THE HELPER COMPONENT-PROTEINASE OF COWPEA APHID-BORNE MOSAIC VIRUS

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STATEMENTS

1. Sequence data cannot entirely substitute for biological properties in differentiating distinct potyvirus species and their strains.
   Ward *et al.*, (1992). Arch Virol [suppl 5]: 283-297
   Berger *et al.*, (1997). Arch Virol: 142: 1979-1999

2. A transgene encoding a viral suppressor of posttranscriptional gene silencing (PTGS) can also induce PTGS and virus-induced gene silencing (VIGS).
   This thesis

3. The 'distinct' role of HC-Pro in replication, movement, pathogenicity determination and synergy is an indirect effect of its ability to suppress a host defense response.
   This thesis
   Anandalakshmi *et al.* (1998). Proc. Natl. Acad. Sci. U. S. A. 95: 13079-13084
   Brigneti, *et al.* (1998). EMBO J. 17: 6739-6746
   Kasschau and Carrington (1998). Cell 95: 461-470

4. Current models of virus movement will have to be revised in line with recent reports of viral movement proteins that interfere with PTGS.
   Carrington J. C. (1999). Microbiol. 8: 312-313
   Voinnet *et al.* (2000). Cell 103: 157-167

5. Any envisioned solution to Africa's food security problems that does not fully consider the often intricate causes of the status quo, could exacerbate the gap between the rich and the poor.

6. Western democracy in the 21st century: a dictatorship of public opinion.

Statements from the thesis entitled:
"The Helper Component-Proteinase of Cowpea Aphid-Borne Mosaic Virus"
Sizolwenkosi Mlotshwa, Wageningen, 8 December 2000.

The Helper Component-Proteinase of Cowpea Aphid-Borne Mosaic Virus

Sizolwenkosi Mlotshwa

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus van
Wageningen Universiteit,
Dr. ir. L. Speelman,
in het openbaar te verdedigen op
vrijdag 8 december 2000
des namiddags om vier uur in de aula

The work presented in this thesis was carried out at the Laboratory of Molecular Biology, Wageningen University and Research Centre, The Netherlands; and Department of Biochemistry, University of Zimbabwe. Financial support was provided by the Netherlands Foundation for the Advancement of Tropical Research (WOTRO).

The Helper Component-Proteinase of Cowpea Aphid-Borne Mosaic Virus / Sizolwenkosi Mlotshwa.

Thesis Wageningen – with references – with summaries in English and Dutch.

ISBN 90-5808-340-3

Subject headings: cowpea, potyvirus, pathogenicity, silencing, virus resistance.

For my parents to whom I owe the gift of life.

ACKNOWLEDGEMENTS

Wageningen, October 2000. It is with a sense of exaltation and humility that I conclude four years of my PhD studies by paying tribute to persons and institutions whose invaluable contributions have culminated in this thesis.

My profound gratitude goes to my promoter Prof. Dr. A. van Kammen and co-promoters Dr. J. Welllink and Dr. I. Sithole-Niang for their resilience and guidance that has underlined my intellectual development and made the completion of this work possible. Extended thanks to Dr. J. Wellink for the dutch translation of the summary. I thank Prof. Dr. T. Bisseling for the opportunities in the laboratory of Molecular Biology without which this work would not have come to fruition. I celebrate Jan Verver's technical expertise and therapeutic humour that has been a constant source of inspiration during the low moments of the HC-Pro experience. I thank Dr. K. Gopinath for the assistance and helpful hints during the course of the work and for the prophetic reassurance that "nothing will happen". Dr. M. Prins and Dr. A. Mordhorst are acknowledged for generously sharing the blessings of their experience with transgenic work. Dr. B. J. M. Verduin is thanked for the kind material support during the tenure of this project and for the initial introduction to potyvirus research. Kind assistance from Dick Lohuis, Kitty Jansen and Yukun Chen is acknowledged with sincere thanks.

Special thanks to the CPMV group (Jan Carette, Peter Bertens, Jeroen Pouwels, Kerstin Guhl, Noor Kornet) for stimulating discussions and shared experiences outside the lab. Jan and Peter, the unique candour with which you made time for the HC-Pro blues will always be treasured with deep reverence. I wish to thank all members of the embryogenesis-, nodule- and tomato groups for all the shared work experiences and acknowledge timely assistance from Dr. Rene Geurts, Dr. Valerie Hecht, Gustavo Gualtieri, Paul Passarinho and Khalid Shah during the course of the work. Khalid, thanks for sharing your unprecedented aura of exuberance. Boudewijn van Veen is thanked for the constant assistance with the computer network; Tony van Kampen and Marijke Hartog for kind assistance with sequence work; Gerard Moerland for assistance with pictures; Marie-Jose van Iersel and Maria Augustijn for kind assistance with logistics of my stay in Wageningen; Piet and Gerrit for assistance with daily material requirements.

Sincere gratitude is expressed to The Netherlands Foundation for the Advancement of Tropical Research (WOTRO) for funding this work. This thesis was printed by GSC Van Gils b. v. and reproduction costs subsidised by Wageningen University. During the course of this study I had

the privilege to participate in the training programmes of the Graduate School Experimental Plant Sciences (EPS).

At the University of Zimbabwe I am indebted to Prof. S. B. Feresu of the Department of Biological Sciences and Dr. A. I. Robertson of the Department of Crop Science for support and advice over the years, and colleagues in Dr. I. Sithole-Niang's lab in the Department of Biochemistry for helpful discussions.

Sincere appreciation is extended to fellow Zimbabwean, Kenyan, Malawian, South African, Ugandan and Ghanaian colleagues in Wageningen for keeping me in touch with the African experience during the last four years.

Final tribute is paid to my parents to whom this thesis is dedicated, and all my siblings and their families, with whom I share a warm sense of destiny.

Sizo

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OUTLINE OF THE THESIS

Cowpea aphid-borne mosaic virus (CABMV) is an economically important pathogen of cowpeas worldwide and moreso in semi-arid regions of Africa where the significance of cowpea as a food source can not be overstated. At the Department of Biochemistry of the University of Zimbabwe, we are interested in gaining more insight into the molecular properties of CABMV, an understanding of which will benefit ongoing attempts to reduce the impact of the virus on crop yields.

In 1997 the Netherlands Foundation for the Advancement of Tropical Research (WOTRO) of the Netherlands Research Organisation, NWO, granted financial support for a joint research project proposal of the Department of Biochemistry of the University of Zimbabwe (Dr. I. Sithole-Niang) and the Laboratory of Molecular Biology of Wageningen University (Prof. Dr. A. van Kammen) entitled "Characterisation of the helper component-proteinase from cowpea aphid-borne mosaic virus". This subsidy made possible my appointment as a PhD student for four years. In this thesis the results of the research of this project are reported.

In **chapter 1,** a historical overview of CABMV as an important pathogen of cowpeas, the progress made in the understanding of the taxonomic status of the virus, its genome organisation, diversity, epidemiology, and natural sources of resistance to the virus are presented which puts the current research initiative in its perspective.

CABMV is a member of the genus *Potyvirus* in the family Potyviridae. The potyvirus monopartite RNA genome has a length of about 10 kb and encodes a large polyprotein from which 10 mature proteins are cleaved. Of the 10 virus specific proteins, the so-called helper-component proteinase (HC-Pro) is of special interest as it is a multifunctional protein that has roles in different steps of the potyvirus life cycle and appears to be the major determinant of the pathogenicity of potyviruses. The prime focus of the investigations presented in this thesis is the characterisation of the HC-Pro protein of CABMV.

As prerequisite for further characterisations, the cloning and sequence analysis of the coding region of the HC-Pro of CABMV were carried out and this is described in **chapter 2.** Comparisons of the encoded protein with corresponding HC-Pro proteins of other potyviruses are also presented and conserved motifs with demonstrated and proposed functions are identified.

Further detailed molecular characterisation of CABMV including the molecular mechanisms underlying most HC-Pro-mediated events will benefit from elucidation of the complete genomic sequence of the virus. So far only limited genomic sequence information had been determined for

3' proximal regions of CABMV isolates thus constraining the scope of molecular studies. Our research on the cloning of HC-Pro had also produced further clones for the larger part of CABMV RNA. In chapter 3 it is discussed how we have prepared some additional clones and then were able to determine the sequence of 9 465 nucleotides which constitutes the major part of the CABMV genome. The results were used to design a genomic map of CABMV and the results of alignments with other potyviral genomes are presented.

In **chapter 4** we describe how a specific antiserum against the HC-Pro of CABMV was obtained and used for immunolocalisation of the protein in plant cells using at the one hand a transient plant expression system and at the other hand an expression system based on cowpea mosaic virus (CPMV). In addition, we have used a green fluorescent protein (GFP) fused to HC-Pro as a reporter of the subcellular location of the protein. In this chapter we also describe the striking effect of CABMV HC-Pro on the pathogenicity of CPMV.

In **chapter 5** we describe how we have further examined the features of HC-Pro as a determinant of virus pathogenicity. *N. benthamiana* plants were engineered to express HC-Pro sequences and used to study the effects of HC-Pro on the parental CABMV isolate and heterologous viruses, and to explore prospects for HC-Pro derived resistance against CABMV.

In the final **chapter 6**, the results of our research and the conclusions that can be drawn are summarised together with some suggestions for further research.

CHAPTER 1

COWPEA APHID-BORNE MOSAIC VIRUS: GENERAL INTRODUCTION

Mlotshwa, S., Sithole-Niang, I., Van Kammen, A. and Wellink, J.

Cowpea aphid-borne mosaic potyvirus (CABMV) is the causative agent of a serious disease in cowpea, an important legume crop in semi-arid regions of Africa. The virus causes severe crop losses each year either alone or in combination with other viruses. CABMV is readily transmissible by mechanical inoculation and by several common species of aphids in a non-persistent manner. It is also seed-borne in cowpea and has a wide geographical distribution, hence the most widespread viral pathogen of cowpeas worldwide.

1.1 COWPEA *(VIGNA UNGUICULATA)* AS FOOD SOURCE

Cowpea, also called southern pea or black eye pea, is one of the world's most important grain legume crops (Thottapily and Rossel, 1992). It has been cultivated for many centuries in the developing world, with Africa currently producing two thirds of the world's total cowpea production. In Zimbabwe nearly 90% of the small scale farmers produce cowpea for both their consumption and sale at local and urban markets. The crop is particularly important as social and monetary currency for rural women since they are the main producers and processers of cowpea in many countries. The crop is of great subsistence value due to its drought tolerance and high nutritional value. Like all legumes it can, in symbiosis with *Rhizobium*, fix nitrogen and hence grow on nitrogen deficient soils. Including cowpea in crop rotation schemes thus leads to improved soil restoration. Cowpea is an early maturing crop and provides 24 % vegetable protein and 62 % soluble starch in the human diet. As a food source it is taken in the form of cooked grain, fresh green pods or tender fresh leaves. However, despite the high yield potential of cowpea, the actual yield on small scale farms is far much less. This trend is attributed to several factors which include poor soils, drought or excessive moisture, weeds, insects and other pests, bacterial and virus infections. Of at least 20 cowpea-infecting viruses reported to-date (Thottapily and Rossel, 1985, 1992; Allen *et al.*, 1998), CABMV causes the most widespread virus disease. Yield losses of 15-87 % have been attributed to infection by CABMV in Iran (Kaiser and Mossahebi, 1975) and 48-60 % in Zambia (Kannaiyan and Haciwa, 1993). Complete loss of an irrigated cowpea crop in Nigeria was tentatively attributed to CABMV infection (Raheja and Leleji, 1974).

1.2 ORIGINAL IDENTIFICATION OF CABMV

CABMV was first reported as a distinct virus species infecting cowpea in Italy by Losivolo and Conti (1966). Since then virus isolates with properties similar to CABMV have been reported from various countries. Earlier attempts to verify the taxonomic status of the strains was frought with errors and misidentification of isolates owing to overlapping host ranges, similar symptomatology and serological cross reactions (Taiwo *et al.*, 1982; Taiwo and Gonsalves, 1982). For instance this prompted some workers to regard black eye cowpea mosaic virus (BlCMV), earlier described by Anderson (1955), and CABMV as the same virus (Bock and Conti, 1974; Dijkstra *et al.*, 1987). The confusion was further exacerbated as the original isolates were not available for direct comparison, and workers compared their isolates only on the basis of the original descriptions (Shukla *et al.*, 1994). Further investigations employing antisera to N-terminal peptide domains of coat proteins and peptide profiling of coat-protein tryptic digests by high perfomance liquid chromatography (HPLC) gradually supported the distinction of BlCMV and CABMV as separate viruses (Lima *et al.*, 1979; Dijkstra and Khan, 1992; Huguenot *et al.*, 1993; McKern *et al.*, 1994). However, a distinction between the strains of these viruses could still not be sufficiently drawn on the basis of biological and conventional serological criteria alone (Dijkstra and Khan, 1992). Recently, the increased knowledge of molecular sequences has contributed greatly in differentiating between viruses and their strains (Ward and Shukla, 1991; Atreya, 1992; Rybicki and Shukla, 1992; Berger *et al.*, 1997). On the basis of sequence comparisons, BlCMV represented by the Florida isolate and CABMV represented by the Moroccan isolate (Fischer and Lockhart, 1976) have been confirmed as distinct viruses, with BlCMV now proposed as bean common mosaic virus, blackeye cowpea strain (BCMV-BlC) (McKern *et al.*, 1992; Khan *et al.*, 1993). In the absence of the original isolate of Losivolo and Conti, the Moroccan isolate (CABMV-Morocco) (Fischer and Lockhart, 1976), formerly designated as BlCMV-Mor, has become the type isolate of CABMV (Shukla *et al.*, 1994), and some isolates previously misidentified as CABMV including the Nigerian and Kenyan isolates (Bock, 1973) have been established to be BCMV isolates (Taiwo and Gonsalves, 1982; Taiwo *et al.*, 1982).

1.3 HOST RANGE AND SYMPTOMATOLOGY

CABMV has a wide host range covering at least 19 species in 13 genera and 6 families: Leguminosae, Amaranthaceae, Chenopodiaceae, Curcubitaceae, Labiatae and Solonaceae (Losivolo and Conti, 1966; Bock and Conti, 1974). Some useful diagnostic species include *Chenopodium amaranticolor, Gomphrena globosa, Glycine max, Ocimum basilicum, Petunia hybrida, Phaseolus vulgaris and Pisum sativum* (Bock and Conti, 1974; Fischer and Lockhart, 1976; McKern *et al.*, 1994). The virus can be best propagated and maintained on cowpea, soybean, and *Nicotiana benthamiana* and assayed on *C. amaranticolor* as a local lesion host (Losivolo and Conti, 1966; Bock and Conti, 1974; Fischer and Lockhart, 1976). The expression and severity of symptoms due to infection by CABMV largely depends on host cultivar, virus strain and time of infection (Rossel and Thottapily, 1985; Thottapily and Rossel, 1985; Allen *et al.*, 1998). The symptoms include a severe mosaic, dark green vein-banding, leaf distortion, blistering and stunting (Losivolo and Conti, 1966; Bock and Conti, 1974; Fischer and Lokhart, 1976). Interveinal chlorosis and mottling also occur (Allen *et al.*, 1998). Systemic necrosis has been reported in some cowpea cultivars in the field (Kannaiyan and Haciwa, 1993; Allen *et al.*, 1998). On important crop plants such as cowpeas, symptom expression is accompanied with extensive deterioration in agronomic performance (Kaiser and Mossahebi, 1975; Raheja and Leleji, 1974; Kannaiyan and Haciwa, 1993).

1.4 TAXONOMY

CABMV is a member of the genus *Potyvirus* in the family Potyviridae, within the picorna-like supergroup (Goldbach, 1986, 1987; Shukla *et al.* 1994), along with at least 200 distinct species most of which are economically important plant pathogens (Ward *et al.*, 1994). CABMV consists of flexuous, filamentous rod-shaped particles with a length of about 750 nm (Bock and Conti, 1974, Lima *et al.*, 1979). It induces the formation of the pin wheel cylindrical inclusions in the cytoplasm of infected cells, a feature shared by all potyviruses and constituting the single most important phenotypic criterion for assigning viruses into this group (Ward and Shukla, 1991). However, with the advent of molecular techniques, the sequences of 3' non-translated regions (NTRs) and coat proteins (CPs) have been extensively used in potyviral molecular phylogenetics

(Ward and Shukla, 1991; Atreya, 1992; Rybicki and Shukla, 1992; Berger *et al.*, 1997) of which distinct potyviruses show 38-71 % sequence identity in their coat proteins, whereas this is greater than 90 % among strains belonging to the same virus (Khan *et al.*, 1993; Ward *et al.*, 1994). On the basis of CABMV 3' proximal partial sequences reported to-date (Brand *et al.*, 1993; McKern *et al.*, 1994; Sithole-Niang *et al.*, 1996; Pappu *et al.*, 1997), CABMV has been more precisely classified as a member of the bean common mosaic virus (BCMV) subgroup of legume-infecting potyviruses. This corroborates the original classification of CABMV in this subgroup along with BCMV, zucchini yellow mosaic virus (ZYMV), bean necrosis mosaic virus (BNMV), soybean mosaic virus (SMV) and passion fruit woodiness virus (PWV) (Ward *et al.*, 1992; Dijkstra and Khan, 1992; McKern *et al.*, 1994) on the basis of symptomatology, host range and serological relationships.

1.5 GENOME ORGANISATION

The genomic sequence of CABMV has more recently been determined (unpublished). The genome consists of a linear, single stranded, positive sense RNA molecule about 9.465 kb long, and like all 21 or more distinct potyvirus species with documented genomic sequences [for review see Shukla *et al.* (1994)], it has a coding region characterised by a continuous, large single open reading frame (ORF) flanked by 5' and 3' NTRs. The translated ORF of CABMV has conserved proteolytic cleavage sites identified in other potyviruses. This suggests that it is expressed as a large precursor polyprotein from which 10 mature proteins are derived through co- and post translational cleavage as has been experimentally demonstrated by *in vitro* translation studies in other potyviruses (Carrington *et al.*, 1990). The genes encoding the proteins have been delineated for CABMV and are conserved in an order similar to all studied potyviral genomic RNAs. By analogy to other potyviruses, the gene products include, proceeding from the N- to C-terminus of the polyprotein, the first protein (P1), the helper component-proteinase (HC-Pro), the third protein (P3), the first 6 kDa protein (6K1), the cylindrical inclusion protein (CI), the second 6 kDa protein (6K2), the VPg domain of the nuclear inclusion protein a (NIa-VPg), the proteinase domain of the nuclear inclusion protein a (NIa-Pro), the nuclear inclusion protein b (NIb) and the CP (Dougherty and Carrington, 1988; Shukla *et al.*, 1994). The demonstrated and proposed

functions of potyviral gene products, last reviewed by Riechmann *et al.* (1992), are summarised in table 1.

Table 1: Proposed and demonstrated functions of gene products

Protein	Function
P1	polyprotein processing; genome amplification
HC-Pro	polyprotein processing; aphid-mediated transmission; genome amplification; cell-to-cell and long-distance movement; transactivation of replication of heterologous viruses; suppression of post transcriptional gene silencing (PTGS)
P3	cofactor in polyprotein processing
6K1	genome amplification
CI	RNA helicase; ATPAse activity; attachment of replication complexes to membranes; cell-to-cell movement
6K2	genome amplification; intracellular transport of NIa
NIa	polyprotein processing; its VPg domain primes genome replication and has roles in subcellular transport and as host genotype-specific long distance movement factor
NIb	replicase (RNA-dependent-RNA-polymerase)
CP	RNA encapsidation; aphid-mediated transmission; cell-to-cell and long-distance movement

1.6 STRAIN DIVERSITY

Following the first reports of CABMV (Losivolo and Conti, 1966), more isolates have since been identified (Bock, 1973; Fischer and Lockhart, 1976; Brand *et al.*, 1993; Sreenivasulu *et al.*, 1994; Huguenot *et al.*, 1996; Sithole-Niang *et al.*, 1996). At least 14 isolates identified in Africa, where the virus is endemic, have been shown to be distributed among six serotypes designated I (CABMV-Monguno; CABMV-Baga), II (CABMV-Fekan; CABMV-Nkechi's; CABMV-Kano 5; CABMV-IITA3), III (CABMV-70.12), IV (CABMV-Morocco; CABMV-87/14 SA; CABMV-SAPV 92/749), V (CABMV-Maputo; CABMV-931510 SA) and VI (CABMV-Moz 11;

CABMV-IITA 4; CABMV-IITA 5) (Huguenot *et al.*, 1993, 1996). The serodiversity attests to the high variability of epitopes in the coat protein and is consistent with the heterogeneity of CABMV isolates revealed by HPLC analysis of CABMV tryptic peptides (Huguenot *et al.*, 1994).

The identity of some strains has further been authenticated on the basis of their 3'-proximal sequences. These include the Moroccan isolate (CABMV-Mor: Y18634/Af083558) (Fischer and Lockhart, 1976; van Boxtel *et al.*, 2000); the Zimbabwe isolate (CABMV-Z: X82873) reported by Sithole-Niang *et al.* (1996); CABMV-GA (U90326) initially referred to as sesame mosaic virus (SeMV) (Sreenivasulu *et al.*, 1994, Pappu *et al.*, 1997); CABMV-SAP (D10053) (Brand *et al.*, 1993); CABMV-Monguno (Y17822); CABMV-70.12 (Y17824) and CABMV-Ibadan (AJ132414). The unpublished CABMV-TH partial sequence (U72204) of a potyvirus from Thailand shows 99.1 % identity with BCMV-BlC and has thus been established to be a strain of BCMV (van Boxtel *et al.*, 2000).

The comparisons of the core CP sequences of these authenticated CABMV strains revealed a division into subgroups comprising CABMV-GA, -Z and -SAP on one hand and -Morocco, -Ibadan and -Monguno on the other, with significant variability in levels of identity (83-97 %) amongst the strains (van Boxtel *et al.*, 2000). This further corroborated the heterogeneity of CABMV strains previously revealed by serology and HPLC analysis (Huguenot *et al.*, 1993, 1994, 1996). However, while the heterogeneity among CABMV strains seems consistent, their apparent clustering could be a function of limited sequence data than a reflection of true clusters (Berger *et al.*, 1997). It is thus conceivable that as new CABMV isolates continue to be identified and sequenced, the putative clusters would be either re-affirmed or replaced by a continuum of sequences representing the natural heterogeneity within CABMV as suggested for BCMV strains by Berger *et al.* (1997).

1.7 EPIDEMIOLOGY

CABMV has been reported in all cowpea-producing areas worldwide (Allen *et al.*, 1998). These include Africa, India, Indonesia, China, Japan, Iran, Australia, Brazil, USA, The Netherlands, Cyprus, Papua New Guinea and Sri Lanka (Taiwo *et al.*, 1982; Dijkstra *et al.*, 1987). In Africa where the virus is considered endemic, it has been reported in various countries including

Nigeria, Morocco, Zimbabwe, Botswana, Tanzania, Cameroon, Uganda, Egypt, Togo, Mozambique [Reviewed by Thottapily and Rossel, (1985)]. A differential geographical distribution of 6 African serotypes has been reported: serotypes I, II and III are prevalent in West and East Africa, serotypes IV, V and VI have been detected in Southern Africa (Huguenot *et al.*, 1993, 1996).

CABMV is readily transmitted by sap inoculation, and in a stylet-borne, non-persistent manner by several common species of aphids (Atiri *et al.*, 1986). These include *Aphis craccivora* (Koch), *A. fabae* (Scopoli), *A. gossypii* (Glover), *A. spiraecola, A. medicaginis* (Koch), *A. citricola* (van der Groot), *Macrosiphum euphorbiae* (Thomas), *Myzus persicae* (Sulzer), *Rhopalosiphum maidis* (Fitch) *and Cerataphis palmae* (Boisduval) (Bock and Conti, 1974; Thottapily and Rossel, 1985; Atiri *et al.*, 1986; Roberts *et al.*, 1993). It is also seed-borne in cowpea, with transmission rates as high as 40 % (Kaiser and Mossahebi, 1975) depending on the strain and cowpea cultivar (Lapido, 1977; Thottapily and Rossel, 1985). This offers means for distribution of the virus to disparate localities either as infected cowpea seedlots, variety trials, or germplasm (Huguenot *et al.*, 1996; Hampton *et al.*, 1997; Allen *et al.*, 1998). This is supported by the close relatedness between some isolates of distinct geographical origin (van Boxtel *et al.*, 2000), with CABMV-Morocco (Northen Africa) and CABMV-Ibadan (Western Africa) displaying 100% identity, and CABMV-Z (Southern Africa) and CABMV-GA (USA) showing 99.1 % identity in their CP core amino acid sequences (van Boxtel *et al.*, 2000). In Africa CABMV is considered the most widespread and important virus disease of cowpeas (Rossel and Thottapily, 1985; Huguenot *et al.*, 1996).

1.8 CONTROL PRACTICES AND NATURAL RESISTANCE

The control of virus diseases is important for increasing food production in the tropics (Singh *et al.*, 1992). The prerequisite for any effective control initiative is reliable and rapid identification of virus strains. However, the apparent heterogeneity of CABMV hinders the development of a general serodiagnostic test for the virus (Huguenot *et al.*, 1993, 1994). Nevertheless, at least 6 African serotypes have been shown to be detectable using a single combination of monoclonal antibody (mAb) (5H5/6F9) (Huguenot *et al.*, 1993, 1994, 1996). This offers a rapid and reliable means for routine detection of CABMV in Africa (Huguenot *et al.*, 1996). This should benefit

quarantine measures and seed indexing in the control of CABMV which, because of its seed transmissibility, is introduced via germplasm exchange in new localities.

Attempts to reduce CABMV infections include cultural practices such as early sowing, intercropping of cowpeas with cereals, roguing and the use of virus-free seed (Kannaiyan and Haciwa, 1993). Some cowpea lines (K-39, L-868, L-1552 and P 1476) are known to be unable to sustain seed transmission despite being susceptible to CABMV infections (Mali *et al.*, 1983). Thus selection for resistance to seed transmission offers additional means of eliminating seed-borne virus (Allen *et al.*, 1998). The alternative use of chemicals eliminates aphid vectors or restricts acquisition and inoculation of the virus (Atiri *et al.*, 1987). For instance, synthetic pyrethroid cypermethrin has been found to protect against transmission of the virus (Atiri *et al.*, 1987). The utility of meristem tip culture and other tissue culture methodologies in clonal propagation of virus-eliminated planting material for dissemination to commercial and communal farmers could be hampered by high rates of re-infection of virus-cleaned clones in the field. However, to avert the ecologically harmful effects of chemicals and problems of high rates of re-infection of virus-cleaned clones, development of resistance to virus infection through breeding or genetic engineering offers possibilities for a long term solution to yield losses. At the IITA screening of cowpea accessions for natural virus resistance and subsequent selection and dissemination of resistant varieties as source of planting material is one of the co-activities.

The identification and development of improved genotypes with resistance to viral infection has been reported (Singh and Ntare, 1985; Thottapily and Rossel, 1985; Lima *et al.*, 1979; Mali *et al.*, 1981; Patel *et al.*, 1982). Sources of resistance to CABMV infection have been identified in cowpea germplasm from diverse localities including India (Mali *et al.*, 1981), USA (Taiwo *et al.*, 1982), Nigeria (Lapido and Allen, 1979), Brazil (Lin *et al.*, 1981) and Iran (Kaiser and Mossahebi, 1975). Natural resistance is expressed as an immunity specified by a single dominant or recessive gene, and also as development of very mild symptoms with no impact on plant growth, and by tolerance in which no symptoms are associated with systemic infection (Lapido and Allen, 1979; Patel *et al.*, 1982). Several cowpea lines with isolate-specific resistance (e.g Tvu 1593) have been identified (Allen *et al.*, 1998). Lines possessing resistance to a range of isolates of disparate geographical origin have also been reported (Lapido and Allen., 1979; Patel *et al.*, 1982). This includes lines TVu 1582 and TVu 401 from the IITA which have been shown to be

resistant to at least 8 African CABMV isolates (Huguenot *et al.*, 1993). Additionally, several lines combining resistance to CABMV and other distinct viruses including cowpea mosaic virus (CPMV), cowpea golden mosaic virus (CGMV), cucumber mosaic virus (CMV) and southern bean mosaic virus (SBMV) have been identified (Allen, 1983) and improved through conventional breeding (Singh *et al.*, 1997). These include IITA varieties designated IT82D-889, IT83S-818, IT83D-442, and IT85F-867-5, IT81D-1137, IT82E-16, IT84D449 and IT85F-2687 (Singh *et al.*, 1992, 1997). To further enhance breeding for resistance to CABMV progress has been made in recent efforts to develop linkage maps for cowpea based on molecular markers (Fatokun *et al.*, 1997).

Although breeding for resistance against CABMV remains the most feasible measure for controlling the virus, this is nonetheless confounded by the presence of numerous distinct strains, with separate cowpea genes conferring resistance to each strain (Ndiaye *et al.*, 1993). New cowpea lines bred specifically for CABMV resistance were reported to succumb to infections with coexisting pathogenic variants of the virus in the same region (Ndiaye *et al.*, 1993). This complicates the strategy for resistance breeding by creating the need for several gene sources for the development of suitable cultivars (Singh *et al.*, 1992). 'Pyramiding' engineered virus resistance and natural resistance could overcome this problem and increase the range and stability of the resistance (van Boxtel *et al.*, 2000). However, this requires a good understanding of the molecular biology of the virus. Recent progress made in the development of detection methods for CABMV, mapping of its geographical distribution and identification of sources of natural resistance has yet to be complemented with detailed molecular characterisation of the virus. This would provide better understanding of the molecular aspects of the virus-vector, virus-host and virus-virus interactions with respect to aphid transmission, replication, movement and synergy needed to effectively exploit the full potential of pathogen-derived resistance.

