Development of Transgenic Papaya and Its Analysis

A THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR

THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

BY

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FEBRUARY 2006

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**"Development of Transgenic Papaya and Its Analysis"** submitted by Neelima Nivrutti Renukdas was carried out under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune.

(Dr. S. K. Rawal)
Research Guide

Date:

DECLARATION

I hereby declare that the thesis entitled " **Development of Transgenic Papaya and Its Analysis**" submitted for Ph.D. degree to the University of Pune has been carried out at National Chemical Laboratory, under the supervision of Dr. S. K. Rawal. The work is original and has not been submitted in part or full by me for any degree or diploma to this or any other University.

Date: (Neelima N. Renukdas)

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Dedicated

To my

Parents …

ACKNOWLEDGEMENTS

It is indeed a great pleasure to write this small note of appreciation. It is a note to thank people around me for the unstinted help, support and encouragement I received from them throughout the course of my studies.

I would like to express my deep sense of gratitude to Dr. S. K. Rawal, my research supervisor, Head, Plant Tissue Culture Division, National Chemical Laboratory, for his guidance, constant encouragement, discussing the difficulties encountered and giving advise how to solve them, and also for giving all the freedom to perform experiments. His support and patience are appreciated.

I am immensely grateful to Dr. S. S. Khuspe, for his constant support, help, and valuable suggestions during my research tenure.

My special thanks are due to Dr. B.M. Khan for his valuable discussions, encouragement, and helpful hints during the work.

Thanks are due to Dr. Mrs. S. R. Thengane, Dr. Mrs S. Hazra, Dr. D.C. Agrawal, Dr. Mrs. Urmil Mehta and Dr. M. M. Jana for their timely help.

It's my pleasure to offer my thanks to Dr. K. B. Bastawade for his encouragement and support throughout the research tennure.

Thanks are due to Dr. Vidya Gupta and Dr. Narendra Kadoo for providing sequencing facility. I am grateful to Dr. M.V. Deshpande and Dr. Vandana for the usage of fluorescence microscope. I am thankful to Dr. P. K. Chitnis for his valuable suggestions.

I would like to thank to Prof. B.B. Chaugule, Botany department, Pune University, for his constant encouragement and help.

I express my heartfelt thanks to my friends and colleagues Dr. Sucheta, Pallavi, Rohini, Manish, Sushim, Arun, Sameer, Abhilash, Noor, Ruby, Ulfat, Malini, Jay, Santosh, Azfar, Vineet, Randheer, Wahida, Aasiya, Neeraja, Rahul and Mrs. Potdar for pleasant work environment and help. I am thankful to Bharti and Nucks for their help. Thanks are also due to Swapna, Shivraj, Sujata, Bhuvan, Shyla, Swheta and Sunil.

I specially thank my project trainee Shweta, Rimita and Shalu for their help, support and hard efforts.

I would like to thank our gardener staff Vinod and Thombare for taking care of my experimental plants.

Thanks are due to lots many individuals who provided papaya fruits from their gardens at the hour of my need.

My special thanks are due to Dr. Mohan for valuable discussions and offering timely help. I am indebted to Dr. Satish for his friendship and constant support. I wish to thank Drs. Madhu, Vijay, Lata, RC and Laxmi for sharing light moments. I would like to thank my friends Neha, Uttara, Asmi, Anjali, Seem, Anish, Milind, Mahesh, Dinesh, Shekhar, Harshali, Nutan, Sheena, Sandhya, Drs. Manju and Noel.

Last but not the least, it is difficult to word my gratitude towards my family for their love, encouragement and strong support. They had to bear with my vacillating moods throughout my studies and in everyday life. I am grateful to Anna, Akka, Aai, Appa, Jijaji, Gauri and Gayatri for supporting me in every possible way and at all times. I specially thank to my sister Tai, brothers Raj and Umesh for their love and care. I am grateful to my niece Sayali and nephews Shreyas, Pranav and Atharv for maintaining cheerful atmosphere and make me laugh when things got tough.

Finally I would like to thank Dr. S. Sivaram, Director, National Chemical Laboratory and Dr. S.K. Rawal Head, Plant Tissue Culture Division for allowing me to submit my findings in the form of a thesis. The financial support in the form of Senior Research Fellowship by C.S.I.R, New Delhi, is duly acknowledged.

Neelima N. Renukdas

Contents

Key to abbreviations

^{0}C	Degree Celsius
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ANOVA	Analysis of variance
B5	Gamborg's medium (1968)
BAP	6-Benzyl amino purine
BSA	Bovine serum albumin
bp	Base pairs
CaMV	Cauliflower mosaic virus
CTAB	Cetyltrimethylammonium bromide
cv.	Cultivars
Dicamba	3,6 Dichloro-2-methoxybenzoic acid
DNA	Deoxy ribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
GA_3	Gibberellic acid
IBA	Indole Butyric acid
IPTG	Isopropyl-β-D-galactoside
Kb	Kilobases
KDa	Kilodaltons
KIN	Kinetin (6-furfuryl amino purine)
LD_{50}	Lethal dose 50
MBG	MS basal media+B5 vitamins+Glycine
MOPS	3-(N-morpholino) propanesulphonic acid
MS	Murashige and Skoog medium (1962)
NAA	α-Naphthaleneacetic acid
O/N	Overnight
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Picloram	4 amino-3, 4,6-trichloropicolinic acid
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
TDZ	Thidiazuron (1-phenyl-3- (1,2,3-thiadiazol-5-yl) urea
UV	Ultraviolet (light)

Papaya (*Carica papaya* L.) a member of the family Caricaceae, is native to tropical America. It is a popular fruit crop of the tropics and subtropics for its ease of cultivation, rapid growth, quick economic returns and adaptation to diverse soils and climates. Papaya (2n=18) is dioecious as well as hermaphroditic, rapidly growing perennial herbaceous plant with hollow stem, usually unbranched and deeply lobed with palmate leaves. It bears fruits throughout the year, which are a rich sources of Vit. A and Vit. C. Both ripe and unripe fruits are rich sources of pectin also. Two enzymes, papain and chymopapain, have been isolated from the plant latex. Consumption of the fruit is reported to aid digestion because of the papain content.

Papaya ringspot virus (PRSV) is a serious threat to papaya cultivation (Purcifull 1972). It has been reported from N. America (Conover1964), Australia, Africa (Kulkarni 1970), Hawaii (Holmes *et al.*1948), the Caribbean (Acuna and Zayas 1939), South-East Asia and Thailand, Vietnam, China, Japan, Philippines and India (Jain *et al.* 198,Yeh *et al.* 1992, Wang *et al.* 1994). The disease derives its name from the striking symptoms that develop on fruit. It is in the form of concentric rings and spots or c-shaped markings, darker green than the background green fruit colour. Vigour of the trees and fruit set are hampered due to the viral attack. Fruit quality, particularly flavour and shape are also adversely affected. The virus is transmitted by aphids. Using conventional methods it is difficult to control the disease. Alternative method is to use short interference RNA (CP-si) and or to isolate and clone gene from the virus and express these in papaya so as to impair virus assembly or replication or movement.

General Introduction Chapter covers introduction of the genus papaya (*Carica papaya* L) and a thorough literature survey on *in vitro* regeneration and transformation studies in papaya. Objectives and Rationale of the present work are also outlined.

Different methodologies employed in entire tissue culture work, histology and *Agrobacterium tumefaciens* mediated genetic transformation. Experiments towards RNA isolation, PCR, Southern hybridization, Cloning strategies for the coat protein gene (~1.0 kb) and Cpsi are embodied in Chapter 2 Materials and Methods.

The Chapter 3 has been split into two sections: I. Somatic embryogenesis and plant regeneration in papaya and II. *In Vitro* induction of multiple shoots and plant regeneration using immature zygotic embryo explants of papaya. Influence of

phytohormones, either alone or in combination, on induction of somatic embryogenesis and multiple shoots from immature and mature embryo axis explants of three different Indian papaya cultivars Honey Dew, Washington and Co-2 cultivars have been described. Influence of ethylene inhibitors viz. Spermidine, Putrescine, ABA and $AgNO_3$ on the maturation of somatic embryos has been discussed. Studies on influence of boron on somatic embryogenesis and morphological aberrations due to higher concentrations and or long exposure to phytohormones used during embryogenesis are described using techniques viz. histology and SEM (Scanning Electron Microscopy).

Establishment of a plant regeneration protocol *via* multiple shoot induction from immature zygotic embryo of papaya using different media combinations, culture conditions in induction and proliferation of multiple shoots using phytohormones TDZ and combination of BAP: NAA has been described. Conditions for elongation, *in vitro* rooting and hardening of plantlets in green house are also mentioned.

Chapter 4 details *Agrobacterium*-mediated transformation of papaya using green fluorescent protein as the screenable marker. *Agrobacterium tumefaciens* mediated transformation and expression of GFP as a screenable marker has been described. The integration of GFP gene in plant tissue has been confirmed by fluorescence microscope study and DNA analysis.

Chapter 5 on deals with **c**loning, sequencing, characterization and sequence analysis of PRSV Coat Protein (CP) gene.

Chapter 6 describes the use of short interfering RNA (CPsi) for Coat Protein gene silencing. PRSV-CPsi was cloned between 35S promoter and g7 terminator (pKOH-CP-si-F) and mobilized into *A. tumefaciens*. pKOH-CP-si-F was introduced into papaya. The integration of CPsi sequence in plant tissue has been confirmed by fluorescence microscope study and DNA analysis.

CHAPTER 1

GENERAL INTRODUCTION

1. Papaya

Papaya (*Carica papaya* L.) is a member of the family Caricaceae. The plant a native to tropical America, is popular in the subtropics for its easy cultivation, rapid growth, quick economic returns, and adaptation to diverse soils and climates (Harkness 1967, Seelig 1970, Campbell 1984). The genus *Carica* contains 22 species of which *C. papaya* (2n=18) is of major economic importance (Litz 1986). *Carica* species have overlapping distributions in the foothills of the Andes in northwestern South America. Papaya latex contains 4 identified proteolytic enzymes (papain, chymopapain A and B, and papaya peptidase A). Papaya (Fig.1.1) is a small, unbranched, and usually dioecious plant, although hermaphroditic sex types occur (Harkness 1967, Seelig 1970, Samson 1986). The melon-like fruits have a sweet taste and agreeable flavor, and are high in vitamins (A, B_1, B_2, C) and minerals (Ca, K, P, Fe), low in sodium, fat, calories, and contain practically no starch (Seelig 1970, Samson 1986). The plant is valued for its fruit and the proteolytic enzymes papain and chymopapain (Medora *et al.* 1979). Unripe fruit may be cooked as a vegetable or used in salads. For successful cultivation, papaya requires moderate temperature coupled with low humidity and adequate soil moisture. Being a shallow rooted crop, the plant can be grown in soils about 45 cm deep. Papaya thrives best in well-drained fertile soil with pH of 6-6.5. It cannot withstand water logged condition, which results in rotting of stem, yellowing and dropping of lower leaves. A well-drained sandy loam soil, rich in nutrients is the best for papaya cultivation (Singh and Dahiya 1982).

This fruit crop suffers heavy commercial losses due to the papaya ring spot virus disease (PRSV) (Purcifull 1972). The disease has been reported from N. America (Conover1964), Australia, Africa (Kulkarni 1970), Hawaii (Holmes *et al.*1948), the Caribbean (Acuna and Zayas 1939) and the South-East Asian countries *e.g.* Thailand, Vietnam, China, Japan, Philippines and India (Yeh *et al.* 1992, Wang *et al.* 1994, Jain *et al.* 1998, 2004). The disease derives its name from the striking symptoms that develop on fruit in the form of concentric rings and spots or c-shaped markings, darker green than the background green fruit colour. Vigour of the trees, fruit set, fruit quality, particularly flavour and shape are hampered due to viral attack. The virus infection spreads by aphids

from plant to plant. Using conventional methods it is difficult to control the disease. The occurrence of the disease has acted as a disincentive to the cultivators and consequently the area under papaya plantation is shrinking every year. Conventionally, papaya is propagated *via* seed. However, *in vitro* clonal propagation of elite plants and their cultivation can increase crop productivity.

1.1 Origin and History

Papaya, a native of tropical America, was introduced in India by the Portuguese during seventeenth century. Presently papaya is grown extensively in Australia, Hawaii, India, SriLanka, Malaya, Myanmar, Taiwan, Peru, Puerto Rico, Florida, Texas, California, South Africa and Kenya. Papaya has now spread to all tropical and subtropical countries.

1.2 Area and Production

Papaya is grown in 80,000 hectares in India with annual production of 1,850,000 tons. Productivity of papaya is the highest among the fruit crops, which has attracted the growers for its commercial exploitation.

1.3 Current status of papaya in India

In India the area under papaya cultivation is 80,000 ha and the annual production is 1.85 million tons (FAO 2004). Papaya is grown commercially in the states of Andhra Pradesh, Tamilnadu, Bihar, Assam, Maharashtra, Gujarat, Uttar Pradesh, Punjab, Haryana, Arunachal Pradesh, Delhi, Jammu and Kashmir, Mizoram, etc. The state wise area under cultivation for the years of 1995-2004 has been presented in Table 1.1. Tables 1.2 and 1.3 present state wise yields of the fruit crop.

Table 1.1 Papaya Major States by Area in India (1995-2004)

Papaya Major States by Area ('000 hectare)									
	1995-96	**1996-97**	**1997-98**	**1998-99**	**1999-00**	**2000-01**	**2001-02**	**2002-03**	**2003-04**
India	60.9	63.0	69.9	67.6	60.5	70.2	73.7	80.0	80.0
Kerala	13.2	14.0	13.0	12.5	12.3	13.2	13.2		
Orissa	14.0	15.1	16.8	17.9	10.4	10.7	10.7		
Assam	7.3	7.5	7.8	7.3	7.4	7.5	7.5		
Karnatka	5.5	5.6	5.6	6.0	6.6	6.3	3.6		
West Bengal	5.5	5.5	5.5	6.0	6.6	6.7	7.2	8.1	8.3
Maharashtra	1.7	1.9	5.4	5.8	5.7	5.7	5.8		
Gujrat	3.2	3.1	3.8	3.9	4.2	4.0	4.4		
Rajasthan	0.4	0.4	0.4	0.4	0.4	0.2			

Table 1.2 State wise papaya production in India (1995 -2004)

Papaya Major States by Production ('000 tons)									
	1995-96	**1996-97**	**1997-98**	**1998-99**	**1999-00**	**2000-01**	**2001-02**	**2002-03**	**2003-04**
India	1329.7	129.3	1618.8	1582.4	1666.2	1767.1	2590.4	1850.0	1850.0
Karnatka	476.1	487.2	274.7	263.8	302.9	293.1	238.1		
West Bengal	166.5	170.5	177.1	199.1	217.9	220.5	241.9	24.0	260.8
Orissa	181.2	195.7	282.8	285.0	199.5	219.7	217.5		
Gujrat	127.5	123.4	160.1	161.4	176.5	154.5	175.1		
Maharashtra	15.1	17.4	43.3	46.6	171.0	171.0	174.4		
Assam	109.6	110.4	113.9	108.5	107.7	109.3	111.8		
Kerala	57.5	61.5	57.0	56.2	55.2	59.7	59.7		
Rajasthan	11.5	11.3	12.8	15.9	19.4	0.4	3.4		

Table 1.3 Papaya major States by Yield in India (1995 -2004)

Papaya Major States by Yield (Kg/ hectare)									
	1995-96	**1996-97**	**1997-98**	**1998-99**	**1999-00**	**2000-01**	**2001-02**	**2002-03**	**2003-04**
India	21826	20624	23159	23408	27540	25170	35150	23130	23130
Karnatka	86564	87000	48947	43673	45698	46524	66139		
Rajasthan	30000	30182	32323	35368	43900	2692	9075		
Gujrat	39748	39496	42132	41552	42163	38604	39796		
West Bengal	30273	31000	32200	33183	33000	32910	33553	31348	31348
Maharashtra	9053	9158	8018	8034	30000	30000	30069		
Orissa	12960	12960	16833	15922	19182	20533	20327		
Assam	15071	14720	14603	14863	14640	14573	14907		
Kerala	4340	4384	4384	4487	4482	4523	4523		

Source: National Horticultural Board, Ministry of Agriculture, Govt. of India and FAO, 2004

1.4 Plant habit

Papaya is a rapidly growing arborescent dioecious plant, but hermaphrodite forms also occur. The grows with a single straight hollow green or deep-purple stem becoming 30-40 cm or more thick at the base and roughened by leaf scars. It is 2-10 m in height; stem is hollow, unbranched and bears a crown of palmately lobed leaves. The leaves emerge directly from the upper part of the stem in a spiral on nearly horizontal petioles 30-105 cm long, hollow, succulent, green or more or less dark purple. Leaf morphology changes during development from single lobed juvenile leaves to palmate leaves of mature plants (Litz 1986). The life of a leaf is about 4 to 6 months. Laticifers occur in all glands through out the plant (Purseglove 1968).

