

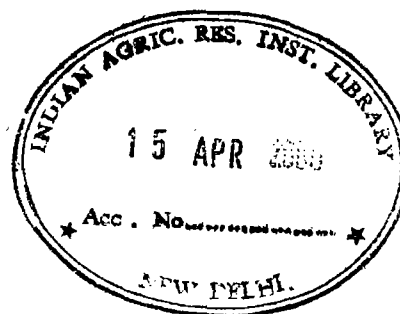
**STUDIES ON THE ROLE OF "PRE COAT PROTEIN"
(ORF AV2) IN *MUNGBEAN YELLOW MOSAIC INDIA*
VIRUS INFECTING GRAIN LEGUMES**

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2006

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(ORF AV2) IN *MUNGBEAN YELLOW MOSAIC INDIA*
VIRUS INFECTING GRAIN LEGUMES**

By

AHMAD ROUHIBAKHSH

A Thesis

**submitted to the Faculty of Post Graduate School,
Indian Agricultural Research Institute, New Delhi,
in partial fulfilment of the requirements
for the award of the degree of**

DOCTOR OF PHILOSOPHY

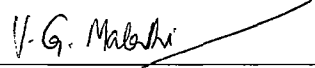
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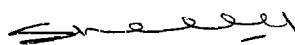
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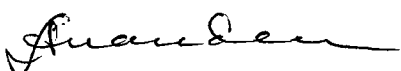

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Dedicated to
Mr. Seied Mohammad Khatami
for inspiring thoughts on
intellectual Freedom and Vibrant
action for Peaceful World

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CERTIFICATE

This is to certify that the thesis entitled "**Studies on the role of 'pre coat protein' (ORF AV2) in *Mungbean yellow mosaic India virus* infecting grain legumes**", submitted to the Faculty of the Post Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy in Plant Pathology** by **Mr. Ahmad Rouhibakhsh** embodies the results of *bonafide* work carried out by him under my supervision and guidance. No part of the thesis has been submitted by him for any other degree or diploma.

I further certify that any help or information received during the work on this thesis has been duly acknowledged.



Place : New Delhi

Date : 23rd February, 2006

(V.G. Malathi)
Chairperson
Advisory Committee

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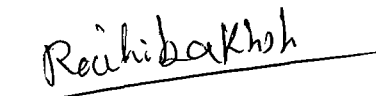
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(AHMAD ROUHIBAKHSH)

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1. INTRODUCTION

Grain legumes, also known as pulse crops, are the second most important group of crops grown essentially in South Asia. They are known to have been cultivated since the Neolithic age. The grain legumes constitute a major caloric and protein source in South Asia especially for the vegetarian population. The grains are rich in starch (60-65%), protein (25%) and have good amount of vitamin A, B₁, B₁₂, niacin, micronutrients and are particularly rich in the amino acid lysine. They are easily digestible and complement the carbohydrate rich cereal diet. The grain legumes are consumed either as sprouts, cooked food (*dhal*) or processed food. Some of them are also known for their medicinal properties. While the bulk of the pulse utilization is for human consumption (70%), the animal feed industry uses 22%.

The most advantageous feature of legume cultivation is its role in maintaining soil fertility by their biological nitrogen fixation activity. By virtue of their ability to fix atmospheric nitrogen enriching the soil fertility, pulse crops remain an integral part of the sustainable agricultural production system of the semi-arid tropics. Globally total grain legume production is estimated to be 58 million tonnes (Belhassen, 2005). Legumes occupy 190 million ha of land, about 13% of the total land under cultivation.

Among the countries in South Asia, India is the key player. About 37% of the total area under pulse cultivation is in India, which ranks first in the world producing more than 25% of the world's food legume. The area under pulse cultivation has not shown appreciable variation and is between 20-25 million ha. The production and productivity stagnate in India at 11-15 million tonnes and 600 Kg/ha, respectively, well below the world average of

846 Kg/ha (Singh and Kochhar, 2005).

Diverse species of legumes are cultivated in India under different agroclimatic conditions (Paroda and Chadha, 1996). They are cultivated round the year in all the four crop seasons, spring (February to March), summer (April to June), *kharif* (July to September) and *rabi* (October to December). They are grown as sole crop or as intercrop and can be grown with sorghum, pearl millet, cotton, and other *kharif* crops.

In spite of the importance of these crops in human nutrition and sustainable agriculture, the productivity is very low, which is attributed to agroecological constraints which include infertile soil, unfavourable weather, varieties, seeds, agronomic practices, strains of *Rhizobium*, socio-economic factors and pests and diseases. Of all the diseases, yellow mosaic diseases caused by whitefly transmitted geminiviruses (WTG) are the major constraints.

Yellow mosaic disease affects a large number of leguminous crops (Fig. 1) grown in India. The diverse crop species affected are: Lima bean (*Phaseolus lunatus*; Capoor and Varma, 1948); dolichos (*Lablab purpureus*; Capoor and Varma, 1950), mungbean (*Vigna radiata*), blackgram (*V. mungo*), soybean (*Glycine max*, Nariani, 1960; Nene, 1972), pigeonpea (*Cajanus cajan*; Williams *et al.*, 1968), horsegram (*Macrotyloma uniflorum*), French bean (*Phaseolus vulgaris*) (Muniyappa *et al.*, 1976), cowpea (*Vigna unguiculata*) (Varma and Reddy, 1984) and mothbean (*V. aconitifolia*) (Satyavir, 1980). The characteristic symptom of the disease as the name implies, is the bright yellow mosaic in the leaf lamina. The fields having infected plants can be easily distinguished from those with healthy plants even at a distance. If the infection occurs at the seedling stage, the plants are extremely stunted. In severe cases, the number of pods is reduced, with shrivelled seeds. The yield

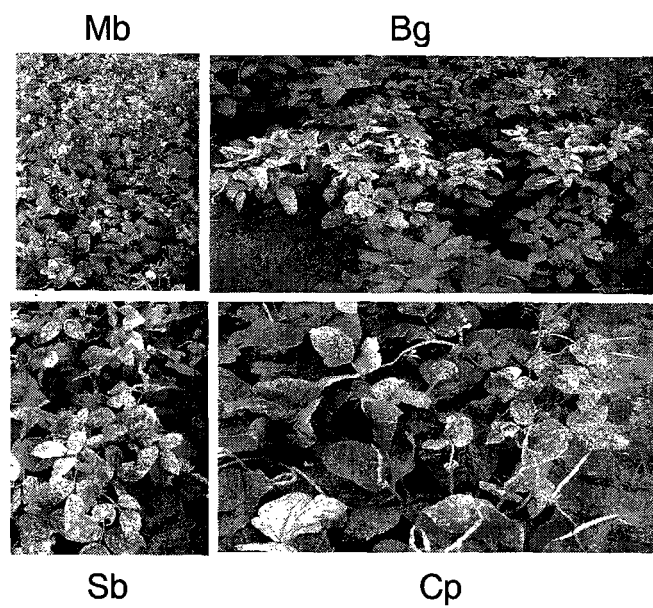


Fig. 1. Legume plants in field showing yellow mosaic.

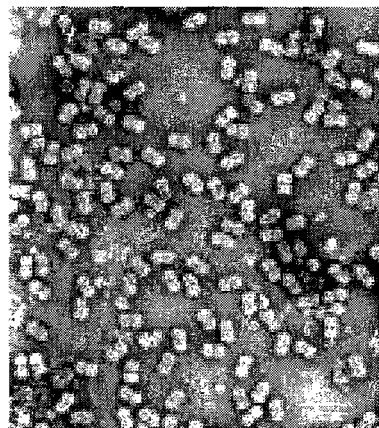


Fig. 2. Electron micrograph of geminate particles of yellow mosaic infected plant by negative staining.

loss due to the disease is considerable. Yield loss in blackgram, mungbean and soybean together is estimated to be around US \$ 300 million in a year (Varma *et al.*, 1992)

The viruses causing YMD are transmitted exclusively by whitefly, *Bemisia tabaci* Genn. and lack sap transmission. However, a virus causing yellow mosaic disease in mungbean in Thailand (Honda *et al.*, 1983) was found to be sap transmissible. In the last decade, molecular cloning and nucleotide sequencing of the genome of more than thirty five virus isolates causing yellow mosaic disease have been completed in the Indian subcontinent and Thailand.

Based on the characteristic whitefly transmission, geminate particle morphology and genome organization, these virus isolates have been identified as virus species belonging to the genus *Begomovirus* of the family *Geminiviridae*. Four virus species, *Dolichos yellow mosaic virus* (DoYMV) infecting dolichos, *Horsegram yellow mosaic virus* (HgYMV) infecting horsegram, *Mungbean yellow mosaic India virus* (MYMIV) infecting all the legumes and occurring in central, eastern and northern India and *Mungbean yellow mosaic virus* (MYMV) occurring in southern and western part of India (Usha Rani *et al.*, 2004) have been recognized.

MYMIV and MYMV, two virus species, share a similar host range and show characteristic features of the genome organization of bipartite begomoviruses. The genome consists of two single stranded circular DNA components, referred to as DNA A and DNA B of 2.6 to 2.7 kb length encapsidated independently.

The DNA A component has two open reading frames (ORFs) in viral sense. ORF AV1, coat protein and ORF AV2 pre-coat protein. The ORF AV2

is present only in Old World Begomoviruses. There are four ORFs in complementary sense. ORF AC1 and ORF AC3 are essential for replication, ORF AC2 is the regulator of transcription of rightward ORFs. The role of ORF AC4 and AC5 is not yet elucidated.

MYMIV and MYMV are distinct from other begomoviruses. The typical feature of begomoviruses is geography related lineages (Harisson and Robinson, 1999). This characteristic geography based affinity between begomoviruses is not exhibited by MYMIV and MYMV (Usha Rani *et al.*, 2004; Fauquet and Stanley, 2003). They are very different both from begomoviruses infecting beans and cowpea in South American and African countries and from Indian begomoviruses (Usharani *et al.*, 2004). Within MYMIV and MYMV, the ORF AV2 showed high degree of diversity. This ORF is absent in New World begomoviruses and exactly what role is played by it in viral pathogenesis is not understood. How does the variability seen in ORF AV2 among MYMIV isolates contribute to the difference in pathogenesis, what is the extent of variability in both ORF AV2 and the ORF AV1 and what are the biochemical functions of the protein ORF AV2 which is present only in Old World begomoviruses are few questions, that need to be addressed.

Keeping these lacunae in view, the following objectives were set for the present study :

- 1. To investigate the function of ORF AV2 by mutagenesis.**
- 2. *In vitro* expression and determination of biochemical functions of ORF AV2.**
- 3. To assess the extent of variability amongst the yellow mosaic virus isolates in ORF AV2 and coat protein regions.**

2. REVIEW OF LITERATURE

Yellow mosaic disease (YMD) caused by whitefly-transmitted geminiviruses is one of the most serious diseases of grain legumes in all of South Asia. Disease incidence, as high as 100% in the farmer's field is common in the Indian subcontinent, often resulting in considerable yield loss. For the past several decades, research has been conducted on symptomatology, yield loss, host range, vector transmission characteristics and management of the viruses causing YMD. In the last decade, progress has been made in molecular characterization of viruses. Understanding of their genome, viral gene function and phylogenetic relationship between the yellow mosaic virus isolates have crystallized. Literature relevant to the work carried out in the present investigation is reviewed below.

2.1 Yellow mosaic disease of legumes

2.1.1 Occurrence and distribution

Yellow mosaic disease affecting pulse crops is endemic to South Asia. Such an endemic distribution of the disease may be due to limited cultivation of these crops in tropical and sub tropical countries in Asia. The disease with characteristic yellow mosaic symptoms, has been recorded (Green *et al.*, 1992, and Green *et al.*, 1998) (Table 1) in Bangladesh, India, Nepal, Pakistan, Sri Lanka and Thailand. In all these countries, disease incidence in mungbean is very high and yield loss is considerable.

YMD is the most destructive disease both in the spring/summer and rainy seasons. It is widely distributed in the north, central and south Indian states (Table 2) (Singh and Asthana, 1997). In the western states of India, the

Table 1. Occurrence and distribution of yellow mosaic disease

Host	Symptom	Country	Reference
Cowpea (<i>Vigna unguiculata</i>)	GM	Pakistan Kenya, Nigeria Tanzania	Ahmad (1978) Singh and Allen (1979)
Mungbean (<i>Vigna radiata</i>)	YM	Bangladesh Sri Lanka Thailand	Jalaluddin and Shaikh (1981) Sivanathan (1977) Thongmeearkom (1981)
Soybean (<i>Glycine max</i>)	YM	Pakistan Sri Lanka	Aftab <i>et al.</i> (1991) Pathirava <i>et al.</i> (1986)

YM : Yellow mosaic; GM : Golden mosaic

disease occurs in sporadic pockets. In the western and southern states, YMD occurrence is more during spring (February-March).

Yellow mosaic diseases (YMD) are the chief limiting factors to economic production of grain legumes in the tropical and sub-tropical regions of the Indian subcontinent. In India, the YMD of grain legume was first reported in Lima bean (*Phaseolus lunatus*) in western India in the 1940s (Capoor and Varma, 1948). Later from Pune, in 1950, similar yellow mosaic symptoms were seen in dolichos (*Lablab purpureus*, Capoor and Varma, 1950). Nariani (1960) observed the symptoms in mungbean (*Vigna radiata*) in Indian Agricultural Research Institute experimental field in the 1950s. The disease was subsequently observed throughout India in almost all the legume crops, such as blackgram (*V. mungo*), soybean (*Glycine max*; Nariani, 1960; Nene, 1972), pigeonpea (*Cajanus cajan*, Williams *et al.*, 1968), horsegram

Table 2. Occurrence and distribution of yellow mosaic disease in India

Host	Symptom	Location	Reference
Blackgram (<i>Vigna mungo</i>)	YM	Southern India Northern India	Nene (1973), Varma <i>et al.</i> (1973), Singh <i>et al.</i> (1979), Murugesan and Chelliah (1977)
Cowpea (<i>Vigna unguiculata</i>)	YF GM	Northern India Northern India	Sharma and Varma (1976) Varma and Reddy (1984)
Cluster bean (<i>Cyamopsis tetragonoloba</i>)	YM	Southern India	Rao <i>et al.</i> (1982)
French bean (<i>Phaseolus vulgaris</i>)	YM	Northern India Western India (Pune)	Singh (1979) Varma <i>et al.</i> (1991)
Groundnut (<i>Arachis hypogea</i>)	YM	Southern India	Sudhakar Rao <i>et al.</i> (1979)
Horsegram (<i>Macrotyloma uniflorum</i>)	YM	Southern India	Muniyappa <i>et al.</i> (1975)
Hyacinth bean (<i>Lablab purpureus</i>)	YM	Several parts of India	Capoor and Varma (1948)
Moth bean (<i>Vigna aconitifolia</i>)	YM	Western India	Satyavir (1980)
Mungbean (<i>Vigna radiata</i>)	YM	Northern India	Nariani (1960), Nene (1972), Mishra <i>et al.</i> (1978)
Lima bean (<i>Phaseolus lunatus</i>)	YM	Western India	Capoor and Varma (1948)
Pigeon pea (<i>Cajanus cajan</i>)	YM	Northern India	Williams <i>et al.</i> (1968)
Soybean (<i>Glycine max</i>)	YM	Northern India Central India Southern India	Keshwal <i>et al.</i> (1988), Suteri (1974), Singh and Mallick (1978)

YM : Yellow mosaic; YF : Yellow Fleck; GM : Golden mosaic.

(*Macrotyloma uniflorum*), French bean (*Phaseolus vulgaris*; Muniyappa *et al.*, 1976) and cowpea (*Vigna unguiculata*; Varma and Reddy, 1984). The YMD of legumes was also reported from Pakistan in cowpea and mungbean (Ahmad and Harwood, 1973), in mungbean from Bangladesh (Jalaluddin and Shaikh, 1981), Sri Lanka (Joseph *et al.*, 1998) and Thailand (Thongameeakom *et al.*, 1981). Details on the distribution of the disease in different states of India and in other countries in Asia and Africa are given in Tables 2 and 3.

2.1.2 Yield loss

It is difficult to estimate the yield loss caused by YMD in grain legumes in the farmer's field. As these losses vary from year to year, cultivar to cultivar, and season to season, attempts have been made by several research workers to estimate the same in experimental trials. The reduction in yields due to YMD depends on the time of infection and severity of disease (Nariani, 1960; Nene, 1973).

When highly susceptible varieties of blackgram or soybean are infected within fifty days after sowing no yield is obtained (Nene, 1969). Infection of susceptible blackgram cultivars during the fourth, fifth, sixth, seventh and eighth week of cultivation causes yield reduction upto 85, 60, 44, 28 and less than 10% respectively. The loss in yield is significant when infection occurs up to 50 days after sowing (DAS) (Nene, 1973). Ahmad (1990) noted a maximum yield reduction of 62.9% when plants got infected earlier (20 days after sowing) and 46.2% at 40 DAS. Singh *et al.* (1982) reported that stunting and reduced branching contributed to yield losses in mungbean. Singh *et al.* (1983) found that the average reduction in yield in soybean varied from 19-21%. YMD also affected the quality and colour of the seeds. In blackgram the infected seeds were small, colour of testa changed to pale yellow and seeds

Table 3. Infectivity of yellow mosaic virus isolates on selected legumes

	Bg	Cp	Do	Hg	Lb	Mb	Mob	Pp	Sb	Mb Pakistan	Mb Thailand
<i>Glycine max</i>	+	0	-	-	-	+	+	+	+	+	+
<i>Lablab purpureus</i>	0	-	+	0	-	0	-	0	-	-	-
<i>Macrotyloma uniflorum</i>	-	-	0	+	-	+	-	+	-	-	-
<i>Phaseolus lunatus</i>	+	-	0	+	+	+	-	+	-	-	-
<i>P. vulgaris</i>	+	+	0	+	+	+	+	+	+	-	-
<i>Vigna aconitifolia</i>	+	-	0	+	0	+	+	-	-	+	-
<i>V. mungo</i>	+	0	0	+	-	+	+	+	+	+	+
<i>V. radiata</i>	+	?	0	+	+	+	+	+	+	+	+
<i>V. unguiculata</i>	?	+	0	0	-	0	0	+	+	-	0

Modified from Varma *et al.* (1992)

- Bg : Black gram (Krishnareddy, 1989; Williams *et al.*, 1968; Dhar, 1990)
 Cp : Cowpea (Srivastava, 1989; Roy, 2000; Roy and Malathi, 2001; Malathi *et al.*, 2005)
 Dol : Dolichos (Capoor and Varma, 1950; Harrison *et al.*, 1991)
 Hg : Horsegram (Muniyappa *et al.*, 1976; Harrison *et al.*, 1991)
 Lb : Lima bean (Capoor and Varma, 1948)
 Mb : Mungbean (Nariani, 1960; Ahmad and Harwood, 1973; Honda *et al.*, 1983; Biswas, 1996)
 Mob : Moth bean (Chakraborty, 1996)
 Pp : Pigeonpea (William *et al.*, 1968; Nene *et al.*, 1973; Mandal *et al.*, 1998)
 Sb : Soybean (Nariani, 1960; Nene, 1972; Anilkumar, 2001)

+ : infection; 0 : not infection; - : Not tested; ? : contradictory results.

were shrunken; also the protein content of infected seeds was 30% higher than healthy ones (Vohra and Beniwal, 1979; Suteri and Srivastava 1975). Late infection, four weeks after germination, also resulted in 39.6% loss in blackgram (Subramanyam, 1979). Reduction in plant height (38.2%) and fresh shoot weight (28.5%) 100-seeds weight (25.7%) contributed to total yield loss of 66.6% in mungbean (Chand and Varma, 1983). Likewise, reductions in number of pods per plant, seed yield and 100-seed weight (Chandrababu *et al.*, 1984) was also seen. The reduction in grain yield ranged up to 57.5% in blackgram which was directly correlated to reduction in plant height, pods per plant, 100 seed weight, net annihilation rate and crop growth rate (Jain *et al.*, 1995). Similar observations on reduction in pod characters were recorded by Ahmad (1990), Khattak *et al.* (2000) and Ghafoor *et al.* (2000).

Krishnareddy (1989) extensively analyzed YMD effect on yield parameters on the basis of disease incidence, infection time, number of pods per plant and severity of the disease and proposed a model for prediction of yield loss. On the basis of this model, Varma *et al.* (1992) suggested that the yield loss due to YMD could be as high as US \$300 million in an epidemic year, taking blackgram, mungbean and soybean together.

2.2 Symptomatology

The first symptom of the disease on the expanding young leaf of legumes is yellow chlorotic spots which start as specks (Nariani, 1960). The small chlorotic areas gradually coalesce to form a large mosaic pattern (Nariani, 1960; Varma and Reddy, 1984). The leaf shows bright yellow patches interspersed with green areas. Some areas of the leaf may be slightly raised showing a puckered appearance. In severe cases, the entire lamina may become yellow as in the case of cowpea, which is called as golden mosaic symptom

(Varma and Reddy, 1984; Roy and Malathi, 2004). In blackgram, two types of symptoms were described by Nene (1973). They are “yellow mottle” and “necrotic mottle”. Necrotic mottle was usually seen only in resistant blackgram cultivars and not in mungbean. Later Nair *et al.* (1973) clarified that the two types of symptoms seen were, more varied reactions of different genotypes to the same virus isolate. In French bean, YMV induces two types of symptoms, downward bending and curling (Honda *et al.*, 1983; Dhar, 1992) as well as yellow mosaic symptom (Singh, 1979). On pigeon pea, bright yellow patches on leaves without leaf distortion were observed by Williams *et al.* (1968). Generally, the leaf size is reduced, the size and number of pods as well as seeds are reduced. In all the cases, infected plants produce fewer flowers and pods.

Recently in cowpea, downward leaf curling, puckering, vein enation and bright yellow mottling along with yellow mosaic symptoms have been reported from northern India (Rouhibakhsh and Malathi, 2005).

2.3 Host range

The isolates of YMV are mostly infectious on limited leguminous crops. In the Indian subcontinent several leguminous species are affected as indicated in the Table 3. Over a period of time, though a large volume of information is available on host range of YMV isolates, it is difficult to get a clear picture due to the following reasons (a) YMV isolates studied by different workers may represent different strains of the virus or different virus species; (b) the susceptibility of host genotypes may differ; unless the inoculations have been done on uniformly established susceptible genotypes, true host range cannot be assessed; (c) the most difficult part in the host range studies on YMV is their transmission exclusively by the vector, whitefly (*Bemisia tabaci* Genn.).

Except for the mungbean isolate from Thailand (Honda *et al.*, 1983 and Thongameeakom *et al.*, 1981), which is sap transmissible, no other isolate is known to be sap transmissible (Malathi and Sumiya, 2005). Net result, host range determination is highly dependent on vector behaviour, like the vector biotype used, since differences in transmission efficiencies exist between vector biotypes. Besides the differences in efficiency, other differences may also come up due to the feeding preference of the whitefly. Thottappilly (1998) and Malathi *et al.* (2005) showed how transmission from cowpea to any other host was difficult due to stenosis caused by feeding on cowpea plants.

Host range differences among YMV isolates are seen even within leguminosae (Table 3). Extreme specificity is shown by dolichos, Lima bean and cowpea isolates. The dolichos isolate was not transmitted by whitefly to Lima bean or other legumes (Capoor and Varma, 1950; Harrison *et al.*, 1991). The Lima bean isolate was also not transmitted to dolichos (Capoor and Varma, 1950). Though, horsegram and pigeonpea isolates are transmissible to Lima bean, isolate from Lima bean is not transmissible to these hosts. However both in the case of dolichos and Lima bean, information available is very limited and with more studies, their host range may get established.

Among other YMV isolates, blackgram, mungbean and horsegram isolates are not transmissible to cowpea and dolichos (Muniyappa *et al.*, 1976; Varma *et al.*, 1991; Dhar, 1992; Biswas, 1996; Chakraborty, 1996; Roy, 2001) but are transmissible to other legumes; however, isolates from pigeonpea and soybean are transmissible to cowpea (Mandal *et al.*, 1997 and Anilkumar, 2001). The perplexing results are seen with cowpea isolates. It was earlier called 'cowpea golden mosaic virus' as it was not transmissible to any hosts (Sharma and Varma, 1976; Varma and Reddy, 1984) other than French bean

and yard long bean (*V. unguiculata* sub species *sesquipedalis*). However, the host range was reexamined through agroinoculation by Malathi *et al.* (2005), who showed that the isolate was infectious on blackgram, mungbean and soybean too.

Whether YMV isolates, infect hosts belonging to other families was the interesting question addressed by some workers. Nene (1971), Rathi (1972), Rathi and Nene (1976) and Aiyathan and Chandrashekharan (1998) reported some non-leguminous hosts.

Yellow streak symptoms in *Brachiaria ramosa*, mild yellowing and severe puckering of leaves in *Xanthium strumarium* and curling of leaves in *Cosmos bipinnatus* were recorded by Nene *et al.* (1971), Rathi (1972) and Nene and Rathi (1976).

Aiyathan and Chandrasekaran (1998) claimed transmission of YMV isolate from mungbean to the weed *Croton sparsiflorus*. They also showed whitefly transmission of the virus associated with yellow mosaic disease of *Acalypha indica*, yellow vein mosaic disease of *C. sparsiflorus* and *Eclipta alba*. However, Dhar (1991) and Mandal (1994) showed that YMV isolates from blackgram and pigeonpea were not transmissible to *E. alba*. In all the earlier studies on host range, nucleotide sequence comparison between the isolates occurring in weeds and legumes were not attempted. Therefore, the non-leguminous host range of the YMV isolates need to be confirmed and revalidated.

2.4 Vector transmission

Yellow mosaic viruses of legumes are transmitted by the whitefly *Bemisia tabaci* Genn. in India, the virus has not been transmitted by sap

(Nariani, 1960; Reddy and Varma, 1988). However, the Thailand isolate of the virus was transmitted by sap to different legumes (Honda *et al.*, 1983). *Bemisia tabaci* is a polyphagous insect with a broad host range (Nene, 1972). The whitefly can acquire and transmit the virus after acquisition access period (AAP) and inoculation access period (IAP) of 15 min, but the optimum time period to achieve more than 60% transmission is an AAP and IAP of more than 4 h. More than 3 h of latent period were required after acquisition by vector, for the virus to be transmissible to a legume host. Pre-acquisition starving of 15 min, increased the ability of the vector to transmit the virus from 5.5 to 39% (Nair and Nene, 1973). A single whitefly can transmit the virus after IAP and AAP of 24 h to 25% of the inoculated plants. The vector could acquire the virus in pre-adult stage and transmit the virus in adult stage (Chenulu *et al.*, 1979). Nymphal stages of the whitefly were able to acquire the virus from infected legume crop (Nene, 1972). Primary and the first trifoliolate leaves were shown to be more susceptible to whitefly inoculation of the YMV (Chenulu *et al.*, 1979; Krishnareddy, 1989). Female whiteflies were more efficient than male for transmission of the virus (Rathi and Nene, 1974). Female whiteflies also retained the virus for a longer period (10 days) than the male whiteflies (Rathi and Nene, 1974). The virus was not transmitted through eggs of *B. tabaci* (Ahmad and Harwood, 1973; Chenulu *et al.*, 1979). Till date, no information is available on the biotype of the vector associated with the epidemics of YMD as seen in the case of tomato leaf curl disease in Kolar district in Karnataka (Banks *et al.*, 2001).

2.5 The causal virus

Whitefly transmissible nature of YMV causing diseases in legumes was demonstrated from early years; however, electron microscopic observation

of geminate particles, physically establishing the presence of 'geminiviruses' within the infected plant took some time, as concentration of virion particles in the infected plant was very low, and they get disrupted very quickly (Honda *et al.*, 1983). Purification and occurrence of quasi isometric particles at a concentration as low as 100 mg/kg tissue was first seen in mungbean isolate from Thailand (Honda *et al.*, 1983). Association of geminate particles in YMD infected plants (Fig. 2) of mungbean, soybean, Lima bean, French bean, groundnut and bambara groundnut was shown by Muniyappa *et al.* (1987). Similar geminate particles were also observed in plants inoculated with YMV isolates from blackgram, cowpea and pigeonpea by immuno-sorbent electron microscopy using polyclonal antibody to *Squash leaf curl virus* (SLCV) for trapping (Srivastava, 1989; Varma *et al.*, 1992). Presence of geminate particles and their reaction with monoclonal antibodies to *African cassava mosaic virus* (ACMV) and *Indian cassava mosaic viruses* (ICMV), positive hybridization with DNA A component of ICMV, finally established the etiological agents of YMD to be a whitefly transmitted geminiviruses, now recognized as a distinct species belonging to the genus, *Begomovirus* of the family *Geminiviridae*.

At present thirty five isolates causing YMD in India, Bangladesh, Pakistan, Nepal, Sri Lanka and Thailand have been cloned and nucleotide sequence completed (Fauquet and Stanley, 2005). Nucleotide sequence comparison revealed that there are four virus species causing YMD in grain legumes in South Asia; they are, *Mungbean yellow mosaic India virus* (MYMIV) that is prevalent in northern India, *Mungbean yellow mosaic virus* (MYMV) occurring in southern India, *Dolichos yellow mosaic virus* (DoYMV) that infects only dolichos, present both in northern and southern India and

Horsegram yellow mosaic virus (HgYMV), infecting horsegram in southern India (Usha Rani *et al.*, 2004; Girish and Usha, 2005; Karthikeyan *et al.*, 2003; Maruthi *et al.*, 2005; Fauquet and Stanley, 2005). The nomenclature followed in this review to refer to different YMV isolates and other geminiviruses are as per the guidelines in the VII report of the International Committee on Taxonomy of Viruses (ICTV), published by Fauquet and Stanley (2003).

2.6 The family *Geminiviridae*

The family 'Geminiviridae' comprises of a unique group of viruses characterized by their twinned particles morphology. Since the earliest report of such structures (Bock, 1974; Mumford, 1974; Bock *et al.*, 1978; Harisson *et al.*, 1977) the members of the family have risen steadily. The distinguishing features are : (a) their twinned icosohedral virion (18 x 30 nm) morphology, based on which the family derived its name from the latin word 'geminus' meaning twin (b) the genome consists of one or two single stranded circular DNA, of size ranging from 2.5 to 3 kb and (c) the replication of genomic DNA is through a double stranded intermediate in a rolling circle fashion, mediated by only one replication initiating protein, Rep. All these features justified the recognition of the members as a separate family '*Geminiviridae*' (Murphy *et al.*, 1995). Members of *Geminiviridae* infecting legume are listed in Table 4.

The family consists of the members, that cause severe outbreaks of diseases (Rybicki *et al.*, 1994) of economic importance, such as the African cassava mosaic disease in the African continent causing losses upto US \$ 1300-2300 million annually (Thresh *et al.*, 1998), Maize streak disease, a serious threat to cultivation of maize in Africa, and Beet curly top disease,

Table 4. Geminivirus species infecting legumes and their accession numbers

Virus name	Acronym	Accession number
<i>Mastrevirus</i>		
<i>Bean yellow dwarf virus</i>	BeYDV	Y11023
*Chickpea chlorote dwarf virus	-	-
<i>Begomovirus</i>		
<i>Bean calico mosaic virus</i>	BcaMV	AF110189-90
<i>Bean dwarf mosaic virus</i>	BDMC	M88179-80
<i>Bean golden mosaic virus</i> -[Brazil]	BGMC-[BR]	M88686-7
<i>Bean golden yellow mosaic virus</i> -[Puerto Rico]	BGYMV-[PR]	M10070, M10080
<i>Cowpea golden mosaic virus</i> -[Nigeria]	CPGMV-[NG]	AF029217
<i>Dolichos yellow mosaic virus</i>	DoYMV	AY309241
<i>Horsegram yellow mosaic virus</i> -[Coimbatore]	HgYMV-[Coi]	AJ627904-5
<i>Mungbean yellow mosaic India virus</i>	MYMIV	AF126406, AF142440
<i>Mungbean yellow mosaic virus</i>	MYMV	D14703-4
<i>Rhynchosia golden mosaic virus</i>	RhGMV	AF239671
*Lima bean golden mosaic virus	-	-
*Lupin leaf curl virus	-	-
<i>Macroptilium golden mosaic</i> [Jamaica 1]	MGMV-[JM1]	AF098940
*Macrotyloma masoic virus	-	-
<i>Wissadula golden mosaic virus</i> -[jamaica 1]	WGMV-[JM1]	U69280-1
<i>Soybean crinkle leaf virus</i> -[Japan]	SbCLV-[JP]	AB050781, AB020977

*Tentative species.

which almost eliminated sugar beet production from western United States in the early 1900s.

The members of *Geminiviridae* are divided into four genera (Table 5) based on host range, insect vector and genome organization (van Regenmortel *et al.*, 2000). The four genera and their characteristics are given below.

Table 5. Genera of family *Geminiviridae*

Genus	Type member	Acronym	Genome	Host name	Insect vector
<i>Mastrevirus</i>	<i>Maize streak virus</i>	MSV	Monopartite	Monocots and few dicots	Leaf-hopper
<i>Curtovirus</i>	<i>Beet curly top virus</i>	BCTV	Mono-partite	Dicots	Leaf-hopper
<i>Begomovirus</i>	<i>Bean golden mosaic virus</i>	BGMV	Mono or bipartite	Dicots	Whitefly
<i>Topocuvirus</i>	<i>Tomato pseudo-curly top virus</i>	TPCTV	Monopartite	Dicots	Tree-hopper

2.6.1 Mastrevirus

The virus species belonging to this genus, have a single genomic component (monopartite) encoding four proteins (Fig. 3a). They usually infect monocotyledonous plants with two exceptions, *Bean yellow dwarf virus* (Liu *et al.*, 1997) from South Africa and *Tobacco yellow dwarf virus* (Morris *et al.*, 1992) from Australia. The type species of the genus is *Maize streak virus* (MSV). The members are transmitted by leafhopper. The genome, a single stranded circular DNA of 2.5-2.8 kb consists of four open reading frames (ORFs) and two intergenic, small (SIR) and large (LIR) regions. Two ORFs in the complementary sense, C1 (Rep A) and C2 (Rep B), together function

Genome Organization of Geminiviruses

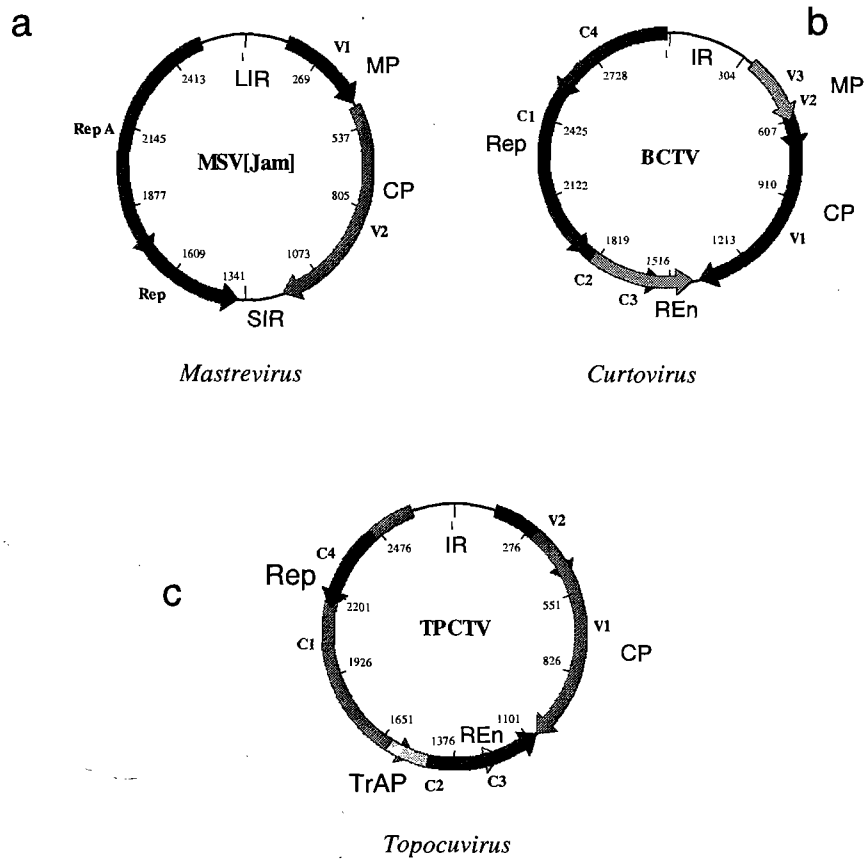


Fig. 3. Genome organization of geminiviruses. Arrows represent open reading frames of virion and complimentary sense. Stem and loop region is indicated; LIR: large intergenic region; SIR: small intergenic region. Names of gene products are given wherever the functions of the genes have been established. CP: coat protein; Rep: replication initiator protein; MP: movement protein.

as replication initiation protein, Rep. Two virion sense ORFs, V1 (movement protein, MP) and V2 (Coat protein, CP), encode for cell to cell movement protein and coat protein respectively. The LIR is similar to the intergenic region seen in other genera, and it is from this region that rolling circle replication is initiated. Transcription is bidirectional, it starts from LIR and terminates at SIR (Rybicki *et al.*, 2001).

There are two legume geminiviruses, *Bean yellow dwarf virus*, and a tentative species, *Chickpea chlorotic dwarf virus* belonging to the genus *Mastrevirus*.

2.6.2 Curtovirus

Members of genus *Curtovirus* have monopartite genome (Fig. 3b) and are transmitted by leafhopper in a circulative manner to dicotyledonous plants. The *Beet curly top virus* (BCTV) is the type species, which infects sugarbeet and has a wide host range.

In BCTV, there are seven ORFs among which three are in virion sense (V1, V2 and V3) and four ORFs in the complementary sense (C1, C2, C3 and C4). The ORF V1 (CP) is responsible for encapsidation, ORF V2 is involved in regulation of the genomic single stranded (ss) DNA accumulation and ORF V3 (MP) is responsible for cell to cell movement of the virus. The complementary sense ORFs are involved in the replication of the virus. ORF C1 functions as replication initiation protein (Rep), C3 as replication enhancer protein (REn) (Hanley-Bowdoin *et al.*, 1999) and C4 plays a role in the initiation of cell division (Hull, 2002). The role of ORF C2 is unknown (Wartig *et al.*, 1997).

2.6.3 *Topocuvirus*

The genus has only one member, namely *Tomato pseudo-curly top virus* (TPCTV) as the type species, which has a monopartite genome and is transmitted by the treehopper to dicotyledonous plants (Pringle, 1999). The genome of TPCTV shows a natural recombination between *Mastrevirus* and an unknown genus (Briddon *et al.*, 1996).

The virus encodes four ORFs in complementary sense named C1, C2, C3 and C4 with similar functions of begomoviruses, responsible for replication of the virus and two ORFs in virion sense called V1 and V2 similar to mastreviruses, which are responsible for movement and encapsidation of the virus respectively (Fig. 3c).

2.6.4 *Begomovirus*

This is the largest genus in the family and consists of 133 recognized distinct virus species and 53 tentative species (Fauquet and Stanley, 2005). The members exhibit a narrow host range among dicotyledonous species. The begomoviruses have either monopartite or bipartite genome. *Bean golden mosaic virus* (BGMV) is the type species. They are transmitted by the whitefly, *Bemisia tabaci* Genn. The genome organization of the genus is discussed in detail as it is relevant to the present study.

Genome organization of *Begomovirus*

Members of the genus *Begomovirus* are the only geminiviruses to have whitefly as vector, all of which belong to only one species *Bemisia tabaci* Genn. Emergence of whitefly transmitted geminivirus diseases as a major constraint to agricultural productivities in tropical and subtropical regions of the World (Varma and Malathi, 2003), increased due to many factors. They

are, worldwide increase in the population and distribution of the vector, extensive monocropping, intensive agricultural endeavour, experimenting with introduction of new crops in unconventional new areas in new seasons and global movement of plant materials.

Diseases caused by members of begomoviruses affect the economy of tropical and subtropical countries (Varma and Malathi, 2003; Rishi, 2004). Typical examples are cassava mosaic disease in Africa (US \$ 2 billion, Thresh *et al.*, 1998), cotton leaf curl disease in Pakistan (US \$ 5 billion, Briddon and Markham, 2000) and yellow mosaic disease of legumes (US \$300 million annually, taking blackgram, mungbean and soybean together (Varma *et al.*, 1992). Due to economic consideration, the viruses have received keen attention from research workers culminating in excellent progress in understanding the gene expression strategies and the evolutionary trends among these viruses. Given below is a brief review of areas relevant to the present study.

Molecular cloning, nucleotide sequencing, Koch's postulate establishment and genome comparisons of more than 133 begomovirus species (Fauquet and Stanley, 2005) have led to the clear understanding of the diversity and complexity of begomoviruses. Among begomoviruses, three types of genome organization are met with (Briddon *et al.*, 2003; Bull *et al.*, 2004). Some Old World and all the New World begomoviruses have two DNA components, referred to as DNA A and DNA B (Stanley, 1985; Harrison, 1985; Stanley, 1991; Lazarowitz, 1992; Hanley-Bowdoin *et al.*, 1999; Harrison and Robinson, 1999; Gutierrez *et al.*, 2002; Gafni and Epel, 2003; Mansoor *et al.*, 2003; Briddon *et al.*, 2003; and Rojas *et al.*, 2005). Among Old World begomoviruses, there are variations in the component. The examples of each type are given below.

Genome Organization of Geminiviruses

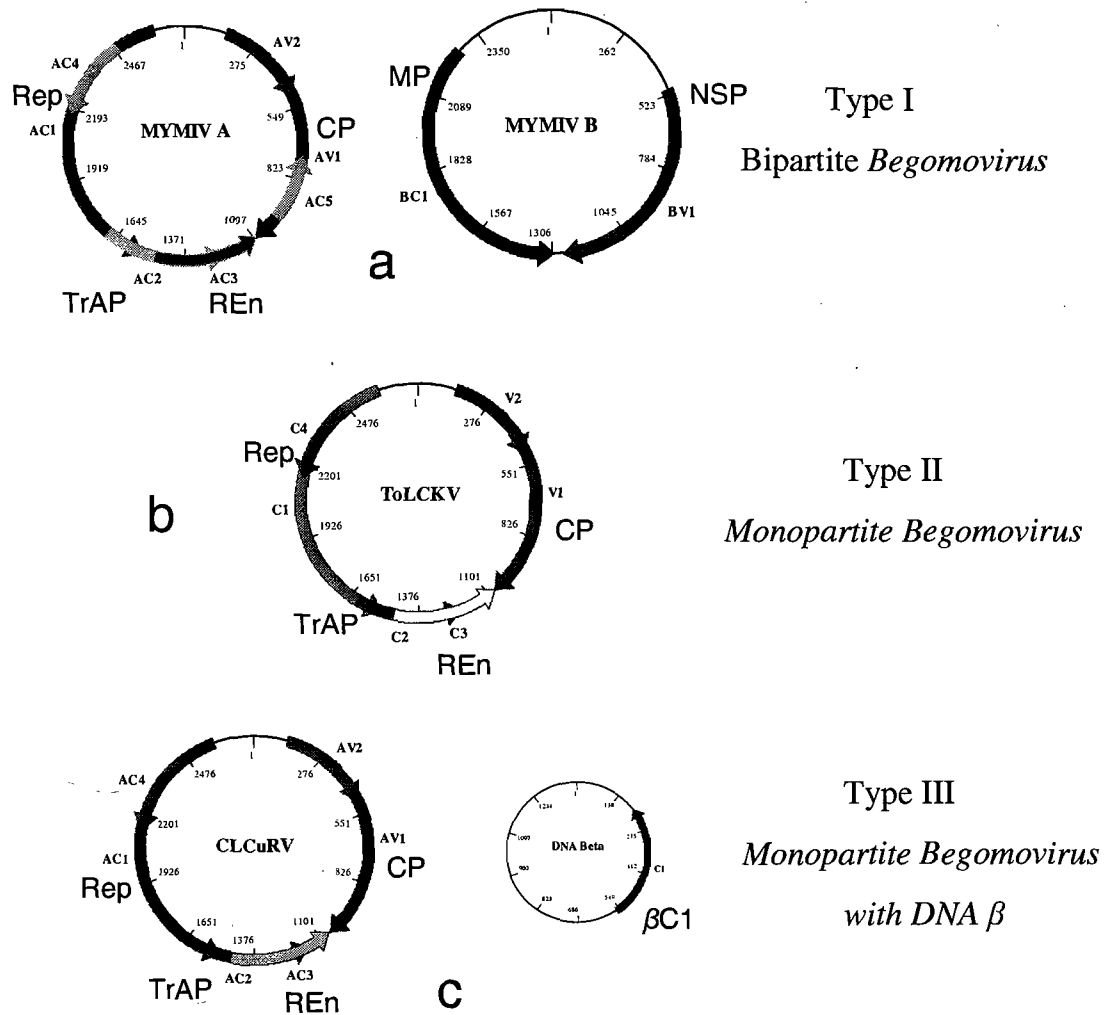


Fig. 4. Genome organization of geminiviruses. Arrows represent open reading frames of virion and complementary sense. Stem and loop region is indicated; CR: common region; IR: intergenic region; Names of gene products are given wherever the functions of the genes have been established. CP: coat protein; Rep: replication initiator protein; TrAP: transcription activator protein; REn: replication enhancer protein; NSP: nuclear shuttle protein; MP: movement protein.

Type I

Begomoviruses having bipartite genome (Fig. 4a); genome consists of two, single stranded DNA components referred to as DNA A and DNA B, of 2.5 to 2.7 kb length. Both DNA A and DNA B are necessary for infectivity and both components are represented in similar amount within infected tissue e.g. *African cassava mosaic virus* (ACMV), *Tomato golden mosaic virus* (TGMV) and *Indian cassava mosaic virus* (ICMV).

Type II

Begomoviruses having monopartite genome; which consists of only one component, DNA A, which has an organization similar to that of DNA A of Type I (Fig. 4b). DNA A alone is infectious and produces severe typical symptoms on experimental assay hosts and primary hosts from which the virus is isolated e. g., *Tomato yellow leaf curl virus-Israel* (TYLCV-[IL]), *Tomato yellow leaf curl Sardinia virus* (TYLCSV) and *Tomato leaf curl Karnataka virus* (ToLCKV).

Type III

Begomoviruses having monopartite genome similar to DNA A of Type I virus; along with it, a satellite DNA component of ~1300 bp length, referred to as satellite DNA β is present (Fig. 4c). DNA A alone is infectious and produces very mild leaf curl symptoms in experimental assay host, but does not cause typical disease symptoms on primary hosts. DNA A along with DNA β when coinoculated, causes typical symptoms of enation, severe leaf curl or yellow vein symptom in the primary host e.g., *Ageratum yellow vein virus* (AYVV), *Bhendi yellow vein mosaic virus* (BYVMV), and *Cotton leaf curl Rajasthan virus* (CLCuRV). The satellite DNA β has no apparent

sequence similarity within the helper virus genome and is fully dependent on the helper virus for replication and vector transmission (Briddon *et al.*, 2003; Monsoor *et al.*, 2003). The DNA β has only one ORF named as β C1, which is a pathogenicity determinant and modulator of symptom development (Saunders *et al.*, 2004). In addition to DNA A and DNA β , a small DNA molecule of 1300 bp called as DNA 1 is also present. DNA 1 resembles DNA 1 component of Nanovirus.

2.7 Genome organization of begomoviruses

DNA A and DNA B encode genes for different proteins necessary for viral encapsidation, replication and movement. Table 6 provides the names of open reading frames (ORF) present in DNA A and DNA B. The nomenclature adopted to refer different ORFs is as given in van Regenmortel *et al.* (2000). DNA A is essential for replication and encapsidation (Rogers *et al.*, 1980; Townsend *et al.*, 1986). In DNA A (Table 6), viral sense strand has two ORFs, ORF AV2, pre-coat protein and coat protein, ORF AV1. In complementary sense, there are totally five ORFs; two ORFs, ORF AC1 and AC3, encode replication initiation protein (Rep) and replication enhancer protein (REn) respectively (Elmer *et al.*, 1988; Etessami *et al.*, 1991). One more important ORF is ORF AC2 which activates the transcription of rightward ORFs of both DNA A and DNA B (ORFs AV1, AV2 and ORF BV1) and so is called as transcription activator protein (TrAP) (Sunter and Bisaro, 1992). In DNA B, there is one ORF in viral sense strand coding for nuclear shuttle protein (BV1, NSP) and one in complementary sense coding for movement protein (BC1-MP) (Lazarowitz, 1992; Frischmuth *et al.*, 1993; Pascal *et al.*, 1994; Ingham *et al.*, 1995).

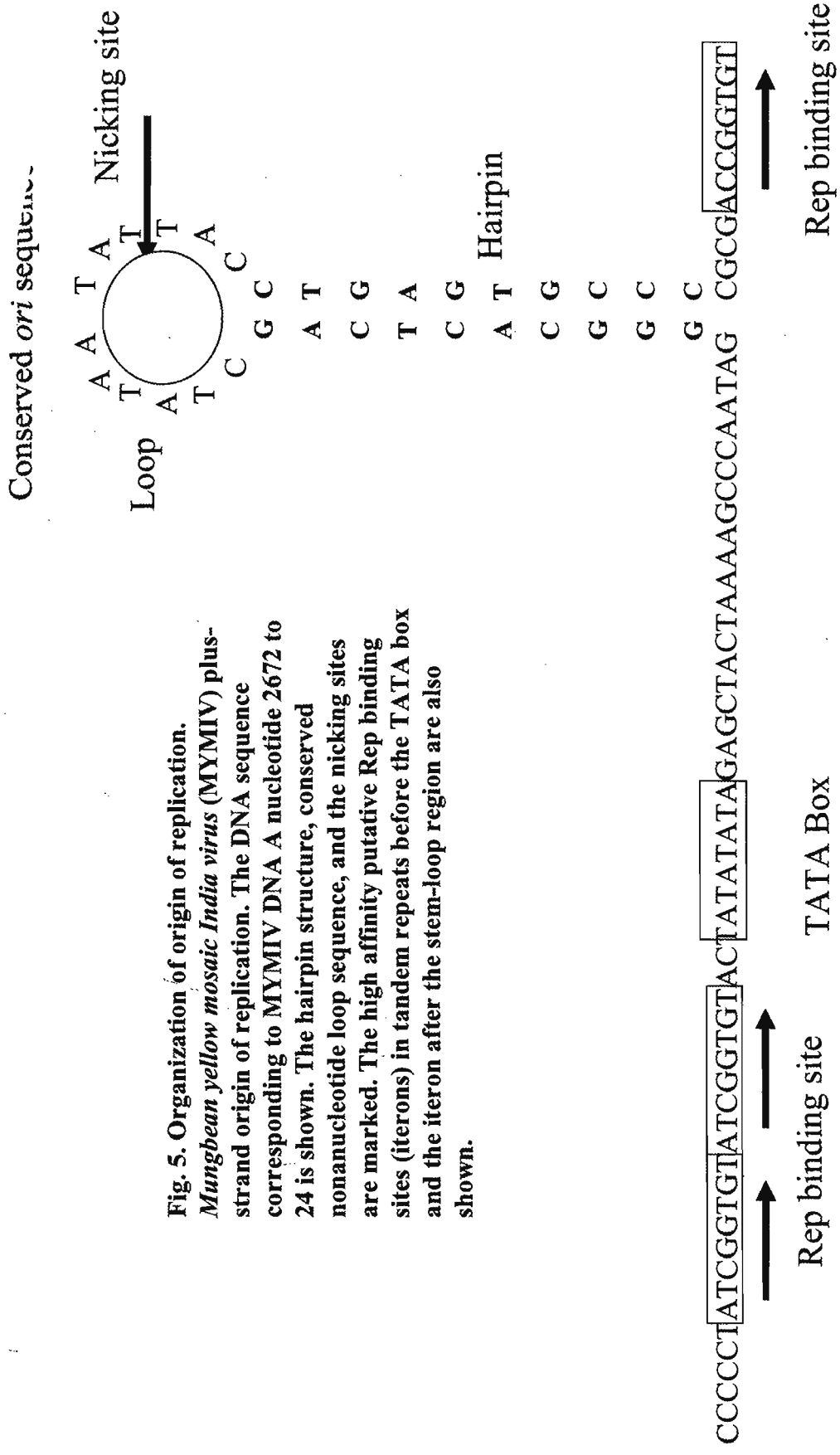


Fig. 5. Organization of origin of replication. *Mungbean yellow mosaic India virus* (MYMIV) plus-strand origin of replication. The DNA sequence corresponding to MYMIV DNA A nucleotide 2672 to 24 is shown. The hairpin structure, conserved nonanucleotide loop sequence, and the nicking sites are marked. The high affinity putative Rep binding sites (iterons) in tandem repeats before the TATA box and the iteron after the stem-loop region are also shown.