CHAPTER 2

CLONING AND SEQUENCE ANALYSIS OF THE HELPER COMPONENT-PROTEINASE OF A ZIMBABWE ISOLATE OF COWPEA APHID-BORNE MOSAIC VIRUS

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Summary

The potyvirus helper component-proteinase (HC-Pro) is a multifunctional protein involved in aphid-mediated transmission, polyprotein processing, genome amplification, long-distance movement and transactivation of replication of heterologous viruses. As prerequisite for further characterisations, we have cloned and sequenced the HC-Pro coding region of a Zimbabwe isolate of cowpea aphid-borne mosaic virus (CABMV-Z). This involved a combined "genome-walking" and direct cloning strategy in reverse transcription and polymerase chain reaction (RT-PCR) with degenerate and/or specific primers. The N- and C-termini were determined by analogy to consensus sequences in other potyviruses. The sequence comprises an open reading frame (ORF) of 1 368 nucleotides encoding 456 amino acids with Mr 51.9 kDa. Alignment of the amino acid sequence with those of other potyviruses identified conserved sequence motifs common amongst members of this group. Overall amino acid sequence identity compared with other potyviral HC-Pros is between 38 and 75 %, with central and C-terminal regions more conserved than the N-terminal region. CABMV-Z HC-Pro displays the highest amino acid sequence identity with sequenced members of the bean common mosaic virus (BCMV) subgroup of potyviruses, but appears to be a distinct potyvirus species within this subgroup.

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1. Introduction

Cowpea aphid-borne mosaic virus (CABMV) infects cowpea (*Vigna unguiculata*) with yield losses of up to 100 % in some parts of Africa (Thottapily and Rossel, 1992). CABMV is a member of the genus *Potyvirus* in the family *Potyviridae*, which is the largest and economically most important group of plant viruses, with at least 200 distinct species (Ward *et al.*, 1994).

Complete genomic sequences of at least 20 distinct potyviral species have been documented (for review see Shukla *et al.*, 1994). The potyvirus genome consists of a linear, single-stranded and positive sense RNA molecule. It is encapsidated in a coat of single species of protein subunits to form the characteristic flexuous rod-shaped potyviral particle. The genomic RNA is about 10 kb long, has a covalently linked 5' terminal viral protein (VPg) and a 3'-terminal polyadenylate tract. The coding region is characterised by a continuous, large single open reading frame (ORF) flanked by 5' and 3' non-translated regions (NTRs). The ORF is expressed as a large precursor polyprotein from which 9 mature proteins are derived through co- and post translational cleavage. The genes encoding the proteins are conserved in the same order on studied potyviral genomic RNAs. Proceeding from the N- to C-terminus of the polyprotein, the gene products include the first protein (P1), the helper component proteinase (HC-Pro), the third protein (P3), the first 6 kDa protein (6k1), the cylindrical inclusion protein (CI), the second 6 kDa protein (6K2), the VPg domain of the nuclear inclusion protein a (NIa-VPg), the proteinase domain of the nuclear inclusion protein a (NIa-Pro), the nuclear inclusion protein b (NIb) and the coat protein (CP). The demonstrated and proposed functions of the gene products are reviewed by Riechmann *et al.* (1992).

Like many potyviral species CABMV is transmitted non-persistently by aphids and through sap inoculation. It is also seed transmissible with rates as high as 40% (Huguenot *et al.*, 1996). Symptoms due to CABMV infection include a severe mosaic, leaf distortion, blistering and stunting (Losivolo and Conti, 1966). Establishment of viral infections involves cell-to-cell movement followed by long distance spread of progeny virions later in infection. For most viruses this process depends on specific virus-encoded proteins, the so-called movement proteins (MPs) (for a recent review see Carrington *et al.*, 1996). A detailed description of potyviral movement functions in infected plants is still lacking, nor has a potyviral gene product with

dedicated movement functions been identified. However genetic and biochemical analyses have recently revealed the dispersion of MP functions among several gene products including the HC-Pro (Cronin *et al.*, 1995; Kasschau *et al.*, 1997; Rojas *et al.*, 1997). In addition to its previously established proteolytic activity and mediation of aphid transmission, other functions recently attributed to HC-Pro include genome amplification (Kasschau *et al.*, 1997) and transactivation of replication of heterologous viruses (Shi *et al.*, 1997). In spite of significant progress recently made concerning the delineation of the functional domains of HC-Pro, little is yet known about the molecular mechanisms underlying most HC-Pro-mediated events (Maia *et al.*, 1996).

We have initiated the cloning and sequencing of the HC-Pro of a Zimbabwe isolate of CABMV (CABMV-Z). This is part of a long term strategy to characterise the gene, study in detail its functions and genetically engineer HC-Pro mediated resistance against CABMV in cowpea. Previously, the sequence of 3'-terminal 1 221 nucleotides spanning the 3' NTR, CP and part of NIb has been reported (Sithole-Niang *et al.*, 1996) and the sequence of full-length NIb (1 550 nucleotides) and part of NIa (327 nucleotides) has recently been determined (Sithole-Niang, person. comm). To clone the HC-Pro we adopted a "genome-walking" strategy combined with direct cloning attempts, using reverse transcription followed by polymerase chain reaction (RT-PCR) with degenerate and specific primers. Sequence analysis of the HC-Pro of CABMV-Z confirmed its taxonomic status as a distinct potyvirus species within the bean common mosaic virus (BCMV) subgroup.

2. Materials and Methods

2.1. CABMV-Z isolate

The isolate of CABMV used in this work was originally collected from infected cowpea (*Vigna unguiculata*) plants grown in a breeder's plot at Gwebi farm, Harare, Zimbabwe (Sithole-Niang *et al.*, 1996). The virus has been maintained through a series of passages on the susceptible cowpea cultivar (California black eye #5) and *Nicotiana benthamiana* by mechanical inoculation. Purified virus, isolated as described for the sweet potato potyvirus from Zimbabwe (SPV-Z) by Chavi *et al.* (1997), was a kind gift from Dr. B. J. M. Verduin (Laboratory of Virology, Wageningen University and Research Centre, The Netherlands).

2.2. Total RNA extraction and cDNA synthesis

Total RNA extractions from CABMV-Z infected plants were carried out with the TRIzol™ Reagent (Total RNA Isolation Reagent) (GIBCO BRL) as described in the protocol supplied by the manufacturer.

First strand cDNAs were synthesised with MMLV reverse transcriptase (Gibco BRL) using purified virus particles as described by Chachulska *et al.* (1997), or with AMV reverse transcriptase (Finnzymes) using total RNA templates. In the MMLV reaction, 10 µg of purified virions were mixed with 1 µg of oligodT p33 (see table 1) in a 10-µl volume, heated 2 min at 50 °C and chilled on ice for 5 min. The remaining components were added: 1.25 mM dNTPs, 20 U RNAsin, 5 mM DTT, 1 X first strand buffer (25 mM Tris-HCl pH 8.3; 75 mM KCl; 3 mM $MgCl_2$) and 200 units of MMLV-RT in a reaction volume of 20 µl. The reaction was incubated at 37°C for 1 h and kept on ice or at -20 °C.

In the AMV-RT reaction, 5 µg total RNA was mixed with 1 µg of primer CI^- or $NIa1^-$ (Table 1) in water, heated 2 min at 70 °C and chilled on ice for 5 min. The reaction components were added: 1 mM dNTPs, 20 U RNAsin, 4 µg BSA, 40 U AMV-RT, 10 X AMV-RT buffer (25 mM Tris-HCl pH 8.3; 50 mM KCl; 5 mM $MgCl_2$ and 2 mM DTT) in a reaction volume of 20 µl. The reaction was incubated at 45 °C for 1 h and kept on ice or at -20 °C.

2.2.1 Primers

OligodT p33 was designed to anneal to the 5' end of the poly A tract. Oligos 2 and 3 flank the HC-Pro and were respectively designed from the conserved P1 FIVRG motif and consensus HC-Pro cleavage site KXYVG/G for the direct cloning strategy. Primers CI^+ and $HC2^+$ were designed for respective use with nested primer sets $NIa1^-$ and 2^-, and $CI1^-$ and 2^- in the "genome walking" strategy. CI^+ was designed from the conserved VATNIIENGVTL motif in the CI central domain, $HC2^+$ from the AELPRILVDH motif in the HC-Pro C-terminal region. Primers hcprofor and hcprorev were designed to flank the HC-Pro for eventual subcloning.

Table 1. Synthetic oligonucleotide primers

Primer name	Polarity	sequence (5 -> 3')
(a) specific primers designed from the CABMV-Z sequence		
p33 (oligodT)	minus	GCCGTCTAGA$(T)_{21}$A
NIa1-	minus	CACTGACACCAATGGTAAACCACA
NIa2-	minus	ATCCCCATCCTGTGTTGAAATCCA
CI1-	minus	GGCTTGTGCCTACCCACACGTCC
CI2-	minus	CTGGATCCTCTCACCGTAGCTCAC
hcprorev	minus	TCATCCTTTGTTGCACACTCCCACCAAC
hcprofor	plus	ATAAATGCCCTTGACTGGTGCAGCGAAC
(b) degenerate primers designed from potyviral conserved regions[1]		
oligo2	plus	GCGGTCGACTTYATHGTNAGRGGNA
oligo3	minus	GCGTCTAGANCCNCCNCYCTRWRYT
CI^+	plus	GCMACIAAYATIATIGARAAYGG
$HC2^+$	plus	GCIGARYTICCIMGIATWYTRGTKGAYCA

[1]IUB codes are used for mixed base sites

2.3. PCR amplification

Taq DNA polymerase (Boehringer Mannheim) and VENT DNA polymerase (NEB) were used in PCR reactions. The 50 µl reactions contained 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.25 µg each of reverse and forward primer (see Table 1), 3 µl cDNA mix, 1 U Taq or vent DNA polymerase, 1 X Taq buffer (10 mM Tris-HCl pH 8.3; 1.5 mM $MgCl_2$ and 50 mM KCl) or vent buffer [10 mM KCl; 10 mM $(NH_4)_2SO_4$; 20 mM Tris-HCl pH 8.8; 2 mM $MgSO_4$; 0.1% Triton X-100]. Amplifications were carried out in a Perkin Elmer thermocycler with the following conditions: 94 °C for 5 min (during which the enzyme was added); 35 cycles including a denaturation step at 94 °C for 30 sec, an annealing step from 50 °C to 65 °C for 1 min, and extension at 72 °C for 2 min. The PCR reactions were terminated with a final extension step at 72 °C for 5 min.

2.4. cDNA cloning

The PCR products were resolved by electrophoresis on 1.0 % agarose gel with ethidium bromide staining. Bands of the expected length were excised and purified from the agarose using glass milk or High pure Plasmid isolation kit (Boehringer Mannheim). Purified PCR fragments were ligated into pGem-T cloning vector (Promega) according to the manufacturer's protocol and electrotransformed (Eurogentec electroporation unit) into *E. coli* DH5∂ for amplification of the constructs. Transformants were screened with ampicillin/Xgal blue-white selection with subsequent restriction analysis of plasmid DNA minipreps.

2.5. Nucleotide sequencing and sequence analysis

cDNA sequencing was performed using the automated Applied Biosystems 373 DNA sequencer (400 ng DNA per sequencing reaction) with universal T7 and SP6 sequencing primers. Both strands of each cDNA clone were sequenced. Nucleotide and amino acid sequence data were assembled and analysed using the Genetics Computer Group Program (GCG). The sequence data of other potyviruses were retrieved using the National Center for Biotechnology Information (NCBI) BLAST search facility and analysed using the DNASTAR program.

3. Results and Discussion

3.1 RNA extraction and cDNA synthesis

The use of high quality RNA is critical for the success of RT-PCR cloning. Extraction of total RNA from infected plants with TRIzol™ Reagent gave good yields of intact RNA free of protein and DNA contamination. Putative secondary structures on purified potyviral RNA tend to limit the processivity of reverse transcriptases in synthesis of long cDNAs. However, at a reaction temperature of 45 °C the cDNAs derived from total RNA using AMV-RT and primers NIa1⁻ and CI1⁻ were suitable for amplification of desired genomic regions in several experiments (see section 3.2). In some instances purified virions were used for the synthesis of cDNAs primed

with oligodT p33 and extended with MMLV-RT at 37 °C as described by Chachulska *et al.* (1997), and that also produced good templates in subsequent PCR reactions (see section 3.2).

3.2 Molecular cloning and sequencing

Initially, we attempted to clone the HC-Pro coding region directly using degenerate primers (oligos 2 and 3) on cDNA derived from total RNA with oligodT p33 but were unsuccessful. We then co-opted a "genome walking" strategy of stepwise cloning of PCR fragments which involved the use of degenerate primers designed from conserved potyviral regions in combination with nested specific antigenome-sense primers complementary to the 5' sequences of each preceding downstream cDNA clone (Fig. 1).

The proposed potyviral genomic map and the strategy used to clone CABMV-Z HC-Pro, along with the positions of different PCR clones is presented (Fig. 1). S1 represents the previously sequenced 3' terminal 1 221 nt (Sithole-Niang *et al.*, 1996), full length NIb (1 550 nt) and part of NIa (327 nt) (Sithole-Niang, person. comm). OligodT p33 was designed to anneal to the 5' end of the poly A tract. Two nested specific primers (NIa1$^-$ and NIa2$^-$) were designed complementary to the 5' end of s1. OligodT p33 was used to prime cDNA synthesis using virion templates, with subsequent extension with MMLV - RT at 37 °C. From this cDNA a fragment with the expected size of about 2.3 kb (clone s2) was amplified with primers NIa2$^-$ and CI$^+$. From the derived partial sequence of clone s2, a second 2-membered nested set of specific antigenome-sense primers (CI1$^-$ and CI2$^-$) were designed to anneal to the 5' end of the clone. Primer CI1$^-$ was used to prime cDNA synthesis using total RNA from infected leaves, with subsequent extension with AMV-RT at 45°C. From this cDNA a 2.7 kb product (clone s3) was reproducibly amplified with primers CI2$^-$ and HC2$^+$.

The derived partial amino acid sequence of clone s3 enabled us to delineate the C-terminus of CABMV-Z HC-Pro as it contained the consensus sequence KXYVG/G of the cleavage site. Meanwhile, continued attempts to directly clone HC-Pro with degenerate oligos 2 and 3 eventually yielded a 1 kb PCR product (clone s4), amplified using cDNA derived from total RNA primed with NIa1$^-$ and extended with AMV-RT at 45 °C. This however, turned out to be the result of a fortuitous nonspecific priming event which we cannot satisfactorily explain, as both

primers annealed upstream of motifs for which they were designed (Fig. 1). This clone spanned the N-terminus of HC-Pro as its derived amino acid sequence contained conserved residues QHY/S of the P1 cleavage site. Having delineated the HC-Pro junctions, HC-Pro specific primers were designed to anneal to the N-terminus (hcprofor) and C-terminus (hcprorev) for subsequent subcloning. Three independent RT-PCR subclones of HC-Pro (clone s5) were amplified with vent polymerase using cDNA derived from total RNA primed with CI1$^-$ and extended with AMV-RT at 45 °C. The sequences of the 3 independent clones exhibited 100 % identity. This was in conformity with the expected fidelity of vent polymerase associated with its 3 -> 5' proofreading exonuclease activity.

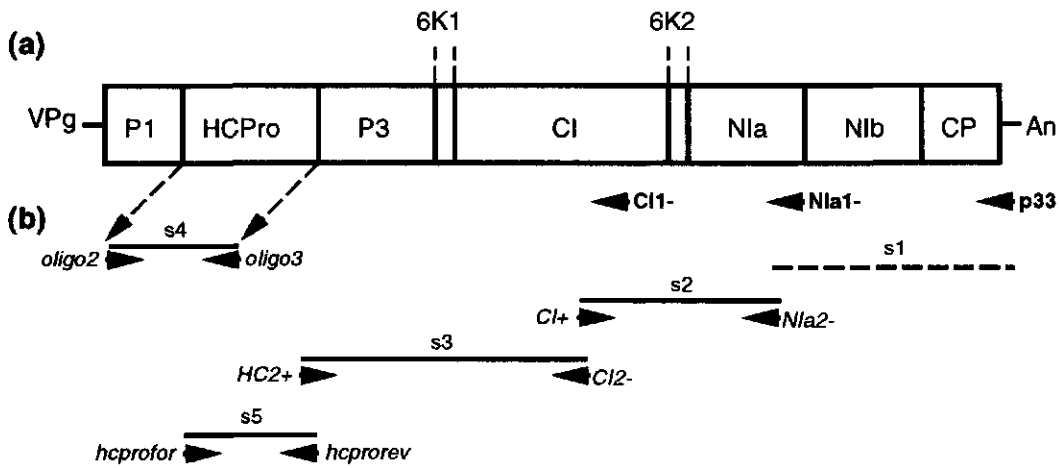

Fig. 1 Cloning strategy for CABMV HC-Pro.

(a) Potyviral genomic organisation. The open bar represents the ORF of the viral RNA. Vertical lines indicate proposed cleavage sites. An: poly (A) tail; VPg: genome linked protein.

(b) Position of the primers and PCR products. The previously sequenced region is shown by the dotted line designated s1. RT-PCR clones are designated s2, s3, s4 and s5. Primers p33, NIa1- and CI1-, used in cDNA synthesis, are shown in bold. Primers used for each amplification are given in italic. Dashed arrows show shift in annealing positions of oligos 2 and 3.

3.3 Analysis of CABMV HC-Pro sequence

3.3.1 Complete CABMV HC-Pro sequence

The complete CABMV-Z HC-Pro nucleotide sequence and deduced amino acid sequence is presented in Fig. 2.

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TCACATCAGTTGGAGGTTCAATTTTTCCAGGGTTGGAAGAAAGTATTTGACAAGCTTGTACCACGCACCCAGGATCATGAGTGCACTGTTGACTACA 97
 S  H  Q  L  E  V  Q  F  F  Q  G  W  K  K  V  F  D  K  L  V  P  R  T  Q  D  H  E  C  T  V  D  Y
GCAATGAGCAATGCGGTGAATTAGCAGCGTCATTGAGTCAAATTCTGTACCCAGTGAAGAAACTATCGTGTAGGCAATGCAGATTCCGCATCAAAGA 194
S  N  E  Q  C  G  E  L  A  A  S  L  S  Q  I  L  Y  P  V  K  K  L  S  C  R  Q  C  R  F  R  I  K  D
TTTGAGCTGGGAAGAATATAAGGAATTTGTTGCAACACACTTTGCATGCTGTGCAGAGACGCTCAAAGAACAACAGAGCGTTGGTTTCAAGAATGTG 291
  L  S  W  E  E  Y  K  E  F  V  A  T  H  F  G  C  C  A  E  T  L  K  E  Q  Q  S  V  G  F  K  N  V
CAAACACTCGTTGAGAGAGCAGTTAGCGAAGATGGAGATATGGAGTTGTCGCTTGAGATCATAAAATTAACACAGAATTACACGAGCACACCAATGC 388
 Q  T  L  V  E  R  A  V  S  E  D  G  D  M  E  L  S  L  E  I  I  K  L  T  Q  N  Y  T  S  T  P  M
TCCAAATTCAAGACATCAATAAGGCTCTCATGAAAGGTAGTTCTGCGAGCAAGCAAGAGCTTGACCAAGCACTCAAGCAGTTGCTAGCAATGACCCA 485
L  Q  I  Q  D  I  N  K  A  L  M  K  G  S  S  A  S  K  Q  E  L  D  Q  A  L  K  Q  L  L  A  M  T  Q
ATGGTGGAAGAATCACATGGATTTAACAGGGGAGGATGCTCTAAAGACTTTTAGAAACAAGAGAGCATCAAAAGCAATACTCAACCCAAGTTTACTT 582
  W  W  K  N  H  M  D  L  T  G  E  D  A  L  K  T  F  R  N  K  R  A  S  K  A  I  L  N  P  S  L  L
TGTGACAATCAGCTTGATAAGAATGGCAATTTCATTTGGGGAGAAAGAGGCAGGCATTCAAAACGATTTTTCTCAAACTTCTTTGAGGAAATTGTTC 679
 C  D  N  Q  L  D  K  N  G  N  F  I  W  G  E  R  G  R  H  S  K  R  F  F  S  N  F  F  E  E  I  V
CATCTGAAGGATATAGCAAGTACACAATCAGACGGAATCCAAATGGTCAAAGAAAGCTAGCTATTGGTTCTCTGGTCGTTCCACTTGACTTGGATAG 776
P  S  E  G  Y  S  K  Y  T  I  R  R  N  P  N  G  Q  R  K  L  A  I  G  S  L  V  V  P  L  D  L  D  R
AGCAAGAGTTTCAATGCAGGGTGAGGGTGTGGCAAGAAAACCACTGACAAAAGCATGTGTTTCGGTTCTTGAGAAAAACTTTGTTTACCCATGCTGC 873
  A  R  V  S  M  Q  G  E  G  V  A  R  K  P  L  T  K  A  C  V  S  V  L  E  K  N  F  V  Y  P  C  C
TGTGTTACATTAGACAATGGACAGCGTCTCTACTCAGAATACAAGAGTCCAACTAAGAGGCACTTAGTTGTCGGCTCATCAGGTGACCCCAAATACA 970
 C  V  T  L  D  N  G  Q  P  L  Y  S  E  Y  K  S  P  T  K  R  H  L  V  V  G  S  S  G  D  P  K  Y
TTGACCTTCCAGCAACTGACTCAGACCGGATGTACATTGCAAAGGAAGGATACTGTTATCTAAATATTTTCCTTGCAATGCTTGTGAACGTCAATGA 1067
I  D  L  P  A  T  D  S  D  R  M  Y  I  A  K  E  G  Y  C  Y  L  N  I  F  L  A  M  L  V  N  V  N  E
AGAAGAGGCTAAGGACTTCACCAAAATGGTTCGAGATGTTTTGGTACCAAAACTTGGAACTTGGCCGACAATGATGGATGTTGCCACTGCAGCATAT 1164
  E  E  A  K  D  F  T  K  M  V  R  D  V  L  V  P  K  L  G  T  W  P  T  M  M  D  V  A  T  A  A  Y
ATGCTCTCAGTTTTTCACCCGGAAACAAAGAGTGCTGAATTGCCAAGAATCCTTGTTGATCACGAGTCGCAGACAATGCATGTCATTGACTCTTTTG 1261
 M  L  S  V  F  H  P  E  T  K  S  A  E  L  P  R  I  L  V  D  H  E  S  Q  T  M  H  V  I  D  S  F
GATCTTTGAACACAGGGTACCACGTACTTAAAGCTGGAACAGTCAACCAACTAATCCAGTTTGCATCAAACGATCTCGATAGTGAAATGAAGTTCTA 1358
G  S  L  N  T  G  Y  H  V  L  K  A  G  T  V  N  Q  L  I  Q  F  A  S  N  D  L  D  S  E  M  K  F  Y
CAAGGTTGGT 1368
  K  V  G
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Fig. 2 Nucleotide sequence of CABMV-Z HC-Pro. The nucleotide sequence of the plus sense cDNA strand is given. The predicted amino acid sequence of the encoded protein is presented under the nucleotide sequence in single-letter code. The conserved amino acid sequences discussed in the text are underlined.

The HC-Pro is known to autocatalytically cleave itself from the polyprotein at a G-G dipeptide which is contained in the consensus sequence K-x-Y-x-V-G*G lying at the C-terminus of HC-Pro (Carrington and Hendorn, 1992). The sequence K-F-Y-K-V-G*G was identified in the CABMV-Z sequence and was designated as the carboxy terminus of the HC-Pro. The N-terminus of HC-Pro is defined by the P1 cleavage site HY*S in tobacco etch virus (TEV), zucchini yellow mosaic virus (ZYMV), johnsongrass mosaic virus (JGMV), plum pox virus (PPV) and peanut stripe virus (PStV). The P1 cleavage site delimiting the N-terminus of CABMV-Z HC-Pro is part of a putative protease recognition site QHY*S also found in soybean mosaic virus (SMV).
The CABMV-Z HC-Pro coding region consists of 1 368 nt. The size falls within the range established from other potyviral HC-Pros (1 368 - 1 383 nt). The base composition of 32.24 % A, 22.88% G, 25.37% U and 19.52% C is similar to other potyviral HC-Pros. The ORF encodes 456 amino acids resulting in Mr 51.9 kDa and bearing motifs conserved in other potyviral HC-Pros.

3.3.2 Conserved motifs with demonstrated and proposed functions

All potyviral HC-Pros have, in the proteinase domain, active site residues C-72aa-H which are required for protease activity. By analogy, we identified C_{343}-72aa-H_{416} as the putative proteinase active site residues for CABMV HC-Pro, with the C residue in the conserved consensus sequence G-Y-C-Y. This is consistent with the established contention that HC-Pro is a papain-like proteinase with an active cysteine (Oh and Carrington, 1989).
A conserved cluster of 5 cysteine residues (C-8aa-C-13aa-C-4aa-C-2aa-C) representing a putative zinc finger metal binding motif was first reported by Robaglia *et al.* (1989) in the N-terminal part of potato virus Y (PVY) HC-Pro. A similar cluster is present in CABMV-Z HC-Pro in the version C-8aa-C-18aa-C-2aa-C, with a C -> V amino acid replacement at the second cysteine site as is also found in BCMV, SMV, ZYMV and PStV, all members of the same subgroup of potyviruses. On the other hand in sweet potato feathery mottle virus (SPFMV-S) and JGMV alanine and glycine respectively replace cysteine at the second C site. Similar cysteine-rich clusters are found in several nucleic acid binding proteins (Evans and Hollenberg, 1988). This is consistent with nucleic acid binding properties of HC-Pro shown by its purification from infected

plant tissue by adsorption to oligo(dT) columns (Thorbury *et al.*, 1985) and *in vitro* nucleic acid binding studies of bacterially expressed PVY HC-Pro (Maia and Bernardi, 1996). Nucleic acid binding properties of HC-Pro are consistent with its recently established role in the replication of potyviral RNA (Kasschau and Carrington, 1995; Kasschau *et al.*, 1997). Besides, in aphid-mediated transmission, HC-Pro was shown to be active as a homodimer (Thornbury *et al.*, 1985), and it was suggested that dimerisation occurred by binding of metal atoms between the cluster of cysteine residues (Robaglia *et al.*, 1985). The KLSC motif at position 53 - 56 together with the FRNK and PTK motifs, respectively at positions 180 - 183 and 309 - 311 in CABMV-Z HC-Pro, have been shown to be involved in aphid transmission in ZYMV (Granier *et al.*, 1993; Huet *et al.*, 1994; Peng *et al.*, 1998).

A conserved CCC box that in CABMV-Z HC-Pro is found at position 291 to 293, has been implicated in long distance movement in TEV (Cronin *et al.*, 1998). Berger and Pirone (1986) have shown that potyvirus HC-Pro is a glycoprotein. By anology with putative glycosylation sites identified for PVY-N by Robaglia *et al.* (1989), in CABMV-Z HC-Pro the motifs NYT and NPS are respectively found at positions 124 - 126 and 191 - 193. These may be important for the functions of CABMV HC-Pro.

3.3.3 Phylogenetic comparisons with other potyviruses

The relationship between CABMV-Z and other potyviruses based on HC-Pro amino acid sequences is shown as a phylogenetic tree (Fig. 3). The tree has been obtained after aligning the HC-Pro amino acid sequences using the DNASTAR program and shows CABMV HC-Pro branching along with all sequenced members of the BCMV subgroup. This strongly corroborates the original classification of CABMV in this subgroup on the basis of symptomatology, host range and serological relationships (Dijkstra and Khan, 1992; Mink and Silbernagel, 1992), later confirmed by CP sequences. It is consistent with the premise that the distinction between potyviruses based upon CP sequences holds for all viral encoded proteins (Shukla *et al.*, 1994), we have also observed this trend with CABMV-Z P3, 6K1, CI, 6K2 and NIa sequences (data not shown).

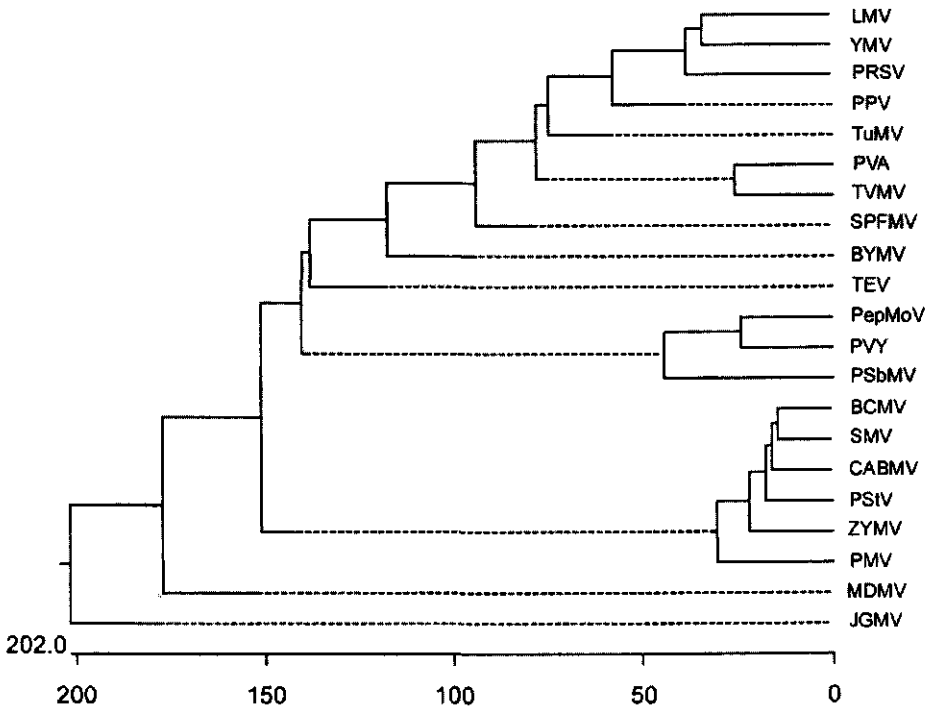

Fig. 3 Phylogenetic tree obtained from the alignment of HC-Pro amino acid sequences of potyviruses. The scale beneath the tree measures the distance between sequences. Dotted lines indicate a negative branch length. Sequences used for alignments were retrieved from the Gene Bank/EMBL sequence data bases using the following accession numbers: PVA (potato virus A) (AJ131400), TVMV (tobacco vein mottling virus) (U38621), LMV (lettuce mosaic virus) (X97704), TuMV (turnip mosaic virus) (P89509), YMV (yam mosaic virus) (U42596), PRSV (papaya ringspot virus) (X97251), BYMV (bean yellow mosaic virus) (D83749), PePMoV (pepper mottle virus) (M96425), PSbMV (pea seed-borne mosaic virus) (X89997), PMV (peanut mottle virus) (056075), MDMV (maize dwarf mosaic virus) (AJ001691), BCMV (Q65399), JGMV (Z26920), PPV (S47508), PStV (U05771), PVY (X97895), SMV (Q90069), SPFMV (D86371), TEV (L38714), ZYMV (P18479).