1.4.1 Flowers

Papaya plants are dioecious or hermaphrodite. Flowers can be produced as early as 4 months after germination of seed in both male (Fig.1.1A) and female plants (Fig.1.1B) (Fitch 1995**).** Male flowers are morphologically distinct from female flowers. Male inflorescence is borne in many-flowered panicles of cymes on horizontal or pendent stalks. The flowers are yellowish, 2-4 cm long. The petals are fused into a long tube, have 10 fertile stamens, and a rudimentary, non-functional ovary. Female inflorescence is much shorter only 3-4 cm long and has fewer flowers. Female flowers are larger, usually white or cream in colour, with five free petals. There are no stamens, but a large ovary with 5 fan-shaped stigmas. The flowers of female plants are usually single whereas the inflorescence of the male plants is cymose (Litz 1986). The dioecious condition has developed from the hermaphrodite condition (Litz 1986).

1.4.2 Fruit

Fleshy berry, 7-30 cm long, weighing up to 0.5- 9 kg, Skin thin, smooth, green, turning yellowish or orange when ripe, flesh yellow to reddish-orange, edible, with mild and pleasant flavour. Fruits from bisexual plants are usually cylindrical or pyriform with small seed cavity and thick wall of flesh. In contrast, fruits from female flowers are nearly round or oval and thin-walled. When the fruit is green it is rich in white latex Seeds are many, parietal, attached in 5 rows to interior wall of ovary, spherical about 5 mm in diameter, black or grayish, wrinkled, enclosed in gelatinous sarcotesta formed from the outer integument.

1.5 Importance and use

Papaya leaves have been employed in place of soap for washing delicate fabrics (Seelig 1970). Papain from the white latex of unripe papayas is reputed as a digestive and has medicinal and industrial uses. In food industries papain is used to tenderize meat and clarify beer; has uses in photography, leather, wool and rayon industries; and has value as a remedy in dyspepsia and similar ailments (Seelig 1970, Poulter and Caygill 1985). Fully ripe papaya can be eaten as a dessert fruit. The fruits are beneficial in piles, dyspepsia of liver, spleen and digestive disorders. Carpaine obtained from papaya is

utilized as a diuretic and a heat stimulant. Ripe fruits are used in preparation of jam, jelly, nectar, soft drinks, ice cream and canned as syrup.

1.5.1 Industrial uses

The latex of the papaya plant contains two proteolytic enzymes, papain and chymopapain. It has varied uses in the beverage, food and pharmaceutical industries, in chill-proofing beer, tenderizing meat, drug preparations for digestive ailments and for the treatment of gangrenous wounds. It is also used in bathing hides, degumming silk and softening wool. Because of its papain content, a piece of green papaya can be rubbed on a portion of tough meat to tenderize it. Sometimes a chunk of green papaya is cooked with meat for the same purpose. Papain is in commercial products marketed as meat tenderizers, especially for home use. Papain has many other practical applications. It is used to clarify beer, also to treat wool and silk before dyeing, to de-hair hides before tanning, and it serves as an adjunct in rubber manufacturing (Morton 1977, Duke 1984). It is applied on tuna liver before extraction of the oil, which is thereby made richer in vitamins A and D. It also finds its use into toothpastes, cosmetics and detergents, as well as pharmaceutical preparations to aid digestion. Papain has been employed to treat ulcers, dissolve membranes in diphtheria, and reduce swelling, fever and adhesions after surgery. Papayas are used in fruit processing industry for making pickles, jam, jelly etc.

1.5.2 Medicinal uses

In tropical folk medicine, the fresh latex is smeared on boils, warts and freckles and given as a vermifuge. The unripe fruit is sometimes hazardously ingested to achieve abortion. Seeds, too, may bring on abortion. The root is ground to a paste with salt, diluted with water and given as an enema to induce abortion. The leaf also functions as a vermifuge and as a primitive soap substitute in laundering. Dried leaves have been smoked to relieve asthma or as a tobacco substitute. Papaya latex is used as anthelmintic (dewormer) in livestock (Satrija *et al.* 1994) and also to cure dyspepsia and is externally applied in treatments of burns and scalds (Reed 1976**).** Apart from all these papaya also finds its use in treatment of cancer, constipation, kidney problems, intestinal disorders, ulcers and urology treatments to name a few. The fruits are beneficial in piles, dyspepsia of liver, spleen and digestive disorders. Papaya has a number of anti microbial uses**.** Latex (with a

minimum protein concentration of 138 μg/ml) and root extracts inhibit *Candida albicans*. However, aqueous extracts are not active. Extracts of pulp and seeds show bacteriostatic properties when tested against *Staphylococcus aureua, Escherichia coli, Salmonella typhi, Bacillus subtilis*, and other bacteria *in vitro*.

1.6 Nutritional quality

Papaya is a very refreshing, delicious fruit and is a rich source of vitamins. Papaya contains about 250 i.u. of vitamin A and 85 mg of vitamin C per 100 g pulp. It is also a rich source of calcium and other minerals. Its riboflavin content is 250 mg/100 g of fruit. Papaya contains 90.8% moisture, protein 0.6%, carbohydrates 7.2%, calcium 0.17%, phosphates 0.13% and iron 0.4%.

1.7 Biotic stresses

1.7.1 Virus and virus like diseases

Papaya ringspot virus: It is caused by papaya ringspot virus. The virus is aphid-borne and spreads rapidly in affected areas. This virus is probably the greatest single threat to papaya production in the world. Fruits from infected trees are marred by greasy ringspot patterns, low sugar content.

Papaya Mosaic: It is caused due to papaya mosaic virus. Leaves become small, curled and wrinkled. The diseased leaves show blister like patches of green tissue. The disease is transmitted through sap, graft and several species of aphids.

Papaya leaf curl: It is caused due to tobacco leaf curl virus. Young leaves at the top of the affected plant become curled, twisted and deformed and deep green. Such plants become stunted and bear no fruit. The vector of this disease is white fly. It is graft transmissible but not by sap.

1.7.2 Insect-Pests

Papaya plants were severely affected by insect pests e.g. Aphids, Red spider mites, White Fly, Fruit fly and Papaya webworm. The pests causes necrotic spots or blistered patches on the leaves and cause damage to papaya production.

1.7.3 Fungal diseases

Papaya fruits, stem and leaves were severely affected at all stages of growth by various fungal diseases viz. anthracnose, collar rot, stem rot, damping off, papaya bunchy top, Phytophthora blight, powdery mildew, black spot, stem rot, root rot and Cercospora leaf spot.

1.8 Abiotic stresses

Papaya plantations need to be protected against strong wind damage by providing effective windbreaks. Low temperature adversely affects the growth and yield of the crop resulting in delayed maturity and ripening. The plants are susceptible to frost. It is shallow rooted crop and highly sensitive to water stagnation even for a short time. It affects the yield whereas in extreme cases the plant may even die. Growth is also hampered by dry climate. Dry climate or meager rainfall tends to add to the sweetness of fruit whereas wet climate with heavy rainfall tends to reduce the sweetness. Dry spells causes abortion of floral and fruit structures leading to sterile phases of fruiting (Muthukrishnan and Irulappan 1987).

1.9 Conventional methods for propagation

Papayas have been seed-propagated, despite the fact that uncontrolled pollinations can cause considerable and rapid genetic drift, particularly in dioecious papaya cultivars (Litz 1986). Rouging unwanted male plants from dioecious plantings and all female plants from hermaphrodite populations is necessary. The hermaphrodite varieties produce true to type seeds if pollinations are controlled, although segregation of sex types does occur (2 hermaphrodite: 1 female). The dioecious papaya cultivars are maintained by means of controlled pollination (sib mating), poly crossing and recurrent selection. Although papaya can be propagated by seed, if pollinations and seed productions are carefully controlled, it is inevitable that there will be continuous genetic drift among dioecious cultivars unless the parental lines are maintained indefinitely or if the cultivars itself can be cloned.

1.9.1 Need for nonconventional methods for propagation

Disease and environmental stresses are usually much more important factors limiting food production in the tropics and subtropics. The most serious threats to papaya production have been virus or virus–like diseases that adversely affect plant development, fruit yields and papain production. Resistance to these diseases is low or nonexistent within the species. Rainy weather can also result in serious losses due to fungal root, stem, leaf and fruit rots. Papayas in coastal region and suffer salt damage from intrusion of seawater into growing regions and in irrigated fields from the slow accumulation of salts in the soil. A need exists for an efficient method to improve quality of papaya fruits and resistance to certain diseases. This may be achieved through modern biotechnology tools. This necessitates methods for nonconventional propagation methods.

1.10 Biotechnological approaches to papaya improvement

1.10.1 *In vitro* studies

Among the *in vitro* approaches, papaya regeneration *via* micro-propagation and somatic embryogenesis method have been developed in few exotic varieties. These, however, are not well adopted in Indian climatic condition. Techniques of anther culture, embryo rescue, and protoplast culture have been developed which should have considerable effect on papaya improvement programs.

1.10.2 Callus induction

The earliest report of *in vitro* studies involving papaya was concerned with callus induction from fruit tissues (Krikorian and Steward 1965). Medora *et al.* (1973) initiated callus from excised stem pieces of aseptically germinated seedlings. Arora and Singh (1978a, b, c) studied induction and growth of seedling stem callus. Yie and Liaw (1977) also induced callus from seedling stem segments. Litz *et al.* (1983) compared callus induction from cotyledon lamina explants with that from vein, midvein explants, and obtained optimum callus and growth from midrib explants. De Bruijne *et al.* (1974) obtained the best conditions for callus growth using stem segments. Callus induction and growth was reported by Litz and Conover (1980).

1.10.3 Somatic embryogenesis

Somatic embryogenesis and plantlet regeneration of papaya has been reported from different explants (Litz and Conover 1981a, b, Rajeevan and Pandey 1983, Cheng *et al.* 1987, Drew 1987, Winnaar 1988, Fitch 1990, Fitch and Manshardt 1990, Zou *et al.* 1992, Fitch *et al.* 1993, Hossain *et al.* 1993, Bhattacharya *et al.* 2002, 2003/4, Renukdas *et al.* 2003, Renukdas *et al.* 2006).

De Bruijne *et al.* (1974) successfully induced somatic embryos from the leaf petiole of papaya in a multistep protocol. These authors obtained somatic embryos but were not able to regenerate whole plants. Yie and Liaw (1977), Medhi and Hogan (1979), Jordan *et al.* (1982), Chen *et al.* (1987), Chen (1988a, b), Yamamoto and Tabata (1989) used seedlings as the explant source for somatic embryo induction. Litz and Conover (1981b, 1982, 1983), Fitch and Mansherdt (1990) and Mansherdt and Wenslaff (1989a, b) regenerated somatic embryos from immature zygotic embryos. Somatic embryos were also induced from the hypocotyl tissues by Fitch (1993). Nevertheless, immature zygotic embryos remain the preferred culture explants (Cai *et al.* 1999).

Immature zygotic embryos of many plant species can be induced to undergo somatic embryogenesis and the phytohormone 2,4-D plays a significant role in the process (Ammirato 1983, Suska-Ard *et al.* 1999). This view was further substantiated by Williams and Maheshwaran (1986) when they induced somatic embryogenesis from immature embryos of twenty dicotyledonous species. Likewise, immature ovular tissues are also considered to be a good source of regenerable cultures (Fitch *et. al.* 1990, Castillo *et al.* 1998a).

Cultures of *C. stipulata* peduncles (Litz and Conover 1980), *C. pubescens* hypocotyl (Jordan *et al.* 1982), and *C. x heilbornii* nm. Pentagona ovules (Vega de Rojas and Kitto 1991) are highly embryogenic. Using the somatic embryogenesis protocols of Litz and Conover (1981b, 1982, 1983) and Mansherdt and Wenslaff (1989a, b) as guidelines, Fitch and Mansherdt (1990) produced somatic embryos for transformation studies. All these reports of papaya somatic embryogenesis studies have been summarized in the Table 1.4.

Table 1.4 Studies on somatic embryogenesis of *Carica papaya*

No.	Explant source	Response	Reference
1	Petiole	Embryogenic callus	De Bruijne *et al.* 1974
2	Seedling stem internodes	Somatic embryos	Yie and Liaw 1977
3	Seedling shoot tips, mature shoots, internodes, seedling internodes	Somatic embryos	Medhi and Hogan 1976
4	Seedling stem, cotyledon, root leaf shoot tip	Somatic embryos	Chen *et al.* 1987, Chen 1988a,b
5	Seedling pieces	Callus	Yamamoto *et al.* 1986
6	Seedling pieces	Somatic embryos	Yamamoto and Tabata 1989
7	Immature zygotic embryos	Somatic embryos	Fitch and Mansherdt 1990
8	Immature zygotic embryos	Somatic embryos	Bhattacharya *et al.* 2001, 2002, Renukdas *et al.* 2003/4, Renukdas *et al.* 2006

1.10.4 Shoot tip culture

Medhi and Hogan (1976) reported the regeneration of single plantlets from shoot tips excised from seedlings. Shoot tip and shoot bud (axillary and lateral bud) may also be used as explants for plant regeneration in papaya (Rajeevan and Pandey 1983, Drew and Smith 1986, Winner 1988, Reuveni *et al.*1990, Mondal *et al.* 1990, Lai *et al.* 1998). Yang and Ye (1992) and Hossain *et al.* (1993) reported plant regeneration from the petiole explants of papaya. Yie and Liaw (1977) could establish papaya seedling shoot tips *in vitro* and obtained proliferated growth. Litz and Conover (1977, 1978a) reported a procedure for establishment and culture of excised shoot tips from field grown mature papayas. They found that establishment time and rate of proliferation were both dependent on age of the stock plant, time of year, sex types and presence of bacterial contaminants (Litz and Conover 1981b). Staminate plants responded more rapidly than pistillate ones and had greater proliferation potential.

1.10.5 Organogenesis

In vitro plant regeneration in papaya has been achieved through organogenesis (Medhi and Hogan 1976, Yie and Liaw 1977). Yie and Liaw (1977) first observed adventitious shoots from callus in a culture medium containing MS basal media supplemented with IAA (0.3 μM) and KIN (4.7 μM) or with KIN (9.4 μM) alone. Regeneration of plants

from callus cultures was also obtained from the stem segments (Arora and Singh 1978b). Arora and Singh (1978) reported that transfer of papaya callus was necessary from a callus induction medium to a shoot induction medium. They could regenerate roots from the callus cultured in media supplemented with KIN and NAA. Reports on micropropagation studies have been summarized in the Table 1.5.

Table 1.5 Studies on micropropagation of papaya

No.	Explant source	Method used	Reference
1	Seedling apices	Micropropagation	Drew and Smith 1986
2	Stem section and Shoot tips	Micropropagation	Medhi and Hogan 1976
3	Six month old axillary rooted cuttings or buds	Micropropagation	Drew 1988
4	Six month old shoots	Micropropagation	Miller and Drew 1990
5	Lateral shoot or mainstem bud	Micropropagation	Reuveni *et al.* 1990
6	Shoot apices	Micropropagation	Litz and Conover 1977
7	Shoot apices mature field grown	Micropropagation	Litz and Conover 1978a
8	Shoot apices	Micropropagation	Litz and Conover 1978b
9	Shoot apices, field nursery plants	Micropropagation	Winnar 1988
10	Seedling stem petiole shoot	Micropropagation	Rajeevan and Pandey 1983
11	Field grown lateral shoots	Micropropagation	Rajeevan and Pandey 1986
12	Shoot buds	Micropropagation	Mondal *et al.* 1990
13	Petioles	Micropropagation	Yang and Ye 1992
14	Petioles	Micropropagation	Hossain *et al.* 1993
15	Petioles	Micropropagation	Lai *et al.* 1998

In papaya tissue cultures, most often, combinations of BAP and NAA have been used for multiple shoot induction (Hossain *et al.* 1993, Rajeevan and Pandey 1983). Thidiazuron (TDZ), a substituted phenyl urea used as a defoliant (Yip and Yang 1986), also exhibits cytokinin like activity (Magioli *et al.* 1998). It has been used to induce adventitious shoots in a number of plant species (Eva 1999, Sujatha and Reddy 1998). Comparative study of multiple shoot induction using combination of BAP: NAA and TDZ have been reported (Bhattacharya *et al*. 2003/4).