Table 6. Gene order in *Begomovirus* DNA A and DNA B components, with their putative protein products and predicted function

ORF	Predicted molecular weight (kDa)	Putative protein	Predicted function
AV1	30.0	Coat Protein (CP)	Encapssidation
AV2	13.0	Pre- coat protein	Cell to cell movement protein
AC1	40.0	Replication initiation protein (Rep)	Replication intiation
AC2	17.0	Transcription Activator Protein (TrAP)	Transcription activation of right ward ORF, supressor of PTGS
AC3	15.0	Replication enhancer protein (REn)	Replication enhancment
BV1	29.0	Nuclear shuttle protein (NSP)	Nuclear trafficking
BC1	32.0	Movement protein (MP)	Cell to cell movement, pathogenicity determinant

2.7.1 Intergenic /common region

Between the start codon of the leftward and rightward coding regions, there is present a non-coding intergenic region (IR). Within the intergenic region is a short stretch of 118-200 nucleotide length segment which is near identical in both DNA A and DNA B components of bipartite begomoviruses and so is called the common region or CR. The nucleotide sequence of CR/IR is highly specific for a given begomovirus. It contains the following elements.

1. A stem and loop structure (Fig. 5). This is the origin element that is common to all geminivirus genome, and also called as hairpin motif

(Arguello Astorga *et al.*, 1994). The hairpin contains a GC rich stem and an AT rich loop. The loop contains a nonanucleotide sequence 5'-TAATATTAC-3' that is conserved among all geminivirus genome (Rogers *et al.*, 1986; Revington *et al.*, 1989; Lazarowitz *et al.*, 1992; Orozco and Hanley-Bowdoin, 1998).

2. The second important feature in the IR/CR is the binding site for Rep. Rep is a sequence specific DNA binding protein that recognizes a 13 bp repeat (iterated, iteron) motif. The repeat element contains two, 5-bp direct repeats (GGTAG) separated by a central core of 3 bp (TAA), GGTAAGGTAG (Bisaro, 1996). These iterons are considered as Rep binding sites. The number of repeats and the way they are arranged in the CR is specific for specific lineages of virus.
3. The segment from the tandem repeat of iteron to end of the stem loop is considered to represent the origin of replications or *ori*.
4. The promoters of both leftward and rightward genes, TATA boxes and sequences recognized by various transcription factors are also present in the IR/CR (Hanley-Bowdoin *et al.*, 1999).

2.7.2 ORF AV1 (Coat Protein)

The geminate particle morphology, that gives the name to the family '*Geminiviridae*', is the typical feature of members of this family. The coat protein is a fascinating multifunctional protein that encapsidates one molecule of single stranded circular DNA. It is required for encapsidation and vector transmission. The viral genome is also protected from nucleolytic digestion, both inside the plant and the vector.

In all the four genera of the family *Geminiviridae* there is only one type of coat protein that forms the twinned structure. The geminate structure of *Maize streak virus* and *African cassava mosaic virus* have been resolved upto 25Å° by cryo-electron microscopy and three dimensional image reconstitution (Zhang *et al.*, 2001; Bottcher *et al.*, 2004). The structure revealed that CP subunits are arranged with quasi-icosohedral symmetry with 110 copies of CP fitted in pentamer capsomers. The tertiary structure was modelled as an eight stranded β barrel motif with N-terminal α helix. It was suggested that the N-terminal helix was involved in maintaining particles structure, encapsidating genomic DNA.

The coat protein plays a vital role in regulation of replication. The disease symptoms are attenuated and the onset of disease is delayed when plants are infected with mutants (Sanderfoot and Lazarowitz, 1996; Unseld *et al.*, 2004). The coat protein of begomoviruses binds to ssDNA preferentially and co-operatively (Palanichelvam *et al.*, 1998; Kirthi and Savithri, 2003; Malik *et al.*, 2005). A putative zinc finger motif corresponding to amino acid residues 65-85 (in monopartite begomoviruses) was considered to play a role in DNA binding (Kirthi and Savithri, 2003; Unseld *et al.*, 2004).

Participation of CP in viral DNA replication was postulated quite early as absence or inactivation of CP resulted in reduced levels of ss DNA without decrease in dsDNA (Briddon *et al.*, 1989; Brough *et al.*, 1988; Padidam *et al.*, 1999). Mutation in the nuclear localization signal (NLS) of Squash leaf curl virus (SLCV) CP reduced accumulation of ssDNA (Qin *et al.* 1998) CP regulation of replication was studied in MYMIV by Malik *et al.* (2005), who suggested that there is a strong interaction between Rep and CP and the nicking and closing activity of Rep is downregulated by CP.

The role played by CP in intracellular, cell to cell and long distance movement, depends on the species and genera of the family. In the case of *Mastrevirus*, CP plays a role in nuclear import and export of viral DNA and systemic movement (Liu *et al.*, 1997; Liu *et al.*, 1999; Liu *et al.*, 2001). In the case of Curtovirus, CP and the ORF V3 bring about systemic infection (Soto, 2001). The most interesting feature is seen among begomoviruses. Among bipartite begomoviruses there is difference over CP requirement. CP-independent movement occurs in a well adapted species and genotypes wherein the non-virion forms are transported across the cells. However, in non permissive species, CP is required (Wang *et al.*, 1999).

In the monopartite begomoviruses, TYLCV, CP plays a major role in movement. Detailed investigation carried out showed that, the nuclear localizing signals (3-20 amino acids and 36-61 amino acid residues) facilitate its nuclear import (Kunik *et al.*, 1998). TYLCV CP was also shown to interact with karyopherin (Kunik *et al.*, 1999). Microinjection and transient expression experiments have revealed that, TYLCV CP also functions in the shuttling of DNA between nucleoplasm and cytoplasms, and acts as homologue of ORF BV1 of bipartite begomoviruses (Rojas *et al.*, 2001). The proteins, V2 and C4 may function as BC1 homologue in mediating the delivery of viral DNA to the cell periphery and plasmodesmata involving ER (Rojas *et al.*, 2001; Rojas *et al.*, 2005).

Begomoviruses are transmitted in a circulative and non-propagative manner. The CP of begomoviruses is essential for transmission and determines insect specificity. Exchange of ACMV CP with that of BCTV altered the specificity of transmission from whiteflies to leafhopper (Briddon *et al.*, 1990). Mutations in the CP of BGMV resulted in the loss of acquisition and

transmission by *B. tabaci* (Azzam *et al.*, 1994). Some of the viruses that infect ornamental plants become non-transmissible due to continuous vegetative propagation like *Abutilon mosaic virus* or *Honeysuckle yellow vein virus* (Bedford *et al.*, 1994; Wu *et al.*, 1996; Hofer *et al.*, 1997). Replacement of the CP of non transmissible AbMV with that of transmissible *Sida golden mosaic virus* CP produced a transmissible chimeric AbMV. Alteration of one amino acid (substitution of an asparagine at 131 position by aspartic acid in *Watermelon chlorotic stunt virus* (WmCSV) restored whitefly transmission (Kheyr-Pour *et al.*, 2000). Alteration of two amino acids in the CP of two TYLCSV isolates reduced transmissibility (Noris *et al.*, 1998). Hohnle *et al.* (2001) showed that the domain from amino acids 123 to 149 in CP as a minimal transmission domain, with the contribution of amino acids 149 to 174 for efficient transmission. Taking together results of TYLCSV, WmCSV and AbMV, it is inferred that no specific domain governs the whitefly transmission. The GroEL homology produced by endosymbionts in *B. tabaci* has been suggested to play an important role in the transmission process of TYLCV-[IL] (Morin *et al.*, 2000). However, GroEL homologue was shown to bind to non-transmissible AbMV as efficiently as TYLCV (Morin *et al.*, 2000).

2.7.3 ORF AV2 (Pre-coat protein)

The ORF AV2, overlapping the ORF AV1 in the viral sense strand of DNA A occurs only in the Old World bipartite begomoviruses and monopartite begomoviruses. The role of ORF AV2 in bipartite begomoviruses was examined only in the case of *Tomato leaf curl New Delhi virus* (Padidam *et al.*, 1996). Abolishing ORF AV2 expression and expression of truncated protein resulted in attenuation of symptoms in *N. benthamiana*

(Padidam *et al.*, 1996). The ORF AV2 mutants showed low level of viral DNA replication in protoplasts (Padidam *et al.*, 1996). AV2 was also found to be present as monomer, dimer and multimer in Western blots. Mutation in AV2 also did not affect CP production in infected plants (Padidam *et al.*, 1996). However, Etessami *et al.* (1989) reported that the absence of ORF AV2 did not affect pathogenicity in ACMV. In a monopartite begomovirus, ToLCV-[AU], inactivation of ORF AV2 led to symptomless systemic infection with reduced titre of all DNA forms (Rigden *et al.*, 1993). It was shown to function as pathogenicity determinant by regulation of ss and dsDNA levels (Wartig *et al.*, 1997).

The pre-coat protein ORF of *Tomato leaf curl virus* (ToLCV-[AU]) when expressed transiently or microinjected, showed peri-nuclear distribution, co-localization with the endoplasmic reticulum. Microinjection experiment showed that it enhanced the CP mediated nuclear export of viral DNA and exhibited limited capacity for cell to cell trafficking through mesophyll plasmodesmata (Rojas *et al.*, 2001).

The analogous proteins in curtovirus, BCTV (V2), and V1 in MSV have interesting role. As such the sequence identity between the ORFs of begomoviruses, curtovirus and mastrevirus are negligible. However, functionally, they seem be similar to monopartite begomoviruses. In BCTV, V2 modulate levels of ss and dsDNA and mutants express phenotypes with highly attenuated symptoms (Rojas *et al.*, 2005). However, it did not affect the cell to cell movement of the virus (Hormuzdi and Bisaro, 1993; Stanley *et al.*, 1992). In the case of MSV, V1 appears not to bind viral DNA; it was proposed that V1 diverts a CP-DNA complex from the nucleus to the cell

periphery, where it mediates movement through plasmodesmata (Liu *et al.*, 2001).

2.7.4 ORF AC1 : Replication initiation protein-Rep

Replication initiation protein is the only viral protein absolutely essential for replication (Elmer *et al.*, 1988). Rep and REN are predicted to bind simultaneously to the stem-loop structure and to upstream Rep-DNA complex (Hanley-Bowdoin *et al.*, 1996).

Rep protein, of molecular weight 40.4 kDa is a multifunctional protein and is conserved in sequence, position, and functional domains. Recombinant Rep protein of Wheat dwarf virus, WDV (Heyerand-Nitschhe *et al.*, 1995), Tomato golden mosaic virus, TGMV (Hanley-Bowdoin *et al.*, 1999), Tomato yellow leaf curl Sardinia virus, TYLSV (Laufs *et al.*, 1995) and Mungbean yellow mosaic India virus, (MYMIV) (Pant *et al.*, 2001; Malik *et al.*, 2005) have been expressed in *E. coli* system. Various functional domains of the protein have been dissected and their ability to function *in vitro* and *in planta* have been ascertained.

Rep specifically recognizes the viral origin (Fontes *et al.*, 1994), binds to specific sequences (iterons) found in the CR and cleaves the phosphodiester bond between the seventh and eighth residue of the conserved nonamer 5'-TAATATT[↓]AC-3' (arrow shows the site of cleavage). Rep binds covalently to 5' phosphate end and 3' end, remains available for rolling circle replication. After one cycle of replication, Rep cleaves once again at the newly generated origin sequence. Then rep ligates the nascent 3' end of DNA with the previously generated 5' end releasing an unit-genome length of circular ss DNA molecule (Bisaro, 1996). Thus Rep acts as an endonuclease and ligase

as it initiates and terminates rolling circle replication (Laufs *et al.*, 1995; Orozco and Hanley-Bowdoin, 1998). The Rep of all the geminiviruses have a NTP binding domain and Rep of TGMV, TYLCV are known to hydrolyze ATP (Orozco *et al.*, 1997; Desbiez *et al.*, 1995). Gorbalenya and Koonin (1993) proposed that Rep might function as a DNA helicase during replication. Pant *et al.* (2001) had shown ATP dependent topoisomerase activity of Rep for blackgram isolates of MYMIV. Rep also acts as a transcriptional regulator to repress its own synthesis (Eagle *et al.*, 1994). Rep has been shown to oligomerize with itself as well as with REn (Settlage *et al.*, 1996).

The domains of binding, cleaving, ligation and oligomerization in Rep protein have been resolved. DNA binding, cleaving and ligation domain of Rep is at N' terminus, oligomerization domain is located almost in the middle and ATP hydrolyzing activity at C' terminus (Orozco *et al.*, 1997). The Rep binds to ss region of TGMV DNA in a sequence specific manner (Fontes *et al.*, 1994) and the binding site is located upstream of the hairpin, between the TATA box and transcription start site of the ORF AC1. The cleavage/ligation activity domain lies between 1-120 amino acid. In TGMV Rep, this region contains the conserved motif I, II and III that are associated with the initiator proteins of other rolling circle replication systems (Ilyina and Koonin, 1992). Mutations in motifs I (FLTY18) and II (HLH60) of TGMV blocked cleavage and replication. The precise way in which they function is not known. Motif III (YXXK₁₀₇) corresponds to the active site for DNA cleavage. The conserved Y₁₀₄ residue is involved in nucleophilic attack of the phosphodiester DNA backbone and is covalently linked to 5' end of cleaved DNA. Amino acids 1 to 130 are sufficient for DNA binding, amino acids 134 to 181 confer oligomerization activity and amino acids 101 to 181 are necessary for ORF

REn interaction (Settlage *et al.*, 1996). The ATPase domain of Rep was identified between amino acid residues 182 to 352 (Orozco *et al.*, 1997); this region contains a conserved motif GXXXXGKT₂₃₀ that corresponds to the P loop of the phosphate binding site of the protein that hydrolyze NTP. While ATPase activity of MYMIV Rep protein (Malik *et al.*, 2005) was shown to be upregulated by ssDNA, it is DNA independent in TYLCV (Desbiez *et al.*, 1995).

Interactions of Rep with host proteins have been studied in recent years. Geminiviruses are totally dependent on DNA replication machinery of the host that makes it an ideal tool to search for cell cycle regulatory proteins. While majority of geminiviruses are confined to the vascular tissue, viruses like TGMV, *Cabbage leaf curl virus* (CbLCV) are found in well differentiated cells throughout the leaf, stem and root (Nagar *et al.*, 1995). These tissues cannot support DNA replication and need to be reprogrammed to produce the nuclear replication enzymes early during infection (Hanley-Bowdoin *et al.*, 1999). Rep protein was found co-localised with DNA polymerase δ accessory factor proliferating cell nuclear antigen (PCNA) (Hanley-Bowdoin *et al.*, 1999; Nagar *et al.*, 1995). Binding of MYMIV Rep with PCNA was shown using yeast two-hybrid system (Malik *et al.*, 2005). Several other host factors like protein kinase, a kinesin and histone H3 have been shown to interact with Rep in yeast two-hybrid system (Kong and Hanley-Bowdoin, 2002). The begomovirus Rep and mastrevirus Rep A proteins interacted with plant homologues of the retinoblastoma protein, a family of proteins involved in the negative regulation of the cell cycle (Gutierrez, 1999).

2.7.5 ORF AC2 : Transcription activator protein-TrAP

The transcription activator protein (TrAP) induces sense promoters in DNA A as well as in DNA B, at the transcription level (Sunter and Bisaro, 1991; Sunter and Bisaro, 1999). Sunter *et al.* (1994) showed that TrAP transactivation was non specific but transactivation by TrAP homologues in curtoviruses C2 protein was not achieved. TrAP is a zinc binding phosphoprotein. They bind to ssDNA with the affinity in a sequence non specific manner (Mories *et al.*, 1996, Sunter *et al.*, 1992). Three conserved domains have been recognized in this protein: a basic domain with a nuclear localization signal (NLS) at the N terminus, a central DNA-binding domain with a non classical Zn-finger motif and an acidic activator domain at the C terminus (Hartiz *et al.*, 1999). TrAP was shown to activate CP expression by two distinct mechanisms, i.e., by activation in mesophyll cells and derepression in phloem cells (Sunter and Bisaro, 1997; Sunter and Bisaro, 2003). The protein was found in both the nuclear and cytoplasmic compartments when expressed in insect and plant cell (van Wezel *et al.*, 2001). Shivaprasad *et al.* (2005) identified the AC2 responsive region in both rightward and leftward promoters in DNA B, which does not overlap the common region that is nearly identical in the two DNA components.

2.7.6 ORF AC3 : Replication enhancer protein-REn

The ORF AC3 encodes the replication enhancer (REn) protein (~15 kDa) that is targetted to the nucleus (Selth *et al.*, 2005) and is involved in upregulation of replication by interacting with Rep and possibly through a role in the recognition of the origin of replication. Replication enhancer protein (REn) regulates viral DNA accumulation. As mutations in REn produced delayed onset and attenuation of symptoms (Sunter *et al.*, 1990). The REn

protein enhances viral infection and symptom development, an effect that is due to its role in induction of viral DNA into replication mode (Elmer *et al.*, 1988; Sunter *et al.*, 1990). The AC3 mutant accumulates reduced amount of viral DNA in plant and protoplasts (Sunter *et al.*, 1990; Etessami *et al.*, 1991; Stanley *et al.*, 1992). REn binds to pRBR and PCNA, suggesting its role in replication and cell cycle modification (Settlage *et al.*, 2001). It also interacts with SINAC I, a family of transcription factors with NAC domain (Selth *et al.*, 2004).

2.7.7 ORF AC4 and AC5

The direct or indirect effect of the ORF AC4 in viral replication is not fully understood. The AC4 protein does not have apparent effect on viral DNA accumulation. In BCTV, due to the effect of C4 protein, the infected leaves showed downward leaf curling, vein swelling and enation (Latham *et al.*, 1997). C4 protein in BCTV has also a role in initiation of cell division (Hull, 2002). Recent findings, have implicated the C4 gene product, to have a role in suppression of silencing (van Wezel *et al.*, 2002). The ORF possibly has a role in replication of begomovirus as identified using a yeast model system that supports MYMIV DNA replication (Raghavan *et al.*, 2004).

2.7.8 The DNA B in bipartite begomoviruses

Begomoviruses multiply in nuclei via double stranded DNA in minichromosomes. Due to their replication in nuclei, geminiviruses need to be exported from nucleus into cytoplasm and transported from one cell to another.

For bipartite begomoviruses, cell to cell and systemic movement depends on proteins encoded by DNA B (Brough *et al.*, 1988; Etessami *et*

al., 1988). In bipartite begomoviruses, DNA B encodes for two proteins, ORF BV1 and ORF BC1 that function in cell to cell movement in infected plants. The BV1 protein is also termed as the nuclear shuttle protein (NSP), as it facilitates the movement of the viral genome from the nucleus to the cytoplasm, whereas the BC1 protein functions as cell to cell, viral movement protein (MP), as it promotes an increase in the size exclusion limit of plasmodesmata (Sanderfoot and Lazarowitz, 1996). BV1 localization to nuclei in infected plant as well as protoplast was shown by immunogold labeling (Sanderfoot and Lazarowitz, 1995; Pascal *et al.*, 1994).

In *Bean dwarf mosaic virus* (BDMV) the BV1 protein exports viral DNA from the nucleus to cytoplasm, and BC1 increases the size exclusion limit of plasmodesmata and mediates the cell to cell movement of viral DNA. Also, in BDMV, both BC1 and BV1 proteins, bind to ssDNA and dsDNA in a form and size specific manner (Rojas *et al.*, 1998).

Coinoculation of ACMV DNA A and DNA B with DNA B mutants containing frame shift mutation, in either BV1 or BC1 allowed local DNA replication, but did not produce systemic infection. Moreover, DNA accumulation was reduced in leaves infected with BV1 mutant, but was normal with BC1 mutants (von Arnim *et al.*, 1993).

The BC1 protein is also a symptom inducing element or pathogenicity determinant in bipartite begomoviruses and mutation studies of BC1 revealed that the 3' region of BC1 gene is associated with symptom development (Duan *et al.*, 1997; Pascal *et al.*, 1994). BC1 proteins of several begomoviruses accumulate in the cell periphery (Lazarowitz and Beachy, 1999; Pascal *et al.*, 1994) to facilitate transport of viral DNA. Zhang *et al.* (2001) showed that a short stretch of BC1 amino acids (pilot domain) is responsible for the

dichotomy of intracellular location whereas another domain (anchor domain) is necessary for cortical and nucleus localization.

2.8 Diagnosis

Detection and diagnosis of begomoviruses and their associated satellite components are essential for managing the devastating diseases caused by them. For begomoviruses, both serology based tests (Sequeira and Harrison, 1982; Cohen *et al.*, 1983; Roberts *et al.*, 1984; Thomas *et al.*, 1986) and nucleic- acid based techniques are used for detection purposes (Harrison and Robinson, 1999). For diagnosis or specific identification of viruses, nucleic acid based techniques like hybridization with radioactive probe to DNA B nucleotide sequence or amplification and cloning of viral DNA followed by sequencing are more reliable.

For any given begomovirus, the epitope profile [patterns of reactivity with a panel of Monoclonal antibodies (MAbs) is specific and hence can be distinguished from other begomoviruses (Harrison and Robinson, 1999). Production of MAbs against ACMV (Thomas *et al.*, 1986) and ICMV (Aiton and Harrison, 1989) revolutionized research on begomoviruses by giving a clue on the identity of the virus and its relationship with other viruses, when cloning and sequencing of these viruses was still at a nascent stage. With the advent of the PCR technique for amplifying specific regions in the viral genome, degenerate primers as well as specific primers were designed for detecting the presence of begomoviruses in infected samples and whiteflies (Deng *et al.*, 1994; Wyatt and Brown, 1996; Brown *et al.*, 2001). Following cloning of begomoviruses, conserved regions were used as radioactive probes in hybridization techniques (Roberts *et al.*, 1984).

Literature regarding diagnosis and detection of YMV isolates are summarized below. Seven WTGs -BYVMV, CYVMV, DoYMV, HgYMV, ICMV, MYVMV and ToLCV were detected by ISEM and DAS-ELISA with polyclonal Abs to ACMV and spot hybridization with probes to ACMV DNA. A. These WTGs were differentiated by their pattern of reactivity in TAS-ELISA with MAbs to ACMV (Harrison *et al.*, 1991). This provided conclusive evidence that MYVMV, CYCMV, DYMV, ToLCV and MYVMV are WTGs at a time when they were not characterized.

Different isolates of YMV were indistinguishable in Enzyme-linked immunosorbent assay (ELISA) with polyclonal antibodies to horesegram isolate of YMV or to Indian cassava mosaic virus, ICMV (Muniyappa *et al.*, 1987; Varma *et al.*, 1989). YMV from Thailand was shown to be serologically related to other WTGs like TGMV, TbLCV and ACMV (Ikegami and Shimizu, 1988). In agar gel double diffusion tests with polyclonal antibodies to YMV isolate of mungbean from Thailand, it was found to be related to Bean golden mosaic virus (BGMV), Tobacco leaf curl virus (TbLCV) from Japan and Tomato yellow leaf curl virus from Thailand (TYLCTHV) (Varma *et al.*, 1992). YMV isolates of French bean from Thailand reacted strongly to polyclonal antibodies to African cassava mosaic virus (ACMV) by double antibody sandwich ELISA (DAS-ELISA) method, whereas YMV isolate of French bean and dolichos from India gave equivocal or no reaction (Harrison *et al.*, 1991). Differences were observed between YMV isolates to panels of monoclonal antibodies to ACMV and ICMV (Varma *et al.*, 1992).

Swanson *et al.* (1992) attempted to differentiate the YMV isolates using panels of MAbs to ACMV and ICMV. Based on epitope profile, they identified two major groups, group one comprised of *Dolichos yellow mosaic virus*

(DoYMV) and group two comprised isolates of *Horsegram yellow mosaic virus* (HgYMV) and isolates from blackgram, cowpea, French bean, pigeonpea, soybean and *Indigofera hirsuta*. The status of YMV isolate of Lima bean was considered uncertain and isolates within each group differed slightly in epitope profile, depending on the source species or geographical origin. In TAS-ELISA with MAbs raised against ACMV and ICMV. Hameed and Robinson (2004) found that the viruses from leguminous hosts like mungbean, cowpea and mothbean were distinct from other cucurbit begomoviruses.

Among different isolates of MYMIV infecting different leguminous hosts, differences were not discernible by NASH using probes to full length DNA A and DNA B components of blackgram isolate of MYMIV (Biswas and Varma, 2000). In NASH tests using DNA A probe to MYMIV causing cowpea golden mosaic disease, Roy and Malathi (2004) detected different WTGs infecting various hosts, other than MYMIV. When probe to DNA B was used, specific detection of MYMIV was made.

Because of the difficulty in purifying begomovirus virion particles, new techniques using nucleic acid based approaches like PCR and radioactive hybridization are being widely preferred for their diagnosis in the place of serological techniques.

2.8.1 Infectivity of cloned components

Majority of the members of '*Geminiviridae*' are not sap transmissible to the host and it is very difficult to study the viral functions using recombinant clones. In this context, two methods of delivery of viral DNA are followed to study the pathogenesis. They are (i) *Agrobacterium tumefaciens* mediated transfer of the viral DNA (ii) biolistic delivery.

Ti plasmid of *Agrobacterium tumefaciens* delimited by border sequences of 25 bp is transferred to the plant cell where it gets integrated with the plant nuclear DNA. Any DNA between the border sequences is also transferred. This transfer region is exploited to introduce the cloned viral genome. The process of reintroduction of viral genome into plants mediated by the Ti plasmid was termed as 'agroinfection' by Grimsley *et al.* (1986) who used the technique first to deliver *Cauliflower mosaic virus* into *Brassica napus*. Since then the technique is used in several geminiviruses to study the viral gene functions (BCTV-Stenger *et al.*, 1991; MSV-Grimsley *et al.*, 1986; WDV-Hayes *et al.*, 1988; BGMV-Morinaga *et al.*, 1988; ACMV-Morris *et al.*, 1988; MYMIV- Mandal *et al.*, 1997; Chakraborty, 1996; Malathi *et al.*, 2005; Usha Rani *et al.*, 2004; Biswas and Varma, 2001; MYMV- Jacob *et al.*, 2003; Karthikeyan *et al.*, 2004; Balaji *et al.*, 2004; PYMV Sung and Coutts, 1995; SLCV Lazarowitz and Lazdins, 1991; TGMV Elmer *et al.*, 1988; Stenger *et al.*, 1992; TYLCV, Kheyr-Pour *et al.*, 1991; Rochester *et al.*, 1990; Czosneck *et al.*, 1993).

In agroinoculation, a partial tandem repeat of a virus is constructed in the T-DNA of a binary vector and introduced into *A. tumefaciens*. Upon inoculation of the plants, the viral genome is released in the plant cell. Two mechanisms are proposed to describe the phenomenon of viral DNA release. In the constructs having two origins of replication, unit genome length is released during replication (Rigden *et al.*, 1996; Stenger *et al.*, 1991). Alternatively unit genome length released due to homologous recombination (Rigden *et al.*, 1996).

Agroinoculation of whole tandem repeat constructs of DNA A and DNA B components of MYMIV was shown by Mandal *et al.* (1997). Biswas

et al. (2001) obtained contrasting results with some genotypes of pigeonpea and soybean, while some genotypes were susceptible to whitefly transmission, with agroinoculation, no symptoms were seen. Agroinoculation of soybean was not efficient (Usha Rani *et al.*, 2004) compared to whitefly inoculation. Balaji *et al.* (2004) performed agroinoculation with one DNA A component of MYMV-[Vig] with multiple DNA B components and differentiated between DNA B, KA22 causing more intense symptoms and high virus titre in blackgram and KA 27 that caused more intense symptoms and high virus titre in mungbean. Malathi *et al.* (2005) showed that the virus causing golden mosaic disease in cowpea is only a variant of MYMIV, i.e., MYMIV-[Cp]. They also showed the adaptation of progeny virus produced after agroinoculation. Surendranath *et al.* (2005) exchanged genomic components of MYMIV and MYMIV-[Cp] and showed that compatible interaction occurred between these two isolates in all the leguminous species except cowpea. Characteristic yellow mosaic symptoms were observed in soybean only after 28 days when inoculated with MYMIV-Sb (MP) (Girish and Usha, 2005). Introduction of repeat construct of A and B components in the same strain of *A. tumefaciens* was found more efficient than delivering it in separate cells (Jacob *et al.*, 2003).

In recent years, alternative methods are also used like biolistic delivery. In this case, there is no need for making repeat construct and the viral DNA either released or unreleased from the cloned vector is delivered into small seedling under electrical pulse. This has been widely used to establish Koch's postulates in BGMV (Gilbertson *et al.*, 1991), PHV (Garzon-Tiznado *et al.*, 1993), Cotton leaf curl virus (Bridson *et al.*, 2000; Mansoor *et al.*, 2003), ToLCGV (Chakraborty *et al.*, 2003), ACMV, ICMV, EACMV, and SACMV

(Chellapan *et al.*, 2004; Vanitharani *et al.*, 2004). Using biolistic delivery or *Agrobacterium* delivery, viral gene function questions have been addressed by various researchers.

2.9 Phylogenic relationship

For begomoviruses, DNA A and their conserved protein products CP and Rep are used for phylogenetic analysis. DNA B sequences are more diverse than DNA A and there are fewer conserved elements in DNA B and hence, in general, are not used for phylogenetic analysis (Harrison and Robinson, 1999). When CP and Rep proteins of different begomoviruses were compared, based on geographical origins, they were divided into the Old World (Asian, African, Mediterranean and Australian) and the New World (American) viruses (Howarth and Vandemark, 1989). This kind of geography-related lineage was confirmed by further analysis including more begomoviruses (Hong and Harrison, 1995; Rybicki, 1994; Padidam *et al.*, 1995; Brown, 2000). Within the Old World branch, three sub-clusters were discerned for CP, one with viruses from African and Mediterranean region, second with Asian viruses and third having viruses from China and Australia (Harrison and Robinson, 1999). The Old World and the New World division of begomoviruses was further established when complete nucleotide sequence of DNA A of 120 geminivirus species were analysed by Fauquet and Stanley (2003). Currently, seven different virus clusters were discerned for begomoviruses. They are, New World viruses comprising meso and Latin American viruses and viruses infecting sweet potato (**Sweepoviruses**); Old World viruses comprising of African, Indian, Asian viruses and viruses infecting legumes in India and Africa (**Legumoviruses**) (Fauquet and Stanley, 2003). The phylogeny of geminiviruses is extremely stable, even when the

viruses used for comparison were increased from 36 in 1989 to 277 in 2002. This aspect of stability in the phylogeny of geminiviruses if understood would help in elucidating their evolution. The significant feature of phylogenetic relationship of YMV isolates is discussed in this review.

The distinct features in the phylogeny of yellow mosaic viruses were discussed by Usha Rani *et al.* (2004) and Fauquet and Stanely (2003). The lineage of YMV is distinct when compared with other begomoviruses. The YMV isolates of legumes and CpGMV from Africa are considered as a separate group, *Legumovirus*. YMV might have diverged earlier from a common ancestor and might have gained their unique features while adapting to the legume hosts during the course of their evolution. Within legumoviruses, the identity of MYMIV and MYMV as separate viruses was also shown in the phylogenetic analysis of Karthikeyan *et al.* (2004) and Girish and Usha (2005).

A notable feature of MYMV is the multiple DNA B components (Karthikeyan *et al.*, 2004; Girish and Usha, 2005). When compared with DNA B of Vig KA27 and MYMV, rest of the DNA B components of YMV isolates have undergone a deletion in the common region (Usha Rani *et al.*, 2004). Apart from differences in the CR, these two isolates are considerably different in the rest of the genome also. These differences are supported by the DNA B phylogenetic tree, where Vig KA27 and MYMV isolates form a separate group. Surprisingly, rest of the DNA B of MYMV isolates (KA21, KA22, KA28, KA34) group with MYMIV suggesting acquisition of a MYMIV DNA B component by MYMV DNA A component resulting in emergence of a variant of MYMV-Vig (Karthikeyan *et al.*, 2004; Girish and Usha, 2005).

Girish and Usha (2005) analysed recombination events in YMV isolates

and detected recombination in the intergenic region and Rep gene in MYMIV. No significant recombination was seen among MYMV isolates.

Perusal of the virus species infecting legumes (Fauquet and Stanely, 2005) show that there is one tentative species of Chickpea chlorotic dwarf virus isolates infecting chickpea and only one established species of *Bean yellow dwarf virus* belonging to the genus *Mastrevirus*. Among begomoviruses, there are six New World virus species, Bean calico mosaic virus, *Bean dwarf mosaic virus*, *Bean golden mosaic virus*, *Bean golden yellow mosaic virus*, *Cowpea golden mosaic virus* and *Rhynchosia mosaic virus*. Among Old World begomoviruses, *Cowpea golden mosaic virus* occurs in Africa, *Soybean crinkle leaf virus* in South East Asia (Thailand) and rest of the species occur in India, Bangladesh, Nepal and Pakistan.

2.9.1 Nomenclature and Taxonomy

Currently, sequence information is available for more than 300 begomovirus isolates. Based on extensive analysis of pair-wise identity and recombination levels between all sequences, a value of 89% identity was established as the threshold between the two taxonomic levels (Stanley *et al.*, 2003). Based on identity in DNA A complete nucleotide sequence, virus isolates having more than 89% identity, were clustered as one strain. The isolates sharing less than 89%, were demarcated as separate species (Stanley *et al.*, 2003; Fauquet and Stanley, 2005). Based on the criterion, 102 distinct begomovirus species were recognized by ICTV (VIIth report, Stanley *et al.*, 2003). Guidelines for improving the descriptors have been proposed recently (Fauquet and Stanley *et al.*, 2005).

MATERIALS AND METHODS

1. Sources of chemical reagents, radioactivity, primers

A comprehensive details of protocols and techniques adopted, along with the materials utilized are given below. Chemicals and reagents used in different experiments were of analytical grade. Information on the reagents and buffers used in molecular studies are given in Appendix I. Glasswares used were of Borosil grade and baked at 180°C for 2 h. Unless specifically stated media, buffers, distilled water and solutions were autoclaved at 120°C for 15 min. Good laboratory practices and the biosafety conditions were strictly followed. For molecular biological work, essentially, the protocols outlined in Sambrook and Russell (2001) were followed. Agroinoculation experiments were done in growth chambers at National Phytotron Facility. Instruction issued by the institute Biosafety Committee were followed in handling the engineered microorganisms and the plants inoculated with different constructs.

2. Sources of plant material

Seeds of different legume cultivars used in this study were collected from sources listed below (Table 7).

3. Yellow mosaic virus isolates used in this study

The clones of DNA A and DNA B components of blackgram and cowpea isolates of Mungbean yellow mosaic India virus i.e., MYMIV and MYMIV-[Cp] (Fauquet *et al.*, 2003) were collected from Plant Virology Unit, Division of Plant Pathology, IARI and used for functional studies on open reading frame ORF AV2 of the virus. Details on the names of the clones, names approved by the study group on the taxonomy of geminiviruses,

Table 7. Source of host plant seeds used in biological studies

Common name	Scientific name	Variety	Source of seeds
Blackgram	<i>Vigna mungo</i>	TN-1	Tamil Nadu Agricultural University
		Co-1	Madurai Kamraj University
		Local variety	Commercial outlet
Cowpea	<i>Vigna unguiculata</i>	Pusa Komal	National seeds corporation
		Sahib lobia	Commercial outlet
		Lal lobia	Commercial outlet
Mungbean	<i>Vigna radiata</i>	Pusa Baisakhi	Division of Genetics, IARI
		Ps-16	Division of Genetics, IARI
		Local variety	Commercial outlet
French bean	<i>Phaseolus vulgaris</i>	Selection 9	National Seeds Corporation
		Pusa Parvathi	National Seeds Corporation
		Chithra	National Seeds Corporation
Tobacco	<i>Nicotiana tabaccum</i>	<i>Xanthi</i>	National Seeds Corporation
Egg plant	<i>Solanum</i> sp.	—	National Seeds Corporation
Cotton	<i>Gossipium hirsutum</i>	—	National Seeds Corporation

accession numbers, acronyms of the clones are given in Table. 8. In this study these are referred to as blackgram and cowpea isolates (Bg and Cp isolates). The names proposed by Fauquet and Stanley (2005), is not followed in the present document as they are not yet approved by ICTV.

The purity and biological integrity of the virus clones were confirmed (vide results 4:1:1) and were sub-cultured every fortnight during the course of investigation.

Table 8. Yellow mosaic virus isolates used in the present study

Virus	Genome components	Acronym	Accession number	Source
<i>Mungbean yellow mosaic India Virus</i>	DNA A	MYMIV	AF126406	Blackgram
<i>Mungbean yellow mosaic India Virus</i>	DNA B	MYMIV	AF142440	Blackgram
<i>Mungbean yellow mosaic India Virus</i> (Cowpea)	DNA A	MYMIV-[Cp]	AF481865	Cowpea
<i>Mungbean yellow mosaic India Virus</i> (Cowpea)	DNA B	MYMIV-[Cp]	AF503580	Cowpea

4. Maintenance of bacterial culture containing constructs

Wild type and mutated clones in the vectors pUC18, pBin19 and pGEMT-Easy, were maintained as plasmid DNA in 50 µl aliquots at -20°C. The bacterial culture, *Escherichia coli* DH5α strain, *E. coli* Rosetta strain and *Agrobacterium tumefaciens* strain EHA 105 were preserved as glycerol stock at -70°C. The cultures which were routinely used were kept at 4°C. For maintenance of *E. coli* and *A. tumefaciens* culture, sub-culturing was done at 20 days interval on Luria Bertani (LB) agar containing appropriate antibiotic.

5. Maintenance of whitefly culture

Non-viruliferous whitefly (*Bemisia tabaci*) cultures were raised from single egg of whitefly and maintained on tobacco (*Nicotiana tabaccum* cv. Xanthi), eggplant (*Solanum* sp.) and cotton (*Gossipium* sp.) seedlings in an insect proof cage. After every month, the colonies were transferred to fresh seedlings of above hosts to maintain the cultures of whiteflies.

6. Miniprep plasmid DNA isolation

Alkaline lysis method (Birnboim and Doly, 1979) was followed to isolate plasmid DNA from *E. coli*. Exposure of bacterial suspension to the strongly anionic detergent like sodium dodecyl sulphate (SDS), at high pH breaks the cell wall; denature chromosomal DNA and proteins and releases plasmid DNA into the supernatant (Sambrook and Russell, 2001).

The isolation of recombinant plasmid DNA was carried out as given below:

- A single colony of transformants was inoculated in 2 ml culture tube containing LB medium having appropriate antibiotics.
- The tubes were incubated at 37°C for 16 h at 200 rpm in a shaker incubator.
- The bacterial cells were harvested by centrifugation at 5000 rpm for 5 min.
- 100 µl of solution I (25 mM Tris-HCl, pH 8.0; 10 mM EDTA; 9 mg/ml glucose) was added to the pellet and was vortexed well for 30 sec.
- 200 µl of solution II, as lysis reagent (0.2N NaOH; 1% SDS) was added, mixed gently and incubated at room temperature for 5 min for cell lysis.
- 150 µl of solution III (3 M Sodium acetate, pH 4.8) was added and mixed by inversion to precipitate bacterial chromosomal DNA and protein. The mixture was incubated on ice for 20 min.
- The complex bacterial chromosomal DNA and debris were pelletized by centrifuging at 13000 rpm for 20 min at 4°C.

- The supernatant was transferred to another fresh tube and centrifuged again at 13000 rpm for 20 min at 4°C.
- The supernatant was collected and 0.8 volume of isopropanol was added and mixed by inversion to precipitate plasmid DNA.
- The mixture was centrifuged at 13000 rpm for 20 min at 4°C.
- The DNA pellet was washed twice to remove salts with 75% and 70% ethanol respectively.
- The pellet was air dried and dissolved in 25 µl of sterile double distilled water (ddH₂O) and stored at -20°C for further use.

7. Large-scale plasmid DNA isolation

Plasmid DNA was isolated from 50 ml bacterial culture by treatment with alkaline lysis solution. The plasmid DNA was analysed by agarose gel electrophoresis and restriction and stored at -20°C till further use. The following were the steps for preparation of large-scale plasmid DNA.

A single colony of selected clone was inoculated into 50 ml LB medium containing appropriate antibiotic. The culture was grown at 37°C with 200 rpm for 14-16 h.

The grown culture was transferred into two 50 ml oak ridge tubes and were chilled on ice for 10 min.

The cells were pelletized by centrifugation at 5000 rpm for 10 min at 4°C.

The pellet was suspended by vortexing for 2 min in 3 ml of solution I, (25 mM Tris-HCl pH 8.0; 10 mM EDTA; 9 mg/ml glucose).

6 ml of solution II (0.2 N NaOH; 1% SDS) was added to above suspension and was mixed gently by inversion and incubated at room temperature for 5 min.

4.5 ml of chilled solution III (3 M Sodium acetate, pH 4.8) was added to lysed cells and mixed by inversion and incubated on ice for 20 min.

The bacterial chromosomal DNA and cells debris were separated by centrifugation at 12,000 rpm, for 30 min at 4°C. The supernatant was removed gently and centrifuged again to remove all debris.

0.8 volumes of cold isopropanol was added to the supernatant, mixed by inversion and incubated at -20°C for >2 hr.

The nucleic acids precipitated were separated by centrifugation at 12000 rpm for 30 min at 4°C.

The DNA pellet was washed with 75 and 70% ethanol respectively, air dried and dissolved in 400 µl of sterile ddH₂O.

The plasmid DNA was further purified using Phenol: Chloroform: Isoamyl alcohol (25:24:1) mixture after RNase treatment. The emulsion was centrifuged at 10,000 rpm for 10 min. at room temperature.

The aqueous phase was taken gently and 1/10 volume of 3 M sodium acetate (pH 4.8) and 2.5 volumes of cold ethanol were added, mixed by inversion and incubated at -20°C for > 5 h.

The plasmid DNA was pelletized by centrifugation at 13,000 rpm for 30 min at 4°C.

The pellet was washed with 70% ethanol twice, air dried and dissolved in 50 µl sterile dd H₂O.

The purity and concentration of plasmid DNA was measured by spectrophotometer and electrophoresis and stored at -20°C for further use.

8. Spectrophotometric quantification of genomic DNA

Quantity of nucleic acids either from plant or plasmid was determined by measuring the absorbance at 260 nm, and the DNA concentration was calculated based on the formula, $1 \text{ OD}_{260} = \text{dsDNA } 50 \text{ } \mu\text{g/ml}$, following are the details.

$$\mu\text{g}/\mu\text{l dsDNA} = \text{OD}_{260} \times 50 \times \text{dilution factor} / 1000$$

9. Restriction analysis of recombinant plasmid DNA

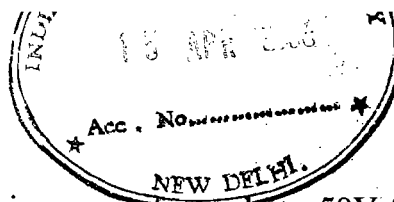
All plasmid DNA that were isolated were subjected to restriction using specific endonuclease. Fragments were separated by agarose electrophoresis to determine the correct recombinant clones. The single restriction reaction mixture was as follows:

Plasmid DNA	5 μl (2 μg)
Restriction enzyme	1 μl (5 units)
10 X reaction buffer	2 μl (1X)
H ₂ O	12 μl
Total volume	20 μl

The restriction reaction mixtures were incubated at 37°C for 5 h. The reaction was stopped by adding 6X loading dye, and electrophoresed on 1% agarose gel.

10. Agarose gel electrophoresis

The plasmid DNA fragments were resolved in 0.8% - 1% agarose gel prepared in 1X Tris acetate EDTA (TAE) buffer, pH 8.8, containing ethidium



bromide (0.5 µg/ml). The electrophoresis was performed at ~50V for 2-3 h using 1X TAE as electrophoresis buffer. In the case of analysis of DNA from infected plants viral DNA forms were resolved in 1.2% agarose gel.

10.1 Gel extraction of DNA fragment

DNA samples were electrophoresed on 1% agarose gel. The desired fragment was scooped from the gel and purified using gel extraction kit as per manufacturer's protocol (Qiagen, MBI, Germany). Three gel volumes of QG buffer was added to one gel weight (W/V), which contained DNA, and matrix was dissolved by heating to 50°C for 10 min. The solution containing DNA was loaded on to Qiaquick spin column and spun for 1 min. The flow through was discarded and the column was washed with buffer PE. The purified DNA was eluted with 50 µl of ddH₂O and checked by electrophoresis and stored at -20°C until further use.

11. Polymerase Chain reaction

Polymerase Chain reaction (PCR) was performed to amplify the full length DNA A and DNA B and selected region in the plasmid or in the viral DNA extracted from plants. Details on primers are given in Table 9. The PCR was performed in a thermocycler (Biometra). A typical reaction mixture had following compositions.

Template plasmid or genomic DNA	100-500 ng
10X Taq DNA polymerase buffer	1X (5 µl)
MgCl ₂	2 mM (4 µl)
dNTP mix	10 mM (1 µl)
Forward primer	100 ng
Reverse primer	100 ng
Taq DNA polymerase	1 unit
ddH ₂ O	make the volume to 50 µl

Table 9. Primers used for amplifying selected regions and full-length DNA A and DNA B components

Primer name	Sequence (5'-3')	Nucleotide co-ordinate
CpV1	5'-TCAAGCTCCCGGTGCATGTTGGCA-3'	203-218
AC-Abut	5'-GTAAAGCTTTACGCATAATG-3'	1125-1107
AV-Abut	5'-AAAGCTTACATCCTCCAC-3'	1117-1134
BV-Abut	5'-CCAGGATCCAATGATGCCT-3'	1347-1329
BC-Abut	5'-ATTGGATCCTGGAGATTCA-3'	1340-1322
MYMIV-AV2 FP	5'-CATGAATTCATGTGGGATCC-3'	156-166
MYMIV-BgAV2 RP	5'-TGAAAGCTTTCAATCTCCTCC-3'	382-369
MYMIV-CpAV2 RP	5'-TAGAAGCTTCTATACAGTCGG-3'	498-487

PCR conditions were: 2 min initial denaturation at 94°C and 30 cycles of, 1min denaturation at 94°C, 2 min annealing at 55°C and 3 min elongation at 72°C followed by a final extension for 10 min at 72°C. Overlapping PCR to generate mutants is described in 12.1.

12. Site directed mutagenesis

In vitro site directed mutagenesis by overlapping PCR (Fig. 6) is an important and easy technique for study of protein function. In this technique the target-DNA is amplified using two overlapping oligonucleotide primers, complementary to each other, containing the desired mutation in the middle of the primers. During thermal cycling, the entire length of both strands of the plasmid DNA are amplified in a linear form and finally amplification, result in the mutated plasmid containing nicks on opposite strands (Fig. 6). The linear dsDNA amplicons are treated with *DpnI*, which specifically cleaves fully methylated and hemimehtyled 5'-Gm6ATC-3' sequences (Nelson and

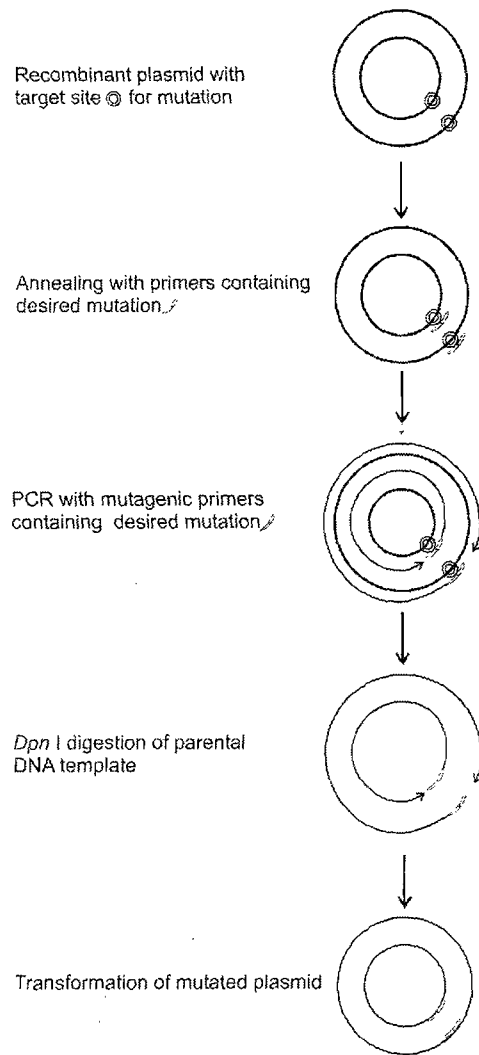


Fig. 6. Diagrammatic illustration of steps involved in site directed mutagenesis by overlapping PCR method.

McClelland, 1992). *DpnI* resistant DNA, which is rich in the desired mutant, is used to transform *E. coli* cells.

12.1 PCR based site directed mutagenesis

Mutagenesis of ORF AV2 in Bg and Cp isolates was carried out using the Quick changeTM site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol. Different primers were designed based on predicted aminoacid sequence of ORF AV2 region of Bg and Cp isolate DNA A. The primers contain the desired substitution, or deletion in the middle of each primer (Tables 10 and 11).

PCR amplification was carried out using the overlapping primers carrying the mutation. The reaction mixture had following composition:

Template double stranded DNA plasmid	100 ng
10X <i>Pfu</i> Turbo DNA polymerase buffer	5 µl (1X)
Forward primer (FP)	125 ng
Reverse primer (RP)	125 ng
dNTP mix	10 mM each
dd H ₂ O	up to 50 µl
<i>Pfu</i> Turbo DNA Polymerase	2.5 unit

The PCR program for site directed mutagenesis was as follows.

(1)	95°C	30 seconds
(2)	95°C	30 seconds
(3)	58°C	1 min
(4)	72°C	5.5 min
(1 min/kb of plasmid length)		

The reaction was performed for 30 cycles from steps 2-4.

The amplified plasmid was given *DpnI* endonuclease treatment as described below.

PCR amplified reaction mix	40 μ l
<i>DpnI</i> enzyme	10 Unit
10X <i>DpnI</i> buffer	5 μ l
dd H ₂ O	up to 50 μ l

The reaction mix was incubated at 37°C for 1 h, which specifically digested methylated or hemimethylated parental non-mutated supercoiled dsDNA. The *DpnI* digested, amplified product was then used for transforming competent DH5 α strain of *E. coli* cells (see below). The transformants were screened and sequenced in an ABI prism automatic sequencer (Delhi University, South Campus).

13. Bacterial transformation

13.1 Preparation of *E. coli* competent cells by CaCl₂ method

10 ml of Luria Bertani (LB) liquid medium was inoculated with a single colony of *E. coli* strain DH5 α overnight at 37°C with shaking at 200 rpm. 500 μ l of this saturated culture was added to 50 ml of fresh LB medium and incubated at 37°C for 90 min (till the OD₆₀₀ reached 0.5). The culture was cooled on ice for 10 min and transferred to a pre-chilled 40 ml centrifuge tube and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was resuspended in 20 ml of chilled 100 mM MgCl₂. The bacterial cells pellet was harvested by centrifugation at 5000 rpm for 10 min at 4°C and resuspended in 20 ml of 100 mM chilled CaCl₂ and incubated on ice for 1 h. The above centrifugation step was repeated and the pellet was dissolved in 1 ml of cold CaCl₂. The competent cells were incubated on ice

for 1 h more. Finally 200 μ l of the competent cells were used for transformation of ligated plasmid DNA.

13.2 Transformation of DH5 α strain of *E. coli* with recombinant DNA

200 μ l of competent cell suspension was added to the 20 μ l of ligation mixture containing recombinant plasmid. Cells were mixed with a pipette tip and the mixture was incubated on ice for 1 h. The cells were subjected to heat shock at 42°C for 2 min and chilled immediately on ice. The cells were allowed to grow at 37°C by adding 1 ml of LB medium and shaking at 200 rpm in a shaker incubator for 1 hour. The cells suspension was centrifuged briefly and 800 μ l of supernatant was removed, finally ~ 200 μ l of resuspended cells were spread on LB agar medium plate containing desired antibiotic (50 μ g/ml), 2% X-gal and 0.2 mM IPTG. The plates were incubated at 37°C for 16 h. From the transformation plate having transformants as well distributed colonies, single colony of more than 25 transformants were picked and inoculated on LB agar medium in a grid format. From this master grid, colonies were analyzed and selected.

