Studies on interference between newly defined bean-infecting potyviruses

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Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 3 november 1993
des namiddags te half twee uur in de Aula
van de Landbouwuniversiteit te Wageningen

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Khan, Jawaid A.

Studies on interference between newly
defined bean-infecting potyviruses /
Jawaid A. Khan. - [S.1 : s.n.]. - III.
Thesis Wageningen. - With ref. - With summary in Dutch
ISBN 90-5485-163-5
Subject headings: French beans / Potyviruses.

Propositions

1. None of the possible mechanisms of cross-protection and interference among plant viruses mentioned in review articles on these subjects, applies to the interference phenomenon studied in this thesis.

   This thesis

2. Recent classification of strains of bean common mosaic virus and blackeye cowpea mosaic virus based on the sequence data from coat protein and 3'-nontranslated RNA does not correlate well with biological properties, in contrast to that of potato virus Y strains founded on the same taxonomic parameters.

   Van der Vlugt, R.A.A. (1993). Engineering resistance against potato virus Y. *Ph.D. thesis*, Wageningen Agricultural University.

   This thesis

3. Characterization and classification of plant viruses are not only prerequisites for successful resistance breeding and better understanding of the epidemiological phenomena, but also for engineered protection to these viruses.

4. In the light of the revised plant virus taxonomic rules recognizing families, genera and species, "subgroups" as defined by potyvirus taxonomists, should obtain the status of subgenera.

   Potyvirus taxonomy. (Ed. Barnett, O.W.). *Archives of Virology* (1992) Supplementum 5. Springer, Wien New York. 445 pp.

5. The claim, that tomato spotted wilt virus causes 86% mortality to immature thrips, needs confirmation.

   Robb., K.L. (1989). Analysis of *Frankliniella occidentalis* (Pergande) as a pest of floricultural crops in California greenhouses. *Ph.D. dissertation.* University of California, Riverside.

6. The proposal given by Adam *et al.* (1993), to define a new species within the Tospovirus genus, is premature.

> Adam, G., Yeh, S.-D., Reddy, D.V.R. and Green, S.K. (1993). Serological comparison of tospovirus isolates from Taiwan and India with Impatiens necrotic spot virus and different tomato spotted wilt virus isolates. *Archives of Virology* 130: 237-250.

7. Very often the term diagnosis is used, where detection is meant.

> Koenig, R. & Burgermeister, W. (1986). Applications of immuno-blotting in plant virus diagnosis. In: *Developments in Applied Biology 1. Developments and applications in virus testing* (Eds R.A.C. Jones & L. Torrance). Association of Applied Biologists, Wellesbourne, U.K. 312 pp.

8. The Jurassic Park rage may instigate scientists to look for the presence of viruses in fossil insects or plants preserved in amber.

9. The world-wide popularity of commercial programmes on T.V./radio might lead to strong capitalism.

10. In the fairly wide coverage of the world by western media, quite often only negative sides of the less industrialized nations are projected.

Jawaid A. Khan
Studies on interference between newly defined bean-infecting potyviruses
Wageningen, 3 November 1993

Contents

Preface

For the successful completion of my Ph.D. research, first I would like to express my sincere thanks to my supervisors Dr Jeanne Dijkstra and Prof. Dr Rob W. Goldbach. Their continuous guidance, stimulating discussions, fruitful criticisms during the whole period is highly appreciated.

Particularly, I would like to thank Dick Lohuis for his excellent technical assistance, and Dr Hans van den Heuvel and Ir. Richard Kormelink for their help during the preparation of the final version of the thesis. The helpful discussions of Dr Bos and Dr Peters are also acknowledged. Thanks are due to all colleagues of the Department of Virology, Wageningen Agricultural University, for their cooperation and a pleasant stay in the laboratory.

Special thanks go to Hinne Wagenaar, Mateen Ahmad and Rosa's Jacob for their moral support, sharing all the times, unconditional help; to Dr Narendra Singh for his affection, criticisms on the social aspects and correcting the text of the thesis. To Antonio, Jordi, Hans, Miranda, Mohammed, Renato, the family Dijkstra Chauhan and my house mates of Rijnsteeg 8-7C for their hospitality and friendship.

I am grateful to the Executive Board of Wageningen Agricultural University for the Research and Ph.D. sandwich Fellowships, Stichting "Fonds Landbouw Export Bureau 1916/1918" for a grant, and contribution of glasshouse staff and photography section for their help.

Finally, to my family for their deep affection and inspiration.

Indeed, Wageningen is a unique town which provides pleasant international academic environment particularly in the agricultural world.

Chapter 1

General Introduction

Bean: cultivation, diseases and pests

Beans, *Phaseolus vulgaris*, constitute one of the most important food crops of the world. They are cultivated in more than 27 million hectares throughout the world *(FAO, Production Year Book 43: 1989)*. Field dry beans rank high as a cheap source of nourishing food. They are rich in carbohydrate, protein, calcium, and iron, and contain a large amount of vitamin B1 (Steibeling & Clark, 1939). Garden beans are credited as a green vegetable and valuable as a source of calcium, riboflavin, and iron. However, these crops are attacked by a large number of pathogens and insect pests which may cause great reduction in yields. Among them viruses, and especially bean common mosaic virus (BCMV), are economically important. Many epidemic outbreaks due to this virus have been reported, with severe strains of BCMV causing upto 68% reduction in seed yield (Hampton, 1975; 1983).

Bean common mosaic virus

The disease caused by bean common mosaic virus was reported for the first time by Ivanovski in 1894 from Russia, and the virus described from U.S.A. by Stewart and Reddick in 1917. Later, the virus has been reported from different parts of the world (Zaumeyer & Thomas, 1957; Lana *et al.*, 1988; Vetten & Allen, 1991). The virus belongs to the genus Potyvirus (family Potyviridae; Barnett, 1991) and is transmitted by aphids in a non-persistent manner.

Like most other potyviruses, BCMV has a limited host range and in nature it has been found to occur in *Phaseolus* species and occasionally in *Lupinus luteus* (Frencel & Pospieszny, 1979) and in wild legumes, such as *Rhynchosia minima* (Meiners *et al.*, 1978). Recently, in a survey conducted throughout Africa on distribution of BCMV strains, it was found that necrotic strains predominate in eastern and southern Africa and non-necrotic strains are prevalent in Ethiopia and perhaps also in Zimbabwe. There are indications that in Zimbabwe intermediate serotypes of BCMV occur (Vetten & Allen, 1991). It was also reported that BCMV strains were found to occur with viruses serologically related to blackeye cowpea mosaic virus (BlCMV) or other viruses. Such mixed infections may result in interactions between the viruses sometimes leading to

antagonistic or synergistic effects. To diagnose such mixed infections a clear distinction between the viruses present is a prerequisite. Accurate virus (strain) identification is also required for understanding the epidemiology of these virus (strains) and for breeding for resistance. Moreover, it forms the basis for sound taxonomy of these viruses and their strains.

In spite of the great economic importance of BCMV, the taxonomic status of its strains and their relationships with other legume infecting-potyviruses especially BlCMV, is not clearly defined. There are no clear-cut differences between these two viruses, due to a large number of variants differing in host range, pathogenicity and serological properties (Drijfhout, 1978; Lana *et al.*, 1988). There is a difficulty in defining the taxonomic position of strains of BCMV and BlCMV by biological and conventional serological properties. Taiwo and Gonsalves (1982) reported a close serological relationship between strains of BCMV and BlCMV. Similarly, Tsuchizaki and Omura (1987) also concluded on the basis of biological characteristics, antigenic and other coat protein properties that BCMV and BlCMV are not distinct viruses. Lana *et al.* (1988) made a detailed comparison of the strains of the two viruses, based on biological and serological properties, but were unable to clearly differentiate them. On the basis of ELISA results with monoclonal antibodies and polyclonal antisera, Wang (1983; 1985) and Vetten *et al.*(1990) placed BCMV-NL3 in serogroup A and BCMV-NL1 and NY15 in serogroup B.

The potyviral genome

The genome of a potyvirus is a single-stranded, positive-sense RNA of approx. 10,000 nucleotides in length. Potyviral genomes resemble those of the plant bipartite comoviruses, nepoviruses, and the picornaviruses on the basis of structure of genome and their organization. In the genomes of all these viruses a cluster of genes is found that encodes a number of non-structural proteins with amino acid sequences which have been shown or suggested to be involved in RNA replication. For this reason, it has been proposed that comoviruses, nepoviruses and potyviruses may be classified in a supergroup of picorna-like viruses (Goldbach 1986; 1987). Molecular studies have shown that potyviral RNA genome is translated into a large polyprotein (Allison *et al.*, 1986)

which is cleaved by virus-encoded proteinases into eight polypeptides (Dougherty & Carrington, 1988; García *et al.*, 1989; Carrington *et al.*, 1990).

The order of gene products in polyprotein is given in Fig. 1 and their functions are described below.

1. The P1 protein. It has been reported that the C-terminal half of P1 protein of tobacco etch virus (TEV) functions as a proteinase in the cleavage of the TEV polyprotein between 35 k Da and HC-Pro proteins (Verchot *et al.*, 1991). On the basis of sequence similarity between P1 protein of tobacco vein mottling virus (TVMV) and 30 kDa movement protein of tobacco mosaic virus (TMV), it has been proposed that P1 protein is involved in the cell-to-cell spread of the infection (Domier *et al.*, 1987; Laín *et al.*1989, Robaglia *et al.*, 1989).

2. The helper component protein (HC-Pro). This viral protein has at least two major functions. It allows aphid transmission (Thornbury *et al.*, 1985) and it is also a protease responsible for the cleavage of polyprotein at the HC-Pro -P3 junction (Carrington *et al.*, 1989). Recently, it has been suggested that HC may have a third function i.e. its central domain is involved in nucleic acid binding (Robaglia *et al.*, 1989; Shukla *et al.*, 1990).

3. P3 protein. Its function is still unknown. Recently, Rodríguez-Cerezo and Shaw (1991) demonstrated the presence of this protein in cells infected with TVMV and suggested it may be an integral membrane protein in the infected cell.

4. Cylindrical inclusion protein (CI). All potyviruses induce the formation of characteristic cylindrical inclusions in the cytoplasm of infected cells (Edwardson, 1974). The morphology of these inclusion bodies is virus-specific which prompted Edwardson and Christie (1978) to propose a scheme that subgroups potyviruses based on inclusion bodies.

A nucleotide binding motif (GAVGSGKTST) located near the N-terminus of the putative CI protein resembles a similar motif in helicase-like proteins (Hodgman, 1988). Laín *et al.* (1990; 1991) have demonstrated that CI of plum pox virus (PPV) unwinds RNA duplexes and acts as an RNA helicase. Its possible function may include unwinding of replicative intermediates, genome recombination or unwinding of single-stranded RNA structures facilitating transcription and/or translation.

5. 6K proteins. The two small proteins 6K1 and 6K2 that are predicted from the amino

acid sequence of the potyviral polyprotein might also play a role in RNA replication, although they have not been identified *in vivo.* (Rodríguez-Cerezo and Shah, 1991).

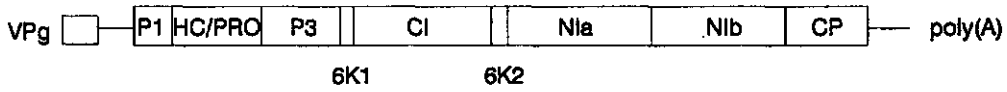
6. The small nuclear inclusion protein (NIa). This protein is frequently found in nucleus where it may give rise to nuclear-inclusion bodies. It acts as a proteinase for the cleavage sites in the C-terminal two thirds of the potyvirus polyprotein. The NIa protein molecule has a two domain structure, the domain located in the C-terminal half being involved in proteolysis (Dougherty & Carrington, 1988). The N-terminal half of NIa protein functions as genome-linked protein (VPg) (Shahabuddin *et al.*, 1988; Murphy *et al.*, 1990). It is covalently linked with the 5'-terminal nucleotide of the genomic RNA (Shahabuddin *et al.*, 1987; Siaw *et al.*, 1985). VPg protects mRNA from exonucleases and is involved in the synthesis of RNA and other steps of replication as a primer for nucleic acid synthesis (Shahabuddin *et al.*, 1988; Baron & Baltimore, 1982; Morrow *et al.*, 1984; Vartapetian *et al.*, 1984).

7. The large nuclear inclusion protein (NIb). This protein has all sequence motifs characteristic of viral RNA-dependent RNA polymerases and, hence, represents the putative potyviral polymerase.

8. The coat protein (CP). This protein is the best characterized gene product of potyviruses. Potyviral coat proteins have a highly conserved core domain but diverge in sequence and length at the amino terminus, which is located on the virion's surface (Allison *et al.*, 1985; Shukla *et al.*, 1988).

One obvious function of CP is to encapsidate the viral RNA. Based on sequence comparisons between aphid-transmitted and non-aphid transmitted isolates of TEV, it was predicted that the N-terminus of TEV CP may also be involved in the CP- HC-Pro interactions, and hence, in aphid transmission (Harrison & Robinson, 1988). This hypothesis was confirmed by Atreya *et al.* (1990) who demonstrated the involvement of coat protein amino acid triplet DAG in the aphid transmission and showed that any change in the triplet may lead to non-aphid transmissibility of TEV.

The non-translated regions (NTRs). These regions are present on 3'- and 5'-ends of potyviral genomes. The 5'-NTR of fully sequenced genomes (TEV, TVMV, potato virus Y) are similar in length but differ in sequences except for a highly conserved region of



A.

B. PRO-1/HC-PRO CLEAVAGE SITES

PepMoV	(286)QY S(288)	Pro3/CI
PVY	(283)QF S(285)	CI/6K
TVMV	(255)HF S(257)	NIa/NIb
PPV	(307)HY S(309)	NIa/CP
TEV	(303)HY S(305)	"6K1"/CI
		NIa internal cleavage

D. PepMoV NIa CLEAVAGE SITES

	P1↓P'1	
	(1151)SGVRHQ SLD	(1159)
	(1785)QFVHHQ SKS	(1793)
	(2271)ESVREQ AHT	(2279)
	(2790)YEVRHQ SSR	(2798)
	(1099)KQVVHQ RST	(1107)
	(2025)EVVKHE AKT	(2033)

C.

HC-PRO/PRO-3 CLEAVAGE SITES

	P4 P2 ↓	
PepMoV	(739)H Y R V G G I V	(746)
PVY	(736)H Y R V G G . V	(742)
TVMV	(709)Q Y K V G G L V	(716)
PPV	(762)T Y L V G G L E	(749)
TEV	(759)T Y N V G G M N	(766)
TEV	(759)T Y N V G G M N	(766)

E.

NIa CONSENSUS CLEAVAGE SITES

PepMoV	A V-HQ/S E G A
PVY	V-HQ/S E G
PPV	A V-HQ/S T G
TVMV	VFRQ/S KT G
TEV	E--Y-Q/S G

Fig. 1: A. Proposed map of the PepMoV polyprotein showing the location of putative cleavage sites and the individual viral polypeptides they demarcate. A comparison of the amino acid sequence of the proposed pro-1 protease cleavage sites of PepMoV C, PVY, TVMV, PPV, and TEV is shown in B. A similar comparison of proposed cleavage sites for the HC-Pro protease is shown in C. (D) The deduced amino acid sequence around the five proposed PepMoV NIa protease cleavage sites. The sequences of two degenerate consensus NIa protease cleavage sites which may delineate the viral $6k_1$ and VP_g proteins are also shown in D. A consensus PepMoV NIa protease cleavage site based on conservation of amino acid sequence among the five sites is shown in E along with the NIa protease consensus cleavage sites of the four other sequenced potyviruses. The locations of amino acid sequences within the various viral polyproteins in B-E are indicated in the parentheses. In each case, the site of cleavage is indicated by an arrow or a diagonal line. Amino acids conserved in all five viral sequences are displayed in bold letters (Reproduced from Vance *et al.*, 1992).
PepMoV = pepper mottle virus; PPV = plum pox virus; PV Y = potato virus Y; TEV = tobacco etch virus; TVMV = tobacco vein mottling virus.

Table 1. *Functions of potyviral gene products*

Gene product	Amino acid sequence feature	(Putative) Function*
P1	Similarity between TVMV P1 and TMV 30 kDa protein; Amino acids typical of serine proteases	Cell-to-cell movement ? Polyprotein processing
HC-Pro	Cysteine-rich region; Amino acids typical of cysteine proteases	Vector transmission Polyprotein processing (Protease)
P3	Similarity with 32 kDa cowpea mosaic virus (CPMV) protein	Polyprotein processing ?
6K1	Stretch of hydrophobic amino acids	Replication?
CI	Nucleotide-binding motif Similarity with helicases	Replication? (RNA helicase)
6K2	Stretch of hydrophobic amino acids	Replication?
NIa	Amino acids typical of serine-like cysteine proteases	Polyprotein processing (Protease), Replication? (VP_g)
NIb	Motifs of RNA-dependent RNA polymerases	Replication (RNA-dependent RNA polymerase)
CP	DAG motif	RNA encapsidation Aphid transmission

* Functions that have not yet been supported by experimental evidence are indicated with a question mark.

12 nucleotides (denoted POTYBOX): UCAACACAACAU (Robaglia *et al.*, 1989; Domier *et al.*; 1986; Maiss *et al.*; 1989, Allison *et al.*, 1986). It was suggested that conserved potybox sequences play a role in viral RNA replication but are not necessary for translation (Riechmann *et al.*, 1991).

Strains of individual potyviruses show a high degree of homology in their 3'-NTR and this sequence can be used as a marker to show the genetic relatedness of the viruses (Frenkel *et al.*, 1989). Sequence comparison of 3'-NTR of a number of potyviruses reveal a conserved nucleotide motif that can form a stem-loop like structure (Bryan *et al.*, 1992). Further, they hypothesized that such conserved stem-loops serve as a recognition site from which viral replicase initiates synthesis of the minus strand. It has also been demonstrated that in the 3'-NTR of TVMV-RNA a determinant of disease symptom severity is located (Rodríguez-Cerezo *et al.*, 1991).

An overview of the functions of potyviral gene products is given in Table 1.

Interference between strains of BCMV

When a plant is infected with a strain of a virus, it may become protected from the effects of subsequent inoculation with another strain of the same virus. This type of interference between virus strains in a plant has generally been described as cross-protection (Price, 1940). Cross-protection has been used to control virus diseases and also as a criterion to establish relationships between viruses.

Bercks (1959) showed that prior inoculation of bean plants with the mild strain P 1075 of BCMV protected the plants against the effects of subsequent inoculation with the severe strain P471 of this virus. However, the degree of protection varied with the time interval between the two inoculations.

In cross-protection experiments with isolates of BCMV, Quantz (1961) found that isolates PV2 and SV1 protected the plants against infection with PV1, but the level of protection was variable. Silbernagel (1969) suggested relationships between Mexican isolate, and the strains NY15 and P1 of BCMV on the basis of observed cross-protection. Prior infection of Bountiful beans with the Florida strain of BCMV gave complete protection against systemic infection of the Mexican strain.

In a preliminary study, it was found that plants of *Phaseolus vulgaris* "Bataaf" inoculated first with strain NY15 of BCMV and later with NL3, then considered to be also a strain of BCMV, did not show systemic necrosis, characteristic of the latter strain (Fig. 2).

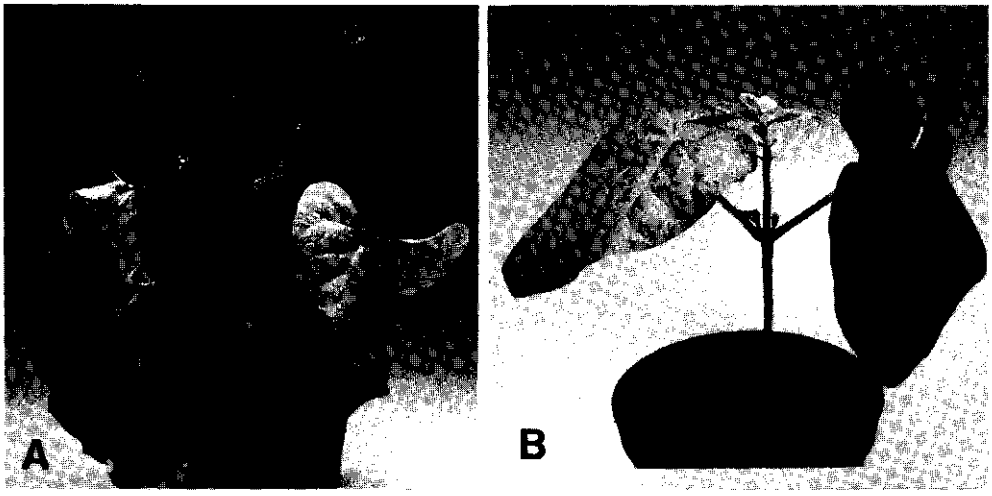


Fig 2. Interference between strains NY15 and NL3 in plants of "Bataaf" bean. The plant inoculated with strain NL3 alone shows top necrosis (A) in contrast to that inoculated first with NY15 and later with NL3 (B).

Objective and an outline of the investigation

In view of the economic importance of BCMV and BlCMV, as pointed out earlier, some of the strains, till now considered to be of these two viruses, were taken up for study with the following objectives:

A. Elucidation of the taxonomic relationships between these two viruses and their strains, preparation of reliable antibodies to distinguish these viruses, thus enabling more efficient control measures and effective breeding for resistance.

B. Gaining insight into the interactions of strains of the above viruses when present in the same plant, both from the practical point of view (diagnosis of mixed infections in nature) and from the fundamental view-point (mechanisms of interactions between viruses and the plant).

Chapter 2 of this thesis deals with the various hypotheses regarding the mechanism of interference among plant viruses. As a first step to study the interference, a clear distinction of the viruses present in mixed infection is a prerequisite. In view of this, novel serological approaches were used to differentiate strains of BCMV and BlCMV as described in Chapter 3. The taxonomic position of strains of BCMV and BlCMV is discussed, and a tentative classification is proposed in Chapter 4. For a sound basis of classification, the nucleotide sequences of coat protein genes and 3'-nontranslated regions have been determined (Chapter 5). A novel type of interference phenomenon, found to occur among different strains of BCMV, has been analysed qualitatively and quantitatively in Chapter 6. Chapter 7 deals with the distribution and localization of BCMV strains in stems of challenge inoculated plants as revealed by light microscopic studies.

References

Allison, R.F., Dougherty, W.G., Parks,T.D., Willis, L., Johnston, R.E., Kelly, M., & Armstrong, F.R. (1985). Biochemical analysis of the capsid protein gene and capsid protein of tobacco etch virus: N-terminal amino acids are located on the virion's surface. *Virology* **147**: 309-316.

Allison, R.F., Johnston, R.E., & Dougherty, W.G. (1986). The nucleotide sequence of the coding region of tobacco etch virus genomic RNA: evidence for the synthesis of polyprotein. *Virology* **154**: 9-20.

Atreya, C.D., Raccah, B., & Pirone, T.P. (1990). A point mutation in the coat protein abolishes aphidtransmissibility of a potyvirus. *Virology* **178**: 161-165.

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

Baron, M.H., & Baltimore, D. (1982). Anti-VP_g antibody inhibition of the poliovirus replicase reaction and production of covalent complex of VP_g-related proteins and RNA. *Cell* **30**: 745-52.

Bercks, R.(1959). Serologische Untersuchungen über das *Phaseolus* -virus 1. *Phytopathologische Zeitschrift* **35**: 105-118.

Bryan, G.T., Gardner, R.C. & Forster, R.L.S. (1992). Nucleotide sequence of coat protein gene of a strain of clover yellow vein virus from New Zealand: conservation of a stem-loop structure in the 3' region of potyviruses. *Archives of Virology* **124**: 133-146.

Carrington, J.C., Cary, S.M., Parks,T.D. & Dougherty , W.G.(1989). A second proteinase encoded by a plant potyvirus genome. *EMBO Journal* **8**: 365-370.

Carrington, J.C., Freed, D.D. & Oh, C.-S.(1990). Expression of potyviral polyproteins in transgenic plants

reveals three proteolytic activities required for complete processing. *EMBO Journal* **9**: 1347-1353.

Domier, L.L., Franklin, K.M., Shahabuddin, M., Hellmann, G.M., Overmeyers, J.H., Hiremath, S.T., Siaw, M.E.E., Lomonossoff, G.P., Shaw, J.G. & Rhoads, R.E. (1986). The nucleotide sequence of tobacco vein mottling virus. *Nucleic Acids Research* **14**: 5417-5430.

Domier, L.L., Shaw, J.G. & Rhoads, R.E. (1987). Potyviral proteins share amino acid sequence homology with picorna-, como-, and caulimoviral proteins. *Virology* **158**: 20-27.

Dougherty, W.G. & Carrington, J.C. (1988). Expression and function of potyviral products. *Annual Review of Phytopathology* **26**: 123-143.

Drijfhout, E. (1978). Genetic interactions between *Phaseolus vulgaris* and bean common mosaic virus with implications for strain identifications and breeding for resistance. *Agricultural Research Reports* 872 *(Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands)*, 98 pp.

Edwardson, J.R. (1974). Some properties of the potato virus Y- group. *Monograph Florida Agricultural Experimental Stations No.* **4**, 398 pp.

Edwardson, J.R., & Christie, R.J. (1978). Use of virus-induced inclusions in classification and diagnosis. *Annual Review of Phytopathology* **16**: 31-55.

Frencel, I., & Pospieszny, H. (1979). Viruses in natural infections of yellow lupin (*Lupinus luteus* L.) in Poland. *Acta Phytopathologica Academiae Scientiarum Hungaricae* **14**: 269-278.

Frenkel, M.J., Ward, C.W. & Shukla, D.D. (1989). The use of 3' non-coding nucleotide sequences in the taxonomy of potyviruses: application to watermelon mosaic virus 2 and soybean mosaic virus-N. *Journal of General Virology* **70**: 2775-2783.

García, J.A., Riechmann, J.L. & Laín, S. (1989). Proteolytic activity of the plumpox potyvirus NIa- like protein in *Escherichia coli* . *Virology* **170**: 362-369.

Goldbach, R. (1986). Molecular evolution of plant RNA viruses. *Annual Review of Plant Pathology* **24**: 289-310.

Goldbach, R. (1987). Genome similarities between plant and animal RNA viruses. *Microbiological Sciences* **4**: 197-207.

Hampton, R.O.(1975). The nature of bean yield reduction by bean yellow and bean common mosaic viruses. *Phytopathology* **65**: 1342-1346.

Hampton, R.O., Silbernagel, M.J. & Burke, D.W. (1983). Bean common mosaic virus strains associated with bean mosaic epidemics in the north western United States. *Plant Disease* **67**: 658-661.

Harrison, B.D. & Robinson, D.J. (1988). Molecular variation in vector-borne plant viruses: epidemiological significance. *Philosophical Transactions of the Royal Society of London Series B* **321**:447-462.

Hodgman, T.C. (1988). A conserved NTR motif in putative helicases. *Nature* (London) **333**: 22-23.

Iwanowski, D. (1894). Ueber die Mosaik Krankheit der Tabakspflanze. *Imp.Akad. Nauk. Izv. (Acad. Imp. Sci. St Petersbourg Bul.)* (n.s.) **35** (3): 67-70.

Laín, S., Riechmann, J.L. & García, J.A. (1989). The complete nucleotide sequence of plumpox potyvirus RNA. *Virus Research* **13** : 157-172.

Laín, S., Riechmann, J.L. & García, J.A. (1990). RNA helicase: a novel activity associated with a protein encoded by a positive strand RNA virus. *Nucleic Acids Research* **18**: 7003-7006

Laín, S., Martin, M.T., Riechmann, J.L. & García, J.A. (1991). Novel catalytic activity associated with positive strand RNA virus infection: nucleic acid-stimulated ATPase activity of the plumpox potyvirus helicase-like protein. *Journal of Virology* **63**: 1-6.

Lana, A.F., Lohuis, H., Bos, L. & Dijkstra, J. (1988). Relationships among strains of bean common mosaic virus and blackeye cowpea mosaic virus-members of the potyvirus group. *Annals of Applied Biology* **113**: 493-495.

Langenberg, W.J. (1986). Virus protein associated with cylindrical inclusions of two viruses that infect wheat. *Journal of General Virology* **67**: 1161-1168.

Maiss, E., Timbe, U., Brisske, A., Jelkmann, W., Caspar, R., Himmler, G., Matlanovichs, D. & Katinger, H.W.D. (1989). The complete nucleotide sequence of plumpox virus RNA. *Journal of General Virology* **70**: 513-524.

Meiners, J.P., Gillaspie, G.A., Lawson, Jr. R.H., & Smith, F.F. (1978). Identification and partial characterization of a strain of bean common mosaic virus from *Rhynchosia minima*. *Phytopathology* **68**: 283-287.

Morrow, C.D., Navab, M., Peterson, C., Hocko, J., & Dasgupta, A. (1984). Antibodies to polio-virus genome-linked protein (VP_g) precipitates in vitro synthesised RNA attached to VP_g-precursor polypeptide(s). *Virus Research* **1**: 89-100.

Murphy, J.F., Rhoads, R.E., Hunt, A.G., & Shaw, J.G. (1990). The VP_g of tobacco etch virus is the 49-kDa proteinase or the N-terminal 24 kDa part of the proteinase. *Virology* **178**: 285-288.

Ponz, F. & Bruening, G. (1986). Mechanism of resistance to plant viruses. *Annual Review of Phytopathology* **24**: 355-381.

Price, W.C.(1940). Thermal inactivation rates of four plant viruses. *Archiv für die gemaste Virusforschung* **1** (3) 1940: 373-386.

Quantz, L. (1962). Zum Nachweis des Gewohnlichen Bohnenmosaikvirus in Bohnensamen mit Hilfe des Schalentest. *Nachrbl. Dtsch. Pflsch. Dienst (Braunschweig)* **14**: 49-54.