1.10.6 Anther culture

Litz and Conover (1978a) first used the technique of anther culture in papaya. Interesting results were obtained by culturing anthers from 16 to 20 mm flower buds in liquid MS

medium supplemented with BA (0.5 mg/l), NAA (1.0 mg/l), and charcoal (10 g/l) following a pretreatment of 4^0C for 3 to 4 days. Only a small number of haploid plantlets have been recovered using these techniques. Chromosome counts verified that the regenerated plants have the haploid number (n=9). Successful regeneration of plants by anther culture was also reported by Tsay (1985).

1.10.7 Protoplast isolation and culture

Protoplasts are considered to be the ideal explants for genetic transformation. Protoplast technique is well established for many plants species and is being routinely used for somatic hybridization and direct gene transfer. Litz and Conover (1979), Litz (1984) first reported the large-scale isolation of papaya protoplasts from papaya cotyledons but with low plating efficiency and limited callus induction (Litz 1986a). Litz (1984) reported a method for efficient isolation of protoplasts from cotyledons of seedlings grown under controlled conditions. However, plant regeneration from *Carica* protoplast was successfully obtained by Chen and Chen (1992**).**

1.11 Transformation studies

Genetic transformation study in papaya was first reported by Pang and Sanford (1988) using *Agrobacterium* mediated gene transfer. However, they could not regenerate plants from the transformed tissues. Following their success, Fitch *et al.* (1990) showed that particle bombardment resulted in stable expression of chimeric genes coding for NPTII and GUS in papaya. Further studies, produced virus resistant papaya plants derived from tissues bombarded with the CP gene of papaya Ring Spot Virus (Fitch *et al.* 1992). Development of transgenic papaya through *Agrobacterium*-mediated transformation (Ye *et al.* 1991, Fitch *et al.* 1993, Yang *et al.*1996, Cabrera Ponce *et al.* 1996) and particle bombardment mediated transformation has been reported (Cabrera Ponce *et al.* 1995, Mahon *et al.* 1996, Cai *et al.* 1999). Success in replicase-mediated resistance against PRSV in papaya has also been reported (Chen *et al.* 2001). Available reports on genetic transformation were summarized in the table 1.6.

Table 1.6 Studies on genetic transformation of *Carica papaya* L.

No.	Explant used	Method used	Reference
1	Leaf disc, stem and petiole	*Agrobacterium*	Pang and Sanford 1988
2	Immature zygotic embryo, hypocotyl, embryogenic calli	Particle bombardment	Fitch *et al.* 1990
3	Somatic embryos	*Agrobacterium*	Ye *et al.* 1991
4	Immature zygotic embryo, hypocotyl, embryogenic calli	Particle bombardment	Fitch *et al.* 1992
5	Hypocotyl	*Agrobacterium*	Fitch *et al.* 1993
6	Immature zygotic embryos	Particle bombardment	Cabrera Ponce *et al.* 1995
7	Leaf disc	*Agrobacterium*	Cabrera Ponce *et al.* 1996
8	Petioles	*Agrobacterium*	Yang *et al.* 1996
9	Immature zygotic embryo	*Agrobacterium*	Cheng *et al.* 1996
10	Immature zygotic embryo	Particle bombardment	Mahon *et al.* 1996
11	Immature zygotic embryo	Particle bombardment	Cai *et al.* 1999
12	Roots and hypocotyls	*Agrobacterium*	Chen *et al.* 2001
13	Somatic embryos	Microprojectile bombardment	Lines *et al.* 2002
14	Hypocotyls	Microprojectile bombardment	Zhu *et al.* 2004
15	Immature zygotic embryo	*Agrobacterium*	Our study

Studies regarding isolation, cloning of PRSV gene and short interfering RNA (CPsi) and its review of literature have been described in the respective Chapters 5 and 6.

1.12 Rationale of thesis

The strategy of combating virus diseases in papaya, or in any other crop, requires thorough knowledge of the pathogens involved. As PRSV appear to be the most wide spread viruses of papaya causing serious disease, knowledge of their molecular variation is essential. Plants transformed with the Coat protein (CP) gene of a pathogenic virus, may be virtually immune to infection by the same or a closely related virus or may show delayed or reduced symptom expression. CP mediated resistance has been the most consistent and effective (Beachy *et al.* 1990). Coat protein mediated protection is found to be very successful for viruses with single stranded RNA genome. Coat protein gene mediated virus resistance studies are going on worldwide. Two transgenic cultivars of

papaya, Rainbow and SunUp, resistant to PRSV in Hawaii were commercialized (Gonsalves 1998, Manshardt 1999). Sun Up was derived from transgenic papaya line 55-1 (Tennent *et al.* 2001) a mild mutant of PRSV-HA strain (Yeh and Gonsalves 1984). It shows resistance to a number of isolates from outside Hawaii. Rainbow is a hybrid of SunUp and the non-transgenic cultivar 'Kopoho'. It is, therefore, hemizygous for the CP gene (Mansherdt 1999). Rainbow and the hemizygous plants of line 55-1 are resistant to PRSV isolates from Hawaii that share at least 97% nucleotide identity to the CP transgene but are susceptible to isolates from outside Hawaii that have 89-94% identity to the transgene.

PRSV strains found in India share 87-93% sequence similarity with worldwide reported CP sequences. The PRSV resistant Hawaiian varieties *viz.* SunUp and Rainbow would have to susceptible to the PRSV strains found in India. Hence, there is reason to believe that to introduce virus resistance in Indian papaya cultivars. PRSV Coat protein gene from the Indian biotypes should be used.

Standardization of the rapid and reproducible regeneration protocols for Indian papaya cultivars is a prerequisite. At the time of initiation of this study, very few reports of plant regeneration with papaya cultivars grown in India were published. Most of the work was done with Australian or Hawaiian varieties. During the present study, the three Indian cultivars Honey Dew, Co-2 and Washington were selected for experiments.

The objectives of the present thesis is, therefore, aimed at fulfilling the prerequisites so that Indian cultivars through biotechnological tools could be developed which exhibit resistance to the PRSV strain found in the Subcontinent. Consequently the aims of the present endeavour were:

- To study *in vitro* regeneration of papaya *via* somatic embryogenesis.
- To develop *in vitro* papaya plant *via* organogenesis.
- To study *Agrobacterium* mediated transformation of papaya using marker gene e.g. Green fluorescent protein (GFP).
- To isolate and clone PRSV Coat protein gene.
- To design short interference RNA (CPsi) for the Coat Protein and clone with the objective of silencing the PRSV Coat protein gene.

CHAPTER 2

MATERIALS AND METHODS

The present Chapter describes techniques routinely followed during the course of work. The material and methods specific to a particular experiment are dealt with in details in the respective Chapters.

2.1 Glassware

Glassware used in the experiments, test tubes, glass bottles, petri dishes, Erlenmeyer flasks and pipettes was procured from Borosil (India).

2.1.1 Preparation of Glassware

All glassware was cleaned by initial boiling in a saturated solution of Sodium bicarbonate for 1h followed by washing with tap water. These were then immersed in 30% nitric acid for 30 min, followed by repeated washings with tap water. Washed glassware was further rinsed with distilled water and dried at room temperature or in an oven at 200^0C. Test tubes and Erlynmeyer flasks were plugged with absorbent cotton. Pipettes and petri dishes were wrapped in brown paper, packed in autoclavable polypropylene bag and autoclaved at 121^0C, 15 psi for 1 h.

2.2 Plasticware

Sterile disposable filter sterilization units and petri dishes were procured from "Laxbro", India. Micro-centrifuge tubes and micropipette tips were procured from "Laxbro" and "Tarsons", India.

2.3 Chemicals

All chemicals used were of analytical grade and obtained from Qualigens, S.D fine Chemicals or Hi-Media (India). Molecular biology related chemicals were obtained from Sigma Aldrich Chemical Co. (USA); Promega (USA) and NEB (USA). Growth regulators and antibiotics were obtained from Sigma Aldrich Chemical Co. (USA). Cefotaxime was procured from Russel India Ltd., Bombay, India. Sucrose, glucose, gelling agent and agar-agar were obtained from Qualigens and Hi-Media, India. Bacto-Agar for microbiological work was obtained from DIFCO laboratories (USA).

2.4 Preparation of culture media

Double distilled water was used for preparation of all the culture media**.** After addition of macro- and micronutrients, vitamins, growth regulators and a carbohydrate source, the pH of the media was adjusted to 5.8 with 0.1N NaOH or HCl. Volume was made up and gelling agent was added as required. The medium was steamed to melt the gelling agent.

The medium was dispensed into culture vessels and sterilized by autoclaving. Thermolabile growth regulators and antibiotics were filter sterilized through a 0.22µm pore size membrane and added to autoclaved medium before dispensing. Salts Composition of Murashige and Skoog (1962), White (1963) and Gamborg (1968) media are given in Table 2.1. Organics Composition of MS, White and Gamborg are given in Table 2.2.

Table 2.1 Composition of salts (mg/l) of the basal media

Macro-element	**MS**	**WH**	**B5**
KNO_3	1900	80	2500
NH_4NO_3	1650	-	-
$CaCl_2.2H_2O$	440	-	150
$MgSO_4.7H_2O$	370	737	250
KH_2PO_4	170	-	-
$NaH_2PO_4.H_2O$	-	16.5	150
$(NH_4)_2\ SO_4$	-	-	134

Micro-element	**MS**	**WH**	**B5**
$MnSO_4.\ 4H_2O$	22.3	6.65	-
$MnSO_4.\ H_2O$	-	-	10
$ZnSO_4.7H_2O$	8.6	2.67	2.0
H_3BO_3	6.2	1.5	3.0
KI	0.83	0.75	0.75
$CuSO_4.5H_2O$	0.025	0.001	0.025
$Na_2MoO_4.2H_2O$	0.25	-	0.25
$CoCl_2.6H_2O$	0.025	2.5	0.025
$FeSO_4.7H_2O$	27.8	-	27.8
$Na_2EDTA.2H_2O$	37.3	-	37.2
MoO_3	-	0.0001	-
$Fe_2(So_4)_3$	-	2.5	-

Table 2.2 Composition of Organics (mg/l) in tissue culture basal media

Organics	**MS**	**WH**	**B5**
Thiamine. HCl	0.1	0.1	10
Pyridoxine HCl	0.5	0.1	1.0
Nicotinic acid	0.5	0.5	1.0
Myo-inositiol	100	-	100
Glycine	2.0	3.0	-

MS-Murashige and Skoog (1962); WH-White (1963); B5-Gamborg *et al.* (1968)

2.5 Collection of Plant material

Seeds of papaya cultivars Honey Dew, Washington and Co-2 were procured locally and planted at National Chemical Laboratory, Pune.

2.6 Preparation of plant material

2.6.1 Surface sterilization of fruits and seeds

Immature and ripe fruits of papaya were washed under running tap water and then in a 1% v/v liquid detergent solution (Labolin, India) for 10 min. Fruits were then rinsed with 70% v/v ethyl alcohol for 30s followed by dipping in Savlon (Johnson & Johnson, India) for 30 min. Fruits were cut aseptically and seeds collected to excise the zygotic embryos.

2.6.2 Inoculation

Zygotic embryo explants were inoculated aseptically in the experimental culture media. All experiments were repeated thrice or otherwise as mentioned. The number of explants and replicates used in each experiment has been specified in the Material and Methods section of respective Chapters.

2.7 Statistical analysis

The data were analyzed using ANOVA techniques and treatment means were compared (Chandel 1993, Sokal *et al.* 1973, Snedecor 1967).

2.8 Culture conditions

The cultures were incubated at 25 ± 2^0C in dark or in light (27 μE m^{-2} s^{-1}) and also in continuous light at an intensity of 27 μE m^{-2} s^{-1}.

2.9 Histological studies

The plant and tissue specimens were fixed in 5-10 ml of FAA (Formalin: acetic acid: 70% ethanol; 5: 5: 90) for 48 hours at room temperature for histological examination. Thereafter, the specimens were washed 3-4 times with glass distilled water and dehydrated by passing through a t-butanol series (Sharma and Sharma 1980). The dehydrated samples were embedding in paraffin wax (melting point 58-60^0C) and 10 μm thick sections were cut using a rotary microtome (Reichert-Jung 2050 Supercut, Germany). Specimens were fixed on slides by mild heating, passed through a xylene: alcohol series (Sharma and Sharma 1980) and stained with 1% Heidenhein's hematoxylin (w/v) in distilled water, matured for one month in light) (Hi-Media, India) for one

minute. The slides were counterstained with 1% eosin (aqueous or alcoholic) for two minute and mounted in DPX mountant. The slides were viewed under a microscope and photographed (Docuval, Carl Zeiss, Germany).

2.10 Hardening of the plantlets

In vitro regenerated plantlets were taken out from the agar medium and gently washed under tap water to remove sticking agar. The rooted plantlets were dipped in 1% aqueous solution of Bavistin®, a systemic fungicide (BASF, India) for 10-15 min and then washed with tap water. The treated plantlets were transferred to pots containing a mixture of autoclaved soil and sand (1:1) or soil: sand: compost (1:1:1mixture). The pots were covered with polypropylene bags, kept in a green house and watered at weekly intervals. After 3-4 weeks, the polypropylene bags were removed and the hardened plants transferred to field.

2.11 Scanning electron microscopy

Samples were prefixed in 2% gluteraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.2) for 48 h at room temperature, and washed thrice with 0.2M Sorrenson's sodium phosphate buffer (NaH_2PO_4: Na_2HPO_4 buffer) and the samples were post fixed with 1% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer for 16 h at 4^0C. The samples were passed through distilled water and then dehydrated in a graded acetone series (15 min each in 30%, 50%, 70%, 90% and 100% acetone). The samples were placed in boats, taking care not to allow drying by keeping them submerged in the transfer liquid (100% acetone), and loaded on the critical point dryer (Polaroid, England), which was maintained at 20^0C. The chamber was filled with substitution fluid (liquid CO_2) and allowed to stand for 1 h for impregnation. The substitution fluid was allowed to evaporate by slowly heating the chamber to 36-38^0C. The CO_2 gas was carefully released, the samples removed and mounted on aluminum stubs using double sticky tapes (Bio-Rad, USA) or high conductivity paint (Acheson Colloids Company, England) and sputter coated with gold-palladium (50-100 A^0) (Polar on coating unit E5000, England). Scanning electron microscope was operated at an accelerating voltage of 10 or 20 kV.

2.12 Microbial and Molecular methods

2.12.1 Bacteriological medium

2.12.2.1 YEB medium

Bacto-Yeast Extract	1 g/l
Beef extract	5 g/l
Bacto-peptone	5 g/l
Sucrose	5 g/l
$MgSO_4$	0.5 g/l

pH was adjusted to 7.2

Antibiotics were added after autoclaving.

2.12.2.2 Luria Bertani Broth (LB)

Bacto-tryptone	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l

pH adjusted to 7.0

Antibiotics were added to the medium after autoclaving.

2.12.2.3 Chemicals

Agarose, ampicillin, Tris, IPTG, X-gal, PEG-8000, bovine serum albumin, EDTA were purchased from Sigma Aldrich (USA). Restriction enzymes, T4 DNA ligase, RNase A, lysozyme were obtained from GIBCO-BRL (USA), Promega (USA), and Amersham (UK). Radiolabelled [α- ^{32}P]-dATP was obtained from BARC (India). X-ray films were obtained from Konica (Japan) or Kodak (USA). All other chemicals were of analytical grade and obtained from Hi-Media, Qualigens Fine Chemicals and E.Merck Laboratories (India).

2.12.2.4 Bacterial strains and plasmids

E.coli JM109 and *E.coli* DH5-α (Promega); *E.coli* XL1-blue (Stratagene); *A.tumefaciens* LBA4404 (Kindly provided by Dr. P. Hooykaas); *A. tumefaciens* GV2260, pCAMBIA11 and 32; pBS+ KS+ (Stratagene); 35SCAT and pAM9 (Dr.S.K.Rawal); pBIN35S-mgfp5-

ER (Kindly provided by Dr. Haseloff, UK) pKOH122 and pKOH200 (Kindly provided by Dr. Holmstrom, Högskolan Skövde, Sweden) were used in the present study.

2.12.2.5 Bacterial culture conditions

E. coli cells were grown at 37^{o}C with shaking at 200 rpm in LB medium and *A. tumefaciens* cells were grown at 28^{o}C with shaking (200 rpm) in YEB broth.