14. Screening of recombinant plasmid DNA

The recombinant transformants were screened by different method to select the correct recombinant clones. They are:

14.1 Rapid analysis of recombinant plasmid DNA

Rapid disruption of bacterial colonies was carried out to screen the recombinant plasmid DNA clone by difference in the electrophoresis pattern in 0.7% agarose gel. Following were the steps for rapid analysis.

An aliquot (tooth pick tip) of bacterial culture was taken from overnight grown master plate cultures and mixed with 50 μ l of 10 mM EDTA (pH 8.0) in a microcentrifuge tube.

50 μ l of lysis buffer (0.2 N NaOH; 0.5% SDS, 20% sucrose) was added into each tube, and vortexed for 30 seconds.

After disruption of bacterial cell wall, the lysate was incubated at 72°C for 5 min.

2 μ l of 4 M KCl and of 0.4% bromo-phenol blue were added into each tube and vortexed for 30 sec.

The disrupted cells were incubated in ice for 5 min. followed by centrifugation at 10,000 rpm for 3 min at 4°C to remove bacterial debris.

20 μ l of supernatant was electrophoresed on 0.7% agarose gel.

The recombinant clones were selected using lysate of bacterial colony with comparable size plasmid as positive control marker. Lysate of bacterial colony with the vector (pUC18 or pBin19) served as negative control.

14.2 Colony PCR

The presence of the recombinant plasmids in transformed bacterial colonies were examined by colony PCR wherein amplification of the inserted DNA was done using specific primers.

Sufficient amount of overnight grown bacterial culture was taken with a sterile toothpick and transferred to a microfuge tube containing 38 μ l ddH₂O, and following reagents were added into the tube and kept for PCR.

Template DNA	Bacterial colony
10X Taq DNA Polymerase buffer	5 µl (1X)
MgCl ₂	4 µl
dNTP mix	1 µl (10 mM each)
Forward primer (FP)	1 µl (100 ng)
Reverse primer (RP)	1 µl (100 ng)
Taq DNA Polymerase	~ 0.5 µl (1 Unit)

The PCR was performed in a thermocycler using the conditions of initial denaturation at 94°C for 2 min and 30 cycles of 1 min denaturation at 94°C, 2 min annealing at 55°C and 3 min elongation at 72°C followed by a final extension for 10 min at 72°C.

15. Agroinoculation

Generally members of begomoviruses are not mechanically transmissible to their host. The ability of *Agrobacterium* to deliver the viral tandem repeats into plant is one of the most routine techniques used by many scientists to understand the role of different viral genes. To facilitate the release of unit genome length viral DNA, it is necessary to introduce more than one copy of the viral genome component. The technique was first developed by Grimsely *et al.* (1986) to transfer CaMV genes into *Brassica napus*. In geminiviruses agroinoculation was first shown for TGMV by Rogers *et al.* (1986).

15.1 Tandem partial repeat constructs in pBin 19

For agroinoculation of infectious clone, a partial tandem repeat of Bg and Cp isolate was constructed in pBin19 vector. Partial tandem repeat constructs of BgA isolate and CpA isolates were 1.6 mers, having one full

length DNA A component along with an additional 1.8 kb *Hind* III/*Bam*H I fragment (0.6 mer), having two *ori*. The cloning of 1.6 mer containing 2 *ori* was done (Fig. 7) in two steps. At first step, the 1.8 kb of *Hind* III/*Bam*H I fragment of the DNA A containing origin of replication was cloned in the multiple cloning site of the binary vector pBin19. The DH5 α strain of *E. coli* cells were transformed with construct. The recombinant clone was screened by PCR and restriction digestion. The 1.8 kb construct in pBin19 was made for CpA and BgA clones separately and was referred to as pBin 0.6 mer of Bg and Cp isolates. 2.7 kb *Hind* III fragment of full length DNA A of either wild type or mutants was released by restriction of the clones by *Hind* III/*Bgl* I and the *Hind* III fragment was ligated with *Hind* III ends of pBin 0.6 mer of Bg or Cp clones.

Ligation of 2.7 kb *Hind*III fragment to 0.6 mer of pBin19 construct gave rise to 1.6 mer of DNA A. Partial tandem repeats were selected after confirming their orientation. The full length DNA B component in the plasmid pBgB12 and pCpP7 was released by PCR using abutting primers. The 2.7 kb fragment was purified by gel extraction and ligated with *Bam*H I ends of pBin19 and *Pst*I end of pCambia1301 in 3:1 insert:vector ratio. Recombinants with two copies of DNA B in pBin19 in tandem orientation were selected.

15.2 Triparental mating

A. tumefaciens EHA 105 strain was grown in 2 ml of LB liquid medium containing rifampicin (50 μ g/ml) at 28°C for 30 h with shaking at 200 rpm. The *E. coli* DH5 α strain containing the tandemn partial repeat construct and the *E. coli* having helper pRK 2013 plasmid were grown in 2 ml of LB containing kanamycin (50 μ g/ml) at 37°C for 24 h with shaking at 200 rpm. 500 μ l of *Agrobacterium* culture, 200 μ l of *E. coli* DH5 α strain having repeat

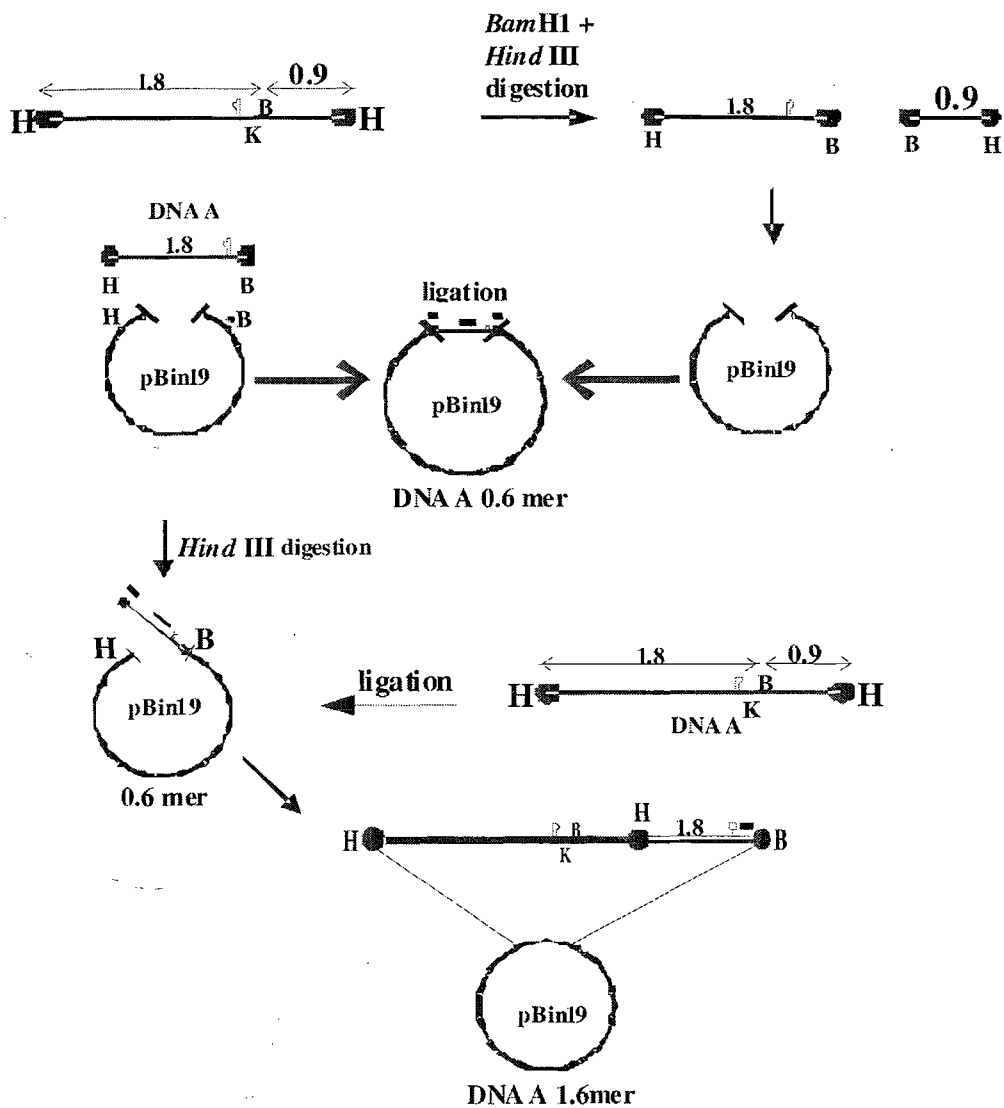


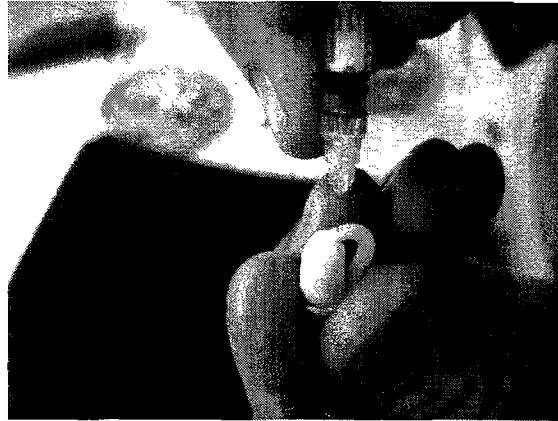
Fig. 7.Diagrammatic representation of steps involved in the preparation of tandem partial repeat Construct of DNA A component of Bg or Cp isolate are shown.

construct and 200 μ l of cells containing helper plasmid were mixed in a microfuge tube. 50 μ l of the mixture were inoculated on 1 square cm of sterile nitrocellulose membrane (NCM), which was placed on plain LB agar plate. The plate was air dried and incubated at 28°C for 24 h. Bacterial cells which had grown on NCM were diluted in 1 ml of LB medium and mixed gently. A serial dilution were prepared in 1 ml of LB, by transferring 10 μ l of the suspension, and 100 μ l of the bacterial suspension at 10^{-3} dilution was spread on a LB agar plate containing kanamycin (50 μ g/ml) and rifampicin (50 μ g/ml). Plates were incubated at 28°C for 48 h till the appearance of single colony. A single colony of the transconjugant was streaked on LB agar plate containing rifampicin and kanamycin and maintained.

16.1 Agroinoculation of tandem repeats on legume hosts

The transconjugant *Agrobacterium* culture with partial or complete tandem repeat constructs were grown on LB (rif-kan) medium for 24 h. About 200 μ l of *Agrobacterium* suspension was spread on LB agar medium (Rif-kan plates), and grown for 48 h at 28°C. The bacterial growth in each LB agar plate were collected with a spatula and transferred to an eppendorf tube containing 500 μ l of B5 medium. The bacterial cells having DNA A and DNA B components were thoroughly mixed and the density of suspension was adjusted to $OD_{600} = 0.8$. In the case of mutation experiments, mutated DNA A constructs were mixed with cells having DNA B constructs. The seeds of test plants were placed on wet filter paper in a Petri dish kept in 28°C for 48 h for germination. Care was taken to maintain sufficient moisture. After 30-48 h, the sprouting seeds were sorted out for different treatments. Testa of the seeds were removed and 10 pin pricks with the needles (G_{22}) were made (Fig. 8a) on the root hypocotyl axis. 40 μ l of *Agrobacterium* cell

a



b

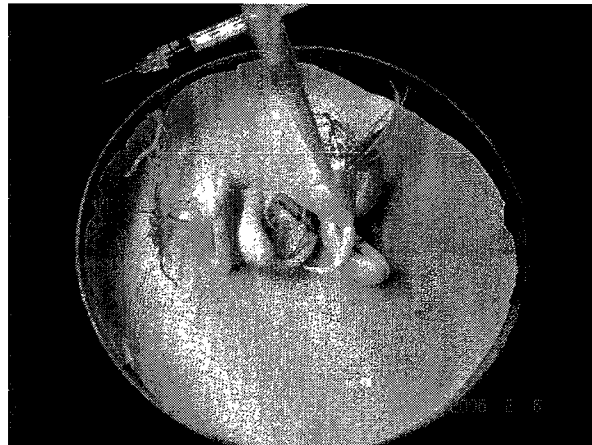


Fig. 8. Sprout-seed method of agroinoculation. A. Pin-pricks on root-hypocotyl region, b. application of *Agrobacterium* cells.

suspension was placed (Fig. 8b) on the pricks. Co-cultivation with the cells was done for 4 h after which sprouting seeds were sown in a pot mix kept in the growth chamber at National Phytotron facility at IARI. Plants were maintained for 30 days at 28°C, 18 h light at 18,000 lux.

16.2 Observations on pathogenesis

The symptoms were recorded every day after the day of inoculation. Following were the parameters considered:

- a. Number of infected plants/Number of plants inoculated to calculate percent infectivity.
- b. Type of symptoms
- c. Reduction in leaf lamina size.

17. Analysis of viral DNA in infected plants

17.1 Total genomic DNA isolation from agroinoculated legume plants

Total DNA was extracted from young leaves of agroinoculated legume plants. Viral DNA was isolated from 100 mg of infected leaves of blackgram, cowpea, French bean and mungbean plants. Total nucleic acid was extracted in the following manner. DNA was extracted from the third trifoliate leaf, 21 days after inoculation (DAI). Care was taken to collect samples from same age and size trifoliate leaves, to prevent error in accumulation of viral DNA replicative forms in Southern blot analysis. Leaf tissue from three plants were pooled and used for analysis. To assess the level of different DNA forms, the analysis was repeated minimum for three times. The leaf tissues were ground in liquid nitrogen using pestle and mortar and the homogenate was transferred to modified extraction buffer (100 mM Tris-HCl, pH 8.0, 1.4M NaCl; 20 mM EDTA, 2% CTAB and 2% β -marcatoethanol BME). The homogenized sap

was transferred into microfuge tubes was incubated at 65°C for > 30 min and mixed by inversion 3-5 times. Equal volume of chloroform, isoamyl alcohol (24:1) was added into each tube and tubes were mixed gently by inversion for 10 min followed by centrifugation at 13,000 rpm for 10 min at 4°C. The aqueous phase was transferred to a sterile microfuge tube. To the aqueous phase, 0.8 volume of isopropanol was added and mixed gently to precipitate the nucleic acids. The DNA was separated by centrifugation at 13,000 rpm for 10 min at 4°C, followed by washing with 80% and 70% ethanol separately. The pellet was air-dried, and dissolved in 100 µl of ddH₂O. The genomic DNA in this step was PCR compatible for amplification of viral DNA. The DNA was further purified by *RNase A* treatment and stored at -20°C for further use.

17.2 Southern transfer of DNA fragments

Total DNAs extracted from plants or plasmid were electrophoresed in 1.2% agarose gel. DNA fragments were transferred to nitrocellulose membrane (NCM, Milipore) or nylon membrane using procedure developed by Southern (Southern, 1975). After electrophoresis, to know the orientation, the gel was given a slant cut at left hand side down corner. The gel was immersed in alkaline denaturing solution (1.5M NaCl, 0.5N NaOH) for 30 min followed by soaking in neutralizing solution (1M Tris-HCl, pH 7.4, 1.5M NaCl) for 30 min and rinsed with distilled water, later the gel was transferred to transfer tank containing transfer buffer (10X SSC) and placed in inverted position on the glass plate over the pre-soaked Whatman paper wig, and the surroundings portion of gel was covered with polythene sheet. Over the gel, pre-soaked nylon membrane and 3 pieces of wetted Whatman paper No. 3 were kept. Then about 40 coarse filter paper folds were placed over the Whatman paper

followed by 0.5-1 kg weight at the top of the filter papers. The capillary transfer of DNA was allowed for 18-20 hours. At the end of transfer, NCM was marked at the well region and was rinsed in 2X SSC to remove traces of agarose sticking to the membrane. Then the DNA on the membrane was fixed by baking at 80°C for 2 h. The NCM was used for hybridization with radiolabelled probe.

17.3 Identification of viral replicative forms by S_1 Nuclease, *Rnase A*, *DNase I* and *Exonuclease III*

To know exact position of the begomovirus viral replicative forms, the total DNA extracted from the infected plants was treated with four different nucleases i.e., S_1 Nuclease, *RNase A*, *DNase I*, and *Exonuclease III* as given below.

(a) *Exonuclease III* treatment

Plant total nucleic acid	5 µg
<i>Exonuclease III</i>	25 units
10 X <i>Exonuclease</i> assay buffer	(1 X) 2.5 µl
H ₂ O	up to 25 µl

The reaction mixture was incubated at 37°C for 30 min.

(b) S_1 nuclease treatment

Plant total nucleic acid	5 µg
S_1 nuclease	20 Units
10 X S_1 nuclease assay buffer	1 X (2.5 µl)
H ₂ O	up to 25 µl

The reaction mixture was incubated at 30°C for 30 min.

(c) RNase A

Plant total nucleic acid	5 µg
RNase A	1.5 µl
H ₂ O	up to 25 µl

The reaction mix was incubated at 37°C for 1 h.

(d) DNase I

Plant total nucleic acid	5 µg
DNase I	5 units
10X DNase I assay buffer	1X (2.5 µl)
H ₂ O	Up to 25 µl.

The reaction mix was incubated at 37°C for 1 h.

The restricted DNA patterns were used as marker for Southern blot analyses of agroinoculated plant genomic DNAs.

18. Hybridisation**18.1 Prehybridization**

The baked NCM blots were kept in hybridization cylinders.

Prehybridization solution was added at the rate of 0.2 ml/sq cm.

The cylinders were then incubated at 65°C for 4h in hybridization oven with gentle rotation.

18.2 Probe preparation**18.2.1 Random primer labelling of DNA**

The random primer labelling reaction was done in 50 µl reaction mixture. 100 ng of DNA template and 1µl of random primer was added into

an microfuge tube containing 34.5 μ l of nuclease free H₂O and boiled for 2 min and immediately kept on ice. 5 μ l of 10 X labelling buffer, 2 μ l dNTPs without dCTP, 2.5 μ l DTT, 2 μ l BSA, 1 μ l [α -³²P] dCTP (10 μ Ci/ μ l, 3000 Ci/mmol) and 1 μ l klenow enzyme were added to the microfuge tube and incubated at 37°C for 1-2 h. The labelled probe was stored at -20°C till further use.

18.2.2 Radiolabelling of DNA by Nick translation method

Nick translation reaction was carried out in a 50 μ l reaction mixture containing ~1 μ g DNA template, 5 μ l dNTPs without dCTP, 5 μ l [α -³²P] dCTP (10 μ Ci/ μ l, 3000 Ci/mmol) and 5 μ l of DNA *Polymerase I : DNase I* mix, containing buffer for 2 h at 15°C as per manufacturer's protocol. Reaction was stopped by adding 5 μ l stop reaction buffer. The labelled product was purified by Sephadex G-50 column and kept at -20°C till further use.

18.2.3 End labelling of DNA by T4 polynucleotide kinase (PNK)

The labelling of 26-mer oligonucleotide was carried out in 20 μ l of reaction mixture containing ~50 ng oligonucleotide, 2 μ l of 10X PNK buffer, one unit of polynucleotide kinase (NEB), 3 μ l of [α -³²P] ATP (10 μ Ci/ μ l; 6000 Ci/mmol). The mixture was incubated at 37°C for 1-2 h. The probe was purified by Sephadex G50 column and stored at -20°C till further use.

18.2.4 Probe purification using Sephadex G-50 Column

The labelled DNA was purified from the unincorporated radio-activity by Sephadex G-50 column chromatography as follows. The bottom of a 1 ml syringe was plugged with autoclaved glass wool. The syringe was packed with 1 ml Sephadex G-50, and equilibrated with T₁₀E₁ buffer pH 8.0. Radiolabelled DNA was loaded onto the syringe column and centrifuged at

3,000 rpm for 4 min at room temperature. The radiolabelled DNA probe eluted out from spun column whereas the free radioactive label was retained in the spun column. The specific activity of purified probe was measured by liquid scintillation counter (Beckman, USA).

18.3 Hybridization

The double stranded α - ^{32}P labelled DNA probe was denatured in a boiling water bath for 5 min.

The denatured probe was added to pre-hybridization solution at 0.5×10^6 cpm/ml concentration. It was allowed to hybridize for 18h at 65°C in hybridization oven with gentle rotation.

18.4 Autoradiography

The hybridization solution was discarded and the membrane was washed 3 times with 2 X SSC, 0.1 percent SDS at 65°C , each time with duration of 15 min.

The washed membrane was dried on a paper towel, then placed within fold of cling film, placed in a lead cassette and exposed to X-ray film (KODAK) for 18h at -70°C .

Autoradiograph was then developed as per X-ray films manufacture's instructions.

19. Whitefly transmission

Adult (5-10) whiteflies were allowed to feed on plants showing good yellow mosaic symptoms for acquisition access period (AAP) of 24 h and on 15 days old healthy mungbean seedlings for inoculation access period (IAP) for 24h in a cage. Then whiteflies were killed and inoculated legume plants

were kept under cages in an insect proof glass house for symptoms development. The symptom appeared 15-30 days after inoculation.

20. Bacterial expression of AV2 protein

20.1 Cloning of ORF AV2 of Bg and Cp isolates in pMAL-p2X and pMAL-c2X expression vector

The p MAL-p2X vector (New England Biolab) was used for cloning of ORF AV2 which was inserted down stream from the *Mal E* gene of *E. coli*. The *Mal E* gene expresses maltose-binding protein (MBP) resulting in the expression of a MBP fusion protein. The vector contains a strong 'tac' promoter and the *Mal E* translation initiation signal to give high level expression of the cloned genes. Finally the fusion protein can be purified using MBP affinity for maltose. The full length ORF AV2 was amplified from the DNA-A component of Bg and Cp isolate using the specific primers (Table 12) for ORF AV2 region of the above isolates.

The sizes of amplicons were 222 and 339 bp for Bg and Cp isolates respectively. The amplified products were gel purified and cloned between the *Hind* III and *Eco*R I sites of the pMAL-p2X expression vector. The recombinant plasmids were used to transform Rosetta strain of *E. coli* cells, which expressed the protein as MBP-AV2 fusion protein.

20.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant MBP-AV2 protein

The presence of protein in the sample and the purity of the fusion protein was checked by SDS-PAGE. Protein samples were mixed with equal volume of 2X SDS-PAGE sample buffer (90 mM Tris-HCl, pH 6.8; 20% glycerol, 2% SDS; 0.02% bromophenol blue; 100 mM DTT or 5%

β -mercaptoethanol). The protein samples were boiled for 5 min in boiling water bath and loaded on the 12% SDS-PAGE and electrophoresed at 50 V/cm, until the dye front reaches the bottom end of the glass plates.

20.3 Coomassie blue staining

For detection of the protein band in SDS-PAGE, the coomassie brilliant blue R-250 which is the most commonly used protein stain was used. The staining solution contained 45% methanol, 10% glacial acetic acid and 0.25% coomassie brilliant blue R-250. The acetic acid-methanol mixture acts as a denaturant to precipitate or fix the protein in the gel, which prevents the stained protein to be washed and removed. The unbound background dye from the gel was removed by adding destaining solution (30% methanol; 10% glacial acetic acid) to the gel for around 1-2 h. For overnight destaining, a mixture of 15% methanol and 5% glacial acetic acid was used as destaining solution.

20.4 Silver staining of protein

The gel following separation of proteins in a 12% SDS-PAGE was fixed in the following fixatives separately for 30 min at room temperature.

- 1) Fixative I (50% methanol, 7.5% acetic acid),
- 2) Fixative II (5% methanol, 7.5% acetic acid),
- 3) Fixative III (10% aqueous glutaraldehyde).

Following fixation, the gel was thoroughly washed using ddH₂O for 60 min in a shaker. The fixed protein was stained by silver nitrate solution [1.4% liquid ammonia, 0.02N NaOH, 0.0064g AgNO₃ (W/V)], with shaking for 30 min at room temperature. This was followed by washing with ddH₂O for 5 min, at room temperature. The protein bands were visualized using developing solution (0.05% citric acid, 2 μ l/ml of 37% HCHO). The

developing was continued till the protein bands appeared. Development of color was stopped by 7.5% acetic acid.

21. *In vitro* expression of recombinant MBP-AV2 fusion protein

About 20 ml of LB medium containing 50 µg/ml ampicillin was inoculated with 200 µl of overnight culture of the Rosetta cells containing fusion plasmid, and grown at 32°C with good aeration until the growth reached OD₆₀₀ ~0.5-0.6 (2X 10⁸ cells/ml). An aliquot of 1 ml of the culture was taken and processed to serve as uninduced control and IPTG was added to remaining culture to a final concentration of 0.4 mM, to express the AV2 protein. One ml of the sample was collected at 1 h interval (up to 5 h) and cells were harvested by centrifugation at 4000 x g for 10 min and the pellet was resuspended in 2 ml (200 µl/ml) of column buffer (50 mM Tris-HCl, pH 7.5; 200 mM NaCl; 1 mM EDTA; 10 mM BME; 10% glycerol; 1 mM PMSF). The cells were incubated at -20°C over night to break the cell by freez-thaw shock. The sonication (10 seconds pulses for 2 minutes) was done with thawed cells on ice. The lysate was subjected to high-speed centrifugation, 9000 x g at 4°C and the supernatant was mixed with amylose resin (5 µl/ml) and mixed by shaking for 2 h at 4°C. The bound protein was loaded onto pre-washed equilibrated amylose resin (New England Biolab) column. The flow through was collected and again passed through column twice. The column was washed with 20 column volumes of column buffer with 0.1% Triton X-100. Ten fractions (100 µl) of the MBP-AV2 fusion protein were eluted with 100 µl of 10 mM maltose in column buffer. The purity of the eluted fractions was checked on a 12% SDS-PAGE gel. Fractions containing the higher amount of fusion protein were pooled and dialysed against 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 40% glycerol. The samples were divided into aliquots

and stored at -20°C until further use. For biochemical activities of the recombinant AV2 proteins of Bg and Cp isolates, the proteins were further purified through Heparin Sepharose and immunoprecipitation.

22. Large-scale preparation of MBP-AV2 fusion protein

500 ml culture of the *E. coli* strain Rosetta, containing fusion AV2-pMAL-p2X (MBP-AV2) vector was grown to OD₆₀₀ ~0.5-0.6, and induced to express the AV2 protein with 0.4 mM IPTG for 6 h at 32°C and for 15 h at 22°C in the case of Bg and Cp clones respectively. The cells were harvested by centrifugation at 4000 x g for 20 min and the pellet was resuspended in 60 ml column Buffer (50 mM Tris-HCl, pH 7.5; 200 mM NaCl; 1 mM EDTA; 10 mM BME; 10% glycerol; 1 mM PMSF). The cells were incubated at -20°C overnight. After freeze-thaw shock, to release the fusion protein from intact cells, the suspension was sonicated. Short pulses of 10 second sonication for 2 minutes, was carried out with thawed cells on ice. The lysate was subjected to high-speed centrifugation, 9000 x g at 4°C for 30 min. The supernatant was collected and bound to amylose resin (equilibrated with column buffer) by rocking for 2 h at 4°C. The column was washed with 20 column volumes of column buffer with 0.1% Triton X-100. The fusion protein was eluted with 4 column volumes of column buffer containing 10 mM maltose. The eluted fractions were checked on a 12% SDS-PAGE. Fractions containing the higher amount of protein were pooled and dialysed against 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 40% glycerol. The samples were divided into aliquots and stored at -20°C until further use.

23. Polyclonal antibody production

Antibody to ORF AV2 of Bg isolate was prepared by immunizing New Zealand white rabbit using purified MBP-AV2 protein in 1X column buffer.

Four intra-muscular injections of 50 to 100 µg purified MBP-AV2 protein in column buffer was emulsified with an equal volume of Freund's incomplete adjuvant. Injections were given at weekly intervals. The rabbit was bled, 10 days after the fourth injection. A booster injection of purified MBP-AV2 protein was given three weeks after the first bleeding and the rabbit was bled 3 weeks after the last injection. The blood samples were kept at room temperature for 4 h to clot, and was followed by overnight storage at 4°C. The sera were collected after low speed centrifugation at 8000 rpm for 10 min. The serum was aliquoted and preserved by mixing with equal amount of 100% glycerol.

23. Preparation of Immuno-globulin G (IgG) by affinity chromatography

For IgG preparation, a glass column was packed with ~200 µl of Protein A Sepharose (Amersham, Biosciences). The column was washed with 10 column volume of 100 mM Tris-HCl, pH 8.0. One ml of crude anti AV2 polyclonal antibody was diluted with 100 µl of 1M Tris-HCl, pH 8.0, and passed through the column. The column was washed with 10 column volumes of 100 mM and 10 mM Tris-HCl, pH 8.0 followed by one column volume of 100 mM glycine pH 3.0 separately. The IgG was fractionated with 400 µl of 100 mM glycine pH 3.0 in eppendorf tubes containing 50 µl of 1M Tris-HCl, pH 8.0.

The quantity of the IgG was estimated by measuring the absorbance at 280 nm ($OD_{280} \cdot 1.33 = 1 \text{ mg/ml protein}$). The purified IgG was stored at 4°C after adding PMSF and benzamidin as protease inhibitor.

24. Western blot analysis using MBP-AV2 antibodies

The protein bands from SDS-PAGE gel were transferred to solid support nitrocellulose membrane (NCM). The transfer was done using semi-transfer electroblot instrument (Bio-Rad) in the presence of 39 mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol as a transfer buffer, at a constant voltage of 15 V and 500 mA for 1 h. At the end of the transfer, the NCM was marked at the well region and rinsed in 1X Phosphate Buffered Saline (1X PBS) (0.02% KH_2PO_4 ; 0.115% Na_2HPO_4 ; 0.8% NaCl; 0.02% KCl (w/v)]. The NCM was incubated in blocking solution (3% BSA or 5% non fat/dry milk powder in 1X PBS) for more than 2 h at 37°C (or 4°C overnight) with gentle shaking. The blocked NCM was washed thrice with 1X PBS each wash being for 10 min. Primary antibody [Anti-MBP, 1:10,000 dilution (NEB) or Anti-AV2] in 1X PBS solution containing 2% BSA 5% dry milk powder was added and incubated at 37°C for 1 h with gentle shaking. The membrane was washed with 1X PBS for 10 min for three times. The secondary antibody (Goat anti rabbit IgG-conjugated with alkaline phosphatase) diluted in 1X PBS with 2% BSA was added and incubated at 37°C for 1-2 h with gentle shaking followed by washing as above.

The colour of protein-antibody complex was developed by using solution containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl_2 with 150 mg/ml (NBT) and 75 mg/ml Bromo chloro indolye phosphate (BCIP). The developing reaction was stopped by fixing solution (10 mM Tris pH 7.5, 1 mM EDTA pH 8.0).

25. Separating the AV2 protein from MBP-AV2 fusion protein after protease cleavage

The pMAL-P2X vector contains the sequence coding for the recognition site of ILe-Glu/Asp-Gly-Arg[↓] located just 5' to the polylinker insertion sites. Specific protease Factor Xa, can cleave this site allowing MBP to be cleaved from the AV2 protein after purification.

The MBP-AV2 fusion protein was subjected to a cleavage using Factor Xa (NEB) protease. The cleaved protein was resolved by 12% SDS-PAGE. The reaction is carried out as below:

MBP-AV2 fusion protein	25 µg
Factor Xa	0.5 µg
Tris-HCl, pH 8.0	20 mM
NaCl	100 mM
CaCl ₂	2 mM
H ₂ O	to make the volume up to 30 µl

The reaction mix was incubated at 23°C for 6 h.

26. Biochemical functions of AV2 protein

26.1 Purification of MBP-AV2 fusion protein using Heparin Sepharose CL-6B chromatography

For biochemical assay of the recombinant MBP-AV2 fusion protein of Bg and Cp isolates, the amylose resin purified proteins were further purified through Heparin Sepharose CL-6B (Amersham Biosciences) column, equilibrated with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl. The protein was bound to the matrix with rocking for 2h at 4°C. The protein bound sepharose beads were washed with 10 column volumes of buffer containing 150 mM

NaCl. The MBP-AV2 protein was eluted with gradient NaCl salt concentration ranging from 150 mM to 1.5 M in the presence of 50 mM Tris-HCl, pH 8.0. The MBP-AV2 fusion protein fractions were collected. The pure protein was eluted at 1.2 M NaCl, 50 mM Tris, which was dialyzed against 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 30% glycerol and used for further study.

26.2 Purification of MBP-AV2 fusion proteins through immunoprecipitation

MBP-AV2 fusion protein was immunoprecipitated using anti AV2 IgG in the presence of Protein A Sepharose (Amersham Biosciences). For this purpose, 50 µg of MBP-AV2 protein was mixed with 20 µg anti AV2 IgG and incubated at 4°C for 30 min. Then 50 µl of Protein A Sepharose slurry (equilibrated with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl) were added to the mixture, and shaken for 30 min at room temperature. The mixture was washed thrice using equilibration buffer and was followed by low speed centrifugation at 3000 rpm for 5 min at room temperature. Finally the matrix pellet was resuspended in 20 µl of 1X ATPase assay buffer.

27. Nucleic acid binding assay using South-Western analysis

MYMIV coat protein gene (CP) DNA fragment (~700 bp) was labelled with [α -³²P]-dCTP using the nick translation method as described earlier. The labeled DNA was purified from the unincorporated radio-activity by Sephadex G-50 column chromatography. Half of the labelled DNA was boiled for 5 min to be made as single stranded DNA (ssDNA) probe, and immediately kept on ice for 10 min. The rest of DNA was used as dsDNA probe. Five µg of MBP-AV2 fusion proteins of Bg and Cp were electrophoresed in SDS-PAGE along with MBP tagged coat protein (MBP-CP, Malik *et al.*, 2005) of

MYMIV and MBP proteins as positive and negative controls respectively. The protein was transferred to nitrocellulose membrane (NCM) using semi blot transfer apparatus (BioRad). The membrane was air dried followed by incubation in pre-hybridization solution [1X binding buffer (10 mM Tris-HCl, pH 7.5; 50 mM KCl, 1 mM DDT), 3% BSA] for 2 h at room temperature. The ssDNA and dsDNA probes were added along with boiled and sheared salmon sperm DNA (50 µg/ml) to hybridization bottle separately. The hybridization was carried out for 3 h at room temperature.

Washing was performed thrice at room temperature using wash buffer (1X Binding buffer, 3% BSA/5% nonfat dry milk, and 0.5 mM DTT). The NCM was air-dried and autoradiographed. The autoradiograph was analyzed using phosphorimager (Amersham Biosciences, Typhoon 9210).

28. Rep protein binding assay

About 1 µg of purified MBP-AV2 fusion protein was incubated with 500 ng to 1.5 µg of 6His-Rep fusion protein in 100 µl binding buffer (25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 1% NP-40 as a detergent) at 37°C for 1 h. The bound protein reaction mix was added to 50 µl of amylose resin (NEB) which was pre-washed and equilibrated with buffer containing 50 mM Tris-HCl, 100 mM NaCl. The mixture was incubated on ice for 1 h with gentle mixing for 5 min. The bound protein was separated from the amylose matrix by centrifugation at 4000 rpm for 3 min. The first supernatant was taken as unbound fraction, and amylose matrix containing bound protein was washed thrice with increasing concentration of NaCl from 200 mM to 500 mM in the presence of 50 mM Tris-HCl, pH 8.0.

The unbound and bound fractions were boiled for 5 min, and centrifuged at 3000 rpm for 3 min. The samples were analysed by SDS-PAGE followed by silver staining and western blotting using anti MBP or anti MBP-AV2 antibody.

29. ATPase assay

To investigate the ability of the AV2 protein of Bg and Cp isolates to hydrolyse ATP, ATPase assay was carried out essentially as described by Begewadi *et al.* (2004). The buffer used for the ATPase reaction contained 20 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 100 mM KCl, 8 mM DTT and 80 µg/ml BSA. Briefly, 1 µl of [α -³²P]-ATP (10 µCi, 6000 Ci/mmol, Perkin-Elmer Life Sciences Inc., Boston, USA) was diluted 50-fold with 5 mM ATP. One µl of dilute radiolabelled ATP was mixed with the desired amounts of protein in ATPase reaction buffer and incubated at 37°C for 30 min. On completion of the reaction, 1 µl of the reaction mix was spotted onto a thin layer chromatography (TLC) plate, polyethyleneimine (PEI, Sigma-Aldrich, USA), air-dried and chromatography was performed using 0.5M LiCl and 1M HCOOH as the running solvent at room temperature for ~25 min. Following completion of chromatography, the TLC plate was air-dried and autoradiographed using phosphorimager (Amersham Biosciences, Typhoon, 9210).

30. Monitoring nicking activity

Nicking activity of MYMIV-Rep protein in the presence of AV2 protein was examined. A 26-mer oligonucleotide containing sequence from hairpin region of MYMIV DNA A, 5'-CGACTCAGCTATAATATTACCTGAGT-3' was used for nicking assay. The 26-mer was 5'-end labelled as described

earlier using T4 polynucleotide kinase. As Rep nicks specifically at TAATATT[↓]AC sequence, the nicked product that could be visualized was a 5'-labelled 18-mer. About 1-2 ng of radiolabelled 26-mer (specific activity ~10,000 cpm/ μ l) was incubated with 50 ng of Rep protein in absence or presence of AV2-MBP fusion protein ranging from 1-4 ng, in 40 μ l reaction mixture in a buffer (25 mM Tris-HCl, pH 8.0, 75 mM NaCl, 2.5 mM EDTA, 10 mM MgCl₂, 5 mM DTT) at 37°C for 1h. The reaction was terminated by adding 5 μ l of stop buffer (1% SDS, 25 mM EDTA, 10% glycerol) and 12 μ l loading dye. The reaction mix was boiled at 100°C for 5 min. The products were resolved on 15% native PAGE and autoradiographed using phosphorimager.

31. Sequencing and sequence analysis

The recombinant plasmid DNA that were screened earlier by rapid analysis, colony PCR and restriction digestion with endonucleases were further confirmed by sequencing in an automated DNA sequencer (at Delhi University, South Campus, New Delhi). Sequencing was done for four to six clones in the case of mutation. In the case of variability studies, sequence was generated at least for two clones in each isolate. The sequence data were assembled and analysed with the software programme BioEdit version 7.04.1 (Hall, 1999). Multiple nucleotide and predicted amino acid sequence alignments were done in CLUSTAL X version 1.81 (Thompson *et al.*, 1997) programme. Comparisons were made with begomovirus DNA sequences obtained from the GenBank sequence database (Table 22). Dendrogram were constructed from the aligned sequences using CLUSTAL X programme at 1000 bootstrap values.

RESULTS

The role of ORF AV2 in viral pathogenesis was investigated in Bg and Cp isolates of MYMIV, the virus isolates which have been characterized earlier (Mandel *et al.*, 1997; Malathi *et al.*, 2005).

4.1. Infectivity of cloned components of Bg and Cp isolates.

The purity of the clones of Bg and Cp isolates and their infectious nature needed to be confirmed before investigation on ORF AV2. The confirmation of the clones are described in this section.

4.1.1. Confirmation of the DNA A and DNA B clones

Large scale plasmid DNA was prepared for DNA A and DNA B components from 100ml culture of *E. coli* DH5 α cells. The plasmids, pBgH13 and pBgB12, the full length DNA A and DNA B clones of Bg isolate and pCpH2 and pCpP7, the DNA A and DNA B clones of Cp isolate were subjected to restriction with different endonucleases to confirm purity of the plasmid DNA. Restriction map of DNA A clones of Bg and Cp isolates are given in Fig 9a for comparison.

Restriction of pBgH13 and pCpH2 (Fig. 9b) with *Hind*III / *Bam*H I released 1.8 kb and 0.9 kb fragment, which hybridized with radio labelled probe to DNA A, confirming the clones to be of DNA A. It was further confirmed by amplification with the DNA A specific abutting primers (AV-abut and AC-abut). In PCR, 2.7 kb full-length product (Fig. 9c) was obtained which was verified to be of viral DNA origin in Southern blots. Similarly, the plasmid DNA, pBgB12 was restricted with *Bam* HI / *Bgl* I and pCpP7 with *Pst* I / *Bgl* I. The Southern blot having DNA was probed with movement

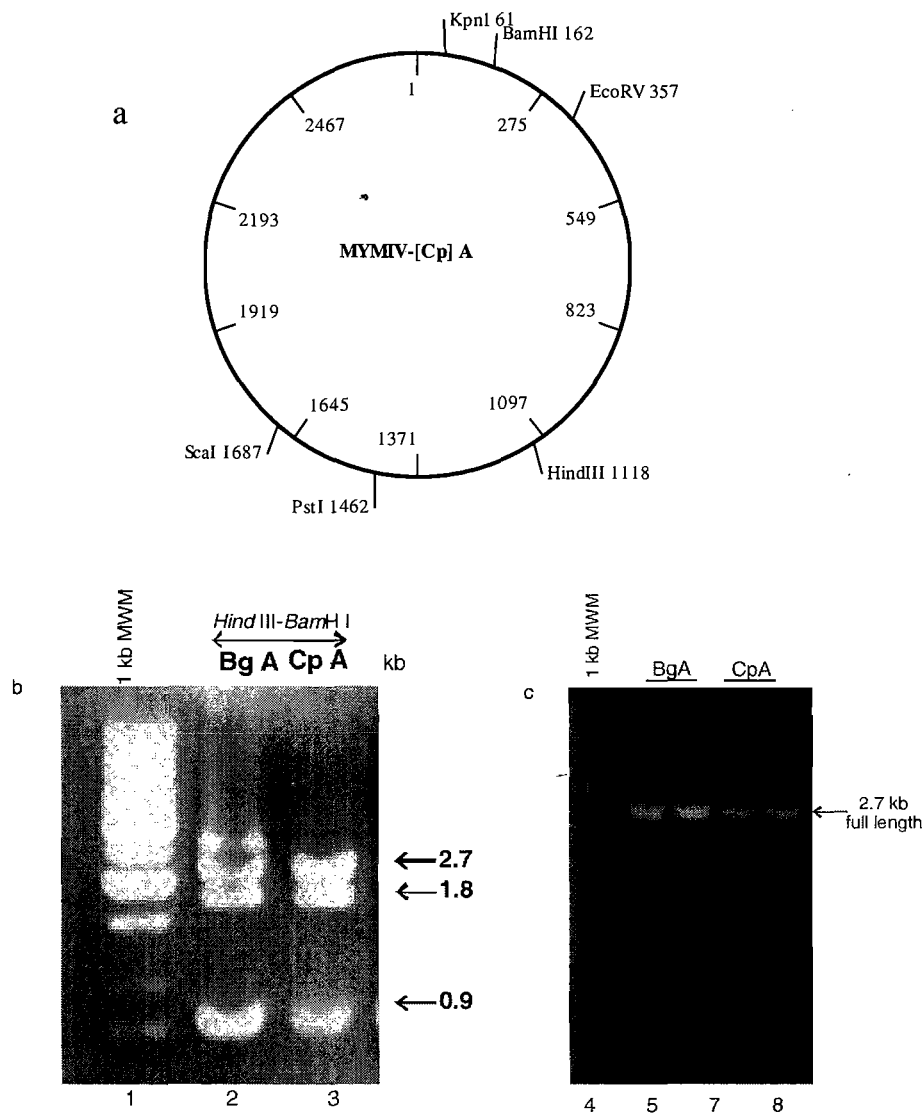


Fig. 9. Agarose gel electrophoresis of genome length PCR products of Bg and Cp isolate and restriction fragments of plasmid DNA . Lanes 1, and 6 : 1 kb DNA ladder; lanes 2 and 4 : DNA A of Bg and Cp amplified with AV/AC abut primers; Lanes 3 and 5 : DNA B of Bg and Cp amplified with BV/BC abut primers; Lanes 7 and 8 : plasmid DNA A of Bg and Cp restricted with *Bam*H I/*Hind* III.

protein gene, (ORF BC1, MP) positive hybridization confirmed the DNA B nature of the clones.

4.1.2. Tandem repeat construct

The partial tandem repeat constructs of DNA A and DNA B components were made as given in Materials and Methods. For preparation of partial tandem repeat, the plasmid pBgH₁₃ (Bg isolate) and pCpH₂ (Cp isolate) of the wild type clone were restricted with *Hind* III/*Bam*H I to release 1.8 kb fragment (Fig. 10b). This 1.8 kb fragment contained one origin of replication. The fragments were separated in 1% agarose gel electrophoresis and the 1.8 kb was cut out from the gel and purified. This fragment was ligated with *Hind* III/*Bam*H I restricted pBin19. Transformants were selected on kanamycin, IPTG/X-gal plates (Fig. 10a).

In the case of Bg isolate, 90 colonies were obtained, out of which 18 colonies were screened, 4 were true transformants which released 1.8 kb fragment (Fig. 10b), when miniprep DNA was restricted with *Hind* III/*Bam*H I and electrophoresed in 0.8% agarose gel. Similarly in the case of Cp isolate 6 colonies were true transformants, which released 1.8 kb insert. The clone pBin19 having 1.8 kb fragment was referred to as pBinBgA 0.6 mer and pBinCpA0.6 mer respectively. One of the transformants was selected. Large scale plasmid DNA of the clone was prepared and used for generating 1.6 mer or head to tail partial repeat constructs. About 100-200 ng of DNA of pBinBgA0.6 mer and pBinCpA0.6 mer were restricted with *Hind* III, treated with calf intestine Alkaline phosphatase. Unit genome length DNA A component (2.7) clones in pUC 18 vector, were released by *Hind* III/*Bgl* I restriction. The restricted fragments were separated in gel electrophoresis, and purified. The genome length DNA A as *Hind* III fragment was ligated with

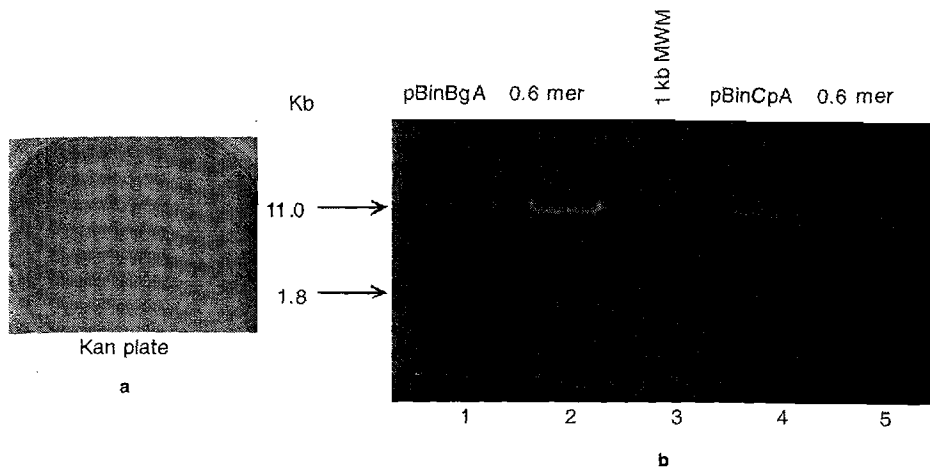


Fig. 10. pBin19 0.6 mer construct of DNA A component of Bg and Cp isolates in pBin19. a. *E. coli* DH5 α transformants having recombinant pBinBgA0.6 mer on kanamycin plate. b. Agarose gel electrophoreses of plasmid DNA of pBinBgA0.6 mer (lanes 1 and 2) and pBinCpA0.6 mer (lanes 4 and 5) restricted with *Bam*H I/*Hind* III releasing 1.8 kb viral insert. Lane 3: 1kb DNA ladder.

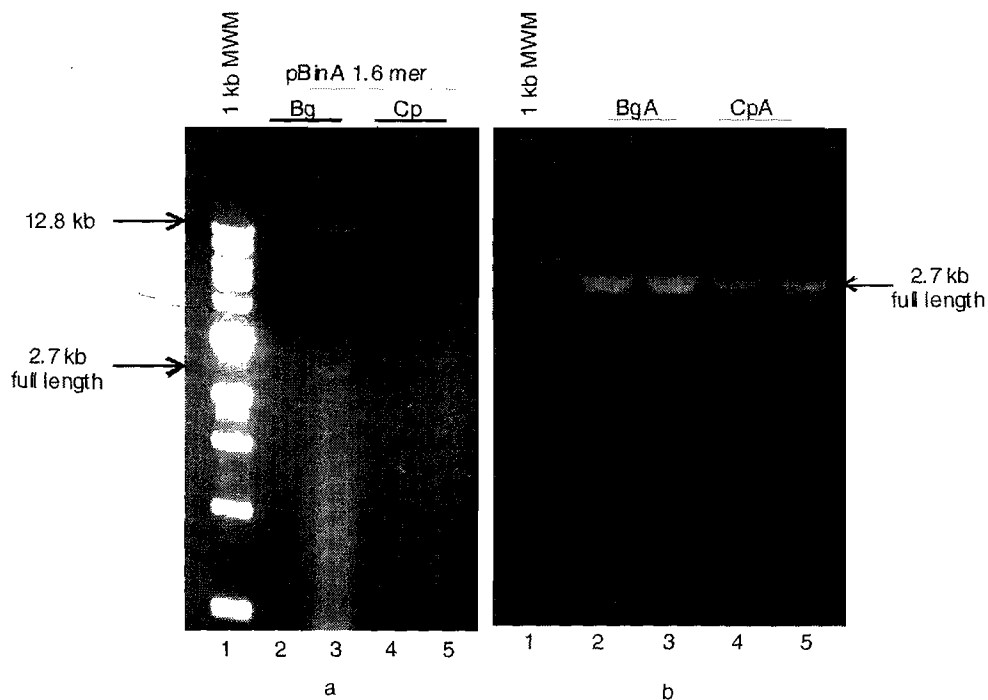


Fig. 11. Agarose gel electrophoresis of PCR products of pBin1.6 mer of Bg and Cp with amplified with AV/AC abut primers. a. Agarose gel electrophoresis of restriction fragments of pBinBgA 1.6 mer (lanes 2 and 3) and pBinCpA 1.6 mer (lanes 4 and 5). Lane 1: 1kb DNA ladder; release of 2.7 kb fragment by *Kpn*I restriction confirmed the tandem orientation of partial repeat.

similarly linearized pBinBgA0.6 mer and pBinCpA0.6 mer. The transformants were selected on kanamycin Luria agar plates. The tandem orientation of the clones were confirmed by colony PCR and restriction with *kpn* I. A diagrammatic representation of steps is given in Fig. 7. The construct for DNA A, had one full length + 0.6 mer of the genome (Fig. 11a) (i.e.1.8 kb fragment, *Bam*H I to *Hind* III fragment in pBin19 (pBinBgA1.6 mer). DNA B of Bg isolate constructs had one full-length plus 0.5 mer (i. e., 1.4 kb fragment from *Bam*H I to *Hind* III (pBinBgB1.5 mer). Cp isolate construct had two complete tandem repeats (*Pst* I to *Pst* I) in the vector pCambia1301 (pCamCpB2.0 mer). The head to tail orientation of the constructs were verified by *Kpn* I restriction for DNA A and *Hind* III restriction for DNA B constructs. The tandem partial repeat construct would release 2.7 kb fragment when the plasmid DNA is restricted with *Kpn* I for DNA A. The Fig. 11a shows 2.7 Kb fragment released in four clones. Similarly restriction of tandem partial repeat construct, pBinBgB1.5 mer released 2.7 Kb band confirming the correct orientation of the construct. The plasmids were mobilized into *Agrobacterium tumefaciens*, strain EHA 105 by triparental mating. The transconjugants were selected on rifampicin, kanamycin plates. PCR amplification with full length abutting/ primers were performed for 16 transconjugants. The colony lysate yielded 2.7 kb fragment which confirmed the viral inserts in the *Agrobacterium* cells.

Agrobacterium cells suspension in B5 medium, were used for inoculation by sprout seed method. About 40 µl of the suspension was placed on pinpricks made at the collar region (Fig. 8).

Three varieties of blackgram (CO1, a local variety, TN-1) mungbean (Pusa Baisakhi, PS-16 and a local variety) cowpea (Pusa Komal, Safed lobia,

Lal lobia), and French bean (Selection 9, Pusa Parvathi, Chithra) were inoculated in order to select highly susceptible variety in which the cloned components will be consistently infectious. Results are summarized below.

4.1.2 Symptoms produced by Bg and Cp isolates

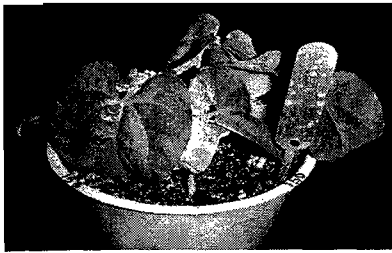
4.1.2.1 Bg isolate

Agroinoculation of Bg isolate produced typical yellow mosaic symptoms, in blackgram and mungbean, 7-10 days after inoculation (DAI). The very first trifoliate leaf showed symptoms, which started as small squarish restricted mosaic, that coalesced and resulted in entire lamina showing bright yellow mosaic (Fig. 12). Among the three varieties tested, variety TN-1 in blackgram and PS-16 in mungbean showed high infectivity, 40 % in TN-1, and 60% in PS-16. They were therefore selected for further pathogenesis work.