Riechmann, J.L., Laín, S., & García, J.A. (1991). Identification of the initiation codon of the plum pox potyvirus genomic RNA. *Virology* **185**: 544-552.

Robaglia, C., Durand-Tarif, M., Tronchet, M., Boudazin, G., Astier-Manifacier, S., & Casse-Delbart, F. (1989). Nucleotide sequence of potato virus Y (N strain) genomic RNA. *Journal of General Virology* **70**: 935-947.

Rodríguez-Cerezo, E. Klein, P.G. & Shaw, J.G. (1991). A determinant of disease symptom severity is located in the 3'-terminal region of the RNA of a plant virus. *Proceedings of the National Academy of Sciences, U.S.A.* **88**: 9863-9867.

Rodríguez-Cerezo, E. & Shaw, J.G. (1991). Two newly detected non-structural viral proteins in potyvirus-infected cells. *Virology* **185**: 572-579.

Shahabuddin, M., Shaw, J.G. & Rhoads, R.E. (1987). Mapping of the tobacco vein mottling virus VP_g cistron. *Virology* **163**: 635-637.

Shahabuddin, M., Shaw, J.G. & Rhoads, R.E. (1988). Mapping of the tobacco vein mottling virus VPg cistron. *Virology* **119**: 150-158.

Shukla, D.D., Frenkel, M.J. & Ward, C.W. (1990). Structure and function of the potyviral genome with special reference to the coat protein coding regions. *Canadian Journal of Plant Pathology* **13**: 178-191.

Shukla, D.D., Strike, P.M., Tracy, S.L., Gough, K.H. & Ward, C.W. (1988). The N and C termini of the coat proteins of potyviruses are surface-located and the N-terminus contains the major virus-specific epitopes. *Journal of General Virology* **69**: 1497-1508.

Siaw, M.F.E., Shahabuddin, M., Ballard, S., Shaw, J.G. & Rhoads, R.E. (1985). Identification of a protein covalently linked to the 5'terminus of tobacco vein mottling virus RNA. *Virology* **142**: 134-143.

Silbernagel, M.J. (1969). Mexican strain of bean common mosaic virus. *Phytopathology* **59**: 1809-1812.

Steibeling, H.K. & Clark, F. (1939). Planning for good nutrition. *U.S. Department of Agriculture Year Book* **1939**: 321-349.

Stewart, V.B. & Reddick, D. (1917). Bean mosaic (abstract). *Phytopathology* **7**: 61.

Taiwo, M.A. & Gonsalves, D. (1982). Serological grouping of isolates of blackeye cowpea mosaic virus and cowpea aphid-borne mosaic virus. *Phytopathology* **72**: 583-589.

Thornbury, D.W., Hellmann, G.M., Rhoads, R.E. & Pirone, T.P. (1985). Purification and characterization of potyvirus helper component. *Virology* **144**: 260-267.

Tsuchizaki, T. & Omura, T. (1987). Relationships among bean common mosaic virus, blackeye cowpea mosaic virus, Azuki bean mosaic virus, and soybean mosaic virus. *Annals of Phytopathological Society of Japan* **53**: 478-488.

Vance, V.B., Moore, D., Turpen, T.H., Bracker, A. & Hollowell, V.C. (1992). The complete nucleotide sequence of pepper mottle virus genomic RNA: Comparison of the encoded polyprotein with those of other sequenced potyviruses. *Virology* **191**: 1931.

Vartapetian, A.B., Koonin, E.V., Agol, V.I. & Bogdanov, A.A. (1984). Encephalomyocarditis virus RNA synthesis in vitro is protein protein-primed. *EMBO Journal* **3**: 2593-98.

Verchot, J., Koonin, E.V. & Carrington, J.C. (1991). The 35 k Da protein from the N-terminus of a potyviral protein functions as a third virus-encoded proteinase. *Virology* **185**: 527-535.

Vetten, H.J. & Allen, D.J. (1991). Recent progress in the identification of viruses of *Phaseolus vulgaris* in Africa. *Bean Improvement Cooperation* 33: 3-4.

Vetten, H.J., Lesemann, D.E. & Maiss, E. (1990). Differentiation of bean common mosaic virus strains.

Abstract P84-008. *VIII th International Congress of Virology*, August 24-26, 1990, Berlin.

Wang, W-Y. (1983). Serology of bean common mosaic virus strains. *M.Sc. Thesis, Washington State University*, Pullman, Washington USA, 52 pp.

Wang, W-Y. (1985). Production and characterization of hybridoma lines and a broad spectum monoclonal antibody against bean common mosaic virus. Ph.D. Thesis, *Washington State University*, Pullman, Washington, U.S.A, 189 pp.

Zaumeyer, W.J. & Thomas, H.R. (1957). A monographic study of bean diseases and methods for their control. *Technical bulletin* no. 868, *U.S. Department of Agriculture*, 255 pp.

Chapter 2

Possible mechanisms of interference among plant viruses: a review of relevant literature

When two viruses are present in the same host plant, they may either interact with each other regarding their multiplication and/or symptom induction, or they may behave in the same way as in a single infection.

In case of interaction, one virus may stimulate multiplication of the other virus, or the overall effect of both viruses on the plant is much stronger than the sum of the effects caused by each of them separately. These types of interaction are called synergism.

Instead of synergism, however, more frequently there is a competition between the two viruses, leading to domination of one, as shown by their ultimate titres and or symptoms in the infected plant. An inhibitory effect of one virus on the other has already been described as early as 1928 and 1929 by Wingard and McKinney, respectively. The latter reported that tobacco plants inoculated with a green strain of tobacco mosaic virus (TMV) produced no further symptoms if subsequently challenge-inoculated with a yellow strain. Similar phenomena in tobacco plants have been described by Thung (1931), Salaman (1933) and by Price (1940) who introduced the term cross-protection for this type of antagonism between virus strains. As the interaction between two viruses described in this thesis is an example of antagonism, this phenomenon will be given further consideration.

Cross-protection in its original meaning was defined as the activity of a virus in a plant preventing the expression of a subsequently inoculated virus (Dodds, 1982). Hamilton (1980) referred to the first introduced virus as the "inducer" and the second introduced virus as the "challenger"; this terminology will be used throughout this thesis.

In the above definition of cross-protection only one aspect of the interaction between two viruses is brought out, viz. the appearance of symptoms but it does not give information on the multiplication of the viruses in the doubly infected plant. It has become clear that the extent of protection may vary considerably, ranging from complete suppression of multiplication of the challenger (*i.e.* no challenger could be detected) to the prevention or delay of symptoms of the challenger, irrespective of its multiplication.

In a few instances, the inducer may make the plant immune to the challenger, such as in case of tobacco infected with tomato ringspot virus being immune to cherry leafroll virus (Fulton, 1975), but in probably most of the cases of cross-protection, there is detectable replication of the challenger. Such a form of protection, which does not imply immunity to, but reduced replication of , the challenger, has been termed interference by Hamilton (1980) who at the same time suggested that the term cross-protection should be reserved for the cases in which either of two viruses used as inducer offers reciprocal protection against symptom expression by the other virus as a challenger.

As formerly cross-protection was thought to occur only between two related viruses, the phenomenon was used to establish relationships between viruses before serological methods became the vogue (Bozarth & Ford, 1988). However, cases have been reported in which strains of the same virus failed to protect (Fulton, 1978) whereas in others protection was induced against unrelated challenger virus (Fulton, 1975).

In most of the studies on interference between viruses, very little attention has been paid to the role of host plant. It is now recognised that in many instances the host may determine the outcome of the interference between viruses, either by a reduced susceptibility to challenger due to the presence of inducer, or by host-mediated substances influencing the result of interaction between inducer and challenger (Blum *et al.*, 1989; Urban *et al.*, 1990)

In the following a review is given of the most important theories which have been proposed to explain the mechanism of interference.

1. Precursor-exhaustion, ribosome-sequestration and limiting cell capacity

The precursor-exhaustion theory starts on the premise that related viruses to some extent use the same cell constituents. In that concept, the inducer when present in most of the cells of an inoculated leaf, will utilize most of the available metabolites thus leaving insufficient amount of precursor material to support the replication of challenger virus.

This theory has been put forward by, among others, Köhler and Hauschild (1947) and Hageman (1964). In experiments with the U_2 strain of TMV-now regarded as a strain of tobacco mild green mosaic virus (Wetter, 1989)- as inducer and the green aucuba strain of TMV as challenger in *Nicotiana tabacum* cv. Turkish, the latter author showed that interference occurred in mature leaves with a low RNA content and a small amount of buffer-soluble RNA. This interference was characterized by absence of demonstrable replication of the challenger. When disks from U_2 -infected, fully protected leaves were transferred to nutrient solution containing salt, malic acid and uracil, a considerable replication of challenger was observed. It is hard to see, however, why this mechanism would hold good for related viruses only, as all viruses require the same selection of precursors.

When the term precursor is being extended to more specific cell constituents necessary for replication of viruses, such as the host-coded subunits of viral replicases (Hariharasubramanian *et al.*, 1973; Zaitlin *et al.*, 1976), the theory might still have some scope. In that case, the inducer might have utilized most of the host component thus depriving a related challenger of this essential virus-specific, host-coded part of its replicase. Also with this theory, however, it can not easily be explained why in a number of cases related viruses fail to protect.

A similar objection might be put forward against the ribosome -sequestration theory proposed by Ross (1974) which suggests that the majority of ribosomes in a cell would be sequestered by a rapid increase in inducer-RNA, irrespective of its relatedness to the inducer.

However, these objections do not take away the fact that the capacity of a cell to support virus multiplication is limited and this aspect deserves more attention. Factors determining the maximum amount of virus in a cell are not identified so far, but some viruses are known to occur in much higher concentrations in plant cells than others. It is, therefore, conceivable that an inducer virus occupying a large number of cells and

present in a high concentration, leaves little or no cell capacity for replication of a challenger virus. This, however, does not explain why cross-protection is more often encountered between strains of the same virus, unless it is assumed that different viruses prefer different tissues for their replication.

2. Role of the coat protein

a. Adsorption to virus aggregates. This theory is based on the assumption that a challenger virus introduced into cells where related inducer virus is present, will be adsorbed to inducer virus-aggregates already formed (Kavanau, 1949). For some viruses with rod-shaped particles which readily form aggregates in a number of cells this may be a valid explanation.

b. Inability of the challenger virus to partially uncoat. After virus particles have been introduced into a cell, they must be uncoated at least partially, before replication can take place. On the basis of *in vitro* experiments by, among others, Wilson (1984) and Roenhorst (1989) it is assumed that partial uncoating takes place at the 5' terminus whereafter the first open-reading frame becomes translated (cotranslational disassembly).

Results of experiments by Sherwood and Fulton (1982) point into the direction of prevention of uncoating by the challenger virus, as an explanation for cross-protection. These authors reported that *N. sylvestris* systemically infected with TMV and showing mosaic patterns, produced local lesions upon challenge-inoculation with a necrotizing strain of TMV on the dark green areas (known to contain little or no virus) of the mosaic only. When, however, RNA of the necrotizing strain was used as challenger, local lesions appeared in both the dark green and the light green areas of the mosaic. When RNA of the necrotizing TMV strain was encapsidated in brome mosaic virus coat protein and used as a challenger, the result was the same as with RNA as inoculum.

Experiments with the tobamoviruses sunn-hemp mosaic virus (SHMV) and TMV showed that prevention of uncoating may be one of the factors, but not the only one,

playing a role in cross-protection (Zinnen & Fulton, 1986). When cowpea plants infected with SHMV were inoculated with RNA of TMV-C encapsidated in either SHMV coat protein or TMV-C coat protein, the heterologously encapsidated RNA was much less infectious than the homologously encapsidated one. When cowpea plants were inoculated with SHMV as inducer and later with either virions or RNA of a necrotizing mutant of SHMV as challenger, the plant did not show any symptom of the latter.

A strong indication that in protection of plants against the effects of a challenger virus coat protein plays an important role is furnished by plants transformed with the coat protein gene of this virus. Register and Beachy (1988) and Register *et al.* (1988) demonstrated that it is the coat protein of the virus expressed in transgenic plants which is responsible for blocking of an early event in the infection process, most likely uncoating of the virus particle. The protection of transgenic plants expressing the coat protein of a particular virus, is very often broken down when instead of the virion, the RNA of this virus is used as inoculum (Nelson *et al.*, 1987; Angenent *et al.*,1990). Also in cross-protection, this phenomenon has been observed, as pointed out earlier. However, transgenic plants expressing coat protein of potato virus X (PVX) were resistant to infection with PVX and PVX-RNA alike (Hemenway *et al.*, 1988). This may imply that besides an effect on uncoating of the virus, coat protein expressed in transgenic plants may also inhibit later stages in the infection process.

c. Complete or partial transcapsidation. Some instances have been reported in which challenger RNA appeared to be encapsidated in coat protein of the inducer (Dodds & Hamilton, 1976). Such a heterologously encapsidated challenger would thus be prevented from replication. Even partial encapsidation of challenger RNA by inducer coat protein might prevent replication, possibly due to blocking of binding sites of the replicase (Horikoshi *et al.*, 1987).

3. Sequestration of antisense or sense RNA

According to Zaitlin (1976) and Palukaitis and Zaitlin (1984) there is a possibility that minus strand copies of the challenger RNA become fully hybridized to plus strand RNA of the inducer, thus rendering the challenger noninfectious (Fig. 1). Such a mechanism would explain how viroids and RNA viruses with defective coat proteins can act as inducers. Additional support for this theory comes from results obtained with transformed plants producing antisense RNAs of a virus. These plants proved to be protected from infection with this particular virus (Matthews, 1991). Also potato and tobacco plants transformed with the coat protein encoding sequence of potato virus Y^N (PVY^N) showed a high degree of protection inspite of the fact that in none of the transgenic plants significant amounts of viral coat protein could be detected (Van der

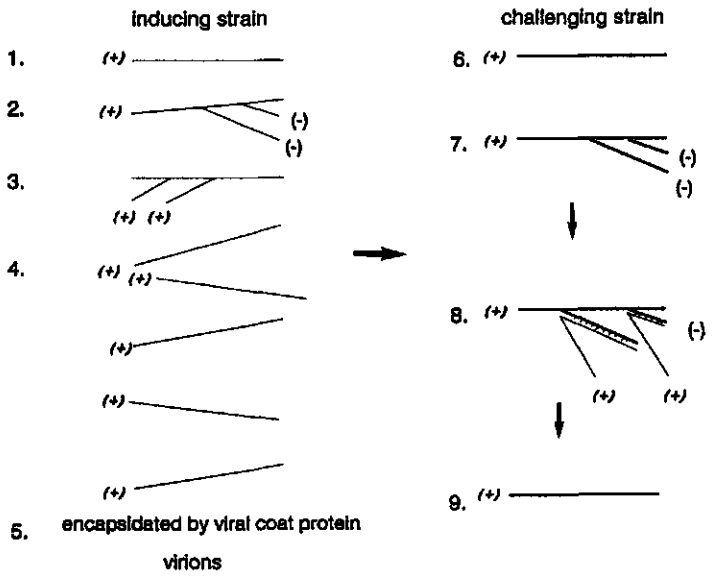


Fig. 2.1 A model to explain the cross-protection shown by plant viruses (adapted from Palukaitis, P. & Zaitlin, M., 1984).

Vlugt *et al.*, 1992). Such an RNA-mediated protection may be based on 'sense-RNA' effect consisting of hybridization of a positive-sense, transgenic RNA to negative-sense viral RNA thus inhibiting virus multiplication.

4. Prevention of systemic spread of the challenger virus

Besides mechanisms which may prevent the initiation of replication or the replication itself, there are also indications that inducer virus allows replication of the challenger virus but inhibits the latter's systemic spread.

Support for involvement of impaired virus movement in cross-protection has been derived from experiments with cucumber mosaic virus (CMV) (Dodds, 1982; Dodds *et al.*, 1985) and with TMV (Urban *et al.*, 1988; Rezende *et al.*, 1992).

The results obtained with CMV in tomato and tobacco plants were, however, somewhat contradictory regarding replication of challenger in the inoculated leaf. In 1982, Dodds reported that the mild strain CMV-S protected tomato plants from becoming systemically infected with the severe challenger strain CMV-P, but both strains were found to accumulate in challenger-inoculated leaves. These experiments were carried out with virions-containing inocula. On the other hand, in a later publication (Dodds *et al.*, 1985) the authors stated that protection was detected in the leaves inoculated with the challenger strain CMV-P and also in later formed leaves. Only when infectious viral RNA instead of virions was used as the challenger inoculum, there was a break-down of protection in the challenger-inoculated leaves. However, irrespective of accumulation of challenger in the inoculated leaves, no accumulation of virus was found in later formed leaves.

Similarly, a strain of TMV used as challenger, multiplied in inoculated leaves of *Arabidopsis thaliana* previously infected with another TMV strain as inducer, but it could not spread systemically in the plant (Urban *et al.*, 1988), at least not in a significant number of the challenged plants (Rezende *et al.*, 1992).

Impaired systemic movement of the challenge virus may point into the direction of involvement of the host producing inhibitory substances. Blum *et al.*(1989) hypothesised that the challenger virus can not synthesize its movement protein because the protecting strain induces the host to produce a substance inhibiting the synthesis of this protein. However, in case of impaired systemic transport of TMV this theory does not hold good, as only for cell-to-cell transport in the inoculated leaf this 30 kDa protein is required whereas for long-distance transport of, among others, TMV and CMV, their respective coat proteins are required.

Although Urban *et al.*(1990) advocate a possible role of pathogenesis-related (PR) proteins by their interaction with the movement protein, also in view of their (so far) unraveled biochemical functions (chitinases, glucanases, etc.) it is hard to see how such proteins can impair cell-to-cell transport of challenger virus which has been shown to accumulate readily in the inoculated leaf. Inhibition of systemic transport of virus by PR-proteins is also not very likely, as these proteins are not transported in a plant. At the most they have been elicited by the inducer virus in the tissues invaded by the latter, but in that case it is not easy to explain why they act only against the challenger virus and not against the inducer itself. Furthermore, prevention of systemic spread of the challenger virus proved to be strain specific. *A. thaliana* plants inoculated with a strain of TMV were protected from superinfection and systemic spread of another strain of TMV, but not from that of CMV (Rezende *et al.*, 1992). Such strain specificity also does not favour the PR-protein hypothesis to explain cross-protection, as induction of PR-proteins is a general defense reaction of a plant and not a virus-specific response.

The cross-protection between two strains of TMV, as reported by Rezende *et al.*, (1992) proved to be host dependent. Protection in *A. thaliana* was characterized by prevention of systemic movement of the challenger from the inoculated leaf, whereas that in *N. tabacum* cvs. Samsun and Xanthi was a result of inhibition of replication of the challenger in the inoculated leaf. The host plant may thus determine the mechanism of

cross-protection.

From the foregoing theories the conclusion may be drawn that most likely not one, but more mechanisms may be involved in interference, and that the role of the host plant should not be underestimated in this respect.

References

Angenent, G.C., Van den Ouweland, J.M.W. & Bol, J.F. (1990). Susceptibility to virus infection of transgenic tobacco plants expressing structural and non-structural genes of tobacco rattle virus. *Virology* **175**: 191-198.

Blum, H., Gross, H.J. & Beier, H. (1989). The expression of the TMV-specific 30-kDa protein in tobacco protoplasts is strongly and selectively enhanced by actinomycin. *Virology* **169**: 51-61.

Bozarth, R.F. & Ford, R.E. (1988). Viral interactions: Induced resistance (cross protection) and viral interference among plant viruses. In: *Experimental & conceptual plant pathology* (Hess, W.M., Singh, R.S., Singh, U.S. & Weber, D.J., eds) vol. 3: 551-567.

Dodds, J.A. (1982). Cross-protection and interference between electrophoretically distinct strains of cucumber mosaic virus in tomato. *Virology* **118**: 235-240.

Dodds, J.A. & Hamilton, R.I. (1976). Structural interactions between viruses as a consequence of mixed infections. In *Advances in Virus Research* (Lauffer, M.A., Bang, F.B., Maramorosch, K., & Smith, K.M. eds). vol. 20: 33-86.

Dodds, J.A., Lee, S.Q. & Tiffany, M. (1985). Cross protection between strains of cucumber mosaic virus: effect of host and type of inoculum on accumulation of virions and double-stranded RNA of the challenge strain. *Virology* **144**: 301-309.

Fulton, R.W. (1975). Unilateral cross protection among some NEPO viruses in tobacco and *Petunia hybrida*. *Acta Horticulturae* **44**: 29-32.

Fulton, R.W. (1978). Superinfection by strains of tobacco streak virus. *Virology* **85**: 1-8.

Hageman, P.C. (1964). Interference of two strains of tobacco mosaic virus. *Ph.D. Thesis*, University of Amsterdam: 1-72.

Hamilton, R.I. (1980). Defenses triggered by previous invaders: viruses. In: *Plant disease* (Horsfall, J.G. & Cowling, E.B., eds) vol. 5: 279-303.

Hariharasubramanian, V., Hadidi, A., Singer, B. & Fraenkel-Conrat, H. (1973). Possible identification of a protein in some brome mosaic virus-infected barley as a component of viral RNA polymerase. *Virology* **54**:190-198.

Hemenway, C., Fang, R.-X., Kanieswski, W.K., Chua N.-H. & Tumer, N.E. (1988). Analysis of the mechanism of protection in transgenic plants expressing the potato virus X coat protein or its antisense RNA. *EMBO Journal* **7** : 1273-1280.

Horikoshi, M., Nakayama, M., Yamaoka, N., Furusawa, I. & Shishiyama, J. (1987). Brome mosaic virus coat protein inhibits viral RNA synthesis *in vitro*. *Virology* **158**: 15-19.

Kavanau, J.L. (1949). On the correlation of the phenomena associated with chromosomes, foreign proteins

and viruses. III. Virus associated phenomena, characteristics and reproduction. *American Naturalist* 83: 113-138.

Köhler, E. & Hauschild, I. (1947). Betrachtungen und Versuche zum Problem der 'erworbenen Immunität' gegen Virusinfektionen bei Pflanzen. *Der Züchter* 17/18: 97-105.

Matthews, R.E.F. (1991). *Plant Virology.* Third ed., Academic Press Inc.

McKinney, H.H. (1929). Mosaic diseases in the Canary Islands, West Africa, and Gibraltar. *Journal of Agricultural Research* 39: 557-578.

Nelson, R.S., Powell Abel, P. & Beachy, R.N. (1987). Lesions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus. *Virology* 158: 126-132.

Palukaitis, P. & Zaitlin, M. (1984). A model to explain the 'cross protection' phenomenon shown by plant viruses and viroids. In : *Plant-microbe interactions* (Kosuge, T. & Nester, E.W., eds) vol. 1: 420-429.

Price, W.C. (1940). Acquired immunity from plant virus diseases. *Quarterly Review of Biology* 15: 338-361.

Register III, J.C. & Beachy, R.N. (1988). Resistance to TMV in transgenic plants results from interference with an early event in infection. *Virology* 166: 524-532.

Register III, J.C., Powell, Abel, P., Nelson, R.S. & Beachy, R.N. (1988). Genetically engineered cross protection against TMV interferes with initial infection and long distance spread of the virus. In: *Molecular biology of plant-pathogen interactions* (Staskawicz, B., Ahlquist, P. & Yoder, O., eds): 269-282.

Rezende, J.A.M., Urban, L., Sherwood, J.L. & Melcher, U. (1992). Host effect on cross-protection between two strains of tobacco mosaic virus. *Journal of Phytopathology* 136: 147-153.

Roenhorst, J.W. (1989). Virus-ribosome complexes from cell-free translation systems supplemented with cowpea chlorotic mottle virus particles. *Virology* 168: 138-146.

Ross, A.F. (1974). Interactions of viruses in the host. In: *Virus diseases of ornamental plants* (Lawson, R.H. & Corbett, M.K. eds): 247-260.

Salaman, R.N. (1933). Protective inoculation against a plant virus. *Nature* 131 :468.

Sherwood, J.L. & Fulton, R.W. (1982). The specific involvement of coat protein in tobacco mosaic virus cross protection. *Virology* 119: 150-158.

Thung, T.H. (1931). Smetstof en plantecel bij enkele virusziekten van de tabaksplant. *Handelingen Nederlandsch-Indisch Natuurwetenschappelijk Congres* 6: 450-463.

Urban, L.A., Sherwood, J.L. & Melcher, U. (1988). The use of *Arabidopsis thaliana* cv. Columbia in plant virus interaction studies. *Phytopathology* 78: 1586.

Urban, L.A., Sherwood, J.L., Rezende, J.A.M. & Melcher, U. (1990). Examination of mechanisms of cross-protection with non-transgenic plants. In: *Recognition and response in plant-virus interactions* (Fraser, R.S.S., ed.) NATO ASI H 41: 415-426.

Van der Vlugt, R.A.A., Ruiter, R.K. & Goldbach, R. (1992). Evidence for RNA-mediated protection of PVY^N in tobacco plants transformed with the viral coat protein cistron. *Plant Molecular Biology* 20: 631-639.

Wetter, C. (1989). Tobacco mild green mosaic virus. *CMI/AAB Descriptions of plant viruses*, no. 351.

Wilson, T.M.A. (1984). Cotranslational disassembly of tobacco mosaic virus *in vitro*. *Virology* 137: 255-265.

Wingard, S.A. (1928). Hosts and symptoms of ringspot, a virus disease of plants. *Journal of Agricultural Research* 37: 127-153.

Zaitlin, M. (1976). Viral cross-protection: more understanding is needed. *Phytopathology* 66: 382-383.

Zinnen, T.M. & Fulton, R.W. (1986). Cross protection between sunn-hemp mosaic and tobacco mosaic viruses. *Journal of General Virology* 67: 1679-1687.

Chapter 3

Distinction of strains of bean common mosaic virus and blackeye cowpea mosaic virus using antibodies to N- and C- or N- terminal peptide domains of coat proteins

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This chapter has been published in a slightly modified version as: Khan *et al.*(1990). Distinction of strains of bean common mosaic virus and blackeye cowpea mosaic virus using antibodies to N- and C- or N-terminal peptide domains of coat proteins. *Annals of Applied Biology* (1990) **117**: 583-593.

Summary

Earlier attempts to discriminate serologically strains NL1, NL3 and NY15 of bean common mosaic virus (BCMV) and strain W of blackeye cowpea mosaic virus (BlCMV) had been unsuccessful. Antibodies directed towards N- and C-, or N-terminal peptide regions of the coat proteins of the above strains enabled the distinction between BlCMV-W, BCMV-NY15 and BCMV-NL3 in electroblot immunoassay and in ELISA. The distinction was better with antibodies directed towards N-termini than with those to N- and C-termini. Strain NL1 of BCMV cross-reacted with both BlCMV-W and BCMV-NY15, but not with BCMV-NL3. Taxonomic implications of these findings are discussed.

Introduction

Common bean (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*) are major protein-producing food legumes in tropical Africa and America. However, these crops are seriously threatened by viruses, especially the potyviruses blackeye cowpea mosaic virus (BlCMV) and bean common mosaic virus (BCMV).

Previous studies (Lana *et al.*, 1988) revealed a close serological relationship between BlCMV and non-necrosis inducing strains of BCMV, particularly NL1 (closely related to the type strain US1) and NY15. In contrast, serological relationships of NL1, NY15 and W to BCMV-NL3 (a necrosis-inducing strain) were more distant when determined in ELISA. This different position of NL3 is also reflected in its biological properties, as it belongs to the pathogenicity group VI of strains causing systemic necrosis in bean cultivars with dominant alleles of the necrosis gene, whereas NL1 and NY15 have been placed in groups I and V, respectively, of strains giving mosaic in these cultivars (Drijfhout, 1978).

In general, there was a difference in pathogenicity to bean and cowpea of BlCMV, on the one hand, and of NL1 and NL3, on the other hand, the former more readily infecting cowpea and the latter being more pathogenic to bean, whereas strain NY15 possessed an intermediate position (Lana *et al.*, 1988).

Recent work on the distribution of strains of BCMV in beans in the tropics shows

that necrotic strains predominate in many areas of tropical Africa, but that there are areas, e.g. Ethiopia, where non-necrosis inducing strains are prevalent (Vetten, H.J. & Owera, S., personal communication, 1989; Morales, F., personal communication, 1990). It has been speculated that the necrotic African strains originated on cowpea, but according to others they may have arisen by mutation from non-necrosis-inducing strains of BCMV (Allen, D. & Morales, F., personal communication, 1990).

Understanding of the epidemiology of these viruses and breeding for resistance require clear differentiation of strains of BlCMV and BCMV. For cross-protection studies, a clear distinction between the strains and quantification of each of them in mixed infections are equally imperative. As attempts to distinguish between BlCMV-W, BCMV-NL1, -NL3 and -NY15 by conventional serology or bio-assay have given ambiguous results, the method of Shukla *et al.* (1989 *a*) was applied, using antibodies to the N- and C- or N-terminal parts of coat proteins of the four virus strains, as on these parts virus-specific epitopes are located (Dougherty *et al.* 1985; Shukla *et al.*, 1988).

Materials and Methods

Virus isolates. The virus strains used were BCMV-NL1, -NY15, -NL3 and BlCMV-W , henceforth referred to as NL1, NY15, NL3 and W respectively (Lana *et al.*, 1988). The BCMV strains were maintained and propagated in young plants of *Phaseolus vulgaris* cv. Bataaf and BlCMV-W in *Nicotiana benthamiana*. Growing conditions of the plants and purification of virus strains were as described by Lana *et al.* (1988). For comparison, NL5, another necrotic strain of BCMV, and B 25 of bean yellow mosaic virus both obtained from Dr L. Bos (IPO-DLO, Wageningen) and strain N of potato virus Y (PVY^N) from our own stock were used.

