplasm. The wall of an Olpidium resting spore was laid down exterior to the thallus plasmalemma, and the wall components appeared amorphous at the initial stages of its formation. However, as characteristic ridges started to develop, at least three layers of the wall were recognizable. The pentamer and hexamer facets delineated by the wall ridges probably provide structural stability as well as flexibility to allow contraction and expansion of the resting spore in response to changing environmental conditions. The facets of air-dried resting spores were seen more deeply sunken than those of resting spores found inside root cells. The recovery of TSV has been reported from viruliferous Olpidium resting spores air-dried for 20 years (Hiruki, 1987). The persistence of TSV in Olpidium-infested soil is explained by the internal association of TSV with resting spores. Moreover, TSV in the resting spores has been shown to withstand not only unfavourable environmental conditions but also certain drastic treatments such as strong acid, alkali and UV irradiation (Hiruki, 1987). However, SEM and TEM study in this investigation failed to detect virus or virus-like particles in Olpidium resting spores. The mechanism of persistent association of TSV with resting spores remain to be determined by further investigation.

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TRANSMISSION OF THE AGENT OF TACHE ORANGÉE OF LETTUCE

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Summary

The tache orangée (TO) disease of lettuce was recovered by growing lettuce bait plants in soil that had been stored air-dry for 3 years. The disease was transmitted by root washings containing, among other organisms, abundant zoospores of O. brassicae. The TO disease was severe on European cultivars, Danilla and Melina, and caused mild symptoms on the North American iceberg lettuce, Climax, and Parris Island Cos. The agent of TO apparently was carried internally in the vector because it was transmitted following acid treatment of dry roots of inoculated plants. Attempts to prove that O. brassicae was the vector of the agent of TO were promising but not convincing: one of 24 single sporangial isolates of O. brassicae transmitted TO and it also transmitted lettuce big vein. A TO-like symptom that consisted of mild lesions on a few outer leaves occurred on lettuces inoculated with a single sporangial isolate of O. brassicae from California.

Introduction

A new disease named "tache orangée" (TO) has caused severe losses in greenhouse crops of lettuce in France in recent years. It was often associated with lettuce big vein (BV) and resembled the disease described as "kringnecrose" in the Netherlands (Huijberts et al., 1983) and "necroses annulaires" in Belgium (Verhoyen et al., 1985). These authors have described the disease as soil-borne, associated with O. brassicae, and graft transmissible, but not mechanically transmissible. The objectives of the present work were to provide proof for the suspected role of O. brassicae as the vector and to compare the disease to lettuce big vein.

Materials and Methods

Samples of soil plus roots of affected plants were collected in 1986 from 3 properties where the disease was observed in France and stored air dry in plastic bags. Trapping of O. brassicae from soil, maintenance of cultures on lettuce, and isolation of single-sporangia were done at Montfavet by established methods (Campbell, 1988). The lettuce cultivars were Climax and Parris Island Cos from the United States and Danilla and Melina from France. A single-sporangium culture (SS 61) of O. brassicae from the Salinas Valley of California was used as a standard for lettuce big vein.

Results and Discussion

Bulk cultures (designated A, G, and T) of O. brassicae were trapped from the three soil samples on lettuce cv. Danilla. Twenty bait plants of each culture were transplanted to pots in an unheated greenhouse. Seventeen of 20 plants of culture G had TO and 2 had both TO and BV. Only half the plants with culture A had symptoms that consisted of both TO and BV on most plants. Plants with culture T remained symptomless in this trial but a few plants showed TO symptoms in a later trial. The SS 61 bait plants developed BV alone or a mixture of BV and a TO-like symptom. This TO-like symptom was a mild, orange, necrotic spotting on the lower leaves, but the severity did not increase as TO did. Non-inoculated controls remained symptomless.

Two bulk cultures (G & T) and SS61 were inoculated to an iceberg lettuce (Climax), to Parris Island Cos (PIC), to a crisphead lettuce (Danilla), and to a butterhead lettuce (Melina). For each cultivar, 10 seedlings were transplanted to pots kept in an unheated greenhouse, and 10 seedlings were put in pots in a plastic tunnel. Results have been combined for both sites. Danilla and Melina were as susceptible to BV as Climax, and PIC was less susceptible (symptom expression was delayed). Typical TO symptoms developed on Danilla and Melina but few plants of Climax or PIC showed mild TO symptoms that were limited to a few leaves. The T culture transmitted TO only to 1 plant of Melina. The G culture transmitted TO to 24 of 40 plants of Melina, 4 PIC and 1 Climax. It transmitted BV to only one plant, a Climax seedling. The SS61 culture transmitted BV to all cultivars and TO-like symptoms to 1 plant of PIC.

A trial was done in California in the winter of 1990 to determine if the TO-like symptoms were transmitted by the original SS61 culture. Inoculated plants of Climax, PIC, and Danilla (8-10 plants/cultivar) were kept in a chamber at 18 C for 33 days and then at 16 C for 16 more days. The Climax and PIC plants had BV but no TO-like symptoms. The Danilla plants had BV on 1 plant, TO-like symptoms on another plant, and both BV and TO-like on 5 plants. Equal numbers of noninoculated plants remained symptomless.

Air-dried roots of 6 plants with TO symptoms from culture G were triturated; one half of each sample was acid-treated (N HCl for 2 hr) and the other half was the water-treated check. O. brassicae was baited from both types of extracts of 5 plants but not from the sixth source plant or 4 non-inoculated checks. Four of 5 cultures baited from acid-treated root extracts transmitted TO to assay seedlings as did 5 of 5 cultures from the water-treated checks. The assay seedlings inoculated with the root washings of three non-inoculated checks remained symptomless, but those inoculated with the fourth check developed TO symptoms due to contamination during the growth of the assay plants.

Single sporangia of O. brassicae were isolated from each of the 3 bulk cultures. None of 16 isolates from culture G or 3 isolates from culture T transmitted TO or BV. One of 4 isolates from isolate A transmitted both TO and BV.

Co-transmission of TO and BV by a single sporangium isolate of O. brassicae, co-transmission of both diseases by bulk cultures containing O. brassicae, and the discovery of a TO-like symptom associated with Californian culture support the hypotheses that O. brassicae may be the vector and that TO and BV may be caused by variants of the same agent.

