CHARACTERIZATION OF PAPAYA RINGSPOT POTYVIRUS AND ITS MANAGEMENT THROUGH CROSS PROTECTION

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CHARACTERIZATION OF PAPAYA RINGSPOT POTYVIRUS AND ITS MANAGEMENT THROUGH CROSS PROTECTION

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This is to certify that the thesis entitled "Characterization of papaya ringspot potyvirus and its management through cross protection", 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, is a faithful record of *bona fide* research work carried out by Shri Gourgopal Roy under my guidance and supervision. No part of the thesis has been submitted 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 : December 7, 2000

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Introduction

INTRODUCTION

Papaya (*Carica papaya* L.), a member of the family *Caricaceae*, is an important fruit crop widely grown in sub-tropical and tropical lowland regions. It is a native of tropical America. It was grown by the Indians in pre-collumbian times during 16th century and was carried from America to Africa, India and the Caribbean Islands by the early Portuguese and Spanish sailors (Kochhar, 1998).

Brazil is the largest producer of papaya followed by India and Mexico. India produces 1.5 million metric tones of ripe fruits per year. Papaya is grown in 69.3 thousand hectares in different parts of the country (Jagadish Chandra and Samuel, 1999; Anonymous, 2000). Bihar, Madhya Pradesh, Uttar Pradesh, Maharashtra, Karnataka, Tamil Nadu, West Bengal, Gujarat and Orissa are the major papaya growing states in the country. There is regional preference for the cultivars and more than 17 cultivars are grown, among which Coorg honeydew, Washington, CO-2, CO-3, CO-4, CO-5, Pusa delicious, Pusa dwarf, Pusa nanha, Pusa majesty and Solo are popular (Jagadish Chandra and Samuel, 1999).

The papaya is a large, fast-growing, lactiferous, hollow-stemmed, and usually unbranched tree (upto 10 m). It has a crown of large, spirally arranged, long-petioled, and palmately-divided leaves, each one exhibiting a pinnate type of branching. The older leaves are shed periodically, leaving behind large orbicular scars on the trunk. The plant is mostly dioecious, though some hermaphroditic or bisexual forms do occur. The sex of papaya plants cannot be deduced at juvenile stage, it can only be deduced

after they have allowed reproductive maturity (6-8 months). Recently, a PCR based seedling sex diagnostic assay (SSDA) has been developed (Parasnis *et al.*, 2000) to overcome the problem of sex identification. The male inflorescence is a long (upto 1 m), auxillary, and pendulous panicle consisting of small tubular flowers. The pistillate flowers are somewhat larger (4-5 cm long) and are borne either singly or in few-flowered cymes in leaf axil. Papaya is largely propagated through seeds sown in seedbeds and 1-2 months old seedlings are transplanted to the field. The first mature fruit can be harvested nine months after sowing seeds, and fruit is produced continuously year-round.

Papaya is grown for its edible, and melon-like fruits and for its milky latex which contains the proteolytic enzyme papain. Papaya is largely consumed as a fresh dessert fruit or the green fruit is often used as salad and can be cooked as vegetables. Ripe fruits contain about 8-10% sugars and are rich in vitamins, particularly A, B_1, B_2 and C, if tapped yield a latex which on drying gives a greenish-grey powder, commercially known as papain. This is used in canned meats and in meat tenderising preparations as well as in pharmaceuticals as a mild laxative. Papain is also used in the manufacture of chewing gum, in the leather industry for treating hides, and in the textile industry, where it prevents shrinkage of wool and silks. Finally, it is a component of some toothpastes for curing pyorrhoea, a disease of gums. Much of the papain imported by the USA is used to produce 'chill-proof' beer since it prevents the cloudiness which results from the precipitation of proteins. An alkaloid 'carpaine' has been utilized as a diuretic and a heart stimulant (Singh *et al.*, 1983). Throughout the tropics, consumption of papaya fruits is considered to be an aid to digestion and an

antihelmintic. Tropical people often wrap a tough chicken in bruised papaya leaves the day before it is to be cooked and then eaten.

The wide acceptance of fruit offers considerable promise for local and export markets and thus making papaya an important crop. However, papaya production is limited due to papaya ringspot virus disease (Varma, 1988; Gonsalves, 1998).

Papaya ringspot virus (PRSV), a definite member of the family *Potyviridae* and the genus 'Potyvirus', is ubiquitous and has become a major limiting factor preventing the realisation of high yield production. It was first described in papaya (PRSV-P) by Jensen (1949a) and in cucurbits by Webb and Scott (1965). In India, papaya ringspot disease was first described by Capoor and Varma (1958) from Bombay, Maharashtra. Since then, the disease has been observed wherever papaya is grown (Singh, 1969; Sureka *et al.*, 1977; Yamewar and Mali, 1980; Khurana, 1984). Although PRSV has been under scrutiny for more than four decades, it continues to be an unsolved and knotty problem. Studies have been mainly confined to symptomatology, host range, vector transmission, physico-chemical properties and serology. PRSV isolates from different papaya growing regions like Bihar, Delhi, Karnataka, Madhya Pradesh, Maharashtra, Rajasthan, Uttar Pradesh and West Bengal have not been adequately characterised. Information on biological, serological and molecular diversity among PRSV-P isolates is completely lacking. In the absence of desired level of intrinsic resistance in papaya germplasm, cross-protection strategy has been exploited to contain PRSV in Taiwan (Yeh *et al.*, 1988). Potential of similar strategy will have to be judged in India to contain PRSV. Recently, a naturally occurring mild strain of PRSV has been identified from

IARI Regional Station, Pune and its potential to protect plants from infection with severe strain will have to be judged. Besides, the complete capsid protein (CP) gene and the 3'-untranslated region (UTR) of the genome of this isolate was cloned and sequenced (Jain *et al.*, 1998). Sequence comparison revealed that the capsid protein (CP) gene of isolate is quite different from this CP genes of other Asian, Australian, Mexican and USA isolates. In order to use this gene as transgene successfully in capsid protein-derived resistance (CPDR), there is a need to sequence CP genes of different isolates and ascertain the homogenity. In order to address aforesaid issues, the present endeavour was undertaken with the following objectives :

(i) To assess biological, serological and molecular diversity of papaya ringspot virus (PRSV) isolates from papaya from different geographic regions.

(ii) To identify capsid protein (CP) gene in the mild and severe strains of papaya ringspot virus (PRSV).

(iii) To demonstrate the potential of cross-protection technology in controlling papaya ringspot virus (PRSV).

Review of Literature

REVIEW OF LITERATURE

Large number of viruses belonging to cucumo-, gemini-, ilar-, poty-, rhabdo-, tobra-, and tospo- virus group have been recorded on papaya (Table 1). Of these, papaya ringspot virus (PRSV), a definitive member of the family *potyviridae* and the genus potyvirus (Fauquet, 1999; Regenmortel *et al.*, 2000), is the major limiting factor affecting the papaya industry worldwide including India (Cook, 1972; Varma, 1988; Gonsalves, 1998). The virus is the causal agent of one of the most economically important diseases of papaya designated as papaya ringspot. The name of the disease has been derived from the ringed spot symptoms on fruit. The disease has been described differently by different workers, such as, mosaic (Capoor and Varma, 1958; Basit, 1974; Marathe and Sammanwar, 1984); leaf reduction (Singh, 1969; Kawano and Yonoha, 1992) and distortion ringspot (Khurana, 1984).

2.1 Occurrence

The virus was first discovered in 1945 (Lindner *et al.*, 1945) and the term "papaya ringspot virus" was coined by Jensen in 1949 (Jensen, 1946, 1949). The virus is cosmopolitan in nature and occurs in tropical and subtropical areas where papaya is grown, including the United States, South America, the Carribbean countries, India, Taiwan, Africa, Japan, Australia, Srilanka, Mexico and other countries (Purcifull *et al.*, 1984; Dahal *et al.*, 1997; Silva-Rosales *et al.*, 2000).

As in the other parts of the world in India too PRSV is the most destructive virus in papaya. In India, natural infection of papaya ringspot

Table 1. Viruses recorded on papaya

Virus group	Disease caused	Recorded from	Reference
Cucumovirus			
Cucumber mosaic virus	Papaya lethal yellowing	Brazil	Loretto *et al.*, 1983
	Papaya mosaic		
Geminivirus			
Papaya leaf curl virus	Papaya leaf curl	India	Nariani, 1956
Ilarvirus			
Tobacco streak virus	—	Brazil	Rezende and Costa, 1986
Nepovirus			
Tobacco ringspot virus	—	Nigeria	McLean and Olson, 1962
Potexvirus			
Papaya mosaic virus	Papaya mosaic	Worldwide	Purcifull and Hiebert, 1971; 1979
Potyvirus			
Papaya leaf distortion mosaic virus	Papaya leaf distortion	Japan	Kawano and Yonoha 1992
Papaya mosaic virus	Papaya mosaic	Pakistan	Basit, 1974
Papaya ringspot virus	Papaya ringspot	Worldwide	Purcifull, 1972
Soybean yellow bud virus	—	Brazil	Rezende and Costa 1986
Rhabdo virus			
Papaya apical necrosis virus	Papaya apical necrosis	Venezuela, USA	Lastra and Quintero 1981
Tobra virus			
Tobacco rattle virus	—	Brazil	Rezende and Costa 1987
Tospovirus			
Tomato spotted wilt virus	—	Hawaii, USA	Trujillo and Gonsalves, 1969
Uncharacterized			
Papaya mild yellow leaf virus	Papaya mild yellow leaf	Venezuela, USA	Mario *et al.*, 1975

virus on papaya was first reported by Capoor and Varma (1958) from Maharashtra. Subsequently, it has been reported from Rajasthan (Sureka *et al.*, 1977) and Uttar Pradesh (Khurana, 1984). Now, the virus seems to be widespread and occurs wherever papaya is grown (Roy *et al.*, 1999). The incidence of PRSV has been reported to be more than 90% or even upto 100% in some of the States in India. However, some of the isolated pockets like southern parts of Karnataka, Tamil Nadu and Kerala are free from the PRSV epidemics so far (Jagadish Chandra and Samuel, 1999).

2.2 Losses

PRSV is the most damaging virus that infects papaya. It has seriously affected the papaya industry in Brazil, Taiwan and Hawaii (Gonsalves, 1998). The destructive effects of PRSV forced the Brazilian papaya industry to move from Southern States of Sao Paulo and Rio de Jeneiro to the northern, more remote States, Espiritu and Bahia. By 1993, papaya cultivation in Sao Paulo and Rio de Jeneiro which accounted for 90% of the total papaya cultivation was reduced to only 1.5%. This migration of the industry is directly attributable to the effects of PRSV (Gonsalves, 1998). Unlike Brazil, Taiwan, being small island, could not escape from PRSV by shifting its industry to disease free areas. PRSV was first recorded in southern Taiwan in 1975 and within four years the total yield of papaya was dropped by 60% by PRSV infection (Yeh *et al.*, 1988). The effect of PRSV in Hawaii was also similar to that in Brazil and Taiwan.

In India too commercial cultivation of papaya has been seriously hampered owing to the severity of the disease which may affect over 90% of grown up plants, reducing latex and sugar contents (Khurana, 1970).

Although there are no reliable estimates of the losses caused by PRSV. The disease has allowed epidemic proportions in some regions and the papaya cultivation is reduced to an annual crop and to kitchen gardens.

2.3 Symptomatology

PRSV induces a variety of plant age, size and vigour, virus strain and temperature-dependent symptoms on foliage, stem and fruits in papaya. Symptoms include vein clearing followed by mottling, intense yellow mosaic and distortion of leaf lamina. The leaf distortion leads to shoe stringing or filiformy on upper surface. The incubation period for primary foliage symptoms lies between 9 to 21 days, but may occasionally go upto 30 days (Jensen, 1949). Spots or streaks with a greasy or watersoaked appearance occur on the stems. Fruit symptom in the field include spots or rings or distortion and thus reducing market quality of the fruits. The presence of abnormal fruit with induced apocarpy and development of a second fruit within the normal one were reported as a consequence of PRSV infection (Khurana and Bhargava, 1970). Infected plants lose vigour and become stunted. The affected plants lose all their older leaves and appear almost denuded except for a tuft of small and upright leaves at the top. In the endemic areas the percentage of infected plants ranges from 75 to 100 per cent. When infected at seedling stage or within two months after planting, papaya plants do not normally produce mature fruits (Lokhande *et al.*, 1992). Some of the PRSV strains cause yellowing, vein clearing, defoliation and wilting. They invariably induced 20 to 60 per cent mortality of the infected plants (Chang, 1979; Jagadish Chandra and Samuel, 1999). Cool weather favours the

development of severe leaf distortion symptoms (Jensen, 1949; Conover, 1964; Lima and Gomes, 1975 and Prasad, 1988).

2.4 Host Range

Like other potyviruses (Shukla *et al.*, 1994) the virus has a narrow host range restricted to the fifteen dicotyledonous species in the families *Caricaceae, Chenopodiaceae* and *Cucurbitaceae* infected by type P isolates and type W isolates are reported to infect 38 species in 11 genera of *Cucurbitaceae* and two species of *Chenopodiaceae* (Table 2, 3) (Purcifull *et al.*, 1984; Brunt *et al.*, 1996).

PRSV is known to invade *Cucurbita pepo, C. mosachata, Cucumis sativus, C. melo, Citrullus vulgaris, Cyclanthera pedata, Melothria pendula, Carica goudotiana, C. cauliflora* and *C. monoica* (Conover, 1962; Herold and Weibel, 1962; DeBokx, 1965; Lima and Gomes, 1975; Sanchez and Lopez, 1976; Wang *et al.*, 1978; Chang, 1979; Barbosa and Paguio, 1982; Wu *et al.*, 1983; Yeh *et al.*, 1984). Wang *et al.* (1978) observed local lesions on both *Chenopodium amaranticolor* and *C. quinoa* in addition to the systemic necrosis on melon and mosaic on other cucurbits. *Chenopodium* species were also found to be hypersensitive to PRSV by Chang (1979) and Yeh and Gonsalves (1984a). However, Sanchez and Lopez (1976b) could not determine any hypersensitive host to PRSV, but did not get systemic lesions on *Carica goudotiana*, watermelon and vegetable marrow. It was observed that despite the systemic hosts not being worthy indicator plants, they could however, be good alternate hosts (Sanchez and Lopez, 1977). *Nicotiana benthamiana* was not susceptible to PRSV as it was to pumpkin mosaic

Table 2. Host range of papaya ringspot potyvirus

Family	Diagnostic hosts		References
	P	W	
Caricaceae			
Carica papaya (Papaya)	+	-	Purcifull *et al.*, 1984
C. cauliflora	+	-	
C. goudotiana	+	-	
C. monoica	+	-	
Chenopodiaceae			
Chenopodium amaranticolor	+	+	Russo *et al.*, 1979
C. quinoa	+	+	Gonsalves and Ishii,1980 Yeh *et al.*, 1984
Cucurbitaceae			
C. metuliferus	+	+	Yeh *et al.*, 1984
Cucurbita pepo	+	+	Gousalves and Ishii,1980 Yeh *et al.*, 1984
Citrullus vulgaris	+	+	Raychaudhuri and Varma, 1975
Cucumis sativus	+	+	Bhargava and Bhargava, 1976
Cucurbita maxima	+	+	Singh, 1981
C. moschata	+	+	Provvidenti *et al.*, 1978; Provvidenti, 1993; Purcifull and Edwardson, 1967
Momordica charantia	+	+	Nagrajan and Ramakrishnan, 1971
Benincasa hispida	+	+	Bhargava and Bhargava, 1976, 1977
Coccinia grandis	+	+	Bhargava and Bhargava, 1976
Cucumis melo	+	+	Mayee *et al.*, 1976
Lagenaria siceraria	+	+	Bhargava, 1977
L. vulgaris	+	+	Bhargava, 1977
Sechium edule	+	+	Singh, 1981
Trichosanthes dioica	+	+	Bhargava and Bhargava, 1976
Cyclanthera pedata	+	+	
Melothria pendula	+	+	
Luffa acutangula	+	+	Milne *et al.*, 1969; Russo *et al.*, 1979

Table 3. Diagnostic host species of papaya ringspot virus

Hosts	P	W
Test species		
Chenopodium amaranticolor	+	-
Citrullus vulgaris	-	+
C. quinoa	+	-
Cucumis anguira	+	-
C. rnelo	+	-
C. satavus	+	-
Propagation hosts		
Carica papaya	+	-
Cucurbita moschata	-	+
Cucurbita pepo	+	+
Cucumis metuliferus	+	-
Assay host		
Chenopodium amaranticolor	+	+
C. quinoa	+	+
Cucurbita moschata	+	-
C. pepo	+	-

virus (=watermelon mosaic virus-2) (Christie and Crawford, 1978). Yeh and Gonsalves (1984b) conducted host range studies on nine PRSV isolates and found them to infect members of the *Caricaceae, Chenopodiaceae* and *Cucurbitaceae.* However, a chinese strain of PRSV could only invade members of the *Cucurbitaceae*, in addition to the *Caricaceae* (Wu *et al.*, 1983). *Cucumis metuliferus* was a better propagation host than *Cucurbita pepo.*

In India, natural infection of PRSV on papaya reported from various regions have been shown to differ in their host range (Roy *et al.*, 1999). Isolates of PRSV reported by Khurana (1984) and Bhat *et al.* (2000) had extremely restricted host range confining to *Caricaceae* but Delhi, Maharashtra and Rajasthan isolates had comparatively wider host range

1958; Sureka *et al.*, 1977 and Roy *et al.*, 1999) and some of the isolates infected plants belonging to *Chenopodiaceae* also (Sureka *et al.*, 1977).

Three distinct pathotypes/strains of PRSV have been recognised on the basis of host range and serology (Table 4). Type P (PRSV-P) is pathogenic to papaya and cucurbits; whereas Type W (PRSV-W) formerly referred to as watermelon mosaic potyvirus-1 (WMV-1) and PRSV-T are pathogeneic to cucurbits and non-pathogenic to papaya. PRSV-P and W are serologically indistinguishable; whereas PRSV-T reported from Guadeloupe FW1 is antigenically unrelated to PRSV-P and W (Purcifull *et al.*, 1984; Quiot-Douine *et al.*, 1986). Studies have shown that PRSV-P (first recorded in Australia in 1991) arose from PRSV-W (first recorded in Australia in 1978) rather than being introduced (Bateson *et al.*, 1994).

Table 4. Pathotype/strains of papaya ringspot virus

Pathotype	Host range*		Serology**		
	papaya	cucurbits	antiserum to PRSV-P	antiserum to PRSV-W	antiserum to PRSV-T
PRSV-P	S	S	+	+	-
PRSV-W	IS	S	+	+	-
PRSV-T	IS	S	-	-	+

*S - Susceptible; IS - Insusceptible
** + = positive reaction; - Negative reaction.

2.5 Inclusions

Cytoplasmic cylindrical pin wheel and scroll types of inclusions, characteristic of potyviruses (Shukla *et al.*, 1994), have also been observed in PRSV-P and W infected tissues (Martelli and Russo, 1976; Barbosa and Paguio, 1982; Wu *et al.*, 1983; Zettler *et al.*, 1986 a,b). Presence

of tubular inclusions as well as short laminated aggregates in sections of PRSV infected papaya leaves have also been reported (Chen, 1984). The cytoplasmic inclusions are proteinaceous in nature and have molecular weight of 70 KD. These inclusions from P and W isolates were serologically indistinguishable (Edwardson and Christie, 1991). However, these were unrelated to their respective capsid proteins also (Yeh and Gonsalves, 1984b).

2.6 Transmission

PRSV is vectored in a non-persistent fashion by numerous aphid species. PRSV-P isolates are transmitted by 21 species in 11 genera (Table 5) including *Myzus persicae*. PRSV-W isolates are vectored by 24 species in 15 genera, including *M. persicae, Aulacorthum solani, Aphis craccivora* and *Macrosiphum euphorbiae* (Wang, 1981; Hwang and Hsieh, 1984; Purcifull *et al.*, 1984). Jensen (1949b) reported *Myzus persicae* to be the main orthopod vector with acquisition access and inoculation access periods of 2 to 5 minutes. PRSV transmission by *M. persicae* under controlled conditions could be achieved upto 6 h after acquisition access, the infectivity of which could be distinguished after a 15 day post inoculation period (Hwang and Hsieh, 1984).

The virus has also been reported to be transmitted by grafting, dodder (*Cuscuta reflexa*) and a bird species (*Saltator coerulescens*). Investigations have failed to demonstrate seed-transmissibility of PRSV (Purcifull *et al.*, 1984). But, according to Bayot and his associates from Philippines (Bayot *et al.*, 1990), the virus is seed-transmissible because 2 of 1355 papaya seedlings from PRSV-infected fruit showed symptoms.

Table 5. Transmission of PRSV-P by insect vectors

Insect	
Aphis gossypii	*A. malvae*
A. medicaginis	*A. citricola*
A. craccivora	*A. nerii*
A. spiraecola	*A. rumicis*
Acyrthosiphon pisum	*Dactynotus ambroisae*
Lipaphis psuedobrassicae	*Macrosiphum sonchi*
M. euphorbiae	*M. solanifolli*
Myzus persicae	*Micromyzus farmosanus*
Pentalonia nigronerrosa	*Rhopalosiphum maidis*
Sinomegoura citricola	*Toxoptera citricida*
Urolecon sonchi	

However, there is a need for systematic study to see whether seed transmission plays a significant role in spreading PRSV or not.

Aphid transmission have also been reported for Indian isolates of PRSV. *Aphis gossypii, A. nerii, Lipaphis pseudobrassicae* transmitted the virus in a non-persistent manner (Mali, 1985).

2.7 Morphology

PRSV particles are flexuous filaments with 760-800 nm in length and 12 nm in diameter having A 260 : 280 1.2 (Chen, 1984; Purcifull *et al.*, 1984). Leaf dip preparations and ultrathin sections of papaya leaves showing ringspot disease disclosed filamentous particles occurring in bundles in the cytoplasm, lying close to the cell wall (Ko *et al.*, 1979). Virions in both P and W isolates have a single polypeptide species of apparent molecular weight of 34 KD in SDS-PAGE

2.8 Physico-chemical properties

PRSV displays a thermal inactivation point (TIP) between 50°C - 60°C, a dilution end point (DEP) of 10^{-2} to 10^{-4} and a longevity *in vitro* (LIV) of 8 to 24 h (Conover, 1962; Zettler *et al.*, 1968b; Lima and Gomes, 1975, Sanchez and Lopez, 1976; Wang *et al.*, 1978; Barbosa and Paguio, 1982; Wu *et al.*, 1983). The two isolates described by Chang (1979) exhibited *in vitro* stability upto 11 days and 8 days in contrast with the other reports where the virus lost infectivity after 12 to 24 hours. Retention of biological activity for PRSV is reported to be over a wide range of pH and concentration.

In squash sap, type W isolates were inactivated by aging for 40-60 days, by heating to 60°C for 10 min, or DEP to 5 to 10^{-4} (Webb and Scott, 1965). Isolates retained infectivity after storage for at least 6 years in $CaCl_2$ dried leaf pieces under vacuum at 4°C

2.9 Serological properties

PRSV particles are strongly imunogenic. Precipitin tests, SDS-immuno diffusion test, ELISA and western blotting have been used to ascertain antigenic relationship of PRSV with other potyviruses using antisera to capsid protein, and cylindrical and amorphous inclusions (Gonsalves and Ishii, 1980; Yeh *et al.*, 1984; Purcifull *et al.*, 1984). PRSV is serologically closely related to Zucchini yellow mosaic virus (ZYMV) and distantly related to Bean yellow mosaic (BYMV), potato Y (PVY) and tobacco etch (TEV) viruses (Edwardson and Christie, 1991).