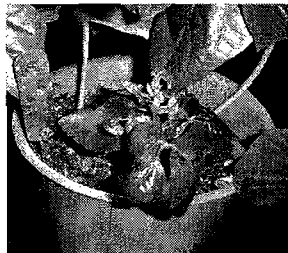
The symptoms in French bean were very distinct and appeared 5-7 DAI; the Bg isolate produced severe stunting (Fig. 12) and downward curling of cotyledonary leaves. The stunting is so severe that shoot elongation was not seen and plants remained aborted in growth and eventually died. Only in few plants trifoliate leaves were produced. The trifoliate leaves if produced showed puckering and were reduced in size. Infectivity percentage was 100% in all the three cultivars tested. The cv. Selection 9 was selected for further work.

The most significant results were seen with cowpea. Inoculations were done on three varieties, Pusa Komal, Safed lobia and Lal lobia. No symptoms were seen in Lal lobia plants. Few leaves showed mild tip necrosis, but no visible symptoms were seen. Contrastingly, the Bg isolate produced atypical

BgA+Bg B



Selection 9



Pusa Komal



Safed Lubia

CpA+Cp B



Selection 9



Pusa Komal



Ps-16

Fig. 12. Symptoms produced by Bg and Cp isolates after agroinoculation. Upper panel: symptoms produced by Bg isolate in French bean, cv. Selection 9, cowpea, cv. Pusa Komal and cowpea, cv. Lal Lobia. Extreme stunting in French bean and atypical symptoms in cowpea cultivars are evident. Lower panel: symptoms produced by Cp isolate in French bean, cv. Selection 9; cowpea cv. Pusa Komal and mungbean cv. Ps 16. Difference in the symptoms in French bean between Bg and Cp may be noted.

symptoms of severe leaf curl (Fig. 12), leaf distortion in the cultivar Pusa Komal and Safed lobia. The symptoms appeared in the second and third trifoliate leaf (15-17 DAI). Two plants out of 6 plants in Pusa Komal, and one plant out of 6 plants in Safed lobia showed these atypical symptoms. In some plants trifoliate leaves appear dark green in color and have asymmetrical leaflets. Extreme reduction in leaf size were also seen. However, in none of the three cultivars tested typical, yellow mosaic or golden mosaic symptoms were seen, with Bg isolate.

4.1.2.2 Cp isolate

Agroinoculation of Cp isolate produced typical golden mosaic symptoms in the cv. Pusa Komal of cowpea. In Pusa Komal, vein clearing of small veinlets started appearing between 12-15 DAI, which intensified, resulting in bright yellow chlorotic area alternating with green area. One peculiar feature of symptoms seen in the present study, which has not been reported by any other worker earlier, was the extreme leaf curling and mottling in young trifoliate leaves. Downward leaf curling (Fig. 12) associated with inward rolling of leaf margin were seen 15 DAI, leaf lamina appeared reduced in size. This kind of severe symptom was seen in 40% of plants inoculated in the cv. Pusa Komal. Safed lobia however, showed less brilliant yellow mosaic and no symptoms were seen in the cv Lal lobia.

In French bean, the CP isolate produced downward leaf curling, puckering and reduction in leaf lamina in the first trifoliate leaf. Symptoms appeared 7-10 DAI. However between Cp and Bg isolate there was difference in severity. Extreme stunting seen in the case of Bg isolate was not seen with Cp isolate. The cotyledonary leaves showed severe backward rolling with Bg isolate but not with Cp isolate. Plants showed limited growth and elongation

of the shoot. First, second and third trifoliolate leaves were produced which showed puckering (Fig. 12) and corrugated symptoms and reduction in lamina size. Infectivity was 100% in all the three cultivars tested.

In both blackgram and mungbean restricted yellow mosaic spots appeared within 7-9 DAI, and coalesced to give rise to bright yellow mosaic throughout the entire leaf. Infectivity rate was 60% in mungbean cv. Pusa Baisakhi.

In both the isolates, infectivity was high (100%) in French bean and low in blackgram.

Viral DNA replicative forms

Five µg of DNA extracted from either healthy or Cp isolate infected French bean plants was electrophoresed on agarose gel in the presence of ethidium bromide. In the gel, two faint bands were visible, in the DNA extracted from infected plant (Fig. 13a), one band was on par with 3 kb band of the molecular weight marker and the second band was on par with 1.6.kb band. No such bands were seen in DNA extracted from healthy plant.

Viral DNA forms were clearly resolved by different nuclease treatment followed by Southern hybridization analysis (Fig. 13b) using full-length DNA A component as probe. Total nucleic acid from infected tissue was hydrolysed by *DNase* I treatment (Fig. 13, lane 7) but were resistant (Fig. 13b, lane 6) to *RNase* A digestion, confirming the viral genome to be DNA and not RNA. In the DNA extracted from infected plants, in addition to two bands seen in the gel two more bands, one on par with 2.7 kb band of linearized plasmid, one closer to 1.6 kb band but slightly faster than 1.6 kb band were seen.

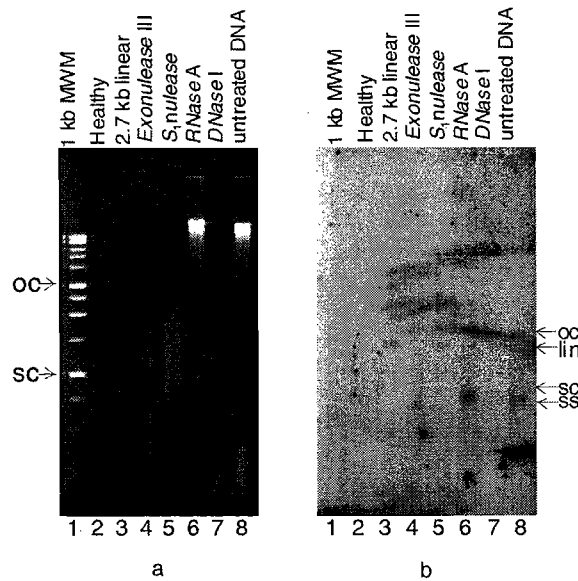


Fig. 13. Analysis of total DNA extracted from French bean plants agroinoculated with Bg isolate to identify the viral DNA replicative forms. Agarose gel electrophoresis (a) and Southern blot analysis (b) of and inoculated plants 5 µg of DNA from uninoculated. Lane 1 : 1 kb DNA ladder; lane 2 : DNA from uninoculated plant; lane 3 : 2.7 kb PCR amplicon of DNA A. 5 µg of DNA was treated with *Exonuclease* III (lane 4), *S₁ nuclease* (lane 5), *RNase A* (lane 6), *DNase I* (lane 7), untreated (lane 8). Blot probed with α -³²P labeled Cp gene fragment.

The majority of the faster migrating band was sensitive to S_1 nuclease (Fig. 13b, lane 5) treatment, and so was identified to represent single stranded DNA. The band above it, was identified as double stranded supercoiled form as it was resistant to S_1 nuclease and *Exonucleases* III treatment (Fig. 13b, lane 4). The slow migrating bands were identified, as double stranded (ds) nicked and linear forms, as they were not digested by S_1 nuclease but were affected by ExonucleaseIII (Fig. 13b, lanes 4, 5).

This identification was useful in assessing the levels of different replicative forms in forth coming experiments.

The viral DNA replciative forms of Bg and Cp isolate inoculated plants were visualized in Southern blot analysis. No such replicative forms were seen in lanes having DNA extracted from healthy plants. Results showed that higher amount (Fig. 14) of linear (lin), open circular (oc), double stranded (ds), super coiled (sc) and single stranded (ss) forms were seen in French bean. Presence of sc and ss forms were less in cowpea and mungbean, contrasting to blackgram and French bean. In the case of mungbean, signal for the replicative forms was less compared to cowpea plants and blackgram when inoculated by Cp isolate. The notable feature was seen in cowpea inoculated by Bg isolate; Trace amounts of replicative forms were seen in all the three varieties of cowpea, though symptoms were atypical. Of the four forms, lin, oc, ds, sc and ss DNA levels were very high in French bean infected by both Bg and Cp isolate (Fig. 14).

4.1.3 Site directed mutagenesis

In order to elucidate the role of ORF AV2, mutagenesis was performed in the ORF AV2. The description of the mutants, position of the codon and the predicted amino acids change are given in Table 10 and 11. Totally five

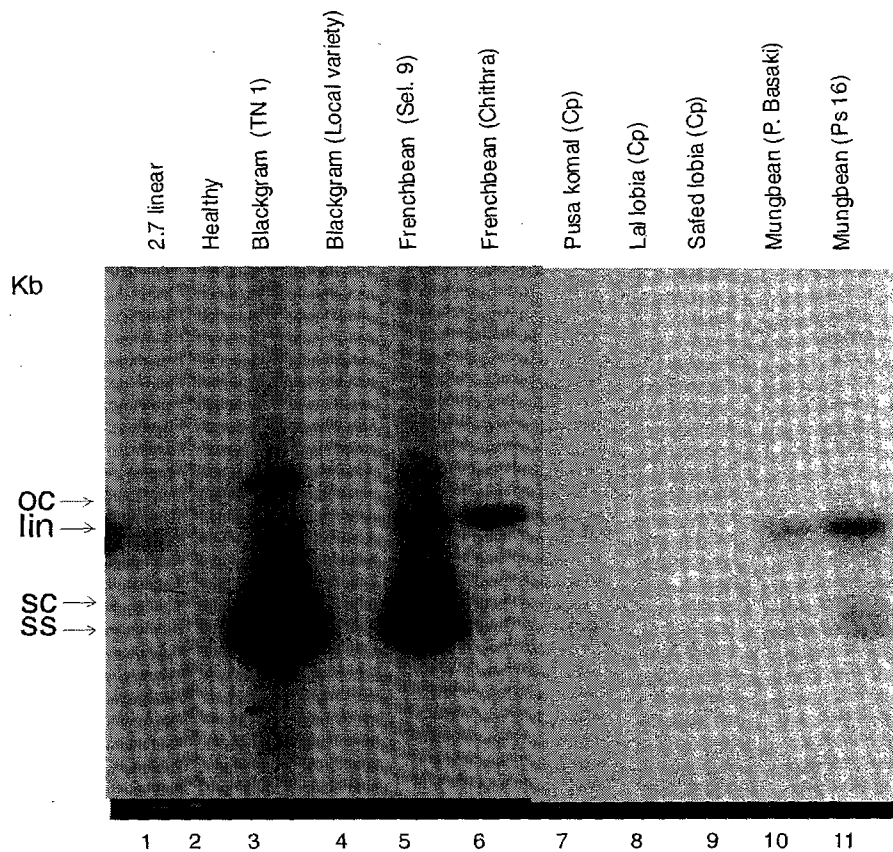


Fig. 14. Southern blot analysis of viral replicative forms in agroinoculated legume plants. DNA extracted from healthy blackgram plant (lane 2). Bg isolate inoculated on blackgram (lanes 3 and 4), French bean (lains 5 and 6), cowpea (lains 7, 8 and 9), and mungbean (lains 10 and 11). lin : double stranded linear; sc : double stranded supercoiled; ss : single stranded DNA forms are indicated.

Table 10. Primers used for site directed mutagenesis of ORF AV2 in blackgram isolate

Primer	Orientation	Sequence (5'-3')	Mutation site	Nucleotide co-ordinates	Codon changed in AV2
BgM ₁	FP	5'-AACCCCACTAACAACTCTGGGATCCATTG-3'	ATG to ATC	144-170	1
BgM ₁	RP	5'-CAATGGATGCCAGATTGTTAGTGGGT-3'	Met to Ile		
Bg del A	FP	5'-GCGGACCTACGA↓TACTGCATTCTAC-3'	Deletion A	324-351	Nucleotide 336
Bg del A	RP	5'-GTGAGAAATGCAGT↓ATACGTAGTCGC-3'			
BgW ₂	FP	5'-CACTAACAAATGTGGGATCCATTGTTG-3'	TGG to TCG	148-173	2
BgW ₂	RP	5'-CCACAAATGGATCCGACATTGTTAGTG-3'	Trp to Ser		
BgH ₁₄ G ₁₅	FP	5'-CCAAATCAGTGCAAGAAATCCGGTGCATG-3'	CAT to CAA (His to Gln)	183-212	14 and 15
BgH ₁₄ G ₁₅	RP	5'-CATGCACCCGGAATTTCTTGCACTGATTGG-3'	GAA to GAA (Gly to Glu)		
BgY ₃₂	FP	5'- GTTCAAGAGAAATTCCCTTCTAAATTC-3'	TAC to TTC	237-282	32
BgY ₃₂	RP	5'- GAATTAGAAGGGAATTTCTCTTGAAC-3'	Tyr to Phe		

The nucleotide changed is shown in *italic bold*.

FP : Forward primer (viral sense), RP : Reverse primer (complementary sense)

Table. 11. Primers used for site directed mutagenesis of ORF AV2 in cowpea isolate

Primer	Orientation	Sequence (5'-3')	Mutation site	Nucleotide co-ordinates	Codon changed in AV2
CpM ₁	FP	5'-CCCAGTAACAATCTGGGATCCAATTG-3'	ATG to ATC	146-170	1
CpM ₁	RP	5'-CAATGGATGCCAGATTGTTACTGGG-3'	Met to Ile		
CpW ₂	FP	5'-CAGTAACAATGTCCGGATCCATTGTTG-3'	TGG to TCG	148-173	2
CpW ₂	RP	5'-CAACAAATGGATCCGACATTGTTACTG-3'	Trp to Ser		
CpH ₁₄ G ₁₅	FP	5'-CCGAATCACTGCAAGAAATTCGGTGTCATG-3'	His to Gln (CAT to CAA)	184-212	14 and 15
CpH ₁₄ G ₁₅	RP	5'-CATGCACCGGAATTCATTGCAATGATTCGGG-3'	GAA to GAA (Gly to Glu)		
CpK ₇₃	FP	5'-TGCACGGAGAGGATTAAACTT-3'	AAG to AGG	363-383	73
CpK ₇₃	RP	5'-AAGTTTAAATCCTCTCCGTGCA-3'	Lys to Arg		
CpC ₈₆	FP	5'-TGCCTGCCGTCACTGGGAGGTGT-3'	TGT to AGT	401-422	86
CpC ₈₆	RP	5'-ACACCTCCCACTGACCGCAGGCA-3'	Cys to Ser		
CpC _{84.86}	FP	5'-TGCTGCCAGCGTCAGTGGGA-3'	TGC to AGC (Cys to Ser)	398-417	84 and 86
CpC _{84.86}	RP	5'-TCCCCACTGACGCTGGCAGCA-3'	TGT to AGT (Cys to Ser)		
CpG ₄₄	FP	5'-GTACCTTACAGACTTAATTCAGG-3'	GCG to GAC	275-200	44
CpG ₄₄	RP	5'-CCTGAATTAAAGTCTGTAAAGGTAC-3'	Gly to Asp		

The nucleotide changed is shown in italic bold; FP : Forward primer (viral sense), RP : Reverse primer (complementary sense).

mutations in Bg isolate and seven mutations in Cp isolates were made by overlapping PCR method using specific primers. Mutations introduced are shown diagrammatically in pairwise alignment of amino acid sequences of Bg and Cp isolates in Fig. 15, details are given in Table 10 and 11.

In mutation 1 (BgM₁ and CpM₁) the start codon methionine is altered; it is expected to give rise to a truncated AV2 protein, with N' terminal deletion of 18/19 amino acid.

Mutation 2 (Bg delA) was introduced only for Bg isolate. Pair-wise nucleotide sequence alignment of Bg and Cp isolates revealed (Fig. 16) presence of nucleotide 'A' at 336 position of the genome; due to which AV2 protein in Bg isolate is truncated. This mutation was done to restore the frame in Bg isolate AV2 and see whether the complement of full length AV2 protein will alter the pathogenesis of Bg isolate.

In other mutations, BgW₂, BgY₃₂, BgH₁₄G₁₅ and CpW₂, CpH₁₄G₁₅, CpK₇₃, CpC₈₆, CpC₈₄₋₈₆ and CpG₄₄ amino acids were substituted as given in Table 10 and 11. In the case of Cp isolate, ORF AV2 overlaps with ORF AV1. The mutations which are introduced after the overlapping region, are CpK₇₃, CpC₈₆, CpC₈₄₋₈₆. Expression strategy of these overlapping ORFs is not yet clear for this virus. Whether the mutations introduced affect the ORF AV1 was verified by change of nt at CpK₇₃, led to change of amino acid K to R (lysine to arginine) in the case of ORF AV2 led to change in R to G (arginine to glycine) in ORF AV1.

4.1.3.1 Overlapping PCR

After PCR amplification for 30 cycles with complementary primers with altered nucleotide, the PCR products were electrophoresed. In gel

	10	20	30	40
MYMIV	MWDPLLND	KSVHGFRC	ML AIKYLQQV	QE NYPSNSLGYS
MYMIV-[CP]	MWDPLLND	ESLHGFRC	ML AIKYLQQV	QE NYPSNSLGFL
	50	60	70	80
MYMIV	YLTGLIQVLR	IRNHAKADLR	--ILHS----	----LPRYRM
MYMIV-[CP]	YLTGLIQVLR	IRNHAQADLR	YGLLYPDIEC	TEKIKLRYPA
	90	100	110	
MYMIV	HGGD-----	-----	-----	---
MYMIV-[CP]	RAACVCGRCP	YQHEEKTLDQ	PPHVEETSVL	PTV

Fig. 15. Pair-wise alignment of the predicted amino acid sequences of the AV2 protein of Bg and Cp isolates. Dashes indicate gaps introduced for maximum alignment. Mutation sites are coloured

electrophoresis a linear band of 5.4 kb (the vector pUC18 along with the full-length viral DNA (2.7 kb insert + 2.7 kb vector) containing the mutation was seen. Initially, plasmid DNA template of concentration 50, 100, 150 ng were evaluated for amplification with *Pfu* DNA polymerase. Amplifications were done for 12-16 cycles for mutation M_1 following manufacturer's recommendations. Number of transformants were less with 12-16 cycles, amplification for 30 cycles gave more transformants which were followed for all other mutations.

4.1.3.2 Restriction with *Dpn*I enzyme to remove the non-mutated wild type DNA

In order to remove the non-mutated wild type DNA of original plasmid from *E. coli*, the PCR product was restricted with *Dpn* I at 37°C for 1 h and an aliquot (10 µl) of DNA was electrophoresed in 1% agarose gel. *Dpn* I treatment had almost removed the template parental methylated DNA as evident from the lack of fluorescence of oc and sc forms of plasmids (Fig. 17a). The *Dpn* I treated PCR product was used for transformation.

4.1.3.3 Transformation

Transformation of *E. coli* strain DH5α (Fig. 17b) with the *Dpn* I treated product, produced more than 40 colonies per plate. In the case of Cp isolate, for mutants CpM₁ and CpW₂, number of colonies were less.

The putative transformants were screened for the presence of unit genome length DNA A molecule. They were screened in following ways.

a. Colony PCR

About 20 colonies of transformants were subjected to colony PCR using AV-abut/AC-Abut primers which were expected to give full length (2.7 kb)

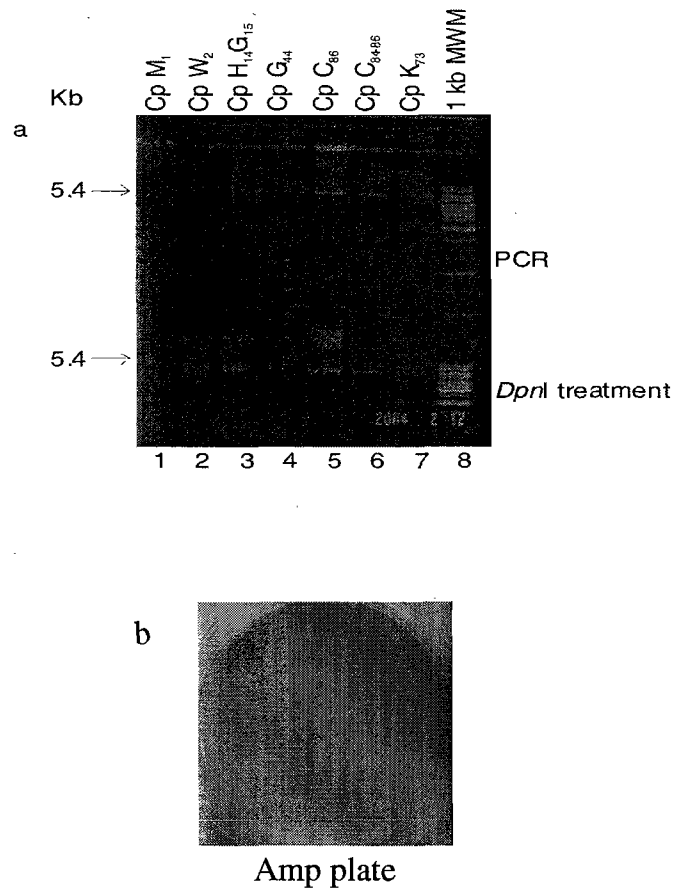


Fig. 17. Agarose gel electrophoresis of overlapping PCR products (a) amplified with primers having desired mutation. pCpH2 plasmid DNA was the template. The products before (upper panel) and after *Dpn* I restriction (lower panel) of different mutations are shown. Lane 8 : 1 kb DNA ladder. 1-7 : mutations of Cp isolates. (b) *E. coli* DH5a cells transformed with CpC₈₄₋₈₆ plasmid. Transformants colonies in ampicillin plates.

product. In each mutation at least 10 colonies were screened, out of which 4-6 were positive showing 2.7 kb molecules.

b. Restriction digestion

The PCR positive colonies were inoculated into Luria broth, and grown at 37°C for 16 h at 200 rpm. Plasmid DNA was prepared and restricted with *Hind* III/*Bgl* I enzymes. *Hind* III/*Bgl* I restriction was expected to release the 2.7 kb viral insert from the vector pUC 18 which was restricted into two fragments of 1.0 and 1.7 kb. Most of the PCR positive clones released, 2.7 kb viral insert.

Four colonies of each transformants confirmed by the three ways, were sequenced (Delhi University, South Campus).

Sequences were compared with the wild type and the clones having desired mutants were selected for further work. The chromatogram of the sequence reactions confirming the mutations are given in Fig. 18 and 19. Normally the frequency of mutation within the transformants were around 25%. For all the mutation study, one of the mutated clones was selected for making head to tail partial repeats of the genome in the Ti plasmid derivative pBin19.

4.1.3.4 Preparation of tandem partial repeats

pBinBgA0.6 mer and pBinCp0.6 mer, having 1.8 kb fragment of DNA A were used to generate tandem repeats. Unit genome length 2.7 kb fragment of DNA containing mutation were released from every mutant by *Hind* III/*Bgl* I restriction. The full length linear *Hind* III fragment was ligated with linearized pBinBgA0.6 mer and pBinCpA0.6 mer to give pBinBgA1.6 mer and pBinCpA1.6 mer. For each mutation, a minimum ten colonies were

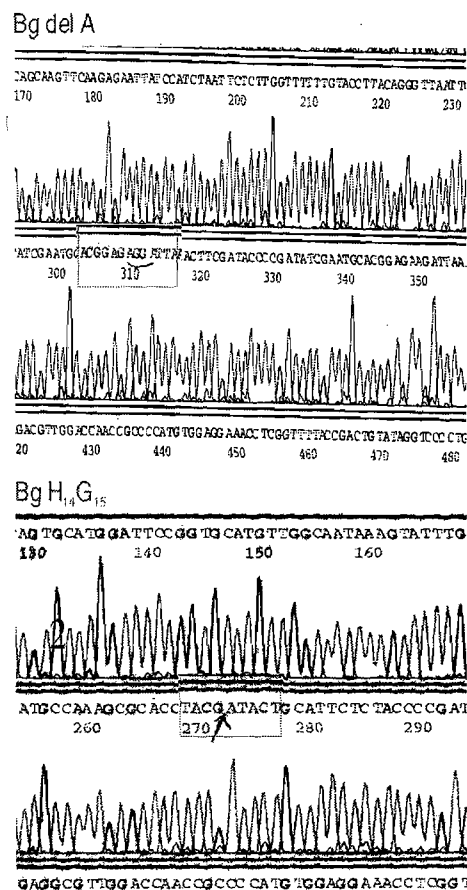
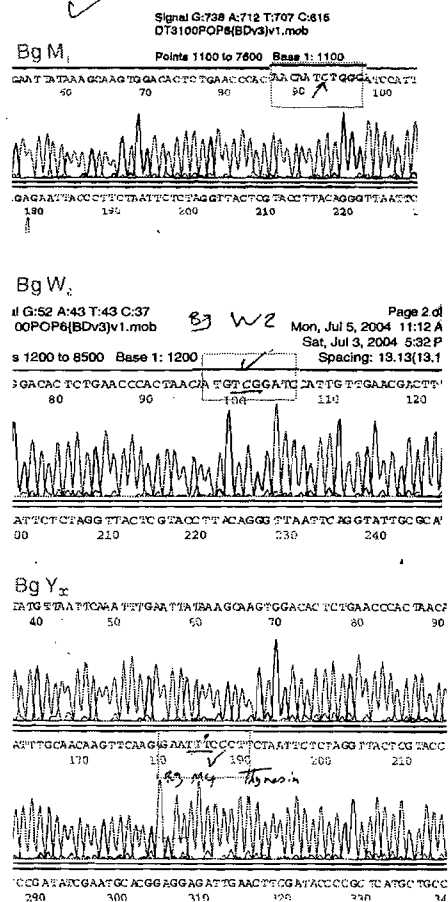


Fig. 18. Chromatogram showing nucleotide sequence of ORF AV2 of Bg isolate. The nucleotide altered after site directed mutagenesis is highlighted.

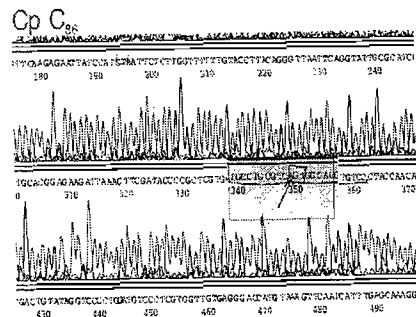
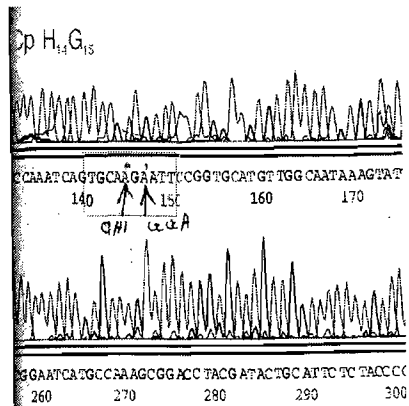


Fig. 19. Chromatogram showing nucleotide sequence of ORF AV2 of Cp isolate. The nucleotide altered after site directed mutagenesis is highlighted.

screened. Miniprep plasmid DNA restriction with *kpn* I and colony PCR were done to confirm the recombinant clones (Fig. 20a, 20b). Restriction with *Kpn* I, as expected gave rise to 2.7 kb length band confirming the tandem orientation of the partial repeats (head to tail) orientation.

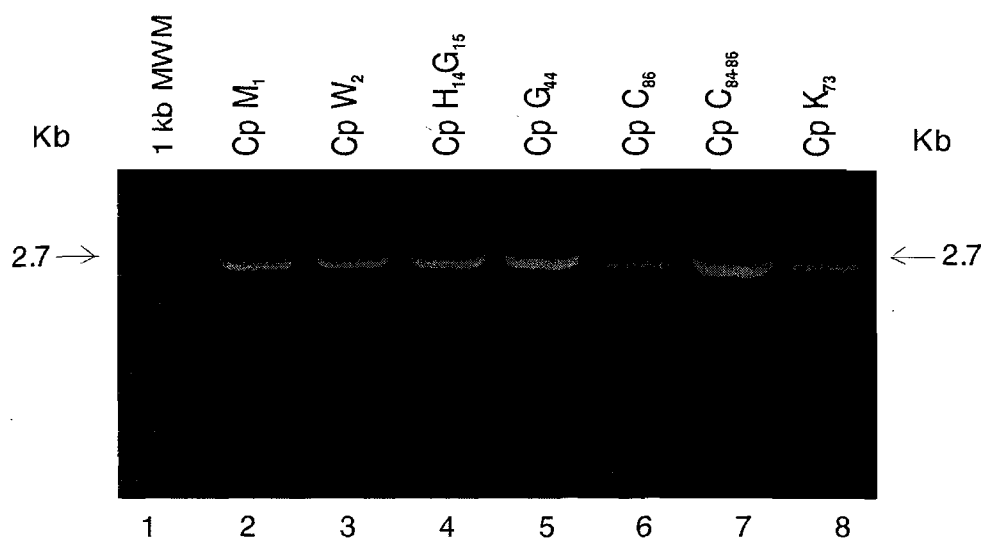
One each, of tandem partial repeats construct of every mutant was selected for mobilization into *Agrobacterium tumefaciens* by triparental mating.

4.1.3.5 Triparental mating

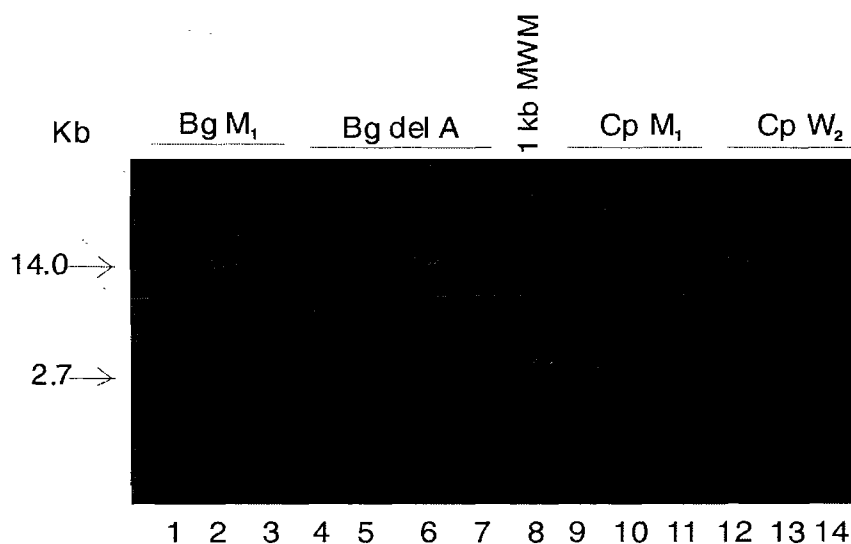
The selected tandem partial repeat constructs of pBinBgA1.6 mer, pBinCpA1.6 mer were introduced into *A. tumefaciens* strain EHA 105 by triparental mating. More than 100 transconjugant colonies were obtained per plate on rifampicin-kanamycin agar. Presence of the constructs in transconjugants were confirmed by PCR and restriction analysis. A 2.7 kb full length amplicon in PCR and a 2.7 kb band with restriction with *Kpn* I confirmed the presence of the partial tandem repeat construct. One of the transconjugant was lifted, streaked on Luria agar plate containing rifampicin-kanamycin, and used for agroinoculation.

4.1.3.6 Agroinoculation

Two hundred µl of overnight grown *Agrobacterium* culture having partial repeat constructs, in Luria broth containing rifampicin-kanamycin, were spread on Luria agar plates having same antibiotics. The 36 h grown *Agrobacterium* culture in Luria agar plate were scrapped and suspended in 500 µl B5 medium per plate. The wild type DNA (pBinBgA1.6 mer and pBinCpA1.6 mer) and the mutants DNA A were mixed separately with DNA B (partial tandem repeat pBinBgB1.5 mer and pBinCp2.0 mer). The mixture



a



b

Fig. 20. Confirmation of tandem partial repeat constructs of mutant DNA A components in pBin19 vector. a : Agarose gel electrophoresis of PCR products of pBinCp1.6 mers of mutants. Amplification was done with AV/AC abut primers. Lane 1 : 1 kb DNA ladder; lane 2 to 8 : amplification from putative 1.6 mer having M₁, W₂, H₁₄G₁₅, G₄₄, C₈₆, C₈₄₋₈₆, K₇₃ mutations. 2.7 kb product confirms full length DNA insert. (b) Agarose gel electrophoresis of restriction fragments of tandem partial repeat constructs of pBinBgA1.6 mers and pBinCpA1.6 mer. Plasmid DNA of 1.6 mer of mutants of BgM₁ (lanes 1-3) Bg del A (lanes 4-7) and CpM₁ (lanes 9-11), CpW₂ (lanes 12, 13) were restricted with *Kpn* I. Release of full length 2.7 kb fragment confirmed the tandem orientation.

of A and B cells were finally suspended in 1.0 ml of B5 medium (500µl each), and used for sprout- seed inoculation as described in Materials and Methods. Germinating seeds (48 h) were given 10 pricks with fine needle (G22) at root hypocotyl region (Fig. 8) on which 40 µl of *Agrobacterium* cells were applied. After 4 h of co-cultivation at 28°C, the seeds were sown. 10-12 germinating seeds of blackgram, cowpea, French bean and mungbean plants were inoculated per mutation in each experiment time. Plants were maintained in growth chambers at National Phytotron Facility at 27±2°C, RH 85 and day light 18000 Lux. Symptom development was monitored for 30 days. Viral replicative forms were analysed by extracting DNA from third trifoliolate leaves pooled from three plants. Southern blot analysis were performed using radiolabelled probe to PCR amplified DNA fragment of CP and MP region of the genome.

The results on infectivity and viral pathogenesis are summarized below.

4.1.3.7 Infectivity of wild and ORF AV2 mutants of Bg and Cp isolates on selected legume hosts

Percent infectivity was computed from the number of plants showing symptoms out of number of plants inoculated. The pooled data of five experiments are given in Tables 12 and 13.

4.1.3.7.1 Bg isolate

There was marginal increase in number of plants showing symptoms in the case of mutants over wild type in blackgram, cowpea and mungbean (Table 12). The increase was more pronounced in blackgram (32 to 40%) and mungbean (64 to 83%). In the case of cowpea, a host in which wild type is not compatible considerable increase from 24.7% to 46% was seen in BgW₂

Table 12. Infectivity of wild type and mutants of Bg isolate on legume hosts by agroinoculation

Virus isolates	Host	No. of plants infected/ No. of plants inoculated	Average infectivity (%)	Symptoms*
*BgA + BgB	Blackgram	11/34	32.3	YM
	Cowpea	5/18	27.7	St, LC, Pk
	French bean	16/16	100.0	St, LC
	Mungbean	35/54	64.8	YM
BgM₁ + BgB	Blackgram	13/32	40.6	YM
	Cowpea	5/19	26.3	St, LC, PK
	French bean	20/20	100.0	St, LC, PK
	Mungbean	36/43	83.7	RYM
Bgdel A + BgB	Blackgram	21/33	63.6	YM
	Cowpea	7/18	38.8	MLC
	French bean	16/16	100.0	St, LC, PK
	Mungbean	27/36	75	RYM
BgW₂ + BgB	Blackgram	13/36	43.3	YM
	Cowpea	7/15	46.6	MLC
	French bean	14/14	100.0	St, PK
	Mungbean	25/30	83.3	YM
BgH₁₄G₁₅ + BgB	Blackgram	17/24	76.0	AYM
	Cowpea	6/17	35.3	MLC
	French bean	15/16	93.0	St, LC
	Mungbean	25/33	76.7	AYM
BgY₃₂ + BgB	Blackgram	18/33	57.6	YM
	Cowpea	5/15	33.3	MLC, RYM
	French bean	15/15	100.0	St, LC
	Mungbean	30/32	93.7	YM

YM = Yellow mosaic; RYM : Restricted yellow mosaic; AYM : Attenuated yellow mosaic;
LC = Leaf curl; MLC : Mild leaf curl; St : Stunting; PK : Puckering.

*Wild type

Table 13. Infectivity of wild type and mutants of Cp isolate on legume hosts by agroinoculation

Virus isolates	Host	No. of plants infected/ No. of plants inoculated	Average infectivity (%)	Symptoms*
Wild type	Blackgram	23/49	46.5	YM
	Cowpea	17/28	60.6	GM, DWLC, VC
	French bean	25/25	100.0	St, DWLC, PK
	Mungbean	32/56	64.0	YM
CpM ₁ + CpB	Blackgram	18/32	57.0	YM
	Cowpea	9/23	39.1	YM, LC, VC
	French bean	14/14	100.0	St, LC, PK
	Mungbean	17/30	56.0	YM
CpW ₂ + CpB	Blackgram	8/21	36.6	RYM
	Cowpea	9/17	52.9	RYM
	French bean	12/12	100.0	St, LC, PK, RYM
	Mungbean	12/17	70.0	RYM
CpH ₁₄ G ₁₅ + CpB	Blackgram	17/34	50.0	AYM
	Cowpea	9/14	64.2	RYM
	French bean	11/12	91.0	St, MLC
	Mungbean	9/16	56.0	RYM
CpK ₇₃ + CpB	Blackgram	9/36	30.0	YM
	Cowpea	10/16	62.5	GM, MLC, VC
	French bean	13/13	100.0	St, MLC
	Mungbean	14/22	63.0	YM
CpC ₈₆ + CpB	Blackgram	10/29	34.4	YM
	Cowpea	9/16	56.2	YM, MLC, VC
	French bean	13/13	100.0	St, RYM, LC
	Mungbean	24/30	80.0	YM
CpC ₈₄₋₈₆ + CpB	Blackgram	9/28	32.1	RYM
	Cowpea	8/3	61.5	RYM, MLC, VC
	French bean	12/12	100.0	St, LC
	Mungbean	15/21	71.0	YM
CpG ₄₄ + CpB	Blackgram	8/25	32.0	YM
	Cowpea	8/13	61.5	YM, MLC
	French bean	14/14	100.0	St, RYM, DWLC
	Mungbean	14/22	63.0	YM

YM = Yellow mosaic; RYM : Restricted yellow mosaic; AYM : Attenuated yellow mosaic; GM : Golden mosaic; LC = Leaf curl; MLC : Mild leaf curl; DWLC : Downward leaf curl; VC : Vein clearing; St : Stunting; PK : Puckering.

mutant. In the case of French bean, except for the mutation BgH₁₄G₁₅ which showed about 7% decrease in the percent infectivity, there was 100% infectivity, in all the cases.

Cp isolates

In the case of Cp isolate, different results were obtained. In the homologous host, in the mutation CpM₁, in which there is N' terminus deletion of 18 amino acid ORF AV2, the percentage infectivity (Table 13) is reduced atleast by 30%. In other mutations there were only marginal variations. In the case of mungbean compared to wild type, there was increase upto 80%, in the mutation CpC₈₄₋₈₆. In CpW₂ the increase was very minimal, other mutants did not have any effect. In the case of blackgram, CpM₁ and CpH₁₄G₁₅ slightly increased the percentage infectivity over wild type. As in the case of blackgram isolate, in French bean, except CpH₁₄G₁₅, which decreased the percentage infectivity (10%) in other mutations, no differences were observed, and 100% infectivity was seen.

4.1.3.8 Symptoms produced by wild and ORF AV2 mutant of Bg and Cp isolates

Symptoms were monitored throughout, upto 30 days. Unlike percent infectivity, ORF AV2 disruption resulted in significant changes in the symptoms. Attenuation of symptoms were seen in most of the mutants, compared to the wild type.

4.1.3.8.1 Bg isolate

Very small restricted yellow mosaic appeared within 7-10 days in first trifoliate leaf, in blackgram and mungbean (Fig. 21 and 24), which did not further intensify and spread. Unlike wild type, the chlorotic area was very

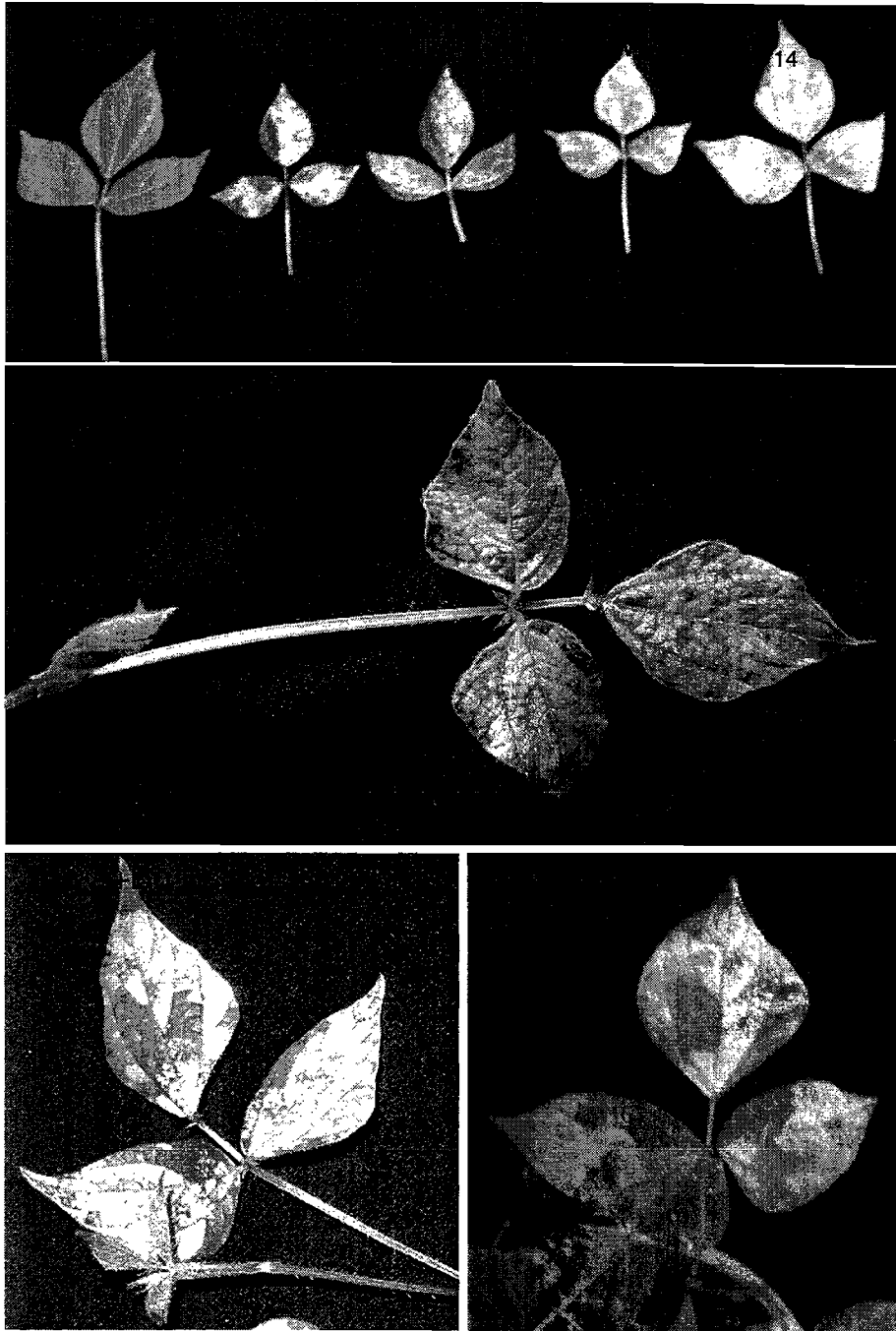


Fig.21. Blackgram (cv. TN-1) plants showing symptoms after agroinoculation with wild type or mutants of Bg isolate attenuation of symptoms in M_1 and $H_{14}G_{15}$.

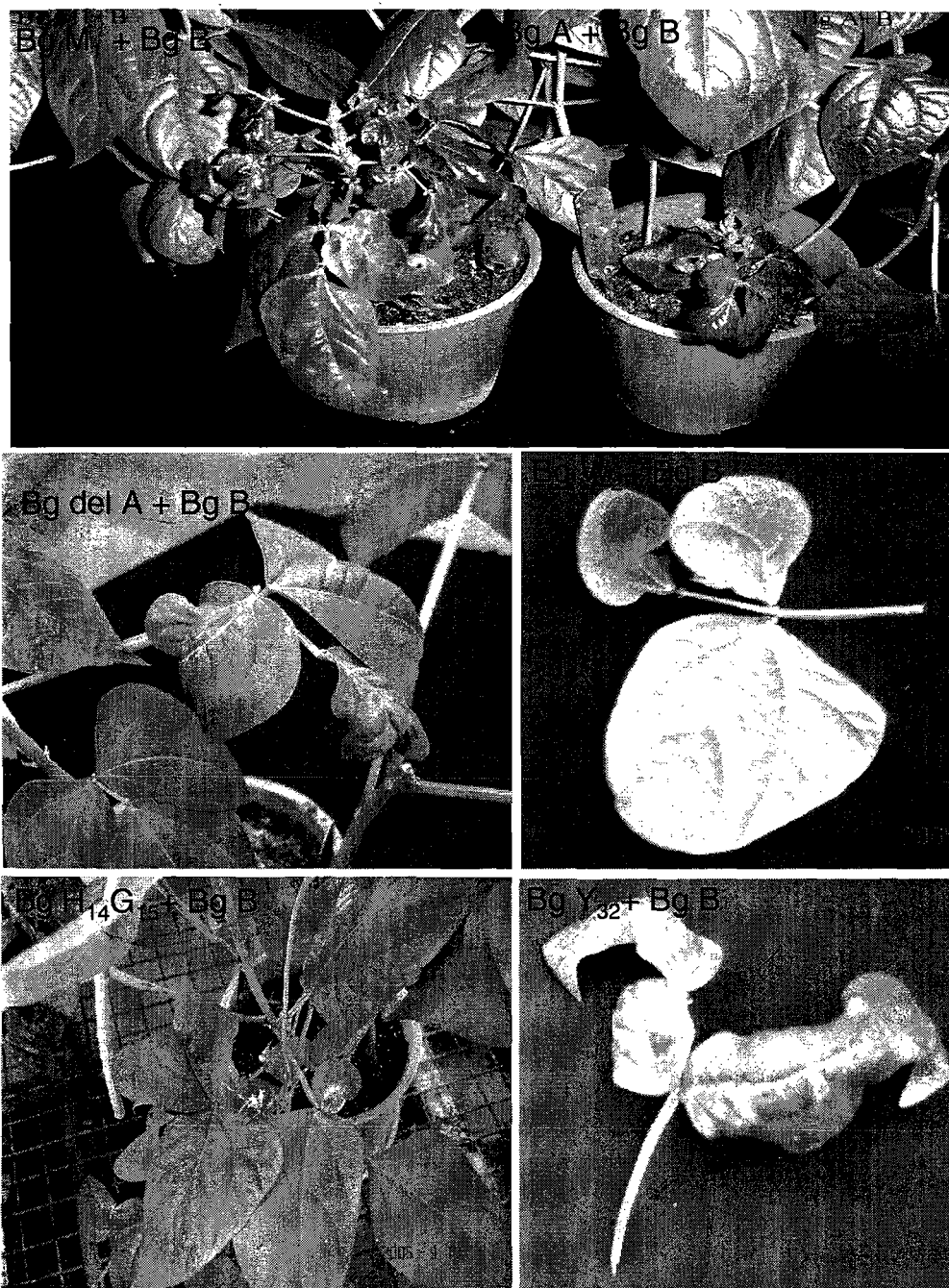


Fig. 22. Cowpea (cv. Pusa Komal) plants showing symptoms after agroinoculation with wild type or mutants of Bg isolate. atypical symptoms in wild type which is mild in the mutants.

faint and appeared scattered. In the case of mutants, BgM₁ and BgW₂ the yellow areas spread only to a limited extent and so blotches of yellow chlorotic area altering with green area were seen. While wild type caused reduction in leaf lamina size, in the mutants BgH₁₄G₁₅, leaf lamina size was not reduced.

In the case of cowpea (Fig. 22), which is not a well adopted host of the Bg isolate, atypical symptoms of leaf curl seen in the wild type is also seen in the mutants. However, leaf curl was mild, stunting was less and asymmetry between the leaflets of the trifoliate leaf, reduction in leaf size and dark green appearance of the leaves were seen randomly in different leaves (Fig. 22). In the mutant BgY₃₂ small chlorotic area appeared, along with curling.

Attenuation was more evident in French bean (Table 12 and 14), wild type produced severe stunting, downward leaf curling of the cotyledonary leaves (Fig. 23). The symptoms are so severe that there is no elongation of shoot, and production of first trifoliate leaf were not seen. However, in the case of mutants, elongation of the shoot occurred and new trifoliate leaves appeared. Stunting was less pronounced, trifoliate leaves though produced, were reduced in size, and showed puckering (Fig. 23).

Compared to wild type, symptoms were very much attenuated in the mutants BgW₂ and BgH₁₄G₁₅. However symptoms were more pronounced in the case of BgY₃₂. Number of plants showing wild type symptoms were more. Both wild type and mutants showed symptoms 5-7 DAI in French bean and cowpea and 7-9 DAI in the case of blackgram mungbean plants.

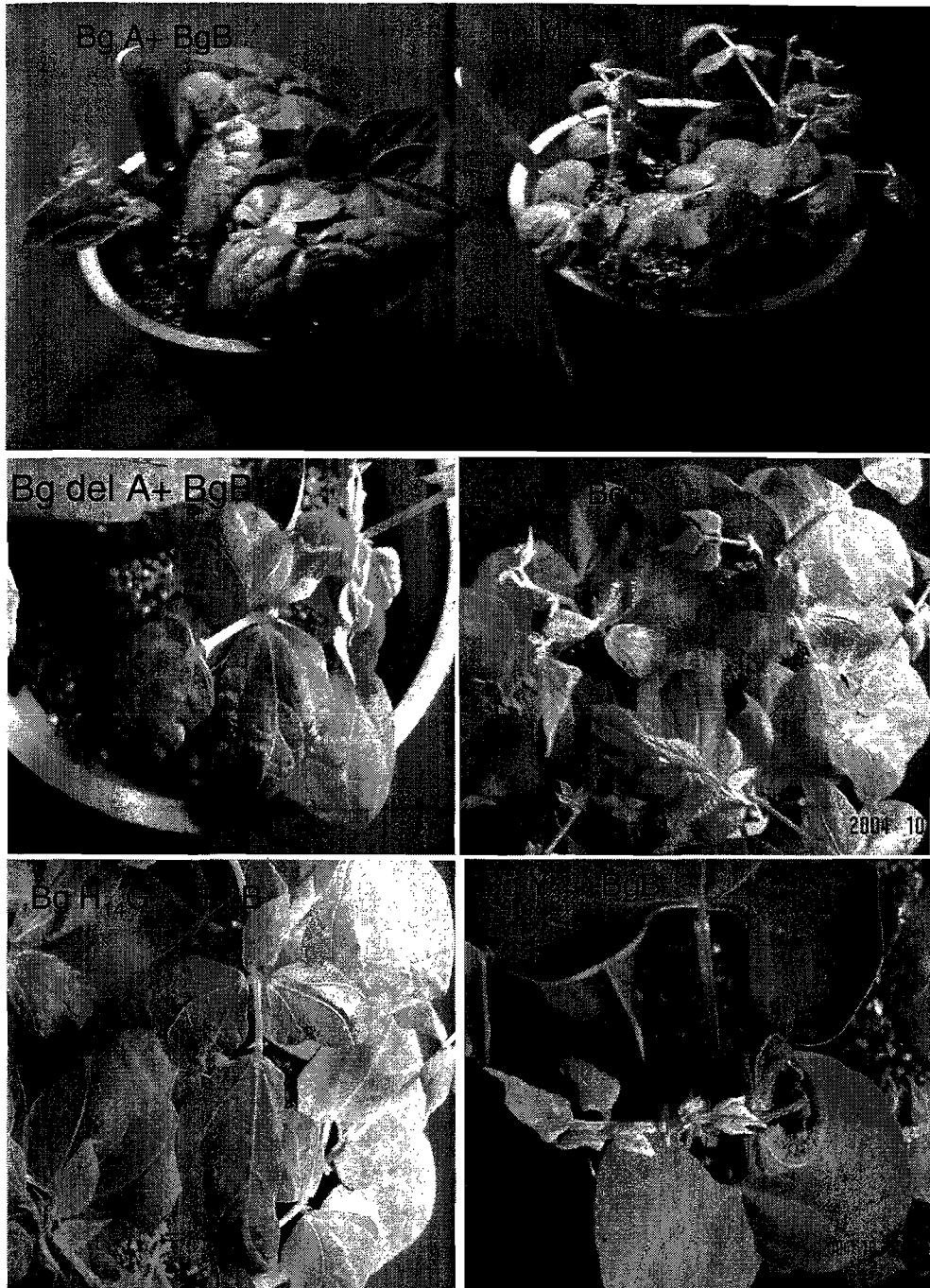


Fig. 23. French bean (cv. Selection 9) plants showing symptoms after agroinoculation with wild type or mutants of Bg isolate. Attenuation of symptoms in mutants.