Antisera. For production of antiserum to each strain, rabbits were injected with freshly purified virus emulsified with Freund's incomplete adjuvant (1:1). Initially, 400 μg of purified virus was injected intramuscularly and subcutaneously; a second injection (400 μg of purified virus) and a third injection (1 mg purified virus) were given intramuscularly 2 and 6 wk respectively, after the first injection. The titre of each antiserum was

determined by microprecipitation test as described by Lana *et al.* (1988). Antiserum from the first bleeding (except with W) made 4 wk after the first injection, was used for the purification of antibodies directed towards N- and C-terminal peptide regions of coat protein.

Removal of N- and C-, or N-terminal peptides from coat proteins. To remove the N-and C-terminal peptides, purified virus (3 mg) was incubated with 30 μg (for NL1,NL3 and NY15) or 90 μg (for W) trypsin (Difco Laboratories) for 45 min at 22° C. To remove the N-terminal peptides, 5 mg purified virus was incubated with 180 μg lysyl endopeptidase (Boehringer, FRG) for 45 min at 25°C. The enzyme-resistant core particles were separated from the N- and C-terminal peptides centrifuging at 4°C in a Beckman ultracentrifuge using an SW-55 rotor at 149000 *g* for 50 min and discarding the supernatants.

Purification of N- and C-terminal specific antibodies. For purification of the N- and C-terminal specific antibodies, the pellet containing the trypsin-resistant core particles was resuspended in sodium phosphate buffer (25 mM, pH 8.0) and dissociated as described by Shukla *et al.* (1989 *a*). The separated protein preparation (2.5ml) was coupled to 0.4 g CNBr-Sepharose gel (Pharmacia, Sweden) according to the manufacturer's instructions. Antiserum (30 μl) to the strains NL1 (titre 32), NL3 (titre 128), NY15 (titre 256) and W (titre 512) was passed through the affinity column to which homologous protein (lacking N- and C-termini) had been coupled, and washed with 10 ml borate saline buffer (prepared by diluting 0.1 M borate-buffer, pH 8.4, in 0.85% saline solution in a ratio of 1:20). The antibodies which did not bind to the columns (i.e. those to the N- and C-terminal peptides) were collected and their concentration measured using ${}^{0}A^{\%}_{10mm,280nm}$ = 1.4 (Clark & Adams, 1977).

For purification of N-terminal specific antibodies, 2.5 ml of the coat protein devoid of N-terminal peptides was coupled to 0.5 g CNBr-Sepharose. Antiserum (40 μl) to each strain was passed through homologous affinity columns. The rest of the procedure was as described for the N-and C-terminal peptides.

Sodium dodecyl sulphate-polyacrylamide gel-electrophoresis (SDS-PAGE). Intact, trypsin- and lysyl endopeptidase-treated virus samples were mixed with an equal volume of denaturing solution (0.5 M Tris-HCl, pH 6.8, 10%(w/v) glycerol, 2% (w/v) SDS, 5% (w/v) ß mercaptoethanol and 0.05% bromophenol blue) and heated in a boiling water-bath for 3 min . Electrophoresis was carried out in 12% (separating) polyacrylamide gels according to the method described by Laemmli (1970) using Bio-Rad Protein II mini gel apparatus. About 0.3 μg of each sample was loaded to each slot of the gel. The marker proteins (Pharmacia, Sweden) used were phosphorylase b (M_r 94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K) and soybean trypsin inhibitor (20.1K).

Electroblot immunoassay (EBIA). EBIA was performed as described by Towbin *et al.* (1979) with some modifications. For each strain, 0.3 μgof purified virus was loaded to each slot of the gel.

After electroblotting, the nitrocellulose membranes were put in phosphate-buffered saline with 0.05% Tween, pH 7.4 (PBS-Tween) containing 5% (w/v) non-fat dry milk as a blocking agent for 1 h at 37°C and then incubated at 80°C for 30 min . Membranes were then saturated in antiserum to intact particles (1μl), antibodies directed towards N- and C-(8 μg) or N-(1 μg) terminal peptide regions of coat protein diluted in 1 ml PBS-Tween and incubated at 37°C for 1 h. The membranes were washed three times (10 min each) with PBS-Tween containing 0.5% non-fat dry milk and incubated in alkaline phosphatase-conjugated secondary antibodies (Tago, U.S.A), diluted 1:3000 in PBS-Tween at 37°C for 1 h and washed as described earlier. A mixture of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BRL) was used as substrate.

Enzyme-linked immunosorbent assay (ELISA). Serological relationships were quantified in direct double antibody sandwich ELISA using purified virus and sap from leaves of healthy and diseased plants of *P. vulgaris* cv. Bataaf and *N. benthamiana*. PBS-Tween was used for extraction and dilution of antigens (1:10). The concentration of immunoglobulins to intact particles used for optimum coating of the wells of the

microtitre plate was 5 μg/ml for NL1, NY15 and W or 1 μg/ml for NL3. The final dilution of conjugated gammaglobulins from antibodies to N-terminal part of coat proteins used was 1:1000 (NL1 and NY15) or 1:2000 (NL3 and W). Readings were made at 405 nm with a Biokinetics Reader EL 312, 2h after adding substrate (p-nitrophenyl phosphate, Sigma, U.S.A.).

Results

Preparation of coat protein core

The molecular masses of intact coat proteins of NL1, NY15 and W were between 36-

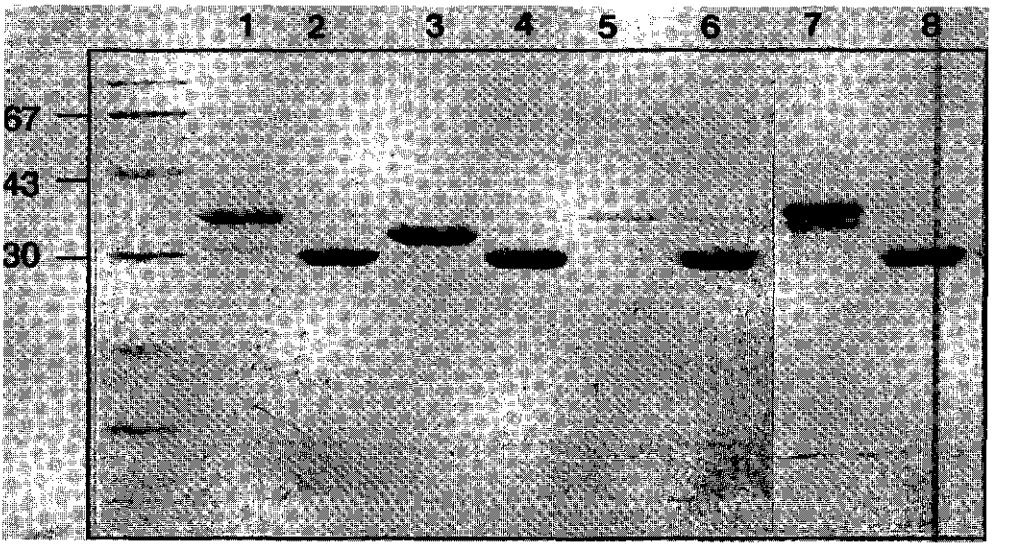

Fig. 1. SDS-PAGE of purified preparations of strains NL1 (1), NL1 lysyl endopeptidase treated (2), NL3 (3), NL3 lysyl endopeptidase-treated (4), NY15 (7), NY15 lysyl endopeptidase-treated (8) of bean common mosaic virus, strain W (5) and W lysyl endopeptidase-treated (6) of blackeye cowpea mosaic virus.
Values to the left are M_r ($x10^{-3}$) of marker proteins in the first lane.

37 K, whereas that of NL3 was about 34 K. After treatment of the virus suspensions with either trypsin or lysyl endopeptidase (Fig.1), the coat proteins of all four strains devoid of either N- and C- or N-termini, respectively, had an M_r of 30K, indicating that only the N- and C-termini vary in length and the remainder of coat protein is of constant length. Further, the enzyme-virus ratios were suited to selectively removing the N- and C- or N-terminal peptide regions of coat proteins as checked by SDS-PAGE and EBIA (Figs 1 & 2).

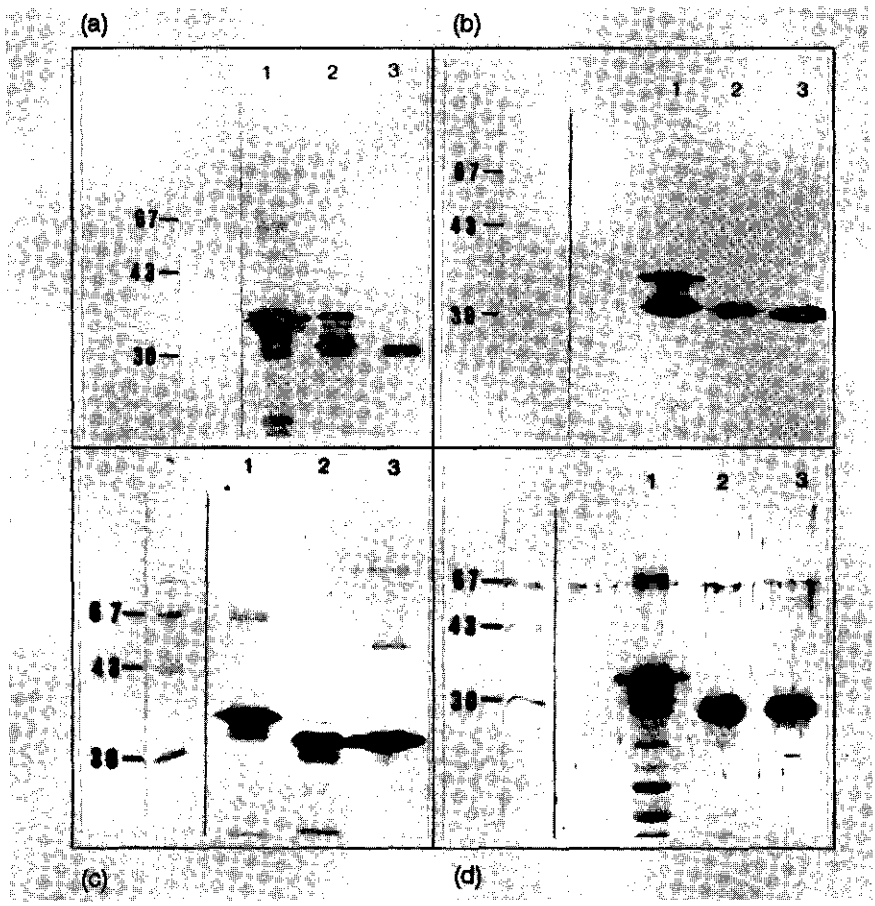

Fig. 2. Electroblot immunoassay of purified preparations of intact (1), trypsin treated (2) and lysyl endopeptidase-treated (3) strains NL1 (a), NL3 (b), NY15 (d) of bean common mosaic virus and strain W (c) of blackeye cowpea mosaic virus reacted with antiserum to their homologous intact particles.
Values to the left are M_r (x 10^3) of marker proteins in the first lane.

EBIA.

In EBIA, antiserum to intact virus particles reacted with all the strains of BCMV and BlCMV in homologous and heterologous combinations and also with the unrelated potyvirus PVY^N (Figs 3 & 6). The reactivity of W with antiserum to intact NL1 was weak due to lower loading and B 25 reacted only with antiserum to intact NL1 (Fig. 3). The polypeptide species with low M_r that showed strong reactions, especially those from NL1, NY15, W and PVY^N, are degradation products of the coat proteins. This heterogeneity of potyviral coat protein molecules is a well known phenomenon (Shukla *et al.*, 1988). Bands in Fig. 6 with high M_r are most likely multimers of coat protein, as they show the same serological properties as monomers.

When, on the other hand, antibodies directed to either the N- and C- termini or the N-termini alone were used, all cross-reactions with viruses like B 25 and PVY^N disappeared and the specificity was such that even some of the serologically closely related BCMV and BlCMV strains could be distinguished. The specificity was slightly greater with antibodies to N-termini alone than with those directed to N- and C-termini (Figs 4 & 5). Antibodies to the N- and C-termini and N-terminal domains, however, showed no reaction with any of the degradation products of the coat proteins which are known to be devoid of N- and C-termini to varying degrees. This shows that affinity absorption had removed all antibodies except those to the N- and C-termini.

Antibodies to the N-terminus of NL1 gave a strong homologous reaction, but weak heterologous reactions with NY 15 and W (Fig. 3); those of NY 15 gave a strong homologous reaction, and an equally strong heterologous reaction only with NL1 (Fig. 5); and those of W gave a strong homologous reaction, but a weaker reaction with NL1 (Fig. 6). In each case, the other heterologous reactions were absent. However, the N-terminal specific antibodies to NL3 showed no heterologous reaction with any of the above strains, but a homologous reaction and a reaction of similar intensity with NL5 only (Fig. 4).

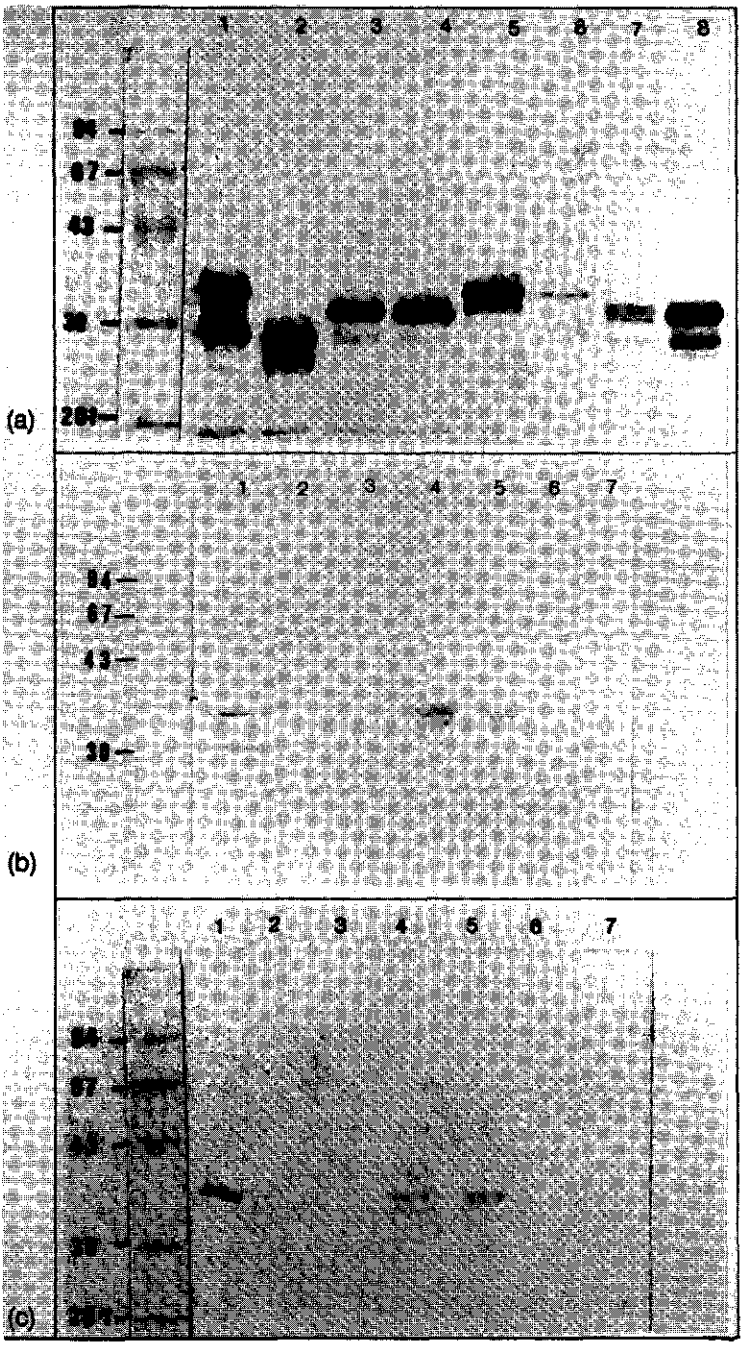

Fig. 3. Electroblot immunoassay of purified preparations of strains NL1 (1), NL1 lysyl endopeptidase-treated (2), NL3 (3), NL5 (4) and NY15 (5) of bean common mosaic virus, strain W (6) of blackeye cowpea mosaic virus, strain B 25 (7) of bean yellow mosaic virus and strain N (8) of potato virus Y reacted with antiserum to intact particles (a); strains NL1 (1), NL1 lysyl endopeptidase-treated (2), NL3 (3), NY15 (4), W (5), B25 (6) & PVY ^{N} (7) reacted with antibodies directed to N- and C-termini (b), and to N-termini (c) of coat protein of NL1. Values to the left are M_r (x10 ^{3}) of marker proteins in the first lane.

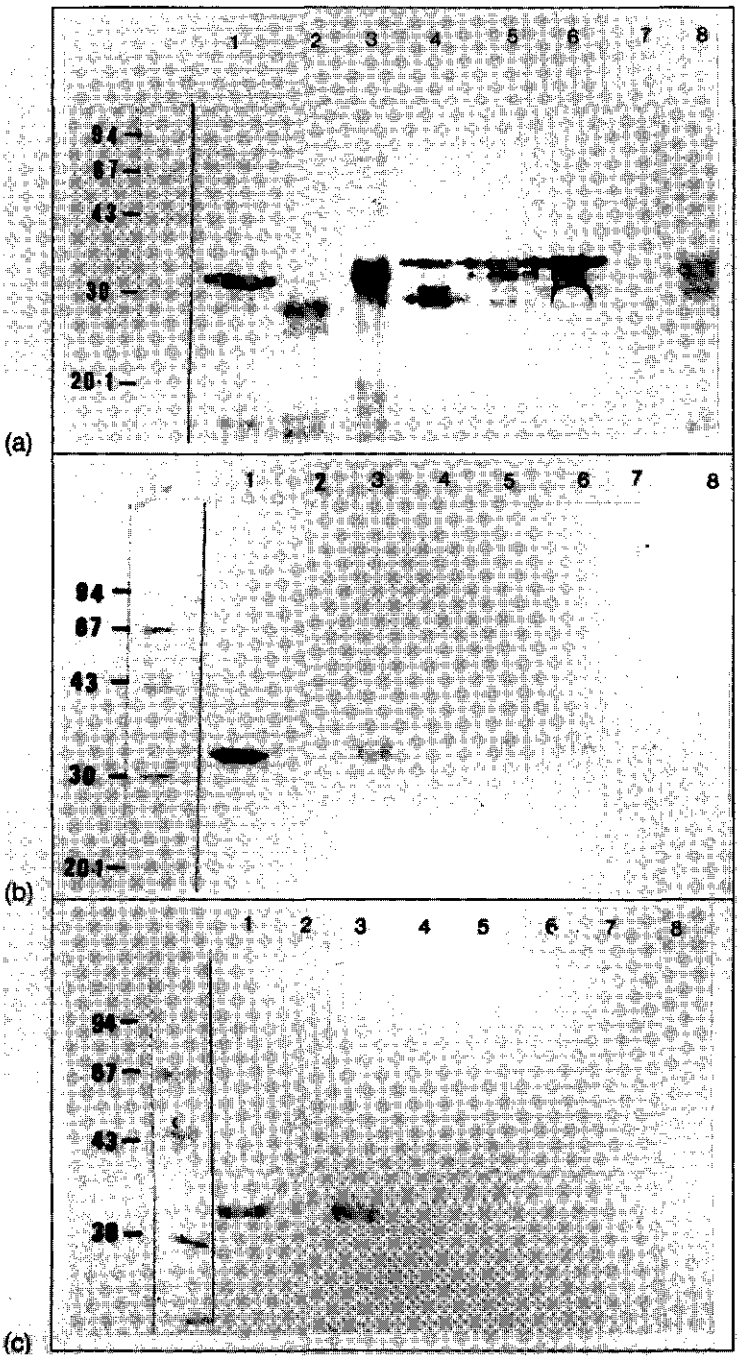

Fig. 4. Electroblot immunoassay of purified preparations of strains NL3 (1), NL3-lysyl endopeptidase-treated (2), NL5 (3), NL1 (4), NY15 (5) of bean common mosaic virus, strain W (6) of blackeye cowpea mosaic virus, strain B 25 of bean yellow mosaic virus and strain N (8) of potato virus Y reacted with antiserum to intact particles (a), antibodies directed to N- and C-termini (b) and to N-termini (c) of coat protein of NL3. Values to the left are M_r ($x10^3$) of marker proteins in the first lane.

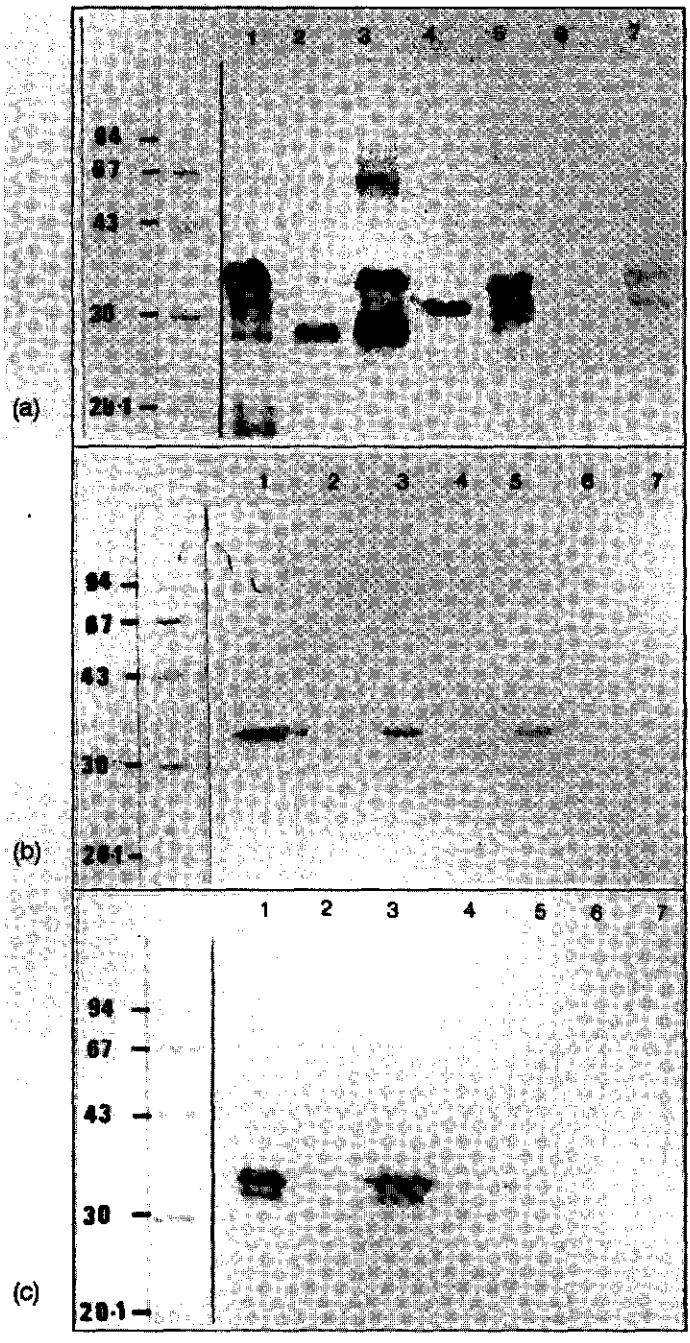

Fig 5. Electroblot immunoassay of purified preparations of strains NY15 (1), NY15 lysyl endopeptidase treated (2), NL1 (3), NL3 (4) of bean common mosaic virus, strain W (5) of blackeye cowpea mosaic virus, strain B 25 (6) of bean yellow mosaic virus and strain N of potato virus Y reacted with antiserum to intact particles (a), antibodies directed to N- and C-termini (b) and to N-termini (c) of coat protein of NY15. Values to the left are M_r (x10 ^{3}) of marker proteins in the first lane.

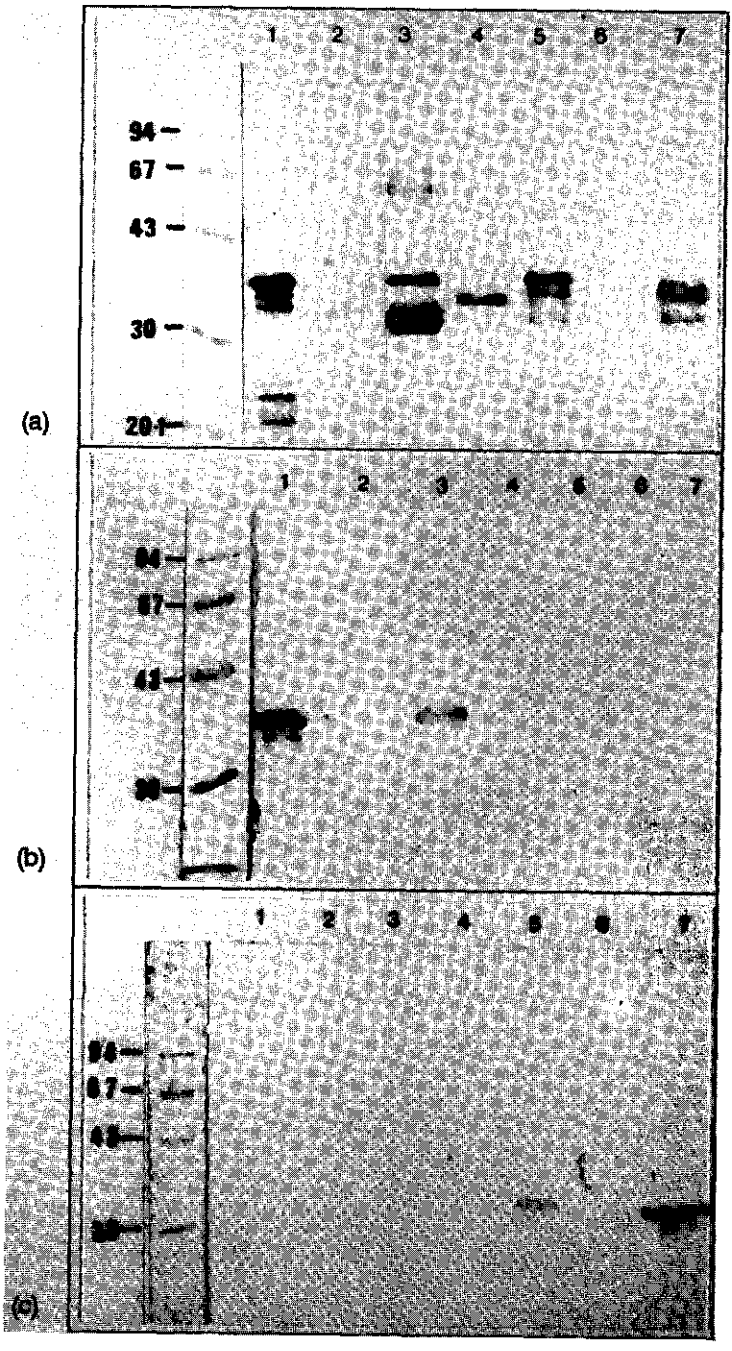

Fig. 6. Electroblot immunoassay of purified preparations of strains W (1), W lysyl endopeptidase-treated (2) of blackeye cowpea mosaic virus, strains NL1 (3), NL3 (4) and NY15 (5) of bean common mosaic virus, strain B 25 (6) of bean yellow mosaic virus and strain N (7) of potato virus Y reacted with antiserum to intact particles (a), antibodies directed to N- and C-termini (b) and to N-termini (c) of coat protein of W. Values to the left are M_r (x 10^3) of marker proteins.

ELISA.

The results obtained in ELISA correlated well with the EBIA results, except in the case of antibodies to the N-termini of NY15 which reacted much less strongly with NL1 in ELISA than in EBIA (Table 1) . However, in EBIA there is always a possibility of difference in loading leading to different reaction intensities.

Table 1. *Reactions of strains NL1, NL3 and NY15 of bean common mosaic virus and strain W of blackeye cowpea mosaic virus to homologous and heterologous antibodies directed towards N-terminal peptide regions of their coat proteins in direct ELISA.*

Antigens Antisera	NL1	NL3	NY15	W
NL1	100	0	48	41
NL3	0	100	0	0
NY15	34	0	100	0
W	48	0	0	100

* A_{405} as a percentage of that of the homologous reaction. Values are the means of three experiments.

Discussion

Previous serological studies, using conventional antisera in both ELISA and SDS-immunodiffusion tests, indicated that strains NL1 and NY15 were closely related to one another and to W, whereas NL3 was only distantly related to these three strains. Hence, NL1, NL3, NY15 and W could not be distinguished clearly from each other using conventional serology (Lana *et al.*, 1988).

By using antibodies specific to the N-terminal peptide regions of the coat proteins, a clear distinction between NL3, NY15 and W has now become possible. The present results offer good perspectives for both applied and fundamental research in which distinction of strains of BlCMV and BCMV is essential. Only NL1 cross-reacted with both W and NY15, but not with NL3. The exclusive reaction of N-terminal specific antibodies to NL3 with itself and with NL5, belonging to the same pathogenicity group as NL3, but not with any of the mosaic-inducing strains of BCMV (NL1, NY15) confirms earlier findings of the special position of this strain.

The question now arises whether the differences in N-terminal peptide regions between NL3, NY15 and W, and the similarities in N-terminal peptide regions between NL1 on the one hand, and NY15 and W on the other, have implications for the taxonomic positions of these strains and viruses. According to Shukla and Ward (1989 *a*) and Shukla *et al.* (1989 *b*), in general, viruses reacting with each other's antibodies to N-termini are strains of one virus, whereas those which do not react are considered to be different viruses. On the basis of this criterion, NL1, NY15 and W should be considered strains of one virus, whereas NL3 may be a different virus. However, it should be borne in mind that a difference in N-terminal regions of coat proteins need not be the sole criterion for classification of potyviruses. For instance clover yellow vein virus and BYMY, biologically and biophysically distinct potyviruses, were found to share N-terminal epitopes (L. Bos, personal communication 1990) and there are more cases where distinction on the basis of N-termini does not work, because of unexpected paired serological relationships (Shukla & Ward, 1989 *b*). Besides coat protein properties, nucleotide sequence information of other parts of the potyviral genome, such as that of the 3'-untranslated region (Frenkel, Ward & Shukla, 1989) may be equally important for classification.