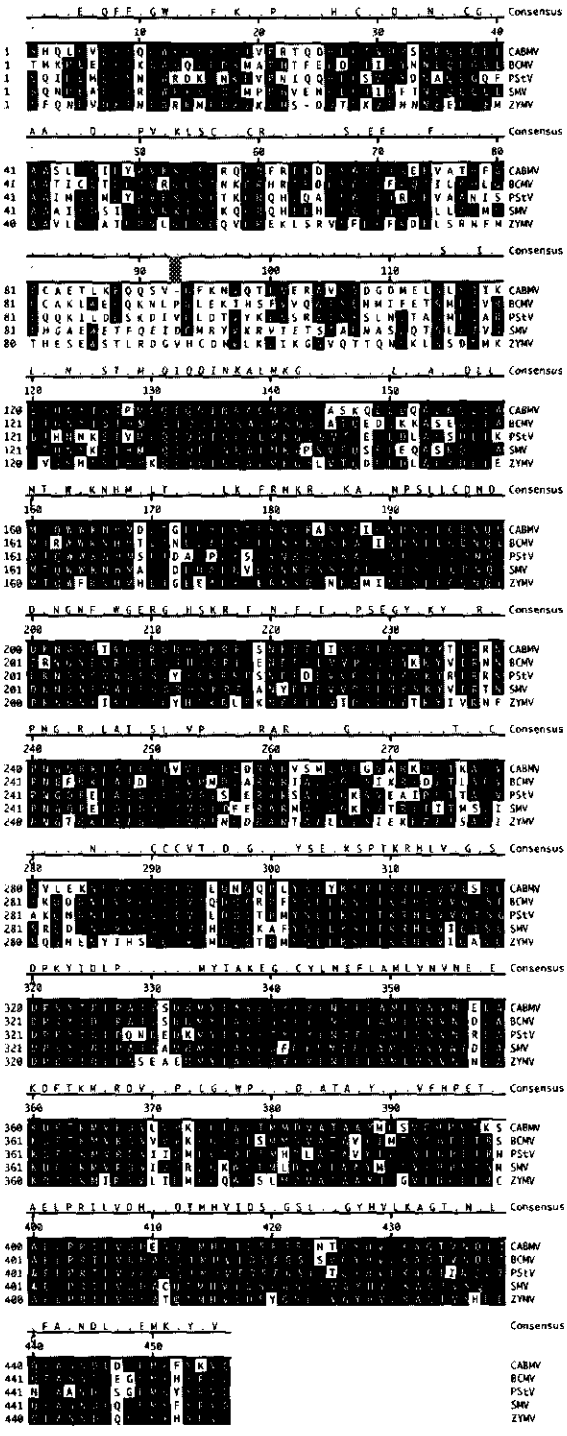

Fig. 4 Alignment of HC-Pro amino acid sequences of potyviruses of the BCMV subgroup using the Clustal method with PAM250 residue weight table. The consensus residues conserved in all sequences are indicated. Residues that are conserved in at least three species are shaded in black.

3.3.4 Comparisons of CABMV HC-Pro with members of the BCMV subgroup

The amino acid sequence relationship between CABMV-Z HC-Pro and sequenced members of the BCMV subgroup are shown as an alignment report (Fig. 4). This has been obtained using the Clustal method of the DNASTAR program.

The overall percentage of amino acid sequence identity of CABMV-Z HC-Pro with other members of the subgroup range from 71% with ZYMV to 75% with BCMV, with the consensus increasing from the N- to C-terminal ends of the HC-Pro proteins. This fits into the differentiation of potyviruses into distinct species sharing close evolutionary relationships on the basis of amino acid sequence homology of their CPs (Ward and Shukla, 1991). Taken together with previous findings (Dijkstra and Khan, 1992; Huguenot *et al.*, 1996; Lima *et al.*, 1979; Mckern *et al.*, 1994), our work further confirms the taxonomic status of CABMV as a distinct species of the BCMV subgroup of potyviruses.

In this work we have succeeded in cloning the HC-Pro gene of CABMV-Z in a combined approach involving direct cloning and genome-walking in RT-PCR with degenerate and specific primers. Further studies will concentrate on studying functions of the gene and engineering virus resistance against CABMV in transformed plants.

Acknowledgements

The authors gratefully acknowledge Dr. B. J. M. Verduin (Laboratory of Virology, Wageningen University and Research Centre, The Netherlands) for kindly providing purified virus. We are indebted to Percy Chimwamurombe for designing oligos 2 and 3. We also thank T. van Kampen for kind assistance with sequencing. This work was supported by a grant of the Netherlands Foundation for the Advancement of Tropical Research (WOTRO).

CHAPTER 3

THE GENOMIC SEQUENCE OF COWPEA APHID-BORNE MOSAIC VIRUS AND ITS SIMILARITIES WITH OTHER POTYVIRUSES

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Summary

The genomic sequence of a Zimbabwe isolate of CABMV was determined by sequencing overlapping viral cDNA clones generated by RT-PCR. The sequence is 9 465 nucleotides in length excluding the 3' terminal poly (A) tail and contains a single open reading frame (ORF) of 9 159 nucleotides encoding a large polyprotein of 3 053 amino acids and predicted Mr of 348 kDa. It is uncertain whether the sequence includes the extreme 5' end of the genome. The putative size of the genome and the encoded polyprotein is in agreement with other potyviruses, with overall amino acid sequence identity of the polyprotein ranging from 39.8% (JYMV) – 69.1% (BCMV). From alignments of the amino acid sequence of the polyprotein of CABMV with other potyvirus polyproteins, nine putative proteolytic cleavage sites were identified. The 10 predicted CABMV proteins contain motifs conserved in homologous proteins of other potyviruses. The P1 and P3 were the most variable proteins while CI, NIb and CP were the most conserved.

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1. Introduction

Cowpea aphid-borne mosaic virus (CABMV) belongs to the genus *Potyvirus* in the family potyviridae, and infects cowpea worldwide. At least 200 potyvirus species have been identified some of which have been fully sequenced (Shukla *et al.*, 1994). Knowledge of complete potyviral genomes has led to a better understanding of their genome structure, biological properties and evolutionary relationships. Complete nucleotide sequences have so far been reported for at least 18 potyvirus species: tobacco etch virus (TEV) (Allison *et al.*, 1986); tobacco vein mottling virus (TVMV) (Domier *et al.*, 1986); plum pox virus (PPV) (Maiss *et al.*, 1989); potato virus Y (PVY) (Robaglia *et al.*, 1989); pea seed-borne mosaic virus (PSbMV) (Johansen *et al.*, 1991); soybean mosaic virus (SMV) (Jayaram *et al.*, 1992); turnip mosaic virus (TuMV) (Nicolas and Laliberte, 1992); pepper mottle virus (PepMoV) (Vance *et al.*, 1992); papaya ringspot virus (PRSV) (Yeh *et al.*, 1992), Johnson grass mosaic virus (JGMV) (Gough and Shukla, 1993); peanut stripe virus (PStV) (Gunasinghe *et al.*, 1994); potato virus A (PVA) (Puurand *et al.*, 1994); bean common mosaic virus (BCMV) (Fang *et al.*, 1995); yam mosaic virus (YMV) (Aleman *et al.*, 1996); bean yellow mosaic virus (BYMV) (Guyatt *et al.*, 1996); lettuce mosaic virus (LMV) (Revers *et al.*, 1997); sweet potato feathery mottle virus (SPFMV) (Sakai *et al.*, 1997); maize dwarf mosaic virus (MDMV) (Kong and Steinbiss, 1998) and Japanese yam mosaic virus (JYMV) (Fuji and Nakamae, 1999).

The potyviral genome consists of a single stranded positive-sense RNA molecule of about 10 kb in length, and has a 5'terminal genome-linked protein (VPg) and 3'-poly (A) tail (Dougherty and Carrington, 1988). Analysis of the published sequences and *in vitro* translation studies revealed a single open reading frame (ORF) encoding a large polyprotein (Allison *et al.*, 1986). The polyprotein is cleaved into 10 mature products at conserved sites by three virus-encoded proteinases (Carrington *et al.*, 1990). The genes encoding the proteins are conserved in the same order in all sequenced potyviral genomes and include the first protein (P1) at the N-terminus of the polyprotein, followed by the helper component-proteinase (HC-Pro); the third protein (P3); the first 6 kDa protein (6K1); the cylindrical inclusion protein (CI); the second 6 kDa protein (6K2); the VPg domain of the nuclear inclusion protein a (NIa-VPg), the proteinase domain of the nuclear inclusion protein a (NIa-Pro); the nuclear inclusion protein b (NIb) and the coat protein (CP) (see review by Riechmann *et al.*, 1996).

The complete nucleotide sequence of CABMV has so far not been reported, only sequences of CP genes and 3' nontranslated regions (NTRs) have been determined for several strains of CABMV (Brand *et al.*, 1993; Mckern *et al.*, 1994; Sithole-Niang *et al.*, 1996; Pappu *et al.*, 1997; van Boxtel *et al.*, 2000). These sequence data have contributed greatly in resolving the taxonomic position of CABMV strains. However, the published sequences account for less than 10 % of the viral genome and offer limited information about the genomic features of CABMV. To understand the organisation of the CABMV genome, we have cloned and sequenced its genome and combined the sequence with the previously published 3'-terminal 1221 nucleotides (Sithole-Niang *et al.*, 1996). In this manuscript we present the deduced genomic sequence of the Zimbabwe isolate of CABMV and compare it with sequences of other potyviruses. Proteolytic cleavage sites and important conserved motifs were identified by analogy with other potyviral genomes. The comparisons revealed that CABMV has a genome organisation similar to other potyviral genomic sequences and confirmed the original classification of CABMV as a member of the bean common mosaic virus (BCMV) subgroup of legume infecting potyviruses.

2. Materials and methods

CABMV isolate

The isolate of CABMV (CABMV-Z) used in this work was described by Sithole-Niang *et al.* (1996). The virus was maintained through a series of passages on the susceptible cowpea cultivar *Vigna unguiculata* California blackeye #5 and *Nicotiana benthamiana* by mechanical inoculation.

Virus isolation and RNA extraction

CABMV was purified from infected *N. benthamiana* leaves as described for sweet potato virus from Zimbabwe by Chavi *et al.*, (1997). Total RNA was isolated as described in chapter 2.

Primer design, RT-PCR, molecular cloning and nucleotide sequencing

The generation of RT-PCR clones s2, s3, s4 and s5, spanning most of the genome of CABMV and the sequencing of s4 and s5 and partial sequencing s2 and s3 clones was described in chapter 2. For further internal sequencing of s2, primers cifor (TTACACCCTCCACCCACTGGAA) and vprev (GTGGGGTGTCCAATCCACTTTGA) were designed. For sequencing of s3, primers p3for (GGCTTCTTTGACATGAATGTGAGTC) and cirev (TAACATTCTCTGCTAGCGGGT CTAA) were designed. The sequencing of both strands of each cDNA clone was performed using the automated Applied Biosystems 373 DNA sequencer.

For cloning of the 5' proximal region, nested primers hcrev1 (GACGCTGCTAATTCAC CGCATTGCTC) and hcrev2 (GTCAACAGTGCACTCATGATCCTGG) were designed from sequences in the HC-Pro region and used together with primer 5'term (AAATTAATAAACAATCTCAATCTACAAAC) designed from conserved nucleotide sequences at the 5' end of potyviral genomes. RT-PCR, cloning into pGEM-T vector and subsequent sequencing of CDNAs was performed as described in chapter 2.

A further set of nested primers, prev1 (AGGAGTTTTGAAACTTATGCTCTCAGA) and prev2 (AGCTGTGTTCAACCCTTGGATG) designed from the P1 region, were used in 5' RACE PCR using the Roche kit (ROCHE).

Nucleotide sequence analysis

Nucleotide and amino acid sequence data were assembled and analysed using the Genetics Computer Group Program (GCG). The sequence data of other potyviruses were retrieved using the BLAST search facility of the National Center for Biotechnology Information (NCBI) and analysed using the DNASTAR program.

3. Results and Discussion

Molecular cloning and sequencing of the genome of CABMV

The sequence of the 3' NTR (231 nt) and the sequence and position of the CP gene (825 nt) at the 3' end of the genome of CABMV have been described before (Sithole-Niang *et al.* 1996). The cloning and sequencing of full-length NIb gene of CABMV (1 554 nt) and part of the 3' terminal region of the NIa gene (327 nt) was described by Dhliwayo (1996).

The generation of RT-PCR clones s2, s3, s4 and s5, spanning most of the genome of CABMV upstream from the 3' end of the NIa gene was described in chapter 2. In chapter 2 the complete nucleotide and amino acid sequence of CABMV HC-Pro gene (clone s5) was also presented.

In addition to the previous clones, two more RT-PCR clones s6 and s7 covering most of the 5' end of the CABMV genome were obtained (Fig. 1). The 1kb s6-clone was amplified with primers hcrev2 (designed from the 5' region of HC-Pro) and 5'term (designed from the first 29 nucleotides at the 5' end of potyviral genomes) on cDNAs synthesised with hcrev1 (designed from the 5' region of HC-Pro) using total RNA from infected leaves as template. The s7 clone (0.7kb) was amplified in a 5' RACE PCR protocol (ROCHE) using the supplied PCR anchor primer and prev2 (designed from the middle of the P1 gene) on cDNAs synthesised with prev1 (designed from the middle of the P1 gene) using total RNA from infected leaves as template. For each of the s6 and s7 clones, 4 independent RT-PCR clones were sequenced, and for each of the s2 and s3 clones two independent RT-PCR clones were sequenced. In all cases both strands of the cDNA were sequenced. The sequences of all 5'RACE clones (s7) terminated at the same region as the s6 clones generated by the 5' end primer (5'term) (Fig. 1), and no extra sequence information could be obtained 5' to clone s6. We are not sure if this means that the 5' end primer (5'term) successfully annealed at the extreme 5' end of the genome, neither could we find the sequence of the 5' term primer in the sequenced clones. We noticed that s7 clones lacked the poly (A) expected from the dA-tailing of the cDNAs thus raising the possibility that the clones were truncated. It is for these reasons that we do not know whether the extreme 5' end of the genome has been reached. It should be further borne in mind that in some potyviral 5'NTRs, secondary structures characterised by hairpin loops are predicted within the first 100 nt (Nicolas and

Laliberte, 1992). The hairpin loops and the presence of the VPg at the 5' end may prematurely stop the reverse transcription reactions.

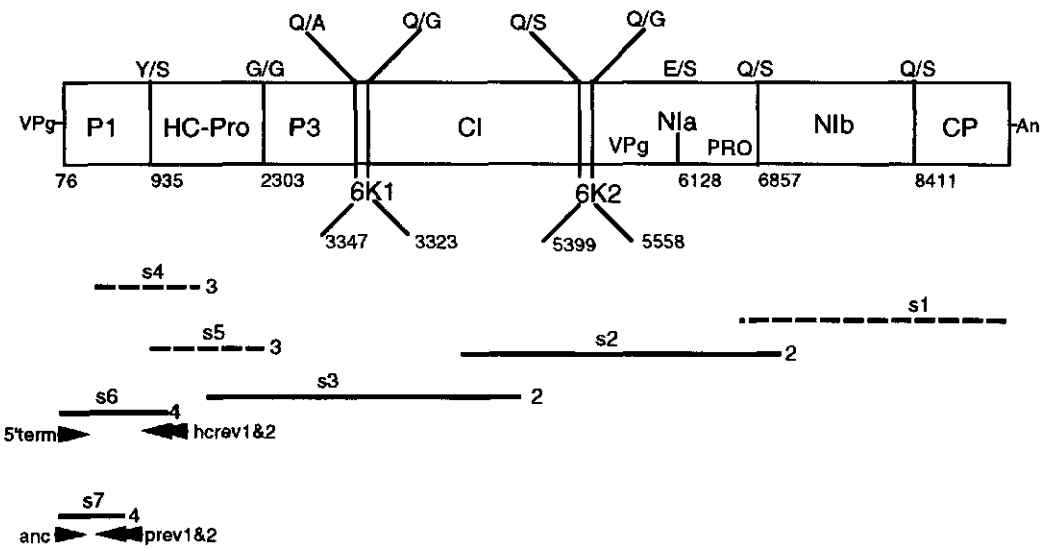

Fig. 1 Proposed genomic map of CABMV. The open bar represents the ORF and NTRs are indicated by a single line. Vertical lines indicate putative cleavage sites within the polyprotein. Amino acids on either side of each cleavage site are shown above the site. Ten mature proteins are predicted from the polyprotein. Numbers below the map indicate the position of the first coding nucleotide of each gene. The dashed line designated s1 indicates the previously sequenced region (Sithole-Niang *et al.*, 1996; Dhliwayo, 1996). The positions of the overlapping RT-PCR clones (s2 - s7) used for sequencing are shown below the genomic map. Clones s4 and s5 indicated in dashed lines were sequenced previously (chapter 2). Numbers behind each cDNA clone indicate the number of independent RT-PCR clones sequenced. The arrow heads show the annealing positions of the primers. Anc: PCR anchor primer supplied in the ROCHE RACE kit. An: poly (A) tail.

Primary structure of the CABMV genome

The assembled genomic sequence of CABMV and the predicted translation product are shown in Fig. 2. The genomic sequence is 9 465 nucleotides in length excluding the 3' terminal poly (A) tail. The single large ORF is 9 159 nucleotides, starting at the AUG (76-78) in the optimal context AAAAUGAUAG providing for an A residue at the -3 and +4 positions as required for initiation of translation (Kozak, 1981; Lutcke *et al.* 1987), and terminated by UAG at position 9 235-9 237, thus encoding a polyprotein of 3 053 amino acids and predicted Mr of 348 kDa. In this case the length of the 5'NTR is 75 nt and the encoded P1 protein is 286 amino acids. The 5' NTR falls short of the 85-205 nt range established for other potyviral 5'NTRs. Whilst the size of the P1 protein falls within the range of 233-663 amino acids established for other potyviruses, it is significantly smaller than the P1 proteins of BCMV (313 amino acids) and SMV (308 amino acids) with which CABMV is closely related. Moreover, for CABMV the assigned initiation methionine is located 27 amino acids upstream of the motif ESKKL, a marked deviation from closely related potyviruses where the methionine is located at least 48 amino acids upstream of the same motif. We thus can not exclude the possibility that the genuine initiation codon is still upstream of the available sequence data, moreso considering that potential initiation codons have been identified downstream of the first initiation codons in other potyviruses (Johansen *et al.*, 1991; Gunasinghe *et al.*, 1994; Guyatt *et al.*, 1996; Kong and Steinbiss 1998).

The 3' NTR is 231 nt in length and has been described previously (Sithole-Niang *et al.*, 1996) and contains a consensus yeast poly (A) signal sequence UAUGA at positions 9 452-9 456 also identified in other potyviruses. The proposed genomic map of CABMV is shown in Fig. 1.

Assignment of proteolytic cleavage sites.

Mature potyviral proteins are produced by proteolytic processing of the viral precursor polyprotein (Riechmann *et al.*, 1992). In other potyviruses the cleavage reactions have been established to be mediated by three virus encoded proteases: the NIa is responsible for seven cis and trans proteolytic activities in the C-terminal 2/3 of the polyprotein while P1 and HC-Pro are responsible for their autocatalytic release from the polyprotein (Carrington *et al.*, 1990). The proteolytic sites are conserved in all potyviruses (Shukla *et al.*, 1994). The CABMV NIa protease

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TATCCAAGTCAAGTTGGAACGACCTCAACTAGCTGGACACTAGTTGACAAGAAACTAGTGGATGAAGGAGTCAAAATGATAG TGGACCTCAAGGATG 97
                                                                    M  I  V  D  L  K  D
AGGTCGAAGAAGCAGCAAAAGTGATTCAAATTCAAAAGGATCACACAAGCTACACTCGAGAAAGCAAGAAATTGCTCTCCGATTACGATGAGGCACA 194
E  V  E  E  A  A  K  V  I  Q  I  Q  K  D  H  T  S  Y  T  R  E  S  K  K  L  L  S  D  Y  D  E  A  Q
AAACAAATTCACTGAAAGCCTTCTGCAACTACCTGGAATGTGGGCAAAACTGCGTATTGATGCATACAGCCCCATTCGTAAGACCAAGAAAGGCGCA 291
  N  K  F  T  E  S  L  L  Q  L  P  G  M  W  A  K  L  R  I  D  A  Y  S  P  I  R  K  T  K  K  G  A
ATCATAAATCGCGTATCATTTAGAAAGGCACAAGAACGCCATGAGGCACGACTCGTGGCAGACAAAGAACTTGCTGCATTTCTTGCTGGCGAATATG 388
  I  I  N  R  V  S  F  R  K  A  Q  E  R  H  E  A  R  L  V  A  D  K  E  L  A  A  F  L  A  G  E  Y
AAAGCGCGGCATACATTGGCTCCATCCAAGGGTTGAACACAGCTCGAAATTCTGAGAGCATAAGTTTCAAAACTCCTTACTACAGGAGGACCCCAAA 485
E  S  A  A  Y  I  G  S  I  Q  G  L  N  T  A  R  N  S  E  S  I  S  F  K  T  P  Y  Y  R  R  T  P  K
GATCCAAAAGCAAAGGATAAAGCCAAAACAAGTTGTTGGTTCATCCCAAGTCCTAAAACAGGTTATGCAGCTCGCTCAGCAAACAGGGATTAGCGTG 582
  I  Q  K  Q  R  I  K  P  K  Q  V  V  G  S  S  Q  V  L  K  Q  V  M  Q  L  A  Q  Q  T  G  I  S  V
GAATTCATCGAGAGGGGCAAGGGAAGAACCCTCAAAGTCAACGTAGTGAAGAAGTACGGTAGCGTACTTCCGAAAATCATTCTACCACATGAGGAGG 679
E  F  I  E  R  G  K  G  R  T  L  K  V  N  V  V  K  K  Y  G  S  V  L  P  K  I  I  L  P  H  E  E
GTGTACATGTTCATGAAGAAGTCAACTACAACAAGCACAAGAACACACTCTTGTTTTTGATTGGGCACAGTTCCTACAAAACCATTCATCAAAGCGT 776
G  V  H  V  H  E  E  V  N  Y  N  K  H  K  N  T  L  L  F  L  I  G  H  S  S  Y  K  T  I  H  Q  S  V
TTTTAGAAAAGGGGATAGTGGGCTTGTTTATCCTACACAGAAAATACAATCACTCCCTGAGCATGACAAAGAGGTATTTGTTGTGAGGGGAAGGTTA 873
  F  R  K  G  D  S  G  L  V  Y  P  T  Q  K  I  Q  S  L  P  E  H  D  K  E  V  F  V  V  R  G  R  L
GATGGATCCCTGATAAATGCCCTTGACTGGTGCAGCGAACCTTCACATGTGCAACATTACTCACATCAGTTGGAGGTTCAATTTTTCCAGGGTTGGA 970
D  G  S  L  I  N  A  L  D  W  C  S  E  P  S  H  V  Q  H  Y  ‖  S  H  Q  L  E  V  Q  F  F  Q  G  W
AGAAAGTATTTGACAAGCTTGTACCACGCACCCAGGATCATGAGTGCACTGTTGACTACAGCAATGAGCAATGCGGTGAATTAGCAGCGTCATTGAG 1067
K  K  V  F  D  K  L  V  P  R  T  Q  D  H  E  C  T  V  D  Y  S  N  E  Q  C  G  E  L  A  A  S  L  S
TCAAATTCTGTACCCAGTGAAGAAACTATCGTGTAGGCAATGCAGATTCCGCATCAAAGATTTGAGCTGCGAAGAATATAAGGAATTTGTTGCAACA 1164
  Q  I  L  Y  P  V  K  K  L  S  C  R  Q  C  R  F  R  I  K  D  L  S  W  E  E  Y  K  E  F  V  A  T
CACTTTGGATGCTGTGCAGAGACGCTCAAAGAACAACAGAGCGTTGGTTTCAAGAATGTGCAAACACTCGTTGAGAGAGCAGTTAGCGAAGATGGAG 1261
  H  F  G  C  C  A  E  T  L  K  E  Q  Q  S  V  G  F  K  N  V  Q  T  L  V  E  R  A  V  S  E  D  G
ATATGGAGTTGTCGCTTGAGATCATAAAATTAACACAGAATTACACGAGCACACCAATGCTCCAAATTCAAGACATCAATAAGGCTCTCATGAAAGG 1358
D  M  E  L  S  L  E  I  I  K  L  T  Q  N  Y  T  S  T  P  M  L  Q  I  Q  D  I  N  K  A  L  M  K  G
TAGTTCTGCGAGCAAGCAAGAGCTTGACCAAGCACTCAAGCAGTTGCTAGCAATGACCCAATGGTGGAAGAATCACATGGATTTAACAGGGGAGGAT 1455
  S  S  A  S  K  Q  E  L  D  Q  A  L  K  Q  L  L  A  M  T  Q  W  W  K  N  H  M  D  L  T  G  E  D
GCTCTAAAGACTTTTAGAAACAAGAGAGCATCAAAAGCAATACTCAACCCAAGTTTACTTTGTGACAATCAGCTTGATAAGAATGGCAATTTCATTT 1552
A  L  K  T  F  R  N  K  R  A  S  K  A  I  L  N  P  S  L  L  C  D  N  Q  L  D  K  N  G  N  F  I
GGGGAGAAAGAGGCAGGCATTCAAAACGATTTTTCTCAAACTTCTTTGAGGAAATTGTTCCATCTGAAGGATATAGCAAGTACACAATCAGACGGAA 1649
W  G  E  R  G  R  H  S  K  R  F  F  S  N  F  F  E  E  I  V  P  S  E  G  Y  S  K  Y  T  I  R  R  N
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  P  N  G  Q  R  K  L  A  I  G  S  L  V  V  P  L  D  L  D  R  A  R  V  S  M  Q  G  E  G  V  A  R
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K  P  L  T  K  A  C  V  S  V  L  E  K  N  F  V  Y  P  C  C  C  V  T  L  D  N  G  Q  P  L  Y  S
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E  Y  K  S  P  T  K  R  H  L  V  V  G  S  S  G  D  P  K  Y  I  D  L  P  A  T  D  S  Q  R  M  Y  I
TGCAAAGGAAGGATACTGTTATCTAAATATTTTCCTTGCAATGCTTGTGAACGTCAATGAAGAAGAGGCTAAGGACTTCACCAAAATGGTTCGAGAT 2037
  A  K  E  G  Y  C  Y  L  N  I  F  L  A  M  L  V  N  V  N  E  E  E  A  K  D  F  T  K  M  V  R  D
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V  L  V  P  K  L  G  T  W  P  T  M  M  D  V  A  T  A  A  Y  M  L  S  V  F  H  P  E  T  K  S  A
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E  L  P  R  I  L  V  D  H  E  S  Q  T  M  H  V  I  D  S  F  G  S  L  N  T  G  Y  H  V  L  K  A  G
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  T  V  N  Q  L  I  Q  F  A  S  N  D  L  D  S  E  M  K  F  Y  K  V  G  ‖  G  S  V  Q  Q  R  M  K  C
GAAACAGCCTTGATAACAAGTATTTTCAAGCCAAAGCGGATGGTTCACATACTTGAAACAGATCCATATGTCCTTCTCATGGGTCTAGTATCTCCCT 2425
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CTCTATTAATCCACATGTTTAGAATGCGGCACCTTGAAAAAGGCGTCCAAATATGGATTAACAAGGAACAGAGCGTGGTAAAGATCTTCTTGATATT 2522
S  L  L  I  H  M  F  R  M  R  H  L  E  K  G  V  Q  I  W  I  N  K  E  Q  S  V  V  K  I  F  L  I  L
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  E  Q  L  T  K  K  L  V  V  T  D  V  L  L  E  Q  L  G  L  I  S  E  A  A  E  P  L  H  S  L  L  L
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  F  S  V  T  W  Q  L  K  R  F  S  D  V  T  E  N  T  L  T  K  K  A  T  V  Q  K  E  K  F  S  K  R
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TTTGTGAGTGCGTGCTTTATGAGTGCCCAAACACACCTGAGAGAAAGCCGTATTTATCTTGCGAATAAGGTACAGCAGTTCTCACATAGGCTTATAG 3007
 F  V  S  A  C  F  M  S  A  Q  T  H  L  R  E  S  R  I  Y  L  A  N  K  V  E  Q  F  S  H  R  L  I
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 G  S  V  C  N  M  F  L  R  F  V  R  R  C  Y  G  D  L  I  F  L  V  N  V  S  I  V  F  S  L  F  I  Q
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CGGATGTATGATCTTTTCATTAAAGGATCAAAAGATCTACCAAGTGCTGGGCAATTTCTCTCTCATGTTGAAGAATTTAGGCCAGACCTGCTCTTAA 3298
 R  M  Y  D  L  F  I  K  G  S  K  D  L  P  S  A  G  Q  F  L  S  H  V  E  E  F  R  P  D  L  L  L
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CGCGTTCAGGGCCTGGATGATCTCAATGATATTGAAGAGTGCAAGAAACTCACAGTTGATTTTGAAGTGTCAACAAGCTATGAACCAACCTCCACAA 3589
 R  V  Q‖G  L  D  D  L  N  D  I  E  E  C  K  K  L  T  V  D  F  E  V  S  T  S  Y  E  P  T  S  T
CCATTGATGTCTCTTTTGAGGGTTGGTGGAATAGACAACTGCAGCAAAACAGGGTTGTGCCACATTACAGATCAACTGGGGAATTCATGGAGTTCAC 3686
 T  I  D  V  S  F  E  G  W  W  N  R  Q  L  Q  Q  N  R  V  V  P  H  Y  R  S  T  G  E  F  M  E  F  T
AAGAGCCACAGCAGCACAAGTCGCTAACTCAATAAGCCTGTCTGCACAGAATGATTTTCTCATTAGAGGAGCTGTCGGCTCTGGTAAATCGACGGGG 3783
   R  A  T  A  A  Q  V  A  N  S  I  S  L  S  A  Q  N  D  F  L  I  R  G  A  V  G  S  G  K  S  T  G
CTACCACACCATTTGGCAAAGAAAGGAAAAGTGTTGCTTCTTGAGCCAACTAGACCGCTAGCAGAGAATGTTAGCAAACAACTGTCACAAGAGCCAT 3880
 L  P  H  H  L  A  K  K  G  K  V  L  L  L  E  P  T  R  P  L  A  E  N  V  S  K  Q  L  S  Q  E  P
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 F  F  Q  N  V  T  L  R  M  R  G  L  S  R  F  G  S  S  N  I  T  V  M  T  S  G  F  A  F  H  Y  Y  V
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   N  N  P  H  Q  L  N  D  F  D  F  I  I  M  D  E  C  H  V  L  D  S  S  S  I  A  F  N  C  A  L  K
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 E  F  S  Y  A  G  K  L  I  K  V  S  A  T  P  P  G  R  E  C  E  F  T  T  Q  H  P  V  K  L  K  V
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 E  E  N  L  S  F  Q  D  F  V  Q  A  Q  G  T  G  T  N  A  D  M  V  Q  H  G  S  N  L  L  V  Y  V  A
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  Y  A  F  D  F  Y  E  V  T  S  K  T  S  D  R  A  R  E  A  I  A  Q  M  K  A  A  A  L  A  N  V  N
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  G  M  T  H  G  Q
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TGCTCAGGGTGACCGTGTCACGCGTAGCTACCGAGATTTGTGTATGAAAACTCTCT 9465
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Fig. 2 The genomic sequence of CABMV (excluding the 3' terminal poly-A tail) and the deduced amino acid sequence of the putative polyprotein. The nucleotide sequence of the plus-sense cDNA is shown. The amino acid sequence is presented under the nucleotide sequence in single-letter code. The positions of the predicted cleavage sites are indicated with double vertical lines. The conserved nucleotide and amino acid sequences discussed in the text are underlined.

cleavage sites at the NIb/CP and NIa/NIb junctions were designated from previous work (Sithole-Niang *et al.*, 1996; Dhliwayo, 1996), and the P1 and HC-Pro autoproteolytic cleavage sites were described in chapter 2. Five more NIa proteolytic cleavage sites conserved in other potyviruses were identified in the polyprotein of CABMV (Fig. 2; Table 1).