Fig. 24. Mungbean (cv. Ps 16) plants showing symptoms after agroinoculation with wild type or mutants of Bg isolate. Attenuation prominent in M_1 and W_1 , $H_{14}G_{15}$.

Table 14. Disease severity of wild type and mutants of Bg and Cp isolates on legume hosts

Virus isolates	Hosts			
	Blackgram	Cowpea*	French bean [#]	Mungbean
BgA + BgB	4	1	4	4
BgM ₁ + BgB	3	1	3	3
BgdelA + BgB	3	1	3	3
BgW ₂ + BgB	2	1	2	2
BgH ₁₄ G ₁₅ + BgB	2	1	2	2
BgY ₃₂ + BgB	2	1	2	2
CpA + CpB	4	4	3	4
CpM ₁ + CpB	3	3	3	3
CpW ₂ + CpB	3	2	3	2
CpH ₁₄ G ₁₅ + Bg	3	2	2	2
CpK ₇₃ + CpB	4	4	3	4
CpC ₈₆ + CpB	4	4	3	4
CpC ₈₄₋₈₆ + CpB	4	4	3	4
CpG ₄₄ + CpB	3	3	3	3

- 1 : < 25% yellow mosaic
 2 : 25-50% yellow mosaic
 3 : 50-75% yellow mosaic, leaf curl
 4 : Bright yellow mosaic, stunting, crinkling
 * : Shows atypical symptoms with Bg isolate
 # : No typical yellow mosaic, grade based on stunting.

4.1.3.8.2 Cp isolate

The differences in symptoms between wild type and mutants were more clear in the case of cowpea and French bean plants (Fig. 27). The blackgram (Fig. 25) and mungbean (Fig. 28) plants showed, well distributed yellow mosaic, throughout the lamina, as comparable to wild type in all the cases except the mutations CpW_2 and $CpH_{14}G_{15}$. In these two mutants, very sparse, restricted small squares of faint yellow chlorotic area were seen (Fig. 28). Symptoms were almost not visible in majority of the plants. However, leaf size and growth of the plants were not affected.

The symptoms in French bean were very interesting. In the case of mutants, stunting and puckering was less severe, compared to Cp wild type, it was less than 50% in the mutant CpW_2 . Interestingly, different type of symptoms were observed. Very small, chlorotic area were seen, which is very different from the wild type. Yellow mosaic, puckering and downward leaf curl symptoms (Table 3 and 14) were recorded in CpC_{84+86} , mild and restricted yellow mosaic was seen in CpG_{44} and CpK_{73} ; this change in the symptom is very significant as MYMIV isolates so far are known to produce only leaf curl and stunting in French bean and not yellow mosaic (Fig. 27). No differences were seen between wild and mutants in number of days taken to produce symptoms after inoculation.

In cowpea (Fig. 26), the downward leaf curl symptoms seen in inoculations with wild type is less prominent in all the mutations. It was mild in all the cases, and was totally absent in the mutants, CPW_2 and $CpH_{14}G_{15}$. In both, leaf size was normal, restricted blotches of yellow mosaic were seen in $CpH_{14}G_{15}$ contrasting to wild type. In wild type small veinlets began to show yellow, leading to broad big veins yellowing and finally yellowing of

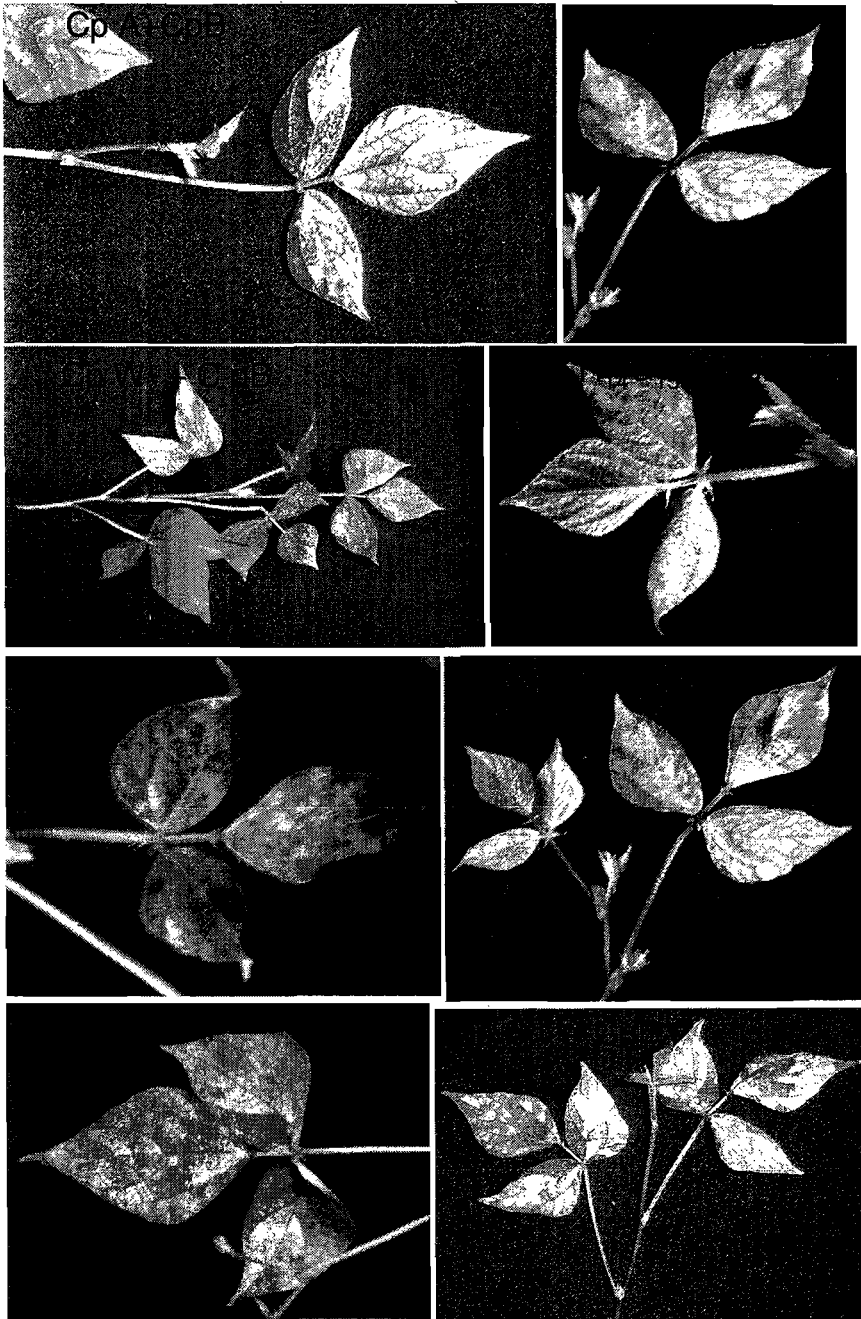


Fig. 25. Blackgram (cv. TN-1) plants showing symptoms after agroinoculation with wild type or mutants of Cp isolate. Very faint yellow specks symptoms in W_1 , $H_{14}G_{15}$.

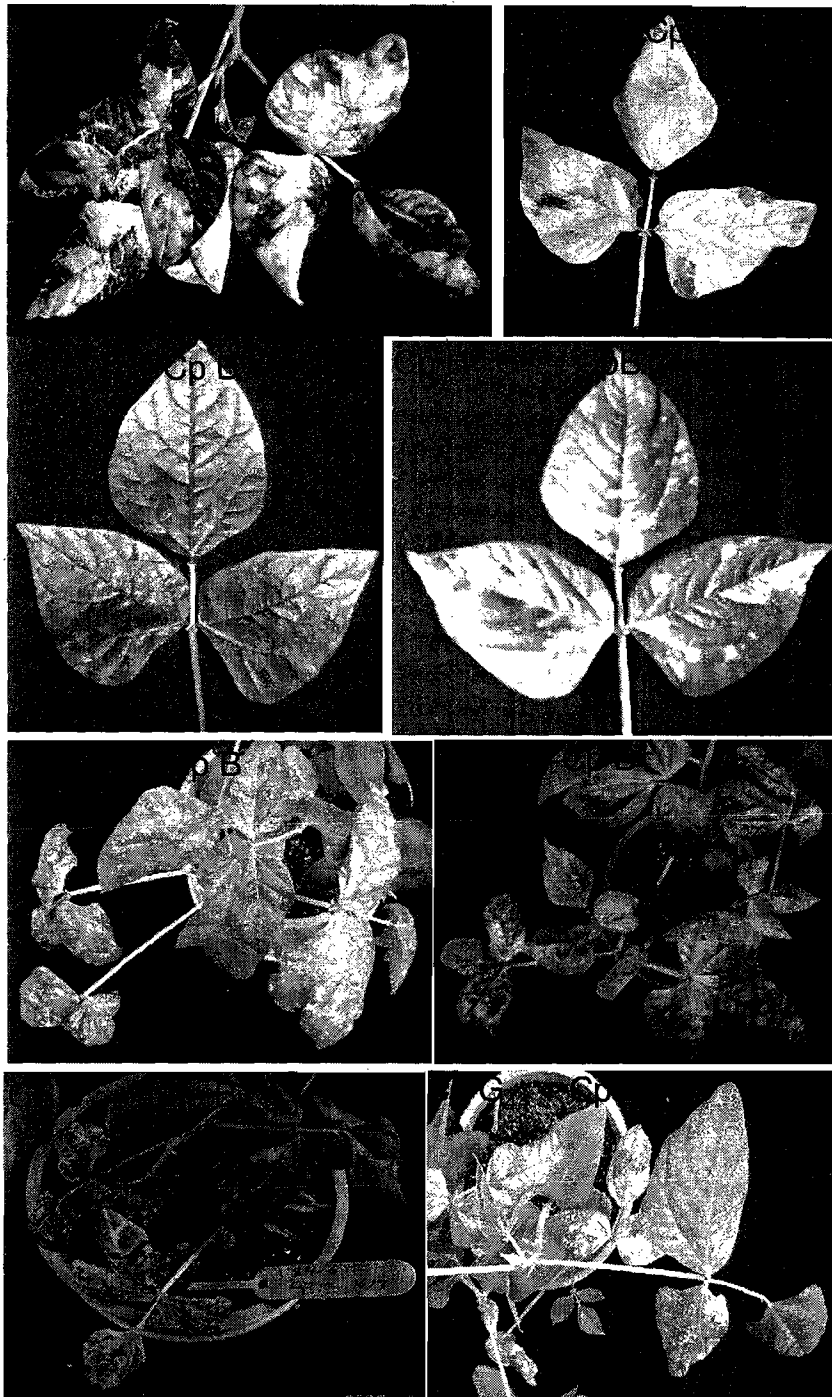


Fig. 26. Cowpea (cv. Pusa Komal) plants showing symptoms after agroinoculation with wild type or mutants of Cp isolate. Absence of leaf curl in W_2 and $H_{14}G_{15}$ vein clearing in C84-86 more. K_{73} and G_{44} -restricted yellow mosaic.



Fig. 27. French bean (cv. Selection 9) plants showing symptoms after agroinoculation with wild type or mutants of Cp isolate. More attenuation in H₁₄G₁₅, yellowing in C₈₄₋₈₆.



Fig. 28 Mungbean (cv. Ps 16) plants showing symptoms after agroinoculation with wild type or mutants of Cp isolate. Attenuation in M_1 , W_2 and $H_{14}G_{15}$.

whole leaf. Vein clearing appeared only in few leaves and golden mosaic predominated. Contrasting to wild type, in the mutants, CPM₁, CPK₇₃, CpC₈₄₋₈₆, vein clearing and yellow net of veins were more prominent. The significant difference in symptoms were seen in the mutant Cp₈₄₋₈₆, which were more severe than wild type.

4.1.3.9 Viral replication in the plants inoculated with wild and ORF AV2 mutants of Bg and Cp isolates

4.1.3.9.1 Bg isolate

Remarkable changes in the level of viral replicative forms were seen in the plants inoculated with the mutants, compared to wild type. The double stranded, open circular (oc), linear (lin), super coiled (sc) and single stranded (ss) forms were identified based on treatment with *Exonuclease III*, *S₁ Nuclease*, *RNase A*, and *DNase I* (Fig. 13). Marked differences were seen in Blackgram and French bean plants inoculated with BgM₁ and Bgdel A. In these two mutations, oc, sc and ss viral DNA were more than wild type (Fig. 29 and 30). It was four folds more than wild type in visual comparison. Characteristic changes were seen in the viral replicative forms (Table 15) in the four host species inoculated by mutants BgW₂, BgH₁₄G₁₅ and BgY₃₂. The blackgram plants inoculated with H₁₄G₁₅ of BgW₂ and BgY₃₂, characteristically supercoiled and single stranded DNA accumulation was less. In the case of French bean plants, reduction in ss DNA level was seen in BgH₁₄G₁₅ and BgY₃₂ mutants and not in BgW₂. As viral replication was poor in cowpea, differences could not be identified. In mungbean samples as such DNA levels were low, however, compared to wild type ss DNA accumulation was low. Results were more significant in DNA A level than in DNA B (Fig. 29 and 30).

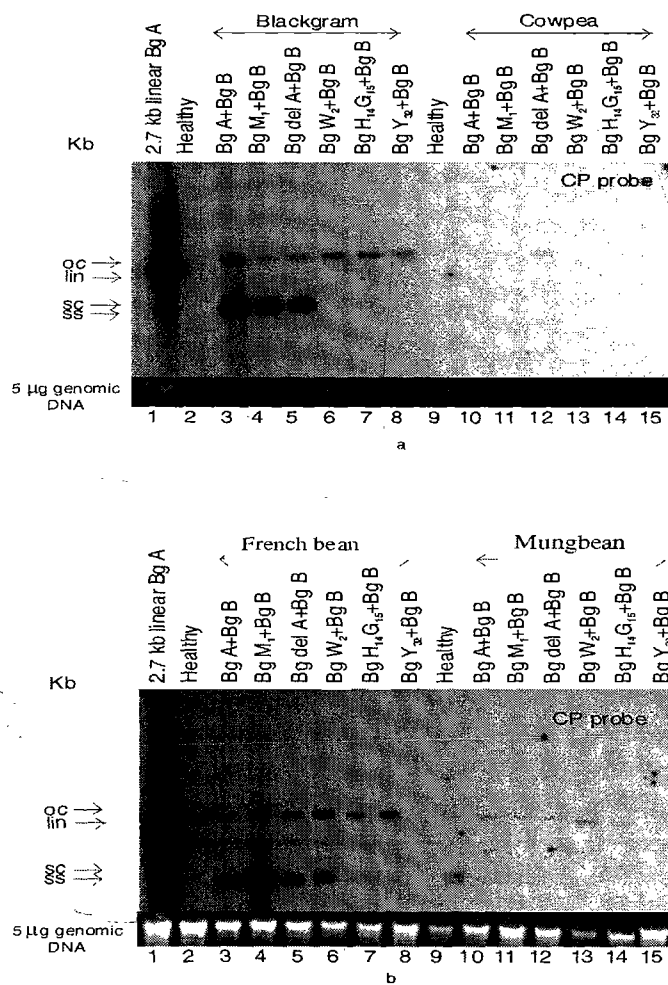


Fig. 29. Southern blot analysis of viral replicative forms in legume hosts, agroinoculated with Bg isolate. Radiolabeled coat protein gene was used as probe. oc- open circular, lin- linear, sc- supercoiled, ss- single stranded.

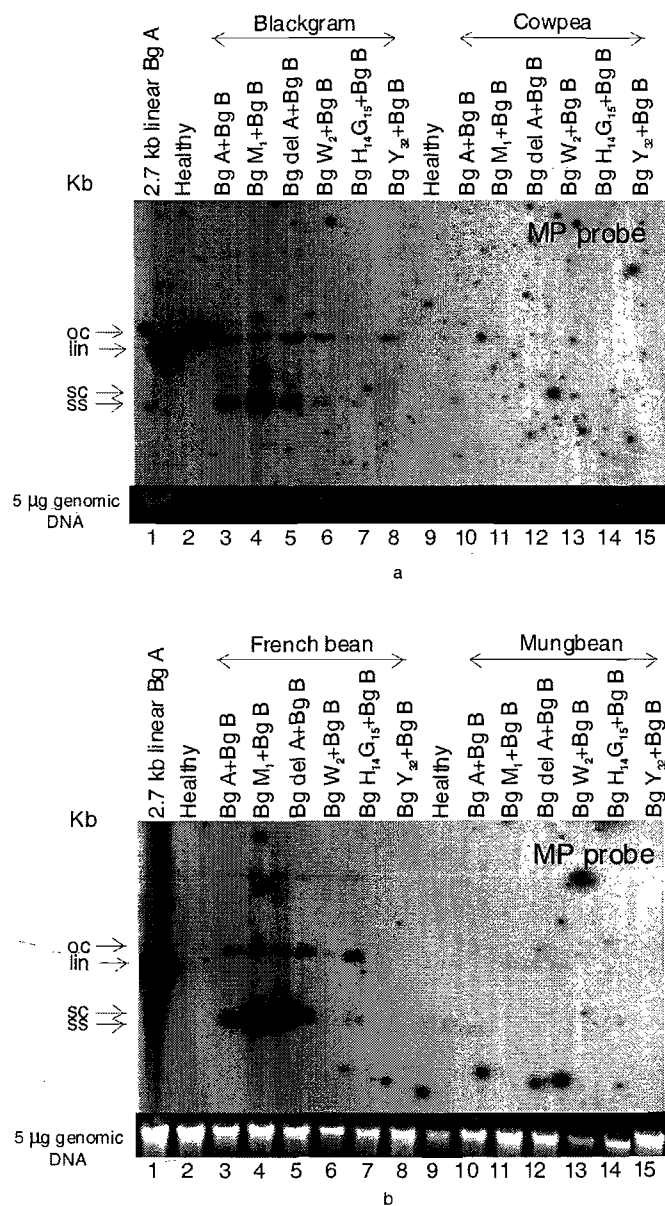


Fig. 30. Southern blot analysis of viral replicative forms in legume hosts, agroinoculated with Bg isolate. Radiolabeled movement protein gene was used as probe. oc-open circular, lin- linear, sc- supercoiled, ss- single stranded.

4.1.3.9.2 Cp isolate

Differences in the viral DNA accumulation was also seen (Fig. 31 and 32) in the case of Cp isolate mutants. However, some of the results were contrasting to what was seen with Bg isolate. For example in the case of French bean, the level of viral DNA forms showed variation. Reduction in sc and ss DNA was very prominent in blackgram and cowpea in the case of CpM₁, CpW₂ and CpH₁₄G₁₅ and in cowpea plants with CpW₂ and CpH₁₄G₁₅. In the mutation CpH₁₄G₁₅, CpM₁ ss and sc DNA were low. In the case of cowpea, there was two folds increase in ds open circular forms in the mutation and four folds increase in ss/ sc forms CpK₇₃ and CpG₄₄ and CpC₈₆, CpC₈₄₋₈₆ in blackgram, cowpea and French bean plants. There was significant reduction in all the DNA forms in blackgram, mungbean plants inoculated with CpH₁₄G₁₅ mutant. In the case of mutants CpW₂ and CpC₈₆ and CpC₈₄₋₈₆, reduction in the accumulation of ssDNA (Table 15) was very clear (Fig. 31 and 32) in the case of mungbean.

4.1.3.10 Reversion of mutation

In the case of plants inoculated with CpM₁ and BgM₁ 27 DAI, six out of 11 plants inoculated showed wild type symptoms. Whether the mutation introduced is maintained at all was verified, ORF AV2 from agroinoculated plants was amplified with AV2 FP and AV2RP primers; PCR products were sequenced and compared with wild type. Unexpectedly in the mutation BgM₁, the original start codon was restored (Fig. 33). In other cases, mutations were maintained.

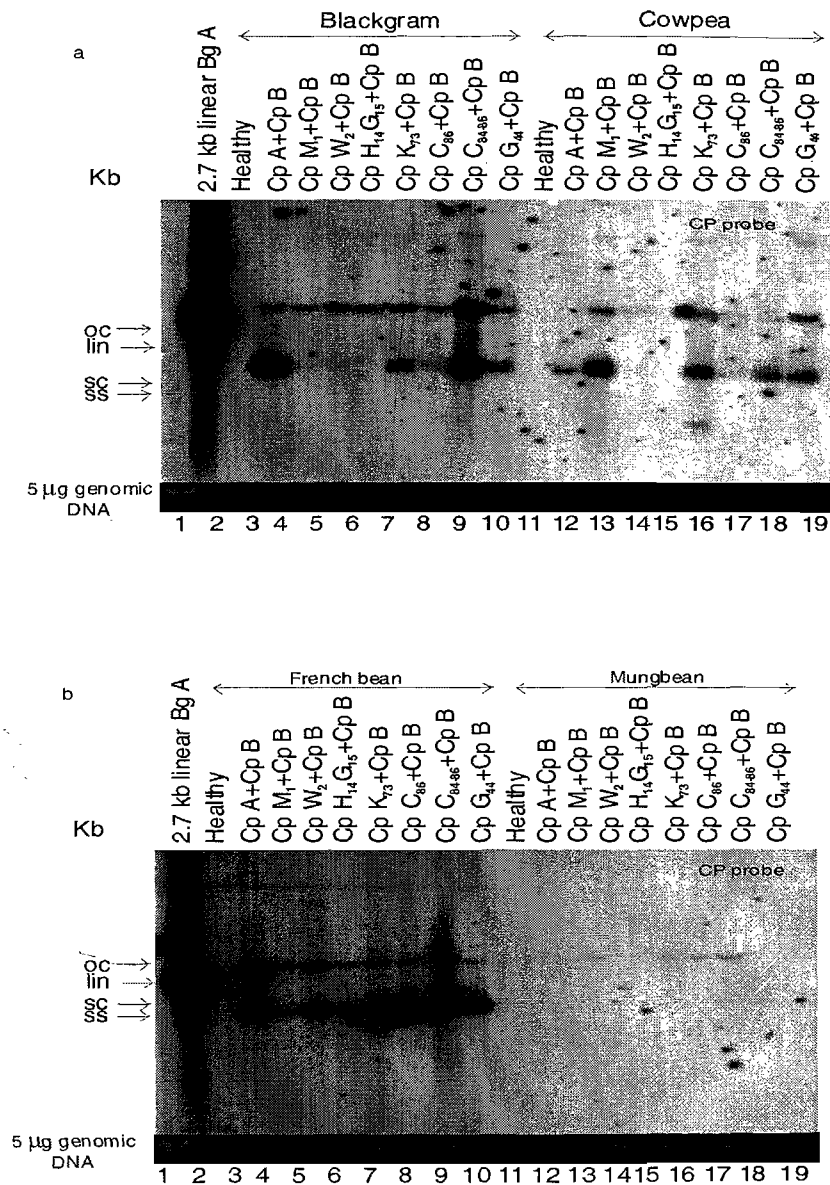


Fig. 31. Southern blot analysis of viral replicative forms in legume hosts, agroinoculated with Cp isolate. Radiolabeled coat protein gene was used as probe. oc- open circular, lin- linear, sc- supercoiled, ss- single stranded.

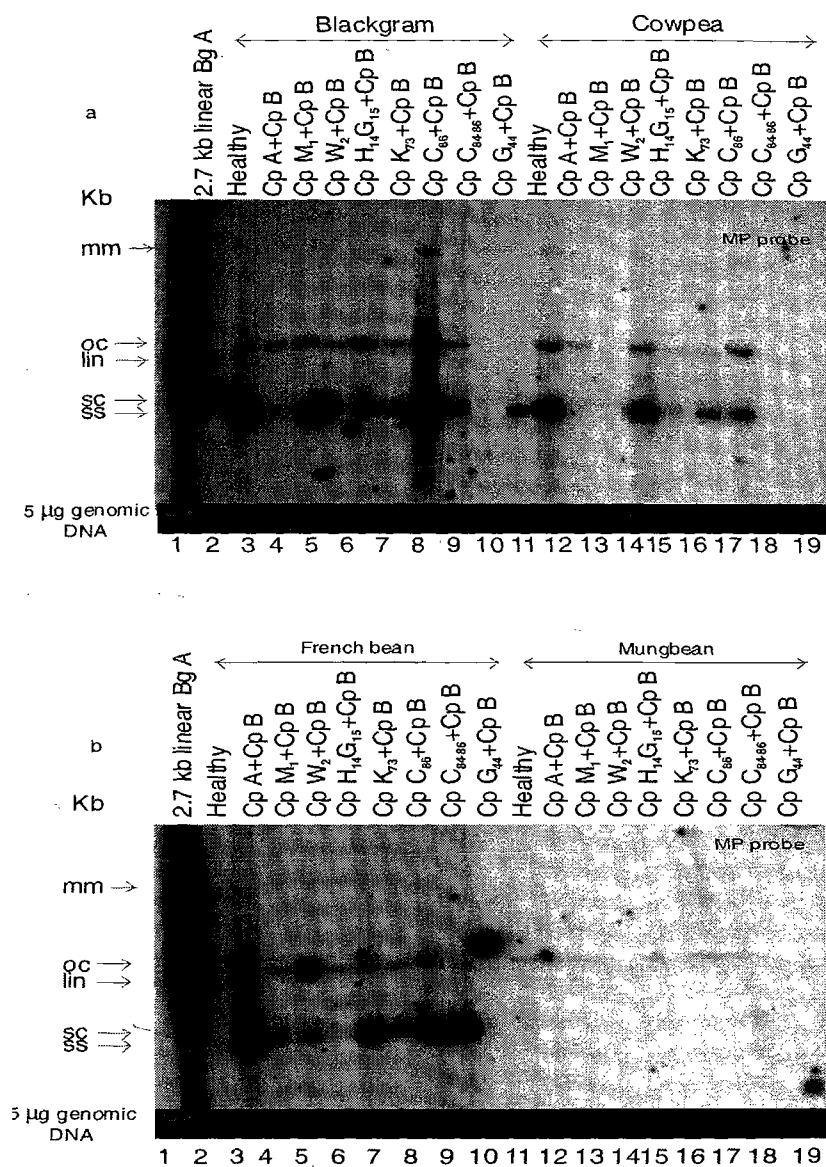


Fig. 32. Southern blot analysis of viral replicative forms in legume hosts, agroinoculated with Cp isolate. Radiolabeled movement protein gene was used as probe. oc-open circular, lin- linear, sc- supercoiled, ss- single stranded.

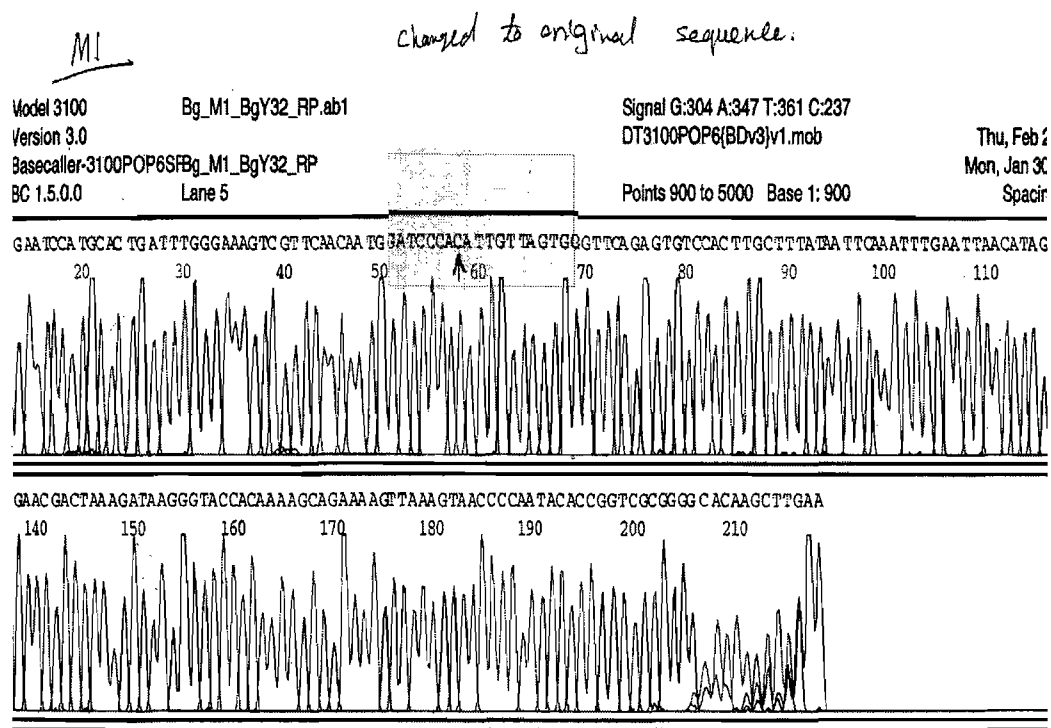


Fig. 33. Chromatogram of nucleotide sequence of ORF AV2 showing the reversion of mutation. The mutated site in BgM₁ (ATG to ATC) is reversed (Sequences are in complementary sense, mutated TAT changed to original sequence TAC).

Table 15. Viral replicative forms in legume hosts agroinoculated with wild and mutants Bg and Cp isolates

Virus isolate	Blackgram				Cowpea				Frenchbean				Mungbean			
	OC	Lin	SC	SS	OC	Lin	SC	SS	OC	Lin	SC	SS	OC	Lin	SC	SS
Bg isolate																
BgA + BgB	++++	++++	++++	++++	+	+	+	++	++++	++++	++++	++++	++	++	+++	+++
BgM ₁ + BgB	++	++	+++	+++	-	-	-	-	++++	++++	++++	++++	-	-	-	-
Bgdela + BgB	+++	++	+++	+++	-	-	-	-	+++	+++	+++	+++	-	-	-	-
BgW ₂ + BgB	++++	++++	-	-	-	-	-	-	+++	+++	++	+++	-	-	-	-
BgH ₁₄ G ₁₅ + BgB	+++	+++	-	-	-	-	-	-	+++	+++	+	+	-	-	-	-
BgY ₃₂ + BgB	++	++	-	-	-	-	-	-	++++	++++	+	+	+	+	-	-
Cp isolate																
CpA + CpB	+++	+++	++++	++++	+	+	+++	+++	++++	++++	++++	++++	+	+	+	+
CpM ₁ + CpB	++	++	-	-	+++	+++	+++	+++	++	++	++	++	++	+	-	-
CpW ₂ + CpB	+++	+++	-	-	+	+	-	-	+++	+++	++	++	+	+	-	-
CpH ₁₄ G ₁₅ + CpB	++	++	-	-	-	-	-	-	++	++	+	+	+	+	-	-
CpK ₇₃ + CpB	+++	+++	++	++	+++	+++	+++	+++	++	++	+++	+++	+	+	-	-
CpC ₈₆ + CpB	+++	+++	++	++	+	+	+	+	++	++	+++	+++	+	+	-	-
CpC ₈₄₋₈₆ + CpB	++++	++++	++++	++++	+	+	++	++	+++	+++	+++	+++	+	+	-	-
CpG ₄₄ + CpB	+++	+++	+++	+++	+++	+++	+++	+++	++	++	+++	+++	+	+	-	-

4.1.3.11 Whitefly transmission

Effect of ORF AV2 in the vector transmission was studied by conducting whitefly transmission in glasshouse condition. Plants agroinoculated with wild type or with mutants served as inoculum source. For both Bg isolate and Cp isolate inoculations were done on mungbean using mungbean plants as source plant. The experiment was done with 6-10 plants, and were repeated twice.

Table 16. Whitefly transmission of wild and mutants of Bg and Cp isolates on mungbean (*Vigna radiata*)

Virus isolates	Number of plants showing symptom/ Number of plants inoculated	Symptom expression (Days after inoculation)
BgA + BgB	6/12	12
BgM ₁ + BgB	2/13	18
BgdelA + BgB	—	—
BgW ₂ + BgB	—	—
BgH ₁₄ G ₁₅ + BgB	2/10	18
BgY ₃₂ + BgB	—	—
CpA + CpB	2/9	12
CpM ₁ + CpB	2/2	14
CpW ₂ + CpB	2/10	—
CpH ₁₄ G ₁₅ + Bg	—	—
CpK ₇₃ + CpB	—	—
CpC ₈₆ + CpB	3/8	12
CpC ₈₄₋₈₆ + CpB	3/9	12
CpG ₄₄ + CpB	6/15	16

Transmission done with 24 h of AAP and IAP.

In the case of Bg isolate, whitefly transmission was less when BgH₁₄G₁₅ mutant was the same and in other cases it was comparable with wild type but took more time (18 days compared to 14 days in wild type) for symptoms development. In the case of Cp isolate, in the mutation CpH₁₄G₁₅ and CpK₇₃, no transmission was seen (Table 16). In other cases it was comparable with wild type.

4.2 Biochemical characterization of ORF AV2 protein

The ORF AV2 is a well conserved gene in the begomoviruses from the Old World. The AV2 ORF overlaps with ORF AV1 in the viral strand of DNA A. Percentage identity between Old World begomoviruses for this region range from 45 to 66 percent. Exact role of ORF AV2 in the Old World begomoviruses is not yet elucidated. It is a small protein of ~ 10.0 kDa and ~ 13.0 kDa in Bg and Cp isolate. Helical wheel structure for the proteins is given in appendix. About 17.5 and 13.2% of the protein is composed of basic amino acid in Bg and Cp respectively. The estimated pI of the protein is 6.47 for Bg and 9.61 for Cp isolates. Except for one motif for phosphorylation site (TNK) in the Cp isolate, no other characteristic motif could be deciphered. Plants inoculated with infectious DNA which contained deletions in AV2 developed attenuated symptoms and accumulated low levels of both single stranded viral DNA. Results obtained in *in planta* system indicated that AV2 protein may play some role in replication. It was therefore, decided to express the AV2 protein in bacterial system and study its interaction with the replication initiation protein (Rep).

4.2.1 Over-expression of ORF AV2 in bacterial system

4.2.1.1 Cloning of ORF AV2 in pQE vector

A 222 bp long DNA encoding the AV2 protein of Bg isolate was amplified by PCR using AV2 FP and AV2 RP primers and cloned in pQE.30-TA vector. Overexpression was done in *E. coli*, M15 strain. A single colony of the clone was grown in Luria broth at 37°C, and induction with IPTG from 0.1 to 1 mM concentration and incubation for 5 h were evaluated for expression. The crude lysate, 5h after induction was analyzed in 12% SDS-PAGE. In SDS-PAGE analysis no protein band of increased intensity was observed. In Western blot analysis using anti 6xHis antibody. His tag-AV2 protein was not detected. Sequence analysis of the construct showed that ORF AV2 was not in frame with the start codon provided in the pQE.30-TA vector.

4.2.1.2 Cloning of ORF AV2 in pMAL vector

A 222 bp and 339 bp long DNA fragments encoding AV2 protein of Bg and Cp isolates respectively were amplified (Fig. 34b) and cloned in pMAL-p2X vector (Fig. 34a) in *Hind* III-*Eco*R I sites, downstream from the *malE* gene of *E. coli* which encodes maltose binding protein (MBP). The insertion of AV2 coding region in pMAL-p2x vector was confirmed by amplification of the plasmid DNA of transformant (Fig. 35a, b) and further by sequencing. One of the confirmed clone in each isolate (MBP-BgAV2 and MBP-CpAV2) was used for overexpression. The recombinant MBP-AV2 fusion protein of 52 kDa molecular weight was overexpressed in *E. coli*, Rosetta strain. The crude lysate of bacterial clone having MBP-BgAV2 and MBP-CpAV2 were electrophoresed in SDS-PAGE. Coomassie blue staining

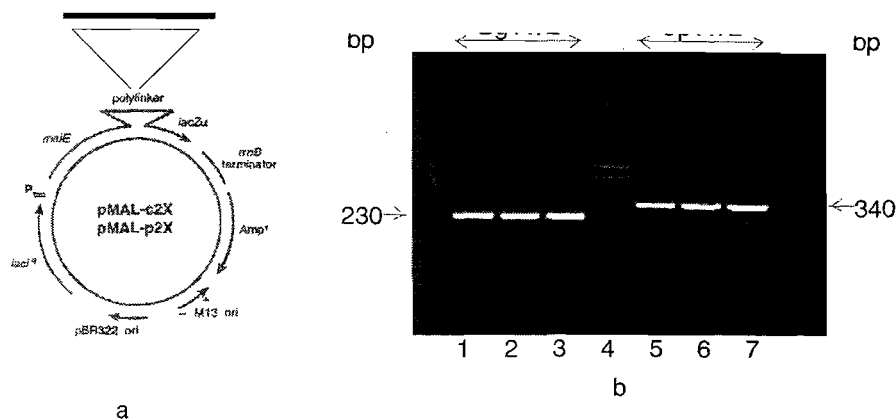


Fig. 34. Cloning of AV2 protein in pMAL vector for overexpression in the *E. coli* (a) physical map of pMAL vector showing *EcoR* I and *Hind* III sites; (b) gel electrophoresis of ORF AV2 amplicons, from Bg (1-3 lanes) and Cp isolate (5-7). Lane 4 : 1 kb DNA ladder

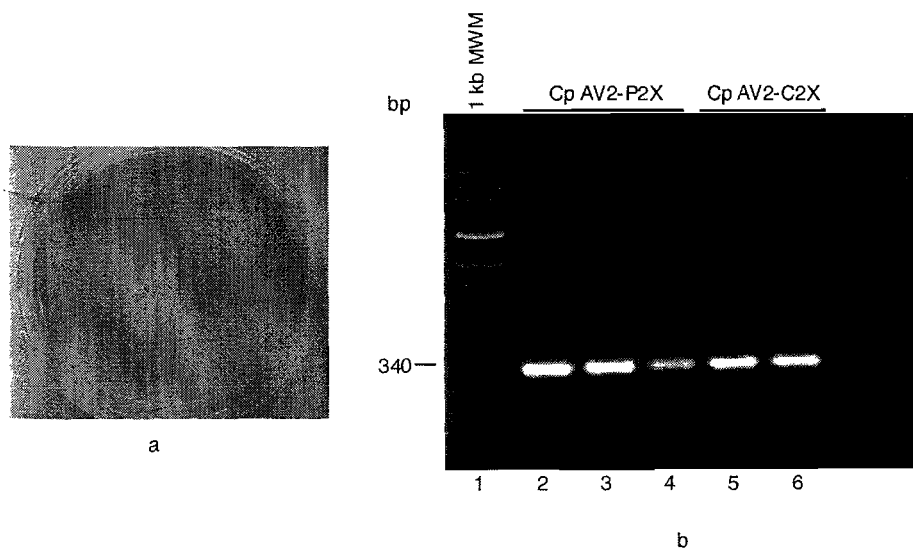


Fig. 35. (a) *E. coli* rosetta strain, transformants having pMAL-AV2 gene inserts, selected on ampicillin plates. (b) Confirmation of *E. coli* rosetta strain transformants for AV2 gene insertion in p2X/C2x vector by colony PCR gel electrophoresis of PCR products amplified with AV2F and AV2R primers.

revealed a band of high intensity in the lysate from induced cells compared to lysate from uninduced cells. The molecular weight of the fusion protein band was 52 kDa in the case of Bg isolate and 55 kDa in the case of Cp isolate.

4.2.1.3 Standardization of conditions required for optimal expression of AV2 protein

Different growth parameters and concentration of IPTG were evaluated for optimum expression of the protein. Concentration of IPTG, 0.1 to 0.5 mM were evaluated for induction and 0.4 mM was found to be highly suitable (Fig. 36).

Growth of the bacteria at different temperatures were attempted to get good expression of MBP-AV2 fusion protein in soluble fraction. After induction, bacterial cultures were incubated at 30-37°C for 6 h and the crude lysate was subjected to SDS-PAGE. MBP-BgAV2 was expressed well at 32°C, grown for 6 h. However, for Cp isolate, the temperature had to be reduced to 22°C for 16 h to produce MBP-CpAV2 fusion protein that are soluble.

To release the fusion protein from the bacterial cells, sonication for different duration and intensities were evaluated. Sonication (Fig. 37a) for 2 min, 10 second pulse each with enough time gap gave good yield of protein in soluble fraction preventing degradation.

4.2.1.4 Purification of the protein through amylose resin

The lysate from 200 ml culture was centrifuged, mixed with 30 ml of column buffer. After freeze thaw shock, the cells were sonicated. The sonicated cells were centrifuged at high speed and supernatant was mixed

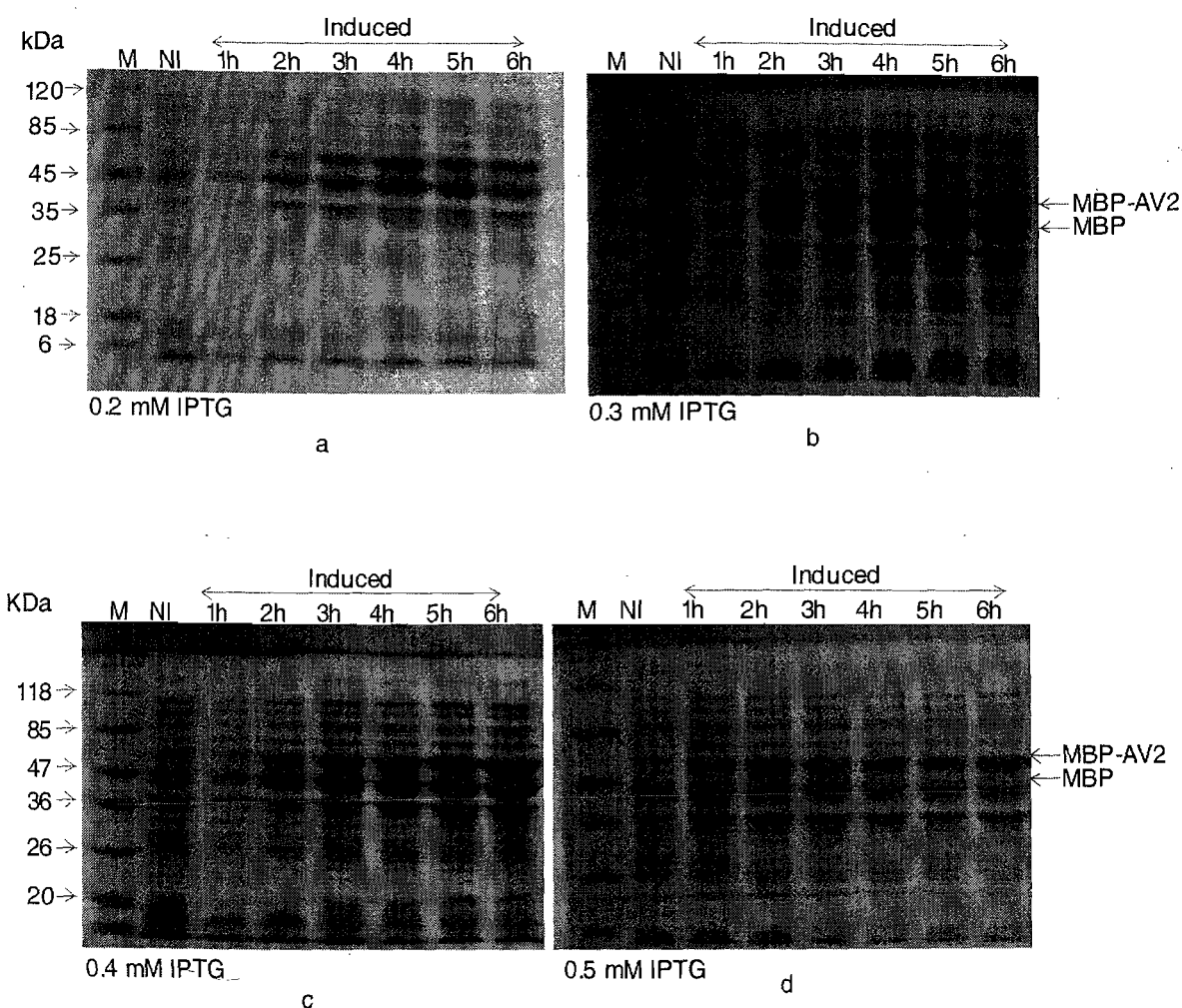


Fig. 36. Overexpression of recombinant AV2 proteins. SDS PAGE electrophoresis of lysate of cells grown at 32°C from 1 to 6 h after induction with different concentration of IPTG. (a) 0.2 mM, (b) 0.3 mM, (c) 0.4 mM, (d) 0.5 mM.

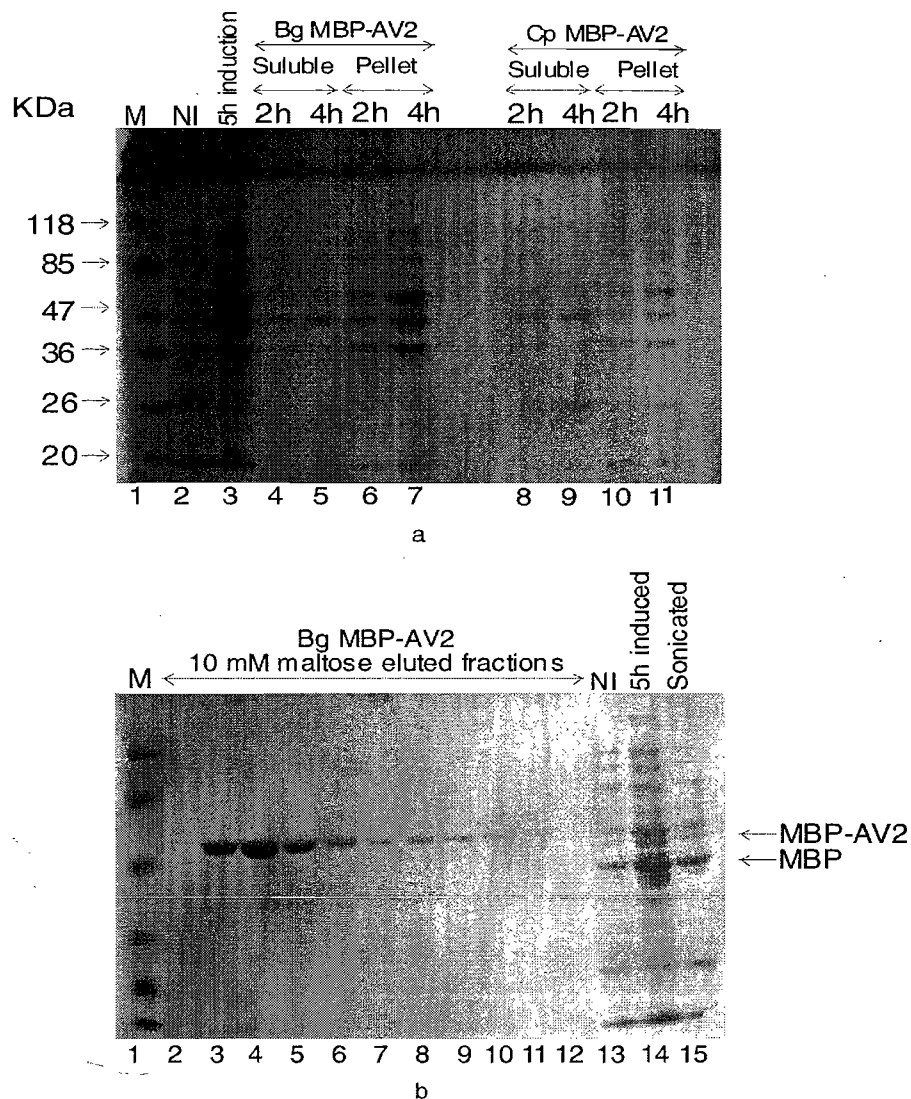


Fig. 37. SDS-PAGE electrophoresis of recombinant AV2 protein of Bg and Cp isolates. Protein released after sonication of lysate for 2 min duration, 10 times with 10 seconds pulse. Lane 1 : MW marker; lane 2 : non-induced culture; lanes 3 : lysate 5 h after induction; lanes 4, 6, 8, 10 : 2 h after induction; lanes 5, 7 and 11 : 4 h after induction with 0.4 mM IPTG. MBPAV2 present both in soluble and insoluble fractions. (b) SDS-PAGE electrophoresis of purified MBP-BgAV2 protein. Recombinant proteins were purified by amylose resin column. The purified protein were eluted from amylose column in different fractions in 10 mM maltose. M : MW marker. lanes 2 to 11 : Aliquots of 10 mM eluted fractions; lane 13 : non-induced; lane 14 : 5 h after induction, lane 15 : 4 h after induction, supernatant of the sonicated lysate.

with 600 μ l amylose resin (Fig. 37b) (3 μ l resin/ml of lysate). Finally after washing MBP-AV2 fusion protein was eluted using 10 mM maltose. Ten fractions, each fraction of 150 μ l were collected. Ten μ l from each fraction was subjected to SDS-PAGE. Electrophoresis (Fig. 37b) revealed two bands of strong intensity, on par with 52 kDa band in the case of MBP-BgAV2 and 55 kDa band in the case of MBP-BgCpAV2. 42 kDa band of molecular weight marker. Both the bands reacted with anti MBP antibody; based on molecular weight, the upper band at 52kDa and 55 kDa is considered to represent the MBP-AV2 fusion protein in Bg and Cp isolate respectively and the common band 42 kDa to represent the MBP.

The amount of expressed AV2 in the soluble protein fraction of *E. coli* was reasonably high for Bg (Fig. 37b) isolate. It was 1 mg/100 ml culture, however the yield for Cp isolate was less. It was only 0.3 mg/100 ml culture. There were always two bands representing the fusion protein and the MBP (Fig. 38a). For biochemical assay, MBP protein alone was expressed and purified (Fig. 38b). In the Western blot, these two bands showed positive reaction with anti MBP antibody (Fig. 39a) confirming the band to be the MBP-AV2 fusion protein.

4.2.1.5 Release of AV2 protein by proteolytic digestion

The fusion protein was incubated with Factor Xa , the protease that would cleave the fusion protein after its four amino acid recognition site at Ile-Glu-Gly-Arg. About 0.5 μ g of Factor Xa was incubated with 20 μ g of MBP-AV2 protein at 37⁰C for 6 h. Products were subjected to SDS-PAGE. Three bands on par with 52 and 55 kDa were seen in the case of MBP-BgAV2 and MBP-CpAV2 respectively (Fig. 39b). Based on molecular weight and the reaction with anti MBP antibody they were identified to represent the

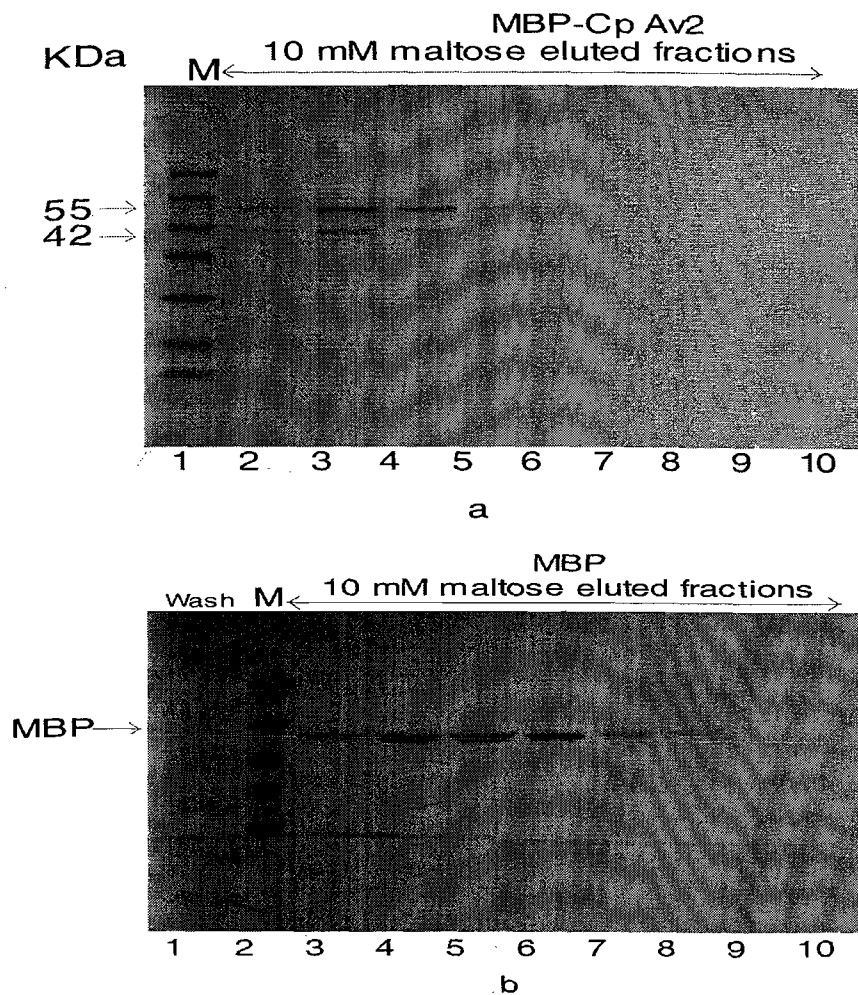


Fig. 38. SDS-PAGE electrophoresis of purified MBP-CpAV2 protein (a) and MBP (b). Recombinant proteins were purified by amylose resin column. The purified protein were eluted from amylose column in different fractions in 10 mM maltose. M : MW marker. lanes 2 to 9 : aliquots of 10 mM eluted fractions.