The serological affinity between PRSV-P and PRSV-W isolates from Florida (USA) (WMV-1) was initially established by Gonsalves and Ishii,

1980. In another study Yeh *et al.* (1984) reported that PRSV isolates collected from different geographical regions were also serologically indistinguishable from the WMV-1. However, another strain PRSV-T, isolated from naturally infected squash on the island of Guadeloupe in the Caribbean Sea, non pathogenic on papaya was serologically distinguishable from P and W (Quiot-Douine *et al.*, 1986). Similarly, PRSV-P and W isolates from Delhi (India) were found to be serologically related. Capsid proteins of both P and W isolates shared antigenic determinant with other potyviruses. P and W appear to be serologically closely related to amaranthus leaf mottle, Egg plant mottle virus and henbane mosaic virus (Roy *et al.*, 1999). PRSV-P and PRSV-W are thus serologically indistinguishable. Their capsid proteins share common antigenic determinants and it is difficult to segregate P and W isolates serologically by using polyclonal antisera. According to Gonsalves (1998), although P and W are serologically related, papaya seems to be the primary and secondary source for the spread of PRSV-P. This is based on the field observations in Thailand and Hawaii that cucurbit plants growing simultaneously with PRSV-P infected papaya were found to have PRSV-W.

2.10 Molecular characterization

Considerable progress has been made in the molecular characterization of PRSV. Virus has a single polypeptide species of about 34 KD encapsidating a single stranded RNA molecule of 10,326 nucleotides and has the typical array of genes found in potyviruses (Shukla *et al.*, 1994).

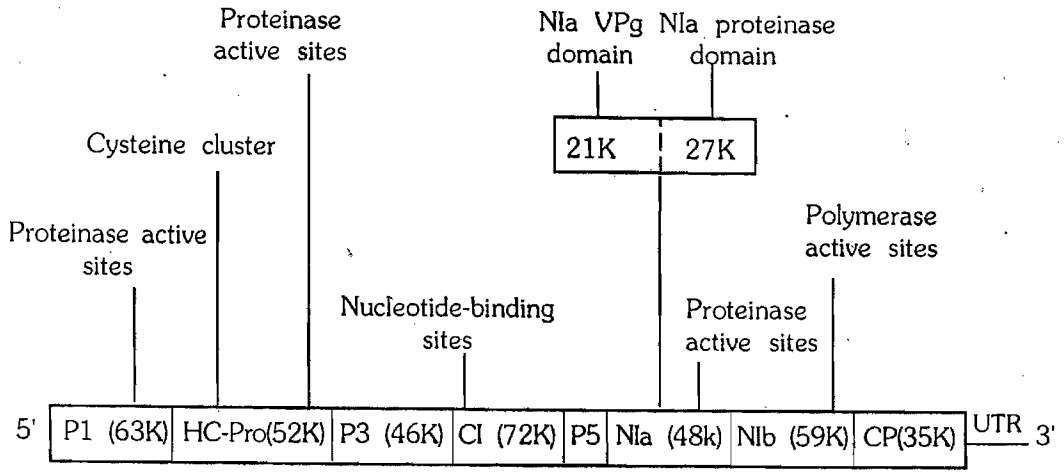


Figure 1. Map of the PRSV HA polyprotein. Solid bars indicate cleavage sites in the polyprotein. Dashed line indicates the potential internal cleavage site of the NIa protein (Source : Wang and Yeh, 1992; Yeh *et al.*, 1992)

The genome organization of PRSV is similar to that of the other potyviruses except that the first protein processed from the N terminus of the polyprotein (NT protein) has an 63 K, 18K to 34K larger than those of the other potyviruses. The most conserved protein of potyviruses appears to be the NIb protein, the putative polymerase for the replication of the potyviral RNA (Yeh *et al.*, 1992). Like potyviruses, the genome has a single open reading frame (ORF) and is expressed via a large polyprotein that is subsequently cleaved to functional proteins (Yeh and Gonsalves, 1985). There are two possible cleavage sites, 20 amino acids apart, for the N terminus of the capsid protein. The upstream site produces a functional nuclear inclusion b (NIb) protein and the other produces an aphid-transmissible capsid protein (Yeh *et al.*, 1992; Wang *et al.*, 1994).

The complete nucleotide sequence of the genome of only two isolates of PRSV-P from Taiwan (PRSV-YK) and Hawaii (PRSV-HA) has been determined (Table 6) (Yeh *et al.*, 1992; Wang and Yeh, 1997). Both

genomes are 10,326 nucleotides long, excluding the poly(A) tail. They encode a polyprotein of 3344 amino acids with a 5' leader of 85 nucleotides and a 3' non-translated region (NTR) of 209 nucleotides. The two genomes shared homology of 83.4% and 90.6% at nucleotide and amino acid levels respectively. Comparison of the similar genes between the two isolates revealed that except 5'-NTR and P1 protein gene other genes shared a high degree of sequence homology of 82.5-92.3% and 91.2-97.6% at nucleotide and amino acid levels respectively, suggesting that they are strains of the same potyvirus (Wang and Yeh, 1997).

Table 6. Comparison of the nucleotide and amino acid sequences of PRSV YK and PRSV HA.

Regions	Percentage identity in	
	Nucleotide	Amino acid
5'NIR	65.0	-
P1	70.9	66.7
HC-Pro	86.5	95.6
P3	87.0	93.5
CIP	87.1	97.6
P5	88.3	91.2
NIa	82.5	94.4
NIb	83.0	95.7
CP	89.8	93.2
3'-UTR	92.3	-

Source : Wang and Yeh, 1997

3'-terminal region comprising nuclear inclusion protein (NIb) gene, capsid protein (CP) gene and untranslated region of large number (17) of PRSV-P and W isolates from different geographic regions such as Asia, Australia, USA and Mexico (Jain *et al.*, 1998) revealed that the coat protein

(CP) genes within as well as between the Australian and US isolates were highly identical both at the nucleotide and at amino acid levels (96% to 100%). The CP genes of Asian isolates shared 86-93% and 89-96% identity at nucleotides and amino acid levels of respectively. CP genes between the Asian and Australian or US isolates also shared similar identity at nucleotide (87-95%) and amino acid (92-97%) levels respectively (Jain *et al.*, 1998). Coat protein sequences of three Mexican PRSV isolates showed higher similarity to isolates from Australia and the United States than to Asian isolates (Silva-Rosales *et al.*, 2000). Among all the known isolates, the CP gene sequence divergence of upto 12% has been observed. Comparison of sequence analysis has also provided insights on the possible origin of PRSV-P from the PRSV-W (Bateson *et al.*, 1994) which was first reported on papaya in Australia in 1991 (Thomas and Dodman, 1993).

2.11 Management

Attempts to develop effective control measures in different papaya growing regions during the last five decades have been unsuccessful. Though host resistance has been the first line of defense and tolerant selections of papaya have been described (Cook and Zettler, 1970; Conover, 1976; Conover and Litz, 1978), but desired level of resistance to PRSV does not occur within papaya (Cook and Zettler, 1970; Conover, 1976; Conover and Litz, 1978; Wang *et al.*, 1978) and these are not commercially desirable. Similarly, a diligent surveying and roguing program was successful to contain the spread of PRSV for 30 years in certain areas of Hawaii (Namba and Higa, 1977). But, roguing of diseased plants is impossible in Taiwan, where the disease has become epidemic. Other

agricultural practices also such as changing planting date, intercropping with maize as a barrier crop, spraying with mineral oils and systemic insecticides and protecting transplanted seedlings with plastic bags have been ineffective or only marginally beneficial (Gonsalves, 1998).

Thus, in the absence of PRSV-resistant papaya cultivars and with restricted host range of PRSV, cross-protection would be an attractive strategy of controlling this virus (Yeh and Gonsalves, 1984a; Wang *et al.*, 1987).

2.12 Cross Protection

Cross-protection, first observed by McKinney in 1929 with tobacco mosaic virus, means the use of a mild virus isolate to protect plants against economic damage caused by infection with a severe chalenge strains) of the same virus. There are few examples where cross protection has been widely used for the control of plant virus diseases (Muller and Costa, 1977; Fletcher, 1978). Papaya ringspot is one of those examples in which cross-protection has been intensively investigated for disease control. The key to these practical applications of cross-protection is the availability of a virus strain that does not cause severe damage and also provides a high degree of protection. The use of cross protection to control PRSV has been investigated in Taiwan and Hawaii (Yeh and Gonsalves, 1984a; Wang *et al.*, 1987; Yeh *et al.*, 1988) and Brazil. A mild strain of PRSV (HA5-1), produced by nitrous acid treatment of PRSV, was used in field experiments in Taiwan. Papaya plants protected by the mild strain of PRSV had 82% greater fruit yield than unprotected trees, resulting in a 111% increase in growers income. Protection was effective when disease pressure in the test orchard was minimised by rouging severely infected

plants upto flowering stage. However, no protection was observed under high disease pressure by other strains from areas near the protected trees. Due to the initial success, more than 1 million papaya seedlings inoculated with mild strain of PRSV were planted in the field in 1986 (Yeh *et al.*, 1988). Further about 100-200 hectares of papaya were protected by PRSV HA 5-1 yearly from 1985-1991 (Yeh and Gonsalves, 1994). Currently, the mild strain is sparsely used, as it does not provide consistent economic returns to the farmers (Gonsalves, 1998). The mild strain did not give good field protection against PRSV strains in Northeast Thailand. However, it performed much better in Hawaii, as it was derived from a Hawaiian isolate. Although field experiments revealed that the mild strain gave good protection on Hawaiian solo cultivar line-8, Kamiya and Sunrise, cross protection has not been widely adopted in Hawaii because (a) CP require extra cultural management and care, (b) reluctance of farmers to infect their trees with a virus and (c) adverse effects of mild strain on sunrise.

In Brazil, preliminary results under greenhouse conditions were encouraging. But, protected plants under field condition showed symptoms after 4-6 months. Failure of protection was attributed to change in the mild strains due to mutation and selective competition (Rezende and Costa, 1987). Level of resistance to plant viruses could be enhanced by "pyramidding" genetically engineered resistance over intensive plant resistance. This approach has been explored in considerable details during the last decade to generate virus resistant transgenic plants (VRTP). In India, investigation on the possibility of using cross protection as a control measure have just started. A naturally occuring mild strain of PRSV which

induces mild mosaic symptoms on papaya has been identified at IARI regional station, Pune. Study on its ability to confer protection is under process. The concept that the viral genes could confer resistance was first documented in 1986 by expressing TMV gene in transgenic tobacco plants (Powell-Abel *et al.*, 1986). Since then other genes from plant viruses are being tested for their ability to confer resistance (Jain and Varma, 2000).

Capsid protein gene is most widely used gene to generate VRTP and the strategy is commonly referred to as capsid-protein mediated resistance (Beachy *et al.*, 1990; Hackland *et al.*, 1994; Pappu *et al.*, 1995). Similar strategy has been employed to generate PRSV virus resistant transgenic papaya.

2.13 Development of transgenic papaya

The work has been started utilizing pathogen derived resistance concept in 1986 by cloning coat protein gene of PRSV HA 5-1 a mild mutant of PRSV HA (Gonsalves, 1998). The gene was engineered as a chimeric protein containing 17 amino acids of cucumber mosaic virus at the N terminus of the full-length coat protein gene of PRSV HA 5-1 (Ling *et al.*, 1991). In tobacco this gene construct had expressed high levels of the coat protein and on infection with tobacco etch virus the transgenic tobacco developed only mild symptoms. Fitch in 1987 started transforming papaya. Cultivars red-fleshed sunrise, the yellow-fleshed sunset and the yellow-fleshed Kapoho, by bombarding embryogenic tissue with tungsten particles coated with DNA of the PRSV HA 5-1 coat protein gene. RO-micropropagated transgenic lines, designated as 55-1, showed excellent resistance to PRSV HA, a severe isolate of PRSV in Hawaii

(Fitch *et al.* 1992). R1 plants were obtained by crossing line 55-1 with non-transgenic sunset and was found to be highly resistant to Hawaii strains but were susceptible to strains outside Hawaii. Field trials with RO plants, however, showed that line 55-1 was effective in controlling PRSV in Hawaii. Subsequently, transgenic plants focussed on transferring resistance into commercial papaya cultivars suitable for Hawaii were developed. The transgenic line 55-1 was renamed as "Sun Up" and a hybrid was made from the cross of the transgenic Sun Up and the non transgenic Kapoho was named as "UH Rainbow" (Manshardt, 1992; 1998). In Hawaii, field test was started in 1992 for the transgenic line 55-1 incorporated with coat protein gene of PRSV. By October 1994, PRSV was widespread in Puna, where 95% papaya were being grown and soon after field trial was started in Puna in 1995 with cultivars 'Rainbow" and sun UP' derived from line 55-1. Excellent resistance were shown by both the crops with good horticultural qualities. The transgenic papaya was commercialized by May, 1998. The continual positive impact of the transgenic papaya on the Hawaiian papaya industry (Gonsalves, 2000) has increased benefits of the farmers in USA. The PRSV-CP gene was successfully integrated into papaya to get PRSV-resistant transgenic plants in Taiwan (Cheng *et al.*, 1996). The transformation method was based on wounding of cultured embryogenic tissues with carborundum in liquid phase. The expressible coat protein gene of a Taiwan strain of PRSV was constructed in a Ti binary vector pBGCP, which contained the NPT-II gene as a selection marker. When transgenic papaya like GCP 17-1 was backcrossed with cultivar 'Sunrise', the segregation ratio of the CP gene was 1:1, indicating that the transgene is inherited like a single dominant gene.

Transgenic papaya developed in Hawaii appeared to be resistant to PRSV HA in Hawaii but was susceptible to a number of PRSV isolates world wide. PRSV recombinants with substitutions of the whole or partial CP sequences and/or 3' non coding region (NCR) from a PRSV strain from Taiwan (YK) were inoculated to UH Rainbow. Recombinants with nucleotide changes in the CP region broke the resistance of Rainbow, including a recombinant with only five nucleotide differences from the transgene (Chiang *et al.*, 2000; Gaskill *et al.*, 2000). Thus, RNA-mediated protection suggests that resistance is dependent on the homology of the transgene and the incoming virus. If the coat protein transgene sequences vary from that of incoming virus, the transgenic plant would be susceptible to the virus. For example, resistance of the line 55-1 was overcome by PRSV isolates from Thailand and Taiwan with coat protein genes with around 90% identity to the transgene (Bateson *et al.*, 1994; Wang *et al.*, 1994).

2.14 Alternative strategies to control PRSV

Development of virus resistant transgenic plants using viral genes might be an ideal approach to develop resistance to a large number of different viruses (Tricoli *et al.*, 1995). However, it is likely that strains may evolve which can overcome the resistance, particularly when CP-MR is used in crop protection and secondly, possibility of recombination for both DNA and RNA viruses make the use of viral genes as source of resistnce to be undesirable. To avoid these difficulties scientists are now looking for alternative strategies to enhance the usefulness of engineered resistance like use of induced hypersensitive response, inhibition of amber codons, movement proteins (Atkins *et al.*, 1995; Cooper *et al.*, 1995; Prins *et al.*, 1995; Martin, 1998).

Materials and Methods

MATERIALS AND METHODS

3.1 Virus Isolates

Papaya ringspot virus (PRSV) infected papaya samples were collected from different growing regions (Table 7). Identity of the virus in symptomatic samples was established on the basis of biological, leaf-dip electron microscopy and immunological assays. Infectivity of the isolates on papaya was checked by sap inoculations using phosphate as extraction buffer (0.01 M, pH 7.0) (Appendix I). Papaya plants (cv. Washington) previously dusted with Celite at 2-3 leaf stage were inoculated with extracts from infected plants. Association of flexuous virus particles was checked through leaf-dip electron microscopy, direct antigen-coated enzyme-linked immunosorbent assay (DAC-ELISA) and immunosorbent electron microscopy (ISEM).

Table 7. Papaya ringspot (PRSV) infected samples collected from different papaya growing regions

No.	Virus isolates	
	Designations	Origin
1.	Andhra Pradesh (AP)	Hyderabad
2.	Bihar (BH)	Ranchi
3.	Delhi (DL)	IARI, Experimental Field
4.	Karnataka-1 (KAR-1)	Bangalore
5.	Karnataka-2 (KAR-2)	IIHR* Experimental Field, Bangalore
6.	Maharashtra-1 (MAH-1)	IARI Regional Station, Pune
7.	Maharashtra-2 (MAH-2)	IARI Regional Station, Pune
8.	Uttar Pradesh (UP)	Gorakhpur
9.	West Bengal (WB)	Bardhaman

* IIHR - Indian Institute of Horticultural Research

3.1.1 Leaf-dip electron microscopy

For ascertaining the type of virus particles associated with infected papaya samples, leaf-dip preparations (Gibbs *et al.*, 1966) were examined under electron microscope (EM) (JEOL 100C x 11) at the Advanced Centre for Plant Virology, IARI, New Delhi-12.

(a) **Grid preparation**

Copper grids (3 mm diameter, 400 mesh) were cleaned with acetic acid and loaded on a filter paper placed at the bottom of a clean petridish with sterile distilled water. A clean slide was then coated with a thin film of carbon in a vaccum coating unit (BIORAD, E 6440, Evaporation PSU) and gently brought in contact with water in the petridish containing grids. The carbon film was gently allowed to float off on the water surface and was adsorbed on the grids when the filter paper was lifted. The carbon coated grids were finally either air-dried in the dark at room temperature for 12-24 h or in an oven at 37°C for 30 min.

(b) Virus extraction

Diseased leaf bits cut with the help of corkborer were macerated on a clean glass slide with a flat ended glass rod in 2-3 drops of phosphate buffer (0.078 M, pH 6.5) (Appendix - I) and left the finally homogenized material for a few seconds. The supernatant was then taken for mounting the grids.

(c) Mounting

A fresh carbon coated grid was taken at the tip of a fine clean forcep. A small drop of the prepared supernatant from the homogenized

virus material was put on the coated side of the grid. Excess of the supernatant was washed off with 5-6 drops of distilled water.

(d) Staining

The carbon coated grid was treated with 2-3 drops of uranyl acetate (aqueous 2%, pH 4.2) for few seconds. Excess stain was removed and blotted dry by touching the edge of the grid with a strip of filter paper. The grid was air-dried for 1-2 min. The negatively stained grid was finally examined under EM.

3.1.2 Direct antigen coated enzyme-linked immunosorbent assay (DAC-ELISA)

DAC-ELISA was performed on polystyrene plate (Corning, New York, USA) as described by Clark and Bar-Joseph (1984). The recipe of various buffers and reagents used is given in Appendix I. Crude antigens were prepared by extracting fresh leaf tissues in coating buffer (1:10 w/v) containing 2% polyvinylpyrrollidone (MW 40 kD) and centrifuging at 5000 g for 10 min. Plates were coated with 200 µl of crude antigen samples and incubated at 37°C for 1 h. Well contents were discarded and washed with PBS-T (flooding with three changes of PBS-T for 3 min each). After final washing, plates were shaken dry over paper towels. Then blocking solution was added (5% w/v; 200 µl per well) and incubated for 1 h at 37°C. Plates were then incubated with antibody for 2 h at 37°C after washing as before. Antiserum to PRSV-P isolate was diluted at 1:1000 using antibody buffer (PBS-TPO). The plates were then washed with PBS-T and incubated with antirabbit immunoglobulin - alkaline phosphatase conjugate (1:20,000 in PBS-TPO) (Sigma, St. Louis, USA.) for 2 h at 37°C. The plates were then washed off with PBS-T as described

earlier. After final washing, 200 µl of substrate (p-nitrophenyl phosphate, 0.5 mg per ml of substrate buffer, Sigma, St. Louis, USA) was added and allowed for colour development at room temperature. Intensity of colour developed was read at 405 nm after 1 h of substrate reaction using ELISA-reader (Sun Rise, TECAN).

3.1.3 Immunosorbent electron microscopy (ISEM)

ISEM decoration test was performed following the procedure described by Milne and Lessemann (1984). PRSV-P antiserum at 1:100 dilution was used for decorating the virus particles with an incubation period of 20-25 min.

3.2 Maintenance of isolates

PRSV isolates were preserved in leaves desiccated at 4^0C over anhydrous calcium chloride and also maintained in the glasshouse by mechanical transmission to papaya cv. Washington after extracting the sap from infected leaves in phosphate buffer (0.01 M, pH 7.0) (Appendix I).

3.3 Biological diversity

Biological divergence among eight PRSV isolates (except PRSV-AP) was ascertained by their reactions on papaya and cucurbits (Table 8). Reaction on five widely grown papaya cultivars and nine different cucurbits species was assessed by visual observations and estimating virus titre. Seeds of papaya and cucurbits were sown in pots in the glasshouse. A minimum of five plants of each were used for inoculation. Sap transmissions were done using phosphate buffer (0.01 M, pH 7.0) as extraction buffer (Appendix I) and celite as abrasive. Papaya plants were

inoculated at 2-3 leaf stage and symptoms were observed at 10 days postinoculation. Cucurbit plants were inoculated at cotelodonary leaf stage and kept under observation for symptom development at 10 and 20 days postinoculation. Virus concentration in both the test species was estimated through DAC-ELISA described earlier (3.1.2) at 10 and 20 days post inoculation.

Table 8. **Different papaya cultivars and cucurbit species used for ascertaining biological diversity of PRSV isolates**

Papaya cultivars	Cucurbits
Barwani CO-2 Coorg honeydew Pusa Nanha Washington	*Cucumis melo* L. *reticulatus* ser. (Muskmelon) *C. melo* cv. utilissimus (Roxb.) Duthie & Fuller (Long melon./Kakri) *C. sativus* L. (Cucumber) *Cucurbita moschata* Duche ex Poir (Pumpkin) *C. pepo* L. (Summer squash) *Lagenaria siceraria* (Mol) Standl. (Bottle gourd) *Luffa acutangula* (L.) Roxb. (Ridge gourd) *L. cylindrica* (L.) Roem. (Sponge gourd) *Praecitrullus fistulosus* Pang. (Tinda)

3.4 Serological diversity

Serological divergence among the capsid proteins of eight PRSV isolates (except PRSV-AP) under study was ascertained by DAC-ELISA and ISEM tests (as described in 3.1.2 and 3.1.3) using antisera against P and W isolates. Antiserum against PRSV-P was produced in our laboratory (ACPV, IARI, New Delhi 110 012) and antiserum against PRSV-W was obtained from D.E. Purcifull (Gainesville, USA).

3.5 Molecular diversity

In order to ascertain the molecular divergence among eight PRSV isolates (except PRSV-AP), a part of the capsid protein (CP) and nuclear

inclusion b (NIb) genes of the PRSV isolates were amplified and compared (Figure 2). The divergence was assessed by comparing the size and single-strand conformation polymorphism (SSCP) analysis of the amplicons.