2.12.2.6 The *A. tumefaciens* strains and plasmids used are listed as below.

Table 2.3 *Agrobacterium* strain and plasmid used in the present studies

Plasmid/*A. tumefaciens*	Culture conditions
1. pBIN 35S-mgfp5-ER/ *A. tumefaciens* LBA4404	YEB + 50 μg/ml Kanamycin +250 μg/ml Rifampicin
2. pCAMBIA-MCS11/ *A. tumefaciens* GV2260	YEB + 50 μg/ml Kanamycin +250 μg/ml Rifampicin

2.12.2 Mobilization of binary vectors into *A. tumefaciens*

The binary vectors (pBIN m-gfo5-ER or pCAMBIA MCS11) were introduced into *A. tumefaciens* strain LBA4404 or GV2260 using the freeze-thaw method described by An *et al*. (1988). *A. tumefaciens* cells were grown overnight at 28^{0}C in 5 ml of YEB medium containing 250 mg/l Rifampicin. Two milliliters of the overnight grown culture was added to 50 ml of YEB medium containing the antibiotics in a 250 ml Erlenmeyer flask and cultured on a shaker at 200 rpm at 28^{0}C until the growth of OD_{600} of 0.5. The cell suspension was centrifuged at 3000X g for 5 min. at 4^{0}C.The supernatant was discarded and the cell pellet resuspended in 1ml of ice cold 20 mM $CaCl_2$. The cell suspension was dispensed into pre-chilled microfuge tubes in 0.1 ml aliquots. One μg of plasmid DNA was added to the cells. The cells were frozen in liquid nitrogen and allowed to thaw at 37^{0}C. The volume of the cell culture was made up to 1 ml with YEB medium and incubated at 28^{0}C for 2-4 h with gentle shaking. The cells were spun down for 30 sec. and the supernatant discarded. The cells were resuspended in 0.1 ml YEB medium and spread on YEB agar plate containing 250 mg/l Rifampicin and 50 mg/l Kanamycin. The plates were incubated at 28^{0}C for 2-3 days. The transformed colonies were selected and

maintained on YEB agar plates containing 250 mg/l Rifampicin and 50 mg/l Kanamycin and stored at 4^0C or as a glycerol stocks at -70^0C.

2.13 Regeneration media

Papaya explants were cultured either on a medium containing Murashige and Skoog's (MS) basal medium supplemented with 3% sucrose and Picloram (4.14 μM) (hereinafter referred to as P medium) or Murashige and Skoog (MS) salts + Gamborg (B5) vitamins + 2.0mg/l glycine supplemented with sucrose 3% and BAP: NAA (4.44 μM: 0.54 μM) (hereinafter referred to as BN medium). Both the media were solidified with 0.75% agar (Hi-Media, India). The cultures were incubated at 25 ± 2^0C under 16 h photoperiod at a light intensity of 27 $\mu E.m^{-2}\ s^{-1}$ by cool white fluorescent lights.

2.14 Determination of LD_{50} of Kanamycin for papaya plants

Kanamycin, was used, as selective pressure, and its LD_{50} was determined by culturing 20 immature zygotic embryo explants in petri dishes in the P and or BN medium supplemented with various concentrations of Kanamycin (25, 50, 75, 100, 200 mg/l).

2.15 Co-cultivation of explants with *A. tumefaciens*

The immature zygotic embryo explants were infected with *A. tumefaciens* LBA4404 or GV2260 harboring the binary vector. The *A. tumefaciens* cells grown in 5 ml YEB medium for 18 h with antibiotics as mentioned above and then centrifuged at 5000 rpm at RT for 10 min. The pellet was resuspended in 2.5 ml of liquid P medium and or liquid BN medium (See section 2.13). This bacterial suspension was then used for co-cultivation experiments. The explants were dipping in batches of 20 in the *A. tumefaciense* suspension for 30 min. The explants were blotted dry on sterile filter paper and inoculated on P medium and or BN medium. After 72 h. the explants were washed with sterile distilled water, blotted dry on sterile filter paper and inoculated on P medium and or BN medium containing 500 mg/l Cefotaxime for one week. After one week of incubation the explants were transferred to P medium and or BN medium containing 250 mg/l Cefotaxime and 50 mg/l Kanamycin for regeneration of somatic embryogenesis or shoots.

2.16 Regeneration of somatic embryos and multiple shoots

Regenerated somatic embryos were transferred to a P medium and multiple shoots on BN medium containing 125 mg/l Cefotaxime and 50 mg/l Kanamycin and incubated for 2

weeks. The surviving somatic embryos and multiple shoots were scored for green fluorescence.

2.17 Rooting and hardening

The surviving putative transgenic somatic embryos were transferred for maturation to modified MS salts+B5 vit. (MSB) medium supplemented with Spermidine (1 μM). The matured somatic embryos were transferred to MSB medium devoid of any growth regulator and incubated for 4 weeks. The somatic embryos developed roots in this medium. Multiple shoots were rooted in the MSB medium with IBA (14.7 μM). The fully developed plantlets were hardened in green house.

2.18 Fluorescence microscopy

Visualization of GFP fluorescence in plant tissues was achieved using a Leica Wild MPS 32 stereomicroscope (Leitz Wetzlar, Germany) fitted with G filter. The excitation wavelength was 395 nm and emission wavelength 509 nm. Photographs were taken using Leica MPS 32 photoautomat camera and Fujichrome 400 ASA film.

2.19 RNA preparation

2.19.1 Materials

2.19.1.1 Glassware: All glassware was treated overnight with 0.1% diethylpyrocarbonate (DEPC) and then autoclaved at 121^0C, 15 psi for 20 min. This was followed by baking at 300^0C for 4 hours.

2.19.1.2 Plasticware: All plasticware was siliconized to render their surfaces hydrophobic. This was followed by treatment with 0.1% DEPC overnight and subsequent autoclaving.

2.19.2 Working Solutions

All working solutions were prepared in deionized 0.1% DEPC treated and autoclaved water and stored in DEPC treated, autoclaved and baked containers. Some plastic wares such as electrophoresis units made from acrylic were found to react with DEPC. These were treated with 3% hydrogen peroxide overnight and subsequently rinsed extensively with DEPC treated deionized water (Sambrook *et al.* 1989).

2.19.3 Isolation of total RNA from PRSV infected leaf tissues and fruits

Grinding Buffer (GB)

50 mM Tris HCL, pH 8.0

7 mM EDTA, pH 8.0

1 mM $MgCl_2$

5 mM β- mercaptoethanol

0.1% BSA

Lysis Buffer (LB)

50 mM Sodium acetate, pH 5.0

1 mM EDTA, pH 8.0

0.5% SDS

Other solutions and reagents

Distilled phenol containing 0.1% 8-hydroxyquinoline and saturated with DEPC treated water. Chloroform: isoamylalcohol in the ratio of 24:1.

2.19.3.1 Total RNA isolation

About 15g tender infected leaf tissue or ring spots from fruit rinds of papaya was transferred to an ice cold mortar and pestle and ground to a fine powder in liquid nitrogen. Grinding buffer (GB) 15 ml was added to the fine tissue powder, allowed to thaw and filtered through muslin cloth. The filtrate was distributed in COREX glass tubes kept on ice. About 2.5 ml of lysis buffer was added to each tube, mixed vigorously and incubated for 10 minutes at room temperature. An equal volume of phenol was added and the mixture centrifuged at 4^0C, 10,000 rpm for 10 min. The supernatant was collected and an equal volume of chloroform: isoamylalcohol added and the mixture centrifuged. After centrifugation the aqueous phase was collected and 1/10 volume 3M NaOAC (sodium acetate) added. The total nucleic acids were precipitated by addition of an equal volume of isopropanol and incubation at -70^0C overnight. The sample was centrifuged at 4^0C at 8,000 rpm for 10 minutes. The precipitate was washed three times by resuspending in 200 µl of 70% ethanol and centrifugation at 4^0C at 8,000 rpm for 10 minutes. The pellet was vacuum dried, dissolved in 500 µl of DEPC water. An equal volume of 4M Lithium chloride was added and incubated at -70^0C overnight. The samples were centrifuged for 15 min at 4^0C at 10,000 rpm. The pellet was washed with 70% alcohol and centrifuged

for 10 minutes at 4^0C at 10,000 rpm, vacuum dried, dissolved in minimum amount of water and run on a formaldehyde/ formamide agarose gel for visualizing the isolated total RNA.

2.19.4 Separation of RNA in denaturing agarose gels

RNA molecules do not have hydrodynamically equivalent conformations in aqueous solutions and therefore several methods are developed in which molecular weights of RNA could be determined by gel electrophoresis under denaturing conditions (Lehrach *et al.* 1977). Formaldehyde (HCHO), the denaturant used for the electrophoretic analysis of RNAs also require the presence of formamide (Lehrach *et al.* 1977).

Reagents used

Reagent A (gel electrophoresis buffer-10X stock)

0.5 M MOPS pH 7.0

0.01 M EDTA pH 7.5

This solution was kept in the dark at 4^0C.

Reagent B

294 µl reagent A+ 706µl distilled water

Reagent C

89 µl HCHO (37%) + 706 µl formamide

Reagent D (gel loading buffer)

322 µl reagent B

5 mg xylene cyanol

400 mg sucrose

178µl HCHO (37%)

500µl formamide

Reagent E

0.5 mg/ml ethidium bromide

Gel preparation

1X MOPS/EDTA

0.5 g agarose

5 ml Reagent A

36 ml distilled water

The MOPS/EDTA, agarose, Reagent A and water was heated to solubilize the agarose and cooled to 60^0C. Nine ml of 37% HCHO was added making the final concentration of HCHO to 2.2 M. This was mixed well and poured into a gel casting mould.

Sample preparation

The RNA sample was vacuum dried and dissolved by sequentially adding 2.2 µl Reagent B, 4.8 µl Reagent C, 1.0 µl Reagent E and heating at 70^0C for 10 min. The samples were quenched on ice. Reagent E (ethidium bromide) was added for better staining of low amounts of RNA without any significant background (Gong 1992). Reagent D (1.5 µl) was added, mixed well and sample loaded on the gel.

2.19.5 Electrophoresis

Electrophoresis was carried out in 1X MOPS/EDTA. Pre-electrophoresis was done at 60V for 30 min. After loading the samples electrophoresis was carried out at 60V for 1 hour and then at 100V for 1-2 hours. Halfway through the process, the electrophoresis buffer was remixed thoroughly and poured back into the electrophoresis unit. Any ionic imbalance at the electrodes was thereby taken care of. The gel was photographed using a GEL DOC unit (BioRad).

2.19.6 RNA observation

The isolated RNA electrophoresed in denaturing formaldehyde-formamide gels run in MOPS/EDTA buffer showed several fluorescent bands. The RNA integrity was judged by the sharpness and presence of 28S and 18S ribosomal RNA bands visualized on the denaturing RNA gel.

2.19.7 Amplification by RT-PCR

The polymerase chain reaction is used to amplify a segment of DNA that lies between two regions of a known sequence (Sambrook *et al.* 1989). Two oligonucleotides set was used as primers for a series of synthetic reactions that are catalyzed by a thermostable DNA polymerase. These oligonucleotides typically have different sequences that lie on opposite strands of the template DNA and flank the segment of DNA that is to be amplified. The template is first denatured by heating in the presence of a large molar excess of each of the two oligonucleotides and the four dNTPs. The reaction mixture is then allowed to cool down to a temperature that facilitates primers annealing which are then extended with the DNA polymerase.

The cycle of denaturation, annealing and DNA synthesis is repeated many times. The products of one round of amplification serve as templates for the next. Each successive cycle therefore, essentially doubles the amount of the desired DNA product. The major product of this exponential reaction is a segment of the double stranded DNA whose termini are defined by the 5' termini of the oligonucleotide primers and whose length is defined by the distance between the two primers (Sambrook *et al.* 1989). In case of reverse transcriptase PCR, the first strand DNA synthesized by reverse transcriptase using mRNA as the template and oligo-dT primers, is used as template for the second strand synthesis using sequence specific primers. The two strands then undergo the routine exponential amplification reaction.

Primers for the PCR were designed on the basis of the sequence data of the coat protein (CP) gene of PRSV available in databases. Forward primer was designated as PRSV-CP-F and PRSV-CP-R as reverse primer.

Set 1: Forward primers

PRSV-CP-F-I: 5'd(TATGGATCCTCCAAGAATGAAGCT) 3'.

PRSV-CP-F-II: 5'd (TATGGATCCAGTCCAAAAATGAAGCTG) 3'

Reverse primer

PRSV-CP-R-I: 5'd(TATGGATCCTTAGTTGCGCATACC) 3'

Set 2:

PRSV 1-F: 5'd(GATCCATGCTGAGAGGTACATTTCAAGAGAATGTACCTCT CAGTA GCATTTTTTTGCTAGCG) 3'

PRSV1-R: 5'd(AATTCGCTAGCAAAAAAATGCTACTGAGAGGTACATTCTCT TGAAATGTAC CTCTCAGTAGCATG) 3'

2.19.8 RT-PCR Reaction

2.19.8.1 First strand synthesis

Total RNA 2 µg

Oligo-dT primers 25 ng/µl

RNase free water

Keep at 70 ^{o}C for 10 min. And then add following:

dNTPs	2 µl (0.2 mM)
$MgCl_2$	1 µl (1 mM)
Taq Pol buffer 10X	2 µl
Forward primer	1 µl (8 picomoles)
Reverse primer	1 µl (8 picomoles)
RT/TAQ (10 units/µl)	0.5 µl

Water to make volume to 20 /µl.

The First strand synthesis reaction was set up as below and run for 35 cycles:

70^{o}C		95^{o}C	42 ^{o}C	/5 min\	
/ 10min \	4 ^{o}C	/ 30 min		\	4 ^{o}C
	5min				∞

2.19.8.2 Second strand synthesis

Template from First strand synthesis (10ng) 5 µl

dNTPs	2 µl (0.2 mM)
$MgCl_2$	1 µl (1 mM)
Taq pol buffer 10X	2 µl
Forward primer	1 µl (8 picomoles)
Reverse primer	1 µl (8 picomoles)
Taq Polymerase (3 units/µl)	0.3 µl

Water to make up volume to 20 µl

The PCR reaction was set up as below and run for 35 cycles:

95^{o}C	95 ^{o}C		72 ^{o}C	72 ^{o}C	
/ 5min	1min \	50 ^{o}C	/ 2min	6 min \	4 ^{o}C
		30 sec			∞

PCR was carried out for 35 cycles. The reaction products were analyzed by electrophoresis on 1% agarose gel in 1X TAE buffer (40mM Tris–acetate, 0.1mM EDTA, pH-8.0)

The desired band was sliced out of the gel and taken in a pre-weighed eppendorf tube. Equal volume of phenol was added to the sample and vortexed for 15 sec. Aqueous phase was recovered and an equal volume of chloroform: isoamyl alcohol (24:1) added. The mixture was centrifuged at 10,000 rpm for 10 minutes at room temperature. The DNA was precipitated from the aqueous phase by adding 1/10 volume of NaOAC, 2.5 volumes of absolute alcohol and incubation at -20^{0}C overnight. The sample was spun at 10,000 rpm for 10 minutes. The precipitate was washed with 70% alcohol, dried in vacuum and then dissolved in sterile deionized water.

2.19.9 Cloning of the RT-PCR amplified product:

2.19.9.1 Bacterial transformation

The PCR amplicon was cloned into the pGEM-T Easy Vector (Promega, USA). *E.coli* DH5α and or JM109 were used as the host cells.

2.19.9.2 Preparation of competent cells

A single colony of *E.coli* JM 109 or DH5-α was inoculated in 2 ml of LB medium and grown overnight at 37^{0}C. About 500 µl of the overnight grown culture was added to 50 ml of fresh LB medium and grown for 2-3 h. Cells were harvested by centrifugation at 4,000 rpm for 10 minutes at 4^{0}C. The cell pellet was suspended in 20 ml ice cold 100 mM $CaCl_2$ and recentrifuged. The pellet was resuspended in 1ml of 100mM $CaCl_2$. This was then dispensed in 200 µl aliquots to eppendorf tubes and kept at 4^{0}C overnight.

2.19.9.3 Transformation of *E.coli*

The competent *E.coli* JM109/DH5α cells were transformed as described (Sambrook *et al.* 1989). DNA (~50 ng) was added to the competent *E.coli* cells, mixed and kept on ice for 30 min. The cells were then incubated at 42^{o}C for 2 min. To each tube 800 µl of LB broth was added and further incubated at 37^{o}C for 1h. The cells were pelleted by centrifugation and resuspended in 200 µl of LB broth and spread on LB medium plates containing appropriate antibiotic, IPTG (40 µg/ml) and 40 µg/ml X-gal.

2.19.9.4 Screening for recombinants

Positive *E.coli* colonies were picked after blue/white selection. Each isolated white colony was grown in 5ml LB-agar amp^{50} overnight. Plasmid DNA was isolated by the alkaline lysis method (Sambrook *et al.* 1989). Positive transformant was characterized for the insert DNA by restriction digestions.

2.19.9.5 DNA sequencing and sequence analysis

The insert DNA was bidirectionally sequenced using Beckman Coulter CEQ™ 8000 Genetic Analysis System. DNA sequencing reactions were set up using CEQ™ DTCS Quick Start Kit Dye Terminator Cycle sequencing kit. Open Reading Frame (ORF) and restriction analysis was done using pDRAW32 version 1.1.61**.** Sequence comparisons and alignments were performed with the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information). Protein parameters were determined by ExPasy software (http://au.expasy.org).