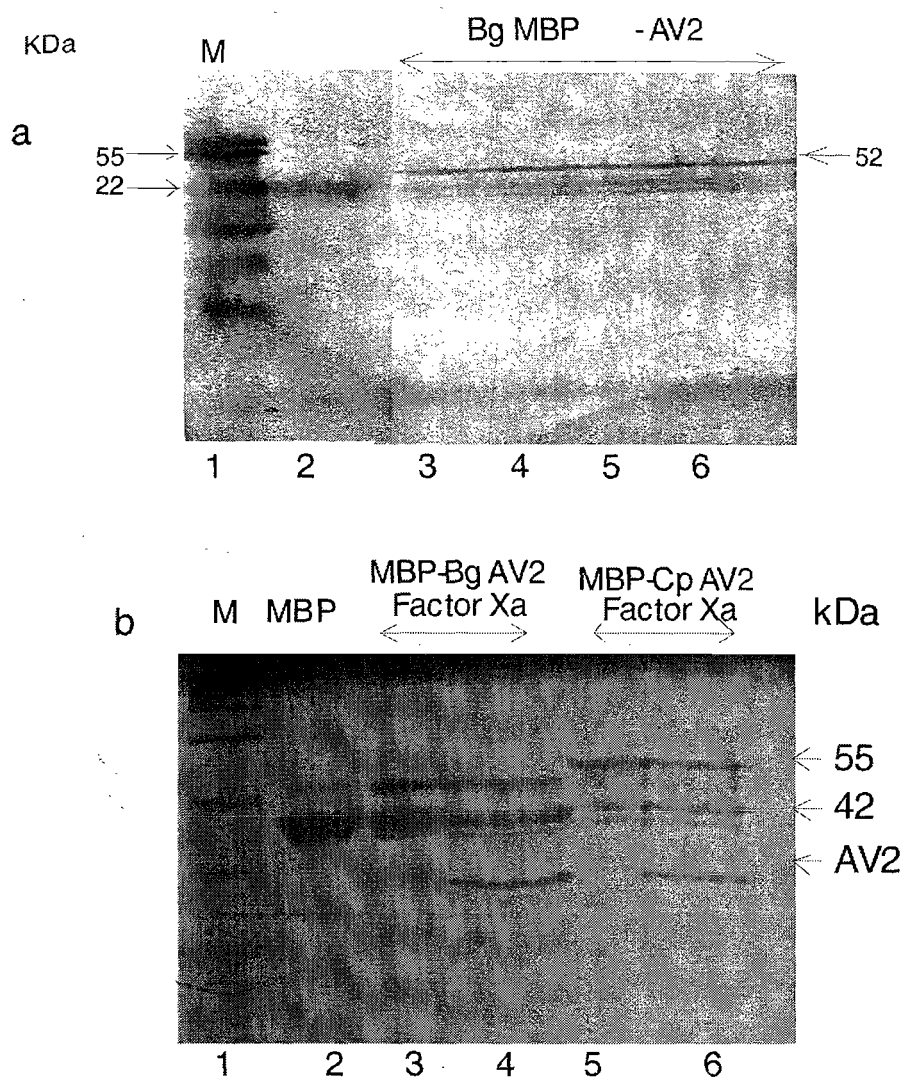


Fig. 39 (a). Confirmation of expression of AV2 protein by Western blot analysis using anti MBP antibody recombinant AV2 protein of Bg isolate were transferred to nitrocellulose membrane after SDS-PAGE. Blot was probed with anti MBP antibody at 1/3000 dilution. The secondary antibody was anti rabbit IgG in goat at 1/10,000 dilution. Positive reaction with recombinant proteins for Bg (lanes 3-6); lane 1 : molecular weight marker; lane 2 : MBP from control plasmid. **b)** SDS-PAGE electrophoresis of recombinant AV2 proteins of Bg and Cp isolates after proteolytic cleavage with Factor Xa. Lane 1 : molecular weight marker; lane 2 : MBP; lane 3-5 : MBP + AV2 fusion protein of Bg and Cp isolate; lane 5 and 6 : cleavage of MBP AV2 fusion protein of Bg and Cp isolate to release AV2 and MBP. Undigested MBP-AV2 fusion protein is also seen.

uncleaved fusion protein at 52-55 kDa, the MBP protein at 42 kDa that is expressed throughout, and the ORF AV2 protein at 10-13 kDa released. The size of AV2 protein was 10 kDa in the case of Bg and 13 kDa in Cp isolates.

4.2.2 Antibody production against MBP-BgAV2

The MBP-BgAV2 protein purified by passing through amylose resin was dialyzed. Aliquots (100 µg) of dialyzed MBP-BgAV2 protein resolved in SDS-PAGE. The MBP-AV2 band in the gel (Fig. 40a and b) was sliced out, homogenized with column buffer, and 100 µg of the fusion protein was emulsified with Freund's incomplete adjuvant. The emulsion was given a quick spin to remove the acrylamide debris and the supernatant was injected into rabbits. Antibody was purified as given in Materials and Methods. The antibody could detect the recombinant MBP-AV2 protein and the AV2 protein in agroinoculated plants in Western blots (Fig. 41). In the case of agroinoculated plants, very faint bands were seen on par with the band of molecular marker 22 kDa. The bands were extremely faint due to low concentration of the virus and these multiples of bands, may represent some read-through proteins or some host protein non-specifically reacting to MBP antibody.

4.2.3 Function of AV2 protein

4.2.3.1 DNA binding activity by South-Western assay

The MBP-AV2 protein was further subjected to Heparin Sepharose CL-6B affinity chromatography (Fig. 42a and b) to remove any contaminating proteins. The nucleic acid binding properties of AV2 protein was examined. Five µg of MBP-AV2 fusion protein of Bg and Cp isolate was resolved on SDS-PAGE (Fig. 43a) and the protein was transferred to nitrocellulose

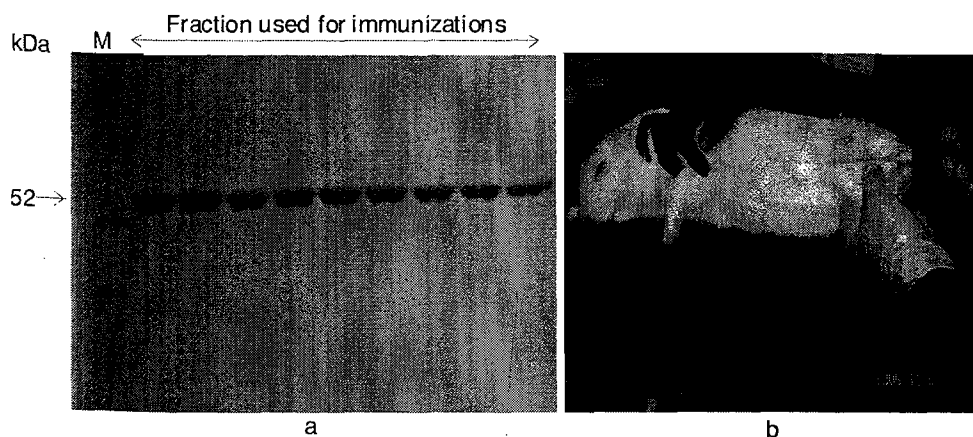


Fig. 40. Production of polyclonal antibody to recombinant MBP-BgAV2 protein. (a) SDS PAGE electrophoresis of fractions of recombinant AV2 proteins, that were pooled for immunization. (b) Injection of female rabbit with emulsified recombinant MBP-BgAV2 protein.

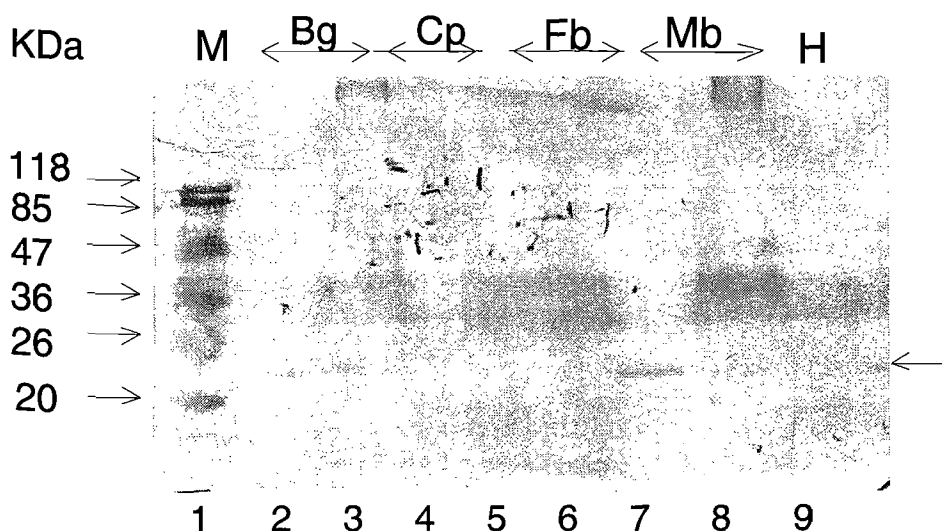


Fig. 41 .Analysis of AV2 protein in diseased legume plants agroinoculated with wild type of Bg. Lanes 2 to 9 are crude protein extracted from healthy and inoculated blackgram (lanes 2-3), cowpea (lanes 4), French bean (lanes 5-6), mungbean (lanes 7-8), and healthy (lane 9); lane 1 : molecular weight marker.

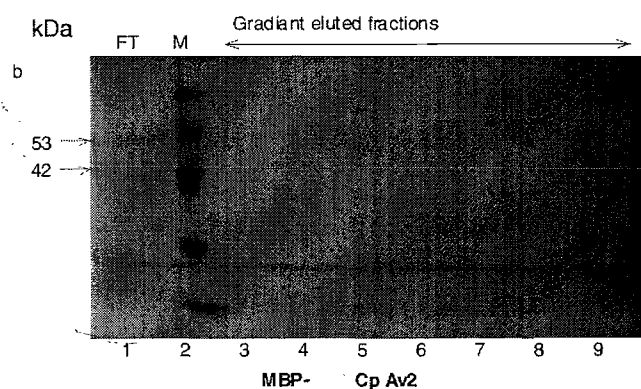
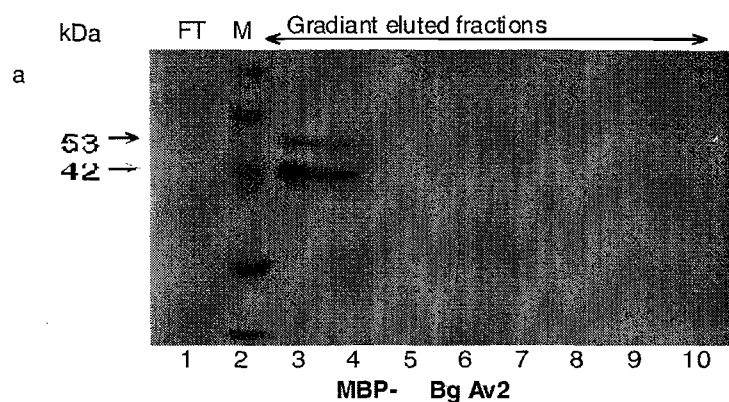


Fig. 42. Purification of recombinant MBP-BgAV2 (a) and MBP-CpAV2 (b) protein through Heparin-Sepharose CL-6B, affinity chromatography. Different fractions eluted with linear NaCl gradients are shown in lanes 3 to 10; lane 2 : molecular weight marker, FT : Flow through.

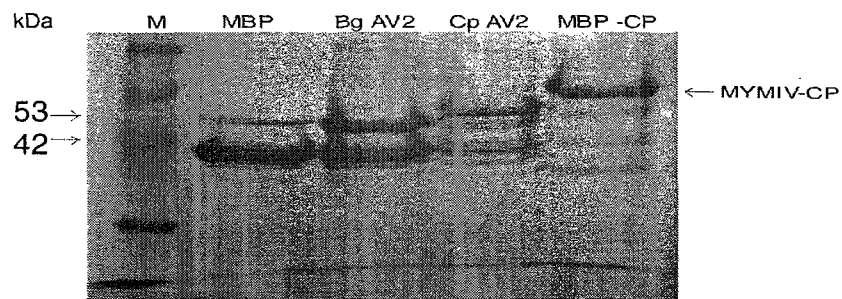
membrane using semiblot transfer apparatus. South-Western assay was done. To the nitrocellulose membrane having AV2 protein, ssDNA and dsDNA (700 bp of the coat protein gene) labelled with [$\alpha^{32}\text{P}$]-dCTP was added separately. Autoradiograms did not show any hybridization signal between the protein and the probe in either case (Fig. 43b). It is inferred that ORF AV2 does not bind strong enough to be detected by the technique employed to either ss or ds DNA.

4.2.3.2 ATPase activity of ORF AV2

The purified protein at 20-50 ng concentration was assayed for ATPase activity. The liberation of radioactive orthophosphate from [$\gamma^{32}\text{P}$]-ATP by AV2 was monitored on a TLC plate. The purified AV2 did not show any appreciable ATPase activity.

4.2.3.3 Interaction between AV2 protein and Rep

Results of experiments on AV2 proteins binding of nucleic acid did not reveal any binding between ssDNA or dsDNA. It was thought that the protein may interact with the replication initiation protein Rep, and thereby influence the viral replication. Whether AV2 protein binds to Rep, and protein to protein interaction takes place was analyzed. Ten μg of AV2 fusion protein (2 μg of AV2 protein) in the amylose matrix was allowed to interact with 0.5, 1 and 1.5 μg of 6xHis-Rep protein at 4°C ; for 1 h with intermittent mixing. After the binding reaction, the matrix was washed with wash buffer thrice by centrifugation at 3000 rpm for 5 min. The bound fractions in amylose matrix (pellet) and the supernatant (unbound fraction) were analyzed in SDS-PAGE. Fig. 44 (a and b), lanes 4-6 represent the His-Rep and MBP-AV2 in the bound fractions which appears as faint bands. These fractions also reacted with anti



a

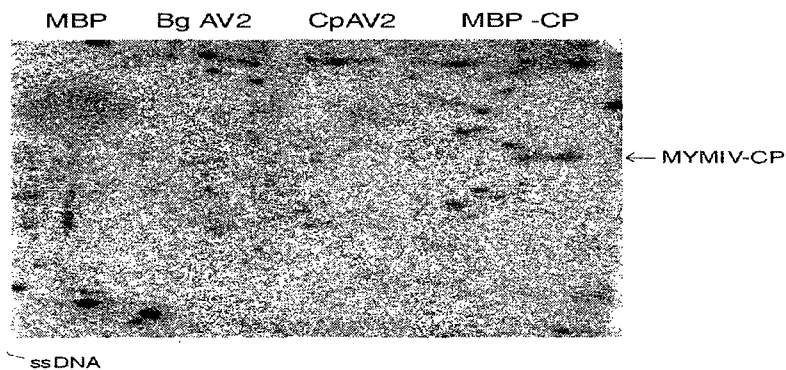


Fig. 43. SDS-PAGE (a) and South-Western (b) blot analysis of interaction between MBP-BgAV2 and MBP-CpAV2 protein with single stranded and double stranded DNA coat protein gene fragment.

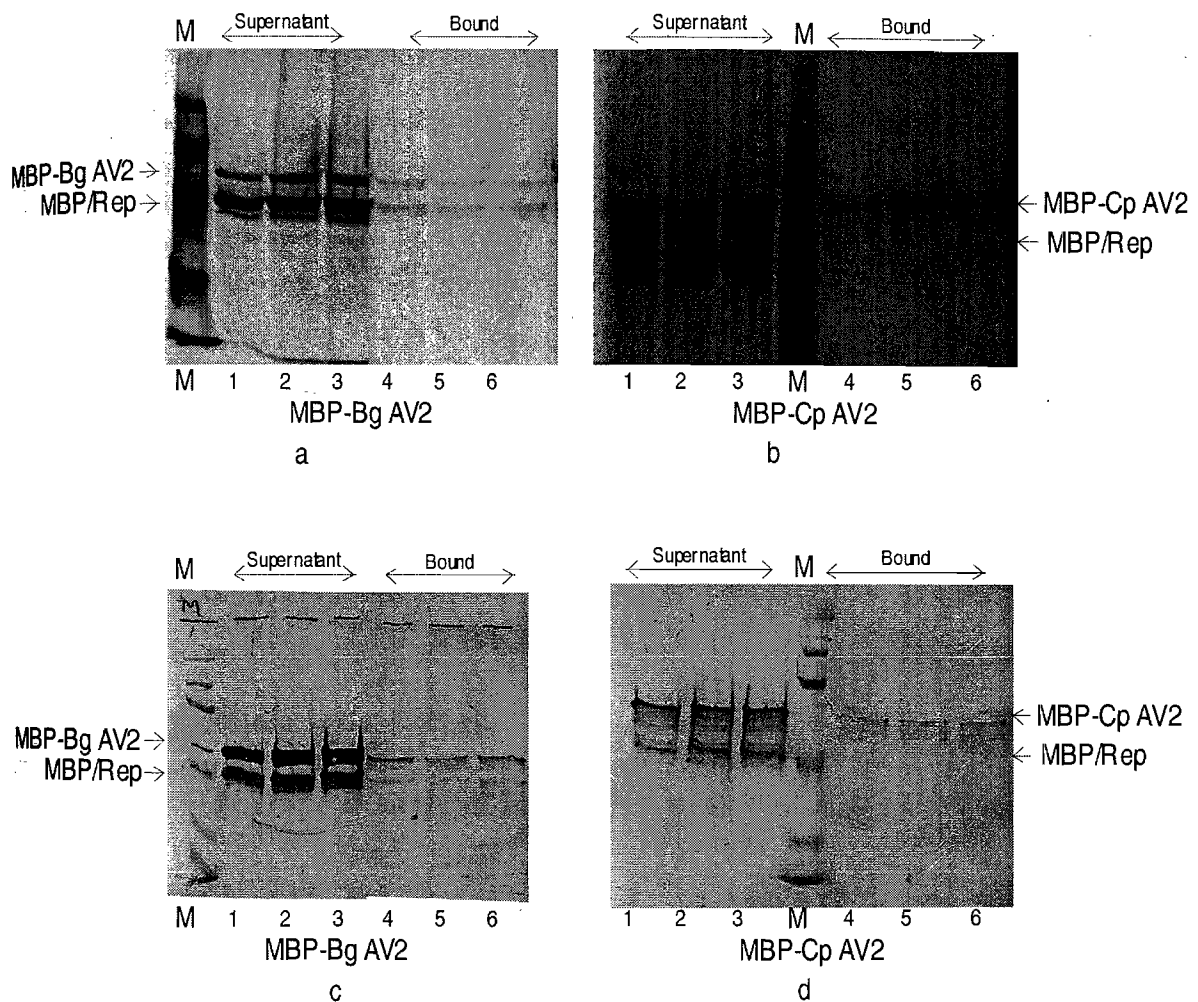


Fig. 44. AV2-Rep interaction. SDS-PAGE (a, b) and Western blot analysis (c, d) of bound and unbound MBP-AV2 and Rep proteins. Lanes 1-3 : unbound Rep, MBP-AV2 fusion proteins present in the supernatant; lanes 4 to 6: Rep and MBP-AV2 fusion protein in the bound fraction in amylose matrix. M : molecular weight marker, anti MBP antibody was used immunoprobe.

MBP and the anti Rep (Fig. 44 c and d) antibody, However the bands of unbound fractions (lanes 1, 2, 3) were very strong, indicating that the weak interaction between Rep and AV2 proteins. Such weak binding was indeed specific since binding was observed after 100 mM stringent NaCl wash.

4.2.3.4 ATPase activity of Rep in the presence of AV2

The release of radioactive phosphate (P_i) from $[\gamma\text{-}^{32}]\text{-ATP}$ by Rep in the presence of AV2 protein was examined. The autoradiogram of TLC plate showed increase in the hydrolysis of ATP by Rep in the presence of AV2. Fig. 45, lane 2 shows ATPase activity by Rep protein alone. Compared to that, in the presence of either Bg AV2 or CpAV2 (Fig. 45, lanes 3 to 6 and 8 to 11) increase in hydrolysis of ATP by Rep was seen. There was at least two fold increase in the presence of AV2. This increase was seen with even as low as 1 ng of AV2 and continued till 2 to 4 ng of AV2, after which the increment reaches a plateau.

4.2.3.5 Nicking activity of Rep in the presence of AV2 protein

Rep protein of geminiviruses which is highly conserved in all members is a sequence specific DNA binding protein with site specific nicking and closing activity. The Rep protein is the only virus encoded protein required for initiation and termination of rolling circle replication. The Rep protein cleaves the phosphodiester bond between the seventh and eighth nucleotide of the nonanucleotide motif (TAATATT[↓]AC). As nicking activity of the Rep protein is its key function, the modulation of such activity in presence of AV2 was checked.

6xHis-Rep was incubated along with AV2 protein at different concentration in a reaction mixture having 5' end labelled 26 mer, containing

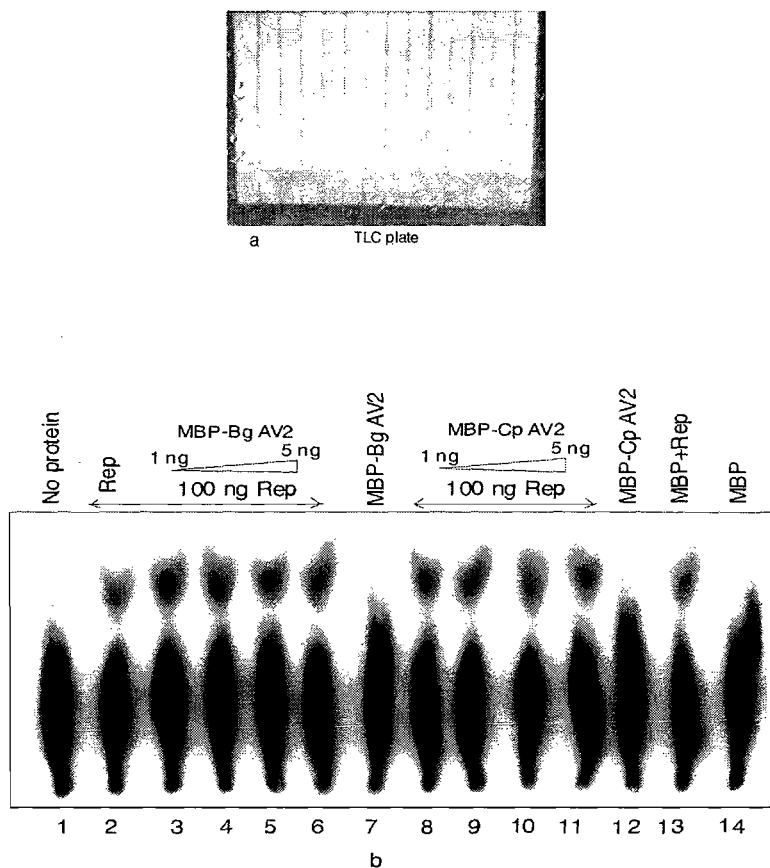


Fig. 45. Modulation of ATPase activity of Rep by AV2. Thin layer chromatography (PEI) plate and an autoradiogram (a and b) showing the release of labeled phosphate from $[\alpha\text{-}^{32}\text{P}]\text{-ATP}$ using 100 ng 6 x His Rep (lanes 3 to 6; 8 to 11) and in the presence of increasing amounts of MBP-BgAV2 (lanes 3 to 6) and Cp (lanes 8 to 11); lane 1 shows the untreated labelled ATP; lane 2 : Rep protein alone; lane 7 and 12 : MBP-BgAV2 and MBP-CpAV2 alone; lane 13 : MBP + Rep; lane 14 : ATP incubated with 100 ng of MBP alone.

the nonanucleotides TAATATT[↓]AC. Sequence nicking at the site indicated by the arrow, the 26 mer was expected to get cleaved into 18 mer and 8 mer. As 26 mer is 5' end labelled, the 18 mer released can be visualized after autoradiography. MBP-AV2 protein alone and MBP with Rep served as controls. Fig. 46, lane 2 shows the nicking activity of 6xHis-Rep, which is considerably low; compared to the activity shown by Rep alone (lane 2), in the reaction having Rep along with MBP-BgAV2 or MBP-CpAV2 protein, there was tremendous (10 folds) increase in the nicking activity as is evident from Fig. 46, lanes 3 to 5 for MBP-BgAV2, lanes 7 to 9 for MBP-CpAV2. The increase in nicking activity was also seen when 60 ng of Rep was mixed with 1-4 ng of AV2 protein (Fig. 47). However, AV2 concentration higher than 20 ng had not increased the cleavage further (Fig. 46, lanes 3 to 5 for MBP-BgAV2 and lanes 7-9 for MBP-CpAV2). In the reaction with Rep protein and MBP protein there was no increase in the release of 18 mer, activity was comparable with activity seen with Rep protein alone (Fig. 47, lanes 11 and 12). MBP protein alone did not show any nicking activity (Fig. 47, lane 13), confirming that in the MBP AV2 fusion protein, it is AV2 which is causing the increase in Rep's nicking activity.

4.3 Genetic variability among YMV isolates

Yellow mosaic diseases are major constraints in improving the productivity of grain legumes in India. In India, the outbreak of the disease in epidemic proportion, is becoming frequent (Varma and Malathi, 2003). The durability of resistance in the genotype is less and there is urgent need to understand the reason behind it. In this context, monitoring the evolutionary trends of the YMV isolates and the possibility of emergence of recombinants causing the outbreak of the disease need to be looked into. With this

5'- GCA CTC AGC TAT AAT ATT↓ACC TGA AT -3' 26 mer
5' labeled

↓ cutting product due to Rep
protein

5'- GCA CTC AGC TAT AAT ATT -3' 18 mer
labelled

+

unlabeled 5 - ACC TGA AT -3' 8 mer

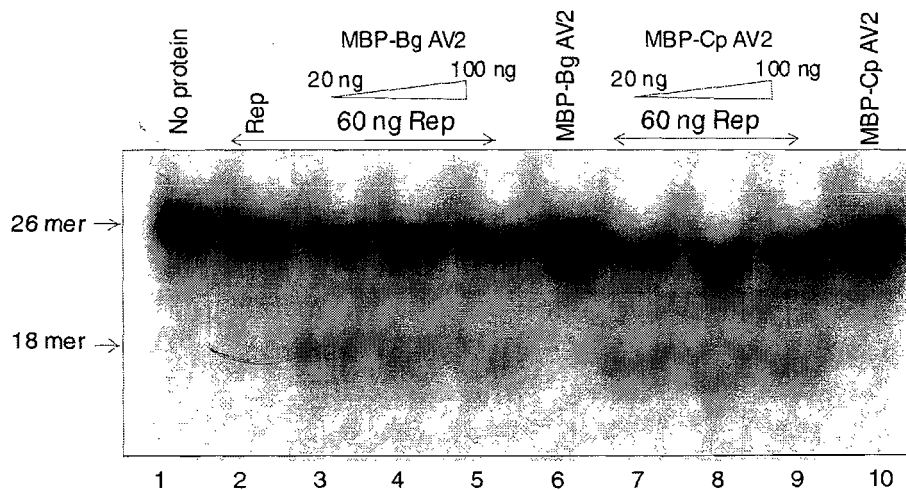


Fig. 46. Modulation of nicking activity of Rep by AV2.

Autoradiogram of a 15% PAGE/urea gel showing the γ - ^{32}P labelled 26-mer substrate (lane 1) and 18 mer cleaved products (lanes 2-5 and 7-9). The substrate was treated with 60 ng of 6 x His Rep alone (lane 2) or in the presence of 20, 60 and 100 ng of MBP-BgAV2 or MBP-CpAV2 proteins (lanes 3, 4, 5 and 7, 8, 9) or with only MBP-BgAV2/CpAV2 (lanes 6, 10).

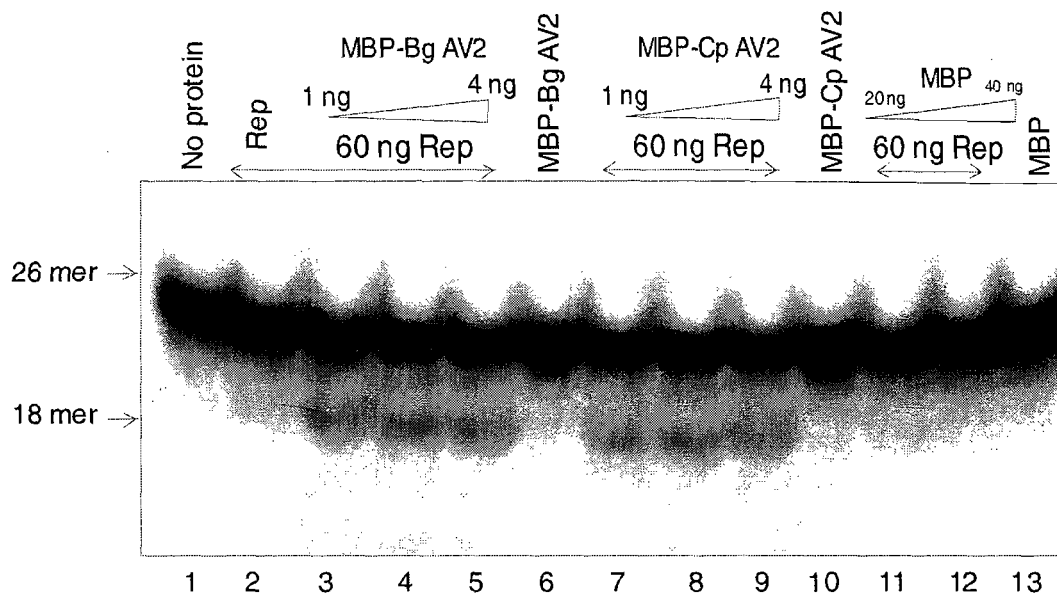


Fig 47. Modulation of nicking activity of Rep by AV2.
Autoradiogram of a 15% PAGE/Urea gel showing 5' ^{32}P labelled 26 mer substrate (lane 1) and the 18 mer cleaved products (lanes 2-5, 7-9, 11 and 12). The substrate was treated with 60 ng of 6 His-Rep alone (lane 2) or in the presence of 1, 2, 4 ng of MBP-BgAV2 (lanes 3, 4, 5) or MBP-CpAV2 (lanes 7, 8, 9) or 20-40 ng of MBP (lanes 11, 12); lanes 6 and 10 : ATP incubated with only MBP-BgAV2 and MBP-CpAV2; lane 13 : ATP incubated with only MBP.

background, it was decided to look into the extent of variability among YMV isolates. The study involved standardization of protocol for extraction of PCR compatible DNA from legumes, collection of samples, PCR amplification of selected region, and analysis of sequences.

4.3.1 Collection of YMV infected legume samples

Leaves from symptomatic leguminous plants exhibiting leaf curling distortion, enation, vein clearing, yellowing, yellow mosaic, crinkling, reduction in leaf lamina and stunting of plants were collected in 2003-2005 (Fig. 48, Table 17). While samples in north India were collected personally, samples from different parts of country were sent by scientists from different institutions. Details on the location from where samples were collected, host and type of symptoms observed at the time of collection are given in Table 17. About 200 samples presenting different geographical regions were studied. Samples were preferably collected early in the morning to prevent starch accumulation interfering with the quality of DNA. The samples were initially tested by Southern/dot blot hybridization to confirm the viral aetiology of the disease.

Most of the samples of blackgram, mungbean, soybean and cowpea showed yellow mosaic or golden mosaic. Samples of Cassia showed yellow mottling. Samples of mothbean from Rajasthan, Frenchbean from Karnataka and cowpea from Gujarat showed puckering enation and raised leaf lamina.

As the present study concentrated on the ORF AV2, the ORF contributing to variable region in the DNA A component, variability in the righthalf of the DNA A component of the genome was studied. The region between the first nucleotide of the genome ('A' after the Rep nicking site) to

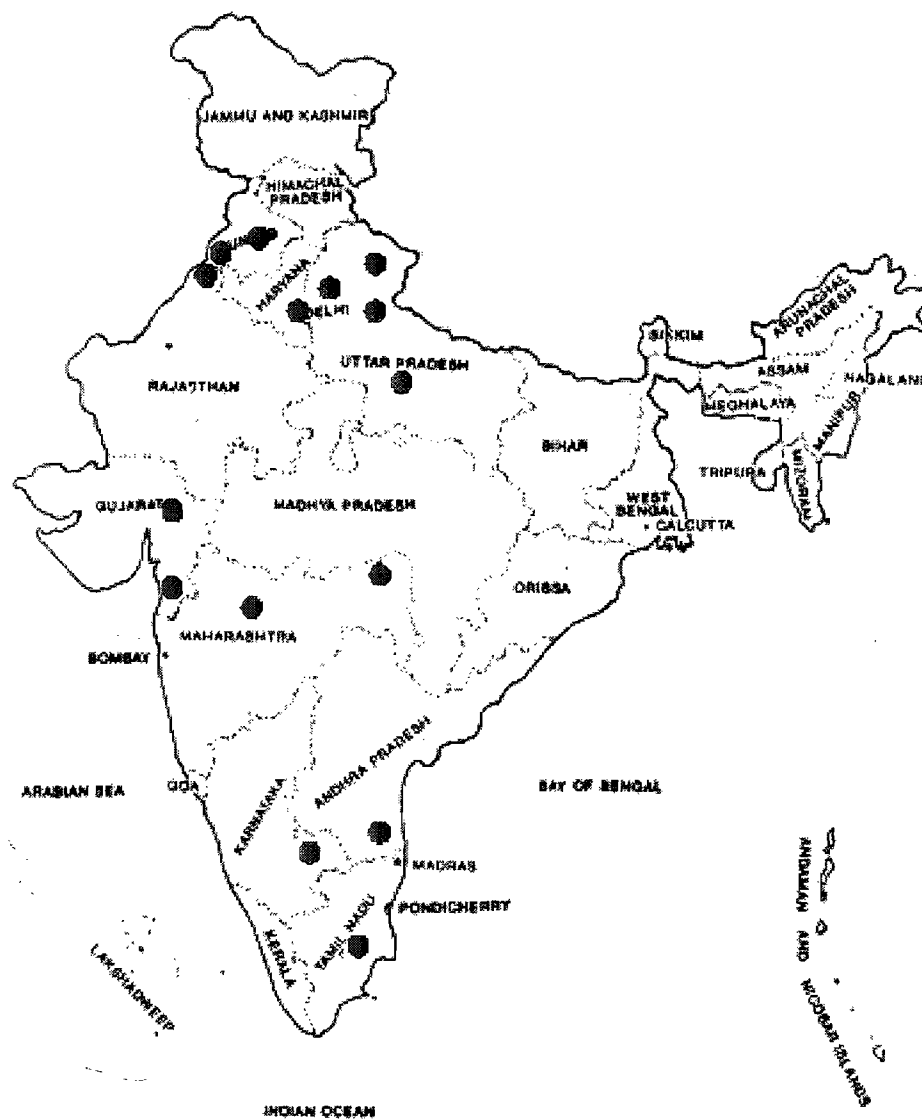


Fig. 48. India map showing the places of collection of yellow mosaic diseased samples. ● Location of sample collection 0

Table 17. Yellow mosaic infected legume samples analyzed

Host	Location	Symptom	No. of samples tested	No. of PCR positive samples		
				A	B	RHA
Cowpea	Delhi	YM, LC, PK	30	12	15	15
	Ludhiana	YM	5	2	2	4
	Meerut	YM	10	5	5	5
	Kanpur	YM,	6	2	2	3
	Anand	YM, LC	8	1	0	3
	Tirupathi	YM	2	0	0	0
	Bangalore	YM	3	0	0	0
	Barielly	YM	5	1	2	3
Blackgram	Delhi	YM, LC	10	4	3	4
	Ludhiana	YM	3	2	2	4
	Kanpur	YM	4	3	1	4
	Anand	YM, LC	3	1	1	2
	Tirupathi	YM	2	0	0	0
	Pantnagar	YM	3	3	2	3
	Tamil Nadu	YM	4	1	1	2
Mungbean	Delhi	YM	20	6	6	10
	Ludhiana	YM	3	1	0	2
	Navasari	YM	3	1	0	1
	Pantnagar	YM	4	2	1	2
	Kanpur	YM	4	0	0	0
	Barielly	YM	4	0	0	0
Soybean	Delhi	YM	12	3	3	6
	Ludhiana	YM	4	1	1	2
	Navasari	YM	4	-	-	2
	Aurangabad	YM	2	-	1	2
	Nagpur	YM	2	-	1	2
	Barielly	YM	4	-	-	3
	Pantnagar	YM	4	-	-	3
Dolichos	Delhi	YM	6	4	0	2
	Ludhiana	YM	3	1	0	1
	Pantnagar	YM	2	1	1	1
	Kanpur	YM	3	0	0	2
	Abohar	YM	2	*1	*1	0
Moth bean	Sri Ganganagar	YM	2	1	1	1
	Anand	YM	2	0	0	0
Cassia	Delhi	YMo	2	1	0	1
Indigofera	Banglore	YM	2	0	0	1
Acacia	Delhi	YM	2	0	0	0
Rhynchosia	Triyendrum	GM	-	0	0	0

YM = Yellow mosaic; GM : Golden mosaic; PK : Puckering; LC : Leaf curl; YMo : Yellow mottle
 - Non-specific or subgenomic DNA.

the end of the coat protein gene was amplified with suitable primers, amplicons were cloned and sequenced and sequences were analyzed. Results are presented below.

4.3.2 Standardization of DNA extraction

The biggest obstacle in the variability study involving field samples was the extraction of PCR compatible DNA from leguminous plants, which are rich in phenols, polysaccharide and have very low concentration of virus. Therefore, a DNA extraction protocol was standardized. To standardize the extraction method, symptomatic leaves of blackgram or mungbean plants naturally infected with YMD were used.

Seventeen methods were evaluated for yield of total nucleic acid (Table 18) and PCR compatible DNA. Four different buffers, Trizol, Tris EDTA, sodium chloride, CTAB, Guanidium thiocyanate buffer and Sodium citrate buffer were employed.

Modifications involved use of different reducing agents at different concentration, dispersing and deproteinising agents. After extensive work involving naturally infected blackgram and mungbean plants, a modified CTAB method was standardized. This method, perfected in the present work is referred to as Gem-CTAB method as it works well for many geminiviruses.

The details of Gem-CTAB method are given in Materials and Methods, results are discussed below.

A good yield of DNA 1 µg/µl (from 100 mg leaf, which was finally dissolved in 100 µl distilled H₂O), two folds more than the normal CTAB method (Dellapora *et al.*, 1983) was obtained in Gem-CTAB procedure. A clear unsheared genomic band was visualized in gel electrophoresis for the

Table 18. Evaluation of different DNA extraction protocols for amplification of geminiviral DNA components

No.	Method	Buffer	Quantity of leaf tissue (mg)	Final volume (µl)	Results	
					Quality of DNA	PCR compatibility
1.	Trizol.1	RLT+Phenol	500	100	Very	-
2.	Trizol.2	RLT+phenol +ME				
3.	Trizol.3	RLT+PVP+ME	500	100	"	-
4.	Trizol.4	RLT+glass milk	500	100	Good	-
5.	Trizol.5	RLT, extract treated with alkali lysis solution				
6.	Trizol.6	RLT, extract treated with alkali lysis solution, cleaned through column	1000	200	bad	-
7.	¹ CTAB-1	TE+2% CTAB	200	100	Good	-
8.	² CTAB-2	TE+2% CTAB +ME	100	50	Very good	-
9.	CTAB-3	TE+2% CTAB extract treated with alkali lysis solution cleaned through column	1000 400	20 125	bad Very good	- +(2/16)
10.	CTAB-4	TE+2% CTAB+ME + (0.65%) sodium sulphite				
11.	CTAB-5	TE+2% CTAB, ME cesium chloride, lithium chloride sodium chloride for precipitation	500 500 450	50 50 50	Good Very Good Very Good	- - -
12.	CTAB-6	TE+2%CTAB + ME sodium sulphite and 2% PVP	400	500	Good	-
13.	Gem. CTAB	TE+2% CTAB+2.5% ME	100	100	Very good	Bg 10/16
14.	GTC	GTC, +0.1% DTT	1000	200	Good	Mb 6/16
15.	Sodium citrate-PEG ³	Citrate + ME+PEG	5000	250	bad	-
16.	Triton-X	TE+Triton x 100, passed through membrane	100	20	good	-
17.	Qiagen DNeasy kit	---	100	200	Good	Bg 3/16

Bg : Blackgram, Mb : Mungbean, TE : Tris-HCl; EDTA, ME : β-mercaptoethanol

¹ Dellaportia (1983), ² Lodhi *et al.* (1983); ³ Jose and Usha (2000).

DNA extracted by all the methods except methods 5, 6, 9, 15. However, with samples extracted by CTAB or CTAB in citrate buffer method, Trizol or GTC no amplification was seen in 16 samples evaluated (Table 18). In samples extracted by commercial kit, amplification was seen only in three YMD samples out of 16 tested. A good amplicon of 1.1 kb with AC-abut CPV2 primers, 2.7 kb with AV-abut/AC-abut primers were seen only with DNA extracted by Gem-CTAB method. In the case of samples from mungbean, β -mercaptoethanol concentration was increased from 1 to 5% which helped in reducing the phenol oxidation (Fig. 49a). As the DNA yield was 2 to 3 folds more in Gem-CTAB method than in other methods, 50 mg of leaf tissue was sufficient to get amplicons. In the case of mungbean samples, where the concentration of virus was low, around 500 mg of leaf was processed. Increasing the ratio of buffer to leaf amount (15 : 1 or more) helped to get clean DNA.

Following are considered as critical steps in the extraction protocol standardized.

- (a) collection of samples in the early morning hours.
- (b) addition of warm extraction buffer to the frozen powder, suspension as rapidly as possible to bring the temperature of suspension from -80°C to room temperature and not allowing it to thaw exposed to air.
- (c) deproteinization in the first step itself before separating the extract from cell debris.
- (d) concentration of β -mercaptoethanol, which can be adjusted between 2 to 5 percent.

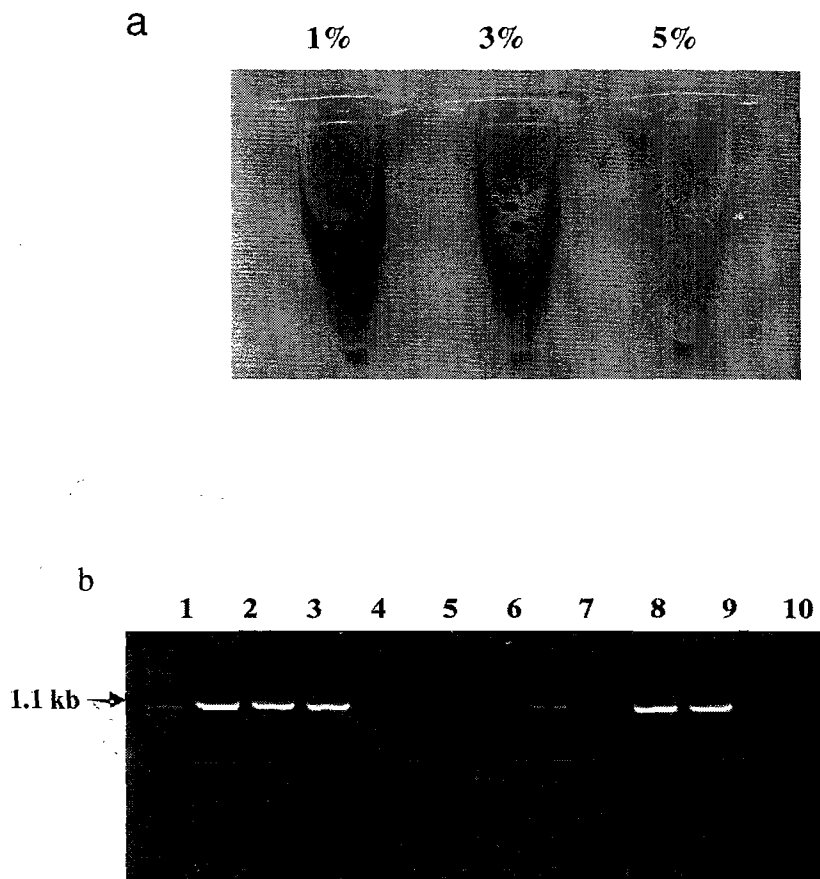


Fig. 49. a) DNA extraction from yellow mosaic affected mungbean plants by Gem-CTAB method. Extract having 1,3 and 5% β -mercaptoethanol. Browning is absent in 5% β ME. **b)** Agarose gel electrophoresis of right half of the DNA A components (1100) amplified with AC abut and CpV2 primers. Lanes 1 - 12 : amplicons obtained from YMD affected blackgram (2) cowpea (3 and 4), dolichos (5, 6), soybean (7, 8, 9 and 10) and cassia (11, 12). 1 : 1 kb DNA ladder.

- (e) removal of CTAB by washing, twice, first in 80% and then in 70% alcohol.
- (f) sufficient time to solubilize the pellet which was colourless and did not contain any jelly like polysaccharides.

Table 17 gives, total number of samples tested in each leguminous species and the number of samples that were PCR positive. It is evident from the data that the protocol works with naturally infected plants from fields.

DNA extracted from leaf tissue collected from different leguminous plants were subjected to amplification with three sets of primers (Table 9) to generate amplicons of full-length DNA A, full-length DNA B and the right half (RH) of the DNA A genome. As the data show, amplifications were obtained in more than 50% of samples in the case of blackgram, cowpea, Cassia, soybean and dolichos. A good amplicon of 1100 bp representing RH region of DNA A was seen in most of the samples (Fig. 49b, Fig. 51), which were cloned and sequenced and taken for variability analysis. However in Dolichos, Cassia and Indigofera samples while RH region were amplified, amplification was not seen for full-length DNA A component (Fig. 50) indicating differences in the sequences. Interestingly in Dolichos samples full-length DNA A amplification were obtained for six samples. Full length DNA B amplifications was not seen in any of the samples. For sample from Vamban, Tamil Nadu, the amplicon was very faint which could be improved by annealing at 53⁰C, amplification of DNA A full-length was successful but it failed in DNA B for three samples. It is suggestive of altogether different DNA B component or a DNA B showing variations, at primer annealing sites. In the case of mungbean, of 38 samples tested, only ten could be amplified for DNA A and seven for DNA B. Most of the time, around 1.1 kb in DNA

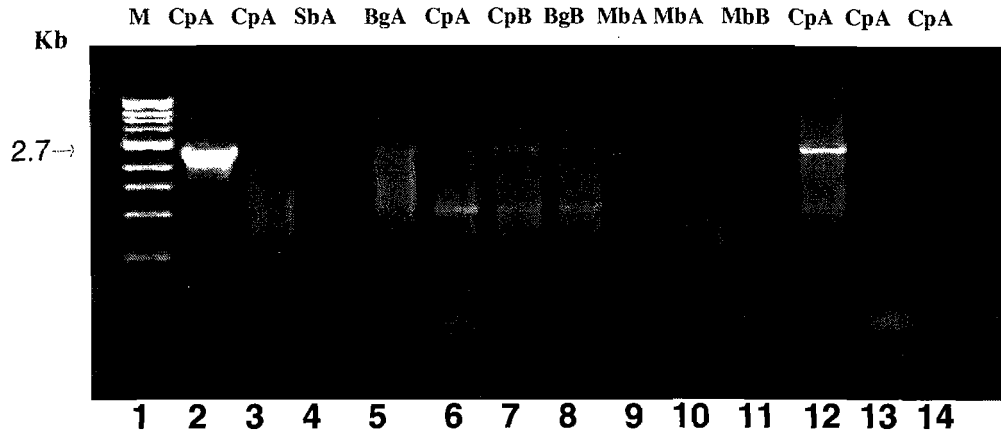


Fig 50. Agarose gel electrophoresis of PCR amplified fragments of DNA A and DNA B from field samples

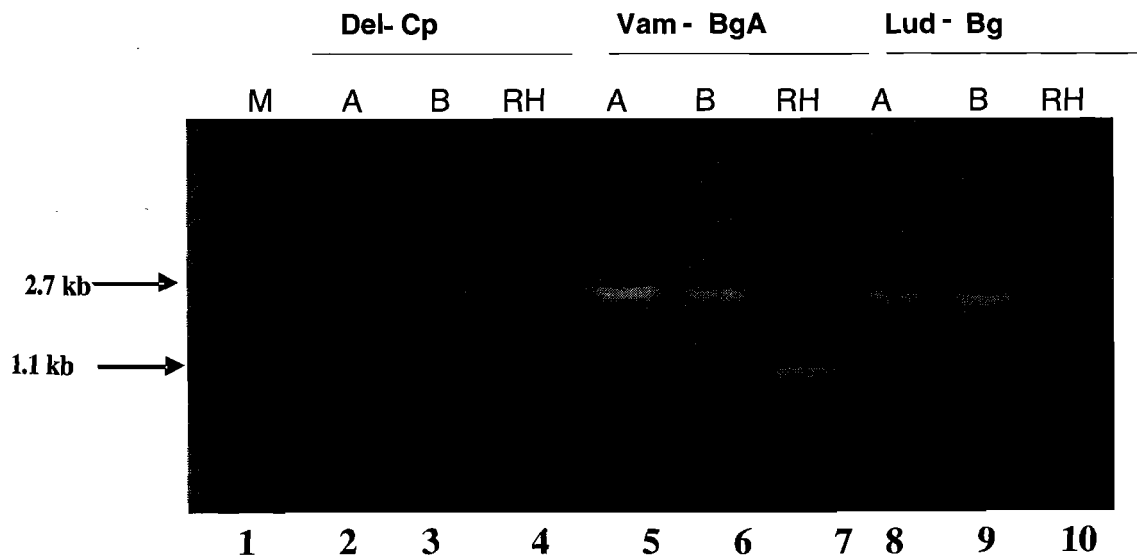


Fig. 51. Agarose gel electrophoresis of DNA A and DNA B components (2.7 kb) amplified with AC abut and AV abut primers, and right half of the DNA A components (1100 bp) amplified with AC abut and CpV2 primers. Lane 1: 1 kb DNA ladder, Lanes 2 - 10: amplicons obtained from YMD affected cowpea from Delhi (2,3 and 4), blackgram from Vamban (5,6 and 7), and blackgram from Ludhiana (8,9 and 10).

A and 1.2 kb amplification in DNA B were seen, which could represent subgenomic DNA A or DNA B in DNA samples.

The results could be improved by taking more amount of tissue, increasing β mercaptoethanol concentration and adjusting DNA concentration.

4.3.3 Cloning of full length and right half of DNA A genome of YMV isolates

In order to confirm the viral origin of 2.7 kb amplicons of DNA A and DNA B components, full-length DNA A and DNA B amplicons of samples of cowpea, from Delhi, Kanpur and Ludhiana and Vamban were cloned in pGEMT-Easy vector. The recombinant clones were analyzed by rapid lysis, colony PCR and miniprep DNA restriction. Whichever clone released the full length (2.7 kb) viral insert from the vector (3 kb) by restriction of plasmid DNA with *Hind* III was considered to represent the putative DNA A component. These virus clones were referred to as DNA A component of Del-Cp, Kan-Cp, Lud-Bg and Vam-Bg isolates.

Full-length DNA B amplicons of cowpea from Delhi and DNA B amplicon of blackgram from Vamban were similarly cloned in PGEMT-Easy vector. The clones were confirmed by PCR (Fig. 52) and *Bam*H I restriction of plasmid DNA which released the full length 2.7 bp viral insert (2.7 kb) and the vector (3 kb).

1.1 kb length, amplicons of samples from different parts of the country representing right region of DNA A genome (from origin of replication to the end of ORF AV1) of kn were cloned in PGemT-Easy vector. The miniprep plasmid DNA was restricted (Fig. 53) with *Eco*R I-*Hind* III, which released 1.1 kb viral insert from 3 kb vector. The samples used for cloning and name given to the clones are given in Table 19.