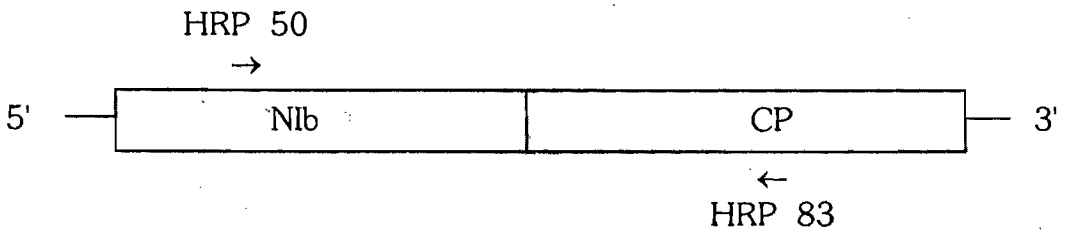


Figure 2. **Schematic of amplification strategy used to amplify a part of the capsid protein and nuclear inclusion b genes of PRSV isolates**

3.5.1 RNA extraction

Total RNA from fresh desiccated infected tissue was extracted using an RNeasy kit according to the manufacturer's instructions (Qiagen Inc., Chatsworth CA 91311, USA, cat No. 74904). Initially 10 mg of tissues were taken for each isolate for isolating the RNA. The powdered samples were immediately soaked with 450 µl lysis buffer (RLT buffer) (containing 1% β-mercaptoethanol), vortexed for 10 sec. and incubated at 56°C for 3 min in waterbath. Clear lysate was then passed through spin column and centrifuged at 8,000 g for 2 min in a benchtop centrifuge at room temperature. The flow through was transferred to a new eppendorf and half volume of (225 µl) chilled ethanol (absolute) was added to clear lysate and mixed by pipetting. It was then passed through another spin column (pink column) and centrifuged for 1 min at room temperature at 8000 g. Flow through was discarded and 700 µl of RW 1 buffer was added to the column and centrifuged for 1 min at same speed. Again flow through and collection tube were dicarded. 500 µl RPE buffer was added to the same column and centrifuged for 1 min. The same was

repeated and centrifuged for 2 min. Finally, RNA was eluted with 54 µl sterile RNase free water by centrifuging at 8,000 g for 1 min and was collected in an eppendorf. This was used as a template in reverse transcription and polymerase chain reaction (RT-PCR). Sap extracts from non-infected tissues were used as control:.

3.5.2 Reverse transcription and polymerase chain reaction (RT-PCR)

RT-PCR was based on the method of Pappu *et al.* (1993). Prior to RT-PCR, the template was incubated at 70°C for 10 min and snap-cooled on wet ice for 2 min. The primers used to prime the amplification of a part of Nlb and CP genes are listed in Table 9. RT-PCR was performed in a single tube and prepared amplification mix by dispensing into 200 µl microfuge tube in the order given in Table 10.

Table 9. **Specific primers* used for amplification of a part of Nlb and CP genes of PRSV isolates**

Primer	Orientation	Position	Sequence	Tm(°C)
HRP 50	Upstream	Nlb region	5' ATGATAGAGTCATGGGG 3'	50
HRP 83	Downstream	CP region	5' CGCGTTACTGAAGTGAGC 3'	56

* Primers received as gifts from H.R. Pappu, University of Georgia, Tifton, Georgia, USA

The reaction mixture was pulse centrifuged and placed the tube in a thermal cycler (Bio Rad) set at the amplification conditions chosen for reaction (Table 11).

Table 10. Composition of amplification mix used for RT-PCR

Reagents	Volume required (µl per reaction)
10 X PCR buffer	10
5 X Q solution	20
10 mM Dithiotheritol (DTT)	10
100 µM dNTPs	3
Primer HRP 50, 200 pM (100 pM/µl)	2
Primer HRP 83, 200 pM (100 pM/µl)	2
Omniscript reverse transcriptase 2 units (4U/µl)	0.5
Taq DNA polymerase 2.5 units (5U/ml)	0.5
Template (RNA)	52

Table 11. The thermal cycling profile chosen for RT-PCR

Steps involved	Temperature (°C)	Duration (min)	Number of cycles
Reverse transcription	42	45	1
Denaturation	94	0.5	40
Primer annealing	46	2	
Synthesis		72	1
Final extension	72	60	1

3.5.3 Analysis of PCR products

Following PCR, amplicons (10 µl) were analysed by 0.7% agarose gel electrophoresis in Tris-Acetate EDTA (TAE) containing ethidium bromide (Appendix II) (Sambrook *et al.*, 1989).

0.35 g agarose (Mol. Biology grade, SRL) was melted in 50 ml 1X TAE running buffer (Appendix II) and 2 µl ethidium bromide (0.5 µg/ml) was added to the agarose after cooling to around 50°C. It was then

poured into the casting tray for polymerisation after inserting the comb. 10 µl each of the RT-PCR product was mixed with 4 µl of 6X loading dye (Appendix II) and sterilised water to make up the volume to 24 µl. The comb was removed and the gel was then placed on electrophoretic tray filled with 1X TAE buffer. Samples were loaded and the gel was run at 60 V for 2 h (BIORAD). An aliquot (500 ng) of 1 kb DNA ladder (Gene Ruler TM, MBI Fermentas) (Appendix II) was mixed with the dye similarly and electrophoresed to serve as molecular weight marker. After the run the gel was observed under ultraviolet transilluminator and photographed on the thermal paper using gel documentation system (UVP, Germany).

3.5.4 Single-strand conformation polymorphism (SSCP) analysis

SSCP analysis was performed directly on the PCR products of a part of CP and NIb genes of different isolates after purification.

3.5.5 Purification of PCR products

PCR products (100 µl each) of eight different PRSV isolates were purified using QIA quick PCR purification kit (Qiagen, Catalogue No. 28104) following the manufacturer's instructions. Five volumes of buffer PB was added to 1 volume of the PCR product and mixed well. Then the sample was applied to the QIAquick column provided with a 2.0 ml collection tube and centrifuged for 45 s. The flow through was discarded and 750 µl of buffer PE was added to the QIAquick column to wash and then centrifuged for 45 s. The flow-through was discarded and centrifuged for an additional 1 min at 10,000 g in a conventional table top microcentrifuge. Finally, the DNA was eluted by adding 30 µl buffer

EB (10 mM Tris.Cl, pH 8.5) to the Centre of the QIAquick membrane and centrifuging the column for 1 min. 10 µl of each was used for analysis.

3.5.6 Polyacrylamide gel electrophoresis (PAGE)

10 µl purified PCR product was mixed with 40 µl denaturing solution (Appendix III). The mixture was then heated for 10 min at 99°C and plunged into ice prior to loading onto the non-denaturing polyacrylamide minigel. The recipe of various solutions and reagents used is given in Appendix III.

Denatured DNA was electrophoresed in a non-denaturing 5% gel using 1 X TBE as electrophoresis buffer. The gel was run at 250 volts (BIORAD) at room temperature for 3 h until the bromophenol blue dye reached the bottom of the gel. The gels were stained with silver nitrate following the procedure of Beidler and his associates (1982) with slight modification. After the run, the gel was kept overnight at 4°C in glass tank containing fixative 1. The fixative 1 was removed and the tank was refilled with fixative II and kept for 1 h. Gel was then soaked in silver nitrate for 45 min to 1 h. During the first 5 min of exposure to silver nitrate, the gel was exposed to uniform intense light for 5 min by placing the glass tray on a fluorescent white-light box. Excess silver was removed by two quick washes with 500 ml water. These washes were effective in reducing background within the gel and the precipitation of excess silver on the gel surface. Potassium hydroxide (42 g $litre^{-1}$ of water) and 750 of formaldehyde were added to the gel after removing the water from the tray. Yellow colour developed within few minutes. Gel was developed by soaking in 100 ml 0.7% sodium carbonate solution and kept for 2 to 3 min. The solution was changed periodically, when

it became light brown. When the desired intensity was achieved, development was stopped by soaking the gel in 10% glacial acetic acid for 30 min.

3.6 Cloning and Sequencing

In order to characterise PRSV isolates at molecular level, c. 0.8 kb 3'-terminal region comprising of a part of CP and NIb genes of the genome PRSV-UP isolate was amplified, cloned and sequenced. In another isolate, PRSV-AP, the amplified product was directly sequenced.

3.6.1 Cloning

Blunt end cloning strategy was followed to clone the PCR product. Purified PCR product of PRSV-UP (as described in 3.5.5) was cloned as blunt end molecules into *Sma*1 cut dephosphorylated pUC 19 vector (MBI, Fermentas) (Figure 2) and recombinant clones were identified by following standard molecular biology procedures (Sambrook *et al.*, 1991).

3.6.1.1 Blunt ending

Blunt ending of the DNA insert was carried out using T4 DNA polymerase or Klenow fragment. Two separate reactions were set up as indicated. The reaction mixture with T_4 DNA polymerase was pulse contrifuged and incubated at 11°C for 20 min. The mixture was finally incubated at 75°C for 10 min prior to ligation. For Klenow reaction, the insert DNA (~ 0.5 µg) was mixed with 1 µl klenow mixture in an eppendorf. The mixture was incubated for 5 min at 37°C. 2 µl of dNTPs solution (conc. 0.5 mM) was then added to the mixture and incubated for 15 min at room temperature prior to denaturation at 65°C for 5 min. The mixture was snap cooled on ice. The blunt ended DNA mixtures were purified using QIAquick PCR purification Kit prior to ligation.

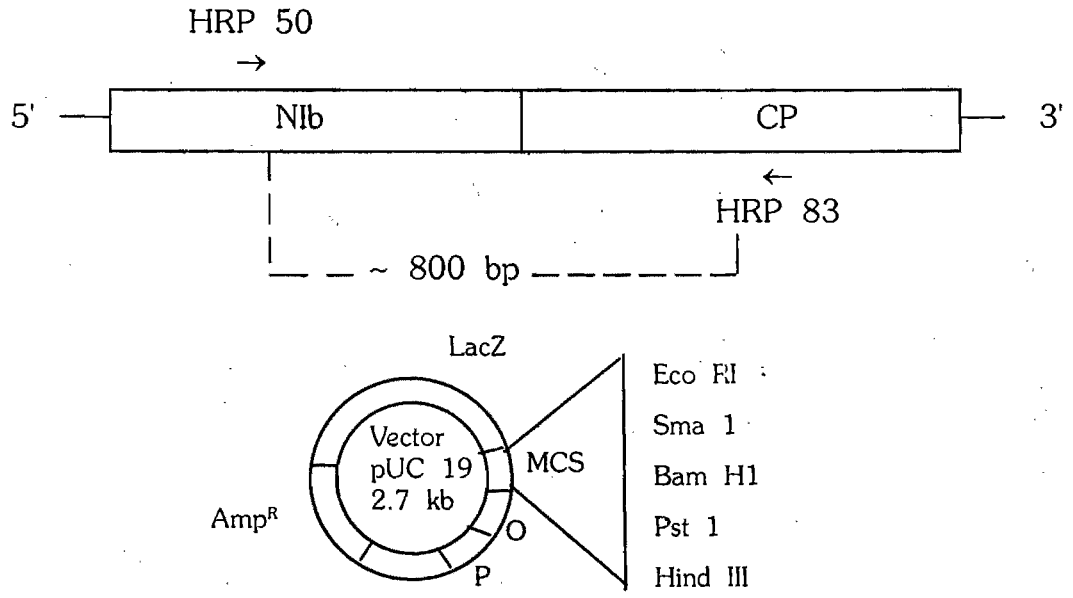


Figure 3. Schematic of cloning strategy of a part of PRSV genome (~ 800 bp) into pUC 19 vector blunt ended by *Sma* 1

T4 DNA polymerse stratgegy		Klenow fragment strategy	
DNA (0.5 µg)	10 µl	Sterile double distilled ater	20 µl
5 x Reaction Buffer	4 µl	1 M $MgCl_2$	6 µl
2 mM dNTPs	1 µl	0.1 M Tris. Cl (pH 7.6)	3 µl
T4 DNA polymerase (10 U/µl)	1 µl	Klenow fragment (*E. coli* DNA polymerase 1, 3U/µl)	1 µl
Sterile double distilled water	4 µl		
	20 µl		30 µl

3.6.1.2 Ligation

Both T4 DNA polymerase and klenow fragment of *E. coli* DNA polymerase 1 treated insert DNA fragments were separately ligated with *Sma* 1 cut dephosphorylated pUC 19 vector. The ligation reaction was set up as indicated.

Recipe for Ligation mix :	
Blunt ended insert DNA	20 µl
Sma 1 cut pUC 19 vector (150 ng)	1 µl
10 X ligase buffer	3 µl
10 mM ATP	3 µl
T4 DNA ligase (5 U/µl)	1 µl
Sterile double distilled water	2 µl
	30 µl

The ligation mixtures were briefly centrrifuged and incubated at 15°C for 18 h.

3.5.1.3 Competent cells preparation

The competent cells were prepared by calcium chloride ($CaCl_2$) method as described by Mendel and Higa (1970).

(1) 50 ml Luria Broth (LB) (Appendix III) was inoculated with overnight grown culture of DH 5 α strain of *Escherichia coli* (Stratagene) and incubated at 37°C for 1.5 h with constant shaking at 200 rpm in a shaker incubator till the bacterial growth reached 0.3 OD at 600 nm.

(2) The culture was then aseptically transferred to 40 ml sterile screw capped centrifuge tubes and kept on ice for 10 min.

(3) The cells were centrifuged at 5000 rpm for 10 min at 4°C in a Sorvall SS-34 rotor to pelletize the cells.

(4) The cells were resuspended gently in 10 ml ice cold 0.1 M $MgCl_2$ solution (Appendix III) and centrifuged at 5000 rpm for 10 min at 4°C.

(5) The pellet was resuspended in 10 ml ice cold 0.1 M $CaCl_2$ solution (Appendix III) and kept on ice for 1 h.

(6) The cells were recovered by centrifuging at 5000 rpm for 10 min at 4°C and the pellets were resuspended in 1 ml of chilled 0.1 M $CaCl_2$ and used for transformation after keeping on ice for 1 h.

(7) Also flash freezed the competent cells in liquid nitrogen and stored at -70°C until needed.

3.6.1.4 Transformation of competent cells

(1) 200 µl competent cells after thawing at room temperaure were added to 20 µl of the ligation mixture in a sterile microfuge tube and incubated on ice for 1 h.

(2) The bacterial cell - DNA mixture was given a heat shock at 42°C for 2 min. and was immediately transferred the tube to ice for 2 min.

(3) Added 1 ml LB medium (Appendix III) to each tube and incubated at 37°C for 1 h in a shaker incubator at 200 rpm.

(4) 200 µl cell suspension were aseptically plated either as such or after concentration on Luria Agar (LA) (Appendix III) plate containing ampicillin, X-gal and IPTG (Appendix III).

(5) The plates were incubated overnight at 37°C with the medium side up.

(6) The transformants were selected on the basis of blue / while colony colour. The white colonies were selected and subsequently plated on IXA (IPTG - X - gal - Ampicillin) plates. This plate having individual transformants in grid served as master plate.

3.6.1.5 Rapid Screening for the Recombinant Clones

To ascertain the recombinant clones with insert, a rapid screening method used was as follows :

(1) A small amount of overnight grown bacterial colonies were picked from master plate individually with the help of sterile tooth pick and mixed with 50 µl of 10 mM EDTA (pH 8.0) (Appendix III) in sterile microfuge tubes. One or two blue colonies of pUC 19 transformed bacterial cells were also taken for control.

(2) 50 µl of fresh lysis solution (2 N NaOH, 0.5 per cent SDS, 20 per cent sucrose) (Appendix III) was added in each tube and vortexed for 30 s.

(3) The mixture was incubated at 70°C for 5 min and immediately cooled down to room temperature.

(4) 1.5 µl 4 M KCl and 0.5 µl 0.4 per cent of bromophenol blue were added to each tube and vortexed for 30 s.

(5) The mixture was then incubated for 5 min on ice and centrifugation was done at 10,000 rpm for 30 min at 4°C in table top centrifuge.

(6) Bacterial cell debris was removed and 30 µl supernatant from each tube along with the control were electrophoresed in 0.7 per cent agarose gel.

(7) The lanes which showed plasmid band higher than the control were considered as recombinant plasmids and the respective clones were selected for isolation of recombinant plasmid DNA.

3.6.1.6 Recombinant plasmid DNA isolation by Miniprep method

Isolation of recombinant plasmid DNA was done by modified alkaline lysis method (Brinboim and Doly, 1979).

(1) Bacterial cells from selected clones containing viral insert were individually inoculated in 2 ml LB medium containing ampicillin (50 mg/ml) (Appendix III) in sterile capped culture tubes.

(2) Tubes were incubated overnight at 37°C at 200 rpm in a shaker incubator.

(3) The overnight grown bacterial cells were then transferred to 1.5 ml sterile eppendorf tube and were harvested by centrifuging in a table top centrifuge for 1 min. Care was taken to remove the medium adhering to the cell pellet.

(4) The pellet was resuspended in 100 µl solution 1 (Appendix III) and mixed vigorously by vortexing.

(5) Then 200 µl freshly prepared lysis solution i.e. solution II (Appendix III) was added and mixed gently.

(6) 150 µl ice cold solution III (Appendix III) was then added and mixed gently with lysed cell suspension and the mixture was kept on ice for 15 min.

(7) The chromosomal DNA and the bacterial cell debris were removed by centrifuging at 15,000 rpm for 15 min at 4°C in a table top centrifuge.

(8) The supernatant was taken and equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) (Appendix III) was added. The mixture was vortexed well and centrifuged in a table top centrifuge for 15 min at room temperature.

(9) The clear aqueous phase was transferred to fresh eppendorf tube.

(10) The DNA in aqueous phase was precipitated by adding 0.8 volume of isopropanol, and kept on ice for 10 min.

(11) This mixture was then centrifuged at 15,000 rpm for 20 min at 4°C.

(12) To the pellet 200 µl of 70% ethanol was added. The tube was rotated well so that the pellet from the wall gets soaked and suspended in alcohol. This ensures removal of adhering salts by 70% alcohol. DNA was then pelletized by centrifuging at 15,000 rpm for 5 min.

(13) The pellet was finally suspended in 30 µl sterile double distilled water.

3.6.1.7 Confirmation of transformants

The presence of insert in transformants was confirmed by restriction analysis and DNA-PCR.

Restriction Analysis

Isolated plasmid DNA was digested with restriction enzymes *Bgl1* and *Pst1* and incubated at 37°C for 4 h. The reaction was set up as indicated.

Plasmid DNA	3.0 µl
10X Reaction Buffer	2.0 µl
Bgl1 and Pst1 (10 U/µl)	1.0 µl each
Sterile double distilled water	20.0 µl

Electrophoresed the digested product at 60 volts on 0.7 per cent agarose gel. Insert size was assessed in comparison with 1 kb molecular weight markers (GeneRuler™, MBI).

DNA- Polymerase Chain Reaction (DNA-PCR).

Assembled various components of DNA-PCR mix on wet ice and three reactions were set up using forward (HRP50) and reverse (HRP 83) primers singly and together by dispensing as indicated

Reagents	I (Forward primer) (µl)	II (Reverse primer) (µl)	III (Forward and reverse primers) (µl)
Template (c-DNA)	1.0	1.0	1.0
Forward primer (100 pM)	1.0	-	1.0
Reverse primer (100 pM)	-	1.0	1.0
10 x PCR buffer	3.0	3.0	3.0
5 X Q solution	6.0	6.0	6.0
0.1 M DTT	3.0	3.0	3.0
10 mM dNTPs (100 µM each)	1.0	1.0	1.0
Taq DNA polymerase (5U/µl)	0.5	0.5	0.5
Sterile double distilled water to	30.0	30.0	30.0

The mixture was vortexed gently and kept in BIORAD PCR machine. The amplification conditions included 25 cycles of 30s at 90°C, 2 min at 46°C and 1 min at 72°C and one final cycle of extension for 10 min at 72°C. Analysed PCR products (3 µl each) by electrophoresing at 60 volts on 0.7% agarose gel.

3.6.1.8 Large scale plasmid DNA isolation

Large scale plasmid DNA preparation of s elected clones were performed following Qiagen Midi plasmid purification protocol (Qiagen Plasmid Midi Kit) (Appendix III). The procedure is described as below :

(1) A single colony from a freshly streaked plate of clone was picked and was inoculated in 50 ml LB medium containing ampicillin (50 µg/ml) in a screw capped conical flask.

(2) The culture was grown for 18 h in shaker incubator at 37°C with 200 rpm rotation.

(3) The bacterial cells were then harvested by centrifugation at 5000 rpm for 10 min at 4°C in RC-5C Sorvall centrifuge with SS-34 rotor.

(4) The pellet was resuspended in 4 ml of buffer PI and vortexed.

(5) 4 ml of buffer P2 was added, mixed gently and incubated at room temperature for 5 min.

(6) 4 ml of chilled P3 buffer was then added to the lysed cell suspension and mixed immediately but gently and incubated on ice for 15 min.

(7) After incubation the bacterial chromosome and debris were removed by centrifugation at 15,000 rpm for 30 min at 4°C.

(8) After removing the supernatant promptly, the same step were carried out once again.

(9) The supernatant was loaded onto Qiagen - tip 100 (previously equilibrated with 4 ml of buffer QBT) and allowed to enter the resin by gravity flow.

(10) Qiagen - tip 100 was then washed twice with 10 ml of buffer QC.

(11) The recombinant plasmid DNA was eluted with 5 ml of buffer QF.

(12) DNA was then precipitated by adding 3.5 ml of isopropanol and centrifugation was done at 15,000 rpm at 4°C for 30 min.

(13) DNA pellet was washed with 2 ml of 70% alcohol and centrifugation was done at 15,000 rpm at 4°C for 10 min.

(14) After air-drying, the pellet was dissolved in 100 µl of sterile double distilled water.

(15) After estimating the DNA concentration, appropriate aliquots were used for various experiments.

3.6.2 Nucleotide Sequencing

Selected recombinant clone with an insert of c. 800 bp of PRSV-UP and purified PCR product of PRSV-AP isolates were sequenced at *Bangalore Genei Pvt. Ltd.*, Bangalore, and Department of Biochemistry, South Campus, University of Delhi, respectively. Sequences were aligned, translated and compared with other PRSV isolates from India (Table 6) using the MacVector (Version 7.0) program (BLAST) and clustalW (version 1.4) multiple sequence alignment cluster dendrograms with pairwise distance matrices were also generated. The nucleotide sequence data was submitted to GenBank under accession No.s AF323638 and AF 32367. Other published PRSV sequences from India were obtained from the National Centre of Biotechnology Information using the GenBank BLAST program (Table 12).

Table 12. Sources of a part of nuclear inclusion b (NIb) and capsid protein (CP) gene sequences of Indian PRSV isolates used in this study for sequence comparisons

Isolate	Pathotype	Origin	Part of NIb - CP region (bases)	N-terminal amino acid	GenBank accession No.	References
PRSV-AP	P	Hyderabad	794	171	AF323637	This study
PRSV-UP	P	Gorakhpur	783	169	AF323638	This study
PRSV-KAR	P	Bangalore	776	165	AF120270	Bhat *et al.*, 2000
PRSV-MAH-1 (MAH.ML)	P	Pune	792	170	AF063220	Jain *et al.*, 1998
PRSV-W	W	New Delhi	782	170	AF063221	Jain *et al.*, 1998

3.7 Management through Cross Protection

3.7.1 Search for Mild strain

A naturally occurring mild strain of PRSV (same as MAH-1/ MAH.ML), identified by Dr. R.D. Ram from IARI, Regional Station, Pune, was used for cross protection studies. The mild strain was maintained in the glasshouse on papaya var. Coorg honey dew by sap inoculation.

3.7.2 Host Susceptibility to mild strain

Susceptibility of various cucurbits and papaya cultivars to mild strain was assessed by sap inoculation. Infected tissues from papaya (var. Coorg honey dew) were ground in 0.01 M sodium phosphate buffer (pH 7.0, 1 g/1 ml) (Appendix 1), and the extracts were rubbed on test plants using Celite. Plants were kept under observation in the glasshouse. Infection by mild strain was checked on the basis of symptomatology, electron

microscopy and direct antigen coated-enzyme linked immunosorbant assay (DAC-ELISA) using the antiserum against PRSV-P.

3.7.3 Cross Protection test under glasshouse condition

About 100 papaya seedlings (var. Coorg honey dew) at 2-3 leaf stage were pre-infected with mild strain by rubbing individual leaves. Each seedling was tested by DAC-ELISA to ensure the infection by mild strain 15 days after inoculation. Pre-infected seedlings were then challenge inoculated with Pune severe (same as PRSV-MAH-2) strain maintained on papaya (var. Coorg honey dew). For challenge inoculation, upper three fully expanded leaves of protected papaya seedlings were inoculated at 15, 20, 25, 30, 35 and 40 days after initial inoculation with mild strain. Six protected plants were used for each challenge test. To determine the combined effect of time and multiple challenge inoculation, two seedlings each were reinoculated with challenge virus at 5 days interval. Thus, seedlings challenge inoculated initially after 15 days received challenge inoculation six times; seedlings challenge inoculated initially after 20 days received challenge inoculation five times and so on. Papaya seedlings inoculated with only Pune mild and severe strain separately served as negative and positive controls, respectively. Breakdown of cross-protection was adjudged by the appearance of severe symptoms and DAC-ELISA on both single and multiple challenge inoculated plants. Observations were taken at 0, 10, 20, 30, 40, 50 and 60 days after challenge inoculation.