Table 1. Putative cleavage sites in the CABMV polyprotein.

P1 cleavage site	P1//HC-Pro	PSHVQHY//S	(280-287)
HC-Pro cleavage site	HC-Pro//P3	MKFYKVG//G	(736-743)
NIa cleavage sites	P3//6K1	GRVGRHQ//A	(1084-1091)
	6K1//CI	AEEVRVQ//G	(1136-1144)
	CI//6K2	LNTVCLQ//S	(1768-1775)
	6K2//VPg	QEEVTTQ//G	(1821-1828)
	VPg//NIa	NEDVGVE//S	(2011-2018)
	NIa//NIb	DDRGCTQ//S	(2254-2261)
	NIb//CP	SEDVVLQ//S	(2772-2779)
Consensus		X-X-V/G-X-X-Q/E//S/G/A	

The seven sites putatively cleaved by CABMV NIa at dipeptides Q/A, Q/G and Q/S conform to the consensus sequence (V/G)-X-X-Q/E//(S/G/A) with valine at -4 position conserved in most sites. The cleavage site at the C-terminus of CABMV P1 at the dipeptide Y/S is in the consensus sequence reported by Mavankal and Rhoads (1991) for these sites. The CABMV HC-Pro cleavage site at the C-terminal G/G dipeptide was identified as part of the conserved motif FYKVG/G (chapter 2). Based on the proposed cleavage sites of gene products, ten mature proteins are predicted for CABMV (Figs. 1 & 2). This is consistent with the expression strategy described for other potyviruses in which the gene order is similarly conserved and the sizes of gene products fall within the same range. Nevertheless, the size of P1 would need to be authenticated in view of the discrepancies mentioned above. The cleavage sites of the CABMV polyprotein were compared with corresponding sites of other members of the BCMV subgroup (Table 2). The dipeptides at HC-Pro/P3, P3/6K1, VPg/NIaPro and NIb/CP junctions were conserved in all members of the subgroup.

Comparison of CABMV proteins with other potyvirus proteins

In addition to putative polyprotein cleavage sites, we identified several important conserved amino acid sequences with proposed functions for other potyviruses. In P1, the amino acids found in the conserved sequence G-D-S-G (238-241) are proposed to represent the serine protease active site responsible for the autoproteolytic activity of P1 (Verchot *et al.*, 1991). The F-V-V-R-G (260-264) motif identified 24 amino acids upstream of the P1 C-terminus is conserved in most potyviruses (Shukla *et al.*, 1994).

For HC-Pro, the conserved motifs and their proposed functions were described in detail in chapter 2. The DPY-$(X)_7$-SP-$(X)_2$-L motif at position 772-786 in the P3 protein suggests a role for CABMV P3 in regulation of proteolytic processing of the potyviral polyprotein as was proposed for a similar motif in the 32K protein of CPMV (Riechmann *et al.*, 1992). For 6K1 & 6K2 roles in potyviral RNA replication was proposed by analogy to picornaviral 2B and 3A proteins respectively (Lain *et al.*, 1989). The motif GAVGSGKST (1227-1235) located near the N-terminus of CI has nucleotide binding properties associated with helicase activity. Other motifs characteristic of helicase proteins (Lain *et al.*, 1990) were identified as clusters VLLLEPTRPL (1247-1256), KVSAT (1343-1347), LVYV (1394-1397), VATNIIENGVTL (1443-1454) and

Table 2. Alignment of the nine putative cleavage sites in the polyproteins of sequenced members of the BCMV subgroup.

Potyvirus	P1/HC-Pro	HC-Pro/P3	P3/6K1	6K1/CI	CI/6K2	6K2/VPg	VPg/NIa	NIa/NIb	NIb/CP
CABMV	SHVQ**HY**/S	**KFYKVG/G**	RVGRH**Q/A**	EEVRVQ/G	NTVCL**Q**/S	**EEVTTQ**/G	EDVG**VE/S**	DRGCT**Q**/S	EDVVL**Q/S**
BCMV	YITE**HY**/T	**KHYRVG/G**	EAVDT**Q/A**	EDVRP**Q**/S	NTVRL**Q**/G	**EPVSTQ**/G	VEVEL**E/S**	SEVSV**Q**/S	ESVST**Q/S**
PStV	YSIH**HY**/S	**KYYRVG/G**	TNVSV**Q/A**	DNVQM**Q**/S	PTVRL**Q**/G	**ESVTTQ**/G	EEVAV**E/S**	GTVAT**Q**/S	ESVHL**Q/S**
SMV	EDIQ**HY**/S	**KFYRVG/G**	EDVSA**Q/A**	EDVKV**Q**/S	NAVQL**Q**/S	**EPVSTQ**/G	ERVEM**E/S**	NTVTV**Q**/G	ESVSL**Q/S**
ZYMV	DDVD**HY**/S	**KHYRVG/G**	EIVTP**Q/A**	ETVRL**Q**/G	KSVVL**Q**/S	**EPVRVE**/S	EHVEL**E/S**	SGVET**Q**/S	DTVML**Q/S**

Sequences used for the comparisons were retrieved from the GeneBank/EMBL sequence data bases using the following accession numbers: BCMV/U19287; PStV/U05771; SMV/S42280; ZYMV/L92569. Bold characters indicate identical amino acids at cleavage sites in CABMV and other members of the BCMV subgroup of legume infecting potyviruses.

GERIQRLGRVGR (1487-1498). The catalytic triad (H_{2063}, D_{2098} and C_{2168}) constituting the active site residues of NIa protease (Dougherty *et al.*, 1989), was found in the carboxy-terminus of NIa. The conserved tyrosine residue required for linking VPg to potyviral RNA was identified at position 1891 at the VPg domain of NIa.

Important conserved motifs in CABMV NIb have been previously identified and described in detail by Dhliwayo (1996). These include the SG-x-x-x-T-x-x-x-NT-x30-GDD (2571-2614) motif conserved in both animal and plant positive stranded viral RNA-dependent RNA polymerases (Kamer and Argos, 1984) and the sequence CHADGS also believed to be involved in putative RNA-dependent RNA polymerase activity. The sequence features of CABMV CP including the aphid transmission DAG motif, and comparisons with the CPs of other strains of CABMV have been described before by Sithole-Niang *et al.* (1996) and more recently by van Boxtel *et al.*, (2000).

The results of alignments of amino acid sequences of the polyprotein and mature proteins encoded by the genome of CABMV with homologous gene products of other potyviruses are shown in Table 3 and Fig. 3.

Overall, the carboxy-terminal 2/3 of the CABMV polyprotein is highly conserved, with highest degree of identity in NIb ranging from 51.7% with PVA to 75.8% with BCMV, followed by CP in which the identity range is 51.6% with SPFMV to 78.9% with BCMV, and the CI protein in which the range is 48.6% with JGMV to 78.5% with BCMV. The HC-Pro is also relatively conserved with an identity range of 42.1% with YMV to 74.1% with BCMV. On the other hand P1 and P3 are least conserved, with identities in P1 ranging from 12.7% with PVY to 33.9% with BCMV, and in P3 the range is 16.7% with TuMV to 53.6% with BCMV. The overall identity of the CABMV polyprotein is lowest with JYMV (39.8%) and highest with BCMV (69.1%) and other members of the BCMV subgroup: SMV (66.5%), PStV (66.3%) and ZYMV (59.9%). The identity range of 39.8% to 69.1% of the CABMV polyprotein with other potyviral polyproteins is in close agreement with the 38-71 % range used to classify potyviruses as distinct species on the basis of amino acid sequences of their coat proteins (Shukla and Ward, 1988, 1989). On the basis of these findings and previous reports, there is no doubt that CABMV is distinct from other potyvirus species.

Table 3. Comparison of the overall amino acid homology of the CABMV polyprotein and the mature cleavage products with proteins of other potyviruses.

Virus	P1	HC-Pro	P3	6K1	CI	6K2	NIaVP	NIaPro	NIb	CP	Polyprotein
BCMV	**33.9**	**74.1**	**53.6**	**78.8**	**78.5**	60.4	**76.2**	**80.7**	**75.8**	**78.9**	**69.1**
BYMV	13.0	45.0	20.7	46.2	53.8	35.8	45.3	42.0	55.8	53.5	42.7
JGMV	15.1	40.6	17.9	42.3	48.6	28.3	49.7	38.6	53.2	57.5	40.0
JYMV	13.3	47.4	21.0	48.1	53.5	26.4	51.1	46.7	54.6	54.9	39.8
LMV	14.3	42.5	19.0	48.1	50.6	39.6	48.4	48.6	57.5	53.8	43.4
MDMV	15.1	44.1	22.2	30.8	52.4	26.4	43.4	42.6	54.6	57.5	42.4
PepMoV	13.3	45.0	23.9	42.3	51.7	30.8	44.7	43.6	52.3	54.9	42.3
PMV	18.9	57.0	25.3	57.7	60.1	41.5	59.5	50.6	62.5	58.5	50.5
PPV	13.6	46.7	23.6	50.0	56.3	35.8	51.1	48.6	60.4	53.1	41.9
PRSV	13.3	45.6	23.2	36.5	51.6	13.2	47.0	45.0	53.9	53.8	42.6
PSbMV	15.7	40.8	19.0	48.1	53.0	39.6	44.2	42.4	54.4	53.1	41.9
PStV	24.8	71.5	49.9	67.3	76.6	**71.7**	75.3	74.1	74.8	69.5	66.3
PVA	14.7	49.3	19.0	44.2	52.8	37.7	48.7	48.1	51.7	55.8	43.6
PVY	12.7	43.2	20.7	46.2	54.3	27.5	45.0	42.8	56.4	56.9	42.9
SMV	30.8	73.5	51.0	71.2	74.2	60.4	75.8	73.7	75.6	70.2	66.6
SPFMV	15.4	50.2	21.0	44.2	55.9	28.3	54.2	51.0	59.5	51.6	42.2
TEV	15.4	45.4	22.2	42.3	51.9	39.6	46.3	46.7	53.1	56.3	43.0
TuMV	14.0	45.4	16.7	50.0	53.8	30.2	53.7	50.2	56.9	56.0	40.1
TVMV	15.0	45.6	22.5	44.2	53.5	32.1	42.6	49.0	54.3	51.7	39.9
YMV	12.9	42.1	22.1	40.0	55.7	34.6	54.7	50.2	58.9	55.3	44.9
ZYMV	20.3	65.1	40.2	69.2	68.6	53.8	66.8	65.0	71.0	67.3	59.9

The figures represent identity (%) in proteins of other potyviruses and corresponding proteins of CABMV, with highest percentage identity shown in bold. Sequences used for comparisons were retrieved from the Gene Bank/EMBL sequence data bases using the following accession numbers: BCMV/Q65399; BYMV/D83749; JGMV/Z26920; JYMV/AB016500; LMV/X97704; MDMV/AJ001691; PepMoV/M96425; peanut mottle virus (PMV)/056075; PPV/S47508; PRSV/X97251; PSbMV/X89997; PStV/U05771; PVA/AJ131400; PVY/X97895; SMV/Q90069; SPFMV/D86371; TEV/L38714; TuMV/P89509;TVMV/U38621; YMV/U42596; zucchini yellow mosaic virus (ZYMV)/P18479.

The phylogenetic tree obtained by aligning the polyproteins using the Clustal method of the DNASTAR program, showed CABMV branching along with other members of the BCMV subgroup (Fig. 3).

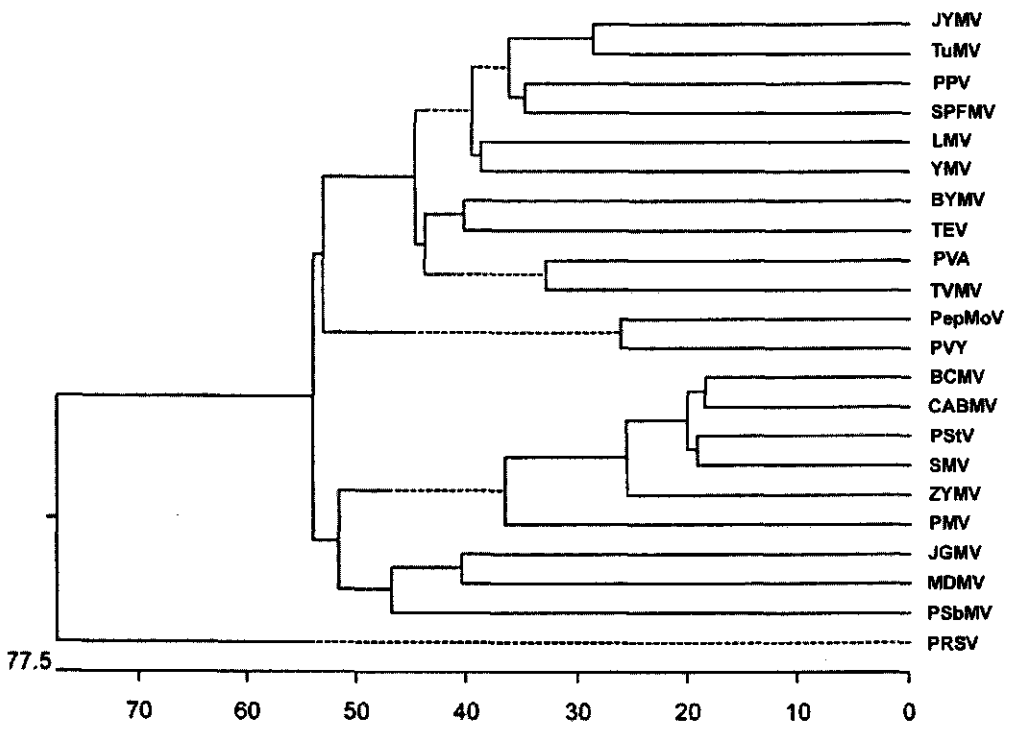

Fig. 3 Phylogenetic tree obtained from the alignment of the complete potyviral polyproteins using the Clustal method of the DNASTAR program. The scale beneath the tree indicates the distance between sequences. Dotted lines indicate negative branch length. Sequences of potyviral proteins were obtained from the Gene Bank/EMBL sequence data bases. The accession numbers of the sequences used are given in the footnote of table 3.

In this chapter, the genomic sequence of CABMV shows that CABMV has a genome organisation that is similar to other members of the genus *Potyvirus*, and contains all functional motifs identified in potyviral proteins as important for gene expression, regulation and virus spread. This was concluded from the consistency in potyviral cleavage sites, the sizes of potyviral proteins and their conservation in the same order on the viral genome. Comparisons of amino acid sequences of polyproteins revealed close evolutionary relationships between CABMV and other sequenced members of the BCMV subgroup. This confirms the original classification of CABMV in the BCMV sugroup.

Acknowledgements

The authors wish to thank Tony van Kampen for kind assistance with sequencing and Rene Geurts for providing synthetic oligonucleotides. Marcel Prins is acknowledged for kind assistance with RACE PCR. This work was supported by a grant of the Netherlands Foundation for the advancement of Tropical Research (WOTRO).

CHAPTER 4

THE HELPER COMPONENT-PROTEINASE OF COWPEA APHID-BORNE MOSAIC VIRUS: SUBCELLULAR LOCATION AND EFFECT ON VIRULENCE OF COWPEA MOSAIC VIRUS

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Summary

In this study we report how we have expressed the cowpea aphid-borne mosaic virus (CABMV) HC-Pro coding region in *E. coli* and used the purified protein to obtain an antiserum specific for HC-Pro and suitable as an analytical tool in further studies on HC-Pro. The subcellular location of HC-Pro in plant cells was then studied by expressing HC-Pro as a free protein or with GFP fused at its N-terminus in cowpea protoplasts using a transient expression system. In both cases the protein showed a diffuse cytoplasmic location similar to HC-Pro expressed in a natural CABMV infection. In an alternative expression system, the HC-Pro coding region was subcloned in-frame between the MP and L genes of RNA2 of cowpea mosaic virus (CPMV). Upon cotransfection of protoplasts with CPMV RNA1, HC-Pro was expressed as part of the RNA2 encoded polyprotein from which it was fully processed. In this case, the protein localised in broad cytoplasmic patches reminiscent of the typical CPMV induced electron dense structures. This suggests an interaction with CPMV proteins. Both on cowpea and *Nicotiana benthamiana* plants, the virulence of CPMV/HCPro was strongly enhanced compared to wild type CPMV and CPMV/GFP, indicating that HC-Pro is a potyvirus pathogenicity determinant.

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1. Introduction

Cowpea aphid-borne mosaic virus (CABMV) is a member of the Potyviridae family of which potato virus Y (PVY) is the type member. Potyviruses have a positive strand RNA genome of about 10 kb, which encodes a single large polyprotein that is processed into 10 mature proteins (see review by Riechmann *et al.*, 1992). The properties and functions of the different gene products have been investigated at length, and from these studies, the nonstructural helper component-proteinase (HC-Pro), has recently emerged as a multifunctional protein that plays a role in different steps of the potyvirus life cycle (see Maia *et al.*, 1996 for review). HC-Pro carries autoproteolytic activity by which it cleaves itself from the polyprotein precursor (Carrington *et al.*, 1989; Oh and Carrington, 1989; Carrington *et al.*, 1990; Thornbury *et al.*, 1993), it has a role in viral RNA replication (Kasschau *et al.*, 1997) and in spread of the virus through its host plant (Cronin *et al.*, 1995; Kasschau *et al.*, 1997; Rojas *et al.*, 1997). Besides, it is involved in aphid mediated transmission of the virus (Atreya *et al.*, 1992; Huet *et al.*, 1994; Wang *et al.*, 1996; Blanc *et al.*, 1998; Flasinski and Cassidy, 1998; Andrejeva *et al.*, 1999) and is a pathogenicity factor (Atreya *et al.*, 1992; Atreya and Pirone, 1993) that can also enhance the pathogenicity of a broad range of heterologous viruses (Pruss *et al.*, 1997). More recently, HC-Pro has been implicated as a suppressor of post transcriptional gene silencing (PTGS) (Pruss *et al.*, 1997; Brigneti *et al.*, 1998; Anandalakshmi *et al.*, 1998). If these findings make HC-Pro a very interesting protein, a detailed picture of the molecular basis of its multifunctional properties is still lacking (Maia *et al.*, 1996).

In our study of CABMV, we are concentrating on the role of the HC-Pro protein. In chapter 2, the cloning and sequencing of the HC-Pro gene of the Zimbabwe isolate of CABMV (CABMV-Z) was presented. Here we report how we have obtained a specific antiserum against the HC-Pro of CABMV that will be an important tool in further studies on HC-Pro. The antiserum was used in subcellular localisation of HC-Pro in protoplasts using a transient expression vector. In an alternative expression system, the HC-Pro coding region was cloned in-frame in RNA2 of cowpea mosaic virus (CPMV). In addition we have used the green fluorescent protein (GFP) fused to HC-Pro as a reporter in studying the subcellular location of HC-Pro. In the course of this

research we ran into a striking effect of CABMV HC-Pro on the virulence of CPMV on cowpea and *Nicotiana benthamiana.*

2. Materials and methods

2.1 Construction of pT_{77}/HCPro and expression in *E. coli.*

Bacterial strains and vectors

The BL21 (DE3) strain of *E. coli* and pT_{77} expression plasmid were kindly provided by B. J. M. Verduin (Laboratory of Virology, Wageningen-UR, The Netherlands).

Primers were designed for subcloning of HC-Pro for expression in *E. coli.* The upstream primer (econdehcF) incorporated an ATG translation start codon in the context of an *NdeI* restriction site, an *EcoR*I site and 31 nucleotides of the 5' coding region of HC-Pro (CCGGAATTCCAT ATGAGCCATCAGTTGGAGGTTCAATTTTTCCAGG). The downstream primer (bamstohcR) incorporated a *BamH*I site, a TAA translation stop codon and the 27 nucleotides complementary to the 3' coding region of HC-pro (CGCGGATCCTTAACCAACCTTGTAGAACTTCATTTCA CTA). The HC-Pro coding region was amplified with primers econdehcF and bamstophcR, digested with *EcoR*I and *BamH*I, and cloned downstream of the phageT7 RNA polymerase promoter in the pT_{77} vector similarly digested with *EcoR*I and *BamH*I. The pT_{77}/HCPro construct was electroporated into the *E. coli* strain BL21 (DE3) and the transformed cells selected on sterile LB medium [0.5 % yeast extract; 1 % tryptone and 0.5 % NaCl] containing ampicillin (100 µg/ml). The transformed BL21 cells were grown overnight in sterile LB medium supplemented with 100 µg/ml ampicillin. The overnight liquid cultures were diluted 100 times in sterile 2YT medium [1.6 % (w/v) tryptone; 1 % yeast extract and 0.5 % NaCl] supplemented with 100 µg/ml ampicillin and grown at 37 ° C in a shaker incubator to OD_{600}0.5. The T7 RNA polymerase expression was induced by addition of 1 mM isopropyl ß-D-thiogalactopyranoside (IPTG) followed by further incubation at 37 ° C for an additional 3-4 hours.

2.2 Purification of the HC-Pro protein

Purification of the HC-Pro from *E. coli* was as described for brome mosaic virus (BMV) MP by Jansen *et al.*, (1998). This involved collection of cells by centrifugation at 10 000 rpm for 10 min at 4 ° C, resuspension in 10 ml lysis buffer [1 % triton X-100; 0.1 mg /ml lysozyme in TBS (137 mM NaCl; 2.7 mM KCl; 25 mM Tris-HCl; 1 mM DDT; 1 mM PMSF; 1 mM EDTA and10 % (v/v) glycerol, ph 7.5] and incubation at 37 ° C for 30 min. The cells were disrupted by sonification on ice in a sonifier (Sonics and Materials Inc.) and the cell lysate centrifuged at 30 000 rpm for 30 min at 4° C to pellet the inclusion bodies. The inclusions were resuspended in buffer C [10 mM Tris-HCl pH 8.0; 1 M NaCl; 10 % (v/v) glycerol and 0.4 % (v/v) Nonidet-P40] and centrifuged as above. The pellets were incubated in buffer B [buffer C + 6 M urea] for 30 min at 37 ° C. The soluble fraction was separated from the insoluble aggregates by centrifugation at 30 000 rpm for 30 min at 4 ° C and re-natured by successive dialysis against buffer C and D [10 mM Tris-HCl pH8.0; 0.1 M NaCl; 10 % (v/v) glycerol and 0.4 % (v/v) Nonidet-P40].

2.3 Preparation of HC-Pro specific antiserum

The re-natured HC-Pro solution was resolved with SDS-PAGE and the 52 kDa band excised from the KCl-stained SDS-polyacrylamide gel. About 250 μg of the gel-excised denatured protein was divided into 3 equal parts that were separately injected into a rabbit at two-week intervals (Eurogentec, Belgium). The rabbit was bled three times and immunoglobulin fractions isolated and tested for immunoreactivity on western blots.

2.4 SDS-PAGE and western blot analysis

Protein samples were boiled for 5 min in protein loading buffer [0.31 mM Tris-HCl pH 6.8; 50 % (v/v) glycerol; 5 % (w/v) SDS; 10 % (v/v) ß-mercaptoethanol and 0.1 % (w/v) bromophenol blue] and separated by SDS-PAGE on 12 % SDS-polyacylamide gel as described in Sambrook *et al.*, (1989) and protein bands visualised with Coomassie blue staining. For western immunoblot analysis, proteins were transferred from SDS-polyacrylamide gel to nitrocellulose membranes

using a Biorad transblot apparatus at 50 volts overnight and the blots stained in Panceau to check the efficiency of transfer.

Nonspecific binding sites were blocked for 1 hour in TBS [50 mM Tris pH 7.5; 150 mM NaCl] containing 0.2 % (v/v) Tween 20 and 5 % (w/v) skimmed milk (TBST-elk). The blots were incubated for 1 hour with primary antiserum diluted 1:5000 in TBST-elk followed by three 5-minute washes in TBS containing 0.2 % (v/v) Tween 20 (TBST) and finally incubated for 1 hour with goat anti rabbit-alkaline phosphatase (GAR-AP) conjugate, washed twice for 5 min in TBST, once for 5 min in TBS and once for 5 min in alkaline phosphatase (AP) buffer [100 mM Tris pH 9.5; 100 mM NaCl and 5 mM $MgCl_2$]. The blots were subsequently incubated in 10 ml of AP buffer containing 66 µl of 100 mg/ml nitroblue tetrazolium (NBT) and 33 µl of 50 mg/ml 5-Bromo-4-Chloro-3-Indolyl-Phosphate (BCIP) till the protein bands became visible.

2.5 Construction of plant expression plasmids.

pMonHCPro. The construct is based on the transient expression vector pMon999 (van Bokhoven *et al.*, 1993). The HC-Pro coding region was amplified with primers econdehcF and bamstophcR (section 2.1) to incorporate a translational start codon (ATG) in-frame at the 5' end and a stop codon (TAA) at the 3' end of the HC-Pro gene. The PCR product was cloned as an *EcoRI-BamHI* fragment between the enhanced 35S promoter (e35S) and the nopaline synthetase (NOS) terminator in *EcoRI-BamHI* digested pMon999 vector resulting in the construct pMonHCPro (Fig. 2a).

pMonGFPHCPro. The construct contains a GFP-HC-Pro fusion under the control of the e35S promoter and NOS terminator. It was engineered by excising the 60K gene of CPMV from the pMonGFP60K construct (kindly provided by J. Carette) as an *EcoRI-BamHI* fragment and replacing it with HC-Pro excised as an *EcoRI-BamHI* fragment from plasmid pMonHCPro (Fig. 2b).

pM19HCPro7 and *pM19HCPro2A.* The constructs are based on CPMV RNA2 vectors pM19-7 and pM19-2A described by Gopinath *et al.*, (2000). The HC-Pro coding region was amplified with primers econdehcF and the primer xbabamhcR (TGCTCTAGAGGATCCACCAACCTTGT

AGAACTTCATTTCACTA) designed from the 27 nucleotides of the 3' coding region of HC-Pro and incorporating *BamH*I and *Xba*I sites. The amplified fragment was cloned in-frame as an *EcoR*I-*Xba*I fragment in *EcoR*I-*Xba*I-digested pM19-7 or pM19-2A vector. This resulted in plasmids pM19HCPro7 and pM19HCPro2A in which the HC-Pro is inserted in-frame between the MP and L genes of CPMV RNA2 (Fig. 2c & 2d).