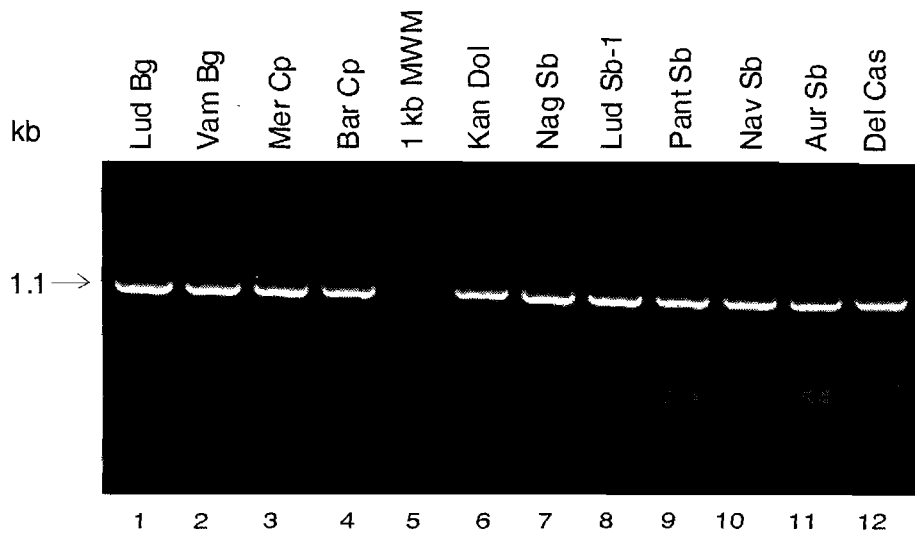


Fig. 52. Agarose gel electrophoresis of right half of the DNA A components (1100 bp) amplified with AC abut and CpV2 primers. Lanes 1 - 11 : amplicons obtained from YMD affected blackgram (1 and 2) cowpea (3 and 4), dolichos (6), soybean (7, 8, 9 and 11) and cassia (12). MW : 1 kb DNA ladder.

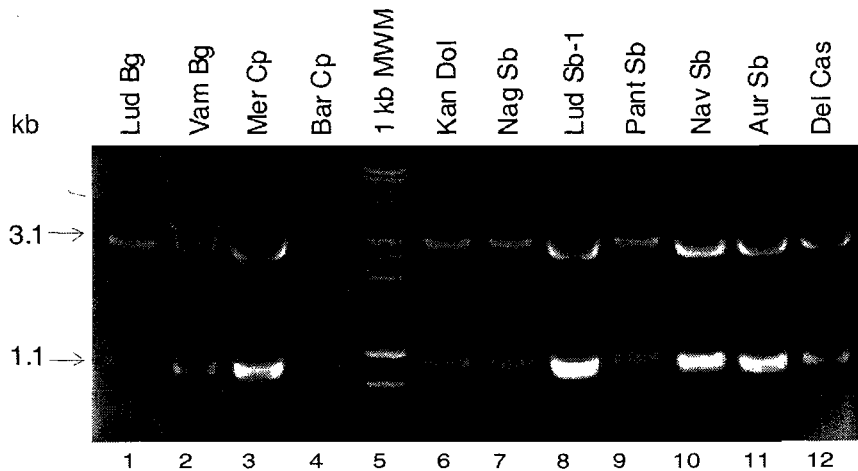


Fig. 53. Agarose gel electrophoresis of plasmid DNA of recombinants having right half of DNA A insert. Plasmids were restricted with *EcoR* I and *Hind* III to release 3.0 kb vector and 1.1 kb viral insert. Lane 1-4 : Lud-Bg, Vam-Bg, Mer-Cp, Bar-Cp (lanes 6-12) kan-Dol, Nav-Sb, Lud-Sb, Plant-Sb, Nav-Sb, Aur-Sb; Del-Cas; lane 5 : 1 kb DNA ladder.

Table 19. Yellow mosaic virus isolates characterized in this study with the GenBank accession numbers

Virus isolate characterized	Host isolated from	Virus species identified as	GenBank accession number	
			DNA A	DNA B
Full length				
Del-Cp	Cowpea (Delhi)	MYMIV	DQ389153	AY939925
Lud-Bg	Blackgram (Ludhiana)	MYMIV	DQ400847	—
Kan-Cp	Cowpea (Kanpur)	MYMIV	DQ389154	—
Vam-Bg	Blackgram (Vamban)	MYMV	DQ400848	DQ400849
Right half of DNA A				
Del-Cp	Cowpea (Delhi)	MYMIV	DQ389153	
Bar-Cp	Cowpea (Barielly)	MYMIV	DQ389145	
Nag-Sb	Soybean (Nagpur)	MYMIV	DQ389146	
Mer-Cp	Cowpea (Meerut)	MYMIV	DQ389147	
Kan-Dol	Dolichos (Kanpur)	MYMIV	DQ389148	
Del-Cas	Cassia (Delhi)	MYMIV	DQ389149	
Lud-Sb1	Soybean 1 (Ludhiana)	MYMIV	DQ389155	
Lud-Sb2	Soybean 2 (Ludhiana)	MYMIV	DQ389151	
Pant-Sb	Soybean (Pantnagar)	MYMIV	DQ389152	
Kan-Cp	Cowpea (Kanpur)	MYMIV	DQ389154	
Lud-Bg	Blackgram (Ludhiana)	MYMIV	DQ400847	
Nav-Sb	Soybean (Navasari)	MYMV	DQ389144	
Vam-Bg	Blackgram (Vamban)	MYMV	DQ400848	
Aur-Sb	Soybean (Aurangabad)	MYMV	DQ389150	

— Not cloned.

4.3.4 Sequencing of full-length and right half of the genome of YMV isolates

DNA A components of Del-Cp, Kan-Cp, Lud-Bg and Vam-Bg and DNA B component of Del-Cp and Vam-Bg were sequenced, end to end by primer walking strategy. In the case of RH, sequences were generated in three reactions, using T7, SP6 primers and a primer CpV2 in the middle of RH. Two clones were sequenced for each isolate. The sequences were analyzed as given in Materials and Methods. Results are summarized below.

4.3.4.1 Sequence analysis of full-length DNA and DNA B components of YMV isolates

The nucleotide sequence of DNA A components of Del-Cp, Kan-Cp, Lud-Bg and Vam-Bg, and DNA B components of Del-Cp and Vam-Bg were aligned. ORFs were deciphered and the genome organization was found to have typical features of begomoviruses (Fig. 54). Sequences are available in the DDJB, EMBL, NCBI GenBank data bank under accession numbers indicated in the Table 19. Genome length, base composition and gene ordering of the isolates are given in Table 20. The genome is diagrammatically represented in Fig. 54. Complete nucleotide sequence analysis of DNA A of Del-Cp, Kan-Cp and Lud-Bg isolates showed > 89% identity with *Mungbean yellow mosaic india virus* (MYMIV) and hence considered as isolates of MYMIV as per the guidelines suggested by ICTV study group on *Geminiviridae*. Contrastingly, the DNA A of Vam-Bg showed > 89% identity with *Mungbean yellow mosaic virus*-[Vigna] (MYMV-[Vig]) from Tamilnadu, and therefore was identified as an isolate of MYMV. DNA A components of all the four isolates have two virion-sense (Table 19) (AV1 and AV2) and five complementary sense (AC1, AC2, AC3, AC4, and AC5) open reading frames with minor variation in Del-Cp.

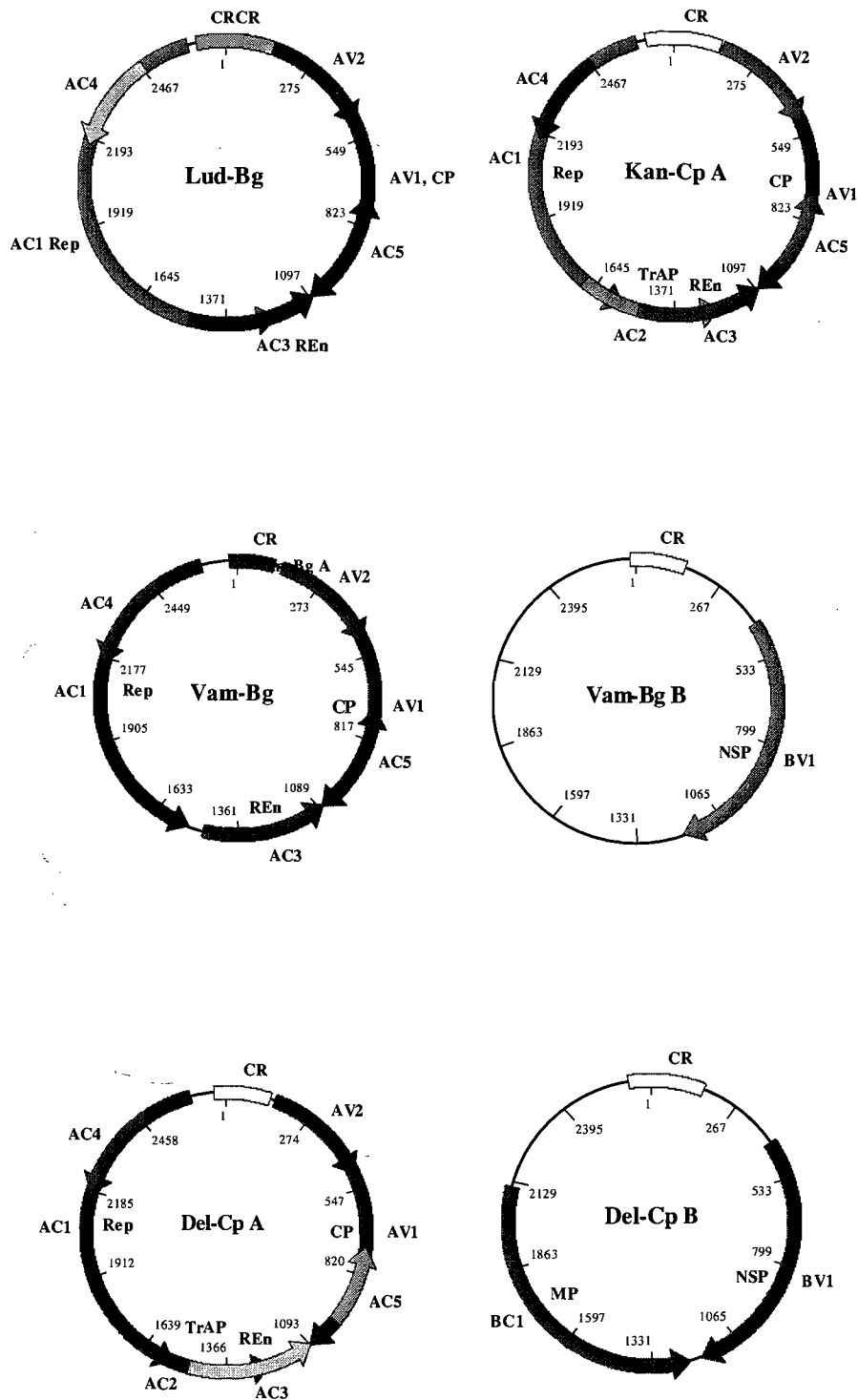


Fig. 54. Genome organization of DNA A and DNA B components of Del-Cp, Kan-Cp, Lud-Bg and Vam-Bg. Names of ORFs and their functions are as described in the text.

Table 20. Open reading frames (ORF) in DNA A and DNA B components of YMV isolates

ORF	Nucleotide co-ordinate							
	Del-cp		Vam-Bg		Kan-Cp		Lud-Bg	
	Start	Stop	Start	Stop	Start	Stop	Start	Stop
AV1	316	1089	302	1075	317	1090	316	1089
AV2	156	497	142	492	157	498	156	497
AC1	2626	1538	2612	1524	2627	1539	2625	1216
AC2	1680	1228	?	?	1681	1229	?	?
AC3	1490	1086	1476	1072	1491	1087	1490	1086
AC4	2475	2176	2461	2162	2476	2177	2474	2175
AC5	984	733	970	719	985	734	984	733
BV1	415	1185	419	1189	—	—	—	—
BC1	2113	1217	?	?	—	—	—	—

? : ORF not clear; — : Not cloned.

The nucleotide sequence analysis of DNA B components of Del-Cp and Vam-Bg showed genome organization similar to DNA B components of bipartite begomoviruses. There were only two ORFs, one in viral and one in complementary sense coding for nuclear shuttle and movement proteins (Table 20 Fig. 54). Nucleotides sequence comparison revealed > 90% identity between Del-Cp and MYMIV and 97% identity between MYMV-[Vig] and Vam-Bg. Based on these identities in A and B genome, Del-Cp was identified as an isolate of MYMIV and Vam-Bg as on isolate of MYMV.

4.3.4.1.1 Common region organization in Del-Cp and Vam-Bg

Full-length DNA A and DNA B components of Del-Cp and Vam-Bg were aligned pairwise (A with B) to identify the common region (CR). The

non-coding region between ORF AC1/AV2 in DNA A and ORF BC1/BV1 in DNA B were aligned, and region showing maximum identity was identified as CR region (Fig. 55). For Del-Cp it extends from nt co-ordinate No ATCGGTGTACAC to nt co-ordinate No ACCGGTGTAT. For Vam-Bg it is from nt co-ordinate No ATCGGTGTCTCT to ACCGGTGTA No. In begomoviruses, CR is highly specific for a virus and near identical between DNA A and B components of the same virus. Unlike other begomoviruses, in Del-Cp and Vam-Bg, there is 19 and 17% divergence between DNA A and B components in the whole CR, and 32 and 13% divergence in the origin of replication respectively. Divergence included 3 deletions, 20 substitutions in DNA B with reference to DNA A in Del-Cp, and 4 deletions and 12 substitutions in DNA B in Vam-Bg. The Rep binding site iteron (RBS) in the CR was predicted to be AT/CCGGTGT and in Del-Cp occurs as an invert repeat followed by a tandem repeat before the TATA box, but in Vam-Bg 4 RBS are in same orientation, Table 21.

Table 21. Iteron characteristics and percentage nucleotide sequence divergence in the common region between DNA A and DNA B components of Del-Cp and Vam-Bg isolates

Virus isolates	Iteron		Percent Divergence	
	Sequence	Position	Whole CR	<i>Ori</i>
Del-Cp	ATCGGTGT GGTGT	→ ← → → TATA→	19	32
Vam-Bg	ATCGGTGT GGTGT	→→→→ TATA→	13	17.5
MYMIV	ATCGGTGT GGTGT	→ ← → → TATA→	16	25
MYMV-[Cp]	ATCGGGTGT GGTGT	→ ← → → TATA→	17	24

Comparison of CR between Del-Cp and Vam-Bg revealed, less than 60% identity between them, and each one has characteristic feature differentiating from the other.

Nucleotide percent identity of the CR between Del-Cp and Vam-Bg with other YMV isolates showed that Del-Cp shared 96% identity with MYMIV and only 60% with that of MYMV isolates. Contrastingly Vam-Bg showed 98% identity with MYMV-[Vig] and less than 60% with MYMIV.

4.3.4.1.2 Regulatory elements in the CR of Del-Cp and Vam-Bg

The common region has the characteristic stem-loop region, 22 bp length stem and invariant nonanucleotide forming the loop. In begomoviruses the transcription of CP and Rep genes are governed by bidirectional promoter that is present in the CR. Fig. 55 gives the details on location of TATA box and other proximal control elements (CAAT Box and GC box). An initiator element, *Inr* with slight variation from the consensus sequence (CCACTAAC) was found encompassing virion sense ORFs. The virion sense core promoter is a composite promoter having both TATA box and Initiator element.

List of geminiviruses used for sequence analyses and phylogenetics comparison are represented in Table 22. Complete nucleotide sequence of DNA A and DNA B of Del-Cp and Vam-Bg, Kan-Cp, Lud-Bg were compared with other YMV isolates (Table 23 and 24). Lud-Bg and MYMIV (Bg isolate used in this study) showed 98% sequence identity in DNA A; Kan-Cp and Del-Cp shared maximum identity with MYMIV-[Cp] (Cp isolate used in this study). Sequence identity matrix drawn shows (Table 23) how MYMIV and MYMV isolates shared high sequence identity in DNA A component among them. While they showed 80% sequence identity with *Horsegram yellow*

	→		→	→
Vam-Bg A	ATCGGTGTCT	CTCTAAAAGT	CCTATGTATC	GGTGTATCGG TGTCTTATTT
Vam-Bg B	ATCGGTGTCT	CTCCAAAAGT	CCTATGTATC	GGTGTATCGG TGTCT---- T
Vam-Bg A	ATATGTATGA	GAGTTACTAA	AACCCTCAGG	GGTCCTCAGC AATAATATTA
Vam-Bg B	ATATATACTA	GAGCTACTAA	AAGCCTTAGG	GGCACTCAGC TATAATATTA

	→
Vam-Bg A	CCTGAGGACC CCGCG ACCGG TGT
Vam-Bg B	CCTGAGTGCC CCGCG ACCGG TGT

	↔	→	→
Del-Cp A	ATCGGTGTAC	ACCGATTGCC	TTCTCTAGCC CCCTATCGGT GTATCGGTGT
Del-Cp B	ATCGGTGTAC	ACCGATT-CA	TTCTCTAGCC CCCTATCGGT GTATTGGTGT

Del-Cp A	ACTATATATA	CTAGAGCTAC	TAAAAGCCTC	TAGGGGCACT	CAGCTACCTG
Del-Cp B	ACTATATATA	GTAAAGTTAC	TAGGGGCTCT	CAGATATAAT	--ATTACCTG

	→
Del-Cp A	AGTGCCCCGC GACCGGTGT
Del-Cp B	AGAGCCCCGC GACCGGTGT

Fig. 55. Nucleotide sequence comparison of the common region of DNA A and DNA B of Vam-Bg and Del-Cp isolate. The conserved nonanucleotide sequences are shown in blue color, bold italic. TATA box is also shown in a box. The horizontal arrows indicate the sequences and orientation of the Rep binding iteron.

Table 22. Geminiviruses with their accession numbers used for sequence analysis and phylogenetic comparison

Virus name	Acronym	Accession number
<i>Cotton leaf curl Rajasthan virus</i>	CLCuRV	AF363011
<i>Indian Cassava mosaic virus-[Maharashtra]</i>	ICMV-[Mah]	AJ314739
<i>Mungbean yellow mosaic India virus</i>	MYMIV	AF126406, AF142440
<i>Mungbean yellow mosaic India virus-[Cowpea]</i>	MYMIV	AF481865, AF503580
<i>Mungbean yellow mosaic virus</i>	MYMV	D14703-4
<i>Mungbean yellow mosaic virus-[Vigna]</i>	MYMV-[Vig]	AJ132574-5
<i>Tomato leaf curl New Delhi virus [AVT1]</i>	ToLCNDV-[AVT1]	AY428769
<i>Horsegram yellow mosaic virus-[Coimbatore]</i>	HgYMV-[Coi]	AJ627904-5
<i>Dolichos yellow mosaic virus</i>	DoYMV	AY309241
<i>Maize streak virus-A [South Africa]</i>	MSV-A	Y00514
<i>Maize streak virus-[Jam]</i>	MSV-[Jam][ZA]	AF329887
<i>Tomato leaf curl Bangalore virus-[Ban 5]</i>	ToLCBV-[Ban 5]	AF295401
<i>Tomato leaf curl virus</i>	ToLCV	S53251
<i>Tomato leaf curl Karnataka virus</i>	ToLCKV	U38239
<i>Tomato Pseudo-curlytop virus</i>	TPCTV	X84735
<i>Beet curly top virus</i>	BCTV	AF379637

selected began using the

[illegible]

Table 24. Matrix of pair-wise identity percentage of complete DNA B nucleotide sequences of YMV isolates. Acronyms are as given in text. * indicates the homologous comparison. Matrix was generated using the CLUSTAL algorithm

YMV	MYMIV	MYMIV-[Cp]	Del-Cp	MYMV	MYMV-[Vig]	Vam-[Bg]	HgYMV
MYMIV	*	92	92	67	86	86	64
MYMIV-[Cp]		*	97	69	88	88	67
Del-Cp			*	69	88	88	67
MYMV				*	69	69	65
MYMV-[Vig]					*	97	66
Vam-[Bg]						*	66
HgYMV							*

mosaic virus, it was only around 60% with other begomoviruses occurring in India.

4.3.4.1.4 Novel features seen in the genome of Del-Cp

Critical analysis of sequence of Del-Cp DNA A revealed some unusual novel features which have not been so far met with any begomovirus.

- (1) Absence of the invariant nonanucleotide sequence : putative stem-loop region of Del-Cp DNA A lacked the typical nonanucleotide TAATATTAC sequence. As is evident from chromatogram (Fig. 56) nucleotide sequence in that region is lacking the typical TAATATTAC sequence, but the nicking site is retained.
- (2) The leftward ORFs show difference from other begomoviruses. The predicted ORF for AC1 does not have a stop codon but extend in-frame

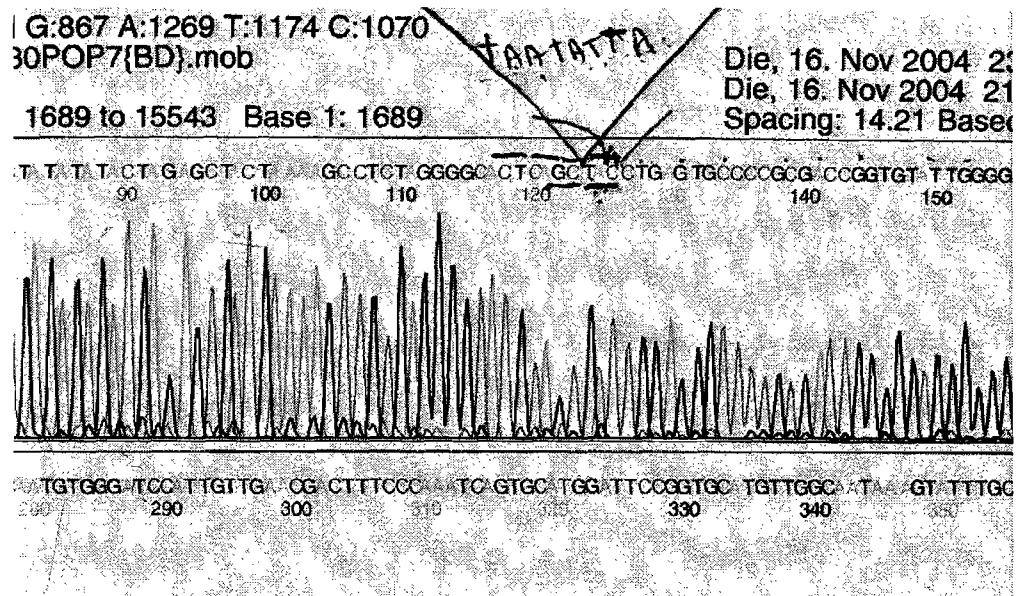


Fig. 56. Chromatogram showing deletion of 'TAATAT' sequences from nonanucleotide TAATATTAC sequences of Del-Cp isolate from Delhi.

ORF AC2 which has putative start codon GUG. The diagrammatic representation is shown in Fig. 54.

Sequence of these two regions were verified twice to confirm these features.

Phylogenetic analysis (Fig. 57) of complete DNA A nucleotide sequence clearly brought out distinct nature of YMV isolates. Bifurcation of YMV isolates into two species MYMIV and MYMV is very evident from the tree constructed based on complete DNA A sequence. The Del-Cp, Kan-Cp, Lud-Bg, all three isolates clustered with MYMIV (Bg isolate), which is the type species. Del-Cp is different from other MYMIV isolates as it branches separately. Among MYMIV isolates, the isolates from cowpea and blackgram, again get separated. YMV isolates are differentiated as only four species MYMIV, MYMV, HgYMV and DoYMV.

Phylogenetic study based on comparison of DNA A nucleotide sequence of YMV isolates with other begomoviruses revealed an unique feature. Members of the genus *Begomovirus* are known to form clusters according to their geographical region. However, in the present phylogenetic study, the YMV isolates occupied a distinct branch well separated from other begomoviruses of Indian origin. For example they are well separated from TOLCNDV, CLCuRV, ToLCBV. They are also distinct from other legume viruses of both Old World (CpGMV from Nigeria, SLCV from Thailand) and New World (BGMV, BDMV from America).

4.4.4.1.4 Complete nucleotide sequence analysis of DNA B component of Del-Cp and Vam-Bg isolates

Total nucleotide sequence of DNA B of Del-Cp and Vam-Bg isolates were analyzed for translation products. The organization was similar to

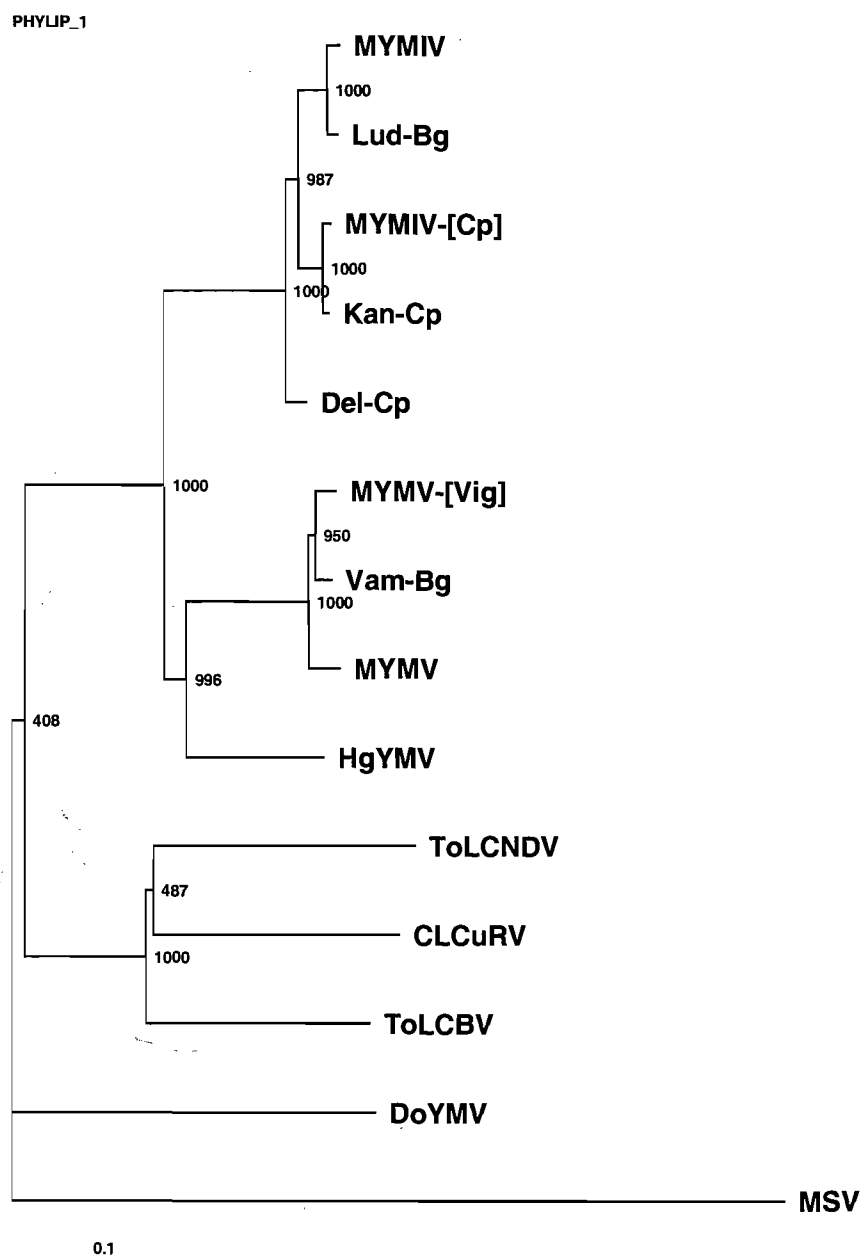


Fig. 57. Phylogenetic relationship between yellow mosaic virus isolates based on complete nucleotide sequence of DNA A. Dendrogram was constructed using the neighbour-joining Phylogenetic relationship between yellow mosaic method with bootstrapping (1000 replicates) in Treecon windows version 1.3b on sequences aligned using CLUSTAL X. vertical distances are arbitrary, horizontal distance are proportional to genetic distances.

organization of DNA B component of bipartite begomoviruses. There is one ORF each in viral sense (ORF BV1) and complementary sense (ORF BC1) with a large (971 nt) non coding region upstream in the case of Del-Cp. However, in the case of Vam-Bg, ORF BC1 could not be deciphered. The predicted amino acid sequence of the protein products (BV1 and BC1) showed motifs for nuclear localizing and nuclear export signal in the case of Del-Cp.

The most significant results were seen when the total nucleotide sequence of DNA B was compared with that of selected YMV isolates (Table 24). Del-Cp shared 97 percent identity with MYMIV-[Cp] and both the cowpea isolate showed slightly lesser percent identity with the type species MYMIV (92%). Contrastingly Vam-Bg isolate showed 97 percent identity with MYMV-[Vig] and considerably less (86-88%) with both MYMIV and Thailand isolate of MYMV. *Horsegram yellow mosaic virus* was very distinct from all the YMV isolates and shared only 66 percent identity with all of them.

The phylogenetic tree constructed based on complete DNA B nucleotide sequence (Fig. 58) clearly brought out the trichotomy among the YMV isolates. All the isolates of northern India (MYMIV, MYMIV-Cp and Del-Cp) formed one clade, MYMV-[Vig] and Vam-Bg formed second clade, MYMV from Thailand, a third clade. HgYMV was very distinct and separated from others.

4.3.4.2 Variability in the right half of the DNA A (RHA) of YMV isolates

Genetic variability studies on YMV isolates investigated earlier clearly showed, how the ORFs in viral sense strand, ORF AV1 and ORF AV2 (CP and pre-coat protein) show differences among them. As ORF AV2 was also

the focus of the present study, it was decided to investigate the extent of variability occurring in YMV isolates in the right half of DNA A. To achieve this, leaf samples were collected from YMD affected legumes from different agricultural fields. DNA was extracted and the right half of the genome that includes, non coding region (NCR-nucleotide 1 to start codon of ORF AV2), ORF AV2 and ORF AV1 was amplified with the AC-abut and CPV2 primers (Table 9). PCR fragments were cloned and nucleotide sequences were determined. Two samples per isolate were sequenced. Sequences were analyzed for translation of ORFs and finally were compared with two isolates of MYMIV i.e., MYMIV and MYMIV-Cp and two isolates of MYMV i.e. MYMV and MYMV-[Vig]. Results are given below.

4.3.4.2.1 Comparison of nucleotide sequence of NCR of YMV isolates

For begomoviruses, the sequences within the intergenic region is a useful marker to analyze diversity. The region selected in the present study represents the non-coding region after the first nucleotide. The isolates which were characterized are listed in Table 19. Totally ten isolates from different legumes like blackgram, cowpea, cassia, dolichos, mungbean and soybean were analyzed. The analysis included right-half of four full-length DNA A clones of Del-Cp, Kan-Cp, Lud-Bg, Vam-Bg, described elsewhere in this chapter.

Comparison of the nucleotide sequence of RHA and identity matrix drawn (Table 25) revealed that irrespective of the host species from which isolated, the YMV isolates from northern India (Delhi, Bareilly, Kanpur, Ludhiana and Pant Nagar) and central India (Nagpur) shared 92-97 percent identity, with MYMIV and MYMIV-[Cp]. The YMV isolates from western India (Navsari, Aurangabad) and one from southern India (Vamban) showed

95-97 percent with MYMV-[Vig]. All the 14 isolates showed only 70 percent identity with HgYMV; the lowest identity percentage was seen with DoYMV. This was true of sample from dolichos collected from Kanpur (Kan-Dol). Thus the NCR of DNA A contributed to differentiate the begomovirus species.

The multiple alignment of ~ 150 nt of NCR region (Fig. 59) shows the changes occurring in the YMV isolates. Especially in the case of isolates belonging to MYMIV group. They show blocks of deletion compared to MYMV isolates. Variability in this region is more pronounced between the MYMIV and MYMV species. Though it is a non-coding region, considerable blocks of homology were also evident amongst the YMV isolates.

Genetic distances between each pair of isolates were estimated and phylogenetic relatedness were estimated (Fig. 60) for this region. In the case of NCR, bifurcation into two major groups was seen, one group having HgYMV and MYMV isolates and the other group comprising MYMIV isolates. Within the MYMIV group, the isolates Lud-Sb1, Lud-Sb2 and Del-Cp were very distinct. They occupied a separate branch, deviating from all the other MYMIV isolates.

4.3.4.2.2 Comparison of predicted amino acid sequence of coat protein of (AV1) YMV isolates

Predicted amino acid sequences of coat protein gene of 14 YMV isolates described in the present study were compared with that of MYMIV, MYMIV-[Cp], MYMV, MYMV-[Vig], DoYMV and HgYMV (Table 26). The pairwise percentage identities of amino acid sequence is shown in Table 26. Isolates from northern India and central India showed only 84/85 percent identity with the type species MYMIV, but shared near 100 percent identity with MYMIV-[Cp]. All these isolates characteristically shared 80-85 percent

MYMV	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
MYMV-[Cp]	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Del-Cp	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Bar-Cp	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Nag-Sb	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Mer-Cp	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Kan-Del	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Del-Cat	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Lud-Sb1	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Lud-Sb2	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Pant-Sb	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Kan-Cp	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Lud-Bq	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
MYMV	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
MYMV-[Mg]	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Nag-Sb	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Bar-Cp	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
Lud-Bq	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
DoYMV	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT
MYMV	ACCTGAGTGC	CCCGCGACCG	GTGTATTGGG	GTTACTTTAA	CTTTT	CTGC	TTTTGTGGTA	CCCTTATCTT

MYMV	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
MYMV-[Cp]	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Del-Cp	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Bar-Cp	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Nag-Sb	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Mer-Cp	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Kan-Del	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Del-Cat	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Lud-Sb1	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Lud-Sb2	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Pant-Sb	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Kan-Cp	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Lud-Bq	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
MYMV	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
MYMV-[Mg]	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Nag-Sb	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Bar-Cp	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
Lud-Bq	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
DoYMV	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC
MYMV	TAGTCGTTCA	ATCAGAAGCG	CTCCTCAGCG	CTATGTTAAT	TCAAATTTGA	ATTATAAAGC	AAGTGGACAC

MYMV	TCTGAACC	ACTAACA
MYMV-[Cp]	TCTGAACC	ACTAACA
Del-Cp	TCTGAACC	ACTAACA
Bar-Cp	TCTGAACC	ACTAACA
Nag-Sb	TCTGAACC	ACTAACA
Mer-Cp	TCTGAACC	ACTAACA
Kan-Del	TCTGAACC	ACTAACA
Del-Cat	TCTGAACC	ACTAACA
Lud-Sb1	TCTGAACC	ACTAACA
Lud-Sb2	TCTGAACC	ACTAACA
Pant-Sb	TCTGAACC	ACTAACA
Kan-Cp	TCTGAACC	ACTAACA
Lud-Bq	TCTGAACC	ACTAACA
MYMV	TCTGAACC	ACTAACA
MYMV-[Mg]	TCTGAACC	ACTAACA
Nag-Sb	TCTGAACC	ACTAACA
Bar-Cp	TCTGAACC	ACTAACA
Lud-Bq	TCTGAACC	ACTAACA
DoYMV	TCTGAACC	ACTAACA
MYMV	TCTGAACC	ACTAACA

Fig. 59. Multiple alignment of nucleotide sequence of the right half, non-coding region of DNA A of yellow mosaic virus isolates.

PHYLP_1

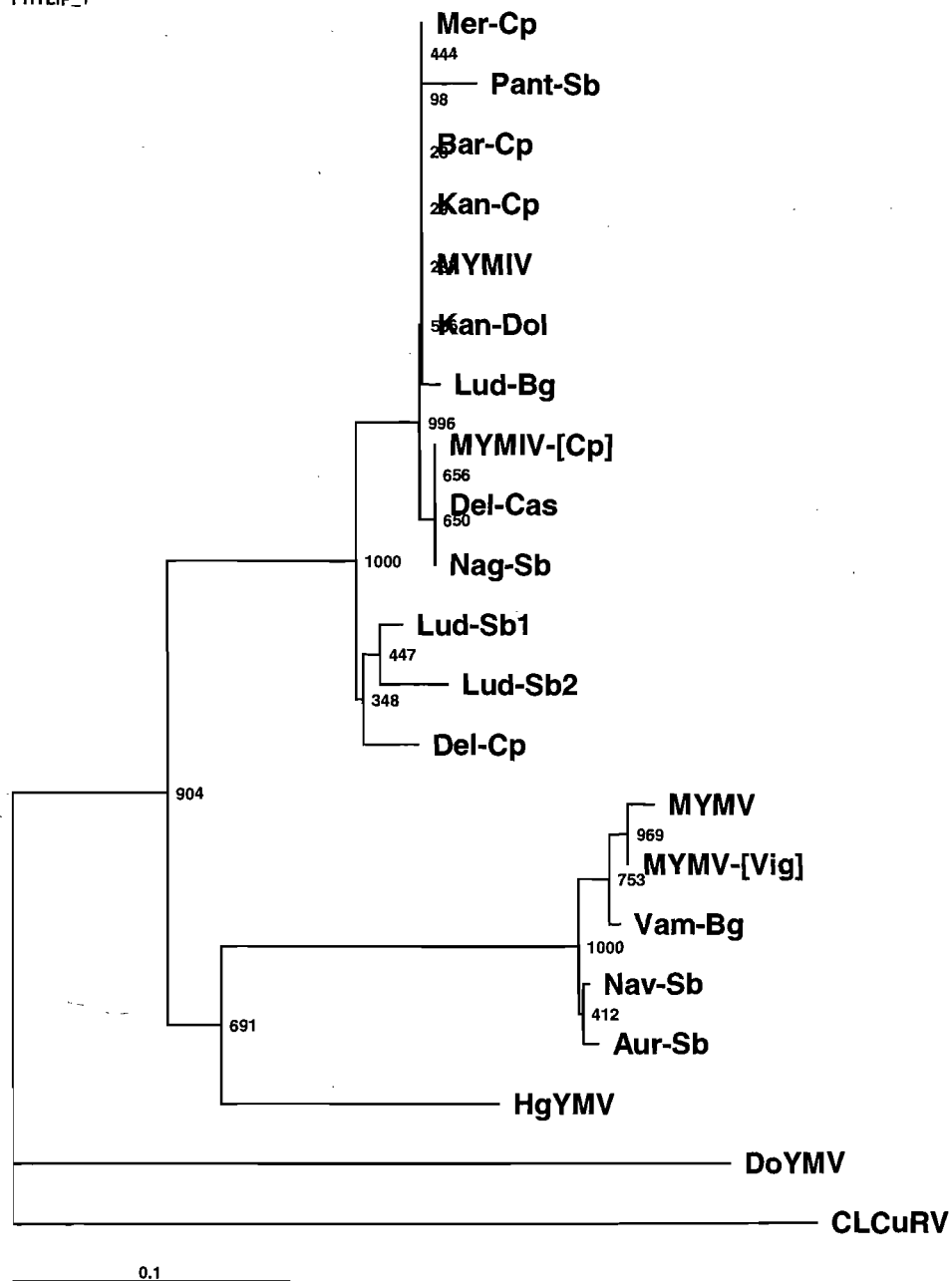


Fig. 60. Phylogenetic relationship between YMV isolates based on the nucleotide sequence of the right half of non-coding region. Dendrogram was constructed using the neighbour-joining method with boot strapping (1000 replicates) in Treecon windows version 1.3b on sequences aligned using CLUSTAL X. vertical distances are arbitrary, horizontal distance are proportional to genetic distances.

identities with MYMV, MYMV-[Vig] and HgYMV. The lowest percent identity was seen with *Dolichos yellow mosaic virus*. As in the case of NCR, in coat protein region too, the isolates from western India (Navsari, Aurangabad) and south India (Vamban) showed nearly 99 percent identity with MYMV-[Vig]. The isolate Aur-Sb was identical to MYMV-[Vig]. Both the groups showed 77 percent identity with DoYMV. However with HgYMV, while isolates belonging to MYMIV showed 85 percent, isolates belonging to MYMV group shared 91 percent sequence identity.

In the case of coat protein gene, it is the N' terminal amino acid from 1-60, that are highly variable. Variability among the isolates was examined in a multiple alignment of predicted amino acid sequence of first sixty amino acid in the N' terminal region. The Fig. 61 shows the details. Less identity seen between type species MYMIV and other MYMIV isolates is understood from the Fig. 61.

The type species MYMIV (Bg isolate used in the present study) ORF starts from a down stream AUG resulting in deletion of first 27 amino acids, present in other isolates. Likewise the type species MYMV also lacks first 13 amino acids contributing to variation. Other than these two major differences, all the isolates show remarkable identity except in few positions (T/N at 5th, I/M at 14th and A/V at 17th, N/T at 22nd, V/S at 28th, S/T at 32nd, G/S at 35th, T/A at 38th, N/S at 39th and F/Y at 56th amino acids). Alignment (Fig. 61) also revealed interesting changes seen in the four isolates, Del-Cp, Lud-Sb1, Lud-Sb2 and Lud-Bg. There is a substitution of M in the place of V at 28th; and A in the place of V at 33rd amino acid. These four isolates also show near 100 percent identity between them in the amino acid sequence.

Nav-Sb isolate showed substitution of D in the place of N unlike any other YMV isolates.

The phylogenetic tree (Fig. 62) designed based on the amino acid sequence of the coat protein confirmed the conclusions derived from multiple alignment analysis. Bifurcation of YMV isolates into two species, MYMIV and MYMV (that includes, HgYMV) were evident. Within MYMIV group, three clusters were seen, the type species MYMIV, the three isolates Lud-Sb1, Lud-Sb2, Del-Cp forming second cluster, all the other MYMIV isolates forming the third cluster.

4.3.4.2.3 Comparison of predicted amino acid sequence of AV2 and protein of YMV isolates

The ORF AV2 in the viral sense strand is present only in Old World begomoviruses. Identification of its role in viral pathogenesis and its contribution to evolutionary progress of the virus are the focus of the present investigation. Identity in predicted amino acid sequence was computed by pairwise comparison.

From the comparison, it is apparent that the two type species, MYMIV (Bg isolate studied in the present work) and MYMV (Thailand isolate) have undergone significant changes, due to considerable C1 terminal deletion. They both seem to have truncated version of AV2 protein. Therefore, here results are discussed in comparison with the other well established species, MYMIV-[Cp] and MYMV-[Vig]. The interesting division can be seen among isolates collected from north and central India. The isolates, Bar-Cp, Nag-Sb, Mer-Cp, Kan-Dol, Del-Cp, Pant-Sb and Kan-Dol share 95-100 percent identity with MYMIV-[Cp]. On the contrary, the isolates Del-Cp, Lud-Sb1, Lud-Sb2,

PHYLIP_1

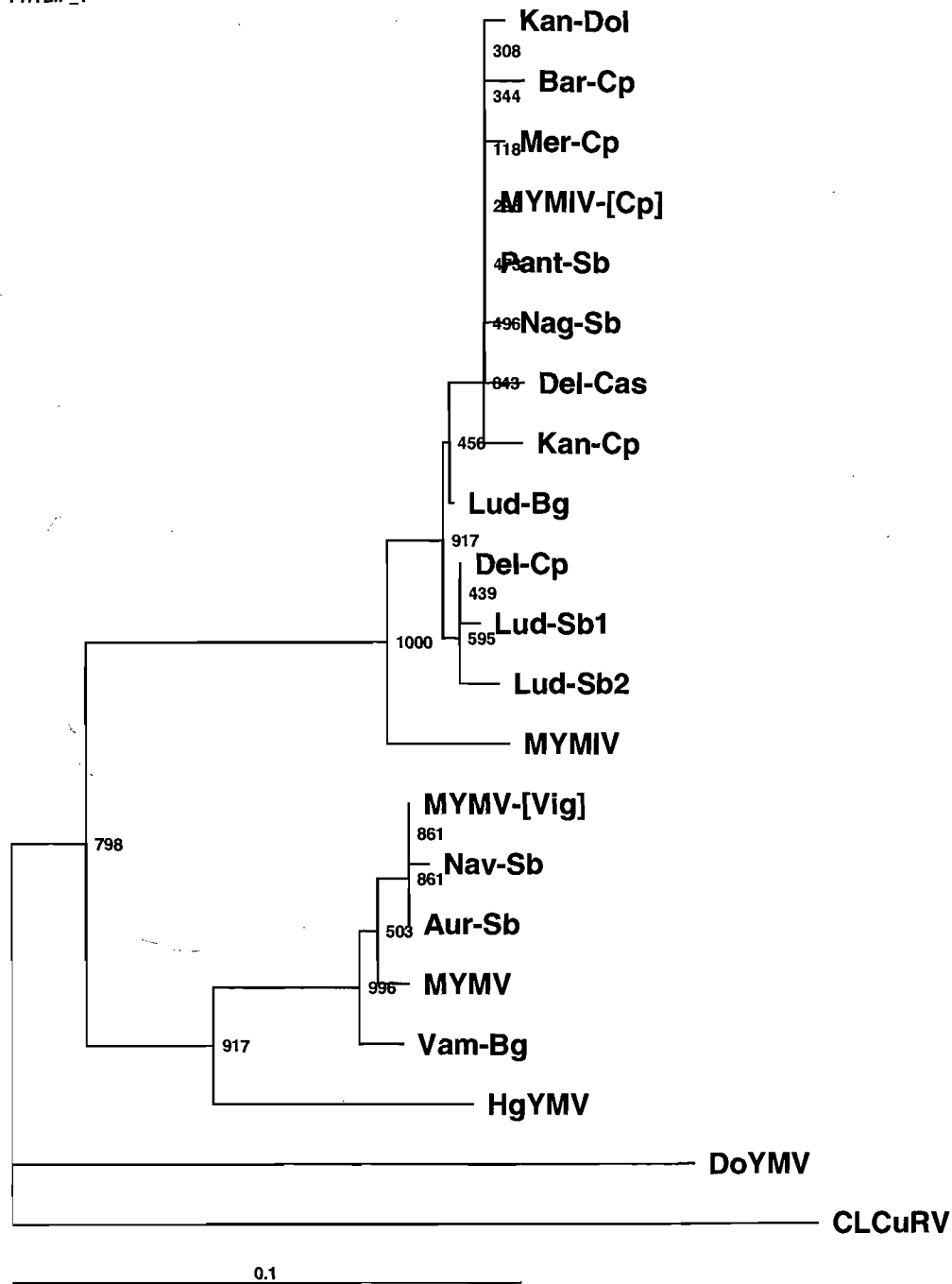


Fig. 62. Phylogenetic relationship between yellow mosaic virus isolates based on predicted amino acid sequence of coat protein (ORF AV1). Dendrogram was constructed using the neighbour-joining method with boot strapping (1000 replicates) in Treecon windows version 1.3b on sequences aligned using CLUSTAL X. vertical distances are arbitrary, horizontal distance are proportional to genetic distances.

Lud-Bg showed only 85 to 88 percent identity. The isolates, Aur-Sb, Nav-Sb and Vam-Bg typically showed 94 to 97 percent identity with MYMV-[Vig] (Table 27).

Multiple alignment (Fig. 63) of predicted amino acid sequence of AV2 protein indicated how, this ORF is under evolutionary pressure. Deletions in C-terminal region was seen in MYMIV, MYMV and in DoYMV. The alignment also showed amino acid substitutions in the isolates Del-Cp, Lud-Sb1, Lud-Sb2 and Lud-Bg. These four isolates shared identical substitutions, K in the place of Q at aa56, H in the place of R at aa81 and P in the place of A at aa83 and L or F in the place of V at aa85.

The grouping of isolates (Fig. 64) into three major clusters were very evident in the phylogenetic tree. The four isolates Del-Cp, Lud-Sb2, Lud-Bg, Lud-Sb1 separate from other MYMIV isolates. Among them too, further division into branches are seen indicating evolutionary divergence.

		10	20	30	40
MYMV	MWDPLLNDFP	KSVHGFRCML	AIKYLQOVQE	NYPSNSLGYS	
MYMV-ICPI	MWDPLLNDFP	ESLHGFRCML	AIKYLQOVQE	NYPSNSLGFL	
Del-Cp	MWDPLLNDFP	KSVHGFRCML	AIKYLQOVQE	NYPSNTVGYG	
Bar-Cp	MWDPLLNDFP	ESLHGFRCML	AIKYLQOVQE	NYPSNSLGFL	
Naq-Sb	MWDPLLNDFP	ESLHGFRCML	AIKYLQOVQE	NYPSNSLGFL	
Mer-Cp	MWDPLLNDFP	ESLHGFRCML	AIKYLQOVQE	NYPSNSLGFL	
Kan-Dol	MWDPLLNDFP	ESLHGFRCML	AIKYLQOVQE	NYPSNSLGFL	
Lud-Sb1	MWDPLLNDFP	KSMHGFRCML	AIKYLQOVQE	NYPSNSLGFS	
Del-Cas	MWDPLLNDFP	ESLHGFRCML	AIKYLQOVQE	NYPSNSLGFL	
Lud-Sb2	MWDPLLNDFP	KSVHGFRCML	AIKYLQOVQE	NYPSNSLGYS	
Pant-Sb	MWDPLLNDFP	ESLHGFRCML	AIKYLQOVQE	NYPSNSLGFL	
Kan-Cp	MWDPLLNDFP	ESLHGFRCML	AIKYLQOVQE	NYPSNSLGFL	
Lud-Bq	MWDPLLNDFP	KSVHGFRCML	AIKYLQOVQE	NYPSNSLGYS	
MYMV	MWDPLVNDFP	KSLHGFRCML	AIKYLQYIQE	NYPSNSLGYS	
MYMV-[Vig]	MWDPLVNDFP	KSLHGFRCML	AIKYLQYIQE	NYPSNSLGYS	
Nav-Sb	MWDPLVNDFP	KSLHGFRCML	AIKYLQYIQE	NYPSNSLGYS	
Aur-Sb	MWDPLVNDFP	KSLHGFRCML	AIKYLQYIQE	NYPSNSLGYS	
Vam-Bq	MWDPLVNDFP	KSLHGFRCML	AIKYLQYIQE	NYPSNSLGYS	
DoYMV	MWDPLVNDFP	DTLHGLRCML	AVKFFVQELLE	TYPRDSNGRI	
HqYMV	MWDPLLNDFP	KTVHGFRCML	AIKYLQOVQE	NYSTNSVGF	
CLCuRV	MWDPLLNDFP	DTVHGFRCML	SVKYLQLLSQ	DYSPDTLGYE	
		50	60	70	80
MYMV	YLTGLIOVLR	IRNHAKADLR	ILHSLP----	-----RYRM	
MYMV-ICPI	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEKIKLRYP	
Del-Cp	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEEIELRYP	
Bar-Cp	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEKIKLRYP	
Naq-Sb	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEKIKLRYP	
Mer-Cp	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEKIKLRYP	
Kan-Dol	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEKIKLRYP	
Lud-Sb1	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEEIELRYP	
Del-Cas	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEKIKLRYP	
Lud-Sb2	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEEIELRYP	
Pant-Sb	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEKIKLRYP	
Kan-Cp	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEKIKLRYP	
Lud-Bq	YLTGLIOVLR	IRNHAKADLR	YGLLYPDIEC	TEEIELRYP	
MYMV	YLTGLIOVLR	IRKHAKAELR	S-----	-----PPS	
MYMV-[Vig]	YLTGLIOVLR	IRKHAKAELR	YRLLYPDVEC	AEEDLRHP	
Nav-Sb	YLTGLIOVLR	IRKHAKAELR	YRLLYPDVEC	AEEDLRHP	
Aur-Sb	YLTGLIOVLR	IRKHAKAELR	YRLLYPDVEC	AEEDLRHP	
Vam-Bq	YLTGLIOVLR	IRKHAKAELR	YRLLYPDIEC	AEEDLRHP	
DoYMV	LLLEELIOVLR	CKRYAKAOF	YGVFVTKAHN	TSKGNLRRPV	
HqYMV	YLTGLIOVLR	IRKHAKAELR	YRLLYPDIEC	SSSETDLRHP	
CLCuRV	LIRDLICILR	SRSYVEASCR	YRHFYARVES	TPASELROP	
		90	100	110	
MYMV	HG-----GD-	-----	-----	-----	
MYMV-ICPI	RAAC-VCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Del-Cp	HAPC-LCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Bar-Cp	RAAC-VCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Naq-Sb	RAAC-VSGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Mer-Cp	RAAC-VCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Kan-Dol	RAAC-VCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Lud-Sb1	HAPC-LCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Del-Cas	RAAC-VCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Lud-Sb2	HVPC-FCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Pant-Sb	RAAC-VCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Kan-Cp	RAAC-VCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
Lud-Bq	HAAC-LCGR	PYOHEEKTLD	OPPHVEETSV	LPTV-----	
MYMV	LPRCRMCGGG	-----	-----	-----	
MYMV-[Vig]	FLTC-HCGKC	PCOREKEEVD	OPTHVEETEI	LSVIPLS--	
Nav-Sb	FLTC-HCGKC	PCOREKEEVD	RPTHVEETE	LSVIPLS--	
Aur-Sb	FLTC-HCGKC	PCOREKEEVD	OPTHVEETE	LSVIPLS--	
Vam-Bq	FLTC-HCGKC	PCOREKEEVD	OPTHVEETE	LSVIPLS--	
DoYMV	RNTD-----	-----D	SSSNSEQOSS	-----	
HqYMV	GATC-FCGR	SCOREKTSVD	QPSHVEETE	LSVVSLS--	
CLCuRV	HOPC-CCPH	PRHKTGTGMDK	QAYEOEAQDV	QDVOKSRCS	

Fig.63. Multiple alignment of predicted amino acid sequence of the ORF AV2 protein of yellow mosaic virus isolates.