2.20 PRSV Coat Protein Short interfering sequence (Cpsi)

2.20.1 Mobilization of pKOH-si-F (35SP-F-si-T cassette) in *Agrobacterium*

pKOH-CPsi-F plasmid harboring the CPsi sequence was mobilized into *A. tumefaciense* GV2260 by the freeze-thaw method (See section 2.12.3).

2.21 DNA preparations

2.21.1 Genomic DNA isolation

Solutions

Extraction buffer: 20 mM Na-EDTA (pH 8.0), 100mM Tris-Cl (pH 8.0), 1.4 M NaCl and 2.0% (w/v) CTAB (Cetyltrimethylammonium bromide). Dissolve CTAB by heating to 60^{o}C. Store at 37^{o}C. β-mercaptoethanol to 0.2% just before use.

TE buffer : 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0)

1.5M *NaCl*; *Chloroform*; *Isoamyl alcohol*; *70% Ethanol*

Genomic DNA from papaya was extracted by using the protocol by Lodhi *et al.* (1994). Papaya leaves from control and transformed plants were crushed in liquid nitrogen. This

crushed powder then added to extraction buffer with PVP 10%. The mixture was kept at 55^{o}C for 30-45 min. and then centrifuged for 15 min, 6000 rpm at RT. Half volume of Chloroform:Isoamyl alcohol (24:1) was added and contents centrifuged at 6000 rpm for 15 min. The supernatant was transferred to a fresh tube and half volume of 1.5M NaCl and 2 volumes ethanol added to precipitate DNA, which was spooled out. The DNA was washed with 70% ethanol, vacuum dried and dissolved in 1-2 ml of 10 mM Tris-1mM EDTA, pH 8.0 (TE) buffer at 55°C.

The gDNA obtained by this method was essentially free of RNA. Alternatively, to make the samples RNA free, RNase A (1 mg/ml) was added to the restriction enzyme (RE) digestion reaction mix or RNase A treatment was given separately and the g-DNA was purified by extraction with chloroform.

2.21.2 Plasmid DNA isolation

Solutions:

Solution I: TEG buffer

50mM	Glucose
25mM	Tris-Cl (pH 8.0)
10 mM	EDTA (pH 8.0)

Solution II

0.2N	NaOH
1%	SDS

Solution III

60 ml	5M Potassium acetate (pH 4.8)
11.5 ml	Glacial acetic acid
28.5 ml	Distilled water

Solution IV

13%(v/v)	PEG-8000
1.6M	NaCl

The alkaline lysis method of Sambrook *et al.* (1989) was improvised upon so that samples are processed conveniently for plasmid DNA extraction, with yields of 5-30 μg per 1.5 ml culture depending on the host strain and the plasmid vector. Another feature of this protocol was the use of PEG-8000 for purification, which resulted in precipitation of high quality super-coiled plasmid DNA free of contamination (PRISM^{TM} 1995). This method is recommended for *E.coli* cell lines JM109, XL1 Blue, and MV 1190 and highly recommended for DH5α and HB101.

The bacterial cultures were grown overnight (O/N) at 37°C in LB (Luria Bertani) broth, with appropriate antibiotic. About 1.5 to 3 ml culture was pelleted for 1 min at 4,000 rpm in a micro-centrifuge. The bacterial pellet was resuspended in 200 μL of TEG buffer by pipetting up and down. Solution II (300 μL) was added and mixed by inversion till the solution becomes clear and incubated on ice for 5 min. The above solution was neutralized by adding 150 μL of Solution III, mixed well and incubated on ice for 5 min. The cell debris was removed by centrifuging for 1 min at 10,000 rpm at room temperature. The supernatant was transferred to a clean tube, RNase A to a final concentration of 20 μg mL^{-1} (Sambrook *et al.* 1989) was added and incubated at 37°C for 20 min. To the above solution 600 μL of chloroform was added, mixed for 30 s and centrifuged for 1 min at 10,000 rpm. The upper aqueous layer was transferred to a clean tube. Equal volume of isopropanol was added with mixing and centrifuged immediately for 10 min, 12,000X g at room temperature. The pellet was washed with 70% ethanol and dried under vacuum for 3 min. The dried pellet was dissolved in 40 μL of deionized water and to it 40 μL of solution IV was added. The mixture was incubated on ice for 20 min and the plasmid DNA pelleted out by centrifugation for 15 min, 4°C at 10,000 rpm. The supernatant was aspirated carefully, the pellet washed with 70% ethanol and dried. The dried pellet was resuspended in 20 μL of deionized water and stored at -70°C.

2.21.3 Nucleic Acid Blotting

2.21.3.1 Southern blotting

Solutions

1X TAE: 0.04 M Tris-Acetate (pH 8.0), 0.001 M EDTA (pH 8.0)

20X SSC:	3 M NaCl, 0.3 M Sodium citrate (pH 7.0)
Depurination solution:	0.25 N HCl
Denaturation solution:	1.5 M NaCl, 5 M NaOH
Neutalization solution:	0.5 M Tris-HCl (pH 7.4), 3 M NaCl
Gel loading dye (6X):	0.25% Bromophenol blue in 40% (w/v) sucrose in water

For Southern hybridization (Southern 1975) the DNA samples were electrophoresed in an agarose gel in 1X TAE buffer. It was rinsed with deionized water (DW) and placed in the depurination solution for 10 min. The gel was rinsed in DW and immersed in the denaturation solution for 30 min with gentle shaking. The gel was rinsed with DW, transferred to neutralization solution for 15 min and set up for capillary transfer of DNA to solid membrane support.

A tray was filled with the transfer buffer (20X SSC). A platform was made and covered with a wick made from 2 sheets of Whatman 3MM filter paper saturated with transfer buffer and the gel was placed on it. It was surrounded with Saran Wrap to prevent the transfer buffer being absorbed directly by the paper towels stacked above the membrane. A sheet of Hybond-N^+ membrane (Amersham, UK) of the exact gel size was wetted with deionized water followed by transfer buffer (20X SSC) and placed on the top of the gel. A glass rod was rolled over the membrane to remove any trapped air bubbles. Two pieces of Whatman 3 MM paper wetted with 2X SSC were placed on the membrane. A stack of absorbent paper towels was placed on top of the 3 MM Whatman papers. A glass plate with ~0.5 kg weight was placed on the top of the paper towels. Transfer of DNA was allowed to proceed for 18 h. The membrane was marked for orientation, removed carefully and washed with 6X SSC. The membrane was air dried and baked for 2 h at 80°C to immobilize DNA.

2.21.3.2 Probe Preparation

Random primer labeling

Random primer labeling of the DNA probe was done using the Megaprime DNA labeling system (Amersham, UK). Reaction (50 μL) was set up as follows:

25 ng of DNA (used as probe)	5.0 μL
Primer solution (Random hexanucleotides) (3.5 A_{260} units)	5.0 μL

Above mixture was heated in a boiling water bath for 10 min and cooled to room temperature to facilitate primer annealing.

10X reaction buffer (500 mM Tris-HCl, pH 8.0; 100 mM $MgCl_2$	5.0 µL
10 mM DTT; 0.5 mg mL^{-1} acetylated BSA)	
0.5 mM of dATP, dGTP, dTTP solutions (333 mM Tris-Cl	12.0 µL
pH 8.0; 33.3 mM $MgCl_2$; 10 mM β-Mercaptoethanol)	(4.0 µL each)
[α-^{32}P-dATP (Sp. activity 3000 Ci $mmol^{-1}$)	3.0 µL
Sterilized deionized water	16.0 µL
Exonuclease free Klenow fragment (2 U μL^{-1})	2.0 µL
TOTAL VOLUME	50.0 µL

The reaction was carried out at 37°C for 45 min. The reaction was stopped and probe denatured by incubation in a boiling water bath for 10 min and snap chilling on ice.

2.21.3.4 Hybridization

Solutions

20X SSC:	See Solutions from Southern blotting
Hybridization buffer:	1% BSA; 1.0 mM EDTA, pH 8.0; 0.5 M Sodium phosphate, pH 8.0; 7% SDS
Low stringency wash buffer:	2 X SSC; 0.1% SDS
High stringency wash buffer:	0.2 X SSC; 1% SDS

The blots were prehybridized at 55°C in 30 mL hybridization buffer for 6-8 h in a hybridization incubator (Robin Scientific, USA). The buffer was decanted and fresh buffer along with the denatured radiolabelled probe was added. Hybridization was carried out at 55°C for 14-18 h. The solution was decanted and the membrane washed with low stringency buffer at 57°C for 15 min. A high stringency wash at 57°C for 15 min followed.

2.21.3.5 Autoradiography

The moist blot(s) was wrapped in Saran Wrap and exposed to X-ray film at -70 ^{o}C in a cassette with intensifying screen.

CHAPTER 3

IN VITRO STUDIES

I. SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN PAPAYA

II. INDUCTION OF MULTIPLE SHOOTS AND PLANT REGENERATION IN PAPAYA

I. SOMATIC EMBRYOGENESIS AND WHOLE PLANT REGENERATION IN PAPAYA

3.1 Introduction

Somatic cells of a plant contain all the necessary genetic information to form a complete and functional plant (Merkle *et al.* 1995). Plant cell, tissue and organ culture is based on the concept of totipotency of the plant cell. *In vitro* system provides an alternative for those cell-biological experiments that are difficult to tackle in intact plants. Somatic embryogenesis is a direct approach to plant regeneration from single cells, wherein embryonic cells undergo polarized and highly controlled cell divisions. The embryonic cells resemble meristimatic cells and the behaviour of early-stage somatic embryos is similar to predetermined meristems. The fusion of the gametes is not involved and differentiation of plants is through characteristic developmental patterns not observed in the zygotic embryogenesis (Tisserat *et al.* 1979, Williams and Maheshwaran 1986, Rangaswamy 1986, Zimmerman 1993, Merkle *et al.* 1995).

The initiation of somatic embryogenesis occurs with the termination of the existing gene expression pattern in the explant tissue and its replacement with an embryogenic gene expression program (Merkle *et al.* 1995). Embryogenic cells after many divisions in culture and require growth regulators not only for their entry in mitosis again but also for the determination of the embryogenic state termed as IEDC's (induced embryogenic determined cells). Formation of IEDC's happen in case of indirect embryogenesis (Sharp *et al.* 1980*,* Williams and Maheshwaran 1986). Conversely, direct embryogenesis in culture, proceeds from cells, which are pre determined (PEDC: pre-embryogenic determined cells) for embryogenic development. They require an external stimulus, either in the form of growth regulators or favourable conditions for the induction and development of embryos (Sharp *et al.* 1980*,* Williams and Maheshwaran 1986*,* Carman 1990). The formation of somatic embryos is also dependent on the epigenetic state of the explant (Merkle *et al.* 1990*,* Litz and Gray 1995). Embryogenic and juvenile tissues of plants may be easily coaxed to form embryos in comparison to differentiated vegetative cells (Thorpe 1994).

The coordinated behaviour of the neighbouring cells as morphogenic groups will determine single or multiple cell origin of the somatic embryos (Williams and Maheshwaran 1986). Determined cells may operate singly or in groups to form embryos.

In case of indirect somatic embryogenesis (either through callus or suspension culture), the origin of the embryos is from a clump of embryogenic cells called the proembryonal mass (PEM) (Williams and Maheshwaran 1986). Direct somatic embryogenesis may be of multicellular origin (Haccius 1978) or may arise from a single superficial cell (Konar and Nataraja 1965) or by both of these pathways (Williams and Maheshwaran 1986).

The potentials of somatic embryogenesis are derived from a number of factors that involve high regenerative capacity, the ability to regenerate from single cells in both the gametophyte and the sporophyte tissues, the bipolarity of embryos and compactness and broad metabolic potential of the embryogenic tissue (Janick 1993). Induction of somatic embryogenesis in plants is one of the significant achievements of experimental embryology and biotechnology.

3.2 Review of Literature

Somatic embryogenesis, organogenesis and plantlet regeneration of papaya has been reported from different explants (Litz and Conover 1981, Rajeevan and Pandey 1983, Cheng *et al.* 1987, Drew 1987, Winnaar 1988, Fitch 1990, Fitch and Manshardt 1990, Zou *et al.* 1992, Fitch *et al.* 1993, Hossain *et al.* 1993, Bhattacharya *et al.* 2002, 2003/4, Renukdas *et al.* 2003/4, Renukdas *et al.* 2006). Morphogenesis from petiole, seedling shoot apices and seedling internode callus cultures (De Bruijne *et al.* 1974, Medhi and Hogan, 1976, Arora and Singh, 1978, Kumar *et al.* 1992). Plantlet production from the shoot buds (Mondal *et al.* 1990, 1994) of *Carica papaya* has also been reported.

De Bruijne *et al.* (1974) successfully induced somatic embryos from the leaf petiole of papaya in a multistep protocol. These authors obtained somatic embryos but were not able to regenerate whole plants. Yie and Liaw (1977), Medhi and Hogan (1979), Jordan *et al.* (1982), Chen *et al.* (1987), Chen (1988a, b), Yamamoto and Tabata (1989) used seedlings as the explant source for somatic embryo induction. Litz and Conover (1981b, 1982, 1983), Fitch and Mansherdt (1990) and Mansherdt and Wenslaff (1989 a, b) regenerated somatic embryos from immature zygotic embryos. Somatic embryos were also induced from the hypocotyl tissues by Fitch (1993). Nevertheless, immature zygotic embryos remain the preferred culture explants (Cai *et al.* 1999). Likewise, immature

ovular tissues of papaya are also considered to be a good source of regenerable cultures (Fitch 1990, Castillo *et al.* 1998a).

Immature zygotic embryos of many plant species can be induced to undergo somatic embryogenesis and the phytohormone 2,4-D plays a significant role in the process (Ammirato 1983, Suska-Ard *et al.* 1999). This view was further substantiated by Williams and Maheshwaran (1986) when they induced somatic embryogenesis from immature embryos of twenty dicotyledonous species.

The present endeavour was directed at the *in vitro* induction of somatic embryos in Indian papaya cultivars. The main objective of the study was the development of a rapid and reproducible plant regeneration system using immature zygotic embryo explants of the Indian papaya cultivars HoneyDew, Co-2 and Washington. The present chapter embodies:

A. Influence of phytohormones on the induction of somatic embryogenesis
B. Influence of ethylene antagonists on the maturation of somatic embryos,
C. Influence of boron on somatic embryogenesis and
D. Morphological aberrations of somatic embryogenesis.

3.3 Materials and Methods

3.3.1 ***Explant preparation*****:** Immature (90-115 days post-anthesis, Fig.3.1A) and mature (130-145 days post-anthesis, Fig.3.1B) fruits of *Carica papaya* L. cvs. Honey Dew, Washington and Co-2 were collected from the field and surface sterilized (see section 2.6.1 in Chapter 2). The seeds (Fig.3.2A, B) were excised under aseptic conditions. From these seeds, zygotic embryos (Fig.3.2C) were taken out and used as culture explants.

3.3.2 ***Media and Culture conditions***: Modified MS medium containing (MSB) MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg *et al.* 1968) and 30 g/l sucrose and was further supplemented with growth regulators and gelled with 7.5g/l agar. Phytohormones, Picloram (0.41-62.11 µM), 2,4-D (0.45-90.50 µM), 2,4,5-T (0.39-78.28 µM), Dicamba (0.45-135.75 µM) and combinations of Zeatin (0.46-68.42 µM) and 2,4-5-T (1.96 µM) were used. The cultures were incubated as described in Chapter 2, Section 2.8.

Boron concentration in the basal MSB medium was varied to study its influence on somatic embryogenesis. Boric acid at concentrations of 30, 60, 100, 150, 180, 200, 240, 320, 400, 450, 500 mg/l was incorporated into the embryo induction medium. Embryo induction medium with 6.2 mg/l boric acid served as the control.

The influence of the ethylene antagonists was studied on the maturation of somatic embryos. MSB medium supplemented with Spermidine, ABA, Putrescine or $AgNO_3$ individually (at concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 μM) was used for somatic embryos maturation studies.

3.3.3 ***Experimental design:*** Ten mature and or immature zygotic embryos each in triplicate was cultured on 30 ml of the medium. The experiments were repeated three times.

3.3.4 ***Statistical analyses*****:** The efficiency of somatic embryogenesis was defined as the percentage of explants forming somatic embryos. The data on somatic embryogenesis rate (%) was subjected to statistical analysis of variance (ANOVA) and the treatment means were compared with Student's *t* test (Snedecor *et al.* 1967*,* Sokal and Rohlf 1973*,* Chandel 1993). In the two-way ANOVA the response of the papaya cultivars to different concentrations of growth regulators was tested. The three-way ANOVA was used to study the interactions of the growth regulator, culture condition and the cultivars.