2.6 Protoplast isolation and transfection

Cowpea protoplasts were isolated from 10-day old plants as described by Rezelman *et al.*, (1989) and aliquots of 150 µl each containing 10^6 protoplasts were transfected either with 10 µg plasmid DNA, 1-2 µg *in vitro* transcripts or 50 µl of homogenate prepared from virus-infected leaves as previously described by van Bokhoven *et al.*, (1993). *In vitro* transcripts of cDNA clones were prepared using T7 RNA polymerase as described by Vos *et al.* (1988). Samples were incubated in sterile 24-well tissue culture plates under continuous illumination at 25 °C for 24-48 hours.

2.7 Ethanol fixation of protoplasts and immunostaining

Clean glass slides were coated with 15 µl 0.05 % (w/v) poly-L-lysine and left to dry for 15 min. Quantities of 20 µl of protoplast suspensions were added to each coated slide and left to settle for 8 min. The slides were immersed in 96 % ethanol for 20 min, washed twice in phosphate-buffered saline (PBS) (100 mM sodium phoshate; 1.5 M NaCl, pH 7.2) for 15 min and blocked for 15 min in PBS supplemented with 4 % BSA. Excess liquid was drained from the slides and the protoplasts incubated for 1 hour with 35 µl of primary antiserum diluted 1:500 in blocking solution (PBS/1 % BSA). The samples were then washed 3 times for 5 min in PBS. Excess water was drained from the slides and the protoplast incubated for 1 hour with 35 µl of goat anti-rabbit antibodies conjugated to fluorescein isothiocyanate (GAR-FITC) (spun for 5 min prior to use and diluted 1:60 in blocking solution). After 3 washes with PBS, a drop of Citifluor was added to each sample and cover slips placed on the protoplast samples.

2.8 Paraformaldehyde fixation of protoplasts and immunostaining

Cover slips were coated with 20 µl of poly-L-lysine and protoplast suspensions allowed to settle on the coated cover slips. An equal volume of fixing solution [4 % paraformaldehyde; 0.1 % glutaraldehyde; 0.25 M mannitol; 50 mM sodium phosphate] was added to protoplast suspensions followed by incubation for 15 min after which the liquid was replaced with fresh fixing solution and samples incubated for 30 min. The samples were washed 3 times in PBS pH 7.4 and permeabilised in 0.5 % (v/v) Triton X-100 in PBS for 10 min. Non-specific binding sites were blocked for 10 min in blocking solution (PBS containing 1 % BSA and 0.8 % gelatin). The protoplasts were incubated for 1 hour in 20 µl of dilutions of primary antiserum, washed twice in PBS and finally incubated for 1 hour in 20 µl of 1:60 dilutions of GAR-FITC. The protoplasts were washed twice with PBS and the cover slips mounted on microscope slides using Citifluor.

2.9 Immunofluorescence and confocal microscopy

Fixed and live protoplasts were observed with a fluorescence microscope equipped with FITC/UV filters (Nikon Optiphot 2), and with a Zeiss LSM confocal microscope using the standard filters to detect FITC in fixed protoplasts or GFP in living protoplasts: excitation wavelength 488 nm and emission band pass filter 505-550 nm.

2.10 Inoculation of plants and analysis of infected plants

To release the virus particles, a pellet of 10^6 protoplasts was suspended in 100 µl of PBS and disrupted by repeated uptake through a fine needle attached to a syringe as described by Verver *et al.* (1998). The protoplast extracts or homogenate prepared from infected leaf material were inoculated onto primary leaves of 10-day old cowpea or 20-day old *N. benthamiana* plants pre-dusted with carborundum. Virus was purified from infected leaves using the polyethylene glycol method described by van Kammen (1967). RNA was isolated from virus particles by phenol/chloroform extraction and ethanol precipitation, analysed by agarose gel electrophoresis and cDNAs amplified by RT-PCR as described by Sijen *et al.*, (1995). The inoculated plants were examined for symptom expression over a 3-week period.

2.11 Preparation of subcellular extracts from virus-infected leaves

About 1 g leaf material was ground in 10 ml HB buffer (50 mM Tris pH 7.4; 10 mM KAC; 1 mM EDTA; 5 mM DTT; 0.8 mM PMSF) and homogenate centrifuged at 3,000g for 15 min at 4 ° C. The supernatant was collected and further centrifuged at 30,000g for 30 min at 4 ° C to obtain the P30 pellet enriched for membranes, and the S30 supernatant fraction containing soluble proteins. Aliquots of 20 µl of the p30 fraction (re-dissolved in minimum volume of HB) and S30 fraction were separately mixed with equal volumes of protein loading buffer for SDS-PAGE and immunoblot analysis.

3. Results

3.1 Purification of HC-Pro expressed in *E. Coli*

To obtain large quantities of the CABMV HC-Pro protein for the production of polyclonal antiserum, *E. coli* BL21 (DE3) cells transformed with plasmid pT_{77}/HCPro were used to overproduce the HC-Pro protein. The induction of T7 RNA polymerase expression resulted in high level synthesis and accumulation of the HC-Pro protein which had the expected size of 52 kDa as shown by SDS-PAGE analysis and Coomassie staining of cell lysates (Fig 1a, lanes 1-4). The expressed protein accumulated in inclusions which were readily separated from *E. coli* proteins by centrifugation of cell lysates followed by further removal of bacterial proteins by washing the pellets in 1 M NaCl. Solubilisation of purified inclusions in 6 M urea yielded about 10 % soluble protein (Fig 1b, lane 4-5) while most of the protein remained in the insoluble fraction (Fig 1b, lane 2-3). In addition to the major 52 kDa band, the soluble fraction had minor bands possibly resulting from degradation or contamination with *E. coli* proteins. To ensure the complete homogeneity of the HC-Pro preparation to be used for raising antiserum, the 52 kDa band of the soluble fraction of the purified protein was excised from the gel and used to raise an antiserum in rabbits.

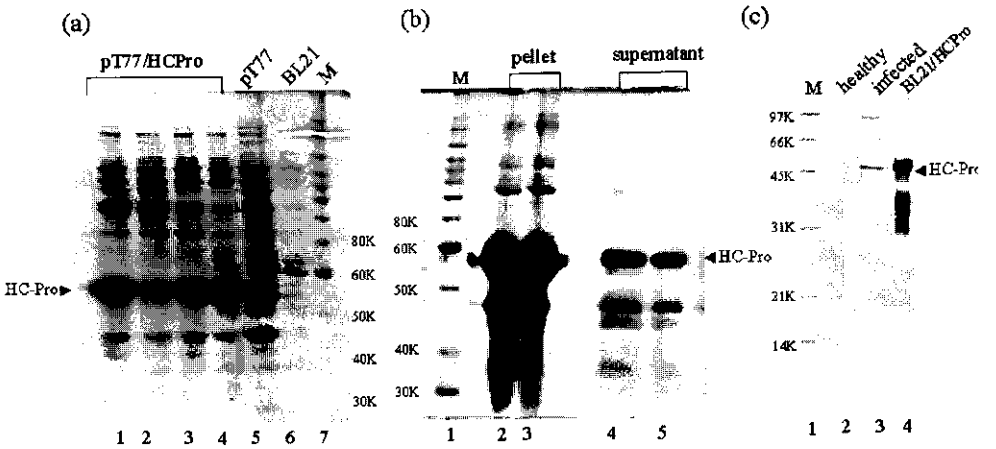

Fig. 1 SDS-PAGE analysis of CABMV HC-Pro expressed in *E. coli* (a, b) and Western blot evaluation of HC-Pro antiserum (c).

(a) Lane 1-4, cell lysates of different cell cultures of pT_{77}/HCPro-transformed BL21 after induction of expression. Lane 5, cell lysate of BL21 transformed with the pT_{77} vector. Lane 6, cell lysate of non-transformed BL21. Lane 7, proteins used as molecular weight marker.

(b) Lane 1, proteins used as molecular weight marker. Lane 2-3, Insoluble pellet remaining after solubilisation of protein aggregates. Lane 4-5, Purified HC-Pro protein obtained by solubilisation of protein aggregates.

(c) Lane 1, protein molecular weight marker, marker sizes are indicated in kDa: 97 kDa (phosphorylase b); 66 kDa (bovine serum albumin); 45 kDa (ovalbumin); 31 kDa (carbonic anhydrase); 21 kDa (soybean trypsin inhibitor); 14 kDa (lysozyme). Lane 2, protein extract prepared from uninfected leaves. Lane 3, protein extract prepared from CABMV-infected leaves. Lane 4, crude lysate of HC-Pro expressed in BL21 cells.

3.2 HC-Pro antiserum raised against the *E. coli* expressed protein

The HC-Pro antiserum was tested for immunoreactivity and specificity to the HC-Pro protein on western blots. With dilutions up to 1:5000, the antiserum was highly reactive against the HC-Pro expressed in *E. coli* (Fig 1c, lane 4) or with homogenate prepared from leaves infected with CABMV (Fig 1c, lane 3), and not with uninfected leaves (Fig 1c, lane 2). Thus the antiserum had a high titer of immunoreactive antibodies specific for the HC-Pro protein and was suitable for immunodetection and localisation of the protein in protoplasts and plants.

3.3 Expression and subcellular location of HC-Pro in protoplasts.

Following its evaluation, the HC-Pro antiserum was used in immunofluorescence assays to determine the subcellular location of HC-Pro in cowpea protoplasts infected with CABMV in which HC-Pro is expressed with other viral proteins, or using a transient expression system in which HC-Pro was expressed under the control of the enhanced 35S promoter seperate from other viral proteins (Fig. 2a). The transient expression system was also used to express a GFP-HC-Pro fusion protein to study the subcellular location of HC-Pro in living cells (Fig. 2b).

An alternative expression system was also used for which the HC-Pro coding region was subcloned in-frame between the MP and L genes of RNA2 of CPMV (Fig 2c & d) (Gopinath *et al.*, 2000). After transfection of protoplasts with this construct together with CPMV RNA1, in which viral replication functions reside (Fig. 2e), the HC-Pro is expressed with the CPMV proteins.

For immunofluorescence assays, the transfected protoplasts were either fixed with ethanol or paraformaldehyde as an alternative fixative in which the integrity of protoplasts is better preserved. The fixed protoplasts were immunostained with the HC-Pro antiserum combined with GAR-FITC secondary antibody and the expression and subcellular location of HC-Pro traced by immunofluorescence using confocal microscopy.

Typically 10-30% of the protoplasts showed immunofluorescence or GFP fluorescence 2 days post-transfection with 10 μg of either pMon/HCPro, pMon/GFPHCPro, pM19HCPro7 or 1 μg of *in vitro* transcripts of pM19HCPro2A.

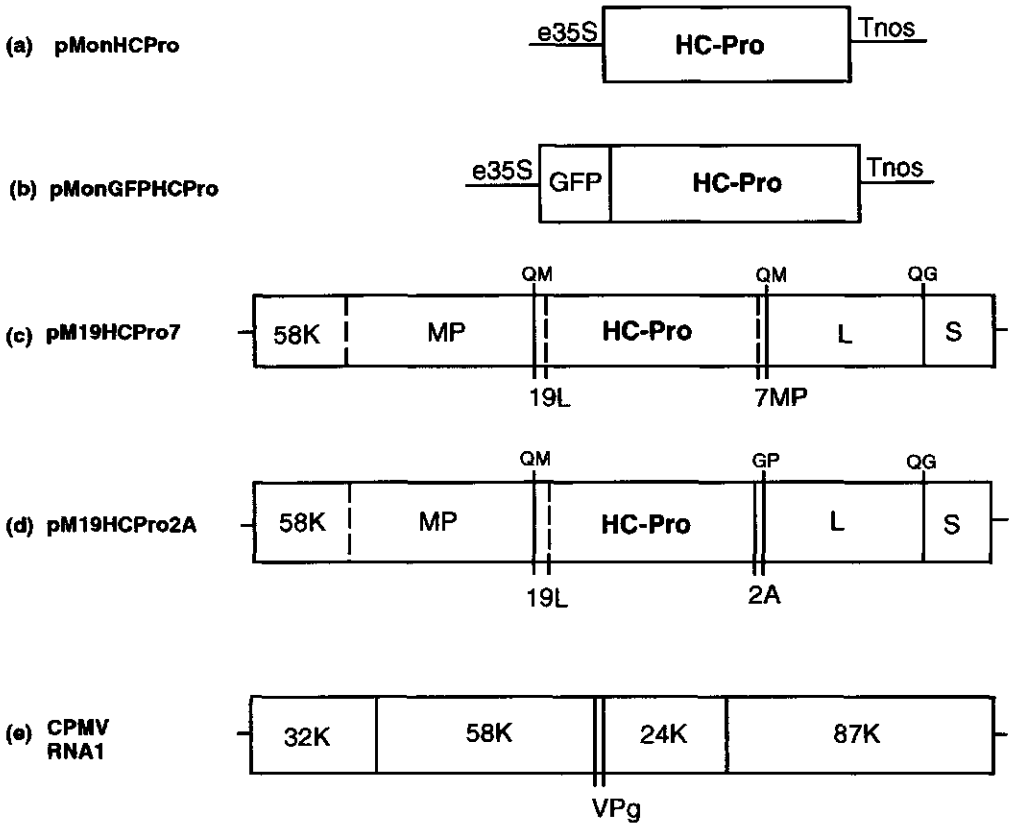

Fig. 2 Constructs used in HC-Pro localisation studies in cowpea protoplasts.

HC-Pro sequences cloned between the enhanced 35S promoter (e35S) and nopaline synthetase terminator (Tnos) in pMon transient expression vector comprise:

(a) The full-length HC-Pro coding region.

(b) GFP-HCPro (GFP fused at the 5' end of the full-length HC-Pro coding region).

In an alternative expression system, HC-Pro was cloned in-frame between the MP and the L coat protein in CPMV RNA2-based vectors:

(c) M19-7 and

(d) M19-2A.

(e) RNA1 of CPMV co-transfected with constructs (c) and (d).

In (c), (d) and (e) the open bars represent the ORF and the solid vertical lines indicate cleavage sites. 19CP: 19 N-terminal amino acids of the L protein; 7MP: 7 C-terminal amino acids of the MP. 2A: foot and mouth disease virus (FMDV) 2A protease. QM & QG: cleavage sites of the CPMV RNA1-encoded 24-kDa protease. GP: cleavage site of the 2A protease of FMDV.

HC-Pro produced with other viral proteins during a natural CABMV infection was diffusely distributed throughout the cytoplasm (Fig 3c). Similar results were obtained when HC-Pro was expressed separate from other viral proteins using the transient expression system in which HC-Pro was produced either as a free protein or with GFP fused at its N-terminus (Fig 3a and 3b). GFP alone, expressed in the same transient expression system, was mainly localised in the nucleus (not shown).

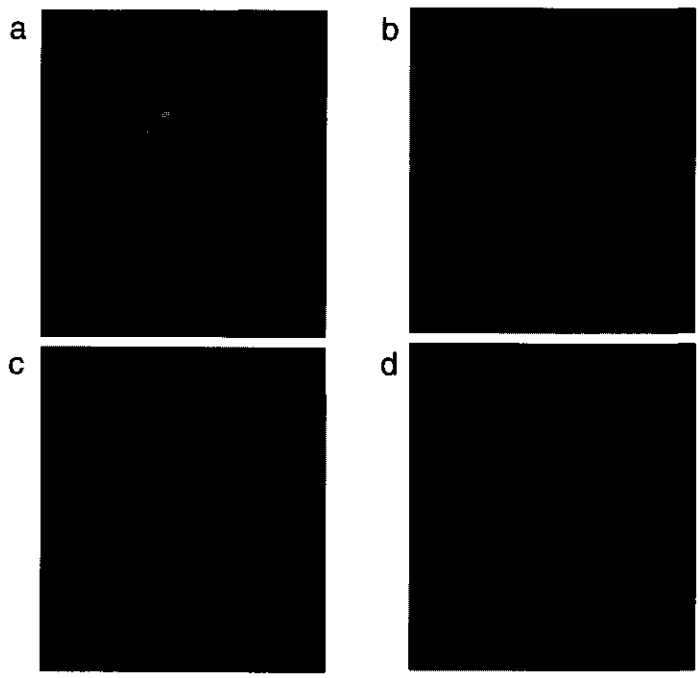

Fig. 3 Confocal fluorescence micrographs showing the intracellular distribution of CABMV HC-Pro in cowpea protoplasts. The images were collected with focal depth of 1 μm.

(a) Image of pMon/GFPHCPro-transfected protoplast (24 h post-transfection) collected using standard FITC filter settings to detect GFP (pseudo-colored green) and standard rhodamine filter settings to detect autofluorescence of the chlorophyll (pseudo-colored red). (b) Image of pMon/HCPro-transfected protoplast. (c) Image of CABMV-transfected protoplast protoplast. (d) Image of CPMV RNA1 + M19HCPro7-transfected protoplast. In b, c, & d protoplasts were ethanol-fixed 48 h post-transfection and immunostained with anti-HC-Pro primary antiserum combined with GAR-FITC secondary antibody and confocal images collected using standard FITC filter settings to detect FITC (pseudo-colored green).

On the other hand, HC-Pro expressed in CPMV vectors (pM19HCPro7 or pM19HCPro2A) was localised in confined cytoplasmic patches close to the nucleus (Fig. 3d). The RNA2-encoded 58K protein was localised in the nucleus, the RNA1-encoded 110K in confined cytoplasmic patches close to the nucleus, and the L protein was distributed throughout the cytoplasm as tested by immunofluorescence with antibodies directed against these proteins (not shown).

3.4 HC-Pro expressed in CPMV is fully proteolytically processed and enhances the virulence of CPMV

Subsequently, cowpea and *N. benthamiana* plants were inoculated with homogenate of either pM19HCPro7- or pM19HCPro2A-transfected protoplasts (NB: in these CPMV constructs the prefix "p" included when referring to the plasmid constructs, will be omitted when referring to the progeny virus).

Seven days after infection, a homogenate was prepared from the infected leaves and resolved into a S30 fraction containing soluble proteins, and a P30 fraction enriched for membrane bound proteins. Western blot analysis of the fractions using HC-Pro antiserum revealed that HC-Pro co-purified with the membrane fraction (Fig. 4a, lane 4) and no protein was detectable in the S30 fraction (Fig. 4a, lane 3) thus indicating an association between HC-Pro and cytoplasmic membranous components. The HC-Pro protein was detected as a single band of molecular weight of 52 kDa thus showing that it was fully processed from the RNA2 encoded polyprotein (Fig. 4a, lane 4).

The blots were further probed with antiserum raised against the L protein of CPMV. In the P30 fraction of homogenate prepared from leaves infected with M19HCPro7, a single band of the fully processed L protein with molecular weight of 37 kDa was detected (Fig. 4b, lane 2). However, in a homogenate prepared from leaves infected with M19HCPro2A and in virus particles purified from this homogenate, two forms of the L protein were detected (Fig. 4b, lanes 3-4): the fully processed 37 kDa L protein released from the polyprotein by the proteolytic activity of the FMDV 2A protease at the G/P cleavage site at the N-terminus of the L protein and of the CPMV protease at the Q/M cleavage site at the C-terminus, and a larger 2A-L fusion protein released from the polyprotein by the proteolytic activity of HC-Pro at the N-terminus of the 2A protein and of the CPMV protease at the C-terminus of the L protein.

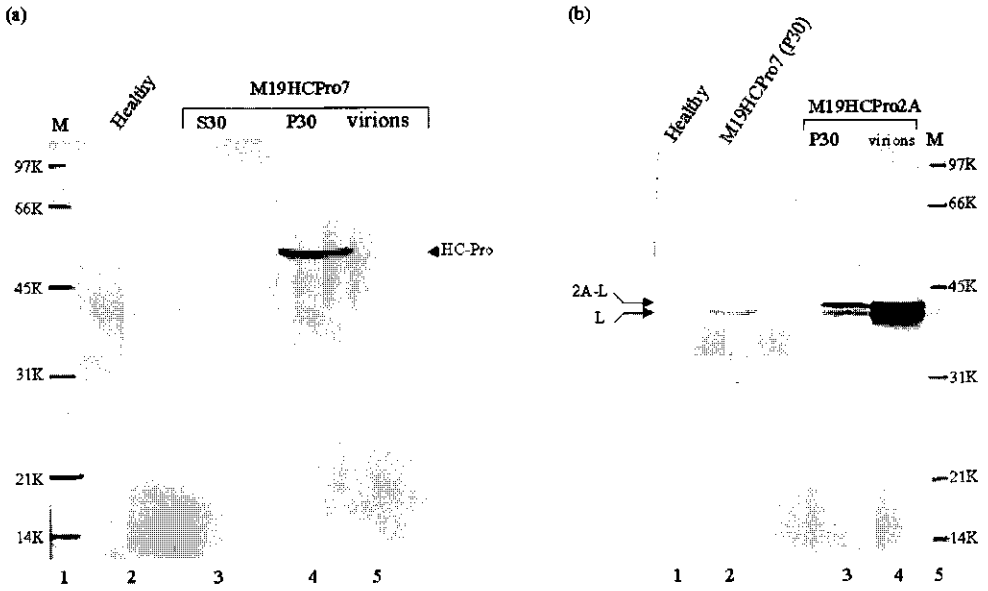

Fig. 4 Western blot analysis of proteins from homogenate of cowpea leaves infected with either M19HCPro7 or M19HCPro2A and of the purified virus particles.

(a) Protein extracts of M19HCPro7-infected plants probed with HC-Pro antiserum. Lane 1, protein molecular weight markers in kDa: 97 kDa (phosphorylase b); 66 kDa (bovine serum albumin); 45 kDa (ovalbumin); 31 kDa (carbonic anhydrase); 21 kDa (soybean trypsin inhibitor); 14 kDa (lysozyme). Lane 2, protein extract of uninfected plant. Lane 3, S30 fraction of infected plant. Lane 4, P30 fraction of infected plant, Lane 5, virus particles purified from M19HCPro7 infected plants.

(b) Protein extracts of M19HCPro7- and M19HCPro2A-infected plants probed with CPMV CP antiserum. Lane 1, protein extract of healthy plant. Lane 2, P30 fraction of M19HCPro7-infected plant. Lane 3, P30 fraction of M19HCPro2A-infected plant. Lane 4, virus particles purified from M19HCPro2A infected plants. Lane 5, protein molecular weight marker. The identities and sizes of the marker proteins are as indicated above.

To study the effect of HC-Pro on the virulence of CPMV, cowpea and *N. benthamiana* plants were inoculated with M19HCPro7 or M19HCPro2A and examined for symptoms over a 20-day period. For controls, plants were either inoculated with wild type CPMV, CABMV, CABMV + CPMV, CPMV expressing GFP (M19GFP7, Gopinath *et al.* 2000) or mock inoculated with water. In cowpea plants inoculated with wild type CPMV, a yellow mosaic appeared on systemic leaves 5 dpi (symptoms 14 dpi are shown in Fig. 5a) and in *N. benthamiana* a mild mosaic appeared 8 dpi (symptoms 14 dpi are shown in Fig. 5f), but in both species plant growth was not significantly affected throughout the 20-day period. In plants infected with M19GFP7 systemic green fluorescence was visible using a hand-held UV lamp 6 dpi followed by a mild yellow mosaic without any significant effect on plant growth (not shown).

However, both on cowpea (Fig. 5b) and *N. benthamiana* (Fig. 5g), CPMV expressing HC-Pro (M19HCPro7 or M19HCPro2A) showed enhanced virulence compared to wild type CPMV (Fig. 5a & 5f), CPMV expressing GFP (M19GFP7) (not shown) or CABMV (Fig. 5c & 5h). The enhanced virulence resulted either in death of cowpea plants 10 dpi as shown 14 dpi in Fig. 5b or an extensive yellow mosaic and severe reduction in growth of *N. benthamiana* (Fig. 5g). This pattern of severe symptoms was also observed in plants doubly infected with wild type CPMV and CABMV (Fig. 5d & 5i). Healthy cowpea and *N. benthamiana* are shown in Fig. 5e & 5j respectively.

4. Discussion

This study investigates the subcellular location of the HC-Pro of CABMV in cowpea protoplasts and its effect on the virulence of CPMV. For this, the HC-Pro was expressed in cowpea protoplasts either with other viral proteins in a natural CABMV infection (Fig. 3c), or separate from other viral proteins using a transient expression system in which HC-Pro was expressed either as a free protein (Fig. 3b) or with GFP fused at its N-terminus (Fig. 3a). In all cases the protein was diffusely distributed throughout the cytoplasm suggesting that the localisation of HC-Pro is not influenced by other CABMV proteins. The cytoplasmic location of HC-Pro has previously been demonstrated by ultrastructural studies which showed that HC-Pro-containing cytoplasmic amorphous inclusions (AIs) occur in cells infected with plum pox virus (PPV) (Riedel *et al.*, 1998), papaya ringspot virus (PRSV) and pepper mottle virus (PepMoV) (De Meija

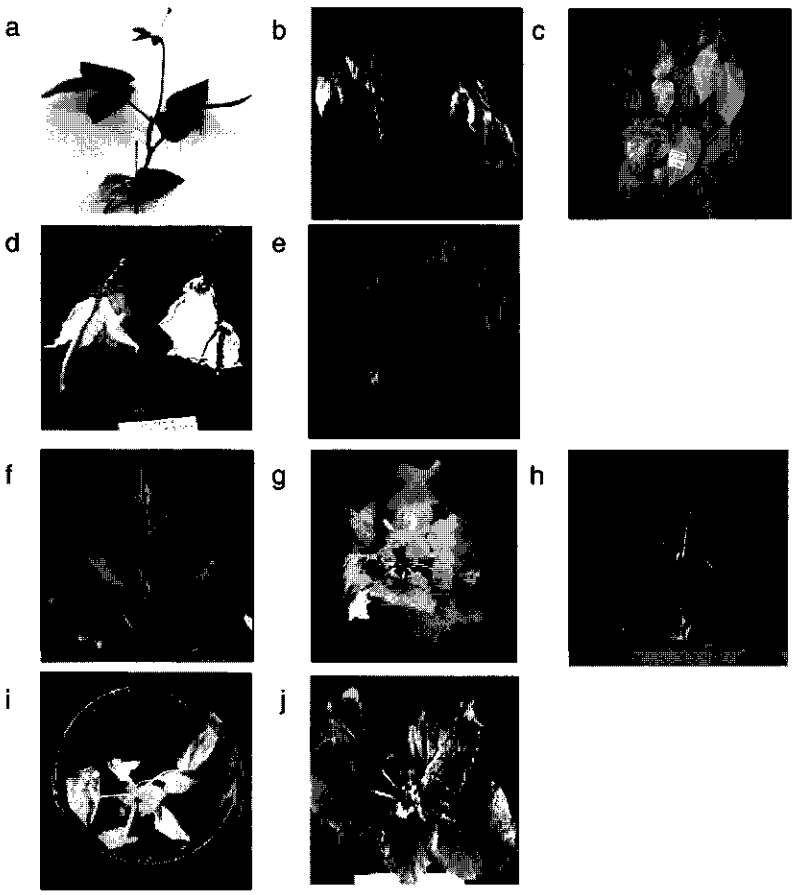

Fig. 5 Effect of HC-Pro on the virulence of CPMV.

In all cases the symptoms are shown 14 dpi.

(a) CPMV wild type symptoms on cowpea.

(b) Enhanced symptoms on cowpea plants inoculated with homogenate of protoplasts transfected with recombinant CPMV expressing HC-Pro (M19HCPro7).

(c) CABMV wild type symptoms on cowpea.

(d) Enhanced symptoms on cowpea plants doubly infected with CABMV and CPMV.

(e) Healthy cowpea plant.

(f) CPMV wild type symptoms on *N. benthamiana*.

(g) Enhanced symptoms on *N. benthamiana* inoculated with homogenate of protoplasts transfected with M19HCPro7.

(h) CABMV wild type symptoms on *N. benthamiana*.

(i) Enhanced symptoms on *N. benthamiana* doubly infected with CABMV and CPMV

(j) Healthy *N. benthamiana* plant.

et al., 1985a and De Meija *et al.*, 1985b). Whilst the cytoplasmic accumulation of HC-Pro could be essential for its role in aphid transmission, targeting to specific subcellular structures might be masked due to accumulation of excess protein. For instance, it is conceivable that due to its role in cell-to-cell spread (Rojas *et al.*, 1997), HC-Pro should be targeted to the cell periphery at an early stage of infection. However this could not be demonstrated even at early times post transfection maybe because of the transient nature of the event.