Acknowledgements

The authors are very grateful to Dr Dick Peters for his valuable help with injection of rabbits. We also thank Dr L. Bos, Research Institute for Plant Protection (IPO-DLO) and Dr M. Fortass, Department of Plant Pathology, Meknes, Morocco, for helpful discussions during the meetings of the Potyvirus Working Group at Wageningen. The viruses (NL1, NL3 and W) were kindly supplied by Dr L.Bos.

References

Clark, M.F. & Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses.*Journal of General Virology* 34: 475-483.

Dougherty, W.G., Willis, L. & Johnston, R.E. (1985). Topographic analysis of tobacco etch virus capsid proteins epitopes. *Virology* **144**: 66-72.

Drijfhout, E. (1978). Genetic interaction between *Phaseolus vulgaris* and bean common mosaic virus with implications for strain identification and breeding for resistance. *Agricultural Research Reports* 872 (*Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands*), 98 pp.

Frenkel, M.J., Ward,C.W. & Shukla, D.D. (1989). The use of 3' non-coding nucleotide sequences in the taxonomy of potyviruses: Application to water melon mosaic virus 2 and soybean mosaic virus-N. *Journal of General Virology* **70**: 2775-2783.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680-685.

Lana, A.F., Lohuis, H., Bos, L. & Dijkstra, J. (1988). Relationships among strains of bean common mosaic virus and blackeye cowpea mosaic virus-members of the potyvirus group. *Annals of Applied Biology*, **113**, 493-505.

Shukla, D.D., Jilka, J. Tosic, M. & Ford, R.E. (1989 *a*). A novel approach to the serology of potyviruses involving affinity-purified polyclonal antibodies directed towards virus-specific N termini of coat proteins. *Journal of General Virology* **70**: 13-23.

Shukla, D.D., Strike, P.M., Tracy, S.L., Gough, K.H. & Ward, C.W. (1988). The N and C termini of the coat proteins of potyviruses are surface-located and the N terminus contains the major virus-specific epitopes. *Journal of General Virology* **69**: 1497-1508.

Shukla, D.D., Tosic, M., Ford, R.E., Jilka, J., Toler, R.W. & Langham, M.A.C. (1989 *b*). Taxonomy of potyviruses infecting maize, sorghum and sugarcane in Australia and the United States as determined by reactivities of polyclonal antibodies directed towards virus- specific N-termini of coat proteins. *Phytopathology* **79**: 223-229.

Shukla, D.D. & Ward, C.W. (1989 *a*). Structure of potyvirus coat proteins 2 and its application in the taxonomy of the potyvirus group. *Advances in Virus Research* 36: 273-314.

Shukla, D.D. & Ward, C.W. (1989 *b*). Identification and classification of potyviruses on the basis of coat protein sequence data and serology. *Archives of Virology* 106: 171-200.

Towbin, H., Staehelin, G. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Science of the USA* **76**: 4350-4354.

Chapter 4

A proposal for a bean common mosaic subgroup of potyviruses

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This chapter has been published in a slightly modified version as : Dijkstra, J. & Khan, J.A. (1992). A proposal for a bean common mosaic virus subgroup of potyviruses. Archives of Virology (Suppl 5): 389-395.

Summary

In order to elucidate the taxonomic positions of bean common mosaic virus (BCMV) and blackeye cowpea mosaic virus (BlCMV), several strains of these viruses were compared on the basis of host ranges, antigenic properties, established with antisera to virions and to the N-terminal peptide domains of their coat proteins, and high performance liquid chromatographic peptide profiles. The comparison includes three strains of BCMV, viz. NL1, NL3 and NY15, four strains of BlCMV, viz. Fla, Ind, NR and W, and a Moroccan isolate (Mor) of cowpea aphid-borne mosaic virus (CAMV), formerly designated as BlCMV-Mor. Based on these parameters, Fla, NR and W are strains of one virus, whereas NL3, Ind and Mor (and possibly NL1, NY15) are separate viruses. In view of these characteristics which allow similar viruses to be separated, we propose that these viruses be included in a bean common mosaic subgroup of the genus *Potyvirus*.

Introduction

A cluster composed of viruses closely related to bean yellow mosaic virus was proposed by Randles *et al.* (1980). This idea has been expanded in a number of subsequent studies and a bean yellow mosaic subgroup of potyviruses accepted. Uyeda (1992) found a sequence common to viruses in this subgroup.

The genus *Potyvirus* contains a large number member and possible member viruses. While subgroups have no formal taxonomic status with the International Committee on Taxonomy of Viruses, a subgroup makes dealing with closely related viruses easier. For some purposes, diagnosis to the subgroup level will be enough. Once a diagnosis to the subgroup level is made, other diagnostic techniques can be used to differentiate individual species and strains. We are proposing a bean common mosaic subgroup here.

Bean common mosaic virus (BCMV) and blackeye cowpea mosaic virus (BlCMV) are two major viruses of French bean (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*), respectively, each with a large number of strains. The BCMV strains have been distinguished mainly on the basis of their genetic interaction with cultivars of

French bean (Drijfhout, 1978), and host ranges and serological properties (Lana *et al.*, 1988; Wang, 1983; Wang, 1985).

Strains of BCMV have been arranged in three main groups according to symptoms in bean cultivars: *(i)* strains that never induce systemic necrosis, but mosaic; *(ii)* strains that never induce systemic necrosis in cultivars of some **II** resistance groups, according to temperature (temperature-dependent, necrosis-inducing strains); *(iii)* strains inducing local and systemic necrosis at all temperature in **II** genotypes susceptible to the strain concerned (temperature-independent, necrosis-inducing strains), Drijfhout (1978). Also, serologically groups 1 and 2 on the one hand and group 3 on the other hand, can be distinguished and have been designated serotype B and A, respectively (Wang, 1983, 1985).

Distinctions between BlCMV strains are also based on host ranges and antigenic properties (Dijkstra *et al.*, 1987; Lana *et al.*, 1988; Taiwo *et al.*, 1982). However, a lack of clear differences between these viruses and their strains has made it obvious that their taxonomic status could not be determined by biological and conventional serological criteria alone (Lana *et al.*,1988; Shukla *et al.*, 1990).

Recently, more information on the structure of their coat protein has led to a better distinction between the different strains. Use of antibodies to the N-terminal parts of coat proteins makes it possible to distinguish between a number of strains of BCMV and BlCMV (Khan *et al.*,1990). Peptide profiling of coat protein tryptic digests by high performance liquid chromatography (HPLC) has also contributed to further classification of strains of both BCMV and BlCMV (McKern *et al.*, 1992; D.D. Shukla, personal communication).

To assign a definite taxonomic status to a virus species, knowledge of complete nucleotide sequence of its genome is desirable. In the absence of the latter, a combination of other parameters, such as host ranges, conventional serology with antibodies to virions, N-terminal serology and HPLC might be used to distinguish between the viruses and their strains. On the basis of these parameters, an effort is made in this paper to tentatively classify the BCMV strains NL1, NY15, (both belonging to serotype B), NL3 (a serotype A strain), the BlCMV strains Fla, Ind, NR, W and the

Moroccan isolate (Mor) of cowpea aphid-borne mosaic virus (CAMV), earlier described as BlCMV-Mor (Dijkstra *et al.*, 1987; Lana *et al*; 1988) and henceforth referred to as Mor.

Parameters:

Host range

All the bean cultivars in Table 1 were infected by NL3 and most of them by NL1 and NY15. Resulting infection was usually systemic, with or without symptoms. Strain W

Table 1. *Reactions of bean cultivars to bean common mosaic virus (BCMV) strains NL1, NL3, NY15 and to blackeye cowpea mosaic virus (BlCMV) strains Fla, Ind, NR, W and cowpea aphid-borne mosaic virus (CAMV) isolate Morocco (Mor).*

Host group[a]	Differential bean cultivar	Strains of BCMV			Strains of BlCMV				Mor
		NL1	NL3	NY15	Fla	Ind	NR	W	
1.	Dubbele Witte	S	S	S	S	S,SN	–	s,S	L
	Stringless Green Refugee	S	S	S	.	.	.	L	.
	Bountiful	S	S	S	.	.	.	S	.
	Saxa	S	S,SN	S	S	S,SN	S	L	L
2.	Redlands Greenleaf C	L	s,S	S	.	.	.	L	.
	Puregold Wax	–	s,S	S	.	.	.	L	.
	Imuna	L,s	s,S	–	.	.	.	–	.
	Bataaf	S	S,SN	S	L	L	L	L	L
3.	Redlands Greenleaf B	L,s	S	L	.	.	.	L	.
	Great Northern UI 123	L,s	s	–	.	.	.	–	.
4.	Sanilac	L	S	S	.	.	.	–	.
	Michelite	s,S	S	S	.	.	.	L	.
	Red Mexican UI 34	L,s	S	S	.	.	.	L	.
5.	Pinto	L,s	S	S	L	L	L	L	L
6.	Monroe	L,s	L	L	.	.	.	L	.
	Great Northern UI 31	L	s	L,s	.	.	.	–	.
	Red Mexican UI 35	L	S	L	.	.	.	L	.
8.	Widusa	–,s	SN	–	.	.	.	–	.
	Black Turtle Soup	–	SN	–	.	.	.	–	.
9a.	Jubila	–	SN	L	.	.	.	–	.
9b.	Topcrop	s,S	SN	–	.	.	.	–	.
	Improved Tendergreen	–	SN	–	.	.	.	–	.

Adapted from Dijkstra *et al.* (1987) and Lana *et al.* (1988); [a] host group based on resistance to BCMV

L = local symptoms; s = symptomless infection or very weak symptoms;
S = systemic, non-necrotic symptoms; – = no infection;
SN = systemic, necrotic symptoms; . = not tested

induced mostly local symptoms or did not infect some cultivars at all. This response proved true for Mor, as well as Fla, Ind, and NR, but the number of bean cultivars tested with these strains was too small to justify such a conclusion.

The cowpea genotypes inoculated with NL1 and NL3 were either uninfected or had mostly symptomless infection. NY15 induced clear mosaic symptoms in 'California Blackeye' and a number of TVu lines, as did all the BlCMV strains and Mor (Table 2).

Of the non-legumes, *Chenopodium amaranticolor* and *C. quinoa* were not infected by NL1 and NL3, but reacted with local lesions after infection with NY15, all BlCMV strains and Mor. *Nicotiana benthamiana* was symptomlessly infected by NL1, not infected by NL3, but showed symptoms with NY15, all strains of BlCMV and also Mor.

Table 2. *Reactions of cowpea cultivars and lines to bean common mosaic virus (BCMV) strains NL1, NL3, NY15, and to blackeye cowpea mosaic virus (BlCMV) strains Fla, Ind, NR, W and cowpea aphid-borne mosaic virus (CAMV) isolate Morocco (Mor).*

Cowpea cultivars and lines	Strains of BCMV			Strains of BlCMV				Mor
	NL1	NL3	NY15	Fla	Ind	NR	W	
California Blackeye	S	–	S	S	S	S	S	S
Early Red	L	–	–	–	–	–	–	S
IITA TVu 196	I	–	S	S	s,S	S	S	s,S
IITA TVu 401	–	–	s	–,S	–,S	–,S	–,S	–,S
IITA TVu 1582	s	s	s,S	S	s,S	S	S	–
IITA TVu 1593	s	s	s,S	–,S	L,s	S	S	–
IITA TVu 2460	–	–	–	.	SN	.	S	.
IITA TVu 2657	s	–	–	–	s,S	–	–,S	–,S
IITA TVu 2740	s	–	–	–,S	I,L	–	–,S	s
IITA TVu 2845	S	S	S	S	S	S	S	S
IITA TVu 3270	–	–	–	–,S	S	–	–,S	S
IITA TVu 3433	s	s	–	S	–	–,S	–	S

Adapted from Dijkstra *et al.* (1987) and Lana *et al.* (1988).
I = symptomless local infection. For other legends, see Table 1

Conventional serology

Homologous reactions among NL1, NY15, Fla, NR, and W were recorded in reciprocal SDS-immunodiffusion tests, but not in direct DAS-ELISA. The relationship between NL3, Ind, and Mor, as opposed to the other strains of BCMV and BlCMV, was non-reciprocal (Table 3).

Table 3. *Reactions of bean common mosaic virus (BCMV) strains NL1, NL3, NY15 and of blackeye cowpea mosaic virus (BlCMV) strains Fla, Ind, NR, W and cowpea aphid-borne mosaic virus (CAMV) isolate Morocco (Mor) to homologous and heterologous antisera in SDS-immunodiffusion tests (D) and direct ELISA (EL).*

	Antigens															
	Strains of BCMV						Strains of BlCMV									
	NL1		NL3		NY15		Fla		Ind		NR		W		Mor	
Antisera	D	EL[a]	D	EL	D	EL	D	EL	D	EL	D	EL	D	EL	D	EL
NL1	+++	3	++	3	+++	3	+++	2	+++	1	+++	2	+++	2	+++	2
NL3	++	1	+++	3	++	1	++	1	++	1	++	1	++	1	++	1
NY15	+++	2	+	2	+++	2	+++	2	++	1	+++	2	+++	2	++	2
Fla	+++	2	–	1	++	2	+++	3	++	1	+++	3	+++	3	+++	3
Ind	+	1	+	1	+	1	++	1	+++	3	++	1	++	1	++	1
NR	+++	3	++	3	++	3	+++	3	+	2	+++	3	+++	3	+++	3
W	++	3	++	2	++	3	+++	3	++	1	+++	3	+++	3	+++	3
Mor	+	1	–	1	+	1	+	1	+	1	+	1	+	1	+++	3

Adapted from Lana *et al.* (1988)

[a] Absorbance values at 405 nm as percentage of that of the homologous reaction arranged in three groups, viz. 1 (1-15%), 2 (15-50%), 3 (50-100%).

+ + + = reaction of homology or identity; + = weak heterologous reaction with spur formation;
+ + = strong heterologous reaction with spur formation; - = no reaction

N-terminal peptide domains of the coat proteins

When using N-terminal specific antibodies in electroblot immunoassay and direct DAS-ELISA , NL1 reacted reciprocally with NY15 and W, but NY15 and W did not react

with each other's antiserum, and NL3 showed reactions only with homologous antiserum (Khan *et al.*, 1990). Mor also did not react with any of the N-terminal specific antibodies to the other strains (the reciprocal test has not been carried out).

High performance liquid chromatography

It has been reported that the coat proteins of Fla and W had similar amino acid compositions (McKern *et al.*, 1991). The peptide profiles and amino acid compositions of some peptides of BCMV and BlCMV strains revealed a great similarity between NY15, Fla and W; the peptide profiles of NL1, NL3 and Mor differed greatly from each other and from NY15, Fla and W (D.D. Shukla, personal communication).

Generalizations

Results from use of these four parameters allow the following generalizations. Strains NL1, NL3 and NY15 usually induce distinct systemic symptoms in susceptible bean cultivars and latent infections in a number of cowpea genotypes. However, NY15 causes mosaic symptoms in the latter, thus resembling in this respect Fla, Ind, NR, W and Mor. In SDS-immunodiffusion tests and ELISA, NL1 and NY15 are closely related to each other, and to Fla, NR and W, but there is a non-reciprocal relationship to NL3, Ind and Mor. In N-terminal serology, NL1 and W cross-react with each other, but not with NL3 and Mor. However, there is no reaction between NY15 and W. HPLC results show great similarity between NY15, Fla and W, but not between these strains and NL1, NL3 and Mor, and also not among the latter three themselves.

Conclusions

The above findings suggest Fla, NR, and W are strains of one virus and NL3, Ind, and Mor need designations of their own. In conventional and N-terminal serology NL1 resembles NY15 and W, but not in HPLC analysis. On the basis of conventional serology and HPLC profiles of coat proteins, NY15 and W are strains of one virus, but not in N-terminal serology. Therefore, for the time being, it is advisable to also give both NL1 and NY15 taxonomic positions of their own. Since these viruses are closely related

biologically and by serological, chemical and physical properties and yet can be distinguished, we propose that these viruses be placed in a subgroup of the potyvirus genus. While a subgroup has no official taxonomic status, its use recognizes the similarities among the viruses so grouped.

References

Dijkstra, J. Bos, L., Bouwmeester, H.J., Hadiastono, T. & Lohuis, H. (1987). Identification of blackeye cowpea mosaic virus from germplasm of yard-long bean and from soybean, and the relationship between cowpea mosaic virus and cowpea aphid-borne mosaic virus. *Netherland Journal of Plant Pathology*, 93: 115-133.

Drijfhout, E. (1978). Genetic interaction between *Phaseolus vulgaris* and bean common mosaic virus with implications for strain identification and breeding for resistance. *Agricultural Research Reports 872 (Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands)*: 98 pp.

Khan, J.A., Lohuis, H., Goldbach, R.W. & Dijkstra, J. (1990). Distinction of strains of bean common mosaic virus and blackeye cowpea mosaic virus using antibodies to N- and C- or N-terminal peptide domains of coat protein. *Annals of Applied Biology*, 117: 583-593.

Lana, A.F., Lohuis, H., Bos, L. & Dijkstra, J. (1988). Relationships among strain strains of bean common mosaic virus and blackeye cowpea mosaic virus -members of the potyvirus group. *Annals of Applied Biology*, 113: 493-505.

McKern, N.M., Shukla, D.D., Barnett, O.W., Vetten, H.J., Dijkstra, J., Whittaker, L.A. & Ward, C.W. (1991). Structural properties of coat proteins suggest that azuki bean mosaic virus, blackeye cowpea mosaic virus, peanut stripe virus and three potyvirus isolates from soybean are all strains of the same potyvirus. *Intervirology*, 33:121-134.

Randles, J.W., Davies, C., Gibbs, A.J. & Hatta, T.(1980). Amino composition of capsid protein as a taxonomic criterion for classifying the atypical strain S strain of bean yellow mosaic virus. *Australian Journal of Biological Sciences*, 33: 245-254.

Shukla, D.D., McKern, N.M., Barnett, O.W.& Ward, C.W. (1990). Identification and classification of potyviruses infecting tropical legumes. *Abstract No. W87-8, VIIIth International Congress of Virology, Berlin*, August 26-31.

Taiwo, M.A. & Gonsalves, D. (1982). Serological grouping of isolates of blackeye cowpea mosaic virus and cowpea aphid-borne mosaic viruses. *Phytopathology*, 72: 583-589.

Taiwo, M.A., Gonsalves, D., Provvidenti, R. & Thurston, H.D. (1982). Partial characterization and grouping of isolates of blackeye cowpea mosaic and cowpea aphid-borne mosaic viruses. *Phytopathology*, 72: 590-596.

Uyeda, I. (1992). Bean yellow mosaic virus search for the group specific sequences in the 3'terminal region of the genome. *Archives of Virology* (Suppl 5): 377-385.

Wang, W-Y. (1983). Serology of bean common mosaic virus strains. *M.Sc. Thesis*, Washington State University, Pullman, Washington.

Wang, W-Y. (1985). Production and characterization of hybridoma cell lines and a broad spectrum monoclonal antibody against bean common mosaic virus. *Ph.D. Thesis*, Washington State University, Pullman, Washington.

Chapter 5

Sequence data to settle the taxonomic position of bean common mosaic virus and blackeye cowpea mosaic virus isolates

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This chapter will be published in a slightly modified version as: Khan *et al.* (1993). Sequence data to settle the taxonomic position of bean common mosaic virus and blackeye cowpea mosaic virus isolates. Journal of General Virology **74**: (in press).

Summary

The nucleotide sequences of the coat protein genes and 3'-nontranslated regions (3'-NTR) of three isolates of bean common mosaic virus (NL1, NL3 and NY15) and one isolate of blackeye cowpea mosaic virus (W) were determined. Comparison of these sequences revealed that the coat proteins of NL1, NY15 and W were identical in size (287 amino acids) and exhibited an overall sequence similarity (94-97%) and (84-98%) in their N-terminal regions. Furthermore, their 3'-NTRs were very similar in length (253-256 nucleotides) and sequence (93-96% similarity). In contrast, the coat protein of NL3 had only 261 amino acids and showed 87-89% similarity with NL1, NY15 and W while its N-terminal region revealed only 46 to 61% similarity. Also the 3'-NTR of NL3 displayed appreciable differences, both in length (240 nts) and sequence (56-63% similarity). These results, in combination with earlier serological findings, justify the conclusion that NL1, NY15 and W are considered strains of the same virus, viz. bean common mosaic virus and NL3 a strain of a different potyvirus for which the name "bean black root virus" is proposed.

Introduction

Bean common mosaic virus (BCMV) and blackeye cowpea mosaic virus (BlCMV), both members of the genus Potyvirus, family Potyviridae, (Barnett, 1991), have been informally placed in the bean common mosaic virus subgroup (Dijkstra & Khan, 1992; McKern *et al.*, 1992 a; Mink & Silbernagel, 1992). In spite of their economic importance, the taxonomic status of BCMV and BlCMV has so far remained unclear. Classical taxonomic parameters such as biological properties and conventional serology have not led to a proper distinction of strains or species within the BCMV subgroup. In the past, the main reason for classifying BCMV and BlCMV as two distinct viruses was the pathogenicity of BCMV to bean and that of BlCMV to cowpea (Drijfhout, 1978; Lana *et al.*, 1988). Mink and Silbernagel (1992) using polyclonal and monoclonal antibodies stated that inspite of all the work done in several laboratories, confusion still exists in labelling

isolates or strains within azuki bean mosaic virus-BCMV-BlCMV-cowpea aphid-borne mosaic virus cluster of viruses.

Recently, the increased knowledge of coat protein structure has contributed greatly in differentiating potyviruses and their strains properly. Extensive comparisons of a large number of potyviruses have revealed that distinct potyviruses show 38 to 71% (average 54%) sequence identity in their coat proteins, whereas this identity is more than 90% among strains belonging to the same virus. Especially, the N-terminal domain of the coat protein differs markedly between individual viruses, while the central core and C-terminal domain are highly conserved (Shukla & Ward, 1988; Shukla & Ward, 1989). Furthermore, it has been shown that the 3'-NTRs of different potyviruses also display a high degree of sequence variation (homology of 39 to 53% only) whereas this sequence is highly conserved between virus strains (homology 83% and more) (Frenkel *et al.*, 1989). With N-terminal targeted serology it has been possible to distinguish some strains of BCMV and BlCMV (Khan *et al.*, 1990). Using antibodies directed towards N-terminal peptide domains of the coat proteins in ELISA and EBIA, BCMV-NL1 reacted with antibodies to BCMV-NY15 and BlCMV-W, whereas BCMV-NY15 did not react with those to BlCMV-W or *vice versa*. Furthermore , BCMV-NL3 reacted with none of the antisera to the isolates tested except with that to BCMV-NL5. Therefore, judging from the N-terminal domains of the coat proteins, BCMV-NL3, BCMV-NY15 and BlCMV-W should all be regarded as different viruses (Shukla *et al.*, 1989; Shukla & Ward, 1989). However, on the basis of High Performance Liquid Chromatography (HPLC) profiles of digested coat proteins it was concluded that BCMV-NL1, BCMV-NY15 and BlCMV-W are strains of one virus, whereas BCMV-NL3 is a strain of another virus (McKern *et al.*, 1992 a,b).

In view of these conflicting results the sequences of the coat protein genes and 3'-NTRs of BCMV strains NL1, NL3 and NY15 and BlCMV strain W have now been determined. Their implications for the taxonomic status of these viruses and their strains are discussed.

Material and methods

Virus purification and RNA extraction. The BCMV strains NL1, NL3, NY15 (henceforth referred to as NL1, NL3, NY15) were isolated from leaves of infected *Phaseolus vulgaris* and BlCMV-W (denoted as W) from *Nicotiana benthamiana*, as described earlier (Khan *et al.*, 1990). RNA was extracted by incubation with 1% SDS followed by phenol/chloroform extraction and subsequent ethanol precipitation (Maniatis *et al.*, 1982).

Primer design. Initially, potyvirus group specific degenerate primers U1000, positioned in the polymerase gene, U341 and D341 positioned in the core of the coat protein gene (Langeveld *et al.*, 1991), kindly supplied by Dr Simon Langeveld, were used to determine the sequences from the core and polymerase of NL1, NL3 and NY15. Subsequent designs were based on determined partial sequences (Table 1).

Amplification of coat protein genes including 3'-NTRs of NL1, NL3 and NY15 by polymerase chain reaction (PCR). First strand cDNA synthesis was performed using 0.5 μg viral RNA and oligo(dT)$_{12\text{-}18}$ as primer. For amplification of cDNA representing the part of 3'-NTR, the C-terminal region and core of coat protein, primer U341 and oligo(dT) were used. For amplification of cDNA representing the N-terminal region, core of coat protein and part of polymerase gene, primers DW48 and UW19 were used. PCR was performed using 5 μl of first strand DNA synthesised from viral RNA, 50 pmol of each primer and 1 U super Taq Polymerase. Thirty five reaction cycles were performed with periods of 1 min 30 sec for annealing at 50°C, 1 min 30 sec for synthesis at 72°C and 1 min for melting at 94°C. Samples (5μl) were analysed by agarose gel electrophoresis.

Cloning and nucleotide sequencing of PCR-amplified fragments. PCR-amplified DNA fragments of NL1, NL3 and NY 15 were resolved on 1% agarose gels and subsequently isolated by freeze-squeeze (Tautz & Rez, 1983). The fragments were ligated in a T-vector (Marchuk *et al.*, 1990) and transformed into E. coli DH5 α cells. Recombinants were

selected and verified by restriction enzyme analysis. Sequence analysis was performed by the dideoxy nucleotide chain termination method (Sanger *et al.*, 1977) using a PCR-Kit (New England Biolabs). For sequencing, universal primers M13, Blue script KS/SK or internal synthetic oligonucleotides complementary to previously described sequences were used. The nucleotide sequence was determined several times for two independent clones from each of two PCR reactions.

cDNA synthesis, cloning and sequencing of isolate W. First strand cDNA to BlCMV-W was primed with oligo $(dT)_{12\text{-}18}$. Second stranded synthesis was performed according to the method of Gubler and Hoffman (1983). Double-stranded cDNA was made blunt-ended using T4 DNA polymerase and subsequently cloned into Sma I site of blue script SK^+ (Stratagene). Following transformation into *E.coli* DH5 α cells, recombinants were selected from filter replicas using radiolabelled (^{32}P ATP) first strand DNA as a probe. Two clones, pWCP-13 and pWCP-8 containing inserts of 1.3 Kb and 0.8 Kb, respectively, were selected for the purpose of sequencing. Sequencing was done as described above.

Comparison of sequences. The nucleotide sequences of the protein genes, their deduced amino acids and the nucleotide sequence data of 3'-NTRs of all four isolates were compiled, analysed and the level of sequence relatedness was compared using the GCG programme package from the University of Wisconsin (Devereux *et al.*, 1984) and where necessary they were aligned manually.

Results

Amplification and sequence analysis of coat protein genes and 3'-NTRs of NL1, NL3, NY15 and W.

The oligonucleotide sequences and positions of downstream and upstream primers are shown in Table 1. Primers UW48 and DW19 amplified DNA fragments of about 600

Table 1. *Oligonucleotide primer sequences*

Primer *	Sequence	+ Position
UW 18	5'CGGACTACTTCGGAATTTG 3'	609-625
DW 19	5'CTTGCTCATCTCCATCCATC 3'	459-478
UW 48	5'CAGCACTTAAAAATCTTTACAC 3'	
DW 49	5'CCTTTCACCATGGGCAAGTTC 3'	294-314
U 341	5'CCGGAATTCATGRTITGGTGYATIGAIAAYGG 3'	477-501
D 341	5'CGCGGATCCGCIGYYTTCATYTGIRIIWKIGC 3'	697-722
U 1000	5'ACIGTIGTIGAYAAYWSIYAGGG 3'	

* U and D are upstream and downstream primers,respectively ; + Positions of the primers is based on nucleotide sequence of BCMV-NY15

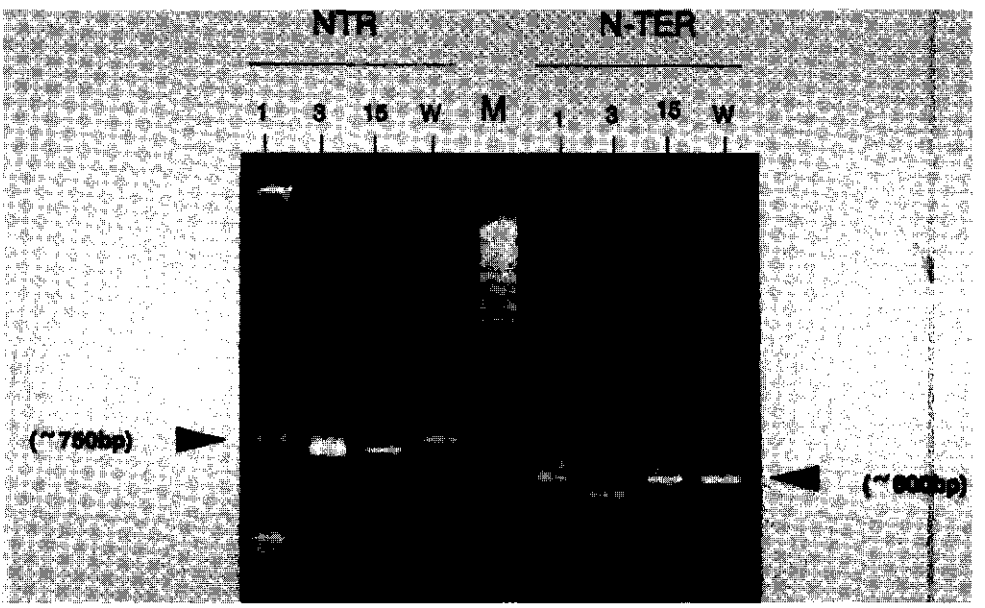

Fig. 1 PCR amplification of fragments of BCMV-NL1 (1), -NL3 (3), -NY15 (15) and BICMV-W (W) containing their 3'-nontranslated (NTR) and the coat protein N-terminal (N-TER) regions. A 5 µl aliquot was analysed on agarose gel containing ethidium bromide.

base pairs long covering part of the polymerase gene and the N-terminal half of the coat protein gene. Primers U341 and oligo-$dT_{12\text{-}18}$ amplified DNA fragments of about 750 base pairs long covering the C-terminal half of the coat protein gene and the 3'-NTR (Fig.1 and 2).