Results

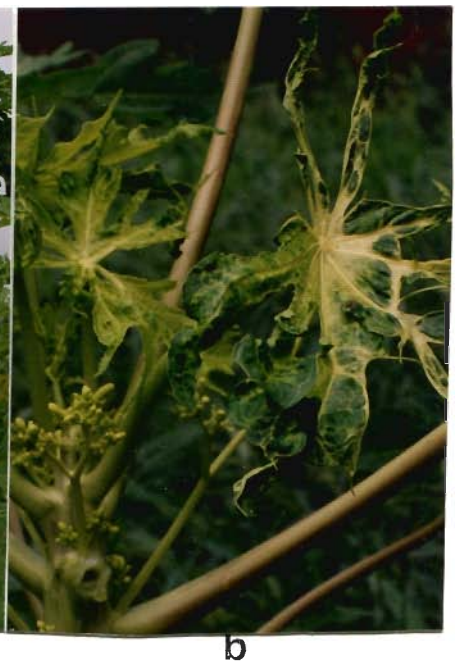
RESULTS

4.1 Virus Isolates

Present investigation was based on nine papaya ringspot virus (PRSV) isolates collected from different papaya growing regions. PRSV infected papaya samples collected from different regions exhibited a variety of symptoms (Figure 4). Foliar symptoms varied from mild mottling (MM) to yellow mosaic (YM), vein clearing (VC), leaf blistering (LB), leaf distortion (LD) and shoe stringing (SS) (Table 13). Isolates incited their own combination of symptoms on the leaves. Symptomatic samples collected from Bihar (BH), Delhi (DL), Maharashtra-2 (MAH-2) and Uttar Pradesh (UP) showed YM, VC, LB and LD with or without SS symptoms. In contrast, symptomatic samples collected from Andhra Pradesh (AP) and Karnataka (KAR-2) showed only YM, LD with or without SS; whereas samples from Karnataka-1 (KAR-1) and West Bengal (WB) and Maharashtra (MAH-1) showed only YM and MM symptoms respectively.

Leaf-dip electron microscopy revealed the association of flexuous virus particles with syptomatic papaya samples collected from different regions (Fig. 5a). Flexuous particles from these samples were detected and decorated in DAC-ELISA and ISEM tests (Fig. 5b) respectively by PRSV-P antiserum. Extracts from field collected samples from different regions reproduced disease symptoms on papaya.

a



b

c

d

Papaya plants infected with papaya ringspot virus (PRSV) at I.A.R.I. experimental field (a), showing symptoms on foliage (b), stem (c) and fruit (d).

Table 13. Symptoms of PRSV isolates on papaya under natural conditions

Virus origin/ Isolate designation	Foliar ymptoms*					
	MM	YM	VC	LB	LD	SS
Andhra Pradesh (AP)	-	+	-	-	+	-
Bihar (BH)	-	+	+	+	+	+
Delhi (DL)	-	+	+	+	+	-
Karnataka-1 (KAR-1)	-	+	-	-	-	-
Karnataka-2 (KAR-2)	-	+	-	-	+	+
Maharashtra-1 (MAH-1)	+	-	-	-	-	-
Maharashtra-2 (MAH-2)	-	+	+	+	+	+
Uttar Pradesh (UP)	-	+	+	+	+	+
West Bengal (WB)	-	+	-	-	-	-

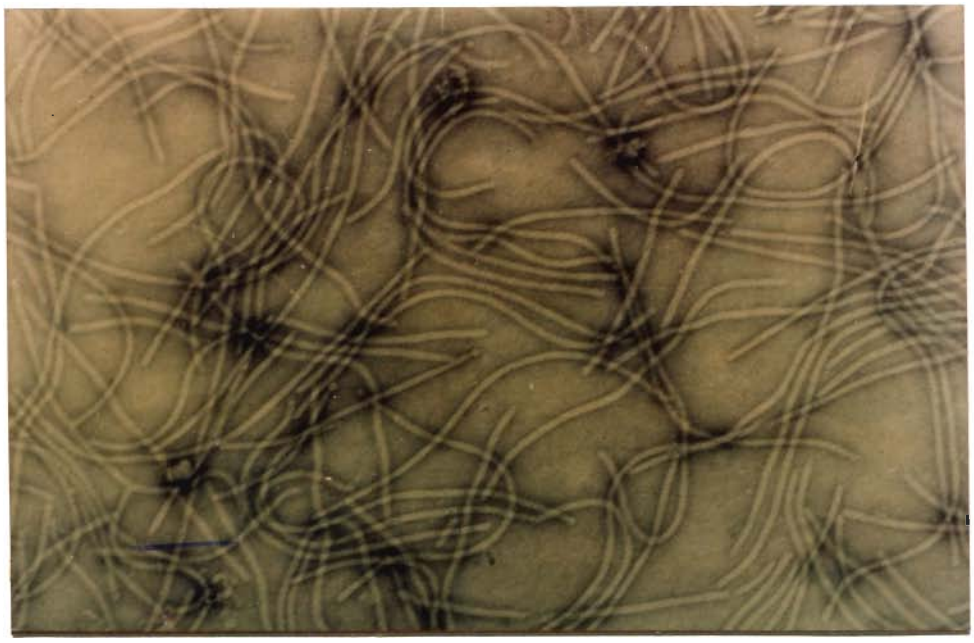
* MM = Mild mottling; YM = Yellow mosaic; VC = Vein clearing; LB = leaf blistering; LD = Leaf distortion; SS = Shoe stringing

4.2 Biological diversity

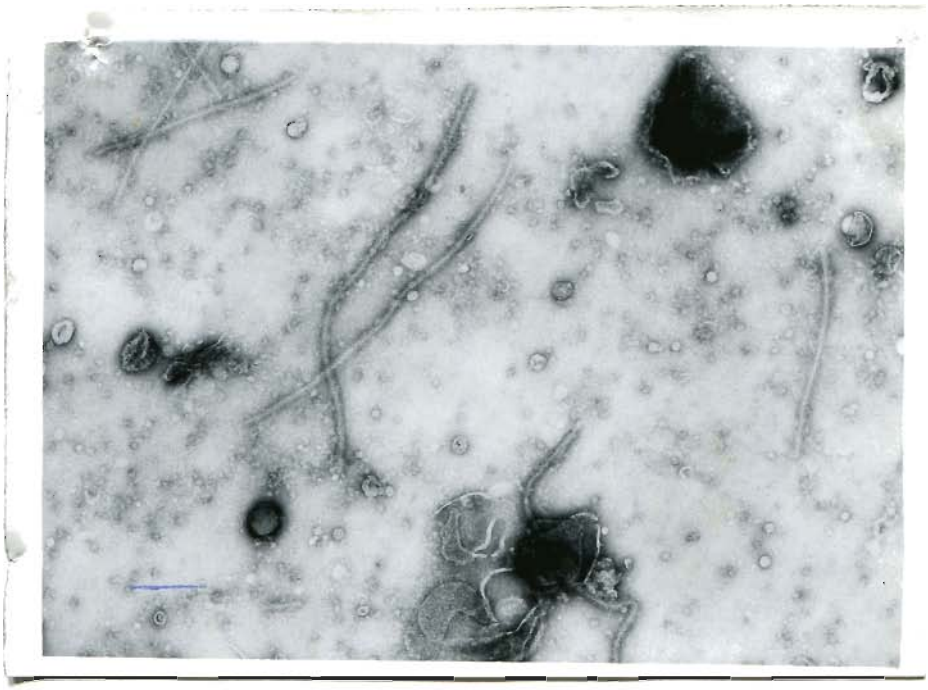
Biological divergence among eight PRSV isolates (except AP) was assessed by sap inoculation of different papaya cultivars and cucurbit species. Both symptomatology and virus multiplication in hosts were compared.

4.2.1 Reaction on papaya cultivars.

Divergence of eight PRSV isolates was assessed on five papaya cultivars by symtomatology and ELISA reaction. Isolates varied in the ability to infect papaya varieties. Foliar symptoms ranging from mild mottling (MM) to vein clearing (VC), stunting (ST), leaf distortion (LD) and shoe stringing (SS) were observed 10 days after inoculation. Of the eight isolates tested, Bihar (BH), Karnataka-1 (KAR-1) and Maharashtra-1 (MAH-1) induced mild mottling (MM) on compatible papaya cultivars. Delhi



a



b

ure 5. Leaf-dip electronmicroscopy of papaya ringspot virus (PRSV) infected papaya sample showing flexuous particles (Bar 100 nm) (a) and particles decorated with PRSV-P antiserum in immunosorbent electron microscopy (ISEM) (b).

(DL), Karnataka-2 (KAR-2) and West Bengal (WB) isolates induced ST and/ or LD with MM on compatible papaya cultivars, whereas Maharashtra-2 (MAH-2) and Uttar Pradesh (UP) isolates caused foliar symptoms ranging from MM to LB, LD, SS, ST and VC depending on compatible papaya cultivars (Table 14; Figures 6, 7). ELISA reactions with a few exceptions were consistent with symptoms observation. Symptomatic papaya plants were ELISA positive; whereas the non-symptomatic plants gave negative reactions (Table 14). Intensity of the reactions varied and isolats gave A_{405} nm values between 0.11 and 0.94. BH, KAR-1, KAR-2 and WB isolates gave weak reactions only (A_{405}, 0.11 and 0.24) on compatible papaya cultivars; whereas DL, MAH-1, MAH-2 and UP isolates gave medium to very strong reactions depending upon the papaya cultivars (A_{405}, 0.38 and 0.94) (Table 14).

4.2.2 Reaction on cucurbits

Divergence of eight PRSV isolates (except AP) was assessed on nine different curcurbitaceous host species by symptomatology and ELISA reactions (Table 15, 16). Isolates varied in their ability to infect cucurbit species and foliar symptoms ranging from mottling (MT) to mosaic (M), vein banding (VB), vein clearing (VC) and leaf blistering (LB) were observed 10-20 days after inoculation. Of the eight isolates tested, Bihar (BH) and Maharashtra-1 (MAH-1) failed to produce symptoms on any of the cucurbits tested; whereas West Bengal (WB) isolate could infect only *Praecitrullus fistulosus* (Tinda). Further, Maharashtra-2 (MAH-2) were able to infect five; Karnataka-1 (KAR-1), Karnataka-2 (KAR-2) and Uttar Pradesh (UP) three and Delhi (DL) two of the nine cucurbits tested (Table 15; Figure 8). ELISA reactions with a few exceptions were consistent with symptoms

Table 14. Reactions of PRSV isolates on different papaya cultivars

Isolates*	Observations**	Cultivars				
		Barwain	CO-2	Coorg honey dew	Pusa nanha	Washington
BH	1	NS	MM	NS	M	MM
	2	-	+	-	+	+
DL	1	LD,MM	MM	MM	NS	LD,MM
	2	+++	-	+	-	++
KAR-1	1	NS	MM	MM	MM	MM
	2	-	+	+	+	+
KAR-2	1	MM	MM	MM	ST	LD
	2	+	-	+	+	+
MAH-1	1	MM	NS	MM	NS	MM
	2	++	+	++	+	+++
MAH-2	1	MM	LD	SS,LD	VC,MM	LD,SS,ST
	2	+++	+++	+++	++	++++
UP	1	LB,LD,SS	NS	MM	NS	NS
	2	++++	-	+	-	+
WB	1	MM	NS	MM	ST	MM
	2	+	-	+	+	+

* BH = Bihar; DL = Delhi; KAR-1 = Karnataka-1; KAR-2 = Karnataka-2; MAH-1 = Maharashtra-1; MAH-2 = Maharashtra-2; UP = Uttar Pradesh; WB = West Bengal

** 1 = Symptomatology; NS = No symptom; LD = Leaf distortion; LB = Leaf blistering; MM = Mottling; VC = Vein clearing; SS = Shoe stringing; ST = Stunting; 2 = OD values based on DAC-ELISA at A_{405} nm after 1 h substrate reaction; A_{405} values were classed as : - = < 0.10 (no reaction); + = 0.10-0.30 (weak reaction); ++ = 0.31 - 0.50 (medium reaction); +++ = 0.51 - 0.70 (strong reaction); ++++ = > 0.70 (very strong)

Table 15. Reactions* of PRSV isolates on different cucurbits

Hosts	Days after inoculation	Isolates**							
		BH	DL	KAR -1	KAR -2	MAH -1	MAH -2	UP	WB
Cucumis melo L.	10	-	-	-	-	-	-	-	-
var. reticulatus ser.	20	-	-	-	-	-	-	M	-
(Muskmelon)									
C. melo L. var. *utilissimus*	10	-	-	-	-	-	-	-	-
(Roxb.) Duthie & Fuller	20	-	M	-	M	-	-	-	(Long
melon/Kakri)-									
C. sativus L.	10	-	-	-	-	-	M	-	-
(Cucumber)	20	-	-	M	M	-	M	-	-
Cucurbita moschata	10	-	-	-	-	-	-	-	-
Duche ex Poir (Pumpkin)	20	-	-	-	-	-	-	-	-
C. pepo L.	10	-	-	-	-	-	M	-	-
(Summer squash)	20	-	M	M	-	-	M	M	-
Lagenaria siceraria (Mol)	10	-	-	-	-	-	-	-	-
Standl. (Bottle gourd)	20	-	-	M	-	-	-	-	-
Luffa acutangula L. (Roxb.)	10	-	-	-	-	-	-	-	-
(Ridge gourd)	20	-	-	-	-	-	M	-	-
L. cylindrica L. Roem.	10	-	-	-	-	-	-	-	-
(Sponge gourd)	20	-	-	-	-	-	M	-	-
Praecitrullus fistulosus Pang.	10	-	-	-	-	-	-	-	-
(Tinda)	20	-	-	-	VC	-	LB, MT	LB VB	M

* - = No symptom; M = Mosaic; VB = Vein banding; MT = Mottling; LB = Leaf blistering; VC = Vein clearing

** BH = Bihar; DL = Delhi; KAR-1 = Karnataka-1; KAR-2 = Karnataka-2; MAH-1 = Maharashtra-1; MAH-2 = Maharashtra-2; UP = Uttar Pradesh; WB = West Bengal

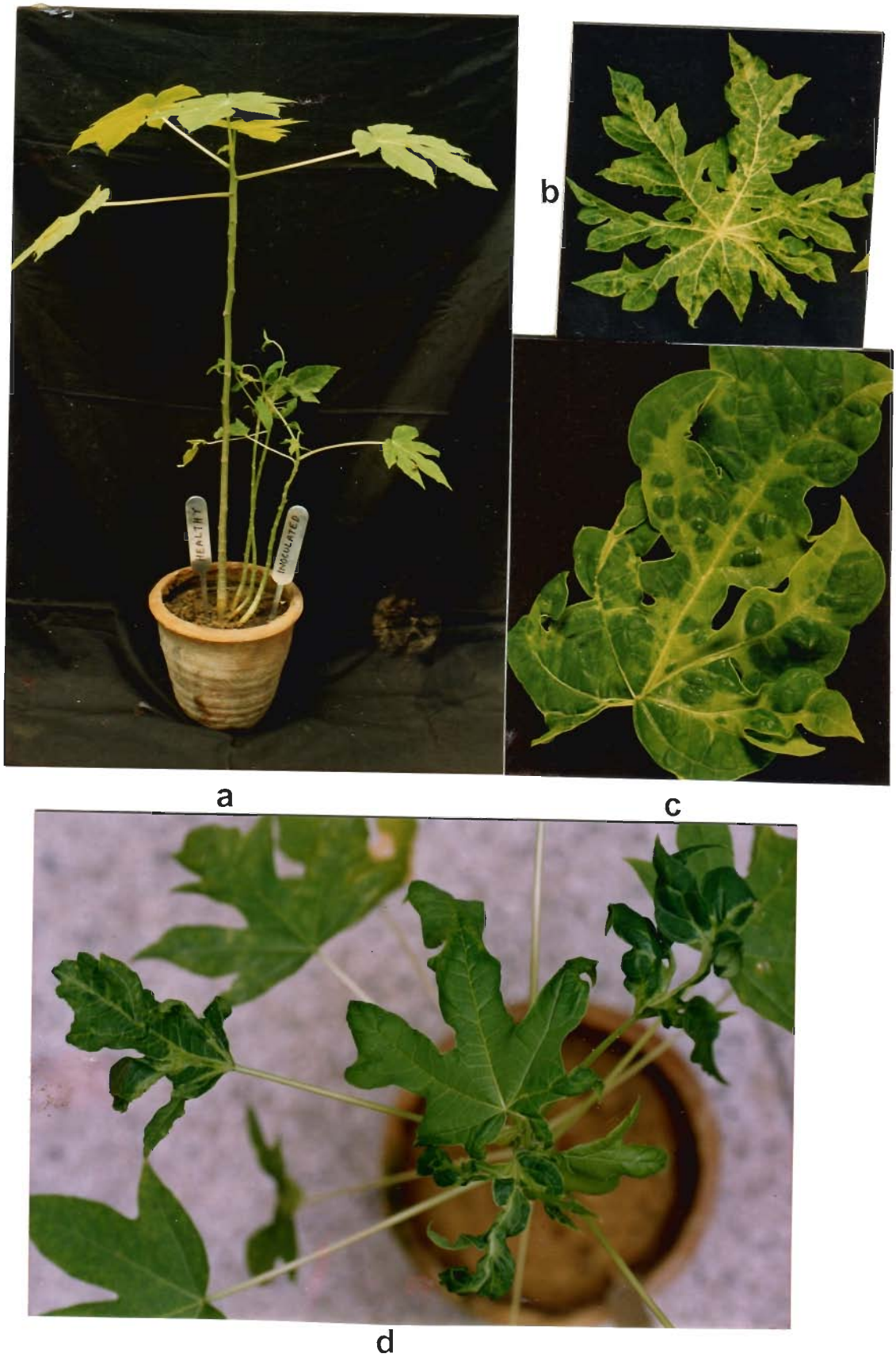


Figure 6. Symptoms induced by papaya ringspot virus (PRSV) isolates: stunting - MAH-2 (a); mottling and vein clearing – MAH-2 (b); leaf blistering – UP (c) and leaf distortion – DL (d).

a

b

Figure 7. Shoe stringing symptoms induced by MAH-2 (a) and UP (b) isolates of papaya ringspot virus.

a

b

c

Figure 8. Cucurbitaceous host species infected with papaya ringspot virus isolates: *Cucurbita pepo* – MAH-2 (a) ; *Cucumis melo* var. *reticulatus* – UP (b) and *Praecitrullus fistulosus* – MAH-2 (c).

observations. Symptomatic cucurbits plants were ELISA positive; whereas non-symptomatic plants gave negative reactions (Table 16). Intensity of ELISA reactions varied and isolates gave A_{405} between 0.10 and 0.85. DL, MAH-1 and WB isolates gave weak reaction only (A_{405} 0.10 and 0.20) on compatible cucurbits; whereas KAR-1 and KAR-2, MAH-2 and UP isolates gave weak to very strong reactions depending on the cucurbits tested (0.16 and 0.85) (Table 16).

4.3 Serological diversity

Serological divergence of eight PRSV isolates (except AP) was assessed by DAC-ELISA and ISEM tests, using antisera to PRSV-P and -W isolates. The results observed are shown in Table 17 and 18. All the eight isolates reacted uniformly with polyclonal antiserum to capsid protein of P or W isolates in DAC-ELISA and ISEM tests. Isolates gave A_{405} mm values between 0.67 and 0.80 (strong to very strong reaction) with PRSV -P antiserum; 0.15 and 0.49 (weak to medium reaction) with PRSV-W antiserum (Table 17).

Similarly all the eight isolates showed strong decoration with antisera to PRSV-P, whereas weak to strong decoration with PRSV-W antiserum in ISEM decoration test (Table 18; Figure 9).

4.4 Molecular diversity

Molecular divergence of all the PRSV isolates was assessed by amplifying 3'-terminal region of viral genome comprising a part of the capsid protein (CP) gene and nuclear inclusion b (NIb) gene.