In an alternative expression system, the HC-Pro coding region was subcloned in-frame between the MP and L genes of RNA2 of CPMV. Upon transfection of protoplasts with CPMV RNA1, the HC-Pro was produced as part of the RNA2 encoded polyprotein prior to proteolytic processing. In this case, the protein localised in broad cytoplasmic patches. The localisation was remarkable in that it is reminiscent of the typical CPMV induced electron dense structures described by De Zoeten *et al.* (1974). It is unlikely that the additional CPMV derived amino acid residues at the termini of HC-Pro (19 from L and 7 from MP) had any significant influence on its localisation since the same residues had no effect on the localisation of GFP expressed using the same system (Gopinath *et al.*, 2000). The HC-Pro antiserum did not cross react with host cowpea or CPMV proteins thus excluding the possibility of nonspecific staining. By showing complete processing of HC-Pro from the M19HCPro7 encoded polyprotein on western blots of protein extracts prepared from cowpea leaves infected with M19HCPro7 (Fig. 4a, lane 4), we could further exclude the possibility that the co-localisation with CPMV proteins was due to incomplete processing of HC-Pro from the polyprotein. The localisation patterns of CPMV proteins (MP, CP and 110K) were found to be unaffected by the expression of HC-Pro in CPMV (not shown). Overall, these results suggest a putative interaction between HC-Pro and CPMV proteins localised in the cytopathic structures in which viral replication occurs.

The recombinant CPMV virus expressing the HC-Pro of CABMV (M19HCPro7 or M19HCPro2A) showed a strongly enhanced virulence on cowpea and *N. benthamiana* in a manner reminiscent of co-infections with wild type CPMV and CABMV. Potyvirus-associated synergisms, characterised by increase in symptom severity and accumulation of co-infecting heterologous species are well documented (Rochow and Ross, 1955; Vance, 1991; Anjos *et al.*, 1992). This phenomenon was shown to be mediated by the expression of potyviral 5' proximal sequences encoding P1, HC-Pro and a fraction of P3 in potato virus X (PVX)/potyviral synergism (Vance *et al.*, 1995; Pruss *et al.*, 1997) and to be abolished by mutations in the region encoding

the central domain of tobacco etch virus HC-Pro (Shi *et al.*, 1997). The increased virulence conferred on CPMV by CABMV HC-Pro is consistent with the effect of HC-Pro encoded by TEV on the pathogenicity of heterologous viruses PVX, cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV) (Pruss *et al.*, 1997; Shi *et al.*, 1997) and fits into the role of HC-Pro in synergistic disease in which it interdicts a host defense mechanism recently shown to be based on PTGS (Pruss *et al.*, 1997; Brigneti *et al.*, 1998; Anandalakshmi *et al.*, 1998). Our results add to the contention that the defense mechanism is shared by different hosts and acts against taxonomically disparate viruses. However, it has yet to be established if movement related functions of HC-Pro are separable from its role in suppressing host antiviral defense responses and if the domains involved in synergy and PTGS overlap completely. Thus expression of HC-Pro derivatives in CPMV constructs or transgenic plants would be useful in defining minimal residues involved in synergy and/or suppression of PTGS.

Acknowledgements

The authors wish to thank B. J. M. Verduin and J. Carette for kindly providing the biological material indicated in Materials and Methods. This work was supported by a grant of the Netherlands Foundation for the Advancement of Tropical Research (WOTRO).

CHAPTER 5

TRANSGENIC *NICOTIANA BENTHAMIANA* PLANTS EXPRESSING THE SEQUENCES OF THE HELPER COMPONENT PROTEINASE GENE OF COWPEA APHID-BORNE MOSAIC VIRUS

Mlotshwa, S., Verver, J., Sithole-Niang, I., Prins, M., Van Kammen, A. and Wellink, J.

Summary

To study the functions of HC-Pro, *N. benthamiana* plants were engineered to express HC-Pro sequences under the control of the enhanced 35S promoter. Most lines expressing HC-Pro (h-lines) showed enhanced symptoms after infection with the parental CABMV isolate and also after infection with heterologous potyviruses PVYn and SPV-Z or nonpotyviruses CPMV, CMV and AMV. On the other hand, none of the lines expressing nontranslatable HC-Pro (t-lines) or translatable HC-Pro with a deletion of the central domain (d-lines) showed enhanced symptoms after infection with the parental CABMV isolate and heterologous viruses. Severe symptoms in plants infected with CABMV were followed by brief recovery and subsequent re-establishment of infection, possibly indicating counteracting effects of HC-Pro expression and a host defense response. One of the h-lines was found to be resistant to CABMV and to CPMV expressing HC-Pro which indicates that the line was putatively silenced for the HC-Pro transgene. The silenced line did not show enhanced symptoms after an infection with CPMV. The same silenced line was found not to be resistant to PVY and SPV-Z but instead showed enhanced symptoms compared to nontransgenic plants. This could be due to relief of silencing of the HC-Pro transgene by HC-Pro expressed by PVYn or SPV-Z.

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1. Introduction

The potyvirus cowpea aphid-borne mosaic virus (CABMV) is a major pathogen of cowpea worldwide. The potyviral genome consists of a single stranded RNA molecule of positive polarity that encodes a large polyprotein. The biological properties of potyviruses are determined by ten mature proteins cleaved from the polyprotein by three virus encoded proteinases: the P1 proteinase, the helper component-proteinase (HC-Pro) and the nuclear inclusion a-proteinase (NIa-Pro) (reviewed in Riechmann *et al.*, 1992). The HC-Pro has attracted special attention due to its multifunctionality and its role at different steps of the potyvirus life cycle (for review see Maia *et al.*, 1996). The functional regions of HC-Pro are broadly recognised as the N-terminal, the central and C-terminal regions (Urcuqui-Inchima *et al.*, 2000). The C-terminal region of HC-Pro contains a cysteine-type proteinase domain which catalyses its autoproteolytic release from the polyprotein as was demonstrated for tobacco etch virus (TEV) by Carrington *et al.* (1989). The N-terminal and central regions of the protein act cooperatively with the coat protein (CP) in acquisition and transmission of the virus by aphids (Huet *et al.*, 1994; Andrejeva *et al.*, 1999).

Nucleic acid binding properties of HC-Pro have been mapped in the central region of the protein in potato virus Y (PVY) (Maia and Bernardi, 1996; Urcuqui-Inchima *et al.*, 2000). In TEV, the central domain of HC-Pro has been shown to act cooperatively with P1 to enhance levels of genomic RNA accumulation over extended periods post infection (Kasschau *et al.*, 1997). The central region of TEV HC-Pro has also been shown to facilitate movement through vascular tissue in tobacco (Cronin *et al.*, 1995; Kasschau *et al.*, 1997). In tobacco vein mottling virus (TVMV) the HC-Pro was further established to be a pathogenicity factor, with determinants involved in pathogenicity mapped in the central domain (Atreya *et al.*, 1992; Atreya and Pirone, 1993).

HC-Pro was also shown to be involved in potyvirus-associated synergism in plant virus infection, and heterologous viruses have been shown to accumulate to higher levels regardless of whether HC-Pro was encoded by a co-infecting potyvirus, a transgene, or by the genome of a recombinant virus (Vance *et al.*, 1995; Pruss *et al.*, 1997; Shi *et al.*, 1997).

The stimulatory effect of HC-Pro was suggested to be a consequence of modulation of host functions in which HC-Pro interdicts a natural plant defense mechanism targeted against viruses (Shi *et al.*, 1997). Posttranscriptional gene silencing (PTGS), a phenomenon in which expression

of transgenes and endogenous genes containing sequences homologous to the transgene can be suppressed, has been linked to natural defense against viruses both in transgenic (Angell and Baulcombe, 1997) and nontransgenic plants (Covey *et al.*, 1997; Ratcliff *et al.*, 1997).
Recently, the HC-Pro encoded by TEV or PVY was shown to interfere with establishment or maintenance of PTGS (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998), whereas the 2b protein, a pathogenicity factor encoded by cucumber mosaic virus, was found capable of blocking the initiation of PTGS at growing points of the plant (Brigneti *et al.*, 1998).

To study the functions of the HC-Pro of cowpea aphid-borne mosaic virus (CABMV), *Nicotiana benthamiana* plants were engineered to express HC-Pro sequences under the control of the 35S promoter. The plants were transformed with either a construct carrying a translatable or nontranslatable full-length HC-Pro gene, or a HC-Pro gene in which the central region was deleted. The plants were assayed for their responses to inoculations with either the parental virus, heterologous potyviruses or cowpea-infecting nonpotyviruses.

2. Materials and Methods

Plants and viruses

CABMV-Z was described by Sithole-Niang *et al.* (1996). Sweetpotato virus (SPV-Z) (Chavi *et al.* 1997) and the N strain of potato virus Y (PVY) were kindly provided by Dr B. J. M. Verduin (Laboratory of Virology, Wageningen University and Research Centre, The Netherlands). The construction of recombinant CPMV (M19HCPro7) expressing the HC-Pro of CABMV-Z, was described in chapter 3. The strain of cucumber mosaic virus (CMV) isolated from lily, was kindly provided by Yukun Chen (Laboratory of Virology, Wageningen University and Research Centre, The Netherlands). Cowpea mosaic virus (CPMV) and alfafa mosaic virus (AMV) were virus strains of Wageningen and were maintained on *Nicotiana benthamiana* by mechanical inoculation. *N. benthamiana* plants were used for the production of transgenic plants.

Bacterial strains and plasmids

The binary vector pBin19 was used for cloning cDNA fragments for plant transformation. The plasmid contains within the left and right T-DNA border sequences, a polylinker, a neomycin phosphotransferase II (nptII) gene that allows selection of transformed cells on kanamycin and a lacZ∂ fragment as a genetic marker for selection in *Escherichia coli*. The plasmids were amplified in *E. coli* DH5∂ and transferred into *Agrobacterium tumefaciens* strain LB4404 (Ditta *et al.*, 1980) for plant transformation, by conjugation in the presence of the helper *E. coli* strain pRK2013 (Horsch *et al.*, 1985).

Construction of plasmids

pBin/HCPro

The construction of pMon/HCPro in which the HC-Pro coding sequence was cloned under the control of the enhanced 35S (e35S) promoter and NOS terminator was described in chapter 3. The expression cassette comprising HC-Pro cloned as an *EcoRI-BamHI* fragment between the enhanced 35S promoter and nos terminator was excised from the pMon/HCPro plasmid as a 2.353 kb *Not*I fragment and ligated into *Not*I digested pBSK- vector, resulting in plasmid pBSK/HCPro. The expression cassette was subsequently excised from pBSK/HCPro as a *Sal*I-*Sst*I fragment and cloned into *Sal*I-*Sst*I digested pBin19 vector, resulting in the binary construct pBin/HCPro.

pBin/TAAHCPro

To incorporate a stop codon at the start of the HC-Pro coding region, the HC-Pro gene was amplified with primers ecostophcF (CCGGAATTCTAATCACATCAGTTGGAGGTTCAATT TTTCC) incorporating an *EcoR*I site and bamstophcR described in chapter 3. The amplified product was subcloned as an *EcoR*I-*BamH*I fragment between the e35S promoter and NOS terminator in *EcoRI-BamH*I-digested pMon999 vector, resulting in plasmid pMon/TAAHCPro. The expression cassette was excised as a 2.353 kb *Not*I fragment and ligated into similarly digested pBSK- vector, resulting in plasmid pBSK/TAAHCPro from which the expression

cassette was subsequently excised as a *SalI-SstI* fragment and cloned into *SalI-SstI* digested pBin19 vector, resulting in the binary construct pBin/TAAHCPro.

pBin/HCProDel

The 5' terminal one third of the HC-Pro coding region was amplified with primers econdehcF, described in chapter 3, and hc466del (CGCGGATCCTTATCTAGAACGCGTTTGGTCAAG CTCTTGCTTGC) designed from nt 441-466 in the HC-Pro coding region and incorporating an *MluI* site, and the 3' terminal one third amplified with primers bamstophcR described in chapter 3 and hc912del (CCGGAATTCATGACGCGTTACAAGAGTCCAACTAAGAGGC) designed from nt 912-937 in the HC-Pro coding region and incorporating an *MluI* site. The two fragments were respectively digested with *EcoRI-MluI*, and *MluI-BamHI* and cloned as two fragments between the e35S promoter and NOS terminator in *EcoRI-BamHI*-digested pMon999 vector, resulting in plasmid pMon/HCProDel. From this plasmid the expression cassette was isolated as a 1.9 kb *NotI* fragment and cloned into similarly digested pBSK- vector, yielding pBSK/HCProDel. The expression cassette was then isolated from pBSK/HCProDel as a *SalI-SstI* fragment and cloned into similarly digested pBin19, resulting in the binary construct pBin/HCProDel.

Triparental mating and colony PCR

The binary constructs were transferred from *E. coli* DH5∂ into *A. tumefaciens* LBA 4404 through triparental mating in the presence of the helper *E. coli* strain PRK2013. For this the *A. tumefaciens* strain was grown overnight at 28 °C in LB medium containing 50 mg/l rifampicine. The *E. coli* helper strain pRK2013 and *E. coli* DH5∂ carrying the binary contructs were grown overnight at 37 ° C in LB medium containing 100 mg/l kanamycin. The overnight *A. tumefaciens* culture was diluted 10 times in fresh LB medium without antibiotics and the *E. coli* strains diluted 50 times. The *E. coli* cultures were grown for 4 hours at 37 °C and the *A. tumefaciens* at 28 °C. The bacterial cultures were mixed in 1.5 ml Eppendorf tubes in the the ratio of 1 volume each of the *E. coli* cultures and 2 volumes of *A. tumefaciens*. As a control the *A. tumefaciens* was mixed with only the *E. coli* helperstrain. Quantities of 50 µl were spotted on sterile nitrocellulose filter on a dry LB agar plate and incubated overnight at 28 °C. The bacteria were streaked on LB

agar plates containing kanamycin and rifampicin and incubated for 2 days at 28 °C. The individual colony forming units were subcultured and checked for the presence of the binary constructs by PCR using primers (econdehcF and bamstophcR) specific for HC-Pro sequences. For this, each colony was picked with sterile toothpick, suspended in 5 µl sterile water in 0.5 ml PCR tubes, denatured for 5 min at 94 °C and mixed with 45 µl of PCR reaction mix containing 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.25 µg each of primers, 1 U Taq DNA polymerase, 1x Taq buffer [10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 50 mM KCl] and amplifications carried out using the Perkin Elmer thermocycler with cycling conditions: 94 °C for 5 min; 35 cycles of denaturation (94 °C for 30 secs), annealing (50 °C for 1 min) and extension (72 °C for 2 min). The reactions were terminated with an extension step at 72 °C for 5 min.

Transformation, selection and regeneration

Quantities of 250 µl of overnight cultures of *Agrobacterium* containing the binary constructs were diluted in 25 ml of LB3 (0.1% tryptone, 0.5% yeast extract, 0.4% NaCl, 0.1% KCl, 0.3% $MgSO_4$.7H2O) supplemented with 50 mg/l rifampicine and 100 mg/l kanamycin. The cultures were grown for 18 hours at 28 °C and 150 rpm to OD_{600}0.5-0.6. The cells were then diluted to 10^7 cells in 50 ml MS medium (Murashige and Skoog, 1962) and cocultivated for 15 min with 0.25-1.0 cm^2 explants punched from sterile *N. benthamiana* plants. The explants were dried briefly on sterile filter paper and transferred to plates with MS medium containing 0.8 % agar and supplemented with 1.0 mg/l 6-benzylaminopurine (BAP) and 0.1 mg/l naphthaleneacetic acid (NAA) followed by cocultivation for 2 days at 25 °C and 16 hours of light to allow for transfer of the plant expression cassette into plant tissue. This was followed by counterselection of *A. tumefaciens* on MS medium containing BAP, NAA and 250 mg/l carbenicillin for 2 days at 25 °C and 16 hours continuous light. Kanamycin resistance was used to select for transformed calli. For this, the explants were cultured on MS medium containing BAP, NAA, carbenicillin and 150 mg/ml kanamycin at 25 °C and 16 hours of light for at least 3 weeks. Shoots were excised and rooted on hormone-free MS medium solidified with 0.7% agar and supplemented with 150 mg/l kanamycin and 250 mg/l cefotaxime. After root formation the To-generation plants were transferred to soil in pots and grown at 25 °C and 16 hours of continuous illumination. Leaf samples were taken for further analysis and seeds were harvested.

PCR analysis of transgenic plants

Samples for PCR were taken from the plants by punching out a disk of leaf material using the lead of an Eppendorf tube. The leaf discs were frozen in liquid nitrogen and ground in Eppendorf tubes using micropestles. The ground material was suspended in 400 µl PCR DNA extraction buffer [200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5 % SDS]. The tubes were centrifuged at 15 000g for 1 min and 300 µl of supernatant transferred into fresh tubes containing an equal volume of isopropanol. The tubes were incubated at room temperature for 2 min and centrifuged at 15 000g for 10 min . The pellets were washed in ethanol, vacuum dried and suspended in 100 µl of sterile distilled water. 2 µl of the DNA was used in 50 µl-PCR reactions containing 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.25 µg each of primers econdehcF and bamstophcR, 1 U Taq DNA polymerase, 1X Taq buffer [10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 50 mM KCl] in 0.5 µl Eppendorf tubes. The reaction mixtures were overlayed with a drop of mineral oil and amplifications carried out as described for colony PCR.

Segregation analysis

For sterilisation, seeds were immersed in 70 % ethanol for 20 s, rinsed in sterile water and then immersed in 4% bleach solution for about 20 s. The seeds were washed 3 times in sterile water, suspended in 400 µl of MS medium containing 0.1 % Dashin agar and spread on MS plates containing 150 mg/ml kanamycin using sterile pipettes. The plates were sealed with cling wrap and transferred to a growth cabinet set at 25 °C, 66 % humidity and 16 hours of light and left to germinate for two weeks.

RNA extraction and RT-PCR analysis

Total RNA extractions from transformed plants were carried out using the TRIzol™ reagent (Gibco BRL) as described in the protocol supplied by the manufacturer. First strand cDNAs were synthesised with MMLV reverse transcriptase (Gibco BRL). About 5 µg of total RNA was mixed with 1 µg primer bamstophcR in a 12 µl volume, heated for 3 min at 70 °C then chilled on ice for 5 min and mixed with 8 ul of cDNA synthesis reaction mix containing 1.25 mM dNTPs, 20 U

RNAsin, 5 mM DTT, 1 X first strand buffer [25 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$] and 200 units of MMLV-RT. The reactions were incubated for 1 hour at 37 °C and 5 µl of first strand cDNA mix used in 50 µl PCR reactions containing 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.25 µg each of primers econdehcF and bamstophcR, 1 U Taq DNA polymerase, 1X Taq buffer [10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$ and 50 mM KCl] in 0.5 µl Eppendorf tubes. The reaction mixtures were overlayed with a drop of mineral oil and amplifications were carried out as earlier described for colony PCR.

Western blot analysis

Leaf tissue was frozen in liquid nitrogen, ground into a fine powder and resuspended in 2 volumes (2 ml/g tissue) of extraction buffer [50 mM Tris-acetate pH 7.4, 10 mM potassium acetate, 1 mM EDTA, 5 mM DTT, and 0.5 mM PMSF]. The homogenate was transferred into 1.5 ml Eppendorf tubes and centrifuged at 3000g for 15 min at 4 °C. Aliqouts of 80 µl supernatant were mixed with 20 µl 5X SDS-PAGE sample buffer [0.31 M Tris-HCl pH 6.8, 50 % (v/v) glycerol, 5 % (w/v) SDS, 10 % (v/v) ß-mercaptoethanol, and 0.1 % bromophenol blue] and boiled for 5 min. Proteins were electrophoretically separated by SDS-PAGE on 12 % polyacrylamide/SDS gels and blotted overnight onto nitrocellulose membranes using the Biorad transblot apparatus at 50 volts. The blots were briefly stained in Ponceau to check the efficiency of transfer, blocked with 5% (w/v) nonfat milk powder and probed with either polyclonal antibodies raised against CABMV, CPMV or CABMV HC-Pro and antirabbit igG coupled with alkaline phosphatase.

Plant infections

Virus inoculum was prepared by homogenisation of infected leaf material in PBS (100 mM sodium phosphate, 1.5 M NaCl, pH 7.2). Twenty-day old *N. benthamiana* plants were pre-dusted with carborundum and inoculated with equal amounts of inoculum and briefly rinsed with tape water prior to transfer into a growth cabinet set at 25 °C, 66 % humidity and 16 hours of light and the development of symptoms monitored for at least 20 days.

3. Results

Construction of transgenic *N. benthamiana* expressing HC-Pro sequences

To study the activity of CABMV HC-Pro, *N. benthamiana* plants were transformed with HC-Pro sequences. Three binary constructs, pBin/HCPro, pBin/TAAHCPro and pBin/HCProDel, were designed to express the HC-Pro sequences in plants under the control of the e35S promoter and NOS terminator (Fig. 1).

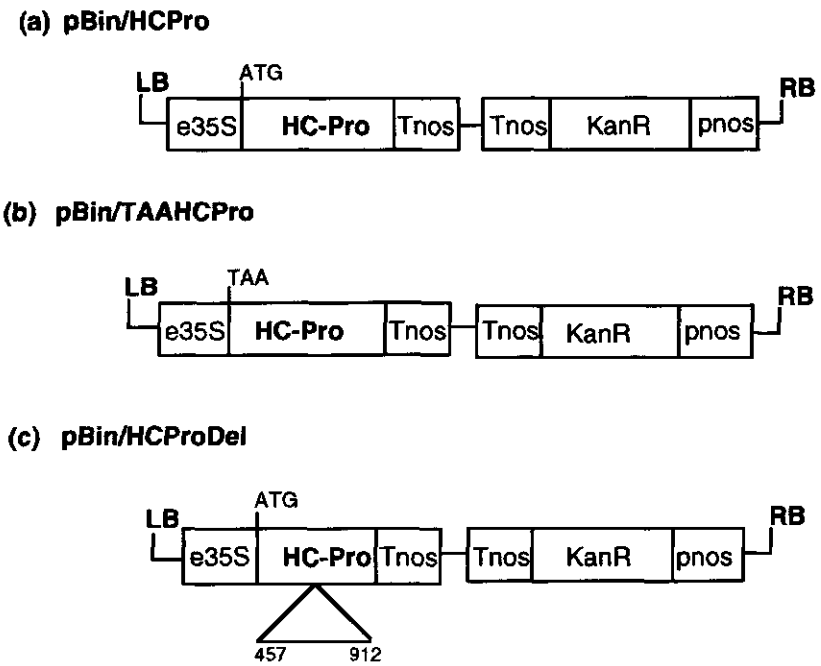


Fig. 1 Constructs used for plant transformation with the binary vector system of *Agrobacterium tumefaciens*.
The HC-Pro sequences cloned under the control of the enhanced 35S (e35S) promoter and the nopaline synthetase terminator (Tnos) in pBin19 binary vector comprise:
(a) The full-length HC-Pro coding region
(b) The full-length nontranslatable HC-Pro coding region
(c) HC-Pro with a deletion in the central region (from nucleotide 457 to 912).
LB and RB: left and right border sequences of the T- DNA respectively; KanR: kanamycin resistance gene under the control of the nopaline synthetase promoter (pnos) and Tnos.

In the case of pBin/HCPro, the expression cassette containing the complete HC-Pro gene was derived from plasmid pMon/HCPro which had been shown to produce large amounts of the protein upon transient expression in cowpea protoplasts (chapter 3). In pBin/TAAHCPro the AUG initiation codon, introduced at the start of the HC-Pro coding region in pBin/HCPro, was replaced by a UAA stop codon thus making the transcribed RNA untranslatable. The construct pBin/HCProDel contained a HC-Pro gene with a deletion in the middle, in this case the translation product lacks 152 amino acids of the central domain of the HC-Pro protein.
Following *A. tumefaciens*-mediated transformation, kanamycin resistant shoots were selected (the T0-generation plants) (Fig. 2).

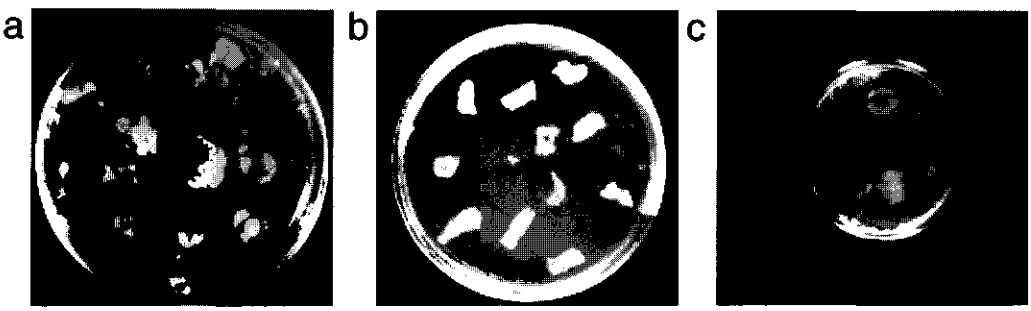


Fig. 2 Selection and regeneration of transgenic *N. benthamiana* plants transformed with HC-Pro sequences.
(a) Multiple shoots induced on transgenic calli after two weeks of incubation on MS medium containing BAP, NAA, carbenicillin and kanamycin.
(b) Bleached nontransformed explants (as negative control to test effectiveness of selection) after two weeks of incubation on MS medium containing BAP, NAA, carbenicillin and kanamycin.
(c) Plantlets growing in MS medium containing kanamycin and cefotaxime during induction of root formation, 3 weeks after excision of the shoots from (a).
All incubations were carried out at 25 °C and 16 hours of continuous light.

Plants transformed with pBin/HCPro, pBinTAA/HCPro or pBin/HCProDel were respectively designated h-, t- and d-lines. Following transfer of plants to soil, the presence of the HC-Pro transgenes was verified by PCR in each line (Fig. 3).

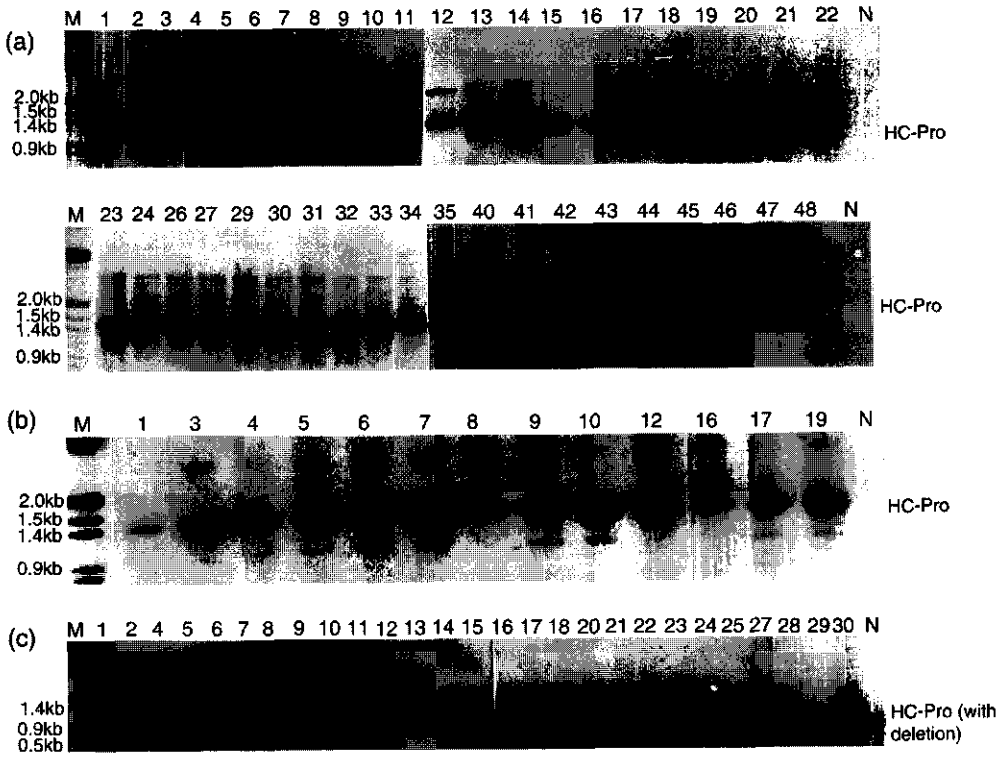


Fig. 3 PCR analysis for the presence of the transgene in T0-generation plants transformed with HC-Pro sequences.

(a) h-lines (transformed with pBin/HCPro)

(b) t-lines (transformed with pBin/TAAHCPro)

(c) d-lines (transformed with pBin/HCProDel).

DNA was isolated from transgenic lines indicated on the top of the lanes and amplified by PCR using HC-Pro specific primers econdehcF and bamstohcR. M: λ-DNA digested with *EcoRI* and *Hind III* as a marker. N: nontransformed plant.

Overall, 42 h-lines, 13 t-lines and 27 d-lines contained HC-Pro sequences (Fig. 3). The phenotypes of most T0-transformants were indistinguishable from untransformed *N. benthamiana,* except for line h30 which showed retarded growth, line h24 with reduced seed production and h25 which was sterile. The plants were left to self pollinate after which T1-generation seeds were harvested per plant. At least 100 seeds per line were sterilised and germinated on kanamycin to determine the proportions of resistant and sensitive plants (Table 1). The segregation ratios were used to estimate the number of T-DNA insertions in each line, with 3:1 ratios indicating insertions at one or more loci on a single chromosome, and larger ratios indicating multiple insertions on different chromosomes. From Table 1 it is clear that T1-generation plants from lines h19, -33, -41 and -45 and d9 and d25 were all sensitive to kanamycin and presumably had not inherited the T-DNA. These lines were not studied further. According to the segregation data at least 1/4 of the lines apparently contained T-DNA insertions at single chromosomes and the rest contained T-DNA insertions in at least 2 or more chromosomes.

Table 1. Assessement of segregation of kanamycin resistance in the T1 progeny of (a) h-lines, (b) t-lines and (c) d-lines.