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FLAME CHLOROSIS: A NEW, SOIL-TRANSMITTED, VIRUS-LIKE DISEASE OF CEREALS IN MANITOBA, CANADA

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Summary

"Flame Chlorosis" (FC) is a new, virus-like, disease that has been identified in spring barley, wheat and oat in Manitoba, Canada. FC is novel and unique among cereal diseases on the basis of: a) striking, characteristic leaf symptoms visible as early as the one-leaf stage; b) transmission to spring cereals from soil and association with the zoosporic fungus, *Olpidium brassicae*; c) cytopathology involving extensive vesiculation and peripheral distortion of chloroplasts and mitochondria, but not inclusions of virus-like particles or pinwheels typical of known soil-transmitted viruses of cereals; and d) presence in infected tissue of disease-specific sets of double-stranded (ds) RNAs of ca. 350 to 3500 base-pairs (bp).

Introduction

In June, 1985 spring barley plants in two adjoining fields near Newdale, Manitoba, Canada were observed with striking symptoms (the basis of the name 'flame chlorosis')(Fig. 1) that were unlike those of any known disease or condition of cereals (Haber et al., 1990). A survey for cereal virus diseases across western Canada in July, 1985 failed to find the new disease at any other location. Surveys (cf. Fig. 2) in 1986 and 1987 identified scattered diseased plants in a small number of barley fields near the original Newdale site. In 1988 and 1989, the disease was identified at many more locations near Newdale, and at a few sites was responsible for economic losses. In addition, FC was recorded for the first time in regions of Manitoba outside the Newdale area (Haber, 1990).

Field experiments carried out every summer since 1986 showed that FC could be transmitted by planting seed of barley or wheat in soil where plants with symptoms of flame chlorosis had grown earlier, with symptoms appearing as early as the one-leaf stage (Haber et al., 1990). This observation and an apparent link between continuous cultivation in spring cereals and occurence of FC suggested the disease agent was transmitted from soil (Haber et al., 1990). Other experiments, as well as light- and electron microscopy of diseased and healthy plant tissues from fields with FC plants, ruled out transmission by seed, insects or mechanical means and excluded fungi, bacteria or fastidious prokaryotes as likely causes of the disease (Haber et al., 1990).

Exhaustive examination of FC leaf and root tissue by electron microscopy failed to detect virus-like particles, but instead revealed striking ultrastructural features that include massive vesiculation and peripheral distortion of chloroplasts and mitochondria by invagination (Haber et al., 1990). In roots, the cytopathology of peripheral distortion and vesiculation in mitochondria and leukoplasts was similar to that seen in mitochondria and chloroplasts of leaf specimens (Haber *et al.*, 1990).

Flame chlorosis can be considered a virus-like disease because other causes have been ruled out, and because consistent sets of dsRNAs are found in diseased tissue but not in healthy tissue (Haber et al., 1990; Morris and Dodds, 1979). The emerging picture of flame chlorosis contains novel elements for a disease of cereals: a) association with virus-like dsRNAs but no virus-like particles; b) field transmission of the virus-like FC agent from soil to spring cereals in contrast to the known soil-transmitted cereal viruses which only occur in winter cereals in natural infections (Adams et al., 1986). We report here on our recent studies of the zoosporic fungi associated with flame chlorosis, and of the variations in

Figure 1. Flame chlorosis (FC) symptoms

Detached leaves of barley cv. Argyle; the leftmost leaf is from a healthy plant, the others are from cultured transplants of different FC isolates. Note the clear variegation between chlorotic and green areas.

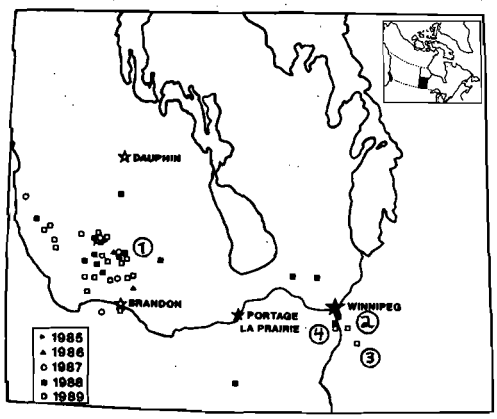


Figure 2. Distribution of flame chlorosis (FC) in Manitoba. At the marked sites for each year FC was present in more than 0.1% of plants in the field, and diagnosis was confirmed by observation of FC-specific symptoms in leaves emerging later in representative transplants. Inset shows area of main map in relation to the northern portion of the North American continent.

patterns of FC-specific RNAs found in flame chlorosis of different cereal hosts and from different locations.

Materials and Methods

Soil samples were taken in the course of a 1989 disease survey (Haber, 1990) from FC fields and from fields carefully examined to be free of FC plants. Zoosporic root fungi were isolated and examined using methods described previously (Slykhuis and Barr, 1978).

Isolates of FC tissue were collected from infected spring cereal plants in the field, and/or from infected plants that had been transplanted and maintained as permanent cultures (Haber et al., 1990). This latter approach made it possible to obtain over time sufficient tissue for extraction of FC-specific nucleic acids even from those isolates where there may have been as little as one young seedling to start with. DsRNA was extracted as described previously (Haber et al., 1990; Kim et al., 1988), and fragment sizes estimated by a robust curve-fitting procedure (Plikaytis et al., 1986), using the BRL dsDNA ladder (.5 to 12 kb; BRL, Gaithersburg MD, U.S.A.) as a known size marker.

Results and Discussion

Isolations of zoosporic fungi from FC- and non-FC soils (Table 1) showed a clear lack of association between the disease and *Polymyxa graminis*, the vector of all fungus-transmitted viruses of cereals known to date. By contrast, there was a strong association between FC and the presence of *O. brassicae* and *L. radicicola*. *O. brassicae* is known to vector tobacco stunt- and lettuce big vein viruses in a persistent-, and tobacco necrosis and satellite viruses in a nonpersistent fashion (Hiruki and Teakle, 1987). However, *O. brassicae* has never been reported as a vector of a soil-borne virus that infects cereals.