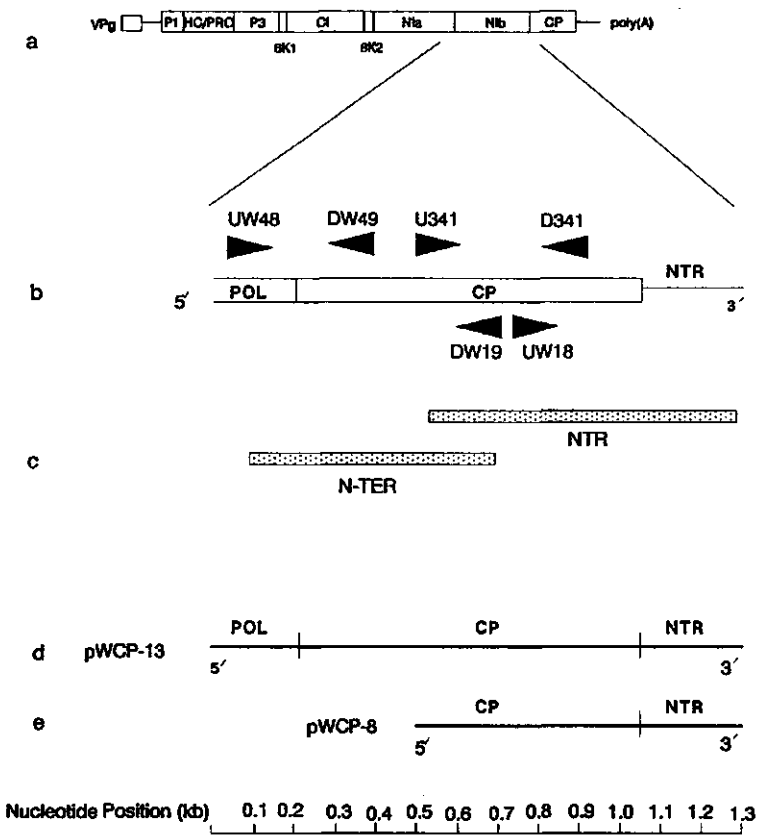

Fig. 2 General potyviral genome map (a); positions of the different PCR primers are shown by arrows (b); N-TER is PCR amplified DNA fragment containing part of the polymerase, the N-terminal region and the core of coat protein and NTR is PCR amplified fragment containing part of the core, the C-terminal region of coat protein and the 3'-nontranslated regions of BCMV-NL1, -NL3, -NY15 and BlCMV-W (c); cDNA clones pWCP-13 and pWCP-8 used to determine the nucleotide sequence of part of the polymerase gene, the coat protein gene and the 3'-nontranslated regions of BlCMV-W are shown in (d) and (e).

After cloning of the various PCR fragments into the T-vector, sequence analysis was performed on isolates NL1, NL3, NY15 and W, according to dideoxy chain termination method (Sanger *et al.*, 1977). The sequence data revealed long continuous open reading frames in all the four viral sequences, terminating with a stop codon TAA at position 944 (isolates NL1, NY15, W) or at position 866 (isolate NL3) shown in Fig. 3-6. The open

reading frames were followed by 3'-NTRs of 253, 240, 256 and 254 nucleotides for NL1, NL3, NY15 and W, respectively (excluding poly (A)-tail).

Comparison of coat protein sequences.

From the nucleotide sequences, the amino acid sequences were predicted. The putative proteolytic cleavage site (ESVXXQ/S) between the NIb protein and the coat protein (Fig. 3-6) was in agreement with molecular weight determinations and sequence comparisons with other isolates of BCMV (Vetten *et al.*, 1992). Fig. 7 shows the multiple alignment of the predicted amino acid sequence data of the coat proteins of NL1, NL3, NY15, and W. The coat proteins of NL1, NY15 and W isolates were identical in size (287 aa), while that of NL3 was shorter (261 aa).

Among isolates NL1, NY15, and W, the N-terminal region of the coat proteins appeared highly conserved, both in length (52 aa) and in sequence (similarity 84-98%). In contrast, the N-terminal domain of NL3 was significantly shorter (26 aa) and had a distinct sequence (similarity to NL1, NY15 and W only 46-61%). Furthermore, the core and C-terminal regions of NL1, NL3, NY15 and W coat proteins were all fairly conserved in length and in sequence (Fig. 7, Table 2). Thus, the overall sequence similarity of the coat proteins of NL1, NY15 and W was quite high (94-97%) and comparable to identities observed between strains of a single potyvirus species (Shukla & Ward, 1988; Ward *et al.*, 1992). On the other hand, since the N-terminal part of the NL3 coat protein showed only limited similarity with those of NL1, NY15 and W, this isolate may be considered a distinct virus (Table 2).

Reference has to be made here regarding the presence of a conserved motif DAG in the N-terminal region of potyviral coat proteins required for aphid transmission (Atreya *et al.*, 1990; Harrison & Robinson, 1988). This motif was present in NL3 and W but absent in NL1 and NY15. Interestingly, NL3 and W were aphid-transmissible while, NL1 and NY15 were not (results not shown). Most probably, NL1 and NY15 lost the aphid-transmissibility due to long maintenance in the laboratory by many serial mechanical inoculations.

```
  K   I   E   E   L   A   K   Y   L   E   V   L   D   F   D   Y   E
AAG ATT GAA GAG TTA GCG AAA TAT CTG GAA GTG CTC GAC TTT GAC TAC GAG
  V   G   C   G   E   S   V   H   L   Q ↓ S   G   P   G   Q   P   Q
GTA GGA TGC GGA GAA TCT GTG CAC CTA CAA TCA GGA CCT GGA CAG CCA CAG
  P   P   I   V   D   A   S   V   G   S   G   K   D   K   K   E   K
CCA CCA ATA GTA GAT GCT AGT GTG GGA TCT GGG AAG GAC AAG AAA GAG AAA
  S   C   K   G   K   D   Q   E   S   R   E   V   A   G   D   N   N
AGC TGC AAA GGA AAG GAC CAA GAA AGT AGG GAA GTG GCA GGA GAC AAC AAC
  R   G   A   G   D   S   A   M   R   D   K   D   V   N   A   G   S
CGT GGT GCA GGG GAT TCG GCA ATG AGA GAC AAG GAT GTG AAT GCA GGT TCC
  K   G   K   V   V   P   R   L   Q   K   I   T   K   R   M   N   L
AAA GGG AAG GTT GTT CCT CGG CTC CAA AAG ATC ACA AAA AGG ATG AAT TTG
  P   M   V   K   G   N   V   I   L   N   L   D   H   L   L   D   Y
CCC ATG GTG AAA GGG AAT GTA ATC TTA AAT TTA GAT CAT CTA TTG GAT TAC
  K   P   E   Q   T   D   L   F   N   T   R   A   T   K   M   Q   F
AAG CCA GAA CAA ACT GAT CTT TTT AAC ACA AGG GCA ACA AAG ATG CAG TTT
  E   M   W   Y   N   A   G   K   A   E   Y   E   I   D   D   D   Q
GAA ATG TGG TAC AAT GCT GGT AAG GCT GAG TAT GAG ATA GAT GAT GAT CAG
  M   S   I   V   M   N   G   F   M   V   W   C   I   D   N   G   T
ATG TCA ATT GTA ATG AAT GGC TTT ATG GTG TGG TGT ATT GAC AAT GGC ACT
  S   P   D   V   N   G   T   W   V   M   M   D   G   D   E   Q   V
TCA CCA GAT GTG AAC GGT ACA TGG GTG ATG ATG GAT GGA GAT GAG CAA GTT
  E   Y   P   L   K   P   M   V   E   N   A   K   Q   T   L   R   Q
GAA TAC CCA CTC AAA CCA ATG GTT GAA AAT GCA AAG CAA ACA CTC CGC CAA
  I   M   H   H   F   S   D   A   A   E   A   Y   I   E   M   R   N
ATC ATG CAC CAT TTT TCA GAT GCA GCT GAA GCA TAC ATT GAG ATG AGA AAC
  S   E   R   P   Y   M   P   R   Y   G   L   L   R   N   L   R   D
TCT GAA AGA CCG TAC ATG CCT AGG TAC GGA CTA CTT CGG AAT TTG AGG GAC
  K   N   L   A   R   Y   V   F   D   F   Y   E   V   T   S   K   T
AAA AAT CTA GCT CGC TAC GTT TTT GAT TTC TAT GAA GTA ACA TCC AAA ACA
  S   D   R   A   R   E   A   V   A   Q   M   K   A   V   A   L   S
TCG GAT CGA GCA AGA GAA GCA GTA GCA CAG ATG AAG GCA GTA GCC CTC AGC
  N   V   S   S   K   L   F   G   L   D   G   N   V   A   T   T   S
AAC GTT AGC AGC AAG TTG TTT GGA CTT GAT GGT AAC GTT GCT ACA ACC AGC
  E   N   T   E   R   H   T   A   R   D   V   N   Q   N   M   H   T
GAG AAT ACT GAA AGG CAC ACT GCA AGG GAC GTC AAT CAG AAC ATG CAC ACA
  L   L   G   M   G   P   P   Q
CTT CTT GGC ATG GGT CCT CCG CAG*  TAAAGGTTGGGTAAACTGACCACAAGTTAGCATC
TGGTCGCTGAATAGTTTCATATAGTAATCTTTTATGTTCTCTTTAGTTTCAGTGTGGTTTTACCACCT
TTGTGTTACTATTGTGATAGCGTGGTTGGTCCACCACATATTGTGAGTACTTTATGTTTATGAGTAAG
CCGGAAGAACCATTGCAATGGTGAGGACATGCAGAGTGATTTCATCACGCTCATGAGGTAGCTACGGC
AATGTTTGTTGTTCC
```

Fig. 3 Nucleotide sequence of the 3'-terminal end of strain NL1 of BCMV. The predicted amino acid sequence is shown above the nucleotide sequence. The putative cleavage of NIa protease is shown in bolds and the actual cleavage site is shown by an arrow (↓). Asterisks (*) indicate a stop codon.

```
  K   M   E   E   M   Q   E   Y   L   K   Q   F   K   F   N   S   N
AAA ATG GAG GAA ATG CAA GAG TAC CTA AAA CAG TTT AAA TTT AAT TCT AAT
  D   E   V   Y   E   S   V   S   T   Q ↓ S   S   K   K   E   E   G
GAT GAG GTG TAT GAA TCC GTG TCA ACA CAA TCC AGC AAG AAA GAA GAA GGG
  K   D   A   G   A   D   E   R   E   K   D   K   G   K   G   P   A
AAA GAC GCT GGG GCC GAT GAG AGA GAG AAG GAC AAA GGC AAA GGC CCA GCG
  D   K   D   V   G   A   G   S   K   G   K   V   V   P   R   L   Q
GAT AAA GAC GTT GGA GCT GGT TCA AAA GGA AAA GTA GTC CCA AGA TTG CAG
  K   I   T   K   K   M   N   L   P   M   V   G   G   R   M   I   L
AAA ATC ACA AAA AAG ATG AAC TTG CCT ATG GTT GGC GGT AGG ATG ATT CTA
  N   L   D   H   L   I   E   Y   K   P   Q   Q   T   D   L   Y   N
AAC TTG GAC CAC CTA ATT GAG TAC AAA CCG CAG CAG ACG GAC TTG TAC AAC
  T   R   A   T   K   A   Q   F   E   R   W   Y   E   A   V   K   T
ACA AGA GCT ACC AAG GCA CAA TTT GAA AGA TGG TAC GAA GCA GTC AAG ACT
  E   Y   E   L   N   D   Q   Q   M   G   V   V   M   N   G   F   M
GAA TAT GAG CTT AAT GAC CAG CAA ATG GGA GTA GTA ATG AAT GGC TTC ATG
  V   W   C   I   D   N   G   T   S   P   D   V   N   G   V   W   V
GTG TGG TGC ATC GAT AAT GGA ACA TCT CCC GAT GTG AAT GGA GTG TGG GTG
  M   M   D   G   D   E   Q   I   E   Y   P   L   K   P   M   V   E
ATG ATG GAT GGA GAT GAG CAA ATA GAA TAC CCA TTG AAG CCA ATG GTT GAG
  N   A   K   P   T   L   R   Q   V   M   H   H   F   S   D   A   A
AAT GCA AAG CCT ACA CTC CGC CAA GTT ATG CAT CAT TTT TCA GAT GCA GCG
  E   A   Y   I   E   M   R   N   S   E   G   F   Y   M   P   R   Y
GAG GCT TAT ATA GAG ATG AGG AAC TCT GAA GGG TCC TAC ATG CCT AGG TAT
  G   L   L   R   N   L   R   D   K   I   L   A   R   Y   A   F   D
GGA CTT CTT CGA AAT TTG AGG GAT AAA ATC CTA GCT CGC TAT GCA TTC GAT
  F   Y   E   V   N   S   K   T   S   D   R   A   R   E   A   V   A
TTC TAT GAG GTT AAC TCA AAA ACA TCG GAT AGA GCC AGA GAA GCG GTT GCT
  Q   M   K   A   A   A   L   S   N   V   N   T   R   L   F   G   L
CAG ATG AAA GCG GCC GCC CTC AGC AAC GTT AAC ACT AGA TTG TTT GGT CTA
  P   G   N   V   A   T   T   S   E   N   T   E   R   H   T   A   R
GAT GGT AAC GTG GCA ACA ACC AGC GAG AAT ACT GAA AGG CAC ACT GCA CGG
  D   V   N   Q   N   M   H   H   L   L   G   M   T   S   G   Q   *
GAC GTC AAT CAA AAC ATG CAT CAT TTG CTT GGT ATG ACT TCT GGG CAG TAA
AGGAGTGGGACAACCCTCTACAGTTAGCATCTCGCGTTCATAGTTTTCTGTATTAGATAGTACGCTT
CAATTCCAGTGTGGTTATACCACCTTGTGTCTATGTAAGTTAGAGAGGCTATGCCACCAGTATGTTA
TGCTATTTCAGTTTATGCGAGCAGGAGGAGCCATTCCAACACCGGAGCTGCCAGTGTGGTTGTATCA
TGAGTG CTGTCCGAGGTGCGGGTATGAATATTCTCC
```

Fig. 4 Nucleotide sequence of the 3'-terminal end of strain NL3 of BCMV. The predicted amino acid sequence is shown above the nucleotide sequence. The putative cleavage of NIa protease is shown in bolds and the actual cleavage site is shown by an arrow (↓). Asterisks (*) indicate a stop codon.

```
  K   I   E   E   L   A   K   Y   L   E   V   L   D   F   D   Y   E
AAG ATT GAA GAG TTA GCG AAG TAT CTG GAA GTG CTC GAC TTT GAC TAC GAG
  V   G   C   G   E   S   V   H   L   Q ↓ S   G   P   G   Q   P   Q
GTA GGA TGC GGA GAA TCG GTG CAC CTA CAA TCA GGA CCA GGA CAG CCA CAG
  P   P   I   V   D   A   S   V   G   S   G   K   D   K   K   E   K
CCA CCA ATA GTA GAT GCT AGT GTG GAA TCT GGG AAG GAC AAG AAA GAG AAA
  S   C   K   G   K   D   Q   E   S   R   E   G   A   G   N   N   N
AGC TGC AAA GGA AAG GAC CAA GAA AGT AGG GAA GGG GCA GGA AAC AAC AAC
  R   G   A   G   D   S   A   M   R   D   K   D   V   N   A   G   S
CGT GGT GCA GGG GAT TCG GCA ATG AGA GAC AAG GAT GTG AAT GCA GGT TCC
  K   G   K   V   V   P   R   L   Q   K   I   T   K   R   M   N   L
AAA GGG AAG GTT GTT CCT CGT CTT CAA AAG ATC ACA AAA AGG ATG AAT TTG
  P   M   V   K   G   N   V   I   L   N   L   D   H   L   L   D   Y
CCC ATG GTG AAA GGG AAT GTG ATC TTA AAT TTA GAT CAT CTA TTG GAT TAC
  K   P   E   Q   T   D   L   F   N   T   R   A   T   K   M   Q   F
AAG CCA GAA CAA ACT GAT CTT TTT AAC ACA CGG GCA ACA AAG ATG CAG TTT
  E   M   R   Y   N   A   V   K   A   E   Y   E   I   D   D   D   Q
GAA ATG CGG TAC AAT GCT GTG AAG GGT GAG TAT GAG ATA GAT GAT GAT CAG
  M   S   I   V   M   N   G   F   M   V   W   C   I   D   N   G   T
ATG TCA ATT GTA ATG AAC GGA TTC ATG GTG TGG TGC ATT GAC AAC GGC ACT
  S   P   D   V   N   G   T   W   V   M   M   D   G   D   E   Q   V
TCA CCA GAT GTG AAC GGC ACT TGG GTA ATG ATG GAT GGA GAT GAG CAA GTG
  E   Y   P   L   K   P   M   V   E   N   A   K   P   T   L   R   Q
GAA TAT CCA CTT AAA CCA ATG GTT GAA AAT GCA AAA CCA ACA CTC CGC CAA
  I   M   L   H   F   S   D   A   A   E   A   Y   I   E   M   R   N
ATC ATG CTC CAT TTT TCA GAT GCA GCT GAA GCA TAC ATT GAG ATG AGA AAC
  S   E   S   A   Y   M   P   R   Y   G   L   L   R   N   L   R   D
TCT GAG AGC GCG TAT ATG CCT AGG TAC GGA CTA CTT CGG AAT TTG AGG GAC
  K   N   L   A   R   Y   V   F   D   F   Y   E   V   T   S   K   T
AAA AAT CTA GCT CGC TAC GTT TTT GAT TTC TAT GAA GTA ACA TCC AAA ACA
  S   D   R   A   R   E   A   V   A   Q   M   K   A   A   A   L   S
TCG GAT CGA GCA AGA GAA GCA GTA GCA CAG ATG AAG GCA GCA GCC CTC AGC
  N   V   S   S   R   L   F   G   L   D   G   N   V   A   T   T   S
AAC GTT AGC AGC AGG TTG TTT GGA CTT GAT GGT AAC GTG GCA ACA ACC AGC
  E   N   T   E   R   H   T   A   R   D   V   N   Q   N   M   H   T
GAG AAT ACT GAA AGG CAC ACT GCA AGG GAC GTC AAT CAG AAC ATG CAC ACA
  L   L   G   M   G   P   P   Q
CTT CTT GGC ATG GGT CCT CCG CAG   *TAAAGGTTGAGGTAAACTGACCACAGTTAGCAT
CTCGCGTCGCTGAATAGTTTCATATAGTAATCTTTTATGTTCTCTTTAGTTTCAGTGTGGTTCTACCA
CCTTTGTGTACTATTGTGATAGTGTGGCTGGTCCACCAACATAGTGTGAGTACTTTATGTTTATGAGT
AAGCCGGAAGAACCATTGCAATGGTGAGGACATGCAGAGTGATTTCATCACGCGTCATGGGGTAGCTA
CGGCAATGTTTGTTGTTCC
```

Fig. 5 Nucleotide sequence of the 3'-terminal end of strain NY15 of BCMV. The predicted amino acid sequence is shown above the nucleotide sequence. The putative cleavage of NIa protease is shown in bolds and the actual cleavage site is shown by an arrow (↓). Asterisks (*) indicate a stop codon.

```
  K   I   E   E   L   A   K   Y   L   E   V   F   D   F   D   Y   E
AAG ATT GAA GAG TTA GCC AAA TAT CTG GAA|GTG TTT GAC TTT GAC TAT GAT
  V   G   C   G   R   S   V   H   L   Q ↓ S   G   T   G   Q   P   Q
GTA GGA TGC GGA GAA TCT GTG CAC CTA CAA TCT GGA ACT GGA CAG CCG CAA
  P   P   I   V   D   A   D   V   G   A   G   K   D   K   K   E   R
CCA CCA ATA GTG GAT GCT GGT GTG GAT GCT GGA AAG GAC AAG AGA GAG AGA
  S   N   R   G   K   D   P   E   S   R   E   G   S   G   N   N   N
AGC AAT AGA GGA AAA GAC CCT GAA AGC AGG GAG GGG TCA GTA AAC AAC AAC
  R   G   A   G   D   S   T   M   R   D   K   D   V   N   A   G   S
CGT GGT GCA GGG GAT TCA ACA ATG AGA GAC AAG GAT GTG AAC GCA GGC TCC
  K   G   K   V   V   P   R   L   Q   K   I   T   K   R   M   N   L
AGG GGA AAA GTT GTC CCG CGG CTT CAA AAG ATC ACA AAA AGG ATG AAC TTG
  P   M   V   K   G   N   V   I   L   N   L   D   H   L   L   D   Y
CCC ATG GTG AAA GGG AAT GTG ATT TTA AAT CTA GAT CAT CTG TTG GAT TAC
  K   P   E   Q   T   D   L   F   N   T   R   A   T   K   D   Q   F
AAG CCA GTT CAA ACT GAT CTT TTT AAC ACA AGA GCA ACA AGG GAC CAG TTT
  E   M   W   Y   N   A   G   K   A   E   Y   E   I   D   D   D   Q
GAA ATG TGG TAC AAT GCT GGT AAG GGC GAG TAT GAA ATA GAT GAT GAT CAG
  M   S   I   V   M   N   G   F   M   V   W   C   I   D   N   G   T
ATG TCA ATT GTA ATG AAC GGA TTC ATG GTG TGG TGT ATT GAC AAT GGC ACT
  S   P   D   V   N   G   T   W   V   M   M   D   G   D   E   Q   V
CCG CCA GAT GTG AAT GGT ACA TGG GTG ATG ATG GAT GGA GAT GAG CAA GTT
  E   Y   P   L   K   P   M   V   E   N   A   K   P   T   L   R   Q
GAA TAC CCA CTC AAA CCA ATG GTT GAA AAT GCA AAG CCA ACA CTC CGT CAA
  I   M   H   H   F   S   D   A   A   E   A   Y   I   E   M   R   N
ATC ATG CAC CAT TTC TCA GAT GCA GCT GAA GCA TAC ATT GAG ATG AGA AAT
  S   E   R   P   Y   M   P   R   Y   G   L   L   R   N   L   R   D
TCC GAA AGG CCG TAC ATG CCT AGG TAC GGA CTA CTT CGG AAT TTG AGG GAC
  K   N   L   A   R   Y   A   F   D   F   Y   E   V   T   S   K   T
AAA AAT CTA GCT CGC TAC GCT TTT GAT TTC TAT GAG GTG ACA TCA AAA ACA
  S   D   R   A   R   E   A   V   A   Q   M   K   A   V   A   L   S
TCG GAT CGA GCC AGA GAA GCA GTA GCA CAG ATG AAG GCA GTA GCC CTC AGC
  N   V   S   S   K   L   F   G   L   D   G   N   V   A   T   T   S
AAC GTT AGC AGC AAG TTG TTT GGA CTT GAC GGT AAT GTT GCA ACA ACC AGC
  E   N   T   E   R   H   T   A   R   D   V   N   Q   N   M   H   T
GAG AAT ACT GAA AGG CAC ACT GCA AGG GAC GTC AAC CAA AAC ATG CAC ACA
  L   L   G   M   G   S   P   Q
CTT CTT GGC ATG GGT TCT CCG CAG* TAAAGGTTGGGTAAACTGACCACAGTTAGCATCT
CGCGTCGCTGAATAATTTCATATAGTAATCTTTATGTTCTCTTTAGTTTCAGTGTGGTTTTACCACC
TTTGTGTTACTATTGTGATAGCGTGGTTAGTCCACCAACATATTGTGAGTACTTTATGTTTATGAGT
AAGCCGGAAGAACCATTGCAATGGTGAGGGCATGCAGAGTGATTTGATCATGTGTCATGAAGTAGCT
ACGGCAATGTTTGTTGTT
```

Fig. 6 Nucleotide sequence of the 3'-terminal end of strain W of BlCMV. The predicted amino acid sequence is shown above the nucleotide sequence. The putative cleavage of NIa protease is shown in bolds and the actual cleavage site is shown by an arrow (↓). Asterisks (*) indicate a stop codon.

```
          1              polymerase                    N-terminus      47
NL1       KIEELAK  YLEVLDFDYE   VGCGESVHLQ   SGPGQPQPPI  VDASVGSGKD
NY15      -------  ----------   ----------   ----------  -----e----
W         -------  ----f----d   ----------   --t-------  ---g-da---
NL3       -m--mqe  --kqfk-nsn   devy---st-   -s...kkeeg  k--g....a-

          48                                              core    97
NL1       KKEKSCKGKD QESREVAGDN NRGAGDSAMR  DK DVNAGSKG  KVVPRLQKIT
NY15      ---------- -----g--n- ----------  --  --------  ----------
W         -r-r-nr--- p----gsvn- -------t--  --  ------r-  ----------
NL3       erek.d---g pa........ ..........  --  --g-----  ----------

          98                                                     147
NL1       KRMNLPMVKGN  VILNLDHLLD YKPEQTDLFN  TRATKMQFEM  WYNAGKAEYE
NY15      -----------  ---------- ----------  ----------  r---v-g---
W         -----------  ---------- ---f------  -----d----  w---v-g---
NL3       -k------g-r  m-------ie ---q----y-  -----a---r  w-e-v-t---

          148                                                    197
NL1       IDDDQMSIVM   NGFMVWCIDN GTSPDVNGTW   VMMDGDEQVE  YPLKPMVENA
NY15      ----------   ---------- ----------   ----------  ----------
W         ----------   ---------- ----------   ----------  ----------
NL3       ln-q--gv--   ---------- ---------v-   --------i-  ----------

          198                                                    247
NL1       KQTLRQIMHH   FSDAAEAYIE   MRNSERPYMP   RYGLLRNLRD  KNLARYVFDF
NY15      -p------l-   ----------   -----sa---   ----------  ----------
W         -p------h-   ----------   -----rp---   ----------  ------a---
NL3       -p----v-h-   ----------   -----gf---   ----------  -i----a---

          248                    core                              297
NL1       YEVTSKTSDR AREAVAQMKA VALSNVSSKL FGLDGNVATT SENTERH  TAR
NY15      ---------- ---------- a-------r- ---------- -------    ---
W         ---------- ---------- a-------k- ---------- -------    ---
NL3       ---n------ ---------- a-----ntr- ---------- -------    ---

          298 C-terminus 313
NL1       DVNQNMHTLL  GMGPPQ
NY15      ----------  ------
W         ----------  ---s--
NL3       -------h--  --tsg-
```

Fig.7 Comparison of amino acid sequences of coat proteins of BCMV-NL1,-NL3,-NY15 and BICMV-W. Homologous amino acids are shown by dashes and gaps are indicated by dots.The putative cleavage site is indicated by the vertical arrow. The locations of the N and C termini of the coat proteins are predicted trypsin-sensitive sites.

Table 2. *Percent amino acid sequence identity and nucleotide sequence identity between the coat proteins and 3'-nontranslated regions of NL1,NL3,NY15 and W.*

STRAINS	NL1	NL3	NY15	W
NL1	-	*53	98	84
		┼87	97	94
		§91	97	97
NL3	#74	-	46	61
	$56		89	89
			92	92
NY15	96	74	-	86
	95	63		95
				97
W	90	75	90	-
	96	61	93	

above the diagonal: amino acid sequence similarity between * = the N-terminal domains, ┼ = the whole coat proteins and § = the cores; below the diagonal: nucleotide sequence similarity between the # = coat protein genes, $ = the 3'-nontranslated regions.

Comparison of the 3'-NTRs.

The 3'-NTRs of NL1, NY15 and W were all similar in length (253, 256, 254 nts, respectively) whereas that of NL3 was only 240 nts long (Fig. 3-6). For strains NL1, NY15 and W the sequence similarities in this region ranged from 93-96% (Table 2). This high percentage was in line with the high sequence identity of their coat proteins. However, the 3'-NTR of the isolate NL3, revealed a lower similarity (56-63%) with those of the other three isolates.

Discussion

The availability of the sequences of both the coat proteins and 3'-NTRs of potyvirus isolates NL1, NL3, NY15 and W allows a reliable establishment of their taxonomic status. The sequence homology found between both the coat protein genes and the 3'-NTRs of NL1, NY15 and W suggests that these isolates are strains of the same potyvirus. The coat proteins of NL1, NY15 and W are identical in size (287 aa), and display high sequence similarity. Furthermore, the number and sequence of nucleotides of their 3'-NTRs are almost identical. The coat protein sequence of NL3 shows an overall sequence similarity of 87-89% with those of NL1, NY15 and W, values which lie between those of different species (38-71%) and strains of the same species (more than 90%). However, most of the sequence differences between NL3 and the other isolates are located in the N-terminal region (sequence similarity 46-61%). Furthermore, both the sequence and size of the 3'-NTR of NL3 differ greatly from those of NL1, NY15 and W, indicating that NL3 should be considered a distinct virus. In a sequence comparison of complete coat proteins of NL1, NY15 and W with that of isolates of peanut stripe virus (serologically closely related to BCMV isolates) and BCMV-NL4, similarity of 92-95% was found and also their 3'-NTRs showed similarity of more than 85-98% (McKern *et al.*, 1991; Vetten *et al.*, 1992; Cassidy *et al.*, 1993). Furthermore, NL3 showed high sequence similarities (98 and 92%) at coat protein and 3'-NTR levels, respectively, with the sequence of BCMV-NL8 (Vetten *et al.*,1992).