Table 16. ELISA reactions of PRSV isolates* on different cucurbit crops

Hosts	Days after inoculation	A_{405} nm**							
		BH	DL	KAR -1	KAR -2	MAH -1	MAH -2	UP	WB
Cucumis melo L. var. *reticulatus* ser. (Muskmelon)	10	-	-	-	-	-	+	+	-
	20	-	-	+	-	+	+	++++	-
C. melo L. var. *utilissimus* (Roxb.) Duthie & Fuller (Long melon/Kakri)	10	-	-	-	+	-	+	+	-
	20	-	+	++	+++	+	+	-	-
C. sativus L. (Cucumber)	10	-	-	-	+	-	++	+	-
	20	-	-	++	++++	+	++	++	-
Cucurbita moschata Duche ex Poir (Pumpkin)	10	-	-	-	-	-	-	-	-
	20	-	-	-	-	+	-	-	-
C. pepo L. (Summer squash)	10	-	-	-	-	+	++++	+	-
	20	-	+	++	-	+	++++	++++	-
Lagenaria siceraria (Mol) Standl. (Bottle gourd)	10	-	-	-	+	-	-	+	-
	20	-	-	++	-	-	+	+	-
Luffa acutangula L. (Roxb.) (Ridge gourd)	10	-	-	-	+	-	-	-	-
	20	-	-	++	+	-	+	-	-
L. cylindrica L. Roem. (Sponge gourd)	10	-	-	-	-	-	+	-	-
	20	-	-	+	-	-	+++	-	-
Praecitrullus fistulosus Pang. (Tinda)	10	-	-	-	-	-	+	-	-
	20	-	-	+	++++	-	++++	++++	+

* BH = Bihar; DL = Delhi; KAR-1 = Karnataka-1; KAR-2 = Karnataka-2; MAH-1 = Maharashtra-1; MAH-2 = Maharashtra-2; UP = Uttar Pradesh; WB = West Bengal

** A_{405} values were classed as : - = < 0.10 ; + = 0.10-0.30 (weak reaction); ++ = 0.31 - 0.50 (medium reaction) ; +++ = 0.51 - 0.70 (strong reaction); ++++ = > 0.70 (very strong reaction)

Table 17. Serological reactions of PRSV isolates* with antisera to capsid proteins in DAC-ELISA

Antisera to	A_{405} nm**							
	BH	DL	KAR-1	KAR-2	MAH-1	MAH-2	UP	WB
PRSV-P	+++	++++	++++	++++	++++	++++	++++	++++
PRSV-W	+	+	++	+	++	++	++	+

* BH = Bihar; DL = Delhi; KAR-1 = Karnataka-1; KAR-2 = Karnataka-2; MAH-1 = Maharashtra-1; MAH-2 = Maharashtra-2; UP = Uttar Pradesh; WB = West Bengal

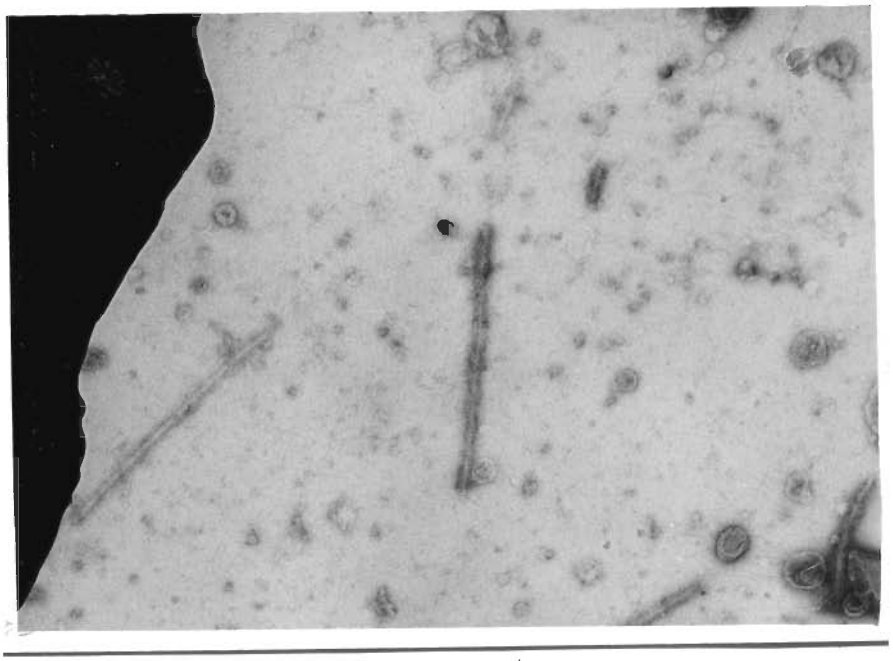
** A_{405} values were classed as : - = < 0.10; + = 0.10-0.30 (weak reaction); ++ = 0.31 - 0.50 (medium rection); +++ = 0.51 - 0.70 (strong reaction); ++++ = > 0.70 (very strong reaction)

Table 18. Serological reactions of PRSV isolates* with antisera to capsid proteins in ISEM

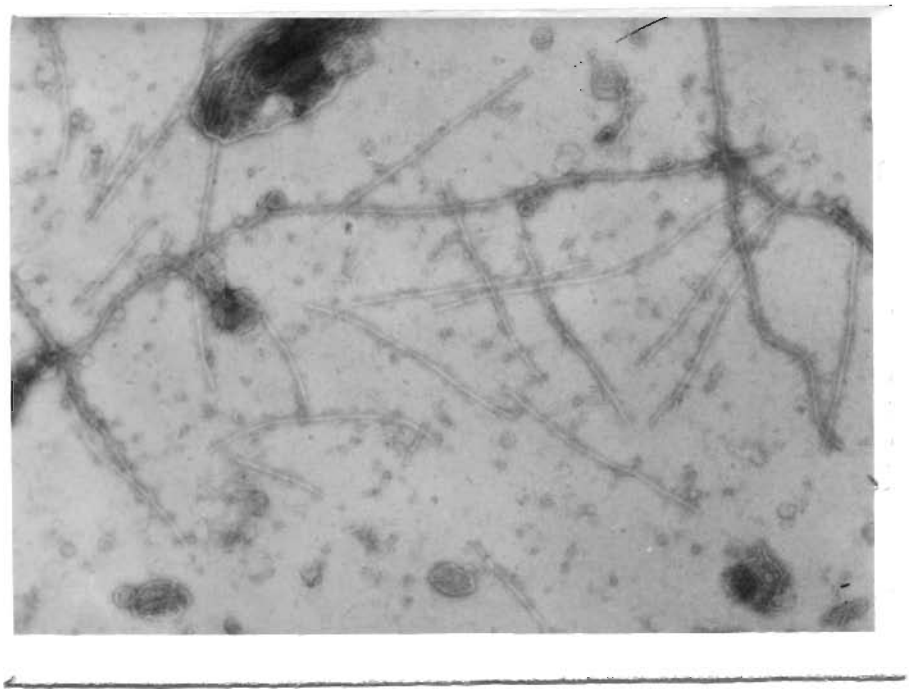
Antisera to	ISEM**							
	BH	DL	KAR-1	KAR-2	MAH-1	MAH-2	UP	WB
PRSV-P	++	++	++	++	++	++	++	++
PRSV-W	+	+	++	+	++	++	++	+

* BH = Bihar; DL = Delhi; KAR-1 = Karnataka-1; KAR-2 = Karnataka-2; MAH-1 = Maharashtra-1; MAH-2 = Maharashtra-2; UP = Uttar Pradesh; WB = West Bengal

** ++ = Strong decoration; + = weak decoration; - = No decoration



a



b

Figure 9. Flexuous particles of papaya ringspot virus (BH) decorated with PRSV-P (a) and PRSV-W (b) antiserum in immunosorbent electron microscopy (ISEM) test.

4.4.1 Reverse transcription and polymerase chain reaction (RT-PCR) analysis

Using primers derived from nuclear inclusion b (NIb) gene and capsid protein (CP) gene, RT-PCR was successful in amplifying NP and CP genes from all the nine isolates. A PCR product of expected size (approximately 800 bp) was observed in all the cases except KAR-2 and MAH-2 which gave less than 800 bp product (Figure 10). No PCR product was obtained from uninfected control samples (data not shown). The identity of the amplicons was confirmed by cloning and sequencing.

4.4.2 Single-strand conformation polymorphism (SSCP) analysis

Results of SSCP analysis performed directly on PCR products comprising a part of coat protein (CP) gene and nuclear inclusion b (NIb) gene of PRSV isolates are shown in Figure 11. Resolution of the two strands of the DNA was better when electrophoresed at room temperature (25-30°C) as compared to 10°C. In the present study, three distinct banding patterns were visible. The mild isolate (MAH-1) showed complete distinct pattern from other PRSV isolates. KAR-2 and MAH-2 together showed similar unique banding pattern while rest of the isolates like BH, DL, KAR-1, UP and WB, gave similar pattern of bands of their own (Figure 11).

4.5 Cloning and Sequencing

4.5.1 Cloning

Cloning of the amplified RT-PCR product (~ 800 bp) comprising a part of CP and NIb genes of the genome from Uttar Pradesh (UP) isolate was successful when blunt end ligation using Klenow fragment was carried out. The presence of the insert in recombinant clones was

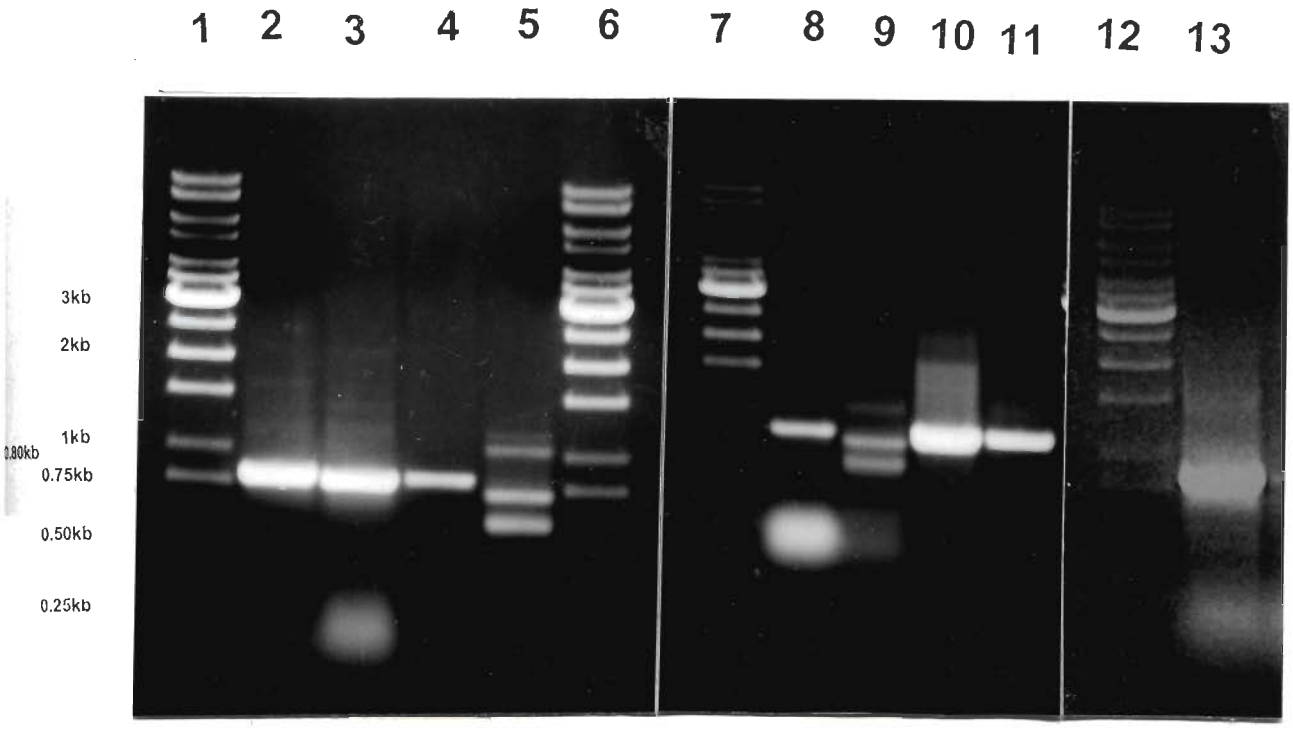


Figure 10. Agarose gel (0.7%) electrophoresis of reverse transcription polymerase chain reaction (RT-PCR) products: lanes 1,6,7 and 12 – 1 kb DNA ladder; lane 2 – Bihar; 3 – Delhi; 4 – Karnataka-1; 5 – Karnataka-2; 8 – Maharashtra-1; 9 – Maharashtra-2; 10 – Uttar Pradesh; 11 – West Bengal; 13 - Andhra Pradesh

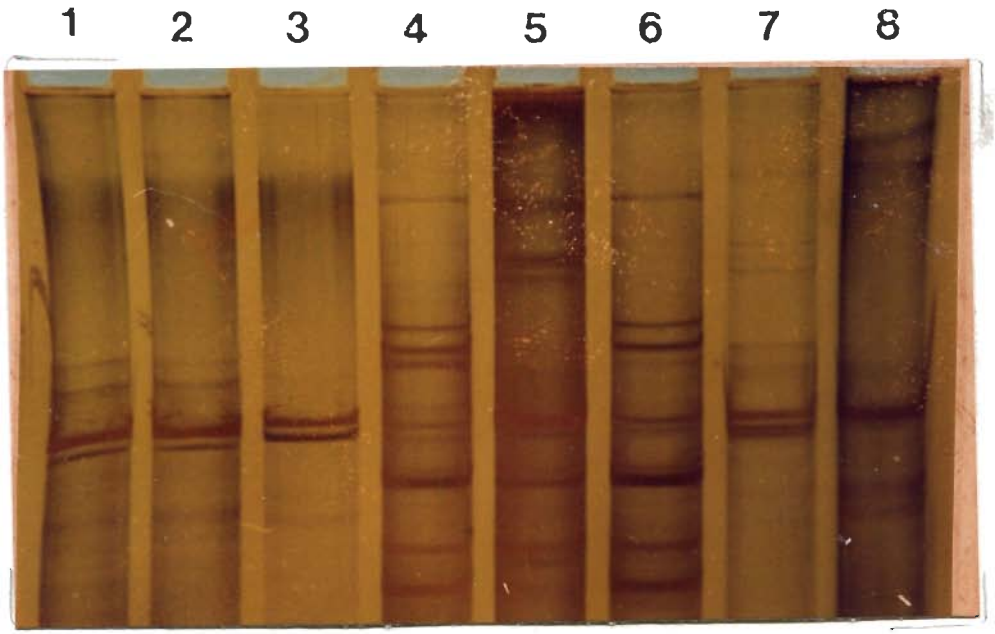


Figure 11. Single-strand conformation polymorphism (SSCP) patterns of RT-PCR products of papaya ringspot virus isolates: lane 1 – Bihar; 2 – Delhi; 3 – Karnataka-1; 4 – Karnataka-2; 5 – Maharashtra-1; 6 – Maharashtra-2; 7 – Uttar Pradesh and 8 – West Bengal.

confirmed through restriction analysis as well as DNA PCR. Restriction of the plasmid DNA from recombinant clone with *Bgl*1 yielded two bands of approximate 2.4 kb and 1.1 kb (Figure 12a), confirming the presence of insert inside the multiple cloning site of the pUC 19 vector. DNA-PCR using plasmid DNA from recombinant clone and both the specific primers resulted in the amplification of a part of CP and NIb genes and approximately 800 bp product was obtained (Figure 12b).

4.5.2 Sequencing

The nucleotide and the translated amino acid sequences of the 3' -terminal regions comprising a part of CP and NIb genes of the genomes from isolates are presented in Figures 13, 14. The sequenced regions contained a single open reading frame (ORF) of 794 and 783 nucleotides that could potentially encode polyproteins of 264 and 260 amino acids in AP and UP isolates, respectively. The polyprotein of AP and UP isolates included a part of the NIb protein consisting of 93 and 92 amino acids, respectively. The protease cleavage site, between glutamine and serine (Q/S) was available in both the isolates. Similarly, other conserved regions along the capsid protein sequence, such as DAG and WCIEN were present in both the isolates. The capsid protein consisted of 171 and 169 amino acids in AP and UP isolates respectively.

4.5.3 Sequence comparison between PRSV isolates from India

Partially sequenced nuclear inclusion b (NIb) and capsid protein (CP) genes of AP and UP isolates were compared with published sequences of Indian PRSV isolates at nucleotide and amino acid levels (Table 19; Figures 15, 16). Comparative sequence analyses showed that PRSV

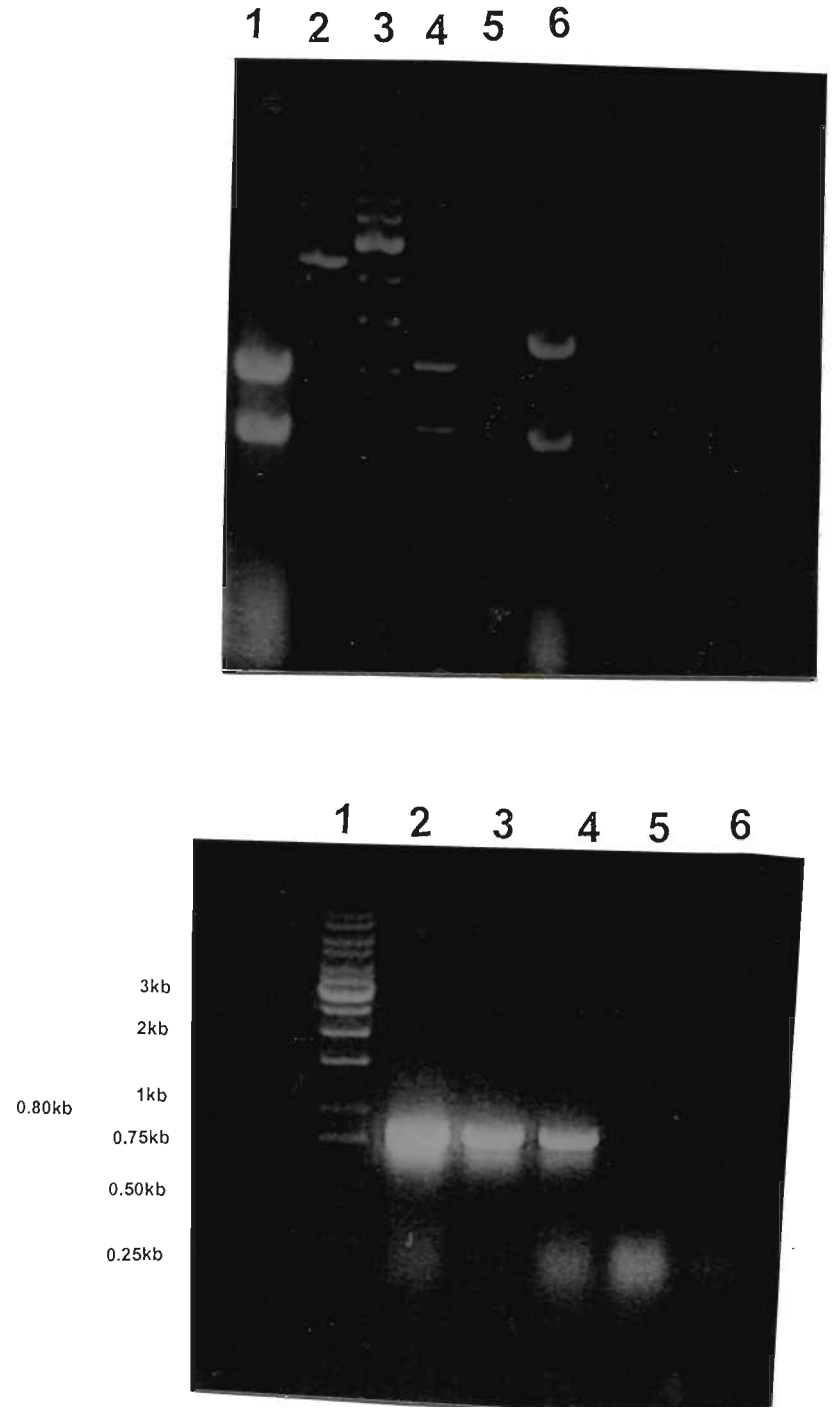


Figure 12. Agarose gel electrophoresis (0.7%) of the recombinant plasmid DNA *[Restricted with Bgl 1: lanes 1,4 and 6 – plasmid vector (pUC 19) cut with Bgl 1; 2 – plasmid vector (pUC 19) cut with Pst 1; 3 – 1 kb DNA ladder and 5 – recombinant DNA cut with Bgl 1 (a); DNA-PCR of the recombinant clone: lane 1 – 1 kb DNA ladder, 2 – RT-PCR product of PRSV-AP, 3 – RT-PCR product of PRSV-UP, 4 – DNA-PCR of recombinant plasmid using both the primers (HRP 50, 83), 5 and 6 – DNA-PCR of recombinant plasmid using single primer (HRP 50 and 83respectively) (b)]*

(a)

```
5'GGTGAACTAACACGCCAGATCCGCAGATTTTACCAATGGG  40
 TTCTTGAGCAGGCTCCATTCAATGAGTTGGCTAGGCAAGG  80
 GAGAGCCCCTTACGTCTCGGAAGTTGGGTTAAGAAGGCTG 120
 TACACTAGTGAGCGTGGTTCGATGGATGAGTTGGAAGCAT 160
 ACATTGATAAATACTTCGAACGTGAGCGTGGAGATTCTCC 200
 TGAGCTATTGGTGTACCATGAATCAAAAAGTTCGGATGAT 240
 CATCAAATTAGCTGTCATATGAATGAACATGTTTATCA fC 280
 AATCCAAAACTGAAGCGGTGGATGCAGGTCTCAATGATAA 320
 GCTGAAAGAGAGGGAAAAAGAAAAAGATAAAGAGAAAGAA 360
 AAAGAAAAGAAAGACAAGAAGGATGCTAGTGACGGAGGTG 400
 ATGTGTCAACTAGCACAAAAACTGGAGAGAGAGATAGAGA 440
 TGTCAATGCTGGAACTAGTGGTACATTTACAGTTCCAAGG 480
 ATCAAGTCATTTACTGATAAGATGATTTTGCCCAGAATTA 520
 AGGGAAAAGTTGtTCTTAATTTGAATCATCTTCTTCAGTA 560
 TAATCCACATCAAATTGACATTTCAAACACTCGTGCCACA 600
 CAATCACAGTTTGAGAGGTGGTATGAGGGAGTGAGGAATG 640
 ACTATGGCCTTAATGATAATGAAATGCAAGTGATGTTAAA 680
 CGGCTTAATGGTTTGGTGTATCGAAAATGGTACATCCCCA 720
 GACATATCTGGCGTCTGGGTTATGATGGATGGTGAAACTC 760
  AAGTCGATTATCCGATTAAACCGTTAATTGAGCA 3'  794bp  800
```

(b)

```
 GELTRQIRRFYQWVLEQAPFNELARQGRAPYVSEVGLRRL  40
 YTSERGSMDELEAYIDKYFERERGDSPELLVYHESKSSDD  80
 HQISCHMNEHVYHQSKTEAVDAGLNDKLKEREKEKDKEKE 120
 KEKKDKKDASDGGDVSTSTKTGERDRDVNAGTSGTFTVPR 160
IKSFTDKMILPRIKGKVVLNLNHLLQYNPHQIDISNTRAT 200
 QSQFERWYEGVRNDYGLNDNEMQVMLNGLMVWCIENGTSP 240
 DISGVWVMMDGETQVDYPIKPLIE 264             280
```

Figure 13: Nucleotide (a) and amino acid (b) sequence of PRSV-AP comprising a part of NIb and CP gene: cleavage site of CP gene (QS), gene for aphid transmission (DAG) and conserved region (WCIEN) have been highlighted

(a)

5′ GACACATCAAATTCGGAGATTCTATCGATGGGTTCTTGAA	40
CAAGCTCCATTCAATGAACTGGCAAGACAAGGCAGGGCTC	80
CTTACGTCTCTGAAGTTGGTCTGAAAAGATTGTACACCAG	120
TGAACGTGGTTCAATGAATGAACTAGAGGCTTACATAAAT	160
AAATACTTTGAGCGTGAAAAAGGAGATTCACCTGAACTAC	200
TTGTGCACCATGAATCAAATGATGCTGCAATAAAGAGGCA	240
TCTTTTATGCAGTAGCTATAAGCACGTATATCACCAGTCG	280
AAGGCTGAGGCTGTGGATGCGGGCTTAAACGATAAGCTCA	320
GAGAAAAAGAACAGAAAGAGAAAGAGAAAAAGAAAGAAAA	360
AGAAAAAGATGAAGCTGGTGGCGGAAATGATGTGTCAACC	400
AGCACGAAAACTGGAGAGAGGGATAGAGATGTTAACGCTG	440
GAACTAGTGGAACTTTTACAGTCCCAAGGATAAAATCATT	480
TACTGACAAGATGGTTCTACCAAGAATTAAGGGAAAAACT	520
GTCCTTAATTTGGATCATCTTCTTCAGTATAATCCACAGC	560
AAATTGACATCTCAAACACTCGTGCCACTCAGTCTCAGTT	600
CGAAAGATGGTACGAGGGAGTGAGGGATGATTATGGTCTT	640
AGCGATAATGAAATGCAAGTGATGTTAAACGGTTTGATGG	680
TATGGTGTATTGAAAATGGTACATCTCCAGACATATCTGG	720
TGTCTGGGTAATGATGGATGGCGACACTCAAGTTGACTAT	760
CCAATCAAGCCTTTGATTGAACA 3′ 783 bp	800

(b)

THQIRRFYRWVLEQAPFNELARQGRAPYVSEVGLKRLYTS	40
ERGSMNELEAYINKYFEREKGDSPELLVHHESNDAAIKRH	80
LLCSSYKHVYHQSKAEAVDAGLNDKLREKEQKEKEKKKEK	120
EKDEAGGGNDVSTSTKTGERDRDVNAGTSGTFTVPRIKSF	160
TDKMVLPRIKGKTVLNLDHLLQYNPQQIDISNTRATQSQF	200
ERWYEGVRDDYGLSDNEMQVMLNGLMVWCIENGTSPDISG	240
VWVMMDGDTQVDYPIKPLIE 260	300

Figure 14: Nucleotide (a) and amino acid (b) sequence of PRSV-UP comprising a part of NIb and CP gene: cleavage site of CP gene (QS), gene for aphid transmission (DAG) and conserved region (WCIEN) have been highlighted.