To provide direct evidence that either *O. brassicae* or *L. radicicola* are the vector of the disease agent, it will be necessary to show that a unifungal isolate can transmit the disease agent from plants previously infected with FC by mechanical means. Attempts to transmit FC mechanically by methods similar to those employed for known soil-transmitted cereal viruses have been unsuccessful (Haber et al., 1990). Since the FC agent does not appear to be encapsidated in virus-like particles, alternative transmission methods are now being explored that introduce intact FC-specific RNA directly into host cells.

Table 1. Presence of root fungi related to flame chlorosis in barley and wheat in Manitoba

Location	FC[a] (host)	No. of samples	Fungi O.b	L.r	P.g.	L.p.	R.g.
Basswood 1	+ (barley)	5	5	3	0	0	2
Basswood 2	+ (barley)	3	3	3	0	0	1
Newdale 1	+ (wheat)	5	5	1	0	0	1
Newdale 2	+ (wheat)	3	3	3	0	0	0
Foxwarren 1	+ (wheat)	3	2	3	0	0	0
Foxwarren 2	+ (barley)	7	7	5	0	0	5
Birtle 1	+ (barley)	3	3	3	0	0	1
St. Adolphe 1	+ (wheat)	3	2	3	1	0	0
Niverville	+ (barley)	5	5	5	0	0	0
Richot	+ (wheat)	1	1	1	0	0	0
Totals		38	36	30	1	0	10
Birtle 2	- (barley)	3	3	3	0	1	0
St. Adolphe 2	- (wheat)	3	0	3	1	0	0
Dauphin	- (barley)	4	1	4	0	0	4
Totals		10	4	10	1	1	4

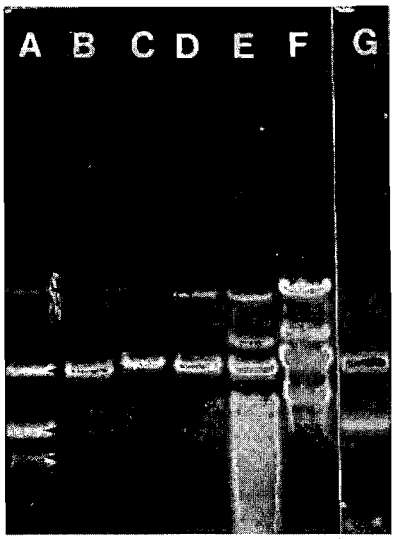
[a] +...flame chlorosis confirmed;
-...flame chlorosis not detected among > 10,000 plants
O.b. *Olpidium brassicae*; L.r. *Lagena radicicola*; P.g. *Polymyxa graminis*; L.i. *Ligniera pilorum*; R.g. *Rhizophydium graminis*

FC-specific dsRNAs were isolated from barley, bread- and durum wheat, and oat that had the characteristic symptoms and cytopathology of the disease. The bread- and durum wheat isolates from western (Basswood) and eastern Manitoba (St. Adolphe) had indistinguishable dsRNA patterns, while there were small variations in the distribution of the smaller dsRNAs between a western (Basswood) and an eastern (Niverville) isolate in barley (Fig. 3). The greatest departure in dsRNA pattern from the 'type' (Basswood barley) FC isolate is seen in the Glenlea oat isolate (Fig. 3).

The oat FC dsRNA was extracted from tissue that had been propagated by repeated teasing apart and re-transplanting of new tiller growth from a single original FC seedling transplanted from the field. Other analyses of FC-specific dsRNA showed that there was no difference between the dsRNA fragment patterns from (more abundant) barley FC tissue harvested directly from the field and that propagated over time from a single plant taken from the same location (Fig. 3).

The close relationship between the putative FC agents of barley, wheat and oat indicated by symptoms, cytopathology, and dsRNA patterns (Fig. 3) appears confirmed in preliminary Northern analyses of hybridization of wheat- and oat FC-specific dsRNAs with labelled probes made from the 1450- and 950 bp fragments of 'type' FC dsRNA. Moreover, no RNAs from healthy cereal tissue are hybridized, further

supporting the concept that the FC-specific dsRNAs are the virus-like causal agent of the disease or its replicative intermediate.



A	B,C,D	E	F	G
			5100	
			3600	
2880		2880	2880	
2430	2430	2430	2430	2430
1750	1750	1750	1750	*1530*
1450	1450	1450	1450	1450
1150	1150	1150	1150	
960		960	960	*1020*
780		*910*	*910*	*880*
710		*750*	*750*	*480*
590				
445			*510*	

A...'TYPE' barley (*Basswood, 1 on map*)
B...bread wheat (*St. Adolphe, 2 on map*)
C...bread wheat (*Basswood, 1 on map*)
D...durum wheat (" " " ")
E...barley (*Niverville, 3 on map*)
F...1 year old plant clone of 'E'
G...oat (*Glenlea, 4 on map*)
arrows indicate bands in 'A'; sizes in bp are estimates based on migration relative to bands of 12 kb dsDNA ladder; numbers offset and in italics denote dsRNAs differing in size from FC-'TYPE'.

Figure 3. DsRNA patterns of flame chlorosis isolates from different hosts and sites

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THE EPIDEMIOLOGY OF WATERCRESS YELLOW SPOT VIRUS

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Summary

In 1989 watercress yellow spot virus (WYSV) infection in experimental watercress beds existed symptomlessly all year round, with symptoms only occasionally seen in the winter months. The distribution of WYSV within the beds was coincident with that of the crook root fungus (Spongospora subterranea f. sp. nasturtii). Zinc treatment reduced the infection levels of both disease agents relative to levels in untreated beds. The distribution of virus particles in plants showing characteristic WYSV symptoms and in symptomless plants suggested symptom expression may be associated with the movement of virus from roots into the aerial parts of plants. Ten watercress lines originating from different regions of the world displayed a range of susceptibilities from very susceptible to slightly resistant with an association between the levels of crook root and virus infection. The results of these experiments provide further circumstantial evidence for the role of S. subterranea f.sp. nasturtii as the vector of WYSV.