The present results corroborate earlier findings that NL3 differs greatly in biological properties from the other BCMV-strains and that NL1, NY15 and W are rather closely related. According to the classification of Drijfhout (1978), NL3 belongs, together with NL5 and NL8, to the pathogenicity group of BCMV strains which cause systemic necrosis (black root) in certain bean cultivars.

Remarkably, the above assignments also correlate well with previous information on serological and other coat protein properties of strains of these viruses (Mink & Silbernagel, 1992; Vetten *et al.*, 1992).

Conventional serological studies showed that NL1, NY15 and W are closely related to

each other (Lana *et al.*, 1988). In contrast, NL3 showed only a distant relationship with NL1, NY15 and W. Such distant relationships may be due to the presence of antibodies directed towards the core region of the coat proteins (Shukla & Ward, 1989). Interestingly, when antibodies directed towards the N-terminal region of NL3 coat protein were used in DAS-ELISA and EBIA, they did not cross react with NL1, NY15 and W but only with the necrotic strain NL5 (Khan *et al.*, 1990).

In contrast to the results with N-terminal serology, comparison of coat protein HPLC peptide profiles revealed a great similarity between coat protein of NY15 and W suggesting they are strains of the same virus (McKern *et al.*, 1992 b). It is worth mentioning that sometimes N-terminal serology may give erratic results (Khan *et al.*, 1990; Shukla *et al.*, 1989) due to unexpected paired relationships between distinct viruses or failed cross-reactions between some strains (Shukla *et al.*, 1992). Indeed, the N-terminal regions of coat proteins of NL1, NY15 and W are highly similar but despite of this, both in DAS-ELISA and EBIA, NY15 and W did not react whereas NL1 did so with antisera to NY15 and W. Presumably, only few potential amino acid residues in an epitope are necessary for antibody binding or, alternatively, the conformation of the epitope involved is changed after removal of the N-terminal region by lysyl endopeptidase (Geysen *et al.*, 1984, 1987, 1988; Shukla *et al.*, 1989). A comparison of amino acid sequences of the N-terminal domains of NL1, NY15 and W showed that W sequence differed from the other strains by six amino acid residues (Thr_{30}, Gly_{41}, Asn_{53}, Ser_{64},Val_{68}, Thr_{75}). These differences may be responsible for the serological reaction of W with NL1, but not with NY15 (Fig. 4). Secondary structure of the N-terminal domains of all these isolates, as determined by Peptide Structure programme of GCG, showed the same folding pattern. However, striking differences were observed with secondary structure of NL3 N-terminal domain (results not shown). Epitope mapping of the mutated N-terminal regions expressed in heterologous systems may give an insight regarding the complexity of these serological results.

In summary, the following conclusions may be drawn with regard to the taxonomic position of NL1, NL3, NY15 and W. Based on sequences of both the coat protein gene and the 3'-NTR, and serological and biological properties, NL3 should no longer be

considered a strain of BCMV but a strain of different potyvirus, as suggested by Dijkstra and Khan (1992), McKern *et al.* (1992 a,b) and Vetten *et al.*(1992). For this virus, McKern *et al.* (1992 a) and Vetten *et al.* (1992) proposed the names "bean necrosis mosaic virus", and "bean necrotic mosaic virus", respectively, indicating that in some bean cultivars it induces systemic necrosis whereas in others it causes mosaic. However, we would favour a name bringing out the most characteristic feature of this virus, viz. its ability to cause severe vascular necrosis in certain bean cultivars, called black root (Grogan & Walker, 1948). We consider the combination "necrosis (necrotic) mosaic" an undesirable one as it is confusing and therefore, we propose to name this virus "bean black root virus". The fact that not all bean cultivars infected with this virus show black root disease need not be an objection. There are many examples, such as tobacco mosaic virus which does not induce mosaic in all tobacco cultivars.

We agree with others (McKern *et al.*, 1992 a,b) that BlCMV and the non-necrosis inducing strains of BCMV, such as NL1 and NY15, should be called strains of one virus, viz. BCMV. However, for relevance to applied virology the strain name must remain recognizable by plant pathologists and breeders. Following the principle of nomenclature proposed by Shukla and Ward (1989), we recommend to retain the addition "blackeye cowpea" (BlC) as a particular strain of BCMV, adding a suffix to indicate the place of isolation, characteristic symptoms on certain plants etc. For example, the present BlCMV-W would then be referred as BCMV, strain BlC/W.

Acknowledgements

We are thankful to Ir. Richard Kormelink for making the cDNA clones to BlCMV-W and to Dr Simon Langeveld for supplying the degenerate primers.

References

Atreya, C.D., Raccah, B. & Pirone, T.P. (1990). A point mutation in the coat protein abolishes aphid transmissibility of a potyvirus. *Virology* **178**: 161-165.

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

Cassidy, B., Sherwood, J.L. & Nelson, R.S. (1993). Cloning of the capsid protein gene from a blotch isolate of peanut stripe virus. *Archives of Virology* **128**: 287-293.

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

Dijkstra, J. & Khan, J.A. (1992). A proposal for a bean common mosaic subgroup of potyviruses. *Archives of Virology* (Suppl) **5**: 389-395

Drijfhout, E.(1978). Genetic interaction between *Phaseolus vulgaris* and bean common mosaic virus with implications for strain identification and breeding for resistance. Agricultural Research Reports 872. Centre for Agricultural Publishing and Documentation, Wageningen, The Netherlands, 98 pp.

Frenkel, M.J., Ward, C.W. & Shukla, D.D. (1989). The use of 3'non-coding nucleotide sequences in the taxonomy of potyviruses: application to watermelon mosaic virus 2 and soybean mosaic virus-N. *Journal of General Virology* **70**: 2775-2783.

Geysen, H.M., Mason, T.J. & Rodda, S.J. (1988). Cognitive features of continuous antigenic determinants. *Journal of Molecular Recognition* **1**: 32-42.

Geysen, H.M., Meloen, R.H. & Barteling, S.J. (1984). Use of peptide synthesis to probe viral antigens for epitopes to a resolution of single amino acids. *Proceedings of National Academy of Sciences U.S.A.* **81**: 3998-4002.

Geysen, H.M., Rodda, S.J., Mason, T.J., Tribbick, G. & Schoofs, P.G. (1987). Strategies for epitope analysis using peptide synthesis. *Journal of Immunological Methods* **102**: 259-274.

Grogan, R.G. & Walker, J.C, (1948). The relation of common mosaic to black root of bean. *Journal of Agricultural Research* **77**: 315-331.

Gubler, U. & Hoffmann, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**: 263-269.

Harrison, B.D. & Robinson, D.J. (1988). Molecular variation in vector-borne plant viruses: epidemiological significance. *Philosophical Transactions of the Royal Society of London* Series B **321**: 447-462.

Khan, J.A., Lohuis, H., Goldbach, R.W. & Dijkstra, J. (1990). Distinction of strains of bean common mosaic virus using antibodies to N- and C- or N-terminal peptide domains of coat protein. *Annals of Applied Biology* **117**: 583-593.

Lana, A.F., Lohuis. H., Bos. L. & Dijkstra. J.(1988).Relationships among strains of bean common mosaic virus and blackeye cowpea mosaic virus- members of the potyvirus group. *Annals of Applied Biology* **113**: 493-505.

Langeveld, S.A., Dore, J.M., Memelink, J., Derks, A.F.L.M., Van der Vlugt, C.I.M., Asjes, C.J. & Bol, J. (1991). Identification of potyviruses using the polymerase chain reaction with degenerate primers. *Journal of General Virology* **72**: 1531-1541.

Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982). Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, New York.

Marchuk, D., Drumm, M., Saulino, A. & Collins, F.S. (1990). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Research* **19**: 1154.

McKern, N.M., Edskes, H.K., Ward, C.W., Strike, P.M., Barnett, O.W. & Shukla, D.D. (1991). Coat protein of potyviruses 7. Amino acid sequence of peanut stripe virus. *Archives of Virology* **119**: 25-35.

McKern, N.M., Mink, G.I., Barnett, O.W., Mishra, A., Whittaker, L.A., Silbernagel, M.J., Ward, C.W. & Shukla, D.D. (1992 a). Isolates of bean common mosaic virus comprising two distinct potyviruses. *Phytopathology* **82**: 923-929.

McKern, N.M., Ward, C.W. & Shukla, D.D. (1992 b). Strains of bean common mosaic virus consists of at least two distinct potyviruses. *Archives of Virology* (Suppl) 5: 407-414.

Mink, G.I. & Silbernagel, M.J. (1992). Serological and biological relationships among viruses in the bean common mosaic virus subgroup. *Archives of Virology* (Suppl) 5: 397-406.

Sanger, F., Nicklen, S. & Coulsen, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of National Academy of Sciences U.S.A.* 74: 5463-5467.

Shukla, D.D., Jilka, J., Tosic, M. & Ford, R.E. (1989). A novel approach to the serology of potyviruses involving affinity purified polyclonal antibodies directed towards virus-specific N termini of coat proteins. *Journal of General Virology* 70: 13-23.

Shukla, D.D., Lauricella, R. & Ward, C.W. (1992). Serology of potyviruses: current problems and some solutions. *Archives of Virology* (Suppl) 5: 57-69.

Shukla, D.D. & Ward, C.W. (1988). Amino acid sequence homology of coat proteins as a basis for identification and classification of the potyvirus group. *Journal of General Virology* 69: 2703-2710.

Shukla, D.D. & Ward, C.W. (1989). Identification and classification of potyviruses on the basis of coat protein sequence data and serology. *Archives of Virology* 106: 171-200.

Tautz, D. & Renz, M. (1983). An optimized freeze-squeeze method for the recovery of DNA fragments from agarose gels. *Analytical Biochemistry* 132: 14-19.

Vetten, H.J., Lesemann, D-E. & Maiss, E. (1992). Serotype A and B strains of bean common mosaic virus are two distinct potyviruses. *Archives of Virology* (Suppl) 5: 415-431.

Ward, C.W., McKern, N.M., Frenkel, M.J. & Shukla, D.D. (1992). Sequence data as the major criterion for potyvirus classification. *Archives of Virology* (Suppl) 5: 283-297.

Chapter 6

Interference between two strains of bean common mosaic virus is accompanied by suppression of symptoms without affecting replication of the challenging virus

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This chapter will be published in a slightly modified form as: Khan *et al.* (1993). Interference between two strains of bean common mosaic virus is accompanied by suppression of symptoms without affecting replication of the challenging virus. Journal of Phytopathology (in press).

Summary

An unusual type of interference between two strains of bean common mosaic virus, viz. NY15 and NL3, infecting bean plants, was investigated. When a primary leaf was inoculated with NY15 as inducer and, one to eight days later, the opposite leaf with NL3 as challenger, the plant did not develop symptoms characteristic of NL3, i.e. systemic necrosis of the stem, in the top and the youngest trifoliolate leaves. A 7-h interval between the inoculations with inducer and challenger already sufficed to reduce the number of plants showing NL3 symptoms. Surprisingly, the amount of NL3 in the challenge-inoculated leaf was not lower than that in the control, while the amount of NY15 in the challenge-inoculated leaf was always lower than in the singly infected control. Furthermore, NY15 could not be detected in the opposite leaf before 8 days post-inoculation and appeared even later in that leaf when challenge-inoculated. Histological studies showed that NL3 appeared later in the xylem of both the petiole and stem between primary leaves and first trifoliolate leaf, as compared with the singly inoculated control.

The results suggest that suppression of NL3 symptoms by NY15 is not caused by impeding its multiplication, but by delaying the transport of NL3 to the xylem of petiole and stem.

Introduction

When a plant is infected with a given virus strain, the plant may become protected against the effects of a subsequent inoculation with another strain of the same virus. This protection is caused by different types of interactions resulting in suppression of symptoms caused by the second (challenger) virus whose replication is often strongly inhibited or completely blocked (McKinney, 1929; Price, 1940; Hamilton, 1980; Bozarth & Ford, 1988).

In a study on the interaction of two bean common mosaic virus (BCMV) strains, NY 15 and NL3 on bean plants, a clear case of interference was observed. NL3 induces a

severe systemic necrosis in *Phaseolus vulgaris* "Bataaf" bean plants. These symptoms do not develop when the plants are previously infected with the mosaic-inducing strain NY15.

To understand this type of interference, the infection events in the primary leaves of which one was challenge-inoculated, has been studied as they constitute the main source from which the whole plant becomes infected. The development of the infection could be monitored as both virus strains can be readily distinguished serologically in mixed infections by using strain-specific antibodies directed towards the N-terminal domains of their coat proteins (Khan *et al.*, 1990).

This paper describes the interaction between these BCMV strains after inoculation on primary leaves in terms of the mutual effects on symptom expression, virus multiplication and types of tissue infected.

Materials and methods

Virus strains, host and inoculation. The BCMV strains NY15 and NL3 used were serologically and symptomatologically characterized by Lana *et al.* (1988). Eight- to ten-day old plants of *Phaseolus vulgaris* "Bataaf" were mechanically inoculated on their fully developed primary leaves with crude sap made by grinding 1 g of infected leaves in 2 ml of deionized water. The plants were grown in a glasshouse in a commercial potting compost and kept at 20-30°C under supplementary illumination.

Singly inoculated plants. For studies on the course of infection, 15 plants were inoculated on one or both primary leaves with either of the two strains. Symptoms were recorded and samples of primary leaves and stem were collected for serological and histological studies at different times after inoculation.

Doubly inoculated plants. For studies on interference, in each experiment, three sets of 15 plants were used. The plants in sets 1 and 2 were first inoculated on one fully expanded primary leaf (P1) with NY15 (inducer), and in set 3 mock-inoculated with

water. After 0, 7 or 18 h, and 1, 2, 3, 4, 6 or 8 days, the opposite primary leaves (P2) were mock-inoculated with water (set 1) or inoculated with NL3 as challenger (sets 2 and 3). Symptoms were recorded and samples of primary leaves, stems and trifoliolate leaves were collected for serological analysis at different times after inoculation of the challenger.

Removal of the first inoculated (P1) leaves at different times after inoculation. To determine the rate of virus transport from the inoculated leaf to the plant parts above the primary leaves, P1 leaves of either singly or doubly inoculated plants were removed at 16, 18, 22, 48 and 72h after inoculation.

Antisera. Antibodies to purified preparations of NY15 and NL3 were raised as described by Lana *et al.* (1988), and to N-terminal peptide regions of coat proteins of NY15 and NL3 prepared as described earlier (Khan *et al.*, 1990).

Enzyme-linked immunosorbent assay (ELISA). The relative amount of virus was estimated by direct double antibody sandwich ELISA as described by Clark and Adams (1977). The concentration of the immunoglobulins used for optimum coating of the wells of the microtitre plate was 1 μg/ml (in experiments with singly inoculated plants) or 5 μg/ml (in experiments on interference). The conjugated gammaglobulins (1mg/ml) to virions and to N-terminal peptide regions of coat proteins were diluted in phosphate-buffered saline with 0.05% Tween 20 (PBS-Tween). The final dilutions were 1:1000 and 1:500, respectively.

For each treatment, one primary leaf from each of three different plants was sampled. The samples were homogenized separately in PBS-Tween in a ratio of 1:2 and the homogenates tested in dilutions x 10 and x 100 in triplicate.

In each plate, a dilution series of standard sap prepared from primary leaves infected with either NY15 or NL3 and stored in Eppendorf tubes at -20°C, was used as a reference to eliminate the effect of differences in microtitre plates by calculating a correction factor based on average optical densities of a dilution (x100 or x330) of

standard sap. Readings were made at 405 nm with a Bio-kinetics Reader EL 312 after addition of substrate at different times.

In situ detection of viral antigen. The spread of virus in the plant was studied by monitoring the virus accumulation in primary leaves and stems of singly inoculated (control) plants and doubly inoculated (challenged) plants by a tissue immunostaining technique as described by Luciano *et al.* (1989) and Lohuis and Dijkstra (1990) with some modifications. Sections of leaves and of the stem between the primary leaves and the first trifoliolate leaf, approximately 1-mm thick were cut at various times after inoculation. After fixation in cold Karnovsky fixative and rinsing with PBS-Tween (3 x 20 min) and with deionized water (2 x 10 min), the sections were decolourised in 96% ethanol for 1 h, rinsed with PBS-Tween and subsequently incubated in PBS containing 5% (w/v) Elk (nonfat dry milk) as a blocking agent, at 37°C for 1 h.

After washing, the sections were incubated with antibodies to N-terminal peptide regions of coat proteins for 1 h, rinsed in PBS-Tween for 1 h and incubated with 1 ml goat anti-rabbit (secondary) antibodies conjugated with alkaline phosphatase (Tago, U.S.A.), diluted 1:3000 in PBS-Tween. The specificity and sensitivity of this serological reaction was enhanced by incubating the sections in a suspension of gammaglobulins (1:1000) prepared from rabbit normal serum (2 µg/ml PBS-Tween) for 1 h and then with labelled secondary antibodies at 37°C for 1 h. After rinsing in PBS-Tween for 1 h, the specimens were incubated in the dark in 10 ml substrate solution consisting of a mixture of 33 µl nitroblue tetrazolium and 25 µl 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BRL). When a violet colour became visible in cells of the diseased plants, the reaction was stopped by replacing the substrate solution with 0.02 M Tris/HCl buffer (pH 7.5), containing 5 mM ethylene diamine tetra-acetic acid. The sections were mounted in a drop of deionized water and viewed in a light microscope or binocular.

Results

Symptomatology of the BCMV strains in bean plants.

The symptoms evoked by the BCMV strains NY15 and NL3 on *P. vulgaris*"Bataaf" plants can readily be distinguished after infection. Inoculation with NL3 resulted in a severe epinasty of the primary leaf three days after inoculation, while a severe stunting of the plant was observed. Top necrosis was noticed five days after inoculation while the first trifoliolate leaf wilted and started to necrotize. The inoculated primary leaf showed some veinal necrosis. Ten days after inoculation almost all plants died from a stem necrosis in late autumn and during winter, whereas in the rest of the year a percentage of 10 to 40 died. The plants which survived, recovered by forming axillary sprouts. Meanwhile, the non-inoculated opposite primary leaf (P2) also had become severely epinastic.

Three days after the inoculation of a primary leaf with NY15 a slight stunting was observed as a first symptom. The inoculated leaf showed chlorotic spots and slight epinasty one day later, followed by a veinal chlorosis in the following days. Seven days after inoculation, a mosaic appeared in the trifoliolate leaves and at 10 days an orange necrosis could sometimes be noticed on the first trifoliolate leaves; the plants were then severely stunted. A couple of days later, new sprouts were formed from buds in the axillae of primary leaves.

When both primary leaves were simultaneously inoculated with either strain, the symptoms were usually more severe. Upon inoculation with NL3 a larger percentage of plants died while the surviving ones recovered slowly. In case of NY15 a lower number of plants formed axillary buds.

Suppression of NL3 symptoms in beans pre-inoculated with NY15.

To determine the minimum time interval after which the inducer (NY15) is able to suppress the appearance of the NL3 symptoms, plants were inoculated with NL3 on P2 at 0, 7, and 18 h and 1 to 8 days after inoculation of NY15 on P1. Symptoms, characteristic of NL3 were only observed in plants which were challenge-inoculated 18h or shorter after NY15 inoculation. Already at six days after challenge inoculation all

plants in the 0-h series showed severe top necrosis, and wilting of the first trifoliolate leaf. With a time interval 7 h and 18 h, 66 and 33% of the plants developed NL3-type symptoms, respectively.

All plants challenged 1 to 8 days after inoculation of NY15 produced symptoms which resembled those induced by NY15 alone. The plants were, in general, slightly more stunted, had a more bunchy appearance and sometimes developed local, necrotic stripes and patches on the epi- and hypocotyls between 10 and 18 days after inoculation.

These results clearly show that the characteristic symptoms of NL3 are suppressed in plants challenged 1 to 8 days after inoculation with NY15.

Accumulation of virus in primary leaves after single inoculations.

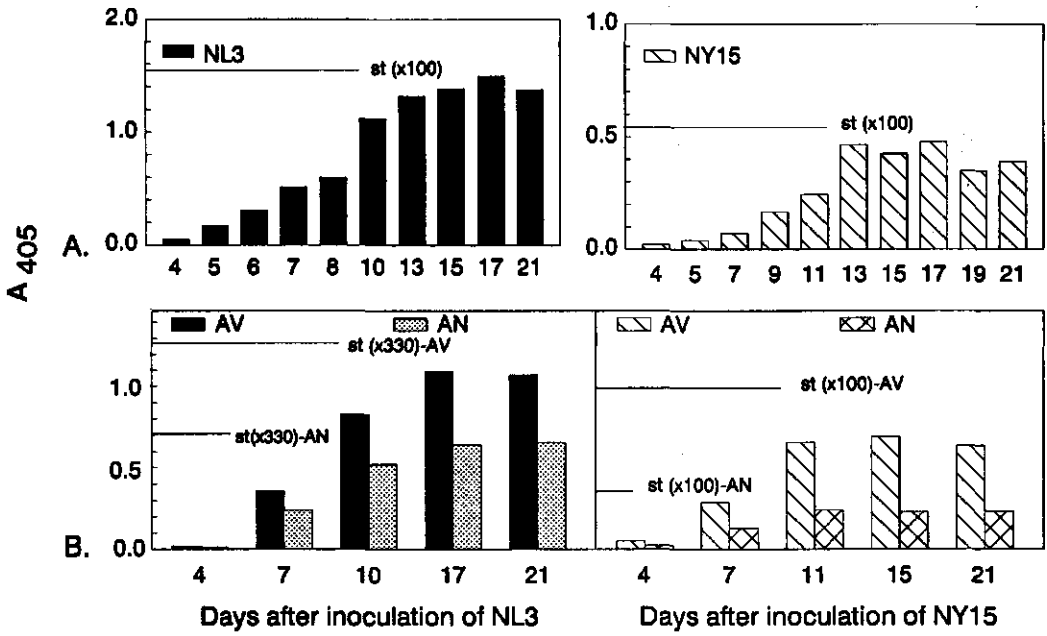

Fig. 1. Detection of BCMV-strains NY15 and NL3 by ELISA in x100 diluted sap from singly inoculated primary leaves of bean at different times after inoculation with BCMV strains NY15 and NL3, using antibodies to either intact virus (A), or using antibodies to either intact virus (AV) or N-terminal peptide regions of the coat proteins (AN) B. The absorbance values of standard sap dilutions, st(x100) and st(x330), are given.

Progeny of both virus strains could be detected as early as four days after inoculation with NY15 or NL3 in P1 leaves using either antiserum (Fig. 1 A). The amount of NY15 and of NL3 increased at similar rates and reached their maximum values approximately two weeks after inoculation. Although the responses were lower, similar results were found when antibodies to the N-terminal regions of the respective coat proteins (AN) of both strains were used (Fig. 1 B). These results show that although the reactivity was lower, the antibodies to the N-terminal region could reliably be used to detect NL3 and NY15 in challenge-inoculated plants.

Accumulation of virus in leaves of doubly inoculated bean plants.

Strikingly, both in the series with 1, 2 and 3 days interval, and in those with intervals of 4, 6 and 8 days, the amount of NL3 in the challenge-inoculated leaves was generally found to be higher than in the P2 leaves of control plants inoculated with NL3 alone (Fig. 2A and B). The latter values, however, were less reliable as they showed a great variation, probably due to the necrotic condition of the leaf. In the 6- and 8-day series, it was even impossible to sample P2 leaves 16 days after challenge inoculation, as most of them had then withered completely.

The amount of NY15 in the P2 leaves of doubly inoculated plants at most times after inoculation was often much lower than that in P2 leaves of the control plants with NY15 alone (determined only in the 1-, 2- and 3-day series) (Fig. 2B). The amount of NY15 in P1 leaves of the doubly inoculated plants did not differ significantly from that in the corresponding leaves of control plants (Fig. 2A and B).

The amount of both inducer and challenger increased with time in trifoliolate leaves. However, reliable estimates of the amounts were hard to obtain, as correct sampling of the trifoliolate leaves was difficult due to sprouting of buds in axillae of primary and secondary leaves. This led to the formation of a number of trifoliolate leaves whose positions could not be established accurately.

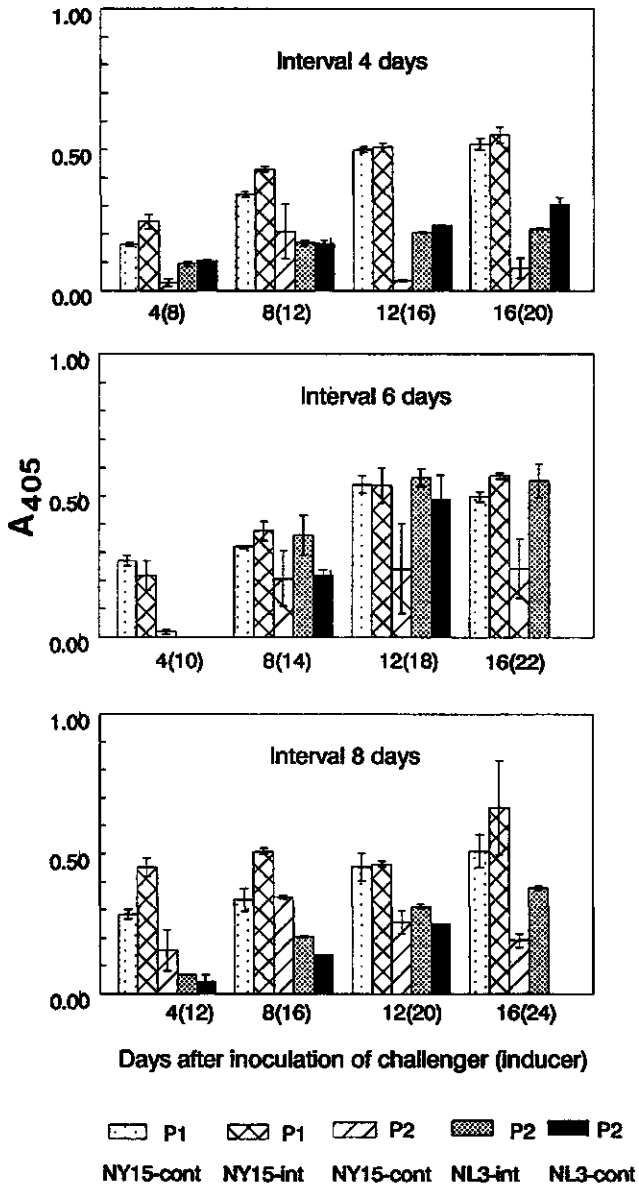

Fig 2 A. Detection of the BCMV- strains NY15 and NL3 by ELISA in primary leaves of bean plants, first inoculated on one primary leaf (P1) with NY15 as inducer and later on the opposite primary leaf (P2) with NL3 as challenger in experiments on interference (int). Primary leaves of plants inoculated with either NY15 alone on P1 or NL3 alone on P2 served as controls (cont). The intervals between the two inoculations were 4, 6, and 8 days, respectively.

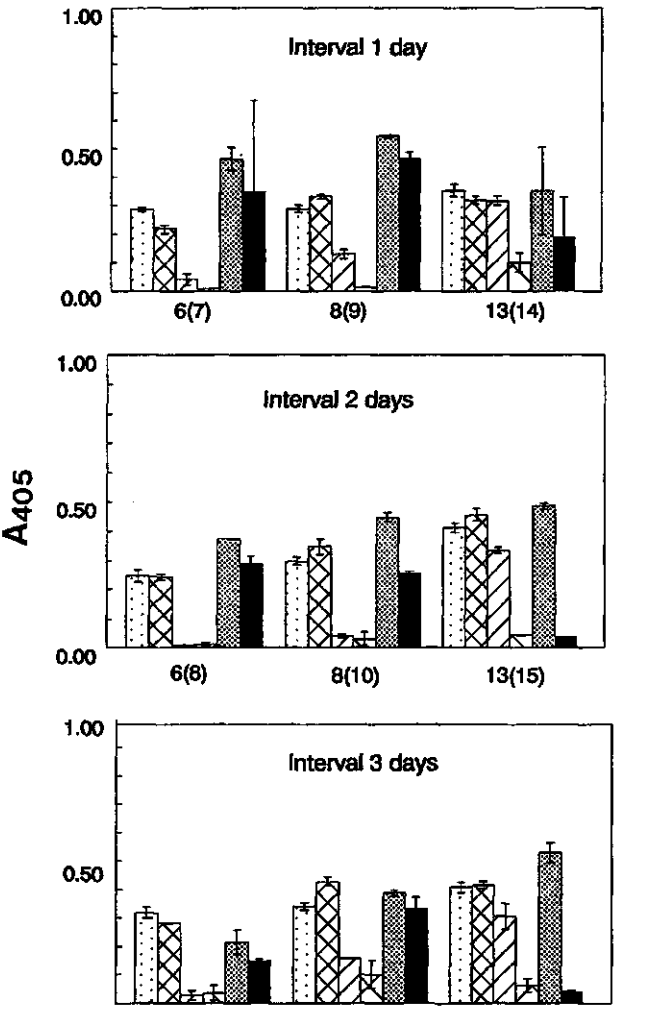

Fig 2 B. Detection of BCMV- strains NY15 and NL3 by ELISA in primary leaves of bean plants, first inoculated on one primary leaf (P1) with NY15 as inducer and later on the opposite leaf (P2) with NL3 as challenger in experiments on interference (int.). Primary leaves of plants inoculated with either NY15 alone on P1 or NL3 alone on P2 served as controls (cont). The intervals between the two inoculations were 1, 2, and 3 days, respectively.