3.3.5 ***Transfer of plantlets to soil****:* Regenerated plantlets were hardened and transferred to soil (See Chapter 2, Section 2.10).

3.3.6 ***Histology:*** Histological studies were carried out as detailed in Section 2.9 Chapter 2 were used.

3.3.7 ***Scanning electron microscopy*** **(SEM):** For SEM tissues were processed and imaged as described in section 2.11 Chapter 2.

3.4 Results and Discussions

3.4.1.A. Influence of phytohormones on the induction of somatic embryogenesis

3.4.1.1 Induction of somatic embryos from immature zygotic embryos

MSB medium supplemented with various phytohormones *viz.* Picloram (0.41-62.11 µM), 2,4-D (0.45-90.50 µM), 2,4,5-T (0.39-78.28 µM), Dicamba (0.45-135.75 µM) individually and combinations of 2,4,5-T: Zeatin (0.46:1.96 µM to 68.42:1.96 µM) were used to study their influence of phytohormones on somatic embryo induction from the immature zygotic embryo explants of papaya.

Globular somatic embryos appeared within 2-4 weeks of culture as round, globular structures (Fig.3.2A, B, Fig.3.3A) loosely attached to the meristimatic region of the immature zygotic embryo. The response was seen in presence of all the phytohormones tested. The response, however, was concentration dependent. The developmental pattern of the somatic embryos was continuous and essentially asynchronous (Fig.3.3B, Fig.3.4A, B). Pale greenish or whitish embryos upon transfer to fresh induction medium produced secondary somatic embryos (SSE) from the apical region of the primary somatic embryos (PSE) (Figs.3.5A-C).

The extent of the somatic embryogenesis response varied with the growth regulator used and its concentration in the medium. Incubation under light or dark conditions also influenced somatic embryogenesis. Picloram (0.41-62.11 µM) showed visually normal somatic embryo induction in all the papaya cultivars and at all the concentrations tested. Picloram at 62.11 µM did not elicite any response from the papaya cultivar Co-2. Somatic embryo induction was observed in presence of 2,4-D (upto 36.19 µM) and 2,4,5-T (upto 39.13 µM). At higher concentrations of 2,4-D and 2,4,5-T the explants turned necrotic. Incorporation of dicamba in the medium elicited a diametrically opposite response as compare to other phytohormones. When Dicamba was used at low concentrations (0.4-4.52 µM) in the medium it induced callus formation. At higher concentrations of Dicamba (22.62-113.12 µM) somatic embryo induction occurred from the apical meristimatic region the immature zygotic embryo explants. The data for the induction of globular stage somatic embryos was scored after 6 weeks of incubation and the results are summarized in Table 3.1.

Table 3.1 Influence of phytohormones and culture conditions on somatic embryogenesis

Phytohormone	Conc. µM	Morphogenetic response in the responding explants					
		Dark Incubation			Light Incubation		
		HD	Wash	Co-2	HD	Wash	Co-2
Picloram	0.41	SE	SE	SE	SE	SE	SE
	2.07	SE	SE	SE	SE	SE	SE
	4.14	SE	SE	SE	SE	SE	SE
	8.28	SE	SE	SE	SE	SE	SE
	20.70	SE	SE	SE	SE	SE	SE
	33.12	SE	SE	SE	SE	SE	SE
	41.41	SE	SE	SE	SE	SE	SE
	62.11	SE	SE	NR	SE	SE	NR
2,4,5-T	0.39	SE	SE	SE	SE	SE	SE
	1.96	SE	SE	SE	SE	SE	SE
	3.91	SE	SE	SE	SE	SE	SE
	7.83	SE	SE	SE	SE	SE	SE
	19.57	SE	SE	SE	SE	SE	SE
	31.31	SE	SE	SE	SE	SE	SE
	39.13	SE	SE	C	NR	SE	C
	58.7	NR	NR	NR	NR	NR	NR
	78.28	NR	NR	NR	NR	NR	NR
2,4-D	0.45	SE	SE	SE	SE	SE	SE
	2.26	SE	SE	SE	SE	SE	SE
	4.52	SE	SE	SE	SE	SE	SE
	9.05	SE	SE	SE	SE	SE	SE
	22.62	SE	SE	SE	SE	SE	SE
	36.19	SE	SE	SE	SE	SE	SE
	45.25	C	SE	C	C	C	C
	67.87	C	C	C	C	C	C
	90.50	NR	NR	NR	NR	NR	NR
Zeatin+2,4,5-T	0.46+ 1.96	SE	SE	SE	SE	SE	SE
	2.28+1.96	SE	SE	SE	SE	SE	SE
	4.56+1.96	SE	SE	SE	SE	SE	SE
	9.12+1.96	SE	SE	SE	SE	SE	SE
	22.81+1.96	SE	SE	SE	SE	SE	SE
	45.62+1.96	SE	SE	C	C	C	C
	68.42+1.96	C	C	NR	C	C	NR
Dicamba	0.45	NR	NR	NR	NR	NR	NR
	2.26	NR	NR	NR	NR	NR	NR
	4.52	C	C	C	C	C	C
	9.05	SE	SE	SE	C	SE	SE
	22.62	SE	SE	SE	SE	SE	SE
	45.25	SE	SE	SE	SE	SE	SE
	67.87	SE	SE	SE	SE	SE	SE
	90.50	SE	SE	SE	SE	SE	SE
	113.12	SE	SE	SE	SE	SE	SE
	135.75	SE+C	SE	C	SE+C	SE+C	C

SE: globular somatic embryo, C: callus, NR: No response, HD: Honey Dew, Wash: Washington.

Plant genotype is a critical factor for induction of somatic embryogenesis (Rangaswamy 1986, Thorpe 1988, Parrott *et al.* 1995). Hence, screening of the genotypes is a prerequisite for intensive studies in somatic embryogenesis. Considering the popularity of the Indian papaya cvs. Honey Dew and Washington these were used for further experiments.

Incubation of zygotic embryos on the basal MSB medium favoured somatic embryo germination. MSB medium supplemented with Picloram or 2,4-D supported direct somatic embryogenesis. In presence of 2,4,5-T or combination of 2,4,5-T: Zeation in the MSB medium, somatic embryos originated both directly and also *via* an intermediary callus phase from the explant. The regeneration response was found to be dependent on the concentration of the phytohormone used. General observation was that lower concentration of Picloram (upto 4.14 µM), 2,4,5-T (upto 3.91 µM), 2,4-D (upto 9.05 µM) favoured direct somatic embryogenesis whereas higher phytohormone concentrations lead to the formation of somatic embryos accompanied by callus proliferation. Dicamba at lower concentration upto 4.52 µM favoured callus formation while at 9.05 µM direct somatic embryogenesis ensured (See Table 3.2 and summary in Table 3.4).

Table 3.2 Influence of phytohormones on somatic embryogenesis in papaya

Phytohormone	Conc. µM	Morphogenetic response of the explants			
		Dark Incubation		Light Incubation	
		HD	Wash	HD	Wash
		SE%	SE%	SE%	SE%
Picloram	2.07	43±6	37±6	27±6	23±6
	4.14	97±6	93±6	70±10	63±6
	8.28	37±6	17±6	13±6	10±0
	20.70	33±6	20±10	7±6	7±6
	33.12	23±6	23±6	3±6	7±6
	41.41	40±10	37±6	7±6	7±6
	62.11	43±6	43±6	17±6	17±6
2,4,5-T	1.96	40±10	33±6	23±6	20±0
	3.91	93±6	90±0	43±6	47±6
	7.83	43±6	47±6	23±6	23±6
	19.57	23±6	23±6	17±6	13±6
	31.31	17±6	13±6	10±0	7±6
	39.13	10±0	10±0	0±0	3±6
	58.7	0±0	0±0	0±0	0±0
	78.28	0±0	0±0	0±0	0±0

2,4-D	2.26	36±6	33±6	27±6	23±6
	4.52	67±6	53±6	47±6	43±6
	9.05	93±6	90±10	73±6	70±10
	22.62	13±6	13±6	10±0	13±6
	36.19	10±0	10±0	3±6	3±6
	45.25	0±0	3±6	0±0	0±0
	67.87	0±0	0±0	0±0	0±0
	90.50	0±0	0±0	0±0	0±0
Zeatin: 2,4,5-T	0.46+ 1.96	90±10	93±6	47±6	43±6
	2.28+1.96	57±6	33±6	23±6	17±6
	4.56+1.96	30±10	27±6	13±6	13±6
	9.12+1.96	23±6	13±6	10±0	7±6
	22.81+1.96	10±0	10±0	7±6	3±6
	45.62+1.96	7±6	7±6	0±0	0±0
	68.42+1.96	0±0	0±0	0±0	0±0
Dicamba	2.26	0±0	0±0	0±0	0±0
	4.52	0±0	0±0	0±0	0±0
	9.05	13±6	13±6	0±0	10±0
	22.62	93±6	90±0	83±6	83±6
	45.25	73±6	67±6	63±6	67±6
	67.87	57±6	53±6	47±6	57±6
	90.50	50±10	33±6	40±10	37±6
	113.12	17±6	23±6	13±6	27±6
	135.75	7±6	10±0	7±6	7±6

SE: somatic embryos; HD: Honey Dew; Wash: Washington

3.4.1.2 Induction of somatic embryos from mature zygotic embryos

Since immature zygotic embryo explants are not available throughout the year, mature zygotic embryos of the two Indian papaya cvs. Honey Dew and Washington were evaluated for their somatic embryo induction potential. The mature zygotic embryo explants were cultured on MSB medium supplemented with Picloram (4.14-41.41 µM), 2,4-D (4.52-45.25 µM), 2,4,5-T (1.96-39.13 µM) and Dicamba (4.52-45.24 µM).

Mature zygotic embryos of cv. Honey Dew and Washington in presence of 2,4-D and 2,4,5-T produced globular embryos directly or indirectly from the zygotic embryo explant. At lower concentration of Picloram, 2,4-D and 2,4,5-T responded with induction of somatic embryos. Dicamba at 22.62 µM also supported somatic embryo induction. After 8 weeks of incubation, globular staged somatic embryos appeared on the shoot pole of the mature embryo explant. These embryos were loosely attached to the explants.

Explants in presence of 20.70 µM, 45.25 µM 2,4-D, and 9.05 µM Dicamba produced loose friable non-embryogenic callus. The results are summarized in Table 3.3.

Table 3.3 Influence of phytohormones on papaya somatic embryo induction from mature zygotic embryo explants

Phytohormones	Conc. µM	Honey Dew	Washington
Picloram	4.14	SE	SE
	8.28	SE	SE
	20.70	C	C
	41.41	NR	NR
2,4,5-T	1.96	SE	SE
	3.91	SE	SE
	19.57	C+SE	C+SE
	39.13	NR	NR
2,4-D	4.52	SE	SE
	9.05	SE	SE
	22.62	C+SE	C+SE
	45.25	C	C
Dicamba	4.52	NR	NR
	9.05	C	C
	22.62	SE	SE
	45.25	SE	SE

SE-Somatic embryos; C-Callus; NR-Not responded

The best somatic embryogenesis response from both papaya cultivars viz. Honey Dew and Washington was elicited from both the immature zygotic embryo and mature zygotic embryo explants in presence of Picloram (4.14 µM) in the MSB medium. The percentage of explant responding with somatic embryogenesis was 97±6% for cv. Honey Dew and 93±6% in cv. Washington from immature zygotic embryos incubated in dark (Table 3.4). While in light 70±6% in cv. Honey Dew and 63±6% cv. Washington immature zygotic embryo explants responded. The percentage of explant responding with somatic embryogenesis from mature zygotic embryo explant was 30±0% for cv. Honey Dew and 27±6% in cv. Washington from mature zygotic embryos incubated in dark (Table 3.4). While in light 23±15% in cv. Honey Dew and 13±6% in cv. Washington responded with somatic embryogenesis. The results are summarized in Table 3.4.

Table 3.4 Comparison of maximum somatic embryogenesis response (%) elicited in presence of various phytohormones.

Phytohormone	Dark Incubation				Light Incubation			
	Immature ZE explant		Mature ZE explant		Immature ZE explant		Mature ZE explant	
	HD	W	HD	W	HD	W	HD	W
Picloram (4.14 μM)	97±6	93±6	30± 0	27±6	70±6	63±6	23±15	13±6
2,4,5-T (3.91 μM)	93±6	90±0	27±15	27±6	43±6	47±6	13±6	7±6
2,4-D (9.05 μM)	93±6	90±6	23±0	10±0	73±6	70±6	13±6	7±6
Zeatin: 2,4,5-T (0.46: 1.96 μM)	90±10	93±10	20±6	12±6	47±6	43±6	10±6	7±6
Dicamba (22.62 μM)	93±6	90±6	26±0	23±6	83±6	83±6	20±10	16±6

Data scored after 6 weeks of incubation, average of three replicates of experiments ±SD.

ZE - Zygotic embryo. HD- Honey Dew. W- Washington variety

3.4.2.B Influence of ethylene antagonists on papaya somatic embryogenesis

Polyamines play a major role in cell division and growth of prokaryotes and eukaryotes (Evans and Malmberg 1989). Polyamines have been related with the induction of somatic embryos in *Daucus carota* (Feirer *et al.* 1985, Fienberg *et al.* 1984, Montague *et al.* 1979), *Medicago sativa* (Meijer and Simmonds 1988), and *Nicotiana tabacum* (Torrigiani *et al.* 1987) besides others. Metabolism of polyamines is connected with several pathways and particularly with ethylene biosynthesis in plants. Ethylene, a gaseous plant hormone, regulates many physiological responses e.g. senescence of oat leaves (Kaur-Sawhney *et al.* 1982), in plants (Feirer *et al.* 1985, Reid 1987). Spermidine, as ethylene antagonists has been implicated in enhancing the regeneration responses in *Brassica campestris* (Chi *et al.*1994). $AgNO_3$ is an ethylene inhibitor, is reported to help in somatic embryogenesis (Songstad *et al.* 1991; Roustan *et al.* 1990). Silver nitrate has been shown to enhance somatic embryogenesis and *in vitro* shoot regeneration in sugarcane, cucumber, carrot, maize, sunflower and wheat tissue cultures (Purnhauser *et al.* 1987). In alfalfa (*Medicago sativa*) ABA enhances somatic embryo quality (Lecouteux *et al.* 1993). It serves as stress agent inducing endogenous ABA accumulation and promotes somatic embryo production *via* an increase in the endogenous ABA levels

(Kong and Yeung 1994,1995). The beneficial effect of ABA on conversion of somatic embryogenesis has been reported in papaya (Castillo *et al.* 1998a; Monmerson *et al.* 1995) and other crops (Roberts *et al.* 1990). Germination is a major problem during somatic embryo development and this is controlled either by increasing osmolarity of the maturation medium with additional sucrose (Carman 1989) or by incorporating ABA in to the medium (Ammirato 1974). ABA prevents precocious germination of the cotyledonary staged embryos. It may be necessary during embryogenesis to initiate the synthesis of proteins and other proteins involved in desiccation tolerance (Galau *et al.* 1990). ABA was also reported to influence development of carrot somatic embryos and in particular affected their capacity to develop functional shoot meristem (Nickle and Yeung 1994).

The induced somatic embryos papaya was transferred to MSB medium supplemented with Spermidine, Putrescine, ABA or $AgNO_3$ individually (each at 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 μM concentrations). The cotyledonary or torpedo stage embryos were maintained for 1 week on these media and then transferred to MSB medium for conversion.