Introduction

Watercress yellow spot virus (WYSV) is a partially characterised virus occurring in France (Spire, 1962) and England (Walsh, Clay and Miller, 1989). It has isometric particles with diameters of 37-38 nm and causes characteristic bright yellow spots and irregular shaped blotches on the leaves of some infected plants at certain times of the year. It is difficult to transmit the virus mechanically or by placing watercress plants in virus suspensions, however, transmission in the presence of the fungus Spongospora subterranea (Wallr.) Legerh. f. sp. nasturtii Tomlinson (the causal agent of crook root disease of watercress) is more effective. S. subterranea is a member of the Plasmodiophoromycetes, members of this group are known to transmit at least eight viruses all of which have labile rod-shaped particles (Brunt, 1988).

Tomlinson and Hunt (1987) described a disease of watercress supposedly caused by watercress chlorotic leafspot virus (WCLV) with symptoms similar to those caused by WYSV. Due to his inability to recover virus particles from infected watercress and inoculated indicator plants Tomlinson (1988) later referred to the causal agent as watercress chlorotic leafspot agent (WCLSA) distinguishing it from WYSV.

This paper describes the natural occurrence of WYSV and crook root fungus in small experimental watercress beds over a full year (1989), the distribution of the virus in plants showing characteristic symptoms and symptomless plants and the response of watercress germplasm from different regions of the world to infection by these pathogens.

Materials and Methods

From January to December, 1989 five experimental watercress beds were monitored at approximately 4-weekly intervals to determine the incidence and distribution of WYSV and crook root fungus in different regions of the beds. This was done by sampling 10 plants randomly across the width of each bed in each of three regions, the inlet region where the spring water enters, the middle and the outlet regions. The plants were assessed visually for crook root, scored for increasing severity on a scale of 0-3, and tested for virus infection by ELISA as described by Walsh, Clay and Miller (1989). Of the five beds used one was studied in greater detail with water temperature and pH measurements made at the time of sampling, two beds had zinc in the form of zinc chloride added to their water supply for crook root control and the remainder were untreated.

To determine the distribution of virus in plants showing characteristic symptoms and in symptomless plants, twelve of each type were collected and their aerial parts (shoots) separated from their roots. These component parts were then tested by ELISA to determine presence/absence of WYSV.

Using a technique developed for screening watercress for resistance to WYSV and crook root in watercress beds (Walsh and Phelps, 1990), the responses of ten different watercress lines (originating from different parts of the world) to WYSV and crook root were studied.

Results

Monitoring of WYSV and crook root incidence throughout 1989 in the experimental watercress beds revealed that the virus existed symptomlessly in all beds for most of the year. Symptoms were only occasionally seen in the winter months. The distribution of WYSV within watercress beds was coincident with that of the crook root fungus. Little or no WYSV infection was found in watercress plants growing at the top (water-inlet region) of the beds, whereas highest levels of WYSV and crook root were found in plants growing in the middle and lower parts (water-outlet region) of the beds (Table 1). Levels of WYSV and crook root were lower in zinc-treated beds than in untreated beds (Table 1).

Table 1. Incidence of WYSV and severity of crook root in untreated and zinc-treated watercress beds in 1989.

	Treatment:	No zinc		Zinc	
	Bed:	E5	E6	E7	E8
Mean percentage WYSV	Top	2.5	3.3	0	0
	Middle	58.3	32.5	18.3	17.5
	Bottom	65.8	40.8	21.7	20.8
	Total	42.2	25.5	13.3	12.8
Mean crook root score	Top	0.21	0.28	0.33	0.05
	Middle	1.85	1.81	1.23	0.98
	Bottom	1.57	1.58	0.82	0.64
	Total	1.21	1.22	0.79	0.56

ELISA tests on plants showing characteristic WYSV symptoms showed that the virus was present in the roots of all plants and in the shoots of half of the plants, whereas tests on symptomless plants showed that the virus was present only in their roots (Table 2).

Table 2. The distribution of virus in watercress plants with and without WYSV symptoms.

	Detection of virus in: Shoots	Roots
Plants with foliar symptoms	6/12	12/12
Symptomless plants	0/12	12/12

The ten different watercress lines that were subjected to WYSV and crook root infection showed a range of susceptibilities from very susceptible to slightly resistant with UK lines being most susceptible to both diseases. Results showed an association between crook root scores and ELISA values (Table 3). In general low crook root scores were associated with low and medium WYSV ELISA categories and high crook root scores with medium and high WYSV categories (Table 3).

Table 3. The association between crook root and WYSV in plants from ten different watercress lines.

		WYSV (ELISA categories) Low	Medium	High	
Crook root	Low	43	54	7	
	Medium	111	804	113	
	High	11	83	32	χ^2 (4DF) = 101.5

Data shown are numbers of plants in each category.

Discussion

The levels of WYSV infection in untreated experimental watercress beds were at least twice as high as those in zinc-treated beds however despite these levels few symptoms were seen. Examination of the raw data showed that the highest levels of infection occurred in the winter months. The incidence of crook root in the same beds was high, with levels in untreated beds higher than those in zinc-treated beds, and the highest levels again in the winter months. Tomlinson (1958) demonstrated that crook root disease could be controlled by the addition of zinc-fritted glass and more recently by adding zinc in the form of zinc sulphate to the water supply of watercress beds (Tomlinson and Hunt, 1987). The zinc applied as zinc chloride solution is the most likely cause of reduced levels of crook root in these experiments. The associated reduction of WYSV in zinc-treated beds provides further circumstantial evidence for the role of the crook root fungus as vector of WYSV. The presence of WYSV particles in the aerial part of some watercress plants showing characteristic disease symptoms and the absence of particles in the aerial part of symptomless plants suggests that symptom expression may be associated with movement of the virus from roots to the shoots. The close association between crook root and WYSV levels in the ten watercress lines that were studied also provides circumstantial evidence for the crook root fungus acting as vector of WYSV, possibly the first record of a plasmodiophorid fungus transmitting an isometric virus.