(a)

line	total no.	R:S ratio
h1	152	3 :1▪
h2	143	8.5:1
h3	188	93:1
h4	171	33:1
h5	190	2.5:1▪
h6	162	3:1▪
h7	155	3:1▪
h8	143	2:1
h9	106	9:1
h10	134	4:1
h11	106	25.5:1
h12	126	30.5 :1
h13	128	31:1
h14	120	3:1▪
h15	116	2:1

[Table 1 (a) cont.]

line	total no.	R:S ratio
h16	124	7:1
h17	144	3.5:1▪
h18	114	3 :1▪
h19	120	0:120*
h20	112	2 :1
h21	124	62:0
h22	122	3:1▪
h23	144	35:1
h24	124	30:1
h26	118	4:1
h27	126	3:1▪
h29	116	3:1▪
h30	112	7 :1
h31	140	3:1▪
h32	128	63:1

[table 1(a) cont.]

line	total no.	R:S ratio
h33	140	0:140*
h34	128	2:1
h35	210	209:1
h40	170	169:1
h41	150	0:150*
h42	174	15:1
h43	170	11:1
h44	203	4:1
h45	126	0:126*
h46	144	144:0
h47	180	2.5:1•
h48	181	10:1

(b)

line	total no	R:S ratio
t1	153	3:1•
t2	155	5:1
t3	186	36:1
t4	192	4:1
t5	159	3:1•
t6	167	14:1
t7	204	204:0
t9	200	16:1
t10	110	17:1
t12	100	2:1
t16	134	18:1
t17	129	5:1
t19	104	25:1

(Table 1 cont.)

(c)

line	total no	R: S ratio
d1	162	11:1
d2	191	1:1
d4	124	2:1
d5	188	18:1
d6	209	11:1
d7	232	6:1
d8	126	24:1
d9	125	0:125*
d10	181	29:1
d11	183	11:1
d12	211	13:1
d13	162	80:1
d14	164	19.5:1
d15	175	3:1•
d16	216	1.5:1
d17	167	4:1
d18	144	4:1
d20	147	1:1
d21	102	16:1
d22	110	6:1
d23	147	48:1
d24	120	29:2
d25	122	0:122*
d27	130	12:1
d28	104	1:2
d29	160	4:1
d30	110	3:1•

* Lines lacking T-DNA inserts.

• Lines containing insertions at single chromosomes

Expression of transgenes

The expression of HC-Pro sequences was analysed by RT-PCR for all lines and by western blotting for a few selected lines. Transgene mRNAs were detected in most h-, t- and d-lines except for lines h48, t1, t4, t10, t16, d2, d15 and d18 which had low levels of amplified product (Fig. 4). To determine the amount of HC-Pro protein in the various lines, extracts of leaves were analysed by western blotting using HC-Pro specific antiserum. The HC-Pro protein was not detectable not only in t-lines containing the nontranslatable version of HC-Pro, but also in h- and d-lines transformed with the translatable gene constructs. The HC-Pro protein was detectable in nontransgenic control plants infected with CABMV (not shown).

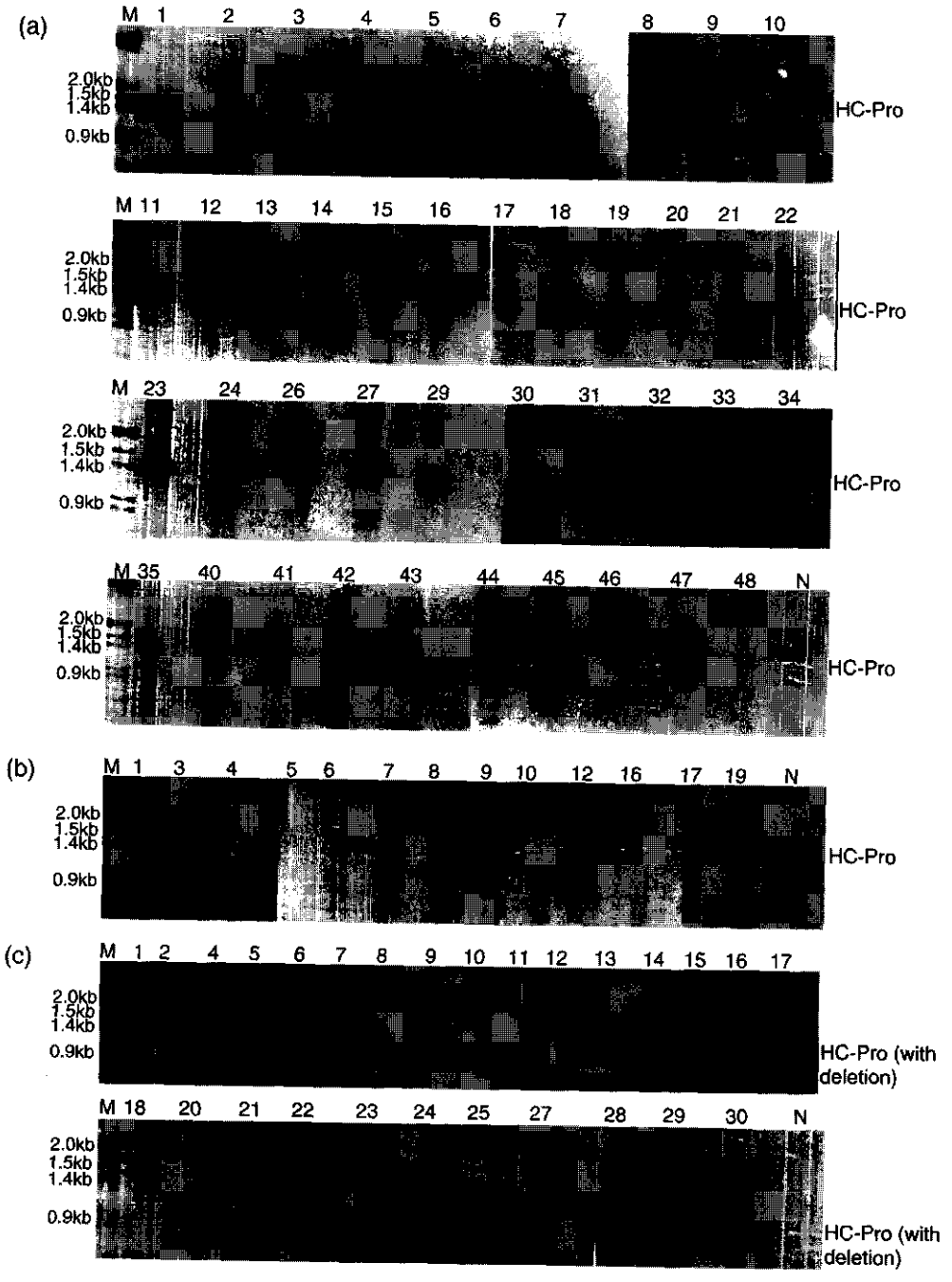
Fig. 4 RT-PCR analysis for the presence of the transgene mRNA in T0-generation plants transformed with HC-Pro sequences.

(a) h-lines (transformed with pBin/HCPro).

(b) t-lines (transformed with pBin/TAAHCPro)

(c) d-lines (transformed with pBin/HCProDel).

In all cases total RNA was isolated from transgenic lines indicated on the top of the lanes and amplified by RT-PCR using HC-Pro-specific primers econdehcF and bamstohcR. The amplified HC-Pro fragment had the expected size of about 1.368 kb in h- and t-lines; in d-lines the amplified fragment (with a deletion of the central domain) had the expected size of 0.912 kb. Lines h48, t1, t4, t10, t16, d2, d15 &18 seem to contain only low or undetectable amounts of transgene mRNA. No transcripts were detected in the nontransformed plant (N). When equivalent amounts of total RNA from the transgenic lines were directly used as template in PCR, no products were amplified as shown in the lane to the right for each line. M: Á-DNA digested with *EcoRI* and *Hind III* as a marker.


(a)
M 1 2 3 4 5 6 7 8 9 10
2.0kb
1.5kb
1.4kb
0.9kb
HC-Pro
M 11 12 13 14 15 16 17 18 19 20 21 22
2.0kb
1.5kb
1.4kb
0.9kb
HC-Pro
M 23 24 26 27 29 30 31 32 33 34
2.0kb
1.5kb
1.4kb
0.9kb
HC-Pro
M 35 40 41 42 43 44 45 46 47 48 N
2.0kb
1.5kb
1.4kb
0.9kb
HC-Pro
(b)
M 1 3 4 5 6 7 8 9 10 12 16 17 19 N
2.0kb
1.5kb
1.4kb
0.9kb
HC-Pro
(c)
M 1 2 4 5 6 7 8 9 10 11 12 13 14 15 16 17
2.0kb
1.5kb
1.4kb
0.9kb
HC-Pro (with deletion)
M 18 20 21 22 23 24 25 27 28 29 30 N
2.0kb
1.5kb
1.4kb
0.9kb
HC-Pro (with deletion)

Response to challenge with parental CABMV

Transgenic h-, t- and d-lines were assayed for their responses to inoculation with CABMV (Table 2). These include 8 h-lines, 7 of which contained detectable mRNA transcripts of the HC-Pro transgene (h35, -40, -42, -43, -44, -46, & 47) and line h48 in which HC-Pro RNA sequences were barely detectable; 6 t-lines and 6 d-lines all of which contained detectable levels of HC-Pro RNA sequences.

At least 15 T1 generation plants of each line were inoculated with sap prepared from *N. benthamiana* leaves infected with the parental CABMV isolate. Six nontransgenic plants inoculated with CABMV and one plant mock inoculated with water served as controls.

Leaf distortion appeared on systemically infected leaves of nontransgenic plants 7 dpi followed by a mosaic 14 dpi (Fig. 5a). In 6 h-lines (h40, -42, -43, -44, -46, -47), the symptom expression was increased in comparison with the nontransgenic plants and typically severe distortion appeared on systemically infected leaves 6 dpi followed by a severe mosaic 10 dpi (the symptoms 14dpi are shown for line h43 in Fig. 5b). Whilst a few infected plants died 14 dpi following severe symptoms, on most plants healthy shoots appeared 28 dpi as shown for h43 in Fig. 5e. However, wild type CABMV symptoms reappeared on the apparently recovered and newly emerging shoots 33 dpi (not shown).

On the other hand most plants of lines h35 and h48 did not develop any symptoms after inoculation with CABMV (Fig. 5c & 5d). A few plants of the lines h35 and h48 did show wild type symptoms (Table 2). PCR analysis revealed that these plants had lost the transgene through segregation.

Some of the CABMV-infected lines (h35, -43, -44 & -48) were assayed for levels of CABMV accumulation on western blots using antiserum against the coat protein to probe homogenate prepared from 1 g of the systemically infected third leaf above the inoculated leaf. The levels of CABMV in transgenic plants exhibiting severe symptoms (h43 and h44) (Fig. 6, lanes 5 and 8) were lower than in nontransgenic plants (Fig. 6, lane 3). In lines h35 and h48 which did not develop symptoms, the virus was not detectable (Fig. 6, lanes 4 & 9). Apparently, lines h35 and h48 are completely resistant to CABMV. In line h43 the virus was barely detectable on healthy looking shoots emerging 28 dpi (Fig. 6, lane 6) but the signal increased with re-establishment of infection (Fig. 6, lane 7).

Table 2. Assessment of responses of h-, t- and d-lines to inoculations with CABMV and CPMV

Line	Insert	HCPro[1] mRNA	R:S ratio[2]	CABMV infection[3]		CPMV infection[3]	
h35	HCPro	+	209:1	r	14/15[a]	wt/s	15/15[b]
h40	HCPro	+	169:1	s	15/17[c]	s	14/16
h42	HCPro	+	15:1	s	12/15	s	13/15
h43	HCPro	+	11:1	s	13/15	s	14/15
h44	HCPro	+	4:1	s	11/15	s	13/15
h46	HCPro	+	144:0	s	13/16	s	12/15
h47	HCPro	+	2.5:1	s	12/15	s	11/15
h48	HCPro	-	10:1	r	14/15	wt	15/15
t3	TAAHcPro	+	36:1	wt	10/10[d]	wt	15/15
t7	TAAHCPro	+	204:0	wt	11/11	wt	14/14
t9	TAAHCPro	+	16:1	r	12/13	wt	15/15
t12	TAAHCPro	+	2:1	wt	11/11	wt	10/10
t17	TAAHCPro	+	5:1	wt	10/10	wt	12/12
t19	TAAHCPro	+	25:1	wt	13/13	wt	13/13
d5	HCProDel	+	18:1	r	11/13	wt	14/14
d6	HCProDel	+	11:1	wt	10/10	wt	13/13
d7	HCProDel	+	6:1	wt	10/10	wt	12/12
d11	HCProDel	+	11:1	wt	12/12	wt	10/10
d12	HCProDel	+	13:1	wt	10/10	wt	11/11
d17	HCProDel	+	4:1	wt	11/11	wt	10/10

(1) +: mRNAs present, -: mRNAs barely detectable.

(2) R:S: segregation ratio on kanamycin.

(3) r: resistant ([a] number of resistant plants out of the total number of inoculated plants, plants that were not resistant showed wild type symptoms), wt/s: slight increase in symptom severity ([b] number of plants showing slightly enhanced symptoms out of the total number of inoculated plants), s: severe symptoms ([c] number of plants showing severe symptoms out of the total number of inoculated plants, plants that did not show severe symptoms had wild type symptoms), wt: wild type symptoms ([d] number of plants showing wild type symptoms out of the total number of inoculated plants).

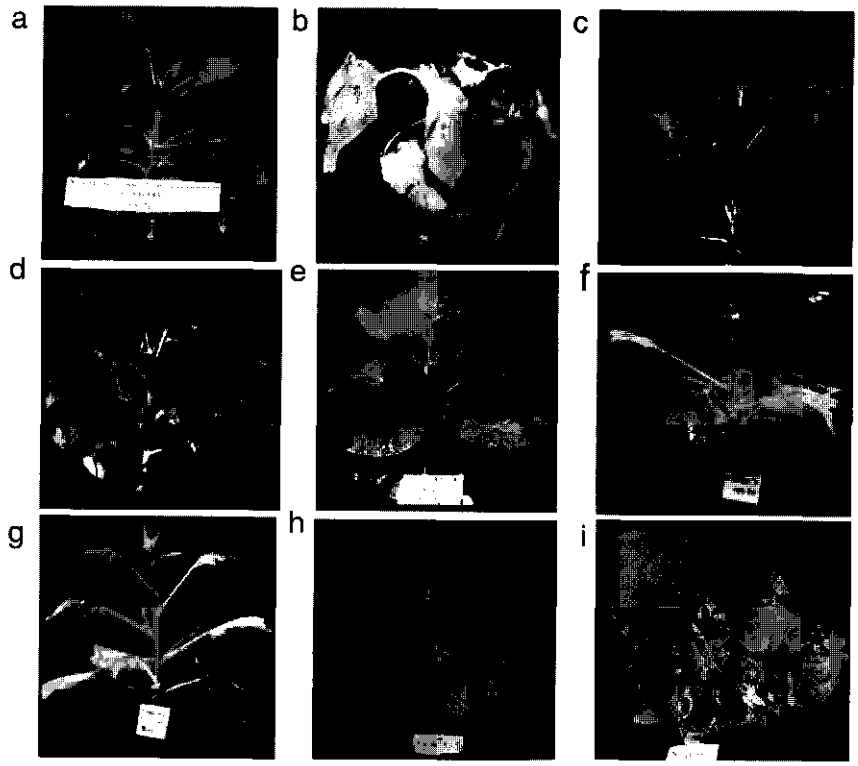


Fig. 5 Effects of the HC-Pro transgene on symptom expression of CABMV in *N. benthamiana.*
Symptoms are shown 14 dpi unless otherwise stated.
(a) CABMV wild type symptoms on nontransgenic plant
(b) Enhanced CABMV symptoms on h43 (HC-Pro expressing line)
(c) Absence of CABMV symptoms on h35 (HC-Pro expressing line)
(d) Absence of CABMV symptoms on h48 (putatively silenced line)
(e) Recovery of emerging upper shoots of h43 to initial CABMV infection (28 dpi)
(f) CABMV wild type symptoms on t3 (line expressing nontranslatable HC-Pro)
(g) CABMV wild type symptoms on d6 (line expressing HC-Pro with a deletion of the central domain)
(h) Absence of symptoms on h48 after inoculation with recombinant CPMV expressing the HC-Pro (M19HCPro7)
(i) Enhanced symptoms of M19HCPro7 on nontransgenic plant

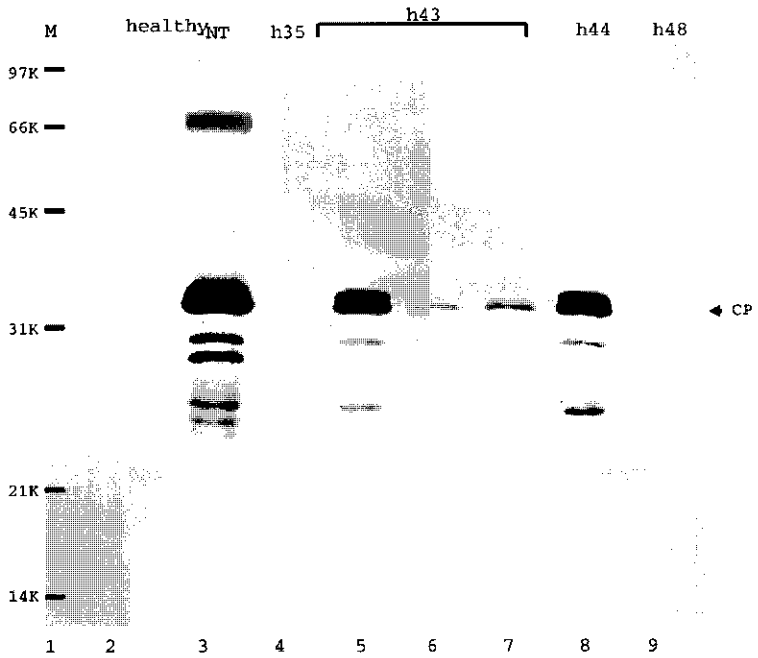


Fig. 6 Western blot analysis of levels of CABMV accumulation in infected *N. benthamiana* plants.

Lane 1, protein molecular weight markers in kDa: 97 kDa (phosphorylase b); 66 kDa (bovine serum albumin); 45 kDa (ovalbumin); 31 kDa (carbonic anhydrase); 21 kDa (soybean trypsin inhibitor); 14 kDa (lysozyme).

Lane 2, protein extract of uninfected plant

Lane 3, protein extract of infected nontransgenic plant during acute phase of infection (10 dpi)

Lane 4, protein extract of h35 (symptom free HC-Pro expressing line) 10 dpi

Lane 5, protein extract of h43 (HC-Pro expressing line) during acute phase of enhanced symptoms (10 dpi)

Lane 6, protein extract of symptom free leaves of h43 following recovery to initial infection (28 dpi)

Lane 7, protein extract of leaves of h43 after re-appearance of symptoms on initially recovered leaves (33 dpi)

Lane 8, protein extract of h44 (HC-Pro expressing line) during acute phase of enhanced symptoms (10 dpi)

Lane 9, protein extract of h48 (symptom free putatively silenced line) 10 dpi.

Most t-lines with an untranslatable HC-Pro gene, and most d-lines with a HC-Pro gene with a deletion in the middle of the gene, showed wild type symptoms similar to those on nontransgenic plants after inoculation with CABMV. Leaf distortion appeared on systemic leaves 7 dpi followed by a mosaic 14 dpi as shown for t3 and d6 in fig. 5f and 5g respectively. None of the plants showed enhanced symptom expression as seen with most h-lines.

However, most plants of one t-line, t9, and one d-line, d5, did not develop any symptoms after inoculation with CABMV (not shown) despite the presence of the transcripts of the transgene in parental T0 plants (Fig. 4b and 4c). Apparently these lines are resistant to CABMV.

The results illustrate the role of HC-Pro as pathogenicity factor in CABMV infection. In the transgenic lines expressing an intact HC-Pro gene, the first reaction is an increase in symptom expression. This is not observed in the transgenic lines expressing untranslatable HC-Pro genes or HC-Pro genes with a deletion. The enhanced symptom expression in the h-lines may therefore be attributed to the HC-Pro protein. Remarkably, it appears that the increased production of HC-Pro in h-lines resulted in an increased defense reaction of the plants resulting in the appearance of healthy shoots, which is not observed in the t- and d-lines and in nontransgenic plants. However the recovery is only temporary as rather soon, symptoms reappear on recovered and newly emerging shoots, even though the symptom expression is less severe than the initial symptoms.

Besides, the results presented in Table 2 indicate that the insertion of HC-Pro sequences in *N. benthamiana* plants can result in resistance to CABMV. If resistance in a line coincides with the absence of HC-Pro mRNA transcripts as observed for line h48, it is tempting to consider the resistance as a result of post transcriptional gene silencing. Remarkable exceptions are lines h35, t9 and d5 that appear resistant to CABMV despite the presence of transcripts of the HC-Pro transgene.

Responses to other viruses

(a) Heterologous potyviruses

After observing enhanced symptoms to CABMV in most h-lines and complete resistance in lines h35 and h48, we challenged these resistant lines and lines h43 & h44 by inoculation with potyviruses PVYn and SPV-Z. At least 10 plants per line were inoculated with each virus strain,

with 6 nontransgenic plants inoculated per strain as control. Remarkably, the 4 lines responded similarly with enhanced symptoms to PVYn and SPV-Z compared to nontransgenic plants. PVYn-infected nontransgenic plants showed necrosis on systemically infected leaves 6 dpi (the symptoms 10 dpi are shown in Fig. 7a). In the h35, -43, -44, & -48 lines, symptoms developed 4 dpi, with plants dying 8 dpi (plants 10 dpi for h43 and h48 are shown in Fig. 7b & 7c). In SPV-Z infections leaf distortion appeared on systemic leaves 12 dpi on nontransgenic lines and already 7 dpi in h-lines (not shown). None of the tested lines showed temporary recovery as observed after infection of lines h43 and h44 with CABMV.

(b) CPMV, CMV and AMV

All the lines listed in Table 2 were challenge inoculated with CPMV in the same way and with the same controls as described above for CABMV. The CPMV inoculum was a homogenate prepared from CPMV infected cowpea leaves. After 8 dpi a mild yellow mosaic appeared on systemic leaves of nontransgenic plants, the symptoms 14 dpi are shown in Fig. 7d. In 6 h-lines (h40, -42, -43, -44, -46, -47), the onset of systemic infection was evident in most plants about 5 dpi as leaf curling, and by 14 dpi the plants were severely stunted, growing tips severely reduced and a severe mosaic developed as shown for h43 (Fig. 7e). A few plants showed wild type symptoms in each line (Table 2). PCR analysis showed that these plants had lost the transgene through segregation.

The CPMV symptoms on line h48 (Fig. 7f) that had been found to be resistant to CABMV, were similar to nontransgenic plants (Fig. 7d) and only a mildly enhanced yellow mosaic developed on line h35 (Fig. 7g), the other h-line found to be resistant to CABMV. None of the lines challenged with CPMV showed the phenomenon of recovery in newly emerging shoots as found with CABMV and accumulation of CPMV in HC-Pro expressing lines h43 and h44 were higher than in nontransgenic plants on western blots (not shown).

Ten plants of line h48, that showed complete resistance to CABMV were further inoculated with recombinant CPMV expressing HC-Pro (M19HCPro7). Nine of the ten plants remained symptom-free (Fig. 5h) on comparison with inoculated nontransgenic plants (Fig. 5i). One plant showed wild type symptoms and had probably lost the transgene through segregation. Apparently the h48 line is also resistant to the CPMV-HCPro recombinant virus.

The CPMV symptoms in both t- and d-lines (Fig. 7h & 7i) were similar to nontransgenic plants (Fig. 7d), with a mild yellow mosaic appearing 8 dpi in all cases. These lines did not show any effect due to the presence of the HC-Pro transgene in their response to CPMV.

For further examination of the responses of the transgenic lines containing HC-Pro sequences, we challenged the lines h35, -43, -44, & -48 by inoculation with two other cowpea infecting viruses CMV and AMV. CMV and AMV elicited very mild symptoms on nontransgenic plants (Fig. 7j & 7l) and on line h48 in which the HC-Pro transcripts were absent or hardly detectable, and which was shown to be resistant to CABMV (not shown). On the other hand infections of HC-Pro expressing lines h43 & h44 by CMV and AMV were characterised by early appearance of significantly elevated symptoms and produced a yellow mosaic in systemic leaves 8 dpi with CMV and mottling of sytemically infected leaves with AMV as shown 14 dpi for h43 (Fig. 7k & 7m). On line h35, containing HC-Pro transcripts but resistant to CABMV, the symptoms of CMV and AMV were intermediate between those on line h48 and h43 or h44 (not shown).

Fig. 7 Effects of the HC-Pro transgene on symptom expression of PVYn, CPMV, AMV & CMV in *N. benthamiana.* Symptoms are shown 10 dpi for PVYn, and 14 dpi for CPMV, AMV & CMV.

(a) PVYn wild type symptoms on nontransgenic (NT) plant

(b) Enhanced PVYn symptoms on h43 (HC-Pro expressing line)

(c) Enhanced PVYn symptoms on h48 (putatively silenced line)

(d) CPMV wild type symptoms on NT plant

(e) Enhanced CPMV symptoms on h43

(f) CPMV wild type symptoms on h48

(g) Enhanced CPMV symptoms on h35

(h) CPMV wild type symptoms on t3 (line expressing nontranslatable HC-Pro)

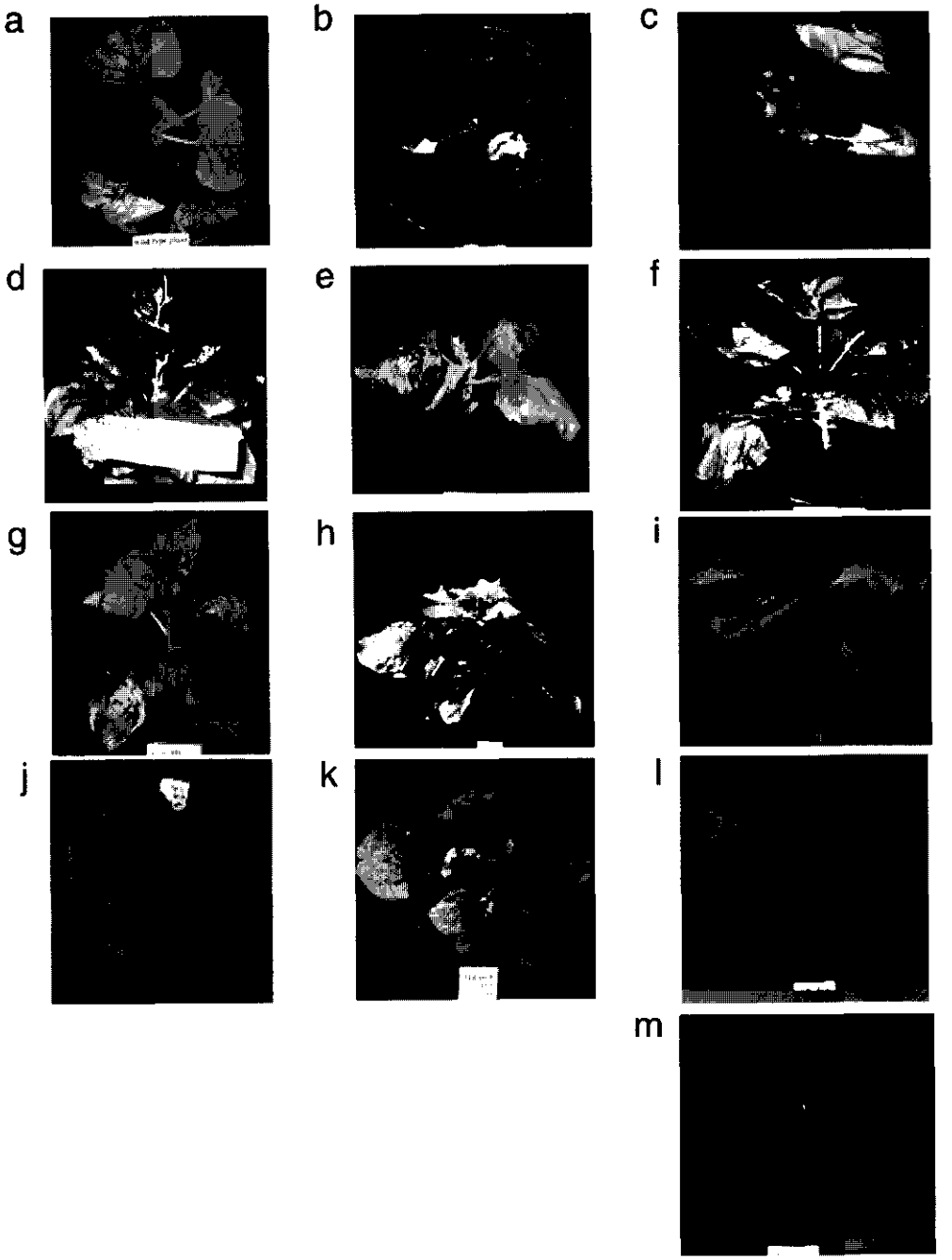
(i) CPMV wild type symptoms on d6 (line expressing HC-Pro with a deletion of the central domain)

(j) CMV wild type symptoms on NT plant

(k) Enhanced CMV symptoms on h43

(l) AMV wild type symptoms on NT plant

(m) Enhanced AMV symptoms on h43.


a
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4. Discussion

The responses of transgenic *N. benthamiana* plants expressing the HC-Pro of CABMV under the control of the 35S promoter upon virus infection prove the function of HC-Pro as a pathogenicity determinant and modulator of plant defense reactions. Although the HC-Pro protein was not detectable on western blots, most transgenic lines expressing HC-Pro (h-lines) showed enhanced symptoms after infection with the parental CABMV isolate as well as after infection with heterologous potyviruses PVYn and SPV-Z or nonpotyviruses CPMV, CMV and AMV. On the other hand, none of the lines expressing nontranslatable HC-Pro (t-lines) or translatable HC-Pro with a deletion of the central domain (d-lines) showed enhanced symptoms after infection with CABMV and other viruses. These results confirm the notion that HC-Pro is able to enhance the pathogenicity of viruses, and that the central domain of the HC-Pro protein is essential for this. Similar enhanced symptom expression has been reported before for PVX, CMV and TMV in tobacco plants expressing the TEV 5' proximal sequence encoding P1, HC-Pro and the N-terminal amino acids of P3 (Vance *et al* 1995; Pruss *et al.*, 1997; Shi *et al.*, 1997).