Spread of NY 15 from inoculated P1 leaves to other plant parts and its effect on the suppression of systemic symptoms.

In order to establish whether the suppression of NL3 symptoms coincided with the spread of NY15 virus particles out of P1, primary leaves of a number of bean plants were inoculated with either NY15 or NL3 and subsequently removed at various times after inoculation. Removal of the inoculated leaf 16 or 18 h after inoculation with NY15 or NL3 prevented systemic infection. When the inoculated leaves were removed 22h after inoculation some six plants showed an NY15 infection, but none with NL3. Finally, all plants, inoculated with either NY15 or NL3, became infected when the leaves were removed 48 h after inoculation.

When the NY15 inoculated P1 leaf was removed after 48h and the opposite primary leaf inoculated with NL3, top necrosis, characteristic for a NL3 infection, was observed in 10 out of 12 plants. However, these symptoms were not observed in any plant when the P1 leaves were removed after 72 h and opposite leaves inoculated with NL3 at the same time.

This delay of NL3 symptom suppression following removal of NY15 inoculated leaves shows that a more than two day long continuous replication of NY15 in a primary leaf is necessary to suppress the typical NL3 symptoms.

Using ELISA, NY15 and NL3 could be detected in stems and top leaves, either with or without top necrosis, of plants of which the leaves were removed 48 or 72h after inoculation of the P1 leaf.

In situ localization of virus.

To investigate whether suppression of NL3 symptoms in NY15-infected plants is due to an altered distribution of NL3 throughout the plant, sections of primary leaves (inoculated with either virus), their petioles and stems were analyzed by an immuno-staining technique 4 to 8 days after the challenge inoculation. The results are visualized after conversion of the qualitative observations in numerical indices (Fig. 3). NL3 could be detected at about the same time in the phloem of midvein and petiole of mock-inoculated and doubly inoculated plants (Table 1). Remarkable differences were found

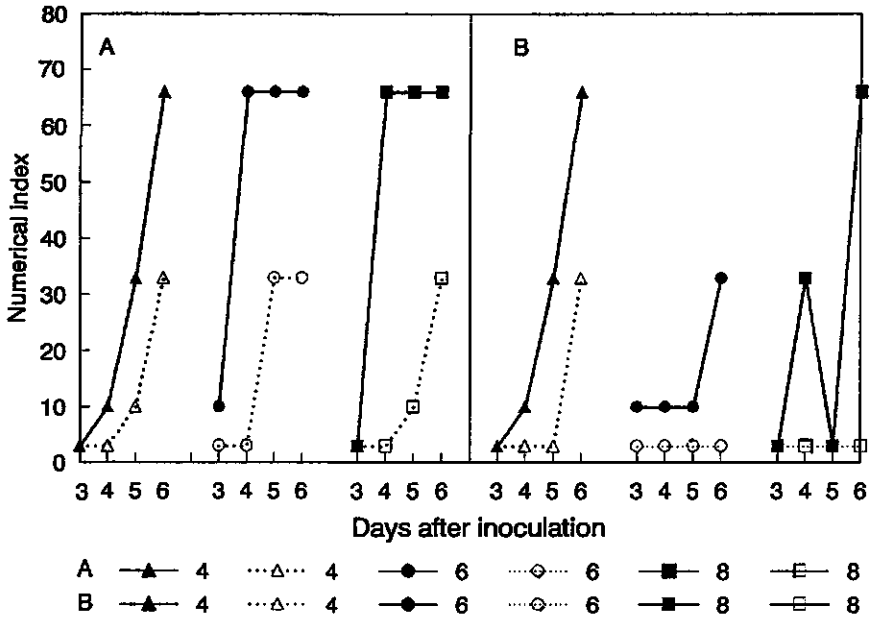

Fig. 3. Graphical representation of the qualitative observations on the occurrence of NL3 in the xylem. The indices 3, 10, 30 and 70 were respectively used to indicate that no cells with virus were found, only a few cells were infected, less than half of the cells were infected and more than half of the cells were infected. (A) refers to the xylem cells of the midvein of the inoculated leaves and (B) to those of the petiole of these leaves. The numbers 4, 6 and 8 refer to the number of days at which the tissues were studied after inoculation. The straight lines refer to NL3-inoculated leaves of control plants.

when such studies were made in the xylem (Fig. 3). NL3 could be demonstrated in the xylem of the midvein of the challenge-inoculated leaf in all three series at about 5 days after challenge-inoculation, and in that of the petiole at least 2 days later compared with the respective controls. Similarly, NL3 was not detectable at the time in the xylem tissues of the stem when it could be observed in the controls.

At the time when NY15 was detected in all tissues of the petiole of P2 leaves, the virus was also present in similar tissues of the stem above the primary leaves of doubly inoculated plants.

Table 1. *Presence of BCMV-NL3 in tissues of challenge-inoculated primary leaves of "Bataaf" bean at different times after inoculation, as determined by immunostaining. The time intervals between inoculations of one primary leaf with the inducer (BCMV-NY15) and the opposite one with challenger (BCMV-NL3) were 4, 6, and 8 days, respectively.*

Time interval (days)	Time after challenge inoculation (days)	Parts of primary leaves [1])					
		Blade			Petiole		
		Ep + mes	Midvein		Ep + cort	X	P
			X	P			
4	3	0(1)	0(0)	0(0)	0	0	0
	4	0(2)	0(1)	0(2)	0(0)	0(1)	0(0)
	5	1(3)	1(2)	1(2)	1(2)	0(2)	1(2)
	6	3(3)	2(3)	2(2)	2(3)	2(3)	2(3)
6	3	1(1)	0(1)	0(1)	0(0)	0(1)	0(1)
	4	2(2)	0(3)	1(1)	0(0)	0(1)	1(1)
	5	2(2)	2(3)	2(1)	0(0)	0(1)	3(1)
	6	1	1	2	0	0(2)	2(2)
8	3	0	0	0	0(0)	0	0
	4	2(2)	0(3)	2(3)	0	0(2)	1(2)
	5	2(2)	1(3)	2(3)	2(2)	0	1
	6	2(2)	2(3)	3(3)	2(2)	0(3)	1(3)

[1]) All observations given in parentheses refer to NL3-inoculated leaves of control plants.
Ep + cort = epidermis + cortex P = Phloem
Ep + mes = epidermis + mesophyl X = xylem
0 = no cells stained; 2 = less than half the number of cells stained
1 = some cells stained; 3 = more than half the number of cells stained

Discussion

This study shows that the BCMV strain NY15 was able to suppress the development of NL3 symptoms in *Phaseolus vulgaris* cv. Bataaf after superinoculating the plant within a day with NL3 on the opposite primary leaf. The suppression of symptoms may be explained by a delayed appearance of the NL3 in the xylem of challenge-inoculated plants. The earlier presence of NY15 may block some sites or mechanisms which may hinder NL3 to develop its adverse effects on the plant. The amount of NL3 in the inoculated primary leaves of control plants appeared to be somewhat lower than in the challenged (Fig. 2). This demonstrates that the inducer does not have an adverse effect on the accumulation of NL3 in the challenge-inoculated leaf. This observation indicates also that symptom suppression is based on another mechanism rather than blocking or inhibition of the replication of the challenging virus. Unexpectedly, except that the NY15 could be detected later in the challenge-inoculated leaf as compared with the controls, the final level of NY15 remained also somewhat lower.

The interference described is characterized by suppression of the symptoms caused by the challenger after short intervals between the inducing and challenging inoculations, while the replication of the challenger is not affected. This phenomenon deviates from other types of interference generated by two related viruses in plants, such as the well described phenomenon of cross-protection and those described by Cassells and Herrick (1977), Nagy and Gáborjány (1991). The former authors showed that the protection generated by a mild strain of tobacco mosaic virus in tomato breaks down as soon as a severe strain starts to replicate in the top leaves in a later stage of infection. The latter authors reported a complete protection between B and H strains of barley yellow stripe mosaic virus. However, when strain W of this virus was used as an inducer, symptoms of the challenger strain B were prevented but it accumulated to a lesser extent.

The effect of NY15 inoculation on development of NL3 symptoms was already perceptible at intervals of 7 and 18 h. Removal of the inducer-inoculated leaf at different times after inoculation delayed the suppression of the NL3 symptoms. The results obtained show that suppression of NL3 symptoms occurred at a higher rate when the

primary leaf inoculated with NY15 remained on the plant, than when it was removed within a few days post inoculation. This may be explained either by the loss of a source from which virus is continuously supplied to other parts of the plant or by a lower replication rate of the virus in the other parts of the plant. These results also demonstrate that the suppression is not caused by a component which is induced during the infection and transported out of the primary leaf prior to translocation of the virus.

Invasion of the plant by NY15 results in a delayed transport of NL3, especially to the xylem. NL3 could be detected in all tissues, except xylem, at a moment that NY15 was already detectable in the xylem of petioles of the challenge-inoculated leaves and stem (8 to 10 days after inducer inoculation). The presence of NY15 in the xylem prior to NL3 may explain the suppression of the development of the NL3 symptoms. An early arrival of NL3 in the xylem, in the absence of NY15, might lead to an impaired water transport resulting in wilting, withering and necrosis of top and youngest trifoliolate leaves, whereas the presence of NY15 may retard or stop invasion of the xylem by NL3 and, hence, blocking of development of its symptoms.

Obviously, the special type of interference, by which a virus strain (NY15) is able to prevent systemic necrosis by another virus strain (NL3) of which its multiplication is not suppressed, requires further investigations into the exact localization and behaviour of NY15 and NL3 in the stems of interfered plants. With the increasing interest to transform plants with virus genes to protect plants from virus infections by interference processes, it is of utmost importance to analyse related interference phenomena in order to understand more appropriately the mechanisms by which plants are protected from virus infections. Studies to analyse the interference phenomenon as described above are in progress.

Acknowledgements

The authors greatly acknowledge the help of Dr J.F.C. Magendans (Wageningen Agricultural University) in interpretation of anatomical data. We also thank Dr L. Bos, DLO Research Institute for Plant Protection (IPO-DLO), and Dr M. Fortass, Department of Plant Pathology, Meknes, Morocco, for inspiring discussions.

References

Bozarth, R.F. & Ford, R.E. (1988). Viral interactions: Induced resistance (cross protection) and viral interference among plant viruses. In: *Experimental & conceptual plant pathology* 3, pp. 551-567. Eds W.M. Hess, W.M., Singh, R.S., Singh, U.S. & Webber, D.J. Gordon and Beach Science Publishers, New York.

Cassells, A.C. & Herrick, C.C. (1977). Cross protection between mild and severe strains of tobacco mosaic virus in doubly inoculated tomato plants. *Virology* 78: 253-260.

Clark, M.F. & Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* 34: 475-483.

Hamilton, R.I. (1980). Defenses triggered by previous invaders: viruses. In: *Plant Disease* 5, pp. 279-303. Eds Horsfall, J.G. & Cowling, E.B. Academic Press, New York.

Khan, J.A., Lohuis, H., Goldbach, R.W. & Dijkstra, J. (1990). Distincton of strains of bean common mosaic virus and blackeye cowpea mosaic virus using antibodies to N- and C- or N-terminal peptide domains of coat proteins. *Annals of Applied Biology* 117: 583-593.

Lana, A.F., Lohuis, H., Bos, L. & Dijkstra, J. (1988). Relationships among strains of bean common mosaic and blackeye cowpea mosaic virus - members of the potyvirus group. *Annals of Applied Biology* 113: 493-505.

Lohuis, H. & Dijkstra, J. (1990). Two immunocytochemical methods for *in situ* detection of antigen with the light microscope. Proceedings of a working group meeting on virus diseases of beans and cowpeas in Africa. Kampala, CIAT Africa Workshop Series No. 13: 6-9.

Luciano, C.S., Gibb, K.S. & Berger, P.H. (1989). A general method for the detection of potyviral gene products in plant protoplasts and tissue. *Journal of Virological Methods* 24: 347-356.

McKinney, H.H. (1929). Mosaic diseases in the Canary Islands, West Africa, and Gibraltar. *Journal of Agriculture Research* (Washington D.C.) 39: 557-578.

Nagy, P.D. & Gáborjányi, R. (1991). Evidence for cross-protection between strains of barley stripe mosaic virus. *Acta Phytopathologica et Entomologica Hungarica* 26: 193-197.

Price, W.C., (1940). Acquired immunity from plant virus diseases. *Quarterly Review Biology* 15: 338-361.

Chapter 7

Distribution and localization of bean common mosaic virus and bean black root virus in stems of doubly infected bean plants

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This chapter has been submitted for publication to the Netherlands Journal of Plant Pathology as: Khan *et al.* (1993). Distribution and localization of bean common mosaicvirus and bean black root virus in stems of doubly infected bean plants.

Summary

Plants of bean (*Phaseolus vulgaris*) inoculated first on one primary leaf with strain NY15 of bean common mosaic virus, as inducer, and after three days, on the opposite leaf, with the strain NL3 of bean black root virus, as challenger, did not show symptoms characteristic of the latter strain, viz. wilting of the youngest trifoliolate leaf followed by top necrosis. This interference phenomenon was studied by determining the amount, distribution and localization of both strains in the part of stem between primary leaves and first trifoliolate leaf in both challenge-inoculated and singly inoculated (control) plants. In dot-blot immunoassay, NL3 was detected seven days after its inoculation, whereas in control plants its presence was established at four days. Immunostained thick sections revealed a large accumulation of NL3 antigen at eight days in both phloem and cambium, but not yet in the xylem and cortex, contrasted with the controls. In immunogold-silver stained semi-thin sections in case of challenge-inoculated plants, most of the NL3 label was present in the companion cells and other phloem parenchyma cells, but in the control plants there was appreciable label also in xylem vessels and xylem parenchyma cells. Inducer strain NY15 was abundantly present in practically all the cells, including xylem vessels, from two days after challenge inoculation onwards. It is concluded that inducer strain NY15 hampers transport of NL3 to, and its spread inside, the stem and prevents the latter strain from exerting its deleterious influence on the water conducting elements.

Introduction

Bean common mosaic virus, a species of the genus Potyvirus (Vetten *et al.*, 1992), possesses a large number of strains which have been classified into seven pathogenicity groups on the basis of symptoms they induce in differential cultivars (Drijfhout, 1978). The majority of strains induce mosaic in most of the bean cultivars, but the strains of pathogenicity groups III and IV cause a lethal systemic necrosis called black root in bean cultivars possessing the dominant alleles I I of the necrosis gene. Also serologically, the

necrosis-inducing strains differ from the only mosaic-inducing ones to such an extent that Wang (1983) classified them in serotype A and serotype B strains, respectively. On the basis of N-terminal serology (Khan *et al.*, 1990), high performance liquid chromatography profiles of digested coat protein (McKern *et al.*, 1992 a), and the nucleotide sequences of the coat protein gene and the 3'-nontranslated regions (Vetten *et al.*, 1992; Khan *et al.*, 1993 a), it was suggested that the two serotypes should be classified as distinct potyviruses (McKern *et al.*, 1992 b; Vetten *et al.*, 1992; Khan *et al.*, 1993 a). The last mentioned authors have proposed to name the serotype A strains bean black root virus (BBRV) and to retain the name bean common mosaic virus (BCMV) for the serotype B strains.

An unusual type of interference between mosaic-inducing strain NY15 of BCMV and necrosis inducing strain NL3 of BBRV has been described (Khan *et al.*, 1993 b). It was demonstrated that plants of *Phaseolus vulgaris* "Bataaf" inoculated first on one primary leaf with strain NY15, as inducer, and after one or more days on the opposite leaf, with strain NL3 as challenger, did not show symptoms characteristic of the latter strain, viz. systemic necrosis of the stem, and the top and youngest trifoliolate leaves preceded by wilting of these leaves. This type of interference observed between the two strains could not be explained by impaired replication of the challenger, as the amount of strain NL3 in the challenge-inoculated leaf was not lower than that in the control.

Histological studies on immunostained, thick sections of primary leaves, petiole and stem part between primary leaves and first trifoliolate leaf of doubly infected plants had shown that strain NL3 appeared later in the xylem of both petiole and stem, as compared with the singly inoculated control (Khan *et al.*, 1993 b). Such delayed appearance of NL3 in the xylem of stem parts of doubly infected plants might be due to either a lower viral content of these plant parts as a result of hampered transport from the primary leaves, and or a different distribution of the virus in this part of the plant as compared to the singly inoculated controls.

In order to gain more insight into the above-described interference phenomenon, following studies were done. The presence of NY15 and NL3 was established in stem parts of doubly inoculated and singly inoculated (control) plants by dot-blot immuno-

assay at different times after inoculation. The distribution of viral antigen in tissues of these plant parts was studied in the first instance by immunostaining. As with this technique the presence of viral antigen, visible as violet stain, could be established with certainty only at the tissue level, further investigations were carried out on the cellular localization of strains NY15 and NL3 in immunogold-silver stained semi-thin sections of stems to establish a possible role of virus in particular cells in the interference phenomenon.

Materials and methods

Viruses and antisera. BCMV strain NY15, strain NL3 of BBRV (formerly considered to be a strain of BCMV), henceforth referred to as NY15 and NL3, respectively, and the immunoglobulins used were as described earlier (Khan *et al.*, 1990; 1993 a).

Inoculation of bean plants. Inoculations were carried out essentially as described by Khan *et al.* (1993 b). Eight- to ten-day old plants of *Phaseolus vulgaris* "Bataaf" were mechanically inoculated on their primary leaves with crude sap from virus-containing bean leaves. In each experiment, three sets of 15 plants were used to study the interference phenomenon. The plants in set 1 and 2 were first inoculated on one primary leaf with NY15, as inducer, and in set 3 mock-inoculated with water. After three days, the opposite primary leaves were mock-inoculated with water (set 1) or inoculated with NL3, as challenger (sets 2 and 3). Samples of stem parts between primary leaves and the first trifoliolate leaf were collected at different times after inoculation of the challenger.

Dot-blot immunoassay. Dot-blot immunoassay was carried out essentially as mentioned by Hibi and Saito (1985). Each sample to be tested consisted of clarified extract from ground stem parts of three plants. Extracts from stems of healthy plants were used as controls. Samples were taken from two to nine days after inoculation of NL3.

In situ detection of viral antigen. Thick sections (approx. 1 mm). Stem parts were collected

from one to nine days after inoculation of NL3. Every time, in each set (1,2,3), sections of stem of three plants have been examined. The experiment has been repeated twice in different times of the year.

Transverse and longitudinal sections of stems of healthy and diseased plants were fixed and immunostained as described by Khan *et al.* (1993 b). Starting on the premise that alkaline phosphatase-labeled anti-rabbit goat IgG used for immunostaining would still possess a number of free antigen-binding sites, semi-thin sections of immunostained specimens were prepared as follows.

Semi-thin sections (approx. 3 μm). Thick sections were incubated for one h in ELK (PBS containing 2% (w/v) sucrose and 5% (w/v) non-fat dry milk to block sites for aspecific globulin attachment. Drops of purified gammaglobulins from normal rabbit serum (1 μg/ml)were placed on the sections and incubation was for one h. Thereafter, the sections were washed 3x10 min in PBS-Tween, incubated with protein A-gold (O.D. $_{520\ nm} = 0.05$) in PBS-Tween, fixed in 1% glutaraldehyde and washed with distilled water 3x10 min, essentially as described by Van Lent and Verduin (1987). All the incubation steps were carried out at 37°C.

The sections were then silver stained in light, using the commercially available Aurion R- Gent (Aurion, Netherlands) kit consisting of separate developer and enhancer components (0.78 g initiator and 0.47 g enhancer were dissolved in 10 ml double distilled water). The initiator and developer were mixed in 1:1, the drops of the mixture were placed on top of the sections and the staining was carried out in a water bath at 17°C for 15 min . The staining was stopped by placing the sections in the fixative for five min and immediately washed with distilled water for 3x15 min .

After silver enhancement, the specimens were stained with 1% (w/v) toluidine blue in distilled water and thereafter embedded and sectioned according to Van Lent and Verduin (1987).

The semi-thin sections thus obtained were examined with epi-illumination in a Leitz Orthoplan microscope equipped with water-immersion objective lenses (x25, x50, x100) and a polarization filter block (epipolarization microscopy) obtained from Leitz, and also

with brightfield transillumination.

Silver stain was observed as light blue precipitates against a black background with epi-illumination. By combining epi-illumination with brightfield transillumination both silver precipitates and the cell morphology could be visualized at the same time.

Results

Bean plants, infected with NL3 alone, developed epinasty of the primary leaves about five days after inoculation, and top necrosis and wilting and withering of the youngest trifoliolate leaf after seven to eight days. The plants, first inoculated with NY15 and three days later with NL3, did not show the above mentioned symptoms, and differed from the mosaic-showing, stunted NY15-inoculated control plants only in more severe stunting and curling of the trifoliolate leaves.

Table 1. *Detection of strain NL3 of bean black root virus (BBRV) and strain NY15 of bean common mosaic virus (BCMV) in stem parts of "Bataaf" bean by dot-blot immunoassay. The bean plants had been inoculated on one primary leaf with strain NY15 and three days later on the opposite leaf with strain NL3 as challenger (NY15+NL3). Plants inoculated with NL3 alone (NL3) were used as controls. Stems were collected from two to nine days after inoculation of NL3.*

Plants inoculated with	Reactions of samples at different times after inoculation of NL3 (days)*1)*							
	2	3	4	5	6	7	8	9
NY15+NL3	-(++)	-(++)	-(++)	-(++)	+(++)	+(++)	+(+++)	+(+++)
NL3	-	+	+	+	+	(++)	(+++)	+(+++)

[1] observations given in parentheses refer to presence of NY15

- = faint purple colour comparable to that of the healthy control
+ = violet colour
+- = light violet colour
++ = dark violet colour
+++ = very dark violet colour

Table 2. *Presence of strain NL3 of bean black root virus in tissues of the stem of "Bataaf" bean first inoculated on one primary leaf with strain NY15 of bean common mosaic virus, as inducer, and three days later on the opposite primary leaf with NL3, as challenger (NY15 + NL3). Singly inoculated plants were used as controls (NY15, NL3). Stem samples were collected 3,4,5,6,7 and 8 days after NY15 control inoculation.*

	Cells with viral antigen[1]														
	NY15					NL3					NY15 + NL3				
Days	3	4	5	7	8	3	4	5	7	8	3	4	5	7	8
Cortex	2	3	3	3	3	0	0	1		3	0(3)	0(3)	0(3)	0(3)	0(3)
Phloem	2	3	3	3	3	1	1	1	1	1	0(3)	1(3)	1(3)	2(3)	3(3)
Cambium	1	1	1	1	1	0	0	0	1	1	0(1)	0(1)	0(1)	1(1)	2(1)
Xylem	3	3	3	3	3	0	0	1	2	2	0(3)	0(3)	0(3)	0(3)	0(3)
Pith	1	2	2	2	3	0	0	0	0	0	0(3)	0(3)	0(3)	0(3)	0(3)

[1] Presence of viral antigen was established by immunostaining technique. Observations given in parentheses refer to presence of the inducer

0 = no cells stained 2 = less than half the number of cells stained
1 = some cells stained 3 = more than half the number of cells stained

In doubly inoculated plants NL3 was detected in stem parts by dot-blot immunoassay seven days after challenge inoculation, whereas its presence in the singly inoculated control could be demonstrated already at four days; the inducer was present in large amount from day two onwards (Table 1). The healthy controls showed only a faint purple colour, both with NY15 and NL3 gammaglobulins.

In thick sections of stems of the challenge-inoculated plants, NL3 was shown to occur first in phloem at day four and at eight it was present in large quantities in phloem and cambium, but not in the other stem tissues (Table 2). Plants inoculated with NL3 alone, on the other hand, showed less viral antigen in phloem and cambium, but appreciable accumulation of virus in cortex and xylem. The number of inducer-containing cells in the

various tissues was comparable to that in the control (Table 2). Immunostained thick sections of stems from healthy plants did not show any violet colour.

In semi-thin sections of stems of challenge-inoculated plants at day one, two and three, the amount of NL3 label was comparable to that of the healthy controls, i.e. a few irregularly scattered minute spots. At day four some label of NL3 was visible, mostly in the phloem cells, both in challenge-inoculated (Fig. 1) and singly inoculated plants, but in the latter there was more label in xylem vessels and xylem parenchyma cells, and also a higher total amount of label.

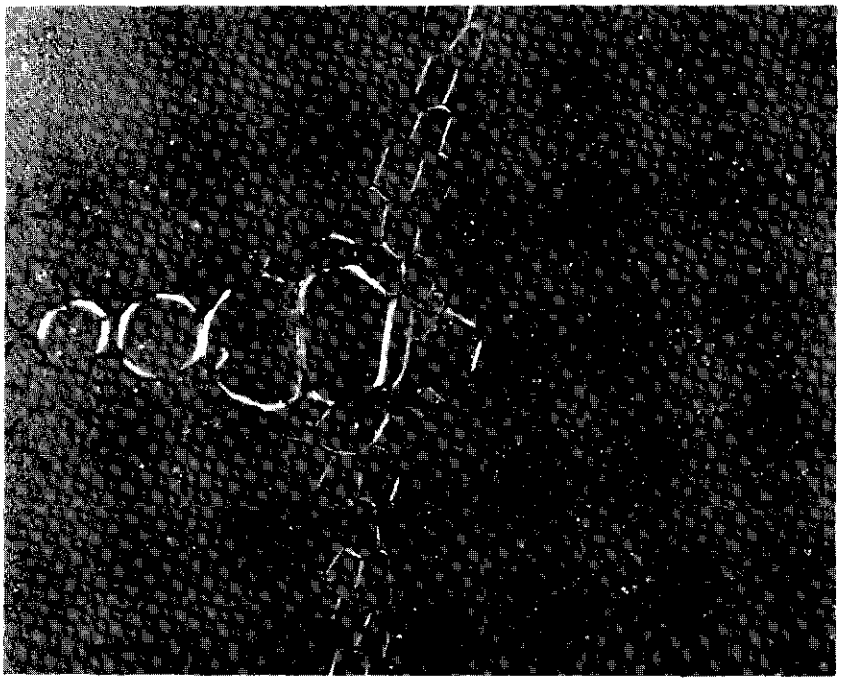

Fig. 1. Transverse section of stem of a bean plant inoculated first with strain NY15 of bean common mosaic virus and three days later with strain NL3 of bean black root virus. Sections of samples, collected four days after NL3 inoculation, were incubated with anti-NL3 and stained with immunogold-silver and toluidine blue. Silver stain is visible as white spots. CP, cortex parenchyma; MP, metaphloem with sieve tubes, companion cells and other parenchyma cells; MX, metaxylem with vessels and parenchyma cells; PP parenchyma cells of pith; PF, immature and mature phloem fibres; SX, secondary xylem. Bar represents 15 μm.

Most of the label in metaphloem cells was found in the companion cells and other phloem parenchyma cells, and very little in the sieve tubes (Fig. 2). At that time (day four), inducer virus was present in large quantities in practically all the cells, including xylem vessels, but also less in sieve tubes (Fig. 3A and 3B). At day eight and nine, label of NL3 in the singly inoculated plant was found to occur in large amounts in cortex parenchyma cells, immature fibres of protophloem, cambium, vessels of metaxylem and secondary xylem and surrounding parenchyma cells (Fig. 4A). The distribution of label was similar, but its amount less, in the challenge-inoculated plants, except in the cortex where it was virtually absent (Fig. 4B).

The fact that at these times, label of NL3 was shown to be present in practically all tissues in the challenge-inoculated plants, indicated the greater sensitivity of the silver staining as compared with the immunostaining technique.

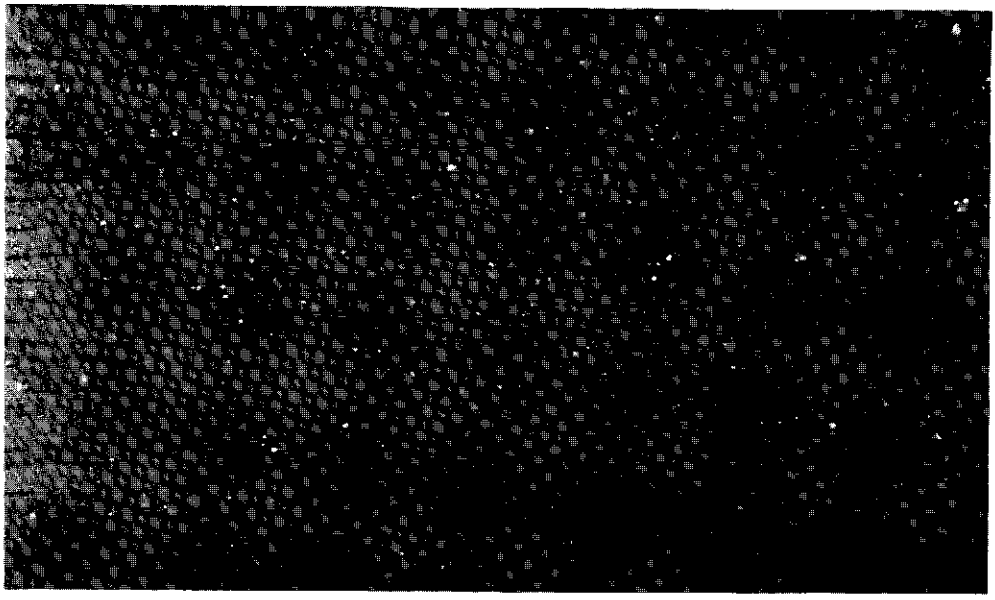

Fig. 2. Longitudinal section of the same stem as shown in Fig. 1. CC, companion cell; PP, phloem parenchyma cells; SP, sieve plate; ST, sieve tube. Bar represents 30 μm.