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THE POTENTIAL AND RISKS OF TRANSGENIC PLANTS FOR RESISTANCE TO VIRUSES

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Summary

Currently there are three basic ways by which plant viruses are controlled: a) removal of sources of infection used mainly for perennial crops, b) cultural practices such as use of insecticides widespread in annual crops, c) breeding in natural virus resistance. The latter has the best long term potential except that in many cases sources of resistance are not available or the resistance breaks down. Recently a new concept has been developed of using virus or virus-related sequences to interfere with viral replication or functions. This has been shown to work, at least for some viruses, by transforming plants to express the viral coat protein which is thought to interfere with an early stage of the viral infection cycle. This concept can now be applied to other viral sequences. The introduction of viral sequences into crop plants, whether it be for conferring non-conventional resistance or as vectors for other genes, raises the possibilities of new disease situations arising. Viral coat protein often confers the transmission characteristics of a virus. In coat protein transgenic plants there is the risk of transcapsidation changing the transmission of viruses against which the plant is not protected. These risks will have to be assessed before widespread field release of such transgenic plants.

Introduction.

Viruses are increasingly being recognized as causing large losses to crops (see Herdt, 1988; Hull, 1984). To counter these losses three basic approaches have been developed. Firstly, sources of infection can be removed, say by eradication or certification schemes (eg. eradication of cocoa swollen shoot infected trees in Ghana, certified virus-free potatoes in the UK). This approach is directed mainly to perennial crops or those which are vegetatively propagated and, with some notable exceptions, has not been particularly successful. The usual approach for annual crops is to prevent virus spread by cultural techniques such as insecticidal control of vectors or disease advoidance by selection of planting time. Once again there are several major disadvantages. For instance, spraying with insecticides does not control certain types of viruses and is becoming increasingly environmentally unacceptable. Control by cultural techniques may break down due to factors such as labour problems or changes in weather. The third approach is to use virus resistance which can be in two forms. Cross protection of infection with a severe strain by inoculating plant with a mild or attenuated strain of that virus has been attempted on a field scale in several cases, eg. tomato against tomato mosaic virus (TMV), papaya against papaya ringspot virus (see Urban et al., 1990). This form of cross protection has not proved fully reliable and attenuated strains can mutate to become severe ones. Also there may be more severe symptoms due to synergistic effects if the crop is infected with another virus against which it is not protected. The other form of resistance is that under genetic control and which can be bred into the crop. This can vary from complete resistance (immunity) in which the virus does not replicate at all to tolerance in which the virus replicates but does not induce marked symptoms. Tolerance can lead to the sort of problems noted for cross protection and the thus, ultimate aim is to have complete resistance. Here the plant breeder comes up against two further problems. Firstly, there are only limited sources of genes which confer immunity and, for many crop-virus combinations, none have yet been found. Secondly, even if resistance is bred into a crop new strains of virus may overcome it. This has been shown well in tomato where one or two amino acid changes in virus-encoded proteins are sufficient to overcome the resistance to TMV (see Hull, 1989). However, recently the whole approach to obtaining virus resistance 'genes' has been revolutionized.

A paper by Sandford and Johnston (1985) encapsulates a new concept that modified pathogen genes could be introduced into the host genome and would confer resistance by interfering with the functioning of the pathogen. This approach is particularly applicable to viruses which have relatively simple genomes amenable to study at the molecular biological level and to plants for which there are techniques being developed for transformation. This concept has already led to non-conventional forms of resistance to plant viruses and has opened up the possibilities of other sources of resistance.

Non-conventional resistance.

1. Current systems.

Three forms of non-conventional resistance have already been studied. Coat protein-induced resistance has proved to be the most successful and is being tested in a whole range of virus-host combinations (for recent review see Nelson et al., 1990). However, so far resistance has only been reported to viruses with plus-strand RNA genomes. If, as has been suggested (Hull, 1990), this form of resistance is coupled with co-translational disassembly (Wilson, 1988) there would be groups of viruses for which it would not operate

Satellite sequences have been shown to reduce symptom expression when expressed in transgenic plants (see Baulcombe, 1989). However, it is necessary for virus replication to 'activate' the satellite system and, as will be discussed later, it is advantageous to prevent virus replication.

The various attempts to use antisense RNA to inhibit virus replication have been unsuccessful. However, all the attempts have so far been aimed at plus-strand RNA viruses. It can be argued that the lack of success is due to the antisense RNA, produced in the nucleus, never making contact with the viral RNA, restricted to the cytoplasm probably as a nucleoprotein complex for most of its replication cycle. In the light of the control of nuclear genes with antisense RNA (see Ecker and Davis 1986; van der Krol et al., 1988) it would be interesting to see if this approach was effective against viruses which had nuclear phases to their replication (eg. caulimoviruses, geminiviruses).

2. Potential systems.

The concept of non-conventional resistance opens up a range of new approaches to making plant resistant to viruses. There are two basic approaches - blocking the interactions of a viral gene (product) or decoying it. The current systems described above exemplify both these approaches. The coat protein-induced resistance is probably being due to the expressed protein blocking an early function in viral replication. Satellite-induced protection is probably due to the satellite sequences decoying viral replication functions.

Thus it should be possible to target any viral gene or control sequence. Fig. 1 illustrates the basic genome organization of a plant virus and identifies potential targets.

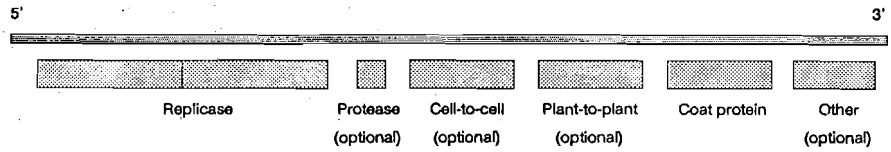

Fig. 1. Basic plant virus genome organization. The top line indicates the viral nucleic acid with the 5' and 3' termini shown. The boxes are the viral gene products which are not necessarily in the order shown.

The replicase, cell-to-cell movement and the plant-to-plant movement proteins could be targetted for interference either by the molecular blocker or the decoy strategies. For instance, if the interactions between the subunits of the replicase were understood it would be possible to transform the plant either to make a defective subunit or to make a molecule that blocked the interaction. The recent finding that transformation of plants with the chalcone synthase gene (Napoli et al., 1990; van der Krol et al., 1990) suppresses the expression of that gene indicates that expression probably has various feedback-type controls. Thus, it may be possible to interfere with, say the replicase, by transformation with replicase genes themselves. The decoy strategy could be effected by transforming the plant to produce the nucleic acid sequence(s) to which the polymerase binds.