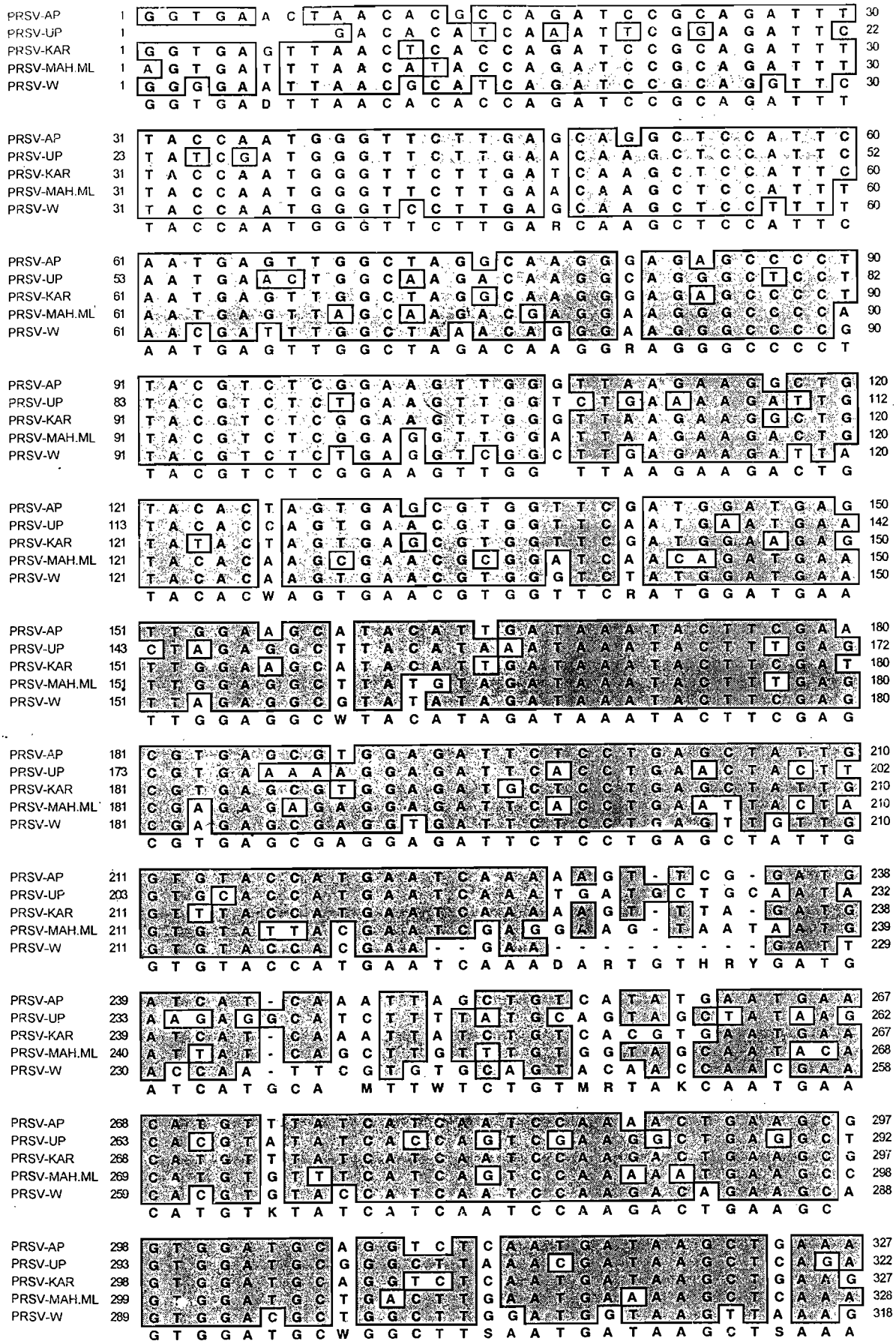
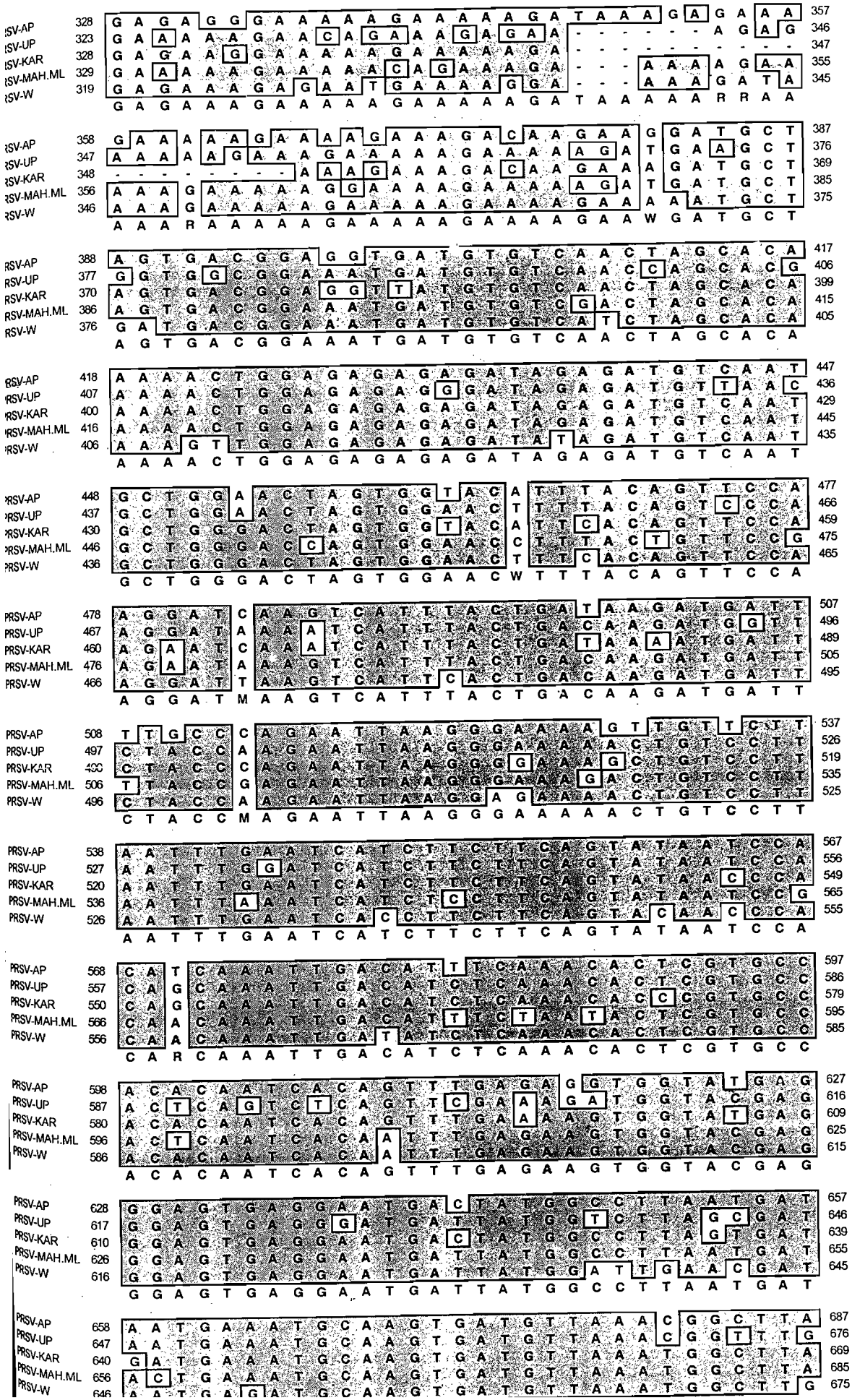
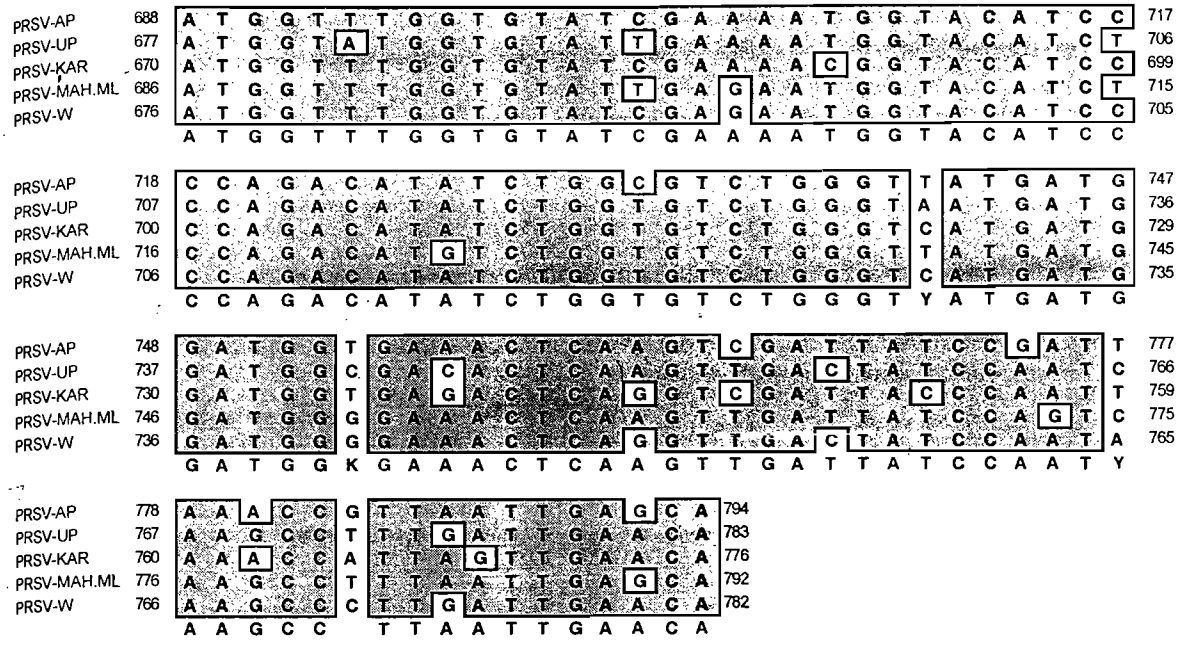


Figure 15. Comparison of PRSV isolates in the nucleotide sequences of a part of NIb and CP genes after pairwise alignment in the MacVector (version 7.0) program.




PRSV-AP 688 A T G G T T T G G T G T A T C G A A A A A T G G T A C A T C C 717
PRSV-UP 677 A T G G T A T G G T G T A T T G A A A A A T G G T A C A T C T 706
PRSV-KAR 670 A T G G T T T G G T G T A T C G A A A A C G G T A C A T C C 699
PRSV-MAH.ML 686 A T G G T T T G G T G T A T T G A G A A T G G T A C A T C T 715
PRSV-W 676 A T G G T T T G G T G T A T C G A G A A T G G T A C A T C C 705
A T G G T T T G G T G T A T C G A A A A T G G T A C A T C C
PRSV-AP 718 C C A G A C A T A T C T G G C G T C T G G G T T A T G A T G 747
PRSV-UP 707 C C A G A C A T A T C T G G T G T C T G G G T A A T G A T G 736
PRSV-KAR 700 C C A G A C A T A T C T G G T G T C T G G G T C A T G A T G 729
PRSV-MAH.ML 716 C C A G A C A T G T C T G G T G T C T G G G T T A T G A T G 745
PRSV-W 706 C C A G A C A T A T C T G G T G T C T G G G T C A T G A T G 735
C C A G A C A T A T C T G G T G T C T G G G T Y A T G A T G
PRSV-AP 748 G A T G G T G A A A C T C A A G T C G A T T A T C C G A T T 777
PRSV-UP 737 G A T G G C G A C A C T C A A G T T G A C T A T C C A A T C 766
PRSV-KAR 730 G A T G G T G A G A C T C A G G T C G A T T A C C C A A T T 759
PRSV-MAH.ML 746 G A T G G G A A A C T C A A G T T G A T T A T C C A G T C 775
PRSV-W 736 G A T G G G A A A C T C A G G T T G A C T A T C C A A T A 765
G A T G G K G A A A C T C A A G T T G A T T A T C C A A T Y
PRSV-AP 778 A A A C C G T T A A T T G A G C A 794
PRSV-UP 767 A A G C C T T T G A T T G A A C A 783
PRSV-KAR 760 A A A C C A T T A G T T G A A C A 776
PRSV-MAH.ML 776 A A G C C T T T A A T T G A G C A 792
PRSV-W 766 A A G C C C T T G A T T G A A C A 782
A A G C C T T A A T T G A A C A

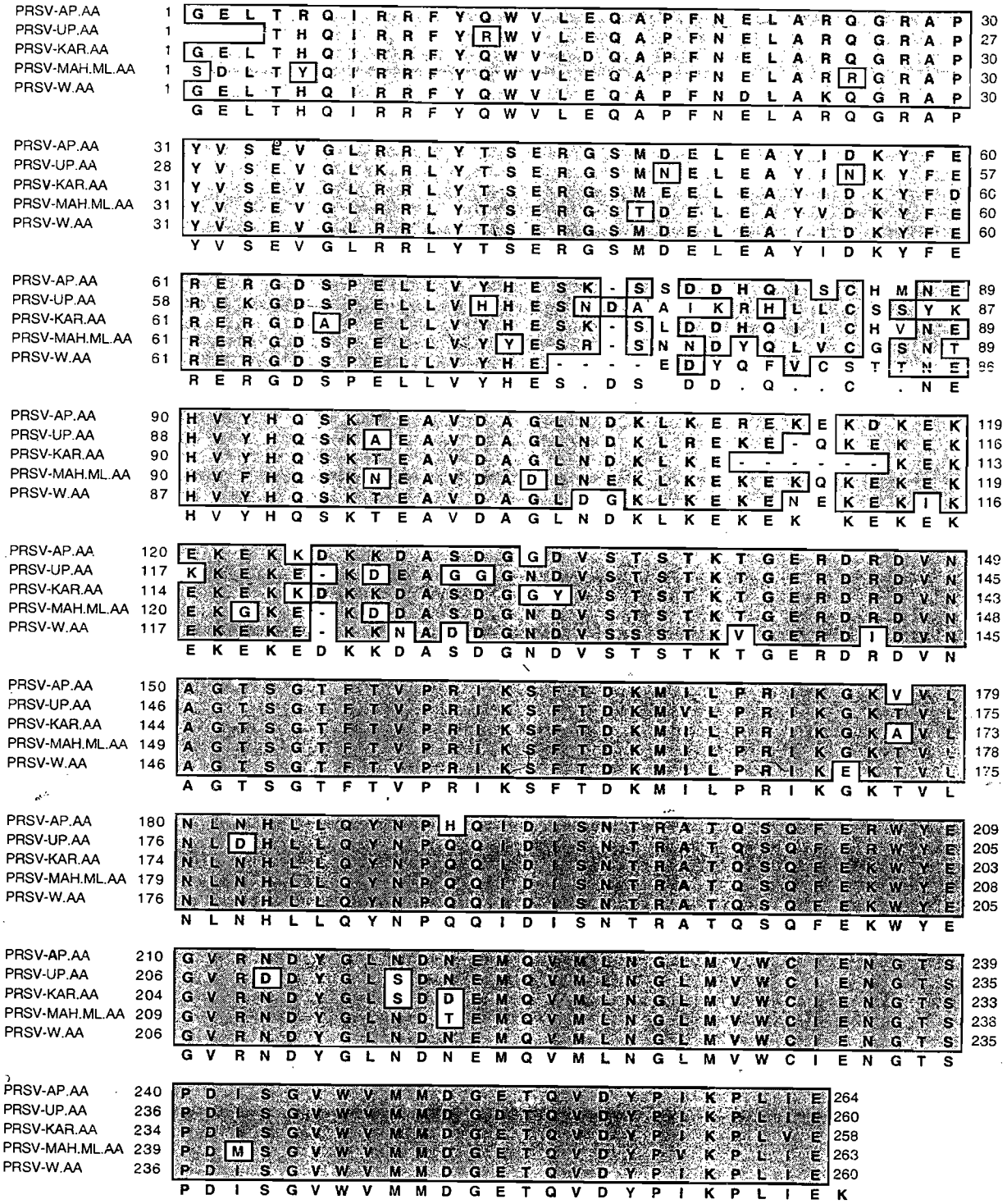


Figure 16. Comparison of PRSV isolates in the amino acid sequences of a part of NIb and CP genes after pairwise alignment in the MacVector (version 7.0) program.

isolates shared 77-91% and 82-92% identity at nucleotide and amino acid levels respectively (Table 19). AP isolate shared highest sequence homology (91% and 92% at nucleotide and amino acid levels respectively) with an isolate from Karnataka forming one cluster (Table 19, Figure 18); whereas, UP isolate shared 78 % and 82% homology at nucleotide and amino acid levels respectively (Table 19).

Comparison of partially sequenced amino terminal regions of capsid protein (CP) gene alone (Table 20; Figure 17) showed that PRSV isolates shared 81-90% and 86-92% identity at nucleotide and amino acid levels respectively (Table 20). AP isolate shared highest homology (90% and 92% at nucleotide and amino acid levels respectively) with an isolate from Karnataka forming one cluster (Figure 18); whereas UP isolate shared 81% and 86% at nucleotide and amino acid levels respectively (Table 20).

When the isolates were compared for the functional NIb protein clevage site located at COOH-terminus of NIb gene, variability was observed (Table 21). The UP isolate was found to show higher sequence divergence from rest of the isolates. The N-terminal CP and functional NIb protein cleavage sites VFHQ/SKNE and VYHE/SNDAA of UP was found to be more variable as compared to others in having one added amino acid, aspartate (D).

Within the N-terminal region of the CP, the PRSV sequences analysed so far have a strech of EK (glutamic acid and lysin) repeats (EK region) starting at the third amino acid after the DAG triplet.

The part of N-terminal CP (7th amino acid from beginning of N-terminus) compared among the PRSV isolates were shown in Figure 19.

Table 19. Percentage identities of total nucleotide (upper half) and amino acid (lower half) sequences (part of NIb and CP) among PRSV-AP, PRSV-UP and other Indian isolates of PRSV, after pairwise alignment of sequences using MacVector (Version 7.0) and ClustalW multiple alignment program respectively. Highest values are within the box and lowest values are underlined

	PRSV-AP	PRSV-UP	PRSV-KAR	PRSV-MAH.1	PRSV.W
PRSV-AP		77*	91*	82*	80*
PRSV-UP	85**		78*	81*	78*
PRSV-KAR	92**	82**		79.5*	79*
PRSV-MAH-1	87**	83**	84**		80*
PRSV-W	87**	83**	84**	84**	

* Nucleotide sequences
** Amino acid sequences

Table 20. Percentage identities of nucleotide (upper half) and amino acid (lower half) sequences of N-terminal part of CP gene among PRSV-AP, PRSV-UP and other Indian isolates of PRSV

	PRSV-AP	PRSV-UP	PRSV-KAR	PRSV-MAH-1	PRSV.W
PRSV-AP		82*	90*	86*	84*
PRSV-UP	87**		81*	84*	83*
PRSV-KAR	92**	86**		82*	84*
PRSV-MAH-1	90**	88**	88**		85*
PRSV-W	89**	87**	87**	89**	

* Nucleotide sequences
** Amino acid sequences

Table 21. Variation of Indian PRSV isolates at the N-terminal part of functional NIb gene

PRSV-AP	V	Y	H	E	/	S	K	-	S	S
PRSV-UP	V	H	H	W	/	S	N	D	A	A
PRSV-KAR	V	Y	H	E	/	S	K	-	S	L
PRSV-MAH-1	V	Y	Y	E	/	S	R	-	S	N
	V	Y	H	E		S	K	*	S	*

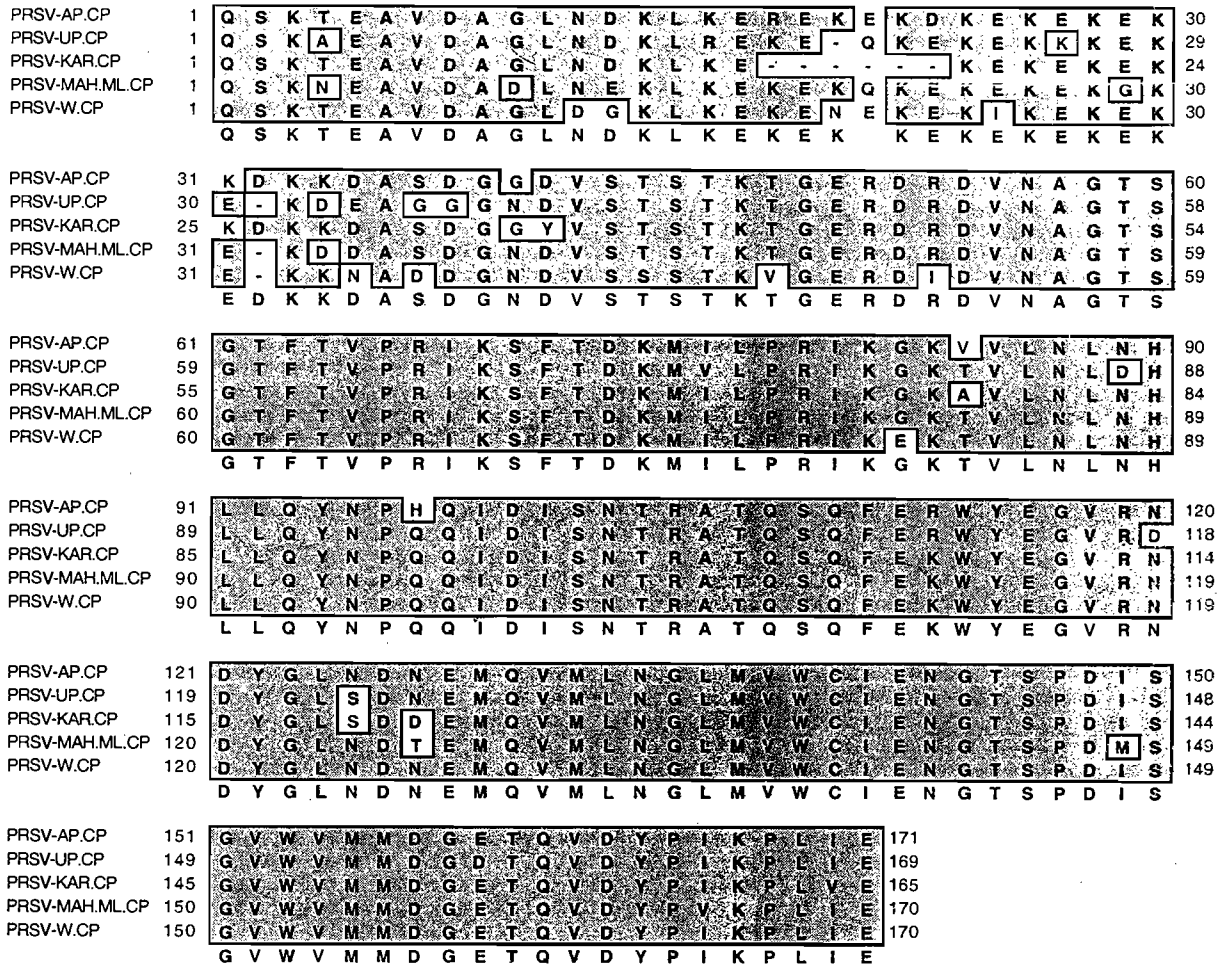


Figure 17. Comparison of PRSV isolates in the amino acid sequences of N-terminal part of CP gene after pairwise alignment in the MacVector (version 7.0) program.