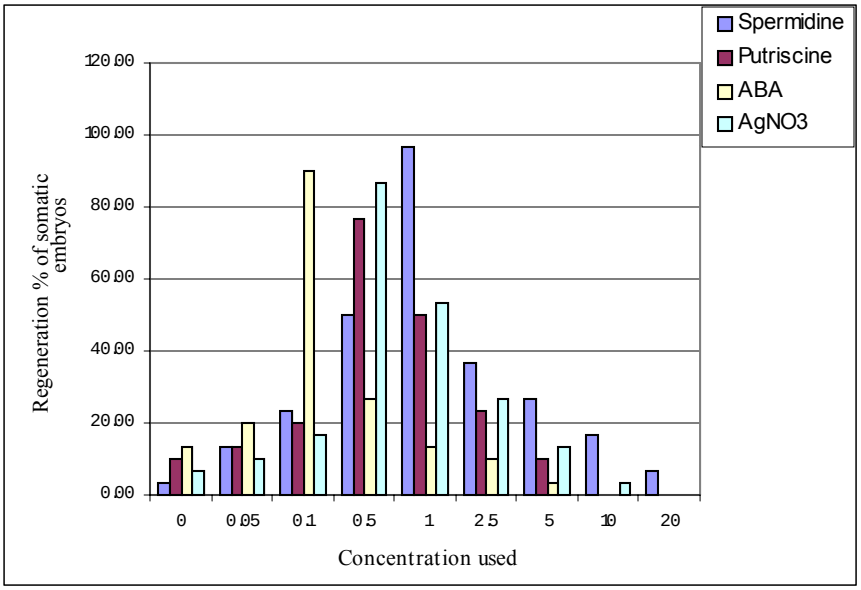
Table 3.5 % maturation of somatic embryos occurred in all the ethylene antagonists

Conc. μM	Spermidine	Putricine	ABA	$AgNO_3$
0	3.33	10	13.33	6.67
0.05	13.33	13.33	20.00	10.00
0.1	23.33	20	90.00	16.67
0.5	50.00	76.66	26.67	86.67
1	96.67	50	13.33	53.33
2.5	36.67	23.33	10.00	26.67
5	26.67	10	3.33	13.33
10	16.67	0	0.00	3.33
20	6.67	0	0.00	0.00

All concentrations of Spermidine (0.05-20 μM) facilitated embryo maturation. The response increased from 13.33 to 96.67% with increasing Spermidine concentrations (0.05-1.0 μM). Thereafter the response decreased with increasing Spermidine concentrations. Likewise the best embryo maturation response supported by Putrescine (0.5 μM) was 76% by ABA at 0.1 μM was 90% and by $AgNO_3$ at 0.5 μM was 86.67%.

Somatic embryo maturation triggered by all the ethylene antagonists was summarized in Table 3.5 and Fig. 3.8.

Fig. 3.8 Influence of ethylene antagonists (μM concentration) on the maturation of somatic embryos



The mature cotyledonary stage somatic embryos (Figs.3.8A, B; 3.9) were transferred to MSB medium for conversion into plantlets. On an average 90% somatic embryos germinated and grew into whole plantlets. Mature somatic embryo was similar to zygotic embryo (Fig. 3.10). The formation of somatic embryos (8-10 per explant) was achieved in 12-14 weeks starting from the initial culture of the zygotic embryos on the induction medium and it required further 6-8 weeks for conversion. After conversion the somatic embryos were germinated (Figs. 3.16A, B; Figs. 3.17A, B, Figs. 3.18A, B). The plantlets were transferred to pots and hardened in green house (Fig. 3.19A-C).

Development of somatic embryos was observed by histological and (Fig.3.3A) and scanning electron microscope studies (Fig.3.3B, Figs. 3.6 A-H) and its origin of induction was confirmed.

In our study we have used cvs. Honey Dew and Washington for the somatic embryogenesis, total average time required for somatic embryo induction to germination

was about 18-22 weeks. In earlier reports the duration has been reported to be 20-30 weeks. A comparative account for the duration of induction, maturation and conversion of somatic embryo is given in Table 3.6.

Table 3.6 Duration required for the induction, maturation and conversion of somatic embryo

Stages of somatic embryo	Mean time required (in weeks)		
	Our study		Earlier reports
	Honey Dew	Washington	
Induction	4-6	4-6	14 Fitch and Manshardt 1990, Fitch 1993) 12 (Litz and Conover 1982) 12 (Castillo *et. al*. 1998)
Maturation	6-8	6-8	4-8 (Fitch and Manshardt 1990, Fitch 1993) 16 (Litz and Conover 1982) 2 (Castillo *et. al.* 1998)
Conversion	2-4	2-4	1-4 (Fitch and Manshardt 1990, Fitch 1993)

3.4.3.C Influence of boron on somatic embryogenesis

Different media constituents, amino acids, carbon sources (Drew *et al*. 1993), inorganic salt concentrations, Iron chelate source (Castillo *et al*. 1997) have been tested for their influence on regeneration of papaya *via* embryogenesis, organogenesis or rooting of shoots (Drew 1987, Drew and Miller 1989).

The micronutrient boron is supplied as boric acid in the plant tissue culture media. It plays an important role in lignin biosynthesis and metabolism of phenolic acids. Boron deficiency results in the death of shoot tip meristems and it has been shown to influence on somatic embryogenesis in rice (Sahasrabudhe *et al.* 1999) and pine (Huang and Li, 1994).

In the present investigation influence of boron on somatic embryogenesis in papaya cv. Honey Dew was studied in presence of the phytohormones Picloram (4.14 µM) and 2,4-D (9.05 µM) using immature zygotic embryo explants (Fig.3.11A). The morphogenetic response to boric acid (30-500 mg/l) concentrations is summarized in Table 3.5.

Table 3.7 Effect of different concentrations of boric acid on somatic embryogenesis

H_3BO_3 mg/l	Embryogenesis Picloram (4.14 µM)	Embryogenesis 2,4-D (9.05 µM)
Control	83.33±5.77	73.33±5.77
30	86.67±5.77	76.66±5.77
62	96.67*±5.77	90.00*±0.0
100	13.33±5.77	10.00±10.00
150	6.67±5.77	3.33±5.77
180	3.33±5.77	3.33±5.77
200	3.33±5.77	0.00±0.0
240	0.00±0.0	0.00±0.0
320	0.00±0.0	0.00±0.0
400	0.00±0.0	0.00±0.0
450	0.00±0.0	0.00±0.0
500	0.00±0.0	0.00±0.0

* Significantly higher at 0.05 probability. (% Explants inducing somatic embryos)

The best response was obtained with supplementation of somatic embryo induction media with 62 mg/l boric acid. Significantly higher frequency of somatic embryogenesis was obtained both in presence of Picloram (4.14 µM) and 2,4-D (9.05 µM). There was drastic reduction in the percentage of somatic embryo induction when boric acid concentration in the medium was increased further. Somatic embryogenesis was not observed when the medium contained 240 mg/l or higher levels of boric acid. Embryogenesis in the form of rosette structure was achieved from the meristimatic regions of the explant. It exhibited a short span of initial stages and then switched over to the cotyledonary stage (Fig.3.11C). Formation of embryos was continuous even after 6-8 months of repeated subculture. Cotyledonary structures obtained were morphologically normal. The normal embryo structures had well-developed shoot and root pole. Morphological and scanning electron micrograph observations (Fig.3.11B) indicate origin of somatic embryos to be from the immature zygotic embryo explant. The somatic embryos germinated and grew into whole plantlets (Fig.3.11D). These germinated plantlets were hardened in greenhouse under controlled conditions (Fig.3.11E). No separate media were required for the maturation and conversion of somatic embryos. While in the control medium, embryogenesis was observed after 6-8 weeks, initiation of

embryogenesis was delayed (8-10 weeks) in the media with higher concentrations of boric acid.

Huang and Li (1994) and Zoglauer *et al.* (1996) have reported enhanced somatic embryogenesis from pine, tea, peanut and potato is a boron concentration depended, as in the present study. This is in contrast to embryogenesis in rice (Sahastrabudhe *et al.* 1999), where the frequency of embryogenesis increased with increasing concentrations of boric acid in the medium.

3.4.4.D Morphological aberrations

In an embryo, the shoot and root meristems are the two primary elements, which produce structures of adult plant (Steeves and Sussex 1989). Aberrant morphologies of torpedo and cotyledonary stage somatic embryos were observed during the course of the present study. High concentration (Picloram 30-60 μM, 2,4.5-T 20-40 μM, 2,4-D 20-40 μM and Dicamba 60-135 μM) and continuous presence of phytohormones affect somatic embryo morphology. Varied morphologies like fused embryos (Fig.3.12A-C, 3.13A-C), embryos with a single cotyledon, multicotyledoary embryos were observed. Upon germination shoot apical meristems with reduced primary leaves (Fig. 3.14A-D) or only with primary roots (3.15A, B) were formed. Often the structures failed to germinate and form proper shoot apex or root. Similar type of abnormal embryo morphology was also reported by Ozias-Akins (1989), Suhasini *et al.* (1996).

3.5 Conclusions

In the present study the potential of immature and mature zygotic embryo explants of papaya to regenerate somatic embryos as influenced by various physiochemical agents was assessed. Among the phytohormones tested Picloram was found to elicit the best somatic embryogenesis response from both immature and mature zygotic embryo explants. Phytohormones 2,4-D, Dicamba, 2,4,5-T and combinations of Zeatin: 2,4,5-T were also induced somatic embryogenesis, however the degrees of response varied with the concentration of the phytohormone used. Picloram at 4.14 μM induced somatic embryogenesis from the maximum number of explants. About 97±6% immature zygotic embryo of cv. Honey Dew and 93±6% in cv. Washington responded with formation of

somatic embryos. The response from mature zygotic embryos was 30±0% in cv. Honey Dew and 27±6% in cv. Washington. Maximum conversion of globular staged embryo to cotyledonary stage embryo was also obtained in presence of Picloram (4.14 μM). Maturation of these embryos was achieved (96.67%) in media supplemented with Spermidine (1 μM).

Increase of boric acid concentration from 6.2 mg/l to 62 mg/l resulted in significantly higher somatic embryogenesis response both in presence of Picloram or 2,4-D. Embryogenesis in the form of rosette structure was observed from the apical meristimatic regions of the explant. It existed for a short span and then switched over to the cotyledonary stage embryos. Formation of embryos was continuous even after 6-8 months of repeated culture. Cotyledonary structures obtained were morphologically normal and developed shoot and root poles.

The use of ethylene antagonists in the medium ensured 10-12 fold enhancement in embryo maturation. Where as only 8.3% of the regenerated somatic embryos matured on the basal MSB medium, the respective percent embryo maturation was 96.67% in the presence of Spermidine (1.0 μM), 76.67% in the presence of Putrescine (0.5 μM), 90.0% in the presence of ABA (0.1 μM) and 86.67% in the presence of $AgNO_3$ (0.5 μM). Maturation of somatic embryos was adversely affected by higher (10 μM) concentration of ethylene antagonists. The mature cotyledonary embryos were transferred to modified MS medium for conversion into plantlets. The normal cotyledonary embryos germinated and grew into whole plantlets.

High concentrations of a phytohormone (Picloram 30-60 μM, 2,4.5-T 20-40 μM, 2,4-D 20-40 μM and Dicamba 60-135 μM) and its prolonged presence in the culture medium affected somatic embryo morphology and the development of abnormal embryos into plantlets. .

The present investigations offer an opportunity to achieve plant regeneration from immature and mature zygotic embryo of papaya. Somatic embryo induction response was genotype independent obtained from both the immature and mature zygotic embryo explants. These regeneration protocols are more amenable for genetic transformation in papaya *via A. tumefaciens* and or particle bombardment.

Parts of this chapter have been published:

- **N.N.Renukdas, M. L. Mohan, S. S. Khuspe and S. K. Rawal (2006). Influence of phytohormones, culture conditions and ethylene antagonists on somatic embryo maturation and plant regeneration in papaya. *Intl. J. Agric. Res.* 1(2): 151-160.**
- **N.N.Renukdas, M.L. Mohan, S.S. Khuspe and S.K.Rawal (2003). Influence of boron on the induction of somatic embryos in papaya (*Carica papaya* L.) cv. Honey Dew. *Biologia Plantarum*, 47 (1): pp.129-132.**
- **Bhattacharya J., S.S. Khuspe, N.N. Renukdas and S.K. Rawal (2002). Somatic embryogenesis and plantlet regeneration in papaya cv. Honey Dew and Co-2. *Indian Journal of Experimental Biology*, 40: 624-627.**

II. INDUCTION OF MULTIPLE SHOOTS AND WHOLE PLANT REGENERATION IN PAPAYA

3.6 Introduction

In vitro plant regeneration or micro-propagation is an important step in the success of any crop improvement program. Plants can be propagated by tissue culture methods in different ways e.g. by inducing the pre-existing shoot primordia, by shoot morphogenesis either directly from the explant or from unorganized tissues and *via* somatic embryogenesis (direct or indirect). The commitment of competent cells for morphogenesis is affected by complex interactions between genotypes, the explant (its stage of development) and the culture medium. Morphogenesis is triggered usually after competent cells are subcultured into a less complex medium allowing the expression of new developmental potential (Thorpe 1983). It is believed that any single factor cannot affect morphogenesis but it is the combination of various stimulating factors, which needs to be applied to the cells not only in right amount and sequence but also under right culture conditions (Stewards *et al.* 1964). The changes in the macro and micro-nutrients and addition of various substances such as charcoal, organic compounds (vitamins, amino acids, polyamines, phytohormones etc), carbohydrates, light intensity, pH, water potential, temperature, gaseous atmosphere and container shape all affect plant morphogenesis (Tran Thanh Van 1981).

In vitro plant propagation mainly consists of induction of shoot buds and their multiplication, elongation and *in vitro* or *ex vitro* rooting of shoots to form plantlets. The process has several advantages. Propagation is simple and rapid, plants obtained are true to type, cultures are initiated from very small segments of the mother plant, propagation is possible throughout the year, greater degree of control over chemical, physical and environmental factors can be exercised and the possibilities of rejuvenation from the mature tissues exist (Ahuja 1986).

3.6.1 *In vitro* plant regeneration in papaya

In vitro plant regeneration in papaya has been achieved through somatic embryogenesis and organogenesis (Medhi and Hogan 1976, Yie and Liaw 1977). Earlier efforts had failed to achieve *in vitro* shoot proliferation in papaya. Subsequently, Litz and Conover (1977, 1978a) developed a procedure of shoot proliferation from the shoot tips of field

grown papaya. Regeneration of plants from callus cultures was also obtained from the stem segments (Arora and Singh 1978b). Shoot tip and shoot bud (axillary and lateral bud) may also be used as explants for plant regeneration in papaya (Rajeevan and Pandey 1983, Drew and Smith 1986, Winner 1988, Reuveni *et al.*1990, Mondal *et al.* 1990, Lai *et al.* 1998). Yang and Ye (1992) and Hossain *et al.* (1993) reported plant regeneration from the petiole explants of papaya. However, shoot regeneration from immature zygotic embryo of papaya has not been reported earlier. Use of immature zygotic embryo as an explant has several advantages: smaller size, amicable to both *Agrobacterium* as well as particle bombardment mediated transformation techniques, takes the least time to develop into shoots compared to several months in case of plant regeneration *via* callus phase and regeneration through zygotic embryo is genotype independent.

Thidiazuron (TDZ), a substituted phenyl urea used as a defoliant (Yip and Yang 1986), also exhibits cytokinin like activity (Magioli *et al.* 1998). It has been used to induce adventitious shoots in a number of plant species (Eva 1999, Sujatha and Reddy 1998). In papaya tissue cultures, most often, combinations of BAP and NAA have been used for multiple shoot induction (Hossain *et al.* 1993, Rajeevan and Pandey 1983).

The aim of the present study was to develop a suitable *in vitro* regeneration protocol and study the potential of immature zygotic embryo as the explant for shoot multiplication for the three Indian papaya cvs. Honey Dew, Washington and Co-2.

3.7 Materials and Methods

3.7.1 Preparation of plant material

Immature fruits were collected from the papaya cvs. Honey Dew, Co-2 and Washington 90–115 days post anthesis. The fruits were surface sterilized, cut transversely under aseptic conditions (Fig 3.20A) and the immature seeds (Fig. 3.20B) collected in Petri dish. Immature zygotic embryos (Fig. 3.20C) were excised from the seeds and used as the explant source.

3.7.2. Media used

MS (Murashige and Skoog 1962), B5 (Gamborg 1968), White (1963) and a combination of MS salts and B5 vitamins (MSB) medium containing 26.6 μM glycine (designated as MBG) were used as the basal nutrient media in the present study.

Different concentrations of TDZ (0.45-22.7 μM) and combinations of BAP (0.2-8.87 μM) and NAA (0.5-2.64 μM) were used as phytohormone supplement.

Half strength MBG medium containing IBA (4.9-19.7 μM) was tested for rooting of the *in vitro* regenerated shoots.

3.7.2.1 Rooting and hardening

The rooted plants were transformed to a soil: sand mixture (1:1) for further growth and hardening.

3.7.2.2 Statistical analysis

Twenty explants in replicates of three were analyzed in each experiment. All experiments were repeated thrice. Data obtained were subjected to ANOVA and Student's t-test and least significant difference was calculated at 5 % level of confidence.

3.7.2.3 Histology and SEM studies

Histology and SEM studies were done as described in Chapter 2.

3.8 Results and Discussion

The immature zygotic embryo explants from the three Indian cvs. Honey Dew, Co-2 and Washington were used for multiple shoot induction using a wide range of concentrations of TDZ and combinations of BAP: NAA.