For viruses, such as comoviruses and potyviruses, which use proteases in their infection cycle decoys of proteolysis sites or blockers of slightly modified sites could be used. Alternatively the activity of the protease could be used to release a molecule in an active form which would block viral replication or kill the cell. This would increase the specificity of the resistance.

3. Non-conventional resistance to fungus-transmitted viruses.

As well as the strategies directed against the virus it should be possible to develop strategies

against fungi which transmit viruses. The same basic approach would have to be used - that of determining interactions which were amenable to being interfered with. The knowledge of the interactions between Plasmodiophora and their plant hosts or the viruses that they transmit is very limited. However, there are specific interactions or stages in the fungal infection cycle which could be targetted. For instance, the zoospores released into the soil from the resting spores are attracted to plant root hairs by certain signals presumably produced by the plant. If these signals could be modified the spores would not be attracted. Then it is likely that there are receptor sites on the root hair or root surface which are involved in the attachment and penetration of the free-living stage of the fungus. Modification of these would interfere with the process of fungal infection. Thus, there is potential for creating non-conventional resistance to both the fungi and the viruses that they carry. However, in developing these systems care must be taken not to interfere with the interactions between plants and beneficial fungi, say of the rhizosphere.

4. Control sequences.

The construct which is transformed into plants would contain, as well as the virus resistance determinants, sequences which controlled their expression. These control sequences usually comprise promoters and terminators for transcription of the primary insert. From the choice of promoter one can determine whether the resistance is expressed constitutively or in response to a certain signal. There has been interest in the promoters of pathogenesis-related proteins for effecting expression in response to infection, but from the considerations in the next section such an approach can be considered disadvantageous. Other promoters control the tissue specificity of expression. These may be useful, say for the root expression of resistance to fungus-transmitted viruses or the phloem expression of resistance to phloem-limited viruses.

5. Non-conventional resistance - ultimate aims.

There are two main aims which should be bourne in mind when developing non-conventional resistance. Firstly, it is important to prevent the virus replicating if possible. When a virus replicates there is the opportunity for it to vary and overcome resistance. Most economically important plant viruses have RNA genomes which replicate RNA to RNA. This form of replication does not have proof reading and the error rate is estimated as being one misincorporated nucleotide per 10^3 - 10^4 nucleotides (Ward et al., 1988). Thus, the particle uncoating stage (at which coat protein-mediated resistance operates primarily) and the nucleic acid replication stage present attractive targets for non-conventional resistance. These considerations also suggest that constitutive expression of the resistance would be better than response expression.

The second aim is to try and confer resistance to a wide range of viruses with a single or few 'gene(s)'. In this the coat protein approach fails as the degree of resistance varies according to the closeness of sequence homology between the coat proteins of the protecting strain and of the infecting strain (Nejidat and Beachy, 1990). Potentially targetting replicase functions should result in broad spectrum resistance. Replication of RNA from RNA is an unusual event in eukaryotes and viruses encode subunits of the enzyme. There is considerable conservation in viral replicases and only three 'families' have so far been recognized (Habili and Symons, 1989). This points to two conclusions. By targetting the replicase one should be able to develop wide spectrum resistance. If the block or decoy is on the conserved sequence it is much less likely that variants of the virus would be able to overcome the resistance.

However, until the interactions involved in viral replication are understood sufficiently to be able to create non-conventional resistance based on them it is important not to ignore systems targetting the other genes and sequences described above. This should then provide material from which 'multigene' resistance could be developed. Such resistance would provide barriers at several levels against resistance being broken by variant viruses.

Risks of use of viral sequences.

Before there can be widespread use of non-conventional resistance to control viruses in the field it has to be determined whether there is any significant risk in growing plants transgenic in virus sequences. Since there is no evidence of humans or other animals suffering directly from plant virus gene products it is unlikely that such transgenic plants will be a health risk. The main risk is in the interactions of the viral sequences or gene products with other viruses whichinfect the plants. Thus the basic question that needs to be addressed is:- 'What is the risk of viral genes or sequences which are used to confer resistance (or as gene vectors) having

significant effects on infection by other viruses?' This question was addressed by Hull (1990) who described various scenarios.

The scenario that should be addressed most urgently is that of the infecting viral genome becoming encapsidated in the expressed coat protein being used to protect against another virus. Such transcapsidation could alter the vector characteristics of the infecting virus. Although this would be for only one transmission passage (in the non-transgenic host the virus would be encapsidated by its own coat protein and revert to its normal means of transmission) the virus might be taken to ecological situations to which its normal vector would not introduce it. Thus, its epidemiology could be changed. If seed transmission was determined by coat protein, or by another gene which was being used for protection, the infecting virus would effectively be taken into the germ line.

Thus, it is important to consider the possible risks of introducing viral sequences into plant to confer resistance. Even if the perceived risks turn out to not be real the experimentation taken to prove them so would not be wasted as it would add considerably to the understanding of viral functions and could possibly lead to further forms of non-conventional resistance.

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INHIBITOR OF VIRUS REPLICATION (IVR)-A STEP TO ENGINEER RESISTANT PLANTS

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Summary

Traditional breeding procedures allow movement of resistance sources only between closely related species, and geneticists lack sufficient sources for their breeding programs. The use of DNA technology to manipulate and move resistance sources against plant pests has been widely advocated. For isolateion of natural resistance genes the following approaches can be considered: via the gene product, insertional mutation with a transposon, or substraction hybridization of mRNA's. One of the most effective natural resistances of plants to viruses is the local lesion response, generally depending on one dominant gene. We have shown that a host-coded protein, termed "inhibitor of virus replication" (IVR), is induced by virus infection in several LL responding tobaccos, and inhibits virus replication. IVR inhibits the replication of a spectrum of viruses. We have purified and characterized this protein and prepared poly- and monoclonal antisera to IVR. These antibodies are used to screen cDNA libraries from resistant plants that express IVR.