Interestingly, upon inoculation with CABMV, the symptoms on most CABMV HC-Pro expressing *N. benthamiana* lines appeared earlier and became more severe than in the nontransgenic plants (Fig. 5), yet tobacco plants expressing the TEV P1/HC-Pro sequence showed a reduced rate of virus spread compared to nontransgenic plants upon infection with TEV (Kasschau and Carrington, 1998). The early appearance of systemic symptoms indicates the additive effect of expression of HC-Pro from both the CABMV genome and the transgene in stimulating a more rapid spread of the CABMV infection. Perhaps this is an indirect effect of enhanced inhibition of a host defense reaction by the presence of HC-Pro both in local and systemic tissue thus conditioning a more rapid and unimpeded cell-to-cell and systemic spread of the virus. These results are consistent with long distance movement-stimulating properties described for TEV HC-Pro (Cronin *et al.*, 1995; Kasschau *et al.*, 1997). However, it is not clear how the expression of HC-Pro in h-lines results in more severe symptoms despite relatively lower levels of virus accumulation as in nontransgenic plants (Fig. 6). It is possible that the severe symptoms are a result of rapid establishment of infection. Previous studies have shown that increased severity of potato virus X symptoms on tobacco plants expressing the TEV P1/HC-Pro

is accompanied by increased levels of the virus (Pruss *et al.*, 1997), this was also the case with severe CPMV symptoms on HC-Pro expressing h-lines.

The severe symptoms in h-lines infected with CABMV were followed by brief recovery in which symptomless new leaves emerged coinciding with a decrease in levels of the virus. Previous studies have shown that recovery to virus infections on transgenic plants is based on PTGS, a natural sequence-specific host surveillance system that targets and degrades RNAs recognised as aberrant either by virtue of expression above permissive threshold levels or due to qualitative features such as self-complementarity (Vaucheret *et al.*, 1998; Waterhouse *et al.*, 1999). A plausible explanation for the brief recovery of h-lines to infection with CABMV is the threshold model in which transgene transcription and virus replication both contribute to reach the level of accumulation of RNA that triggers the PTGS reaction resulting in degradation of both viral and transgene transcripts (Waterhouse *et al.*, 1999). The observation that these HC-Pro expressing h-lines succumbed with enhanced symptoms to challenge with heterologous potyviruses PVY and SPV-Z without showing any recovery indicates that the brief recovery during CABMV infection is dependent on homology of the transgene with the infecting virus and supports the notion that the recovery is due to PTGS.

Observations that viruses can be both inducers and targets of PTGS prompted the idea that PTGS is a manifestation of a natural defense mechanism against virus infections (Baulcombe, 1996; Ratcliff *et al.*, 1999). Consistent with this idea is the recovery induced by nepoviruses and caulimoviruses on nontransgenic plants which has features of PTGS (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). Recovery during potyvirus infections on transgenic plants expressing CP genes has also been shown to condition an immune state in which newly emerging shoots were immune to reinfection by the parental virus (Dougherty *et al.*, 1994). However, contrary to these reports, the recovery phase in h-lines expressing the HC-Pro of CABMV was only brief and subsequent re-establishment of infection occurred in which systemic symptoms appeared on initially recovered and on newly emerging shoots. While at first sight this result seems to be in contradiction with previous observations, it should be borne in mind that some viruses have evolved proteins to counter PTGS. Examples are the potyvirus HC-Pro and cucumovirus 2b proteins which have been shown to suppress PTGS by putatively interfering with an RNA targeting mechanism that is

part of both initiation and maintenance stages of PTGS (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998; Dalmay *et al.*, 2000).

However, despite its role as suppressor of PTGS, it seems that the increased production of HC-Pro elicits a corresponding increase in host defense reactions resulting in temporary recovery in which PTGS overrides the counteracting effects of HC-Pro, at least for a while. The fact that recovery was not sustained showed that PTGS was incomplete as the virus, possibly aided by transgene expressed HC-Pro, continues to mount a counter, a phenomenon that was not observed in plants expressing the CP of TEV where recovery was not only activated but sustained despite production of HC-Pro from the viral genome during the initial susceptible phase (Dougherty *et al.*, 1994). Overall, these results indicate counteracting effects of HC-Pro expression and a host defense response in an ongoing contest between the virus and the host.

Line h48, which was found to contain very low levels of the HC-Pro transgene mRNA, was completely resistant to infections with CABMV and also to the recombinant CPMV containing the HC-Pro sequence (M19HCPro7). Furthermore, h48 did not show enhanced symptoms upon inoculation with CPMV thus indicating that HC-Pro is not produced, and that resistance to M19HCPro7 was sequence specific. As low levels of transcript and immunity against viruses with homologous sequences are hallmarks of PTGS (Kasschau and Carrington, 1998), it is very likely that the HC-Pro transgene in line h48 is posttranscriptionally silenced. Contrary to the recovery phenomenon in HC-Pro expressing h-lines in which PTGS was induced only upon CABMV infection and was incomplete, PTGS in line h48 occurred in the absence of CABMV infection and confers complete resistance to CABMV. Transgene-induced PTGS occurs at a low frequency in transformed plants and was shown to be triggered by highly expressed single transgenes, multiple transgenes or transgenes integrated as inverted repeats (Elmayan and Vaucheret, 1996; Stam *et al.*, 1997). It has been proposed that the RNAs transcribed from such transgene loci either accumulate above a certain threshold level or are aberrant in some way and serve as template for a plant encoded RNA-dependent RNA polymerase which potentiates their degradation via synthesis of complementary RNAs (Dougherty and Parks, 1995).

On the other hand, the silenced line h48 was not resistant to the less related potyviruses, PVY and SPV-Z. This showed that the silencing mechanism could not recognise these viruses. This was not surprising given that PTGS has been shown to be highly sequence specific and only effective against viruses with at least 80 % nucleotide sequence homology to the silenced viral transgene

(Sijen and Kooter, 2000). In this case we expected h48 to respond to PVY and SPV-Z as nontransgenic plants but interestingly, h48 responded with severe symptoms similar to HC-Pro-expressing lines h43 & h44. It is envisaged that the expression of PVY or SPV-Z HC-Pro suppressed the silencing of the CABMV HC-Pro transgene in h48 thus leading to enhanced symptoms similar to HC-Pro-expressing lines h43 and h44. This provided more support for PTGS of HC-Pro in h48 given that reversion of silencing upon infection of silenced plants with potyviruses has been proposed as an assay for PTGS (Marathe *et al.*, 2000).

Overall, in this chapter the role of CABMV HC-Pro as a pathogenicity factor and modulator of host defense reactions has been illustrated. Both functions have been shown to reside in the central domain of the HC-Pro protein. Defining the minimum domain of HC-Pro involved in mediating these responses and cellular factors with which HC-Pro interacts will provide a better understanding of the molecular basis of these virus-host interactions. Besides, PTGS of the HC-Pro transgenes has also been observed notwithstanding its established role as a suppressor of the process, and this has been shown to result in resistance to CABMV. Preliminary data show that some lines are resistant to CABMV in spite of the presence of the HC-Pro transcripts (h35, d5 and t9). It would be of great interest to dissect the basis of this resistance.

Acknowledgements

We are grateful to B. J. M. Verduin and Yukun Chen for kindly providing the virus samples indicated in Materials and Methods. B. J. M. Verduin is further acknowledged for kindly providing the CABMV CP antiserum. Wim van Hof is acknowledged for kind assistance with photography. This work was supported by a grant of the Netherlands Foundation for the Advancement of Tropical Research (WOTRO).

CHAPTER 6

SUMMARY AND CONCLUDING REMARKS

The work presented in this thesis is part of ongoing efforts to gain more insight into the molecular properties of cowpea aphid-borne mosaic potyvirus (CABMV), an important potyviral pathogen of cowpeas. The main focus of our research initiatives has been the helper component-proteinase (HC-Pro), a multifunctional protein that plays a role in different steps of the potyvirus infection cycle and is a major determinant of the pathogenicity of potyviruses. It is envisioned that knowledge gained from this research will contribute to the attempts to protect cowpea against CABMV infection and to minimise the impact of the virus on cowpea yields.

As a prerequisite for detailed molecular characterisations, the cloning and sequencing of the HC-Pro coding region of the Zimbabwe isolate of CABMV was achieved using RT-PCR with degenerate primers designed from conserved potyviral sequences and specific primers designed from the 3' proximal sequences determined earlier by Sithole-Niang *et al.* (1996) and Dhliwayo (1996) (chapter 2). The sequencing of overlapping RT-PCR-amplified cDNA clones of CABMV was continued resulting in determination of 9 465 nucleotides of the genomic sequence of the virus (chapter 3). The deduced genomic organisation was found to be similar to other members of the genus *Potyvirus* and was characterised by a continuous open reading frame (ORF) encoding a large polyprotein from which 10 mature proteins could be predicted on the basis of conserved proteolytic cleavage sites. In the predicted CABMV proteins conserved amino acid sequences with implicated roles in the potyviral infection cycle were identified (chapter 2 & 3). Results of alignments of the amino acid sequence of the polyprotein of CABMV with those of other potyviral polyproteins confirmed the original classification of CABMV as a distinct virus species of the bean common mosaic virus (BCMV) subgroup of legume infecting potyviruses. The knowledge of the genomic sequence opens up prospects for the use of infectious full-length cDNA clones of the genome of CABMV for studies of functions of the gene products using reverse genetic approaches. However, making a full length cDNA clone will have to be preceded by verification of the sequence of the nucleotides at the extreme 5' region of the genome since this remains uncertain (chapter 3).

Following the completion of cloning of the HC-Pro gene, further analyses focussed on gaining insights into the intracellular location of the HC-Pro protein in cells using immunofluorescence and western blot assays. For this it is important to have specific antiserum against the HC-Pro protein. For that purpose, the HC-Pro gene was expressed in *E. coli* and using the purified protein as antigen, a high titer antiserum specific to the HC-Pro protein was obtained (chapter 4). The

HC-Pro protein produced in cowpea protoplasts infected with CABMV, or expressed apart from other CABMV proteins in a transient expression vector was found to be localised in the cytoplasm. This was consistent with the cytoplasmic location of other potyviral HC-Pro proteins revealed earlier by ultrastructural studies (De Meija *et al.*, 1985; Riedel *et al.*, 1998). On the other hand, in an alternative cowpea mosaic virus (CPMV)-based expression system, localisation of the HC-Pro protein was found to be confined in cytoplasmic patches reminiscent of CPMV induced cytopathic structures in which CPMV replication takes place (De Zoeten *et al.*, 1974). This raises the possibility for an interaction between HC-Pro and CPMV proteins or specific host proteins in these structures. The expression of the HC-Pro protein as part of the CPMV genome further revealed its striking ability to enhance the virulence of CPMV on infected plants in a manner reminiscent of co-infections of wild type CPMV and CABMV (chapter 4). The increased severity of the infection might be associated with the putative interaction of HC-Pro with CPMV proteins or specific host proteins and appears consistent with the established role of HC-Pro in promoting virus infections by suppressing host defense responses.

The HC-Pro mediated effects on virus-host interactions and the regions of the protein involved were further studied in transgenic *Nicotiana benthamiana* plants transformed with HC-Pro sequences (chapter 5). This also included exploring the prospects of virus derived resistance using the HC-Pro gene. Upon infection of the transgenic plants with the parental CABMV and heterologous viruses, a symptom enhancing effect was observed in plants expressing the complete HC-Pro gene while the responses of lines expressing a nontranslatable HC-Pro gene or HC-Pro gene with a central domain deletion were similar to those of nontransgenic plants. The analyses of the pattern of symptom expression in infected plants proved the role of HC-Pro as a pathogenicity factor and modulator of host defense responses, as previously proposed for other potyviral HC-Pro proteins (Atreya *et al.*, 1992; Pruss *et al.*, 1997; Shi *et al.*, 1997), and further demontrated the importance of the central region of the HC-Pro in these functions.

The increased systemic symptoms on leaves of HC-Pro expressing lines infected with the parental CABMV isolate was followed by the appearance of healthy shoots, but a few days later CABMV symptoms reappeared on the initially recovered and newly emerging shoots. Recovery was earlier described for transgenic plants expressing potyviral genes encoding the coat protein (CP) (Dougherty *et al.*, 1994), P1 and P3 proteins (Moreno *et al.*, 1998) and was linked to

posttranscriptional gene silencing (PTGS). In all cases it remains unclear how PTGS is activated in newly formed shoots despite the production of HC-Pro during the initial susceptible phase of the infection by a potyvirus. Recovery as described for plants expressing the other potyviral genes is associated with complete immunity to secondary infection by the homologous virus (Dougherty *et al.*, 1994; Moreno *et al.*, 1998), yet recovery in plants expressing CABMV HC-Pro has been found to be transient (chapter 5). This might reflect that the establishment of PTGS was incomplete in these plants possibly due to a combined effect of expression of HC-Pro from both the virus and transgene which finally overcame the defense by PTGS.

It was also shown that PTGS can be induced by a HC-Pro transgene in *N. benthamiana* despite the established role of the protein as a suppressor of PTGS, and this conferred complete resistance to CABMV (chapter 5). The explanation proposed is that transcripts of the HC-Pro transgenes are inactivated before the protein could be produced to functional levels, and that upon virus infection the CABMV genome is inactivated prior to establishment of infection and production of CABMV-encoded HC-Pro.

The results presented in this thesis have implications on the use of HC-Pro for engineering pathogen-derived resistance in plants. In plants expressing the complete HC-Pro gene, interactions between the HC-Pro transgene and parental CABMV and heterologous viruses resulted in enhanced symptoms as has been well illustrated in chapter 5. On the other hand, we have shown that like other potyviral genes, transgene-induced PTGS of HC-Pro can occur and provide excellent resistance against the parental virus. However, in spite of the resistance to CABMV, the line succumbed with enhanced symptoms to heterologous potyviruses potato virus Y (PVY) and the sweet potato virus (SPV-Z). This raised the possibility that the PTGS of the CABMV HC-Pro transgene, which confers specific resistance to CABMV, is reversed in trans by HC-Pro expressed by PVY or SPV-Z thus resulting in enhanced symptoms upon infection with these viruses. These detrimental effects of HC-Pro transgenes make them less suitable for application in effecting pathogen derived-resistance despite that their role in different steps of the potyvirus infection cycle would make them a good target for pathogen-derived resistance. The absence of any synergistic effect in plants transformed with the nontranslatable HC-Pro gene or central domain mutants which were resistant to CABMV showed that attempts to engineer RNA based resistance can be safely achieved in transgenic plants transformed with such constructs.

For the T1 lines that were found to be resistant to CABMV despite the presence of HC-Pro transcripts in parental T0 plants, it would be of interest to study the basis of this resistance (chapter 5). It is possible that these T1 lines have been post transcriptionally silenced since the incidence of PTGS was previously shown to increase upon selfing of hemizygous plants (Goodwin *et al.*, 1996; Jorgensen *et al.*, 1996, Pang *et al.*, 1996).

The number of HC-Pro transformed lines generated in this study was more than could be fully characterised due to temporal and spatial constraints. It would be of interest to study the responses of more lines to virus challenge and characterise in more detail the molecular basis of PTGS and the various resistance phenotypes in these lines.

Overall, the results underline the need for a detailed understanding of the molecular aspects of virus-host interactions as prerequisite for pathogen-derived resistance. In this respect, the cloning and elucidation of the genomic sequence of CABMV has increased the choice of suitable regions for pathogen derived resistance studies and for further molecular characterisation of the virus functions in infection, replication, movement and transmission. The achievements reported in this thesis, combined with preliminary reports of successful genetic transformation and regeneration of cowpea (Kononowicz *et al.*, 1997), further opens up new opportunities for engineering resistance to CABMV in cowpea.

SAMENVATTING

Het onderzoek dat in dit proefschrift beschreven staat maakt deel uit van een samenwerkingsproject tussen de Universiteiten van Wageningen en Zimbabwe dat erop gericht is om meer inzicht te verkrijgen in de moleculaire eigenschappen van cowpea aphid-borne mozaiek virus (CABMV), een potyvirus en de belangrijkste veroorzaker van een infectie-ziekte die op cowpea (kouseband) wordt aangetroffen. Centraal in dit onderzoek staat het helper component-protease eiwit (HC-Pro), een multifunctioneel eiwit dat een belangrijke rol heeft in verschillende stappen in de levenscyclus van een potyvirus en dat ook één van de belangrijkste pathogeniciteits factoren is van potyvirussen. Eén van de doelstellingen van dit onderzoek is dat kennis wordt verkregen die gebruikt zou kunnen worden om cowpea te beschermen tegen infecties met CABMV.

Om het HC-Pro eiwit te kunnen bestuderen was het eerst nodig om de sequentie die codeert voor het HC-Pro van het Zimbabwe isolaat van CABMV te kloneren om vervolgens de basenvolgorde te kunnen bepalen. Hierbij werd gebruik gemaakt van RT-PCR met behulp van gedegenereerde primers die gebaseerd waren op geconserveerde potyvirus sequenties en specifieke primers die waren ontworpen op basis van de sequentie van het 3' uiteinde waarvan de basenvolgorde al eerder bepaald was door Sithole-Niang *et al.* (1996) en Dhliwayo (1996) (hoofdstuk 2). Door ook de basenvolgorde van de andere cDNA fragmenten die met behulp van RT-PCR van het CABMV RNA waren verkregen te bepalen werd een sequentie met een totale lengt van 9 465 nucleotiden van het genoom van het virus verkregen (hoofdstuk 3). De van de sequentie afgeleide organisatie van het genoom leek erg veel op die van andere potyvirussen en bevatte één groot open leesraam dat codeert voor een polyeiwit dat op basis van geconserveerde klievingsplaatsen bestaat uit 10 rijpe eiwitten. Deze eiwitten bevatten korte aminozuur volgordes die ook zijn aangetroffen in de eiwitten van andere potyvirussen en waarvan is vastgesteld dat ze een belangrijke rol hebben in het infectieproces (hoofdstuk 2 & 3). De resultaten van het vergelijken van de aminozuurvolgorde van het polyeiwit van CABMV met polyeiwitten van andere potyvirussen hebben bevestigd dat CABMV een aparte virussoort is binnen de bean common mozaïek virus (BCMV) subgroep van potyvirussen die vlinderbloemige planten infecteren. Met behulp van de kennis van de basenvolgorde van het genoom is het nu mogelijk geworden om een infectieuze cDNA kloon van het genoom van CABMV te maken die gebruikt kan worden voor het bestuderen van functies van de verschillende gen producten via de techniek van omgekeerde genetica. Echter, voordat het

mogelijk zal zijn om deze cDNA kloon te maken, zal eerst moeten worden nagegaan of de sequentie aan het uiterste 5' uiteinde wel volledig is (hoofdstuk 2).

Na het kloneren van het HC-Pro gen was vervolgens de aandacht gericht op de analyse van de intracellulaire locatie van het HC-Pro eiwit door middel van immuno-fluorescentie en eiwitblot analyse. Hiervoor was het belangrijk om over een antiserum te kunnen beschikken dat specifiek is voor HC-Pro. Daarom is het HC-Pro gen tot expressie gebracht in *E. coli*, het eiwit gezuiverd uit een *E. coli* extract en vervolgens gebruikt als antigeen in een konijn. Dit resulteerde in een antiserum met een grote specificiteit voor HC-Pro (hoofdstuk 4). Met behulp van dit antiserum werd aangetoond dat zowel het HC-Pro eiwit dat wordt geproduceerd tijdens een CABMV infectie als HC-Pro geproduceerd met behulp van een transient expressie systeem zonder de andere CABMV eiwitten gelokaliseerd is in het cytoplasma. Dit kwam overeen met de locatie van HC-Pro van andere potyvirussen zoals die was bepaald met behulp van de elektronenmicroscopie (De Meija *et al.*, 1985; Riedel *et al.*, 1998). Als daarentegen HC-Pro tot expressie werd gebracht in een alternatief expressie systeem dat gebaseerd is op het cowpea mozaiek virus (CPMV), hoopte HC-Pro zich op in een specifieke lokatie in het cytoplasma die erg veel leek op pathologische structuren die een rol hebben bij de replicatie van CPMV (De Zoeten *et al.*, 1974). Dit zou er op kunnen wijzen dat er een interactie plaatsvindt tussen HC-Pro en een CPMV eiwit of een gastheer eiwit dat specifiek is voor deze structuren. Het zou zeer interessant zijn om een dergelijke interactie aan te tonen. Door HC-Pro tot expressie te brengen in de CPMV vector werd ook duidelijk dat de virulentie van CPMV hierdoor was vergroot op een manier die deed denken aan een infectie van een plant met zowel CPMV als CABMV (hoofdstuk 4). De verhevigde infectie wordt misschien veroorzaakt door de bovengenoemde interactie tussen HC-Pro en de CPMV eiwitten of gastheereiwitten en is in overeenstemming met de al eerder vastgestelde rol van HC-Pro in het stimuleren van virus infecties door het onderdrukken van de afweerreactie van de gastheer.

De effecten van HC-Pro op interacties tussen virus en gastheer en de domeinen van HC-Pro die hierbij zijn betrokken werden verder bestudeerd met behulp van transgene *Nicotiana benthamiana* planten die getransformeerd zijn met HC-Pro sequenties (hoofdstuk 5). Hierbij werd ook gelet op de mogelijkheid dat het HC-Pro gen gebruikt zou kunnen worden voor virus-afgeleide resistentie. Op planten waarin het complete HC-Pro gen tot expressie was gebracht leidden infecties met CABMV en andere potyvirussen tot verhevigde symptomen, terwijl op

planten waarin een HC-Pro gen was ingebracht dat niet vertaald kan worden in eiwit of een HC-Pro gen met een deletie in het centrale domein de infecties identiek waren aan niet-getransformeerde controle planten. Dit patroon van expressie van symptomen in geïnfecteerde planten laat zien dat HC-Pro een pathogeniciteits factor is en een modulator van de reactie van de gastheer op een infectie, zoals dat al eerder is voorgesteld voor HC-Pro van andere potyvirussen (Atreya *et al.*, 1992; Pruss *et al.*, 1997; Shi et *al.*, 1997), en laat ook zien dat het centrale domein van HC-Pro hierbij een rol speelt.

Nadat eerst verhevigde systemische symptomen zichtbaar waren op bladeren van planten waarin HC-Pro tot expressie was gebracht en die waren geïnfecteerd met CABMV, verschenen vervolgens op de planten gezonde jonge blaadjes die echter een paar dagen later toch weer symptomen van een infectie met CABMV vertoonden. Het herstellen na een infectie met een potyvirus is al eerder beschreven bij transgene planten waarin genen van potyvirussen die coderen voor het manteleiwit (Dougherty *et al.*, 1994), en de P1 en P3 eiwitten (Moreno *et al.*, 1998) tot expressie zijn gebracht. Voor al deze gevallen is het nog onduidelijk hoe post transcriptionele silencing (PTGS) kan worden geactiveerd in de nieuw gevormde jonge blaadjes ondanks de productie van HC-Pro gedurende het beginstadium van de infectie met een potyvirus. Het herstellen van een infectie met een potyvirus zoals beschreven voor transgene planten waarin de andere potyvirus genen tot expressie komen resulteert altijd in volledige resistentie van de plant voor een nieuwe infectie met het zelfde potyvirus, terwijl het herstel van de transgene planten waarin CABMV HC-Pro tot expressie is gebracht maar zeer tijdelijk is (hoofdstuk 5). Het is mogelijk dat door het combineren van het effect van de expressie van HC-Pro van zowel het transgen als van het virus de PTGS in deze planten niet volledig is zodat het virus de kans krijgt de plant opnieuw te infecteren.

De experimenten hebben ook laten zien dat een HC-Pro transgen PTGS kan induceren in *N. benthamiana*, ondanks dat het eiwit PTGS kan onderdrukken, en dat dit kan resulteren in planten die volledige resistentie vertonen tegen een infectie met CABMV (hoofdstuk 5). Het is mogelijk dat in dergelijke planten de boodschapper RNA's die afkomstig zijn van het HC-Pro transgen afgebroken worden voordat ze vertaald kunnen worden in een functionele hoeveelheid eiwit, en dat tijdens een infectie met CABMV het virale RNA direct wordt afgebroken zodat er geen HC-Pro gemaakt kan worden.

De resultaten die beschreven staan in dit proefschrift hebben gevolgen voor het gebruik van HC-Pro voor virus-afgeleide resistentie in planten. In planten waarin het volledige HC-Pro gen tot expressie is gebracht, hebben interacties van het HC-Pro transgen met CABMV of met andere virussen verhevigde symptomen tot gevolg, zoals beschreven in hoofdstuk 5. Toch kan, net als de andere potyvirus genen, het HC-Pro gen PTGS induceren die een plant volledige resistent maakt voor een infectie met CABMV. Als deze planten echter met andere potyvirussen worden geïnfecteerd reageren ze met verhevigde symptomen. Waarschijnlijk wordt dit veroorzaakt doordat de PTGS van het CABMV transgen, die verantwoordelijk is voor de resistentie tegen CABMV, onderdrukt wordt door de HC-Pro van het potyvirus dat de plant infecteert. Ofschoon HC-Pro betrokken is bij verschillende stappen van het infectieproces en daarom een goede kandidaat zou zijn om te gebruiken voor virus-afgeleide resistentie maakt dit nadelige effect het HC-Pro transgen minder geschikt voor deze toepassing. Dat er geen synergistisch effect op treedt in planten die getransformeerd zijn met een HC-Pro gen dat niet vertaald kan worden in eiwit of dat een deletie heeft in het centrale domein maar die wel resistent zijn tegen CABMV, wijst erop dat dit wel een veilige manier is om op RNA-gebaseerde resistentie tegen CABMV in te bouwen.
Het zou van belang kunnen zijn om na te gaan wat de oorzaak is van de resistentie tegen CABMV in die T1 planten die nakomelingen zijn van T0 planten waarin wel HC-Pro transcripten zijn aangetroffen (hoofdstuk 5). Het is mogelijk dat in de T1 planten wel PTGS plaats vindt, omdat in de homozygote situatie de kans hierop toeneemt (Goodwin *et al.*, 1996; Jorgensen *et al.*, 1996; Pang *et al.*, 1996).
Er zijn tijdens deze studie meer met HC-Pro getransformeerde planten gemaakt dan de planten die gezien de beperkte tijd en de beperkte hoeveelheid kasruimte goed zijn bestudeerd. Het zou van belang kunnen zijn om ook van deze andere transgene planten de reacties te bestuderen op infecties met virussen om zo de moleculaire basis van PTGS en andere resistente fenotypes nauwkeuriger te kunnen karakteriseren.
De resultaten laten duidelijk zien dat voor het toepassen van virus-afgeleide resistentie het belangrijk is om goed te begrijpen wat de moleculaire basis is van interacties tussen virus en gastheer. Door het kloneren van het genoom van CABMV is het nu mogelijk om te kiezen uit meer gebieden om te gebruiken voor virus-afgeleide resistentie of om op moleculair niveau de functie te bestuderen van de verschillende gebieden bij de infectie, replicatie, verspreiding door de plant en overdracht. Recentelijk is er ook vooruitgang geboekt op het gebied van transformatie

en regeneratie van cowpea (Kononowicz *et al.*, 1997) en gecombineerd met de in dit proefschrift beschreven resultaten zal het nu ook mogelijk zijn om cowpea resistent te maken tegen CABMV.

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CURRICULUM VITAE

Sizolwenkosi Mlotshwa was born on 6 November 1965 in Lupane, Zimbabwe. He did his secondary education in the city of Bulawayo where he obtained the University of Cambridge Ordinary Level General Certificate of Education at Luveve High School in 1986 and the University of Cambridge Advanced Level General Certificate of Education at Northlea High School in 1988. From 1989 to 1991 he studied science at the University of Zimbabwe where he obtained a BSc Honours degree in Biological Sciences and was awarded the *Clare Swift* prize. As part of the requirements for the BSc Honours degree he did research on the efficacy of disinfectants on raw milk bacterial species under the supervision of Prof. S. B. Feresu (1991). From 1992 to 1994 he worked for the Zimbabwe Ministry of Education. From 1995 to 1996, he studied and obtained an MSc degree in Biotechnology with merit at the University of Zimbabwe on a scholarship provided by the Directorate General for International Cooperation (DGIS) of The Nertherlands Ministry of Foreign Affairs and coordinated by the Centre for Development Cooperation Services of the Free University (VU) in Amsterdam. In accordance with the terms of the MSc degree programme he carried out research for his MSc thesis entitled "Cloning and expression of the coat protein gene of sweet potato feathery mottle potyvirus" in the Laboratory of Virology (Prof. Dr. R. Goldbach) at Wageningen University under the supervision of Dr. B. J. M. Verduin (The Netherlands) and Dr. A. I. Robertson (Zimbabwe) in 1996. In 1997 he started research for his PhD degree in the Laboratory of Molecular Biology (Prof. Dr. T. Bisseling) at Wageningen University (The Netherlands) on a joint project of Dr. I. Sithole-Niang (University of Zimbabwe) and Prof. Dr. A. Van Kammen (Wageningen University) funded by The Netherlands Foundation for the Advancement of Tropical Research (WOTRO).