ClustalW (v1.4) Multiple Alignment Parameters:
Open Gap Penalty = 10.0; Extend Gap Penalty = 5.0; Delay Divergent = 40%
Transitions: Weighted

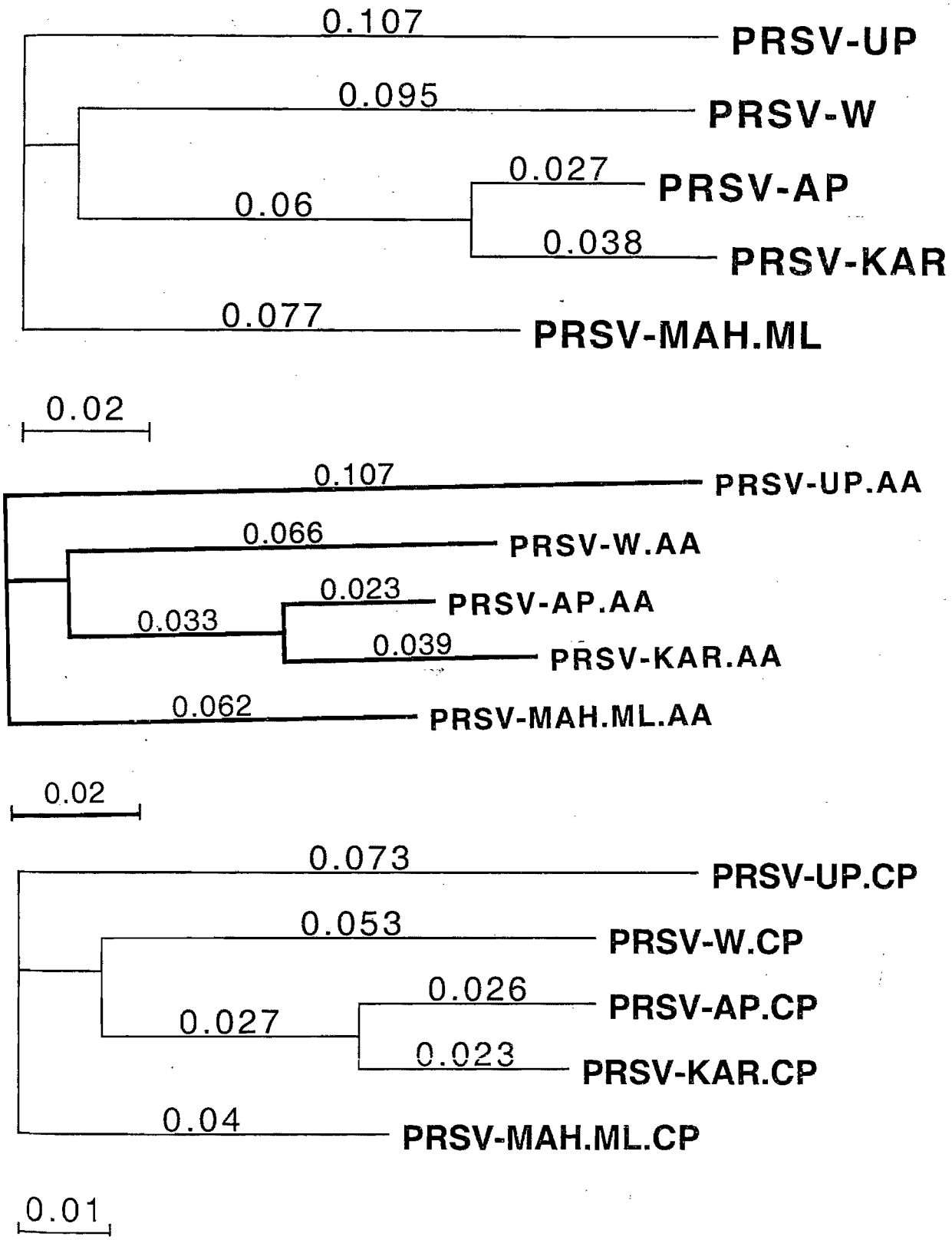


Figure 18. Dendogram of the PRSV isolates for nucleotide sequences of a part of NIb and CP gene (a), amino acid sequences of a part of NIb and CP gene (b) amino acid sequences of N-terminal part of CP gene (c).

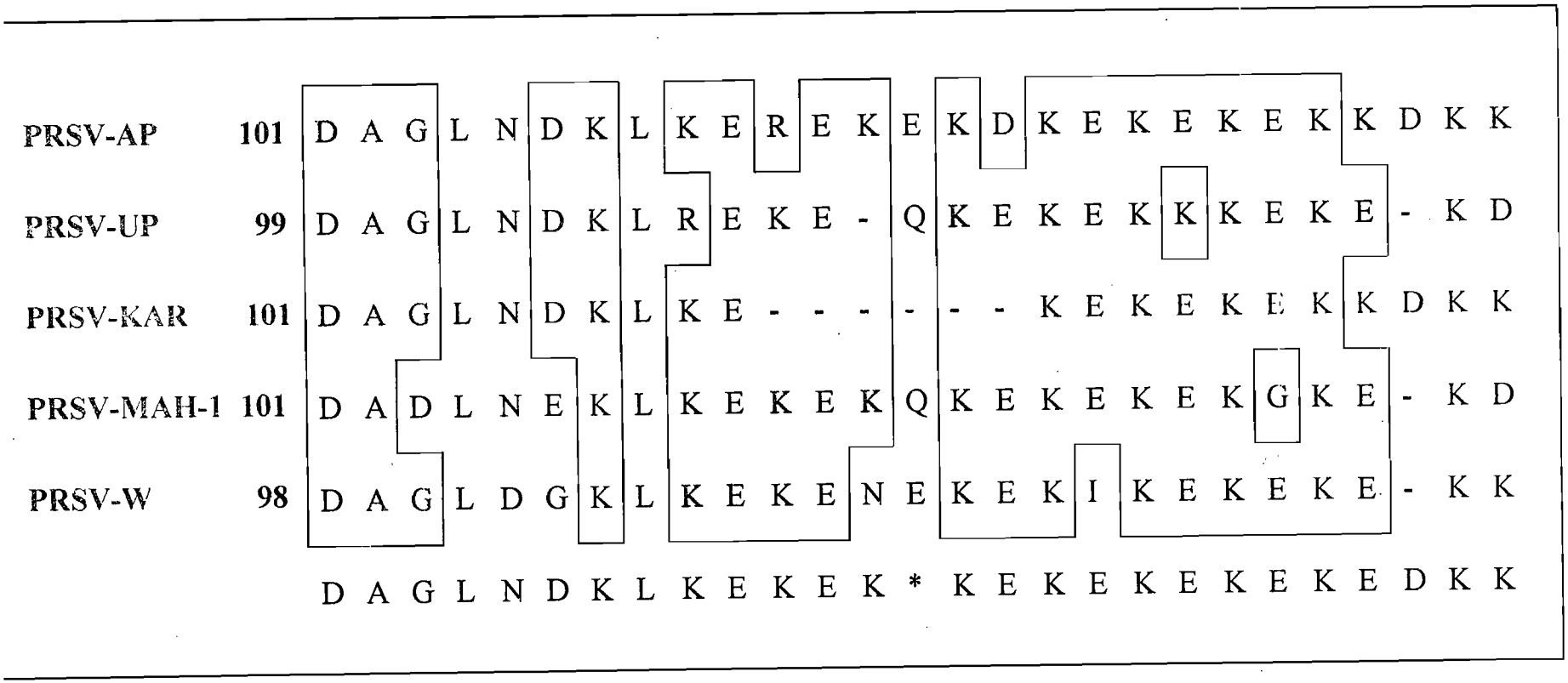
The DAG triplet is present in all the isolates except for PRSV-W. The second box having an DK repeat was identified and repeat consensus KEKEK regions were also observed (3rd and 4th box) with some variability (Figure 19).

4.6 Management through cross-protection

4.6.1 Cross protection tests in glass house

Results of cross-protection effectiveness of mild strain (same as MAH-1) against the severe strain (same as MAH-2) in papaya (cv. Coorg honeydew) under glass house condition are shown in the Table 22a, b; Figures 20. Papaya seedlings preinfected with mild strain (MAH-1) showed only mild mottling symptoms and were ELISA positive; absorbance ranging from 0.71 to 0.76 at 15 days post inoculation. Unprotected plants inoculated with the severe strain (MAH-2) developed severe symptoms ranging from stunting (ST) to leaf distortion (LD) and shoe strinigng (SS) at 10-12 days after inoculation. Complete cross protection was observed only when challenge inoculations were done 15 days after the protective inoculation. These plants did not show severe symptoms until 60 days after challenge inoculation. When challenge inoculations were done 20-40 days after protective inoculation, partial cross protection was observed as some of the protected seedlings gradually showed severe symptoms. For example, 50% of the seedlings challenged at 40 days after protective inoculation were having severe symptoms at 60 days after challenge inoculation.

Effect of multiple challenge inoculations on the effectiveness of cross protection was also examined (Table 22).



igure 19. Glutamic acid and Lysine (EK) repeat patterns in aligned sequences following the DAG triplet at the amino terminus of the PRSV capsid protein from Indian isolates. Numbers indicate respective amino acid position.

Table 22. Evaluation of cross-protection effectiveness of PRSV-mild strain against severe strain in papaya at different time intervals under glass house condition

Days after chalenge inoculation	No. of plants without symptoms, challenged at days after protective inoculation					
	15	20	25	30	35	40
0	6/6	6/6	6/6	6/6	6/6	6/6
10	6/6	6/6	6/6	5/6	4/6	4/6
20	6/6	6/6	6/6	4/6	3/6	3/6
30	6/6	6/6	4/6	4/6	3/6	3/6
40	6/6	5/6	4/6	4/6	3/6	3/6
50	6/6	4/6	4/6	4/6	3/6	3/6
60	6/6	4/6	3/6	3/6	3/6	3/6

(b) Effect of multiple challenge inoculation

Days after challenge inoculation	Number of challenge inoculation				
	6	5	4	3	2
0	0/2*	0/2	0/2	0/2	0/2
10	0/2	0/2	0/2	0/2	2/2
20	0/2	1/2	1/2	1/2	2/2
30	2/2	1/2	2/2	2/2	2/2
40	2/2	2/2	2/2	2/2	2/2
50	2/2	2/2	2/2	2/2	2/2
60	2/2	2/2	2/2	2/2	2/2

* No. of infected plants / total no. of plants

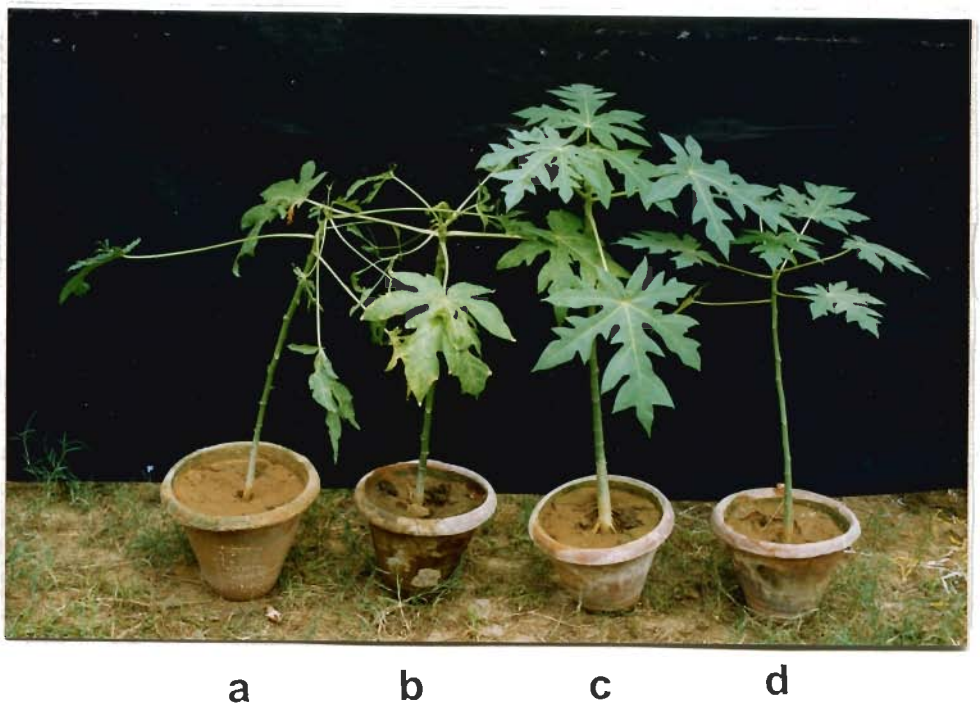


Figure 20. Cross protection effectiveness of papaya ringspot virus MAH-1 (mild strain) against MAH-2 (severe strain) in papaya: papaya (cv. Coorg honeydew) inoculated with severe alone (a); mild- protected plant repeatedly (six times) challenge inoculated (b); cross-protected plant (c) and papaya inoculated with mild alone (d).

When the protected papaya seedling were subjected to multiple challenge inoculation for six times, complete protection was observed upto 20 days. Protection was upto 10 days when the pre-inoculated plants were challenge inoculated thrice with severe strain. 50% of plants got infected after 30-40 days when pre-inoculated plants were subjected to multiple challenge inoculation.

Virus concentration in unprotected, cross protected and pre-infected (mild strain alone) papaya seedlings was monitored throughout the experiment. Irrespective of the time of and after challenge inoculation virus concentration in the unprotected plants was maximum; whereas virus concentration in cross-protected plants and pre-infected plants was almost similar. However, virus concentration in cross protected plants which developed severe symptoms was at par with unprotected plant (Figure 21, 22, 23)

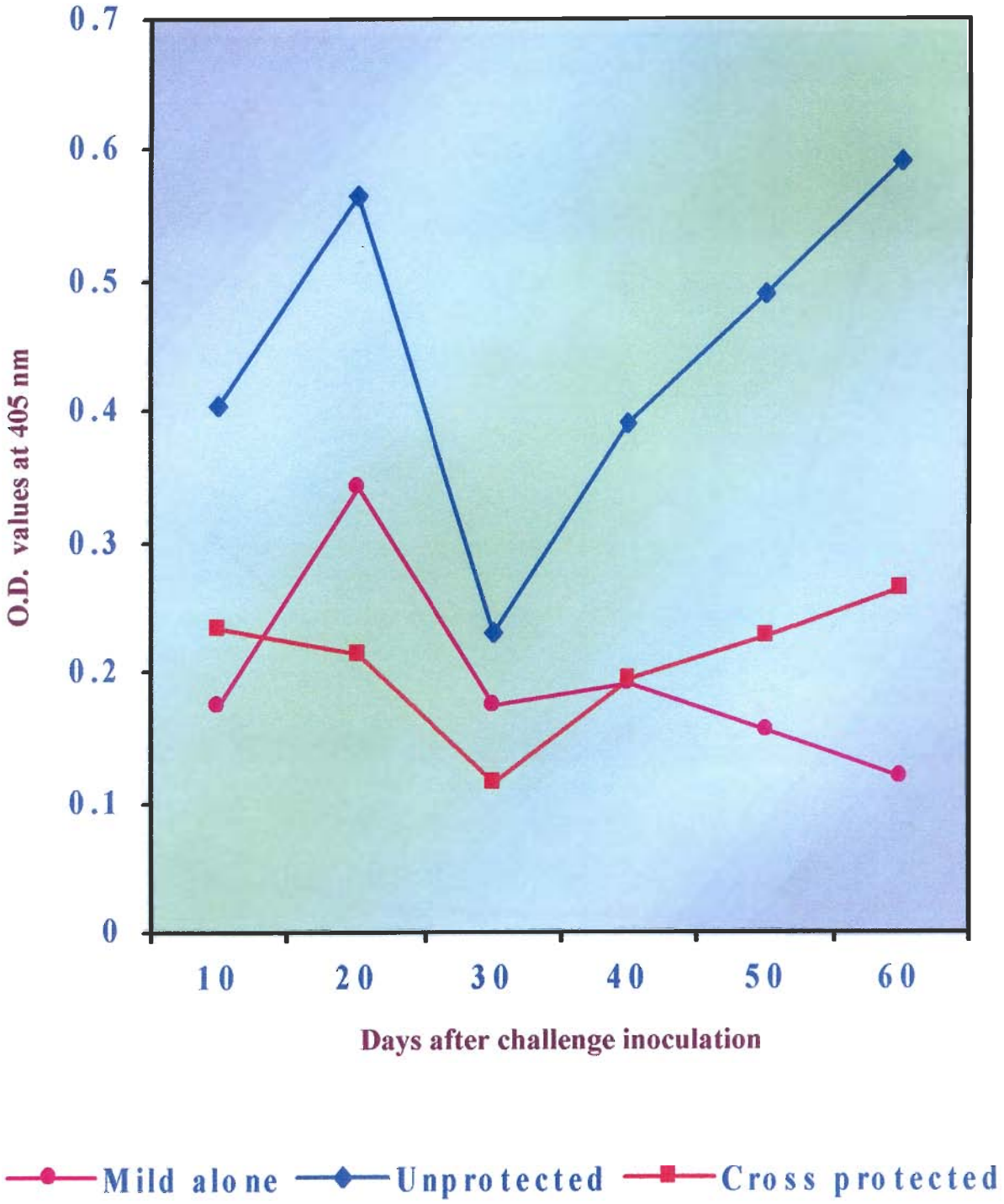


Figure 21 : Cross protection effectiveness of mild strain challenged 15 days after mild inoculation

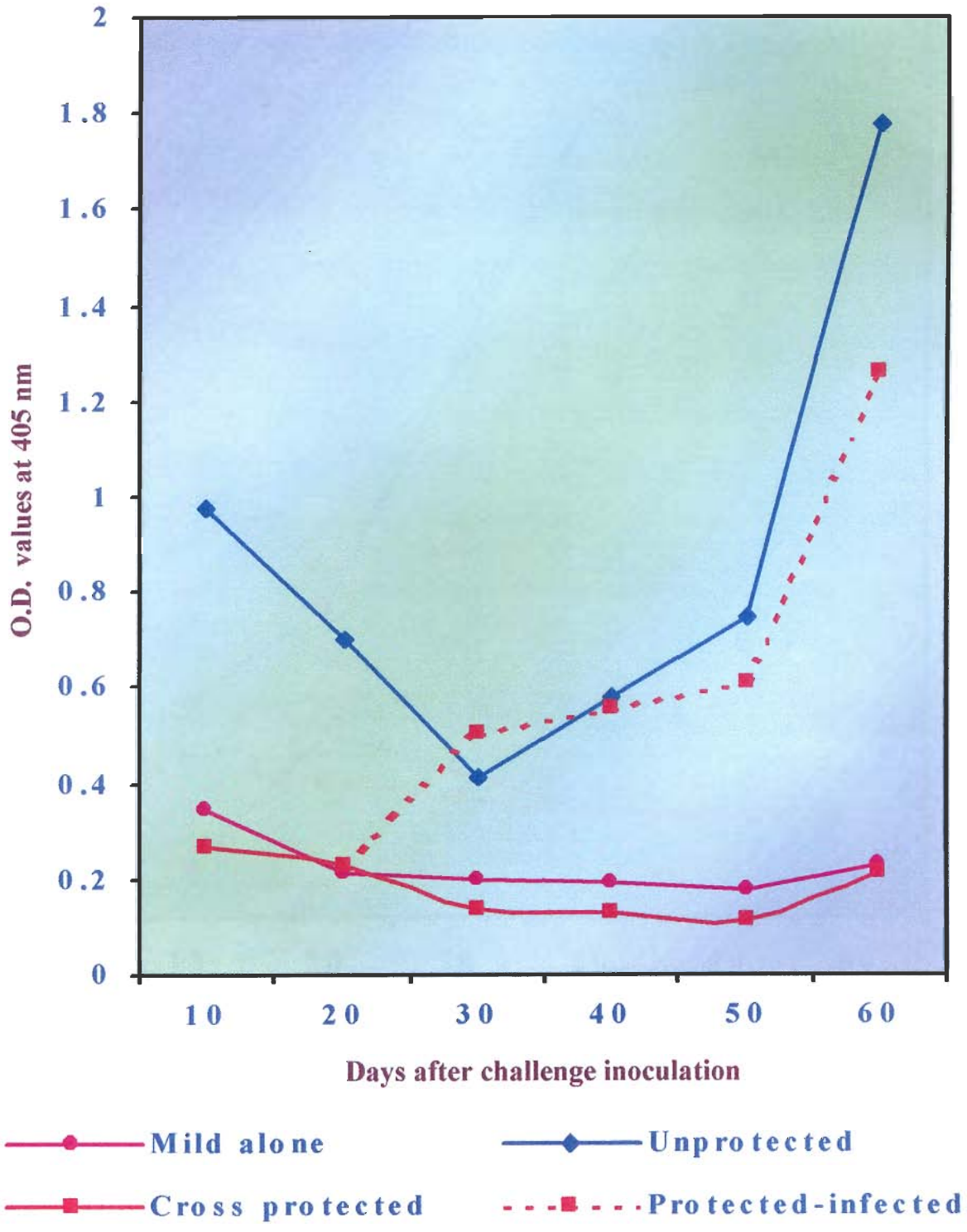


Figure 22 : Cross protection effectiveness of mild strain challenged 25 days after mild inoculation

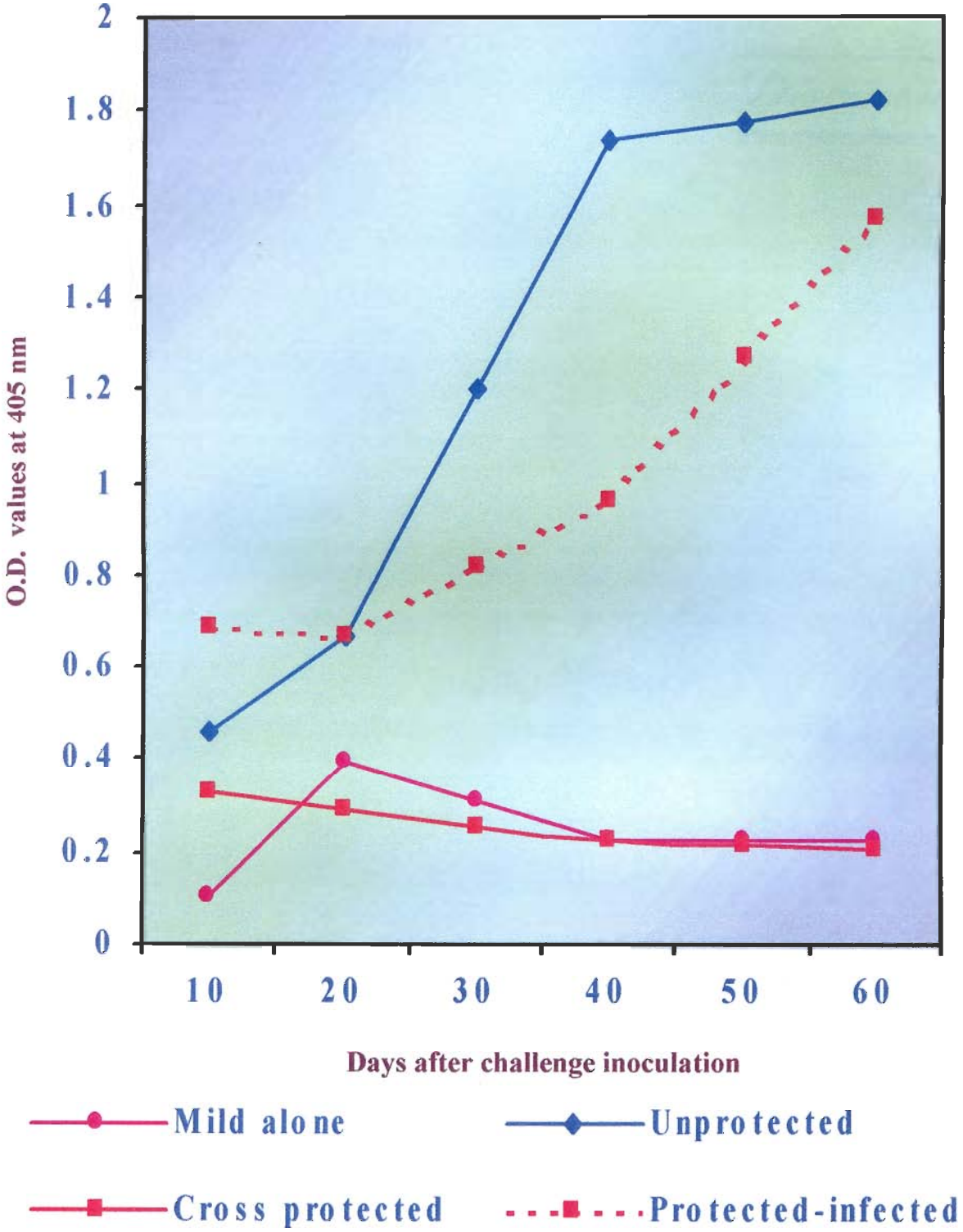


Figure 23 : Cross protection effectiveness of mild strain challenged 35 days after mild inoculation

Discussion

DISCUSSION

Papaya ringspot virus (PRSV), a definitive member of the family '*Potyviridae*' and the genus 'Potyvirus' (Ward and Shukla, 1991; Fauquet, 1999; Regenmortel *et al.*, 2000) is one of the most devastating virus affecting papaya industry in Southeast Asia including Indian sub-continent. The disease incited by the virus is generally characterized by a range of symptoms : mosaic, distortion and shoe-stringing of leaf lamina, water-soaked oily streaks on the petiole, trunk and ringspots on fruits. Even though prevalence of PRSV infection on papaya has been recorded from different geographic regions (Sureka *et al.*, 1977; Yemewar and Mali, 1980; Roy *et al.*, 1999), adequate characterization of PRSV isolates from these regions is lacking. Comparative study on biological, serological and molecular typing of PRSV population is an essential prerequisite for breeding for host resistance. Recently, a naturally occurring mild strain of PRSV has been identified from IARI regional station, Pune and its capsid protein (CP) gene was cloned and sequenced (Jain *et al.*, 1998). The potential of mild strain to contain the disease through cross-protection will have to be judged. The precise aim of the present investigation was thus to evaluate PRSV isolates at biological, serological and molecular levels and to contain the disease through the use of mild strain in cross-protection.