Introduction

Traditional breeding procedures allow movement of resistance sources only between closely related species. Especially against plant viruses, where chemical pesticides are not available, breeding for resistance is of major importance, but geneticists lack sufficient sources for their breeding programs. The use of DNA technology to manipulate and move resistance sources against plant pests has been widely advocated, but with little practical work in this direction. This would be in addition to current approaches of inserting viral genes [coat protein (Nelson et al., 1990), satellite RNA (Gerlach et al., 1987) and antisense strategies (Hemenway et al., 1988)] to obtain resistant plants. The major obstacles to progress in using natural resistance genes in DNA technology is choosing the right gene on one hand, and its isolation on the other hand.

Various resistances against plant viruses are known (Loebenstein et al., 1984). The local lesion response is probably the most notable active resistance phenomenon, whereby after inoculation the virus invades and multiplies in several hundred cells but does not spread to other tissues. It generally depends on one single dominant gene and may therefore be most suitable for isolation and transformation of plants.

Strategies for isolating genes

The following strategies can be considered for the isolation of natural resistance genes:

a. Transposon tagging. The recent isolation and characterization of plant transposable elements offer the possibility to mutagenize resistance genes. In case where the resistance gene is dominant, the transposable element changes the phenotype from resistant to susceptible. Parallel to insertion, the resistance gene also gets ear-marked, and can thus be cloned molecularly, using the transposable element as a probe. This type of approach should allow the isolation of resistance genes in cases where resistance is conferred by a single dominant gene. Transposon tagging could be used to isolate the "N" gene from resistant tobaccos. Insertion of a transposon in this case should change the phenotypic expression from a local necrotic lesion response to tobacco mosaic virus (TMV) to a systemic infection.

b. Differential screening and substraction hybridization.
The isolation of a "resistance" gene might be achieved by obtaining mRNA from the resistant line, which is then reverse-transcribed into cDNA using labeled

nucleotides. This cDNA is then hybridized with a huge excess of mRNA from the susceptible, otherwise isogenic, line. Most of the mRNA sequences in the susceptible and resistant lines are common, so most of the "resistant" cDNA will hybridize with the mRNA from the "susceptible" line. Specific "resistant" cDNA, however, will remain single-stranded, and can be separated from the cDNA-mRNA hybrids by chromatography on hydroxyapatite, which binds double-stranded molecules more avidly than single-stranded molecules. The labeled "resistant" cDNA can then be used to screen a "resistant" cDNA library to obtain a cloned copy of the "resistant" cDNA. By substraction hybridization it is possible to increase the concentration of the desired (resistance associated) sequences (Sargent, 1987). Thus, mRNA from resistant plants is used as a template to produce radiolabeled cDNA. This cDNA is hybridized to an excess of mRNA isolated from the susceptible plant. The unhybridized cDNA from the resistant plant represents an enriched population of sequences expressed in the resistant tissue.

c. Via the gene product. Probably the best option to isolate a gene is via its product, providing that biochemical information on the product is available. In that case an oligonucleotide probe can be synthesized, based on the amino acid sequence of the protein product. If the N-terminal of the protein is not blocked, a sequence of 6-10 amino acid residues is sufficient for preparing a probe with at least 16-20 nucleotides. In case the N-terminal is blocked, partial digestion of the protein is necessary to obtain a polypeptide stretch. Another possibility is to prepare poly- or monoclonal antibodies as a probe. These probes are used to screen a recombinant DNA library, obtained by reverse transcription of poly(A) RNA from resistant plants. Recombinant DNA packaged for example in lambda gt 10 phage, plated on an appropriate Escherichia coli host, can be screened with the nucleic acid probe. When antibody probes are available for screening, lambda gt 11 is used, since it is an expression vector (Jendrisak et al., 1987).

Inhibitor of virus replication (IVR)

We have reported that a substance(s) inhibiting virus replication (IVR) is released into the medium from TMV-infected protoplasts of Samsun NN, a cultivar in which infection in the intact plant is localized (Loebenstein and Gera, 1981). IVR inhibited virus replication in protoplasts from both local-lesion-responding resistant Samsun NN and systemic responding susceptible Samsun plants, when applied up to 18 hours after inoculation (virus being assayed by a local lesion assay, ELISA or a c-DNA probe for TMV). This indicates that IVR acts as an inhibitor of replication and not as an inhibitor of infection or at a later stage (translocation). IVR was also produced by N. glutinosa and Xanthi-nc protoplasts. It was not produced in protoplasts from susceptible plants or from non-inoculated protoplasts of the resistant cultivar. IVR was found to be sensitive to trypsin and chymotrypsin but not to RNAse, suggesting that it has a proteinaceous nature (Gera and Loebenstein, 1983). A specific protein band of an approximate molecular weight of 23K was regularly observed in polyacrylamide gel electrophoresis (PAGE) of crude preparations of IVR (Spiegel et al., 1989; Gera et al., 1990). Electroeluting protein from this band recovered a biologically active fraction with a twenty-five fold increase in specific activity over that of the crude preparation. This fraction revealed only one band at 23K in PAGE, providing evidence that the 23K band is IVR purified to a high degree. Polyclonal (Gera et al., 1990) and monoclonal (Gera et al., 1989) antibodies were prepared, which neutralized IVR's antiviral activity and enabled its detection in immunoblots. The 23K band gave a specific reaction in immunoblots with IVR antiserum (Gera et al., 1990). IVR was found also to inhibit TMV replication in Samsun and tomato leaf tissue disks. IVR also inhibited replication of cucumber mosaic virus (CMV), potato virus X (PVX) (Gera and Loebenstein, 1983), and potato virus Y (Loebenstein and Gera, 1988) in different host tissues, thereby indicating that it is neither virus nor host-specific. Inhibition rates by IVR in both protoplasts and leaf tissue disks were dose-responsive (Gera et al., 1986). IVR was also obtained from the intercellular fluid of Samsun NN tobacco infected with TMV and from induced resistant tissue (Spiegel et al., 1989).