PHYLIP_1

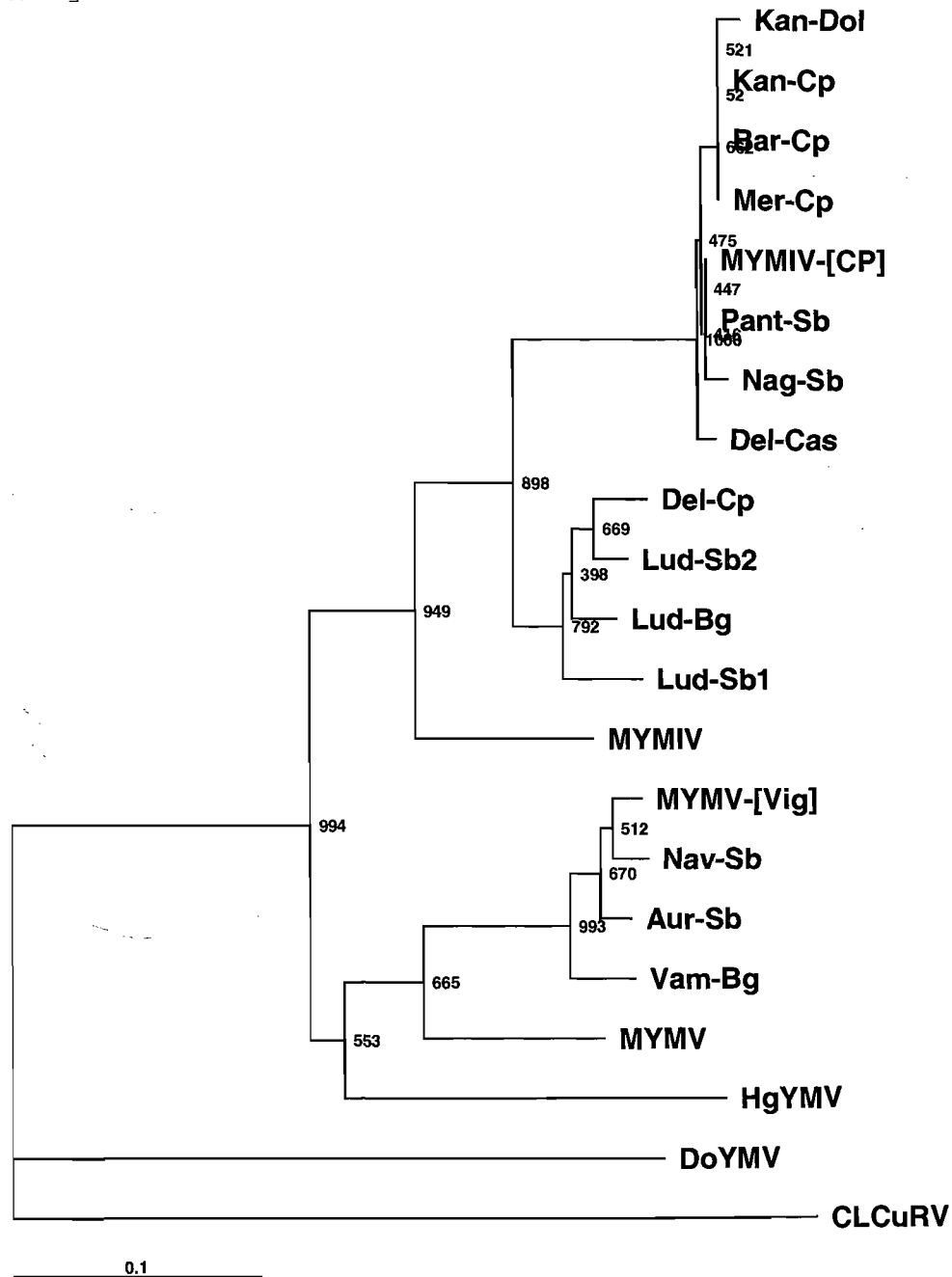


Fig.64. Phylogenetic relationship between yellow mosaic virus isolates based on predicted amino acid sequences of ORF AV2 protein. Dendrogram was constructed using the neighbour-joining method with boot strapping (1000 replicates) in Treecon windows version 1.3b on sequences aligned using CLUSTAL X. vertical distances are arbitrary, horizontal distance are proportional to genetic distances.

DISCUSSION

Pulses and grain legumes, a major source of dietary proteins, are affected by yellow mosaic and golden mosaic diseases causing an annual yield loss of about \$ 300 million (Varma *et al.*, 1992) in the three important pulse crops. Though the disease was noticed in 1950s and whitefly transmission was well established, the yellow mosaic viruses (YMV) causing the disease have been characterized only recently. YMV's belong to the genus *Begomovirus* under the family *Geminiviridae*. They show characteristic features of genome organization of begomoviruses. On the basis of nucleotide sequence identity of more/less than 89% [the cut off for distinguishing species from strains for begomoviruses (Fauquet *et al.*, 2003)], four species are recognized among YMV isolates. They are : *Mungbean yellow mosaic virus*, *Mungbean yellow mosaic India virus*, *Dolichos yellow mosaic virus* and *Horsegram yellow mosaic virus*. Of these four species, bipartite genome having DNA A and DNA B components and their contribution to viral pathogenesis have been established for MYMIV and MYMV. These YMV species that comprise isolates from India, Pakistan, Nepal, Bangladesh and Thailand in South Asia form a distinct phylogenetic lineage, they are not closely related to other begomoviruses from the same geographical area (Fauquet and Stanley, 2003; Usha Rani *et al.*, 2004; Kartikeyan *et al.*, 2004). This distinct nature of YMV offers an opportunity to address various questions relating to viral gene functions.

The genus *Begomovirus* comprises a large number of viruses that cause devastating disease in tropical and sub-tropical countries (Varma and Malathi, 2003). They have characteristic geminate particles (30 x 20 nm) encapsidating

single stranded circular DNA. Begomoviruses are either monopartite or bipartite in genome organization. Bipartite begomoviruses have DNA A and DNA B components each of ~ 2.7 kb in length. In DNA A, ORF's AV1 (coat protein) and AV2 (pre-coat protein) are present in the virion sense. ORF's AC1 (Rep), AC2 (TrAP), AC3 (REn) are present in the complementary sense. In DNA B, ORF BV1 (Nuclear shuttle protein, NSP) is encoded in the virion sense and ORF BC1 (movement protein, MP) in the complementary sense.

Comparison of the coat protein amino acid sequences combined with antigenicity of virus particles from diverse area led to clear perception of demarkation of begomoviruses into two branches, one containing Old World viruses and the other New World viruses (Howarth and Vandermark, 1989; Harrison and Robinson, 1999). Besides the differences in the coat protein amino acid sequences, Old World begomoviruses, differ from New World viruses in having one more ORF in the viral sense (ORF AV2), upstream of ORF AV1 (coat protein) and so called as pre-coat protein.

The function of ORF AV2 has been examined in two bipartite begomoviruses, ACMV and ToLCNDV. While, its mutation did not affect symptom production in ACMV (Etessami *et al.*, 1989), in ToLCNDV, it caused attenuation of symptoms and reduced ss DNA level accumulation. A role in movement of viral genome was speculated (Padidam *et al.*, 1996). In the case of monopartite *Tomato yellow leaf virus*, it is considered to play a role as pathogenicity determinant, possibly *via* the regulation of ss and ds DNA levels (Wartig *et al.*, 1997). Based on another interesting study on subcellular localization of AV2 of TYLCV, Rojas *et al.* (2001) were of the opinion that the AV2 (V1 in this case) enhanced CP mediated nuclear export

of viral DNA and was not efficiently involved in cellular trafficking. According to their findings, V1 (= AV2 protein) was found to facilitate coat protein mediated viral DNA export from the nucleolus to plasmodesmata.

Perusal of the literature clearly reveals that, information available is very limited, especially in the case of Old World begomoviruses. It is especially true for YMV isolates, where the ORF AV2 is variable (Usha Rani *et al.*, 2004) in both MYMIV and MYMV. Keeping above points in view, objectives of the present study were set to identify the role of AV2 protein in viral pathogenesis by site directed mutagenesis. It was also planned to study the biochemical function by overexpressing AV2 protein in bacterial system. As in DNA A genome, the intergenic region especially the non-coding region immediately after *ori* and AV2 show divergence, it was decided to look into the extent of variability in the right half of DNA A component. The significance of the results obtained are discussed in this chapter.

The AV2 function was investigated in the present work by agroinoculating DNA A and DNA B components of *Mungbean yellow mosaic India virus* and the *Mungbean yellow mosaic India virus*-[cowpea] (MYMIV-Cp). These two isolates were used, as they offer certain advantages like (a) agroinoculation using these two virus isolates, Bg (MYMIV - the type species) and Cp (MYMIV-[Cp]) has been well established from our laboratory (Mandal *et al.*, 1997; Biswas and Varma, 1991; Usha Rani *et al.*, 2004, 2005; Surandranath *et al.*, 2005; Malathi *et al.*, 2005), (b) biological characteristics of these two Bg and Cp isolates are very distinct; Bg isolate infected leguminous species blackgram, mungbean, soybean, French bean, mothbean but not cowpea; Cp isolate, though failed to infect blackgram, mungbean through whitefly transmission, by agroinoculation could infect all the above

four species, (c) nucleotide and predicted amino acid sequence comparison of Bg and Cp isolates revealed that Bg isolate had only a truncated AV2 protein. Due to insertion of 'A' at 336 nt position, there is frame shift and protein is terminated prematurely so the isolate offers a chance to study the effect of N' terminal deletion of the protein and to study the effect of this protein without affecting expression of coat protein.

Infectivity of Bg and Cp isolates

Differences in symptoms host reaction

Before mutagenesis, these two isolates were agroinoculated into three cultivars of blackgram, mungbean, cowpea and French bean (Fig. 12) in order to confirm the biological integrity of the clones and to select a suitable susceptible variety. Inoculation revealed some unexpected results.

Yellow mosaic viruses infecting legumes are non-sap transmissible and host range identification depends mainly on the behaviour of the whiteflies, that varies with the host, genotypes, vector biotypes and growth conditions. In such cases agroinoculation offers an alternative method for the introduction of viral nucleic acids into plants (Grimsley *et al.*, 1986). Agroinoculation of isolates of MYMIV (blackgram, Mandal *et al.*, 1997; Chakraborty, 1996; Surendranath *et al.*, 2005; mungbean isolate, Biswas, 2000; Chakraborty, 1996 and cowpea isolate, Malathi *et al.*, 2005; soybean, Usha Rani *et al.*, 2005) have already been established by several workers. There are differences in the infectivity of the isolates. For example, the same clones of Bg isolate, MYMIV-Bg3 has been used by Chakraborty (1996) and Surendranath *et al.* (2005). In both the reports; the isolate was seen to produce either very mild tip bending or blunting (Surendranath *et al.*, 2005) or no symptoms at all

(Chakraborty, 1996) contrasting to results obtained in the present work. This discrepancy is attributed to two things, one the strain of *Agrobacterium* used; Chakraborty used LBA 4404 strain of *Agrobacterium*, compared to which *Agrobacterium tumefaciens* EHA 105 strain is known to be (Hood *et al.*, 1993) a supervirulent strain with higher delivery of T DNA, (2) the *Agrobacterium* strain itself may not explain. The difference in results as Surendranath (2005) used the same EHA 105 strain. Another factor may be the host-genotype. For example jumbled. Surendranath (2005) used the cowpea cv. Lal lobia and got high rate of infectivity and expression of symptoms in the cv. Lal lobia (57%) when inoculated with Cp isolate. In the present study cv. Lal lobia did not get infected at all by the Bg and Cp isolates. On the contrary, Malathi *et al.* (2005) could not get high rate of infectivity in the cv. Pusa Komal, and good expression of symptoms were seen in cv. Lal lobia. It is inferred that the host genotypes in spite of having the name Lal lobia, may not be pure. Though legumes are self pollinated crops ~ 1% cross fertilization is reported, impurity of seeds the genotypes may explain the ambiguity in results.

The results on the agroinoculation of Bg and Cp isolate on three cultivars again showed the importance of host genotype. In the case of YMV, reaction of the host to virus isolate is specific and this combined with vectors biotype may explain why host range of the isolates are very different to estimate (Varma *et al.*, 1992). Transmission to cowpea, or transmission from cowpea to other hosts may be hindered because of above factors. In this context agroinoculation of cowpea plants by Cp isolate is very advantageous. The significant difference between whitefly transmission and agroinoculation is seen in the case of Cp isolate. Cp isolate is not whitefly transmissible to

blackgram and mungbean (Mandal and Varma, 1996; Varma *et al.*, 1992). But through agroinoculation Cp produced typical yellow mosaic in blackgram and mungbean. Delivery of viral components through agroinoculation perhaps ensured introduction of sufficient amounts of viral DNA into the host cells resulting in pathogenesis. Similar results were seen in tomato cultivars showing field resistance to *Tomato yellow leaf curl virus* (Kheyr-Pour *et al.*, 1994). In the present work, even Cp isolate produced a severe leaf curl symptoms in the leaves in cv. Pusa Komal. Such leaf curl symptoms are not seen in the naturally infected plants in the field. Malathi *et al.* (2005) did not report any leaf curl symptoms with Cp isolate. Extreme reduction in leaf lamina, asymmetry in the leaflets size and curling seen here are indicative of viral delivery, which may induce different host response, in cv. Pusa Komal.

Viral DNA accumulation in legume hosts, after inoculation with Bg and Cp isolate showed the differences in host adaptation of the isolate. In cowpea plants, inoculated with Bg isolate that showed mild atypical leaf curl symptoms, viral progeny DNA was detectable at a very low concentration (Fig. 14). High level of viral replicative forms could be seen in blackgram, French bean and very moderate level in mungbean plants. From the study, it was evident that though Bg isolate infects cowpea, it is replication incompetent. On the contrary Cp isolate replicated efficiently in all the four hosts, the absence of pathogenity in whitefly transmission may be only due to inefficient delivery by the vector.

Differences in the times taken for symptom development after agroinoculation and whitefly transmission was described by Biswas and Varma (2001). They also demonstrated how agroinoculation can be used for screening legume cultivars for virus resistance. Jacob *et al.* (2002) showed that when

DNA A and DNA B constructs of MYMV-[Vig] are harboured in one *Agrobacterium tumefaciens* strain, infection was 100%. Girish and Usha showed that characteristic yellow mosaic symptoms were observed 28 DPI when MYMV-Sb was inoculated. Agroinoculations of blackgram and mungbean with partial dimers of multiple DNA B components of MYMV-[Vig] separately along with DNA A caused distinctly different symptoms (Balaji *et al.*, 2004). The DNA B component KA22 caused more intense yellow mosaic symptoms with high viral DNA titre in blackgram; in contrast the DNA B component KA27 caused more intense yellow mosaic symptom with high viral DNA titre in mungbean. Thus, DNA B of MYMV-Vig was shown as an important determinant of host-range between blackgram and mungbean.

Agroinoculation is used routinely to address various questions regarding viral gene function and as a technique to study the assortment of genetic components (Ramos *et al.*, 2003; and references therein). This is more efficient and cheaper to adopt than biolistic delivery.

Role of AV2 (pre-coat) in viral pathogenesis

In the present study, the functions of virion sense ORF AV2 present only in Old World begomoviruses was investigated by site directed mutagenesis. The region upstream of ORF AV1, including ORF AV2 and the non-coding region upstream of AV2 are very important ones having regulatory sequences. In the case of YMV isolates transcriptional strategy of expression of ORF AV2 and AV1 is not well understood. Shivaprasad *et al.* (2005) did extensive analysis of transcription start sites of the viral sense transcripts and suggested that ORF AV2 and ORF AV1 are translated from a dicistronic transcript, which have two different 5' transcription start site, one

at 4 nt above ATG of ORF AV2 and at 107th coordinate above ATG of ORF AV1. However, Usha Rani *et al.* (2006) reported two different transcription start sites for ORF AV1 and AV2, one at 107 nts upstream of ATG of ORF AV1 and another four nucleotide upstream of ATG of ORF AV2. This is suggestive of monocistronic and dicistronic transcripts for viral sense genes. As transcription strategy was not clear, mutations were planned in such a way that ORF AV1, or the coat protein is not at all affected. In Bg isolates, five mutations were done as M₁ (Met to Ile), W₂, H₁₄G₁₅ del A (insertion of A is removed) and Y₃₂. All these cases did not affect CP expression, as in this case, the AV2 ORF ends pre-maturely and does not overlap with ORF AV1. However, in the case of Cp isolate, ORF AV1 overlaps with the C' terminal of ORF AV2. So the mutation at K₇₃, C₈₆ and C₈₄₋₈₆ change of nucleotide in ORF AV2 could change in amino acid. However, only in the case of CpK₇₃ change from K to P (lysine to arginine) were seen in translation analysis.

In both Bg and Cp isolates, there was a marginal increase in percentage infectivity, and a slight decrease (100 to 93%) in French bean. It is negligible and it is inferred that number of plants, the isolates could infect, did not differ between wild and mutants. Mutations totally abolished symptoms in analogous V₁ protein in *Tomato yellow leaf curl virus* (Wartig *et al.*, 1997). In contrast in a mutation that produced truncated protein with premature stop codon, 3 of 10 inoculated plants showed symptoms. In the case of Bg and Cp isolates symptoms were produced, which were faint and attenuated in all the legume hosts. Attenuation was very prominent in the mutation M₁, del A, W₂, H₁₄G₁₅ and G₄₄. Attenuation was prominent in French bean for Bg isolate, where, elongation of shoot, production of trifoliate leaves were seen with mutant, but none in wild type. Symptoms were very insignificant, small squarish

yellow mosaic area faintly merging with green area.

Plants inoculated with BgW₂, H₁₄G₁₅, Y₃₂ showed minimum five fold decrease in the amount of supercoiled and ss DNA levels. While decrease was very apparent in blackgram inoculated by Bg mutants, in DNA A and DNA B it was only 2 to 3 folds decrease in French bean.

Contrasting to these two mutation were the results of CpK₇₃, CpC₈₆, CpC₈₄₋₈₆. In these three cases, the symptoms were severe, it was of different type of increased networking of vein different from the golden mosaic symptoms produced by the wild type.

Strikingly in the case of W₂, H₁₄G₁₅ mutation of Cp isolate, in cowpea other replicative forms oc, lin were also decreased, totally replication seems to be affected.

Just contrasting to decreased ss DNA sc DNA accumulation, increased ss and sc DNA levels were seen in the case of BgM₁ and Bgdel A in French bean, the level differences could be easily perceived in DNA B levels. The impact of K₇₃, C₈₆, C₈₄₋₈₆ were marked, there were minimum five folds increase in sc and ss level of viral replicative forms.

Summary of all the observations clearly indicate ORF AV2 requirement for accumulation of viral DNA and in symptom development. Reduced levels of viral DNA may occur either due to reduced replication or movement. Analysis of the DNA from inoculated plants could not separate effect due to reduced replication or impaired movement. Experiments with protoplasts would have helped to separate the effect on replication from impaired movement. Attempts to transfect French bean protoplast could not succeed

in our laboratory. However, from *in planta* experiments, it is very clear that ss DNA accumulation is affected. Low level of ss DNA accumulation, indicates continuous ds DNA synthesis by increasing the amount of template available for minus-strand synthesis.

This altered ratio of DNA forms suggest a role for AV2 to regulate the switch from ds DNA to ss DNA synthesis. Or as in the case of M13 gene 5 protein, it may participate in the virus assembly (Kornberg and Baker, 1992) whose role is to interrupt synthesis of duplex replicative forms and to prepare the DNA for packaging. In which case AV2 protein and AV1 might interact, as in the case of TLCV shown by Ridgen *et al.* (1993).

Synergistic reduction in ss DNA level of TLCV V1-V2 double mutants compared to single mutant were seen, suggesting a co-ordinated effort between AV2 and Cp (Ridgen *et al.*, 1993).

The mutations BgM₁ and Bg delA caused increase in the level of sc and ss DNA accumulation in French bean compared to Wild type level. This is also seen in black gram, but it is more pronounced in DNA B level of French bean. Mutation M₁ was expected to produce a truncated AV2 protein with N terminal deletion of 18 amino acid. From the results it appears that it is N terminal region that may be important in sequestering ss DNA form from the replication pool, as it is deleted, there is concomitant increase in its level. However, why it is evident in French bean and not in blackgram is not understood. This disparity in the behaviour of the mutant between two leguminous hosts, may indicate how, replication regulation is specific in a host species.

Increase in sc and ss DNA level in the mutants CpK₇₃, CpC₈₆, CpC₈₄₋₈₆ in all the leguminous hosts for both the isolates need careful

examination, as these regions also overlap with the N' terminal regions of coat protein. The results may be *per se* due to the AV2 protein. The fast migrating band represent the overlapping bands of sc and ss forms; based on the S₁ nuclease treatment, in W1 inoculated plants, majority of the DNA in that region are considered to be ss DNA, and as sc form are less in agroinoculated legume plants, ss DNA may be accumulated in these cases. It is hypothesized that AV2 failed to interact with CP protein to sequester the ss DNA, leading to its accumulation.

Alternatively, the more appealing inference based on our results on modulation of nicking activation of Rep by AV2, is as follows; majority of the fast migrating band may represent the ds supercoiled form, which has not been efficiently nicked by Rep, to enable it to enter into rolling circle replication. The AV2 protein is required in extremely small amount and, as such Rep has a minimal nicking activity which can initiate replication, the mutated versions of AV2 may not enhance the nicking activity of Rep resulting in the accumulation of sc DNA.

In the case of Cp isolate, the AV2 protein overlaps with coat protein gene, the mutation K₇₃, has correspondingly caused change of arginine to glycine in coat protein. This substitution might have interfered with coat protein function of sequestering ss DNA from replication pool interaction. Malik *et al.* (2005) have demonstrated that nicking and closing function of Rep in the case of MYMIV (Bg isolate) is down regulated by CP. It will have to be investigated, how AV2 and CP regulate the nicking activity of Rep.

When the AV2 mutants were agroinoculated in legume plants, in some plants inoculated by BgM₁ and CpM₁, some plants developed wild type of

symptoms. Determination of the sequence of the viral progeny in plants displaying wild type symptoms revealed that the introduced mutation had reverted to the wild type sequence, thus restoring ORF AV2. Such reversion of mutants have been seen in other geminiviruses (Stanley *et al.*, 1992; Rigden *et al.*, 1994). Padidam *et al.* (1996) showed that viral DNA without a mutation in AV2 replicates preferentially over viral DNA with a mutation in AV2. All these strengthen the inference that, though the ORF AV2 may have indirect involvement either in symptom production or in replication and its indispensable nature cannot be negated.

Disruption of ORF AV2 in different host legumovirus caused different effects. Though the position is well conserved in all the begomoviruses from the Old World the percentage identity between them is from 45 to 66% (Padidam *et al.*, 1996). Disruption of AV2 in TOLCV-Australia led to symptomless, systemic infection with reduced titre of all DNA forms (Rigden *et al.*, 1993) but the disruption of AV2 ORF in ACMV had no effect on infection (Elessami *et al.*, 1989).

Mutations in the ORF V₂ in MSV and V₁ in BCTV, a ORF present in a similar location in their genome showed that the ORF is required for movement of viral genome in the case of MSV (Boulton *et al.*, 1989; Lazaronitz *et al.*, 1989) and not required for spread in the case of BCTV (Hormuzdi and Bisaro, 1993; Stanley *et al.*, 1992). As sequence similarity between these viruses in this positionally conserved protein is very low, no comparison can be made.

Biochemical function of AV2 protein

Replication of viral genome inside the plant cell, has three overlapping stages of DNA synthesis namely ss-ds DNA, ds-ds DNA and ds-ss DNA (Guiterez, 1999; Saunders *et al.*, 1991; Stenger *et al.*, 1991). Both viral encoded proteins and host factors play roles in modulating synthesis of viral nucleic acid. Since the focus of the present investigation was ORF AV2 of Bg and Cp isolate AV2 proteins were expressed as MBP-AV2 fusion protein. More than 50% of expressed protein were in the insoluble form which could be released by sonication. While in the case of Bg a single band at 54 kDa was seen, in Cp, there was an additional product at ~ kDa due to proteolytic cleavage.

The antibody raised against MBP-BgAV2 detected the fusion protein in the Western blot. However, using this antibody detection of AV2 protein in the plants was difficult. Protein extract from agroinoculated blackgram, cowpea, mungbean and French bean plants showed multiples of bands in a SDS-PAGE gel which are considered as non-specific protein bands. Padidam *et al.* (1996) showed that *in planta*, AV2 protein was present as multimer. They could also detect it in protoplasts. Failure to detect AV2 protein in inoculated plants may be due to low concentration of protein extract. Padidam *et al.* (1996) got the signal in Western blot using 20 µg of protein from infected plant, 100 µg of protein from protoplast. In the work present here only 4 µg of protein were used, which may have to be increased several folds to get signal.

In the binding assay experiment, under the conditions employed, strong binding between DNA fragment and AV2 protein could not be shown. Lack

of DNA binding activity of AV2 (V1 in TYLCV) were also seen in TOLCNDV (Padidam *et al.*, 1996), TYLCV (Wartig *et al.*, 1997). The DNA binding motif, a putative C₃H₁ type Zn-finger motif located in CP is not seen in this protein.

In planta, viral replicative forms levels were affected when agroinoculated with isolates having mutated AV2, which is suggestive of its interaction with replication initiator protein (Rep). Results of protein-protein binding revealed that, interaction between Rep and AV2 protein may be very specific and weak. The interacting domain in Rep and AV2 may have to be studied in detail; that is expected to shed light on how AV2 protein may interfere with replication.

Modulation of nicking and ATPase activity of Rep by AV2 protein was interesting. AV2-Rep interaction at very low concentration of 1-4 ng enhanced the nicking activity atleast by 20 folds. With the increased concentration AV2 protein, further enhancement was not seen indicating how site involved in nicking could be saturated even at low concentration. ATPase activity is only two folds enhanced in presence of AV2 protein and not as dramatic as in the case of nicking activity. These interesting results need further elaborative investigation. No such information is available on any protein that modulate the activities of Rep in other geminiviruses, their activity is readily comparable with any accessory protein. AV2 protein may function as a molecular chaperone to bring Rep and DNA together in a controlled fashion. The very low concentration at which it is efficient, also may explain its lack of detection *in planta* system.

Genetic variability among YMV isolates

The mutational analysis of AV2 proteins indicated how AV2 and coat protein may function in co-ordination. Molecular variability studies in begomoviruses is often done concentrating on N' terminal region of CP, or core CP or entire CP region. This analysis was further extended in the present work. The right half of the DNA A genome consists of non-coding region comprising regulatory sequences of downward transcription, and the ORF AV2 and ORF AV1 that overlap. It was decided to assess the extent of variability in this region among yellow mosaic virus isolates. More than 200 samples were analyzed and finally 14 isolates were cloned, analysis of nucleotide and predicted amino acid sequence of the region indicate how there is geographical gradient of similarity.

Usually begomovirus genome are cloned by either isolating double stranded replicative form by cesium chloride density gradient centrifugation (Stanley and Townsend , 1983) or by amplifying the full-length or partial genome with specific primers. Both these require high quality DNA without polysaccharide or phenolics. An efficient protocol for extraction of PCR compatible DNA was required to be developed. In the present work, several methods were evaluated, and a protocol, named as Gem-CTAB was evolved, that gave PCR compatible DNA in 6 out of 16 samples tested.

Among several approaches, PCR using degenerate primers has been the most useful for the detection of geminiviruses (Deng *et al.*, 1994; Rojas *et al.*, 1995; Rybicki *et al.*, 1990; Wyatt and Brown, 1996). Several modifications in template preparation have been suggested by several workers. The Gem-CTAB method gives a protocol by which both full-length and partial genome could be amplified for YMV infecting legume species. However,

the percentage of success with samples from mungbean was low. It needs modification in the amount mercaptoethanol added. Sample to sample adjustment is required to achieve amplification.

Sequence analysis of the legume infecting begomoviruses reveal that these viruses are unique and have characteristic distribution. In this study, four full-length DNA A and two full-length DNA B components and ten right half of DNA A genome were cloned and sequenced. The phylogenetic tree based on DNA A, DNA B and the RHA clearly showed that the YMV isolates from northern India, Bar-Cp, Nag-456, Del-Cp, Kan-Cp, Mer-Cp, Lud-Bg, Lud-Sb1, Lud-Sb2, Pant-Sb, Kan-Dol belong to MYMIV species and always cluster with the MYMIV, MYMIV-[Cp] group. Whereas, the YMV isolates collected from southern (Vam-Bg, Vamban, Tamilnadu) and western India (Nav-Sb Navsari, Gujarat; Aun-Sb, Aurangabad, Maharashtra) belong to MYMV. From the analysis it is clear that four legume infecting begomoviruses occur in South and South-east Asia, and they have specific distribution. They are very distinct and separated from other begomoviruses occurring in the same location. This conclusion has been demonstrated earlier by Fauquet and Stanley (2003), Usha Rani *et al.* (2004), Girish and Usha (2005), Hameed and Robinson (2004), Karthikeyan *et al.* (2004). However, occurrence of both MYMIV and MYMV has been reported from Pakistan (Hameed and Robinson, 2004); similarly MYMIV occurrence has been reported by Priya (2005) from Gujarat. More number of representative samples may have to be collected to precisely map the distribution of the viruses.

Of the four viruses, DoYMV and HgYMV are very different from MYMV and MYMIV and share only 60-62% nucleotide sequence identity in DNA A. In the study on YMV isolates collected from Dolichos, 11 out of 21

samples amplified with Cp and full-length abutting primers, one of which, Kan-Dol (dolichos sample from Kanpur) has been cloned, sequence and found to belong to MYMIV. However, from Delhi, a YMV from Dolichos has been characterized and found to be distinct as DoYMV (Malathi *et al.*, 2005). It is possible that at least in northern India, dolichos may be infected by both MYMIV and DoYMV which may be present as mixed infection.

For two isolates, full-length DNA A and DNA B components have been cloned. Based on DNA A nucleotide sequence analysis and comparison and as per guidelines laid by ICTV (Fauquet *et al.*, 2003), Del-Cp isolate was identified as MYMIV and so named as MYMIV-Cp [Del2] and Vam-Bg as MYMV-Bg[Vam]. The degree of divergence in the CR and *Ori* were analyzed. Unexpectedly, Del-Cp showed 32% divergence, highest among YMV isolates, Vam-Bg also showed about 19% divergence. A similar divergence is observed between DNA A and DNA B components of all other yellow mosaic virus isolates (Usha Rani, 2004; Usha and Girish, 2005). The divergence emerge because of deletions and insertions. Infact, Usha Rani *et al.* (2004) revealed that compared to MYMV isolate from Thailand, extensive deletions have been met in all the YMV isolates. *Ori* is the hot spot for recombination during replication of circular single stranded DNA of phage M13 (Michel and Ehrlich, 1986) and was a site of forced recombination in ACMV (Stanley, 1995). It is possible, that CR represents the hot recombination site for YMV isolates. Deletions and insertion of sequences are more evident in Del-Cp.

Del-Cp has several non-canonical features in the genome. First, the DNA A component lacks the conserved nonanucleotide sequence TAATATTAC. It has the nicking site, but lacks TAATAT thereby missing

the typical loop structure. Replicative forms of DNA A seen in southern blot analysis with CP probe, clearly indicated that despite the disruption of cleavage site, replication was not affected in Del-Cp. This is quite contrasting to observations of Laufs *et al.* (1995) who considered that change of sequences at +7 or +10 position, may reduce the efficiency of cleavage. Del-Cp also shows another unique feature. The predicted ORF AC1, Rep, does not have a stop codon (Fig. 54) and downstream of this ORF in frame is the ORF AC2 which has a GUG start codon. This is very unusual and has not been reported for any begomoviruses. A non ATG codon is seen in many plant viruses like *Rice tungro virus* (RTBV) (Hull, 2002).

Vam-Bg isolate shows 97% identity in both DNA A and B nucleotide sequence with MYMV-[Vig]. The YMD infected samples in southern India have multiple DNA B components (Karthikayan *et al.*, 2004; Balaji *et al.*, 2004; Usha and Girish, 2005). However, in the isolate Vam-Bg, there was only one DNA B component, which was near identical with MYMV-[Vig].

An extensive analysis of variability among YMV isolates involved right half of DNA A sequences of 14 isolates. Analyses were divided into (1) on non coding region after *ori* (2) predicted amino acid sequences of ORF AV2 and (3) AV1. Of the three regions compared, within the species, identity percentage is less for ORF AV2 region (85-93% in MYMIV, 95% in MYMV); followed by non-coding region, CP region is almost near identical in AV1.

The study hypothesized that ORF AV2 is variable in YMV isolates, and may help in differentiating the variants/strains. As Fig. 63 shows MYMV, MYMIV, DoYMV showed different degree of deletions. The most remarkable is the cluster containing Lud-Sb1, Lud-Sb2, Lud-Bg, Del-Cp isolates. These four isolates showed only 85% identity in predicted amino acid sequence,

with other isolates belonging to MYMIV. All these four isolates collected from blackgram, soybean and cowpea from Delhi, and Ludhiana, showed identical substitution of amino acids. The results clearly revealed that ORF AV2 is undergoing lot of changes especially deletions.

The sequence analysis of Del-Cass established that the YMV causing mottling symptom in this host is MYMIV. Till date there is no record of MYMIV infecting cassia and the present work is the first record, significance of this weed host in perpetuation of virus inoculum will have to be looked into.

The emergence of new diseases caused by begomoviruses is mainly decided by the genetic structure of the population of the virus and genetic variability and structure of vector. While, involvement of different biotype of whitefly vector (biotype K with cotton leaf curl virus in Pakistan, biotype B in Kolar district, *Tomato leaf curl Bangalore virus*). With outbreak of YMD in legumes, new population of whitefly has not been found associated with the disease.

However, there is enough evidence that population structure of the virus is undergoing changes which is evident from divergence in CR, multiple DNA B, and genome modification with alteration in expression strategy. Despite the changes observed, YMV isolates seem to possess genetic stability, unlike begomoviruses infecting tomato or cotton. The present work represents the first analysis including 14 isolates of MYMIV, MYMV and other two virus species HgYMV and DoYMV. Their distinct lineage, different from other begomoviruses is challenging field that needs to be addressed. Implication of our studies is that YMV isolates may have originated independently following a different path of evolution and may not share any common ancestor with other Indian begomoviruses.

SUMMARY

Yellow mosaic disease is the major constraint in improving the productivity of grain legumes. The disease is caused by whitefly transmitted geminiviruses belonging to the genus *Begomovirus* of the family *Geminiviridae*. In India, four species are associated with yellow mosaic disease. They are : *Mungbean yellow mosaic India virus*, 2. *Mungbean yellow mosaic virus*, 3. *Dolichos yellow mosaic virus*, 4. *Horsegram yellow mosaic virus*. All these viruses have characteristic features of genome organization of bipartite begomoviruses. These viruses have an open reading frame in the viral strand upstream of coat protein gene, referred to as pre-coat protein or ORF AV2 protein. ORF-AV2 is present only in Old World begomoviruses, its role in viral pathogenesis and its functions in plants are not elucidated. Therefore, the present study had as its objectives (a) to elucidate the role of AV2 in viral pathogenesis by site-directed mutagenesis, (b) to express the ORF AV2 protein and investigate its biochemical function, (c) to assess the extent of variability in the right half of DNA A component. The results are summarized below :

- * DNA A and DNA B components of MYMIV (Bg isolate) and MYMIV-[Cp] isolates were obtained from Plant Virology Unit, Division of Plant Pathology, IARI and their partial tandem repeat constructs were made in pBin19.
- * Agroinoculation of Bg and Cp isolates were performed by sprout seed method, on three cultivars of blackgram, cowpea, mungbean and French bean.

- * Maximum infection was seen in French bean for both the isolates.
- * Symptoms caused by Cp isolate in French bean were not that severe as Bg isolate.
- * Bg isolate was infectious on cowpea cv. Pusa Komal, produced mild atypical leaf curl and replicate incompetent. Only trace amounts of viral replicative forms were seen.
- * Cp isolate produced a new severe leaf curl symptom in cowpea cv. Pusa Komal. This symptom has not been reported earlier.
- * Cowpea cv. Lal lobia was not susceptible, contrary to earlier reports.
- * Site directed mutagenesis was performed following overlapping PCR method.
- * Around 25% of the transformants had desired mutation as verified by sequencing.
- * Totally five mutations were done for Bg isolate. They were : BgM₁, Bgdel-A, BgW₂, BgH₁₄G₁₅ and BgY₃₂.
- * Similarly, seven mutations were done for Cp isolate. They were : CpM₁, CpW₂, CpH₁₄G₁₅, CpK₇₃, CpG₄₄, CpC₈₆ and CpC₈₄₋₈₆.
- * Agroinoculation of legume hosts with mutants produced attenuation of symptoms in the case of W₂, H₁₄G₁₅ and Y₃₂ mutants. Severe and altered symptoms localized to vascular tissue were seen with CpK₇₃, CpC₈₄₋₈₆.
- * Agroinoculation with mutants of W₂, H₁₄G₁₅ and Y₃₂ resulted in reduced ss and sc DNA levels.

- * Inoculation with CpK₇₃, CpC₈₄₋₈₆, however caused increased ss DNA levels.
- * It is inferred that, ORF AV2 may be affecting the replication of the viral genome, by interacting with Replication initiator protein.
- * AV2 protein of Bg and Cp isolate were expressed in pMAL vector, as MBP fusion protein in *E. coli Rosetta* cells..
- * MBP-BgAV2 and MBP-CpAV2 fusion protein was purified through amylose column, antibody to BgAV2 was raised.
- * AV2 proteins bands could not be clearly detected in Western blots using anti BgAV2 antibody.
- * MBP-BgAV2 and MBP-CpAV2 were further purified and its binding of ss DNA and ds DNA was investigated. In the technique adopted, efficient binding between AV2 protein and DNA could not be seen.
- * Binding between AV2 and Rep protein was found to be very weak.
- * Both Bg and Cp AV2 proteins modulated the nicking and ATPase activity of Rep. About 1-4 ng of MBP-BgAV2 or MBP-CpAV2 could bring 20 folds increase in nicking activity of 60 ng of Rep.
- * Similarly in the presence of AV2 protein two to three fold increase in ATPase activity was observed.
- * It is presumed that AV2 protein may play a role of accessory protein facilitating the activities of Rep.
- * A protocol for extraction of PCR compatible DNA for amplification of geminiviral DNA was evolved. The protocol was named as Gem-

CTAB as it worked well for detection of begomoviruses in sample rich in polyphenol and polysaccharides.

- * Four full-length DNA A, two full-length DNA B and ten right half of DNA A components were cloned from YMD affected samples collected from different places.
- * Of the 14 isolates characterized, eleven isolates collected from northern India were identified as belonging to MYMIV. The other three isolates from western and southern India belonged to MYMV.
- * Full-length DNA A and DNA B components of two isolates Del-Cp, Vam-Bg, Lud-Bg and Kan-Cp were analyzed for genome characterization.
- * Del-Cp showed 32% divergence in the CR and showed read through, AC1/AC2 ORFs organization with AC2 having GUG as start codon.
- * *In silico* analysis of non-coding region, amino acid sequence of ORF AV2 and ORF AV1 of all the fourteen isolates showed that ORF AV2 showed only 85-92% identity within the species MYMIV.
- * Four isolates, Del-Cp, Lud-Sb1, Lud-Sb2 and Lud-Bg showed divergence in ORF AV2 sharing only 85% identity with MYMIV-[Cp].
- * Phylogenetic analysis of DNA nucleotide sequence of all the isolates revealed, the distinct lineage of YMV isolates.

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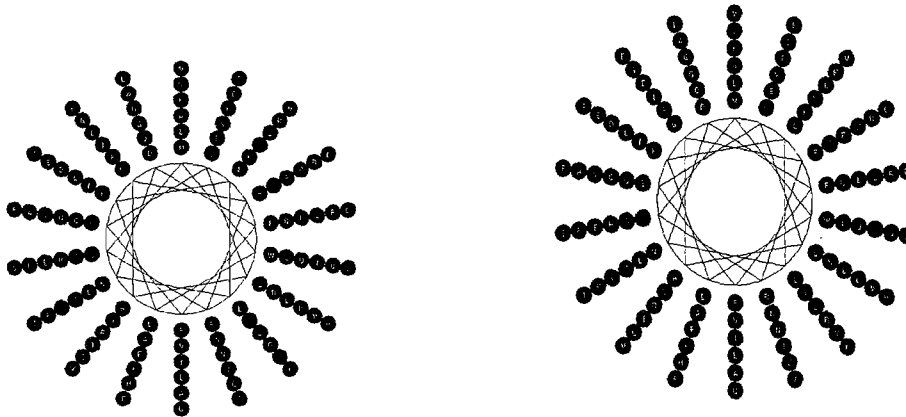
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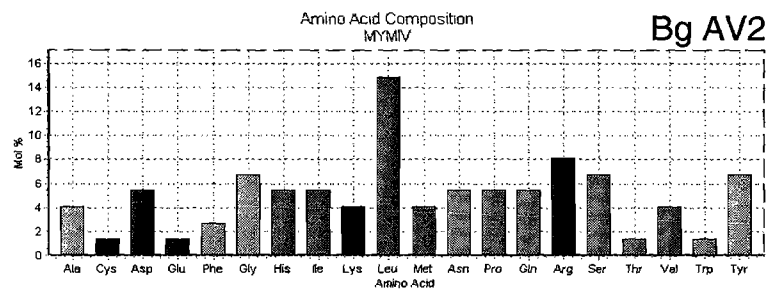
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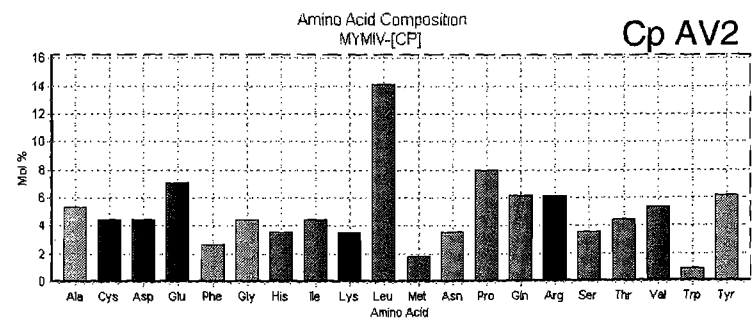
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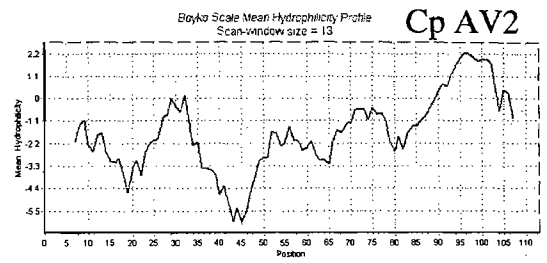
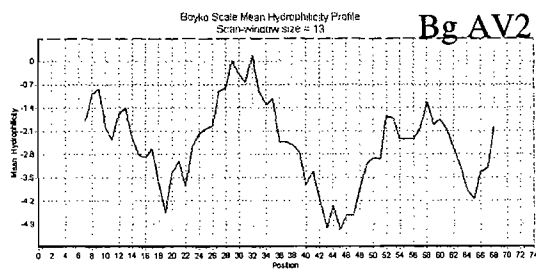
(a). Helical wheel structure prediction for Bg AV2 / Protein
(b) Helical wheel structure tradition for Cp AV2 / Protein



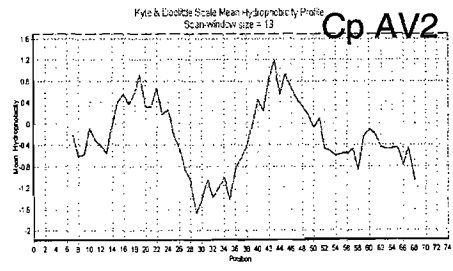
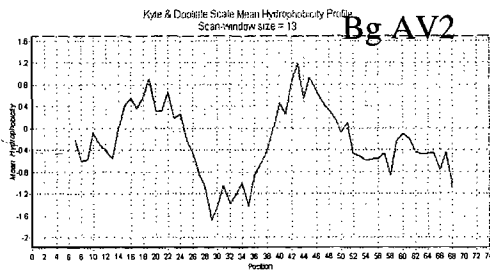
Basic amino acids are shown in Blue (Argenine & Lysine),
and Pink (Histidine).



Acidic amino acids are shown in Red (Aspartic acid &
Glutamic acid)



Hydrophobicity profile amino acid contents are represented.



Hydrophobicity profile amino acid contents are represented.

Appendix

Reagents and Buffers

Antibiotics

Name of antibiotics	Stock Soln. (mg/ml)	Solvent -----
Ampicillin	50	water
Kanamycin	50	water
Rifampicin	50	methanol

All antibiotics, which were water-soluble, sterilized by filtration (0.22 mm pore size) and then stored as frozen stock solution at - 20°C.

Denaturing solution

NaCl	1.5 M
NaOH	0.5 M

Denhardtts solution (50x)

Ficoll (type 400)	1%	5.0 gm
PVP (sigma)	1%	5.0 gm
BSA	1%	5.0 GM
Distilled water		up to 500 ml

DNA Extraction

Buffer

Tri - HCl	100 mM
NaCl	1.4 M
EDTA	20 mM
CTAB	2 %
βME	1-5 %

DNA resuspension

Buffer (pH 7.4)

Tris-HCl	10 mM
EDTA	1 mM

Gel loading Buffer

TAE buffer	6x
Succrose	55 % (w/v)
Bromophenol blue	0.25 % (w/v)

Luria Agar Medium

Bacto tryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	10 g
Agar	15 g

Readymade LA and LB medium was used from Himedia Company.

Luria Brot Medium

Bacto tryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	10 g
Distilled H ₂ O upto	1 lit

**Luria Brot Medium
(for induction)**

Bacto tryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	10 g
Glucose	2 g
Distilled H ₂ O upto	1 lit

**Loading dye
(MBI fermentas)**

1% Bromophenol Blue	200 ml
Glycerol	200 ml
10% SDS	60 ml
0.5 MEDTA	50 ml
10xTAE	60 ml
Water	30 ml

Neutralization solution

NaCl	1.5 M
Tris-HCl (7.4)	1.0 M

**Pre-hybridization
Solution**

Denhardts 's	5X
SSC	6x
SDS	0.2 %
Yeast RNA / Salmon sperm	200 mg/ml

TAE (Tris-Acetate EDTA Buffer, pH 8.0; 50X)

Tris- base	242 g
Glacial Acetic Acid	58.8 ml (of 96.6 %)
EDTA (0.5 M, pH 8.0)	20 ml
dd H ₂ O	up to 1000 ml

Column Buffer

50 mM Tris-HCl pH 7.4
200 mM NaCl
1 mM EDTA
10 % Glycerol
10 mM bME / 1 mM DTT
1 mM PMSF

2X SDS gel-loading buffer

90 mM Tris-HCl pH 6.8
20% Glycerol
2% SDS
0.02% Bromo-phenol Blue
5% β ME / 100 mM DTT

Southwestern 10x Binding Buffer

100 mM Tris-HCl pH 7.4
500 mM KCl
1 mM DTT (just before use)

Southwestern Pre-Hybridization Solution

1x Binding Buffer
5% Nonfat dry milk / 3% BSA
1 mM DTT (just before use)

Southwestern Wash solution

1x Binding Buffer
0.25% Nonfat dry milk / 0.15% BSA
0.5% DTT

10x protein-protein interaction Binding Buffer

250 mM Tris-HCl pH 8.0
750 mM NaCl
25 mM EDTA
50 mM MgCl₂

10% NP-40
DTT

ATPase 5x Buffer

100 mM Tris-HCl pH 8.0
5 mM MgCl_2
500 mM KCl
40 mM DTT
400 $\mu\text{g/ml}$ BSA

Satining solution for Silver staining

Silver staining Fixative I

50% methanol
7.5% Acetic Acid

Silver staining Fixative II

5% methanol
7.5% Acetic Acid

Silver staining Fixative III

10% Aqueous Glutaraldehyde

Preparation of Commonly used stock solution

Solution	Method of preparation
0.1M ATP	60.0mg of ATP was dissolved in 0.8 ml of distilled water. The pH was adjusted to 7.0 with 0.1 N NaOH and volume was made up to 1 ml with sterile distilled water. The solution was dispersed to small aliquots and stored at -70°C.
1 M CaCl₂	54.0 gm of CaCl ₂ ·H ₂ O was dissolved in 200 ml of distilled water and solution was sterilized by autoclaving.
0.5 M- EDTA	186.1 gm of EDTA was added to 200ml of distilled water and stirred vigorously on a magnetic stirrer and pH was adjusted to 8.0 with NaOH and sterilized by autoclaving.
Ethidium Bromide	100 gm of ethidium bromide was added to (10 mg/ml) 100 ml of distilled water and stirred on magnetic stirrer for several hours to ensure that dye has dissolved. The solution was transferred to a dark colored bottle and stored at room temperature.
0.1M IPTG	1.19 g IPTG (isopropyl-β-thiogalactoside) powder dissolved in 40 ml distilled water and volume was made up to 50 ml by distilled water sterilized by filtration by using disposable filter. The solution was dispensed into aliquot and stored at - 20°C.
100mM PMSF	1.74 g PMSF (phenyl methyl sulfonyl fluoride) powder dissolved in 100 ml isopropanol and stored at -20°C.
1M MgCl₂	203.3 gm MgCl ₂ ·6H ₂ O was dissolved into 200 ml of distilled water, the volume was made up to 1000 ml. and Sterilized by autoclaving and stored in cold room.
3M NaOAc pH 4.8 & 5.2	408.1 gm of NaOAc·3H ₂ O was dissolved in 800 ml of distilled water and pH was adjusted to 4.8 and 5.2 with glacial acetic acid volume was made up to 1 liter with sterile water and sterilized by autoclaving.

Phosphate-Buffered Saline (PBS, pH 7.4):

NaCl	137 mM
KH ₂ PO ₄	1.5 mM
Na ₂ HPO ₄	8.0 mM
KCl	2.7 mM
NaN ₃	3.0 mM

Check the pH of the final solution. It may be convenient to prepilre a stock solution at 10 X the desired concentration. In that case, no preservative, such as NaN₃, has to be added. The pH of concentrate will be lower than that of the working solution. Note: Sodium azide is highly toxic and it binds to metals, forming explosive compounds when kept dry. Wear gloves to dispense.

Alkaline Phosphatase (AP) Buffer (pH 9.5)

AP buffer is also referred to as Tris-buffered saline (TBS) substrate buffer.

NaN ₃	3.0 mM
Tris base	0.1 M
NaCl	0.1 M
MgCl ₂ ·6H ₂ O	5.0 mM

Adjust pH to 9.5 with 5 N HCl.

NBT (Nitro Blue Tetrazolium Chloride)

Prepare a stock solution by dissolving 75 mg in 1 ml of N,N- (concentration: 0.092 M).

BCIP (5-bromo-4-chloro-3-indolyl phosphate)

Prepare a stock solution by dissolving 50 mg in 1 ml of N,N-dimethylformamide (concentration: 0.135 M).

Substrate Solution (NBT /BCIP)

This is a mixture of 22 µl NBT and 16111 BCIP in 5 ml AP buffer (final concentrations of NBT and BCIP 0.40 mM and 0.45 mM, respectively). Substrate solution is also commercially available.