Total of nine different isolates from Andhra Pradesh (AP), Bihar (BH), Delhi (DL), Karnataka (KAR-1, KAR-2), Maharashtra (MAH-1, MAH-2), Uttar Pradesh (UP) and West Bengal (WB) were used for comparison. Host- and ELISA reactions were used as parameters to evaluate biological variability of eight PRSV isolates in papaya cultivars and cucurbits. All the

isolates studied varied in their ability to infect papaya and cucurbits. BH, DL and WB isolates were mainly pathogenic to papaya cultivars, suggesting that their host range is restricted to papaya. While KAR-1, KAR-2, MAH-1 and MAH-2 and UP isolates were pathogenic to both papaya and cucurbits; MAH-1 was strongly pathogenic to papaya, KAR-1 and KAR-2 were strongly pathogenic to cucurbits and MAH-2 and UP were strongly pathogenic to both papaya and cucurbits. Our results suggest that while KAR-1 and KAR-2 can infect both papaya and cucurbits, but they are better adapted on cucurbits in nature. This is supported by the field observation that PRSV has been naturally infecting cucurbits while its infection on papaya has been observed recently (Krishnareddy, M., IIHR, Bangalore, Personal Communication). Further, the biological variations which exist in the PRSV population should be taken into account while devising management strategy. Some biological differences in PRSV-P and W population from widely separated geographic regions of the world have also been observed (Yeh *et al.*, 1984; Baker *et al.*, 1991).

Polyclonal antisera against potyviruses not only react with viruses against which they are produced but also with other distinct viruses (Shukla and Ward, 1989a, b). In the present study also, all the isolates cross-reacted uniformly but strongly with 'P' and weakly with 'W' antisera to capsid proteins in DAC-ELISA and ISEM tests. Thus, the isolates collected from Delhi, Bihar, Karnataka, Maharashtra, Uttar Pradesh and West Bengal are serologically indistinguishable and it would be difficult to segregate them using polyclonal antiserum. This is in confirmation with our previous observation that the isolates from Bihar, Karnataka, Madhya Pradesh, Maharashtra, Sikkim, Tamil Nadu and Uttar Pradesh are serologically

population from widely separated geographical regions of the world, such as USA, Taiwan and Ecuador has been reported to possess similar serological properties (Yeh and Gonsalves, 1984b; Quiot-Douine *et al.*, 1990). Despite the limited study, it can be concluded that PRSV population from different regions is antigenically similar. The fact that capsid protein (CP) genes of Asian, Australian, Mexican and USA isolates shared sequence homology c. 90% or more (Bateson *et al.*, 1994; Jain *et al.*, 1998; Silva-Rosales *et al.*, 2000) clearly supports our observation. However, serological variability among Florida isolates of PRSV-W was detected by using a panel of monoclonal antibodies (MAbs) (Baker *et al.*, 1991). Thus, MAbs will have to be used to ascertain the serological variability in PRSV population. Intensive study is required to correlate biological and serological variations.

Reverse transcription - polymerase chain reaction (RT-PCR) and single strand conformation polymorphism (SSCP) analyses were used to evaluate molecular variability among different PRSV isolates. Amplicons of expected size (c. 800 bp) were obtained in all the isolates except KAR-2 and MAH-2 which gave smaller size products. This is in conformity with an earlier study, where < 800 bp product was obtained for another PRSV isolate from Karnataka using same primers (Bhat *et al.*, 2000). Cloning and sequencing of the amplicons revealed substantial deletion of nucleotides in the amino terminus of CP gene. Similarly, deletion in the CP genes of KAR-2 and MAH-2 isolates might have occurred which could be confirmed by cloning and sequencing.

Unequivocally, nucleotide sequencing is the most reliable procedure for evaluating molecular variability, but it is expensive and impractical for routine purposes. SSCP provides a good alternative and has been used to

detect single base mutations in DNA fragments of 135 to 710 bp (Orita *et al.*, 1989a, b; Prosser, 1993; Enomoto *et al.*, 1994, 2000; Febres *et al.*, 1995; Koenig *et al.*, 1995; Rubio *et al.*, 1996; F d'urso *et al.*, 2000). Under the conditions used in this study, three distinct electrophoretic patterns were observed. Besides, several bands instead of two were seen in some isolates. This could be attributed to either error in nucleotide incorporation during the initial cycles of PCR (Hongyo *et al.*, 1993) or due to the presence of more than one stable conformations for one of the strands (Hayashi, 1991) or to an excess of primer that might bind to the DNA strands and form a detectable band (Cai and Touitou, 1993). Electrophoretic conditions will have to be further standardised to make use of this procedure to differentiate PRSV isolates.

Studies on the sequences divergence have a direct bearing on the management of PRSV either through cross-protection or capsid protein derived resistance (CPDR) as these forms of resistance were highly sequence specific (Shukla and Ward, 1989a; Shukla *et al.*, 1994; Lomonossoff, 1995). The different levels of cross-protection or CPDR conferred by a Hawaiian mild isolate against its severe counterpart or the Taiwan and Thailand isolates (Tennant *et al.*, 1994) supports our view point. Eventually, c. 800 bp 3'- terminal region comprising a part of nuclear inclusion b (NIb) gene and capsid protein (CP) gene of AP and UP isolates were sequenced and compared with other Indian PRSV isolates. The ORF's in both the AP and UP could potentially encode a polyprotein of 264 and 260 amino acids respectively. Both AP and UP isolates were characterised by the presence of a DAG sequence near the amino terminus which is associated with aphid transmissibility (Atreya *et al.*, 1990; 1991).

UP isolate showed higher sequence divergence at carboxyl terminus of NIb gene as compared to AP isolate. The amino terminus of CP gene and predicted functional NIb protein cleavage sites, VFHQ/SKNE (Quemada *et al.*, 1990) and VYHE/SRGTD (Yeh *et al.*, 1992) were more conserved in AP isolate; whereas these were more divergent in UP isolate.

The partially sequenced CP genes of AP and UP isolates were 87% identical at amino acid levels, results confirming their taxonomy as serologically related strains of the same species (Shukla and Ward, 1989a, b). The CP genes of AP and UP isolates, like other potyviruses and their strains (Shukla *et al.*, 1994) also possessed variable amino termini. Unlike alternating repeats of K (Lysine) and E (glutamic acid) observed in the amino termini of CP genes of PRSV isolates (Shukla *et al.*, 1994), EK sequence was replaced by DK or GH in the amino termini of CP genes of AP and UP isolates. The divergence in the amino termini of CPs of AP and UP isolates might account for their differences in host reactions on papaya and cucurbits, as suggested by Xiao *et al.* (1993) for sugarcane mosaic potyvirus. In contrast, the amino termini of CPs of PRSV isolates from Australia and the US, irrespective of their host-specificity, were highly conscerved. Intensive study is needed to correlate molecular and biological differences observed in the study.

Among all the known PRSV isolates, the US and Australian isolates are less divergent (up to 4%) and are more closely related to each other than to Asian isolates (up to 12%) (Jain *et al.*, 1998). Sequence divergence seems to have some relationship with geographical regions. Presently, information on sequence divergence within geographical region is only available for Australia and significant differences were not observed between

the six Australian isolates of PRSV (Bateson *et al.*, 1994). However, higher level of divergence (up to 14%) was observed within Indian isolates including AP and UP isolates from this study. The dendograms indicated that AP isolate originating from South India shared a closer relationship with KAR isolate (another isolate from South India) than UP isolate originating from North India. On the other hand, UP isolate (originating from Northern India) shared a closer relationship with other isolates from India. This might be attributed to the differences in wide range of cropping systems and cultivation practices followed in Northern and Southern part of the country, which might have resulted in different levels of selection pressure on the virus. The sequence divergence will have a direct bearing on the management of PRSV either through cross protection or pathogen derived resistance (PDR). Further studies on the divergence of gene sequences of isolates from Northern and Southern India would help in designing a transgene for engineering an effective CPDR. Besides, it will be possible to develop isolate-specific probes/primers to differentiate the isolates.

Despite the potential risks associated with mild strain protection in cross protection studies (Hammond *et al.*, 1998), cross protection strategy has been applied successfully to control PRSV infection in Taiwan (Yeh and Gonsalves, 1984a; Tennant *et al.*, 1994) by using a mild variant obtained from a severe strain from Hawaii (USA) through nitrous acid mutagenesis. However, in the present study a mild strain (MAH-1) which was isolated as naturally occurring variants directly from plants with mild symptoms in the field from IARI, Regional Station, Pune has been used. It induces infection with mild symptoms both on papaya and cucurbits and has not reverted to the severe form so far. The mild virus strain persisted throughout the

experiment and did not require reinoculation. 100% protection under glasshouse was observed in plants which were challenge inoculated 15th day after protective inoculation and the protection maintained even 60th day after challenge inoculation. However, there was breakdown of protection after 20 days when protected plants were challenged repeatedly (six times). Further, partial protection (50%) was observed when protected plants were challenged 40 days after protective inoculation. Eventhough, complete protection has not been observed for a longer period in the mild-inoculated plants, yet the secondary spread of PRSV from protected plants would be lesser as compared to unprotected plants.

Intensive study is needed to judge the potential of cross-protection in managing PRSV infection. Efforts are needed to compare cross-protection effectiveness against different isolates and to correlate the breakdown of cross-protection with inoculum concentration of severe strains. Besides, there is a need to monitor mild strain in the field or to develop a panel of mild strains from severe strains. These efforts would help to minimise the incidence of breakdown of cross protection.

Summary and Conclusions

SUMMARY AND CONCLUSION

Papaya ringspot virus (PRSV), which is recognised as the most widespread and devastating virus in the world including Indian sub-continent affecting papaya production, is characterized by inciting a range of symptoms on papaya : mosaic, leaf blistering and shoe stringing of leaf lamina, water-soaked oily streaks on the trunk and ringspots on fruits. Based on biological properties, PRSV isolates are classified as either papaya infecting (P) or non-papaya infecting (W) types. Although PRSV-P infection has been recorded in different papaya growing regions of India, biological, serological and molecular typing of the PRSV-P isolates have not been done. Besides, potential of cross protection strategy, which has been successfully used in Taiwan to contain the disease has not been judged. The present investigation which was based on nine different isolates from papaya (Andhra Pradesh - AP; Bihar - BH; Delhi - DL; Karnataka - KAR-1, KAR-2; Maharashtra - MAH-1, MAH-2; Uttar Pradesh - UP and West Bengal - WB) is one step in that direction.

1. PRSV isolates collected from different regions incited their own combinations of symptoms. Symptoms included mild mottling, yellow mosaic, vein clearing, leaf blistering, leaf distortion and shoe stringing. One of the isolates from Maharashtra (MAH-1), which incited only mild mottling, was used as mild strain for cross protection studies in the study.

2. Biological variability among eight PRSV isolates was evaluated by host- and ELISA reactions on different papaya cultivars (5) and

cucurbitaceous plant species (9). Some biological differences in PRSV population under study from different regions was observed as isolates differed in their ability to infect papaya and cucurbits. BH, DL and WB isolates were predominantly restricted to papaya. Of the other isolates infecting both papaya and cucurbits, MAH-1 and KAR-1 and 2 were strongly pathogenic to papaya and cucurbits alone respectively; whereas MAH-2 and UP were strongly pathogenic to both.

3. Serological variability among eight PRSV isolates was evaluated by using polyclonal antisera directed against capsid proteins of PRSV-P and W in DAC-ELISA and ISEM tests. Serological differences in PRSV population under study was not observed as all the isolates cross-reacted uniformly but strongly with 'P' and weakly with 'W' antisera in DAC-ELISA and ISEM. The isolates were serologically indistinguishable and did not show antigenic differences.

4. 3'-terminal region of the viral genome comprising a part of nuclear inclusion b (NIb) and capsid protein (CP) genes from all the nine isolates was amplified using PRSV specific primers through reverse transcription polymerase chain reaction (RT-PCR).

5. Molecular variability among PRSV isolates was observed when amplicons comprising a part of NIb and CP genes were subjected to agarose gel electrophoresis or single-strand conformation polymorphism (SSCP) analyses. KAR-2 and MAH-2 differed from other isolates in amplifying smaller RT-PCR products (less than 800 bp). Further differences in the electrophoretic profiles were observed

when the amplicons from different isolates were subjected to SSCP analyses.

6. 3'-terminal regions of the viral genome comprising a part of nuclear inclusion b (NIb) gene and capsid protein (CP) gene from AP and UP isolates were sequenced and compared. The ORFs of both the AP and UP isolates encoded a polyprotein of 264 and 260 amino acids respectively and were characterized by the presence of DAG triplet near the amino terminus which is associated with aphid transmissibility.

7. The partially sequenced capsid protein (CP) gene of both the AP and UP isolates retained conserved sequences WCIEN and KEKEK repeats in the amino terminus region.

8. The amino terminus of CP gene and the predicted functions NIb protein cleavage sites, VFHQ/SKNE and VYHE/SRGTD were more conserved in AP than UP isolate.

9. Comparison of partially sequenced capsid protein (CP) gene of AP and UP isolates with other PRSV isolates from India revealed that the Indian isolates shared 81-90% and 86-92% sequence identity at nucleotide and amino acid levels respectively. Unlike UP isolate, AP isolate shared highest identity of 90% and 92% at nucleotide and amino acid levels respectively with an isolate from Karnataka, thus forming one cluster. Sequence divergence of 14% was observed within Indian isolates including AP and UP isolates from this study.

10. A naturally occurring mild strain (MAH-1) was used for cross protection studies. Complete protection (100%) under glass house condition was observed till 60 days in the plants challenge inoculated at 15th day after protective inoculation; whereas partial protection (50%) was observed when protected plants were challenged 40 days after protective inoculation. There was breakdown of protection after 20 days when protected plants were challenge inoculated six times.

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Appendix

APPENDIX I

1. Extraction buffer Phosphate buffer, pH 7.0, 0.01 M

Ingredients	Amount (g/l)
Potassium dihydrogen phosphate (KH_2PO_4)	1.362
Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$)	1.781

51.0 ml $Na_2HPO_4.2H_2O$ solution mixed with 49.0 ml KH_2PO_4 solution gives phosphate buffer pH 7.0 and 0.01 M

2. **Enzyme-Linked Immunosorbent Assay (ELISA)**

(i) Coating buffer (carbonate buffer), pH 9.2 :

Ingredients	Volume required		
	1l	2l	3l
Na_2CO_3 (g)	1.59	3.18	4.77
KH_2PO_4 (g)	0.2	0.4	0.6
Na_2HPO_4, $12H_2O$ (g)	2.9	5.8	8.7
KCl (g)	0.2	0.4	0.6

Add distilled water to make desired volume

(iii) Wash buffer (PBS - T) :

Add 0.5 ml of Tween-20 to 1000 ml PBS

(iv) Blocking solution :

Add 5 g of Bovine Serum Albumin (BSA) to 1000 ml coating buffer

(v) Antibody conjugate buffer (PBS-TPO) :

PBS - T	1000 ml
Polyvinyl Pyrrollidone (PVP)	20.00 g
Ovalbumin / Egg albumin	2.0 g

(vi) Substrate buffer (pH 9.8)

Diethanolamine 97 ml

Distilled water 800 ml

The pH adjusted to 9.8 with 1 N HCl (about 67 ml) and made upto 1000 ml with distilled water.

APPENDIX II

I. Antibiotics

Ampicillin — Stock solution (50 mg/ml) of the antibiotic was made in double distilled water, filter sterilized (through 0.22 micron filter) and distributed into 200 µl aliquots and stored at -20^{0}C. It was used at a concentration of 50 µg/ml

II. Electrophoresis reagents

50X TAE	Tris base	242.0 g
	Glacial acetic acid	57.1 ml
	0.5 M EDTA (pH 8.0)	100 ml
	Distilled water to 1 litre	
Loading dye	1% Bromophenol blue	200 µl
	Glycerol	200 µl
	10% SDS	60 µl
	0.5 M EDTA	50 µl
	10 X TAE	60 µl
	Distilled Water	30 µl

III. Media

Luria Agar medium	Bacto-Tryptone	10.0 g
	Bacto-yeast extract	5.0 g
	NaCl	10.0 g
	Agar	215.0 g
	Deionized water	950 ml

pH was adjusted to 7.0 with 5N NaOH and volume made upto 1 litre with deionized water. It was dispensed in 100 ml aliquots in 250 ml flasks and was sterilized by autoclaving for 20 min. at 15 p.s.i.

Luria Broth medium	Tryptone	10.0 g
	Yeast extract	5.0 g
	NaCl	5.0 g
	Deionized water	950 ml

pH was adjusted to 7.0 with 5 N NaOH and volume made upto 1 litre with deionized water. The medium was aliquoted into 50 ml in 250 ml flasks and sterilized by autoclaving for 20 min at 15 p.s.i.

IV Plasmid isolation buffers

Solution I (resuspension buffer)	25 mM Tris HCl (pH 8.0)
	50 mM Glucose
	10 mM EDTA
Solution II (lysis buffer)	0.2 N NaOH
	1% SDS
Solution III (neutralization buffer)	3 M Sodium acetate pH 4.8

V. DNA Molecular weight marker

1 kilobase (1 kb) DNA ladder of MBI Fermentas was used as marker. The ladder is formed by fourteen DNA fragments of 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3.0 kb, 2.5 kb, 2 kb, 1.5 kb, 1 kb, 0.75 kb, 0.5 kb and 0.25 kb.

VI. Composition of buffers used in Qiagen plasmid midi kit

Buffer	Composition
Buffer P1 (Resuspension Buffer)	50 mM Tris, Cl, pH 8.0
	10 mM EDTA
	100 µg/ml RNase A

Buffer P2 (Lysis buffer)	200 mM NaOH, 1% SDS
Buffer P3 (Neutralization Buffer)	3.0 M Potassium acetate, pH 5.5
Buffer QBT (Equilibration Buffer)	750 mM NaCl; 50 mM MOPS, pH 7.0 15% isopropanol 0.15% Triton X-100
Buffer QC (Wash Buffer)	1.0 M NaCl 50 mM MOPS, pH 7.0 15% isopropanol
Buffer QF (Elution Buffer)	1.25 mM NaCl 50 mM Tris. Cl, pH 8.5 15% isopropanol

VII Preparation of comonly used stock solution

Solution	Method of preparation
0.1 M Adenosine triphosphate (ATP)	60.0 mg 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 made upto 1 ml with distiled water. The solution was dispensed into small aliquots and stored at - 70^0C
1 M $CaCl_2$	54.0 g of $CaCl_2.2HO_2$ was dissolved in 200 ml of pure water. (Mill-Q or equivalent). The solution was sterilized by pasing through a 0.22 micron filter and stored in 1 ml aliquots at 4^0C.

0.5 M EDTA (pH 8.0)

1.86 g of ethylenediamine tetra acetic acid disodium sald.$2H_2O$ was added to 800 ml of distilled water, stirred vigorously on a magnetic stirrer, pH was adjusted to 8.0 with NaOH (20.0 g of NaOH pellets). Volume made upto 1 L with distiled water, dispensed into aliquots and sterilized by autoclaving.

Ethidium bromide (10 mg ml^{-1})

1.0 g of ethidium bromide was added to 100 ml of distilled water and stirred on a magnetic stirrer for several hours to ensure that the dye has dissolved. The solution was transferred to a dark bottle and stored at room temperature.

Phenol : Chloroform : isoamyl alcohol

Buffer saturated phenol, chloroform and isoamyl alcohol were mixed in the ratio of 25 : 24 : 1. The equilibrated mixture was stored under a layer of 0.01 M Tris-HCl (pH 7.6) at 4^0C in dark glass bottle.

IPTG (isopropyl-β-D-thiogalactopyranoside)

A solution of IPTG was made by dissolving 2.0 g of IPTG in 8 ml of distilled water. Volume was made upto 10 ml with distilled water and sterilized by filtration through a 0.22 micron disposable filter. The solution was dispensed into 1 ml of aliquots and stored at -20^0C.

1 M $MgCl_2$ — 203.3 g of $MgCl_2.6H_2O$ was dissolved in 800 ml of distilled water. The volume was made upto 1L dispensed into aliquots and sterilized by autoclaving.

10% Sodium dodecyl sulphate (SDS)

100.0 g of electrophoresis grade SDS was dissolved in 900 ml of distilled water, heated at 68^0C to assist dissolution and pH was adjusted to 7.2 by adding few drops of concentrated HCl. The volume was made upto 1L with distiled water, dispensed in aliquots.

10 (N) NaOH — Dissolve 400 g of NaOH in 800 ml of distilled water and make up the volume to 1L with distilled water.

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)

The stock solution was made by dissolving X-gal in dimethyl formamide to make a 20 mg/ml^{-1} solution and stored at -20^0C.

APPENDIX III

Denaturing Solution	Formamide	950 ml
	EDTA	20 mM
	Xylene cyanol	500 mg/lt
	Bromophenol blue	500 mg/lt
	Sterile distilled water to make volume upto 1 lt	
1 X TBE	Tris-Borate	89 mM
10 X TBE (Stock)	EDTA (pH 8.0)	2 mM
	Tris base	108 g
	Boric acid	55 g
	0.5 M EDTA (pH 8.0)	9.2 g
	Double distilled water to make volume upto 1000 ml	
Silver Nitrate Staining		
Fixative-I	Rectified spirit	62.50 ml
	Glacial acetic acid	11.75 ml
	Double distiled water	44.25 ml
Fixtative-II	Rectified spirit	12.5 ml
	Glacial acetic acid	1.18 ml
	Double distilled water	104.82 ml
Silver nitrate	0.21 g dissolved in 100 ml distilled water	
Developer		
Solution-I	Potassium hydroxyde	4.2 g
	Formaldehyde (37%)	0.750 ml
	Double distilled water to make volume upto 100 ml	
Solution-II	0.07 M Sodium carbonate	0.74 g
	Double distilled water to make upto volume upto 100 ml	

Preservative 10% Glacial acetic acid

30% Acrylamide solution, pH 7.0 or less

Acrylamide	29 g
Methylene-bisacrylamide	1 g

Double distilled water to make volume upto 100 ml treated at 37^{0}C to dissolve.

Resolving gel (5%)

10% acrylamide solution (pH 7.0)	6.60 ml
10 X TBE	4.00 ml
TEMED	0.05 ml
10% Ammonium persulphate	0.35 ml
Double distilled water	29.01 ml