Recently, we found that IVR is produced constitutively (without infection) in the amphidiploid of N. glutinosa x N. debneyi (Loebenstein et al., in press). This hybrid is highly resistant to infection with TMV (Ahl and Gianinazzi, 1982). IVR has a certain resemblance with a class of proteins termed b or "pathogenesis-related" (PR(b) proteins). Induction of PR(b) proteins in most Nicotiana species requires infection by either viruses, fungi, bacteria or other external stimuli (Van Loon, 1985). The characterized PR(b) proteins, however, occur in the plant in relatively large quantities, while only ngs of IVR can be obtained from one g of leaf tissue. Furthermore, IVR is sensitive to proteolytic enzymes (Gera and Loebenstein, 1983) in contrast to the stability of most of the characterized PR(b) proteins. The major difference between IVR and the PR(b) proteins is that no biological antiviral activity has been reported for the latter. As tested by Dr. S. Gianinazzi (INRA, Dijon, France) no serological reaction was obtained between PR(b) proteins of Xanthi n.c. and IVR antiserum (personal communication).
Recently, the N-terminal sequence for IVR was determined (in cooperation with Prof. Y. Burstein, Weizmann Institute) and no homology was observed with the N-terminal sequences of all the PR-proteins that have been sequenced. It seems therefore that IVR is not related to those PR proteins that have been well characterized. Recently, it was reported that the constitutive expression of several PR-proteins in transformed tobacco had no effect on virus infection (Linthurst et al., 1989).

The recovery of IVR from protoplasts and intact tissue of resistant cultivars, its purification and production of poly- and monoclonal antisera, are the base for isolation and cloning the gene coding for IVR. Advances in this direction will be reported. This gene might then be useful in transforming susceptible plants.

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STRATEGY OF POTATO VIRUS X GENOME EXPRESSION

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The genome of potato virus X (PVX), the type member of the potexvirus group consists of a single-stranded RNA which is capped and polyadenylated. The complete nucleotide sequence of PVX RNA [6435 bases excluding poly (A)] has been reported independently by two groups (Kraev et al., 1988; Skryabin et al., 1988; Huisman et al., 1988).

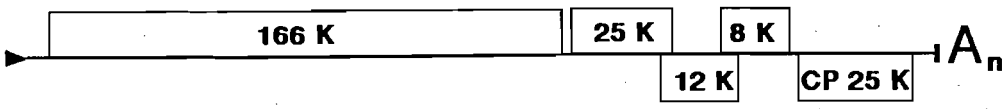

Fig. 1 Schematic representation of PVX genome coding sequences.

The large 5'-proximal ORF corresponds to the 165K protein, the putative RNA replicase containing two domains of homology with other RNA-replicating proteins. The first domain is homologous to the hypothetical NTP-binding domain (NTP ase-helicase consensus) and the second contains the GDD domain also homologous to the analogous sequences of Sindbis-like viruses. The coat protein gene is located at the 3'-proximal position and between these two terminal ORFs a block of three overlapping conserved ORFs occurs (Fig. 1).

The putative 12K and 8K proteins encoded in overlapping ORFs contain blocks of uncharged amino acids and have been speculated earlier to be membrane-bound proteins. Similar triple blocks of ORFs have been shown to occur in genomes of different plant viruses including hordeiviruses, furoviruses and carlaviruses. We suggest that one or more proteins coded by this triple block of genes may be responsible for the cell-to-cell transport function, since it was demonstrated earlier by our group that the transport function of a TMV ts mutant can be complemented by PVX in plants infected by both viruses.

Only the 5'-proximal gene of PVX RNA can be expressed by direct translation (Wodnar-Filipowicz et al., 1980; Bendena et al., 1986; Adams et al., 1987) but the product of this ORF (the 165K protein) migrates in a Tris-glycine-SDS ele trophoresis system not as the 165K but as 210K protein which is due to its anomalous electrophoretic behaviour in this system (Karasev et al., 1989). On the other hand, this protein moves as 165K protein in Tris-phosphate-SDS buffer.

The PVX virions contain only the genomic size RNA, however, several subgenomic 3'-cotermi al ssRNAs were present in the infected cells, the major sgRNAs being of 0.9, 1.4 and 2.1 kb. The ds analogous have been found for all these three sgRNAs. We suggest that these three sgRNAs are used in expression of the 5'-distal genes. It is clear that the 0.9 kb sgRNA is sgRNA for the coat protein and the 2.1 kb sgRNA apparently codes for the 25K protein.

cDNA clones were constructed from which subgenomic copies of viral RNA could be transcribed in vitro under the control of the T7 promotor. These synthetic RNA transcripts of subgenomic size were translated in vitro in RRL and KA systems and directed the 8K, 12K proteins and the coat protein (Morozov et al., 1990).

It was found that the translation products of the 12K and 8K genes are bound to the membraneous fraction in membrane-enriched Krebs 2 extracts. Thus, the in vitro products of the two small PVX genes seem to be membrane-bound proteins (Morozov et al., 1990).

The 5'-untranslated leader sequence of PVX RNA (83 nt apart from the cap structure) consists of two subsequences referred to as α-sequence (41 nt with no G) and β-sequence (42 nt upstream from first AUG). Computer-based folding predictions suggest that the 5'-proximal region of the $\alpha\beta$-leader is unstructured. The second structural feature of the $\alpha\beta$-leader is the presence of sequences apparently complementary to the 3'-terminal region of 18S rRNA. The $\alpha\beta$-leader has been shown to enhance strongly the translation of contigous foreign gene (NPTI) transcripts in cell-free translation systems from rabbit reticulocytes (RRL), wheat germ (WG) and Krebs-2 ascites cells extracts (KA). In comparative experiments PVX RNA $\alpha\beta$-leader was shown to be equally effective as TMV Ω-leader in enhancing the translation of a reporter gene.

In competitive translation PVX RNA inhibited strongly TMV RNA and some other plant virus mRNAs in RRL and WG systems. No competition occurred between PVX and TMV RNAs in the KA system. There was no correlation between the translational efficiency and competitive ability of PVX RNA in different cell-free translation systems.

The competitive ability did not only correlate with the presence of the $\alpha\beta$-leader in mRNA. Evidence is presented to suggest that the $\alpha\beta$-leader together with about 150 nt of the coding sequence is responsible for the translation competitive ability of PVX RNA.

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