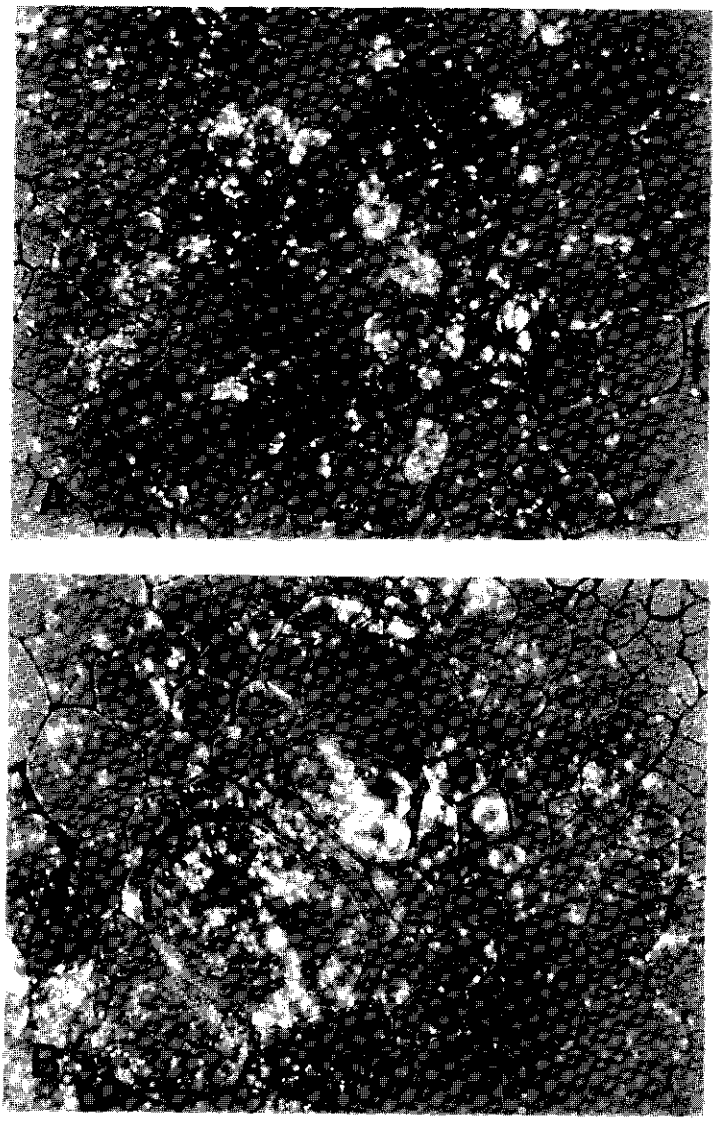

Fig. 3. Transverse section of stem of a bean plant inoculated first with strain NY15 of bean common mosaic virus and three days later with strain NL3 of bean black root virus. Sections of samples, collected four days after NL3 inoculation, were incubated with anti-NY15 and stained with immunogold-silver and toluidine blue. Silver stain is visible as white spots in cortex, phloem and cambium (A) and cambium, xylem and pith (B). For explanation of letter symbols, see Figs. 1 and 2. Bar represents 15 μm.

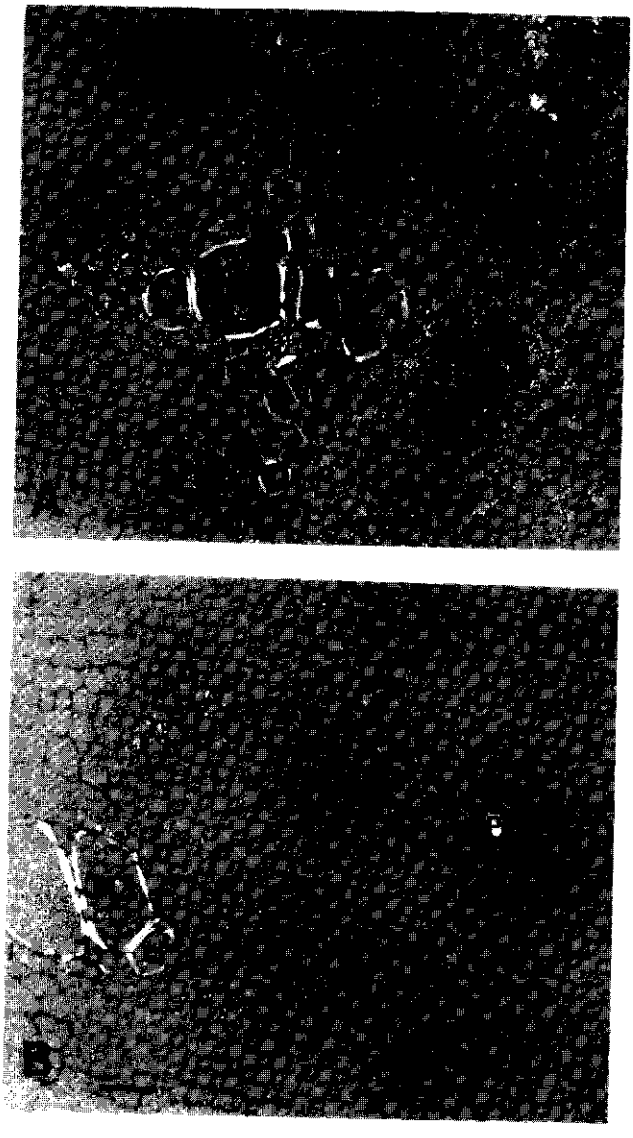

Fig. 4. Transverse sections of stems of bean plants inoculated with strain NL3 of bean black root virus (A) or first with strain NY15 of bean common mosaic virus and three days later with strain NL3 of bean black root virus (B). Sections of samples, collected nine days after NL3 inoculation, were incubated with anti-NL3 and stained with immunogold-silver and toluidine blue. Silver stain is visible as white spots. For explanation of letter symbols, see Figs. 1 and 2. Bar represents 30 μm.

Discussion

Previous investigations had shown that in the challenge-inoculated plants, with NY15 as inducer, NL3 arrived later in the xylem of petiole and stem, as compared with the singly inoculated controls (Khan *et al.*, 1993 b). The present study has confirmed this.

However, contrary to no difference in the challenge-inoculated primary leaf, the amount of NL3 in the stem parts of doubly inoculated plants was less than that in the singly inoculated controls, at least during the first four days after inoculation. This might be explained by a hampered transport from the inoculated leaf to the stem. Assuming that most of the virus transport is via phloem, further spread from this tissue into surrounding tissues of the stem seemed to be impeded as well, as deduced from the results with immunostaining. From the latter experiments it became clear that in the challenge-inoculated plants, NL3 was restricted to phloem in the first five days after inoculation and later spread to surrounding tissues, but to a lesser extent than in the singly inoculated controls.

In general, there was less label of NL3 in the xylem vessels and surrounding cells of challenge-inoculated plants as compared to the singly inoculated ones. The wilting of first trifoliolate leaf and the top necrosis observed in plants inoculated with NL3 only, may be due to blocking of xylem vessels by the virus leading to impaired water transport. The fact that no wilting and necrosis occurred in doubly infected plants might be ascribed to the abundant presence of NY15 in the xylem preventing deleterious effects of NL3 on the water-conducting system.

Although no double labelling has been carried out, from eight days after inoculation onwards both NY15 and NL3 were found to occur in the xylem vessels and surrounding cells in all sections examined. It is, therefore, assumed that in doubly infected stems both viruses are present in the same cells.

Remarkably, the presence of NY15 in the xylem vessels does not seem to have any adverse effects on the functioning of these vascular elements, possibly due to the different genetic make-up of the two viruses.

Possible blocking of xylem vessels by NL3 may play a less important role when the

NL3 infected plant survives and grows older, because by that time the water-conducting role of primary xylem, the metaxylem in particular, has been taken over by vessels of the secondary xylem. That might be the explanation for non-necrotic appearance of the newly formed shoots from axillary buds.

Reference should be made here to the nature of the systemic necrosis encountered with NL3 infection in "Bataaf" bean. There appears to be a discrepancy between the necrosis described here and that mentioned in the literature. According to Drijfhout (1978), "Bataaf" belongs to resistance group 2 of bean cultivars with recessive alles (I^+I^+) of the necrosis gene. These cultivars react with mosaic upon infection with most of the BCMV strains, including the black root- causing NL3. As already reported by Lana *et al.* (1988) "Bataaf" bean reacted to NL3 with severe necrosis of stem, young leaves and top. Light-microscopy studies of stems and roots of such necrotic plants have revealed confinement of necrosis to epidermis, cortex, phloem fibres, primary xylem and pith (unpublished results). However, the vascular necrosis of black root was shown to be restricted to the conducting elements of the phloem, cambial layers and, in a few instances, to the outermost layer of xylem (Jenkins, 1941). Thus the necrosis described in the present study, basically differs from that in the black root-disease affected beans. Also, upon recovery, "Bataaf" plants showed only mosaic and vein banding in the newly-formed leaves and no necrosis whatsoever. Such symptoms have not been reported from bean cultivars with dominant alleles of the necrosis gene.

From the results obtained in this study, the conclusion is warranted that most likely NL3 present in xylem vessels in singly inoculated plants gives rise to wilting and systemic necrotic symptoms, but not in challenge-inoculated ones due to a protective action of NY15 in the same tissue.

References

Drijfhout, E. (1978). Genetic interaction between *Phaseolus vulgaris* and bean common mosaic virus with implications for strain identification and breeding for resistance. *Agricultural Research Reports* 872, PUDOC, Wageningen, 98 pp.

Hibi, T. & Saito, Y. (1985). A dot-blot immunobinding assay for the detection of tobacco mosaic virus in infected tissue. *Journal of General Virology* 66: 1191-1194.

Jenkins, W.A. (1941). A histological study of snap bean tissues affected with black root. *Journal of Agricultural Research* (Washington, D.C.) 62: 683-690.

Khan, J.A., Lohuis, H., Goldbach, R.W. & Dijkstra, J. (1990). Distinction of strains of bean common mosaic virus and blackeye cowpea mosaic virus using antibodies to N- and C- or N-terminal peptide domains of coat protein. *Annals of Applied Biology* 117: 583-593.

Khan, J.A., Lohuis, H., Goldbach, R.W. & Dijkstra, J. (1993 a). Sequence data to settle the taxonomic position of bean common mosaic virus and blackeye cowpea mosaic virus isolates. *Journal of General Virology* (in press).

Khan, J.A., Lohuis, H., Bakardjieva, N., Peters, D., Goldbach, R.W. & Dijkstra, J. (1993 b). Interference between two strains of bean common mosaic virus is accompanied by suppression of symptoms without affecting replication of the challenging virus. *Journal of Phytopathology* (in press).

Lana, A.F., Lohuis, H., Bos, L. & Dijkstra, J. (1988). Relationships among strains of bean common mosaic virus and blackeye cowpea mosaic virus-members of the potyvirus group. *Annals of Applied Biology* 113: 493-505.

Lent, J.W.M. van & Verduin, B.J.M. (1987). Detection of viral antigen in semi-thin sections of plant tissue by immunogold-silver staining and light microscopy. *Netherlands Journal of Plant Pathology* 93: 261-272.

McKern, N.M., Mink, G.I., Barnett, O.W., Mishra, A., Whittaker, L.A., Silbernagel, M.J., Ward, C.W. & Shukla, D.D. (1992 a). Isolates of bean common mosaic virus comprising two distinct potyviruses. *Phytopathology* 82: 923-929.

McKern, N.M., Ward, C.W. & Shukla, D.D. (1992 b). Strains of bean common mosaic virus consist of at least two distinct potyviruses. *Archives of Virology* (Supplement 5): 407-414.

Vetten, H.J., Lesemann, D-E. & Maiss, E. (1992). Serotype A and B strains of bean common mosaic virus are two distinct potyviruses. *Archives of Virology* (Supplement 5): 415-431.

Wang, W-Y. (1983). Serology of bean common mosaic virus strains. *M.Sc. Thesis* Washington State University, Pullman, Washington, USA, 52 pp.

Chapter 8

Summary and Conclusions

Bean common mosaic virus (BCMV) and blackeye cowpea mosaic virus (BlCMV) belonging to the genus Potyvirus of the plant virus family Potyviridae (Barnett, 1991, 1992) are of great economic importance. A large number of strains of BCMV and BlCMV are found to occur in nature, either in single or in mixed infections (Vetten & Allen, 1991). The latter may result in interactions among the strains and sometimes lead to antagonistic (Bercks, 1959; Quantz, 1961) or synergistic effects.

Aim of the present research was to resolve the chaotic and contradictory taxonomic status of different isolates of BCMV (NL1, NY15, NL3) and BlCMV (W) and to gain an insight into the mechanism of antagonism observed in *Phaseolus vulgaris* cv. Bataaf infected with both NY15 and NL3, by using these viruses as models.

When this investigation was started, it was impossible to serologically distinguish the different strains of BCMV and BlCMV. As distinction of viruses in mixed infections was a prerequisite to study their behaviour in the plant, a novel serological technique was applied. To this end, antibodies directed towards N- and C-, or N-terminal peptide domains of the coat proteins of these viruses were prepared. The N-terminal targeted antibodies thus obtained, enabled a clear distinction of the strains in mixed infection. The specificity of these antibodies had some implications on the taxonomy of these viruses (Chapter 3). According to N-terminal serology NL1 of BCMV and W of BlCMV should not be considered strains of the two different viruses but of the same virus viz. BCMV, whereas NL3 should be a strain of a distinct potyvirus. Although N-terminal serology enabled distinction between NY15, NL3 and W in mixed infections, it did not give a clue to their exact taxonomic positions. In some cases there may be unexpected serological relationships (Shukla *et al.*, 1989) whereas in others there is lack of expected serological relationships (Shukla *et al.*, 1992).

The understanding of the coat protein structure in recent years has greatly contributed to potyvirus taxonomy. During the course of this investigation, some information on high performance liquid chromatography (HPLC) digests of the coat proteins of BCMV and BlCMV became available. Therefore, an attempt was made to tentatively classify the strains of BCMV and BlCMV into a proposed BCMV subgroup using biological, serological and HPLC characteristics as taxonomic parameters.

Although subgroups have no official taxonomic status, for practical purposes it is easier to deal with closely related viruses at subgroup level (Chapter 4).

Some of the results obtained with N-terminal serology were contrary to those with HPLC. The former had shown that NY15 and W were different viruses (Chapter 3), whereas according to the latter these were strains of one virus (McKern *et al.*, 1992). We should realise that N-terminal serology and HPLC are both based on characteristics of the coat proteins. To resolve these conflicting findings, nucleotide sequences of the coat protein genes and 3'-nontranslated regions of the genomes of NL1, NL3, NY15 and W were determined (Chapter 5). The deduced amino acid sequences revealed that the coat proteins of NL1, NY15 and W were identical in size and exhibited a high percentage of sequence similarity (94-97%). Moreover, this high percentage of similarity was also reflected in their 3'-nontranslated regions (93-96% similarity) confirming that NL1, NY15 and W are not strains of two different virus species but of one single species only, viz. BCMV. It has been proposed to designate these strains BCMV-NL1, BCMV-NY15 and BCMV-BlC/W, respectively. On the other hand, NL3 had a shorter coat protein and displayed a lower percentage of sequence similarity both in the coat protein (87-89% similarity) and 3'-nontranslated region (56-63% similarity) with the other investigated strains. This different molecular make-up of NL3 combined with earlier serological and HPLC findings justified the conclusion that NL3 should no longer be considered a strain of BCMV, but of a different virus, for which the name bean black root virus (BBRV) has been proposed.

It is clear that the lack of serological relationship between NY15 and W, as reported earlier, can not be attributed to major differences in the nucleotide sequences of the N-terminal part of the coat protein genes and 3'-nontranslated regions. Regarding this, epitope mapping of mutated N-terminal domains expressed in heterologous systems, might give an insight into the complexity of the serological results.

A special type of antagonism, henceforth referred to as interference, between strains NY15 and NL3 has been described in Chapter 6. It was found that, when a primary leaf of *Phaseolus vulgaris* cv. Bataaf was inoculated with the mosaic-inducing NY15 as inducer and one to eight days later with systemic necrosis inducing NL3, as challenger,

on the opposite leaf, the characteristic symptoms of NL3 i.e. wilting of first trifoliolate leaf followed by top necrosis, did not occur. The amount and distribution of both strains were analysed in the primary leaves of protected plants. It was remarkable to note that the amount of NL3 in the challenger-infected leaf of the protected plant was comparable to that of controls. At the histological level it was found, that NL3 arrived later in the xylem of petioles of challenger-infected leaves. In the light of these results, it was concluded that NY15 protected the plant without affecting the replication of NL3 in the inoculated leaf.

Having analysed this unusual interference phenomenon in the primary leaves, the next logical step was to further investigate the exact localization and behaviour of NY15 and NL3 in the stem of protected plants (Chapter 7). Using dot-blot immunoassays, NL3 was detected seven days after its inoculation in protected (infected with NY15 and NL3) plants while in the controls (NL3 infected only) its presence was established at four days. The later arrival of NL3 in the stems of protected plants was in line with the earlier findings and could be explained by a hampered transport of NL3 from the inoculated leaf into the stem. Interestingly, the amount of NL3 in the stem of protected plants was less than that in the singly inoculated controls, in contrast to no difference in challenger inoculated leaf as reported in Chapter 6.

In the light of these observations, it can be hypothesized that an early arrival of NL3 in the xylem might lead to an impaired water transport due to its (NL3) deleterious effects on the water conducting-system resulting in wilting, withering and top necrosis. The fact that no necrosis occurred in protected plants might be due to the abundant presence of NY15 hampering the invasion of NL3 from the inoculated leaf into the stem, thus preventing the deleterious effects of NL3 on the water-conducting system. Furthermore, the presence of NY15 in the xylem vessels did not seem to have any adverse effects on the functioning of these vascular elements, possibly due to the different genetic make-up of the two viruses.

The differences in nucleotide sequences of the 3'-nontranslated genomic regions between NL3 on the one hand, and BCMV strains on the other, may be reflected in their symptomatology. Such a correlation has been established in case of tobacco vein

mottling virus possessing a determinant of disease symptoms in its 3'-nontranslated region (Rodriguez- Cerezo *et al.*, 1991).

Besides the 3'-nontranslated regions, also the coat protein gene may be involved in the physiological interactions between the virus and its host, as has been shown for tobacco mosaic virus (TMV) mutants (Dawson *et al.*, 1988). Moreover, a single point mutation in the coat protein gene of TMV, leading to a single amino acid substitution, has been shown to be responsible for the induction of hypersensitive reaction in *Nicotiana sylvestris*, possessing the N'N' genotype (Culver & Dawson, 1989). In contrast, induction of the N gene-mediated hypersensitive reaction caused by TMV, was mapped to the 126-KDa (polymerase) gene as reported by Padgett and Beachy (1993).

In view of the possible involvement of more than only one single viral gene in symptomatology, site directed mutagenesis in different regions of the BCMV and BBRV genomes might give a clue to the different reactions of these viruses in bean plants.

References

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

Barnett, O.W. (1992). A summary of potyvirus taxonomy and definitions. *Archives of Virology* (Suppl 5): 435-444.

Bercks, R. (1959). Serologische Untersuchungen über das *Phaseolus*-virus 1. *Phytopathologische Zeitschrift* **35**: 105-118.

Culver, J.N. & Dawson, W.O. (1989). Tobacco mosaic virus coat protein: An elicitor of the hypersensitive reaction but not required for the development of the mosaic symptoms in *Nicotiana sylvestris*. *Virology* **173**: 755-758.

Dawson, W.O., Bubrick, P. & Grantham, G.L. (1988). Modifications of the tobacco mosaic virus coat protein gene affecting replication, movement, and symptomatology. *Phytopathology* **78**: 783-789.

McKern, N.M., Ward, C.W. & Shukla, D.D. (1992). Strains of bean common mosaic virus consist of at least two distinct potyviruses. *Archives of Virology* (Suppl 5): 407-414.

Padgett, H.S. & Beachy, R.N. (1993). Analysis of tobacco mosaic virus strain capable of overcoming N gene-mediated resistance. *The Plant Cell* 5: 577-586.

Quantz, L. (1961). Zum Nachweis des gewohnlichen Bohnenmosaikvirus in Bohnensamen mit Hilfe des Schalentest. *Nachrbl. Dtsch. Pflsch. Dienst (Braunschweig)* **14**: 49-54.

Rodriguez-Cerezo, E., Klein, P.G. & Shaw, J.G. (1991). A determinant of disease symptom severity is located in the 3'-terminal region of the RNA of a plant virus. *Proceedings of the National Academy of Sciences* U.S.A. **88**: 9863-9867.

Shukla, D.D., Lauricella, R. & Ward, C.W. (1992). Serology of potyviruses: current problems and some solutions. *Archives of Virology* (Suppl 5): 57-69.

Shukla, D.D., Jilka, J., Tosic, M. & Ford, R.E. (1989). A novel approach to the serology of potyviruses involving affinity-purified polyclonal antibodies directed towards virus-specific N-termini of coat proteins. *Journal of General Virology* 70: 13-20.

Vetten, H.J. & Allen, D.J. (1991). Recent progress in the identification of viruses of *Phaseolus vulgaris* in Africa. *Bean improvement Cooperation* 33: 3-4.

Vetten, H.J., Lesemann, D.-E., & Maiss, E. (1992). Serotype A and B strains of bean common mosaic virus are two distinct potyviruses. *Archives of Virology* (Suppl 5): 415-431.

Samenvatting

Het rolmozaïek van de boon, *Phaseolus vulgaris* is reeds in 1894 door Ivanovski uit Rusland gerapporteerd en is hiermee de oudst bekende virusziekte in dit gewas. Het virus, dat deze ziekte veroorzaakt, is vele jaren later beschreven onder de naam bonerolmozaïekvirus (Eng. bean common mosaic virus; BCMV). Het BCMV is over de hele wereld verbreid en veroorzaakt aanzienlijke economische schade.

Het virus behoort tot het geslacht Potyvirus van de grootste plantevirusfamilie, de Potyviridae. Zoals de meeste andere potyvirussen, heeft BCMV een beperkte waardplantenreeks, met de boon, lupine en enkele wilde vlinderbloemigen als enige natuurlijke waardplanten. Het virus wordt, zoals alle soorten van het geslacht Potyvirus, op non-persistente wijze door verschillende soorten bladluizen overgebracht en bezit de volgende algemene kenmerken:

- De virusdeeltjes zijn draadvormig met een normale lengte van ongeveer 750 nm.
- Het genoom bestaat uit een enkelstrengs RNA met positieve polariteit en heeft een lengte van ongeveer 10.000 nucleotiden. Aan het 5'-uiteinde van het RNA bevindt zich een klein eiwit, VPg ("Viral Protein genome-linked") en aan het 3'-uiteinde een poly-A-staart. Het genoom wordt vertaald in een polyproteïne, dat door drie proteases in acht functionele polypeptiden wordt geknipt. Tot deze eiwitten behoren, behalve het VPg en de bovengenoemde proteases, o.a. het manteleiwit en het virale RNA-afhankelijke RNA polymerase.
- Het virus induceert in het cytoplasma van geïnfecteerde cellen karakteristieke schoepenradvormige insluitsels (Eng. pinwheels), die uit virale eiwitten zijn opgebouwd.

In de natuur wordt een groot aantal stammen van het BCMV gevonden, die afzonderlijk, maar ook gemengd in boneplanten kunnen voorkomen. In het laatste geval kunnen de stammen antagonistische of synergistische effecten teweegbrengen.

Ondanks het economische belang van BCMV bestond er grote onduidelijkheid ten aanzien van de taxonomische positie van stammen van dit virus en van het serologisch verwante blackeye cowpea mosaic virus (BlCMV). Laatstgenoemd virus heeft hoofdzakelijk soorten van het geslacht *Vigna* tot waardplant.

Het onderzoek dat in dit proefschrift wordt beschreven, was gericht op twee

aspecten van bovengenoemde virussen: Enerzijds het ophelderen van de taxonomische positie van de stammen NL1, NY15 en NL3 van BCMV en stam W van BlCMV en anderzijds het verkrijgen van inzicht in het mechanisme van antagonisme tussen de stammen NY15 en NL3, wanneer deze samen in de bonecultivar "Bataaf" voorkomen.

Bij de aanvang van dit onderzoek bleek het onmogelijk om met behulp van de conventionele serologie de verschillende stammen van BCMV en BlCMV van elkaar te onderscheiden. Aangezien voor onderzoek naar het gedrag van meerdere stammen in een plant een dergelijk onderscheid conditio sine qua non is, is aandacht besteed aan het verkrijgen van stam-specifieke antisera. Hierbij werd gebruik gemaakt van antilichamen tegen het N-terminale gedeelte van het manteleiwit, dat gekenmerkt is door een grotere specificiteit dan het resterende deel. Met behulp van deze antilichamen is het gelukt om enkele stammen van elkaar te onderscheiden (Hoofdstuk 3).

De specificiteit van de N-terminale antilichamen had tevens gevolgen voor de taxonomie van BCMV en BlCMV. De met N-terminale serologie verkregen resultaten zijn, in combinatie met biologische en conventioneel-serologische eigenschappen, alsmede literatuurgegevens over peptide-profielen van de manteleiwitten uit "High Performance Liquid Chromatography" (HPLC) gebruikt om de stammen van BCMV en BlCMV voorlopig in te delen in een BCMV-subgroep (Hoofdstuk 4).

De resultaten verkregen met N-terminale serologie en HPLC bleken echter niet altijd met elkaar in overeenstemming te zijn. Zo reageerden NY15 en W niet met elkaars antilichamen, doch ze gaven wel overeenkomstige peptide-profielen. Aangezien door deze tegenstrijdige resultaten geen uitspraak kon worden gedaan over de precieze taxonomische positie van de BCMV en BlCMV stammen, zijn de nucleotidenvolgorden van de manteleiwitgenen en de 3'-niet-vertaalde gebieden (3'-NTR) van de genomen van NL1, NY15, NL3 en W bepaald (Hoofdstuk 5). De afgeleide aminozuurvolgorden toonden aan, dat de manteleiwitten van NL1, NY15 en W identiek waren in grootte (287 aminozuren) en een hoog percentage overeenkomst in aminozuurvolgorde vertoonden (94-97%). Ook in de nucleotidenvolgorden van hun 3'-NTR bleek een grote mate van homologie voor te komen (93-96%). Hieruit kon worden geconcludeerd, dat NL1, NY15 en W stammen van één virus zijn, nl. BCMV. Stam NL3, daarentegen, had een korter

manteleiwit dan de andere stammen (261 aminozuren), een lager percentage overeenkomst in aminozuurvolgorde (87-89%) en een homologie van 56-63% in het 3'-NTR. Deze verschillen, alsmede de resultaten van N-terminale serologie en HPLC hebben geleid tot de conclusie, dat NL3 geen stam is van BCMV, maar van een ander virus, waarvoor de naam bean black root virus (BBRV) is voorgesteld.

Een bijzonder soort antagonisme (interferentie) tussen NY15 en NL3 is beschreven in Hoofdstuk 6. Bij inoculatie van een primair blad van "Bataaf" boon met de mozaïek-inducerende NY15 als "inducer" en een tot acht dagen later op het tegenoverstaande blad met de necrose-inducerende NL3 als "challenger", bleven de karakteristieke symptomen van NL3 (verwelking van het eerste drietallige blad gevolgd door topnecrose) achterwege. Serologische analyse van de primaire bladeren toonde aan, dat in de dubbel geïnfecteerde plant de hoeveelheid NL3 in het blad geïnoculeerd met de "challenger" vergelijkbaar was met die van de controle, die alleen met NL3 was geïnoculeerd. Histologisch onderzoek wees uit, dat NL3 later aantoonbaar was in het xyleem van bladsteeltjes van dubbel geïnfecteerde planten. Uit deze resultaten werd geconcludeerd, dat NY15 de plant beschermde zonder de vermeerdering van NL3 in de primaire bladeren nadelig te beïnvloeden.

Als volgende stap werd op verschillende tijdstippen na inoculatie met behulp van immunogoud-zilverkleuring de verdeling van NY15 en NL3 onderzocht in weefsels van het stengelstuk tussen primaire bladeren en eerste drietallige blad (Hoofdstuk 7). Tevens werd door middel van een "dot-blot immunoassay" een schatting gemaakt van de hoeveelheid virus in deze stengelstukjes. Behalve het reeds eerder geconstateerde, vertraagde transport van NL3 naar het xyleem van dubbel geïnfecteerde planten (Hoofdstuk 6), bleek ook op latere tijdstippen minder virus in de houtvaten en houtparenchymcellen aanwezig te zijn dan in de controle.

Daar op deze tijdstippen "inducer" NY15 in grote hoeveelheden voorkwam in alle weefsels van de stengel, werd geconcludeerd, dat dit virus zowel het transport van NL3 naar, als verspreiding ervan in, de stengel remt. Het uitblijven van verwelking en necrose in de dubbel geïnfecteerde plant kan mogelijk worden verklaard door aan te nemen, dat NY15 de water-vervoerende vaten beschermt tegen schadelijke invloeden van NL3 op

deze vasculaire elementen. Het feit, dat de aanwezigheid van NY15 in vrijwel alle cellen van het xyleem blijkbaar geen nadelig effect heeft op het watertransport, zou toegeschreven moeten worden aan de verschillen in genetische samenstelling van NY15 en NL3.

Curriculum vitae

Jawaid Ahmad Khan was born in Delhi on May 26, 1960. He did his M.Sc. (1981) and M.Phil. (1983) degrees in Botany from Aligarh Muslim University, India. In 1983, he took up a Research Fellowship in the Division of Vegetable Crops, Indian Agricultural Research Institute, New Delhi and served there till 1986. From 1986 to 1988, he worked as a Plant Pathologist for Agricultural Technical Co. (Ltd), Muscat, Oman. Thereafter in October 1988, Jawaid joined the Department of Virology, Wageningen Agricultural University for his Ph.D. work and the results obtained are presented in this thesis.