3.8.1 Influence of TDZ

The number of shoots regenerated increased with the increasing concentration of TDZ from 0.45 to 2.2 μM in the MBG medium. Green shoot initials were induced from the meristimatic region of the immature zygotic embryo in the second week of culture in the TDZ containing media (Fig.3.21A). In all the three papaya cultivars tested, maximum average numbers of shoots regenerated per explant was in MBG basal medium supplemented with 2.2 μM TDZ (Table 3.6). The cultivar response was: cv. Honey Dew 95.5±1.2%, 91.1±0.6% in cv. Co-2 and 94.4±1.5% in cv. Washington. The maximum average number of shoots regenerated per cultured explants were 14±2 for cvs. Honey Dew, Co2 and Washington. TDZ concentrations beyond 4.5 μM resulted in stunting of the regenerated shoots. With further increase of TDZ concentrations in the medium the phenomenon became progressively acute to the extent that at 9.0 μM, the number of the regenerated shoots could not be counted (Table 3.8). Multiple shoot induction was

recorded after two (Fig.3.21A), four (Fig.3.21B) and six weeks (Fig. 3.21C). No multiple shoot regeneration occurred on the MBG medium.

Table 3.8 Effect of TDZ on multiple shoot formation in papaya

Conc. Used (μM/l)	% Explant forming multiple shoot			No. of shoots per explant		
TDZ	**HD**	**Co-2**	**WA**	**HD**	**Co-2**	**WA**
0.0	-	-	-	1.0	1.0	1.0
0.45	62.2±0.6	60.0±1.0	63.3±1.0	4.2±1.3	4.6±1.5	4.53±1.5
0.91	86.6±1.0	80.0±1.0	85.5±1.5	5.6±1.6	5.5±1.58	5.84±1.7
2.2	95.5±1.2	91.1±0.6	94.4±1.5	14.6±1.9	14.5±2.0	14.57±2
4.5	71.1±1.2	66.6±2.0	67.7±2.8	7.6±1.5	7.9±1.7	7.84±1.6
6.8	44.4±0.6	46.6±1.7	47.7±1.5	4.9±1.8	5.1±2.1	5.14±2.0
9.0	44.4±1.5	48.8±1.5	46.6±1.0	*	*	*

*: Unable to count no. of shoots, HD: cv. Honey Dew variety, Co-2: cv. Co-2, WA: cv. Washington

3.8.2 Influence of BAP: NAA combination

Multiple shoot regeneration was observed in presence of a wide range of combinations of BAP: NAA in the MBG medium. Shoot initials were induced from the meristimatic region of immature zygotic embryo explant in the second week of incubation (Fig.3.22A). Multiple shoot induction was recorded after two (Fig.3.22A), four (Fig.3.22B) and six weeks (Fig. 3.22C). The multiple shoot induction was observed in all the combinations of BAP: NAA. The % response forming multiple shoot was maximum in the MBG medium supplemented with BAP (4.44 μM): NAA (0.54 μM). The cultivar wise response was: Honey Dew 92.2±0.6%, 91.1±0.6% in Co-2 and 93.3±3.9% in Washington. Maximum number of shoots regenerated were 14±4 for cvs. Honey Dew, Co-2 and Washington in presence of BAP (4.44 μM): NAA (0.54 μM) in the MBG medium (Table 3.9). Further increase in the BAP: NAA concentration resulted in progressive decrease in the number responsive explants and the number of regenerated shoots. The phenomenon of shoot stunting and fasciations was not observed with the incorporation of BAP: NAA in the medium.

Table 3.9 Effect of BAP: NAA on multiple shoot formation in papaya

Conc. Used (μM/l)	% Explant forming multiple shoot			No. of shoots per explant		
BAP: NAA	**HD**	**Co-2**	**WA**	**HD**	**Co-2**	**WA**
0.22: 0.54	8.8±0.6	10.0±1.0	10.0±0.4	2.8 ± 0.4	2.9±0.4	3.0±0.4
0.22:2.64	20.0±2.0	18.4±1.5	18.9±0.8	3.4± 0.8	3.4±0.8	3.6±0.8
0.44:0.54	48.9±0.6	47.8±0.6	46.7±1.4	4.4 ± 1.4	4.1±1.4	4.2±1.4
0.44:2.69	80.0±1.0	82.2±0.6	81.1±1.7	6.2±1.7	6.0±1.7	6.1±1.7
2.22: 0.54	76.6±1.0	76.7±1.0	77.8±1.5	7.3±1.5	7.0±1.5	7.1±1.5
2.22: 2.69	85.5±0.6	84.4±0.6	83.3±2.5	8.8 ±2.5	8.8±2.5	8.9±2.5
4.44: 0.54	92.2±0.6	91.1±0.6	93.3±3.9	14.3±3.9	14.1±3.9	14.4±3.9
4.44: 2.69	85.3±0.6	82.2±1.1	83.3±2.8	10.1±2.8	9.9±2.8	10.0±2.8
8.87: 0.54	23.3±1.0	22.2±1.1	21.1±1.4	4.03±1.4	3.4±1.4	3.9±1.4
8.87: 2.69	11.1±1.5	12.2±1.1	10.0±1.1	3.42±1.1	3.0±0.0	3.0±0.0

*: Unable to count no. of shoots, HD: cv. Honey Dew variety, Co-2: cv. Co-2, WA: cv. Washington

3.8.3. Elongation of shoots

The shoots regenerated in presence of TDZ (2.2μM) or a combination of BAP (4.44 μM): NAA (0.54 μM) were transferred to MS basal medium supplemented with GA_3 (5.7 μM) for 15 days wherein shoot elongation occurred (Fig. 3.21D and 3.22D).

3.8.4 Effect of various basal media on multiple shoot induction

Various basal media MS, B5, White, 1/2 strength MS and MBG were tested to evaluate the influence on papaya multiple shoot regeneration. These media was supplemented with TDZ (2.2 μM) or a combination of BAP (4.44 μM): NAA (0.54 μM). Response of the explants and shoot proliferation is reported to vary with the composition of the basal medium (Shekhawat *et al.* 1993, Das *et al.* 1996). Among all the media tested, MBG was found to be the most suitable medium for multiple shoot induction, 96% of the explants cultured on this medium responded with shoot regeneration. Lowest response was obtained on ½ MS basal medium. Regeneration of maximum average number of multiple shoot from an explant was also achieved (Table 3.10 and 3.11) with the use of MBG supplemented either with 2.2 μM TDZ (14±2 in Honey Dew, 14±2 in Co-2, 14±2 in

Washington) or a combination of BAP (4.4 μM): (0.5 μM) NAA (14±4 cv. Honey Dew, 14±4 cv. Co-2 and 14±4 cv. Washington).

Table 3.10 Multiple shoots formation in TDZ (2.2μM) using different basal media

Basal media used	HoneyDew	Washington	Co-2
	No. of shoots formed	No. of shoots formed	No. of shoots formed
MS	11±2	11±2	11±2
B5	11±3	12±2	11±2
White's	9±2	9±2	9±2
½ MS	8±4	8±3	8±3
MBG	14±2	14±2	14±2

Table 3.11 Multiple shoots formation in BAP: NAA (4.44μM: 0.54 μM) combination using different basal media

Basal media used	HoneyDew	Washington	Co-2
	No. of shoots formed	No. of shoots formed	No. of shoots formed
MS	12±2	12±2	12±2
B5	13±2	13±2	13±2
White's	8±3	8±2	9±3
½ MS	7±2	7±2	7±2
MBG	14±4	14±4	14±4

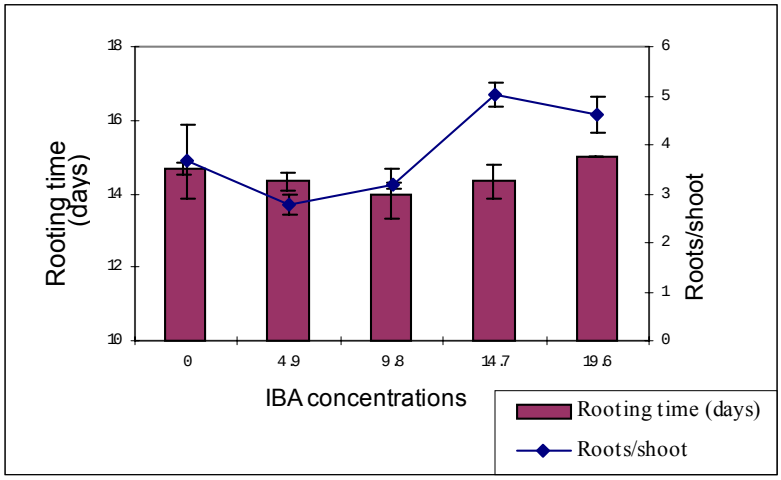
Scanning electron micrograph study (Fig.3.23) and histological examination (Fig. 3.24) revealed the origin of the shoots from the meristimatic region of immature zygotic embryo explant.

3.8.5 Rooting and hardening

Well developed and elongated shoots (3-5 cm in height) were excised after 15-20 days and transferred to half strength MBG medium supplemented with 4.9-19.6 μM IBA and 3 % sucrose. The shoots developed roots after 2 weeks of incubation in presence of all the concentrations of IBA tested. In all the three cvs. Honey Dew, Washington and Co-2 the maximum number of roots per shoot are induced in medium containing 14.7 μM IBA (Figs. 3.25A-C; Figs.3.26A-C). Lowest rooting response was observed on medium

containing 4.9 µM IBA (Fig.3.27). Phytohormone free basal medium served as control where 3.66±0.76 roots per shoot were induced.

Fig. 3.28 Effect of IBA concentrations (µM) on the root formation from *in vitro* shoots of papaya



The rooted shoots were transferred to pots containing a mixture of sand: soil: compost (1:1:1), covered with polypropylene bags (Fig.3.28A) and hardened (Fig.3.28B). About 76% of the plants survived under greenhouse condition and these were later planted in the field (Fig.3.28C).

The present shoot multiplication process in papaya does not require different media for establishment of cultures, and for proliferation and development of shoots as compared with earlier reports (Rajeevan and Pandey 1983, Winnar 1988, Mondal *et al.* 1990, Hossain *et al.* 1993). The procedure has the added advantage of using immature zygotic embryo as the explant source, which is aseptic and hence the chances of contamination are minimized. Embryo axis is also preferred for transformation experiments (Polowick *et al.* 2000, Krishnamurthy *et al.* 2000) due to its smaller size, which favors handling of a large number of explants at one time and also it takes less time to develop shoots. The zygotic embryo explant offers the advantage of direct shoot regeneration, and is not prone to somaclonal variation and chromosomal abnormalities (Saeed *et al.* 1997).

3.9 Conclusions

The present protocol describes a rapid, efficient and reproducible method for the development of papaya plants *via* multiple shoot regeneration using immature zygotic embryo explant from papaya cvs. Honey Dew, Washington and Co-2.

Maximum induction of multiple shoots (~14 shoots per explant) achieved from immature zygotic embryo explants when cultured in MBG medium supplemented either with TDZ (2.2 μM) or BAP: NAA (4.44:0.54 μM). Among all the basal media tested, MBG medium was found to be the most suitable for multiple shoot induction. Elongation of the shoots was obtained in MBG medium supplemented with GA_3 (5.7 μM). The best rooting response of the *in vitro* regenerated shoots was achieved on half strength MBG basal medium supplemented with 14.7 μM IBA.

Survival of tissue culture raised plants was 76% after hardening under greenhouse conditions. Use of immature zygotic embryo explant for multiple shoot development offers the additional advantages of maximum number of regeneration and survival of plantlets.

Part of this chapter has been published:

- **Bhattacharya J. N.N.Renukdas, S.S.Khuspe and S.K.Rawal (2003/2004). Multiple shoot regeneration from immature embryo explants of papaya. *Biologia Plantarum,* 47(3): pp. 327-331.**

CHAPTER 4

TRANSFORMATION STUDIES IN PAPAYA

Agrobacterium-mediated transformation using green fluorescent protein (GFP) as a Screenable marker

4.1 Introduction

Genetic engineering involves manipulation of the genetic material toward a desired end in a directed and predetermined way. It has become an additional tool in plant development (Comai 1993). Unsuccessful crosses and narrow gene pool available within the species has catalysed plant genetic engineering to become a rapidly emerging reality and plant gene transfer is now a fertile field.

Genetic engineering offers opportunities for improving agriculture and public health. An elite variety could be modified for a single trait with the gene/s coding for insect, disease resistance (viral and fungal) or herbicide tolerance from entirely different organisms. Some of the quality traits such as protein, carbohydrate content, modified plant oil, enhanced flavour, texture and longer shelf life could be introduced in recent past (Smith 1994). Potential benefits include higher yields, enhanced nutritional value, reduction in pesticide and fertilizer use or even provide novel pharmaceuticals.

Disease resistance has been targeted as an advantageous trait in major crops and a number of strategies have been employed to achieve this goal (Smith 1994). Virus resistance is important for good crop yields and also to reduce the amount of chemicals required to control the insect vectors that transmit the virus.

In 1983, the era of plant transformation was initiated when *A. tumefaciens*-mediated gene delivery was used to produce transgenic plants (Fraley *et al.* 1983, 1986). Following years of unsuccessful experiments with variations in feeding isolated DNA to plant tissues and organs, gene transfer became a reality soon after it was discovered that the soil bacteria *A. tumefaciens and A. rhizogenes* are natural genetic engineers due to their ability to transfer and integrate DNA into plant genomes through an unique integrative gene transfer mechanism (Jouanin *et al.* 1993).

4.1.1 *Agrobacterium* mediated gene transfer

A.tumefaciens possess the tumour inducing (Ti) plasmid responsible for tumour formation in plants (Zupan and Zambryski 1995). *A. rhizogenes* on the other hand possess the root inducing (Ri) plasmid, which is responsible for DNA transfer and the resulting hairy root formation (Tepfer 1984). Introduction of the Ti plasmid into plant cells occurs without alteration of their normal regeneration capacity (Zambryski *et al.* 1983, 1989). During infection, the bacterium transfers a small section of its own genetic

material (T-DNA) into the genome of the host plant cell (Zambryski 1992). Once inserted, the bacterial genes are expressed by the infected plant cells. During the infection process the plant cell begins to proliferate to form tumours and synthesize arginine derivatives called opines, usually nopaline or octopine depending on the *A.tumefaciens* strain involved. Opines are catabolized and used as nitrogen sources by the infecting bacteria. By understanding and manipulating this process of infection or transformation, researchers have been able to harness these powerful and sophisticated vectors to transfer specific cloned genes of major importance to plants.

T-DNA of *A. tumefaciens* is a small section of the Ti-plasmid about 23 kb in size, which makes up about 10 % of the Ti or the Ri plasmids. This stretch of DNA is flanked by 25 bp repeated sequences, which are recognized by the endonucleases encoded by the *vir* genes. Within the T-DNA, two distinct regions TL and TR have been identified. The T-DNA of nopaline strains can integrate as a single segment, whereas octopine strains frequently integrate as two segments, TL and TR. TL carries the genes controlling auxin and cytokinin biosynthesis and is always present when tumours are formed. Failure of TR to integrate, results in the loss of opine biosynthesis (Webb and Morris 1992). The *vir* (virulence) region of the Ti plasmid contains the genes which mediate the process of T-DNA transfer. *Vir* gene action generates and processes a T-DNA copy and facilitates T-DNA movement out of the bacterium and into the plant cell. Helper plasmids for non-oncogenic plant transformation have been developed to utilize the *vir* gene functions with T-DNAs containing genes of choice (Hood *et al.* 1993).

The removal of the oncogenes from the Ti plasmid results in disarmed strains of *A. tumefaciens* (Klee *et al.* 1987). The oncogenes of *Agrobacterium* are replaced by reporter genes/screenable marker genes (e.g. β-glucuronidase gene (gus), luciferase (luc) gene for analyzing gene expression. Genes conferring resistance to antibiotics (e.g. neomycin phosphotransferaseII (nptII), hygromycin phosphotransferase (hpt), phosphinothricin acetyl transferase (bar) are used to allow selection between transgenic and non-transgenic cells. Oncogenes may be replaced with genes of economic importance (McElroy and Brettel 1994).

Plant species differ greatly in their susceptibility to infection by *A. tumefaciens* or *A.rhizogenes*. Even within a species, different cultivars or ecotypes may show different

degrees of susceptibility. These differences have been noted in a variety of plant species (Gelvin 2000). Though environmental or physiological factors are attributed for these differences, genetic basis for susceptibility has been described in *Arabidopsis* (Nam *et al.* 1997).

Agrobacterium attaches to plant cells in a polar manner in a two-step process. The first step is likely mediated by a cell-associated acetylated, acidic capsular polysaccharide (Reuhs *et al.* 1997). The second step involves the elaboration of cellulose fibrils by the bacterium, which enmeshes large numbers of bacteria at the wound surface (Matthysse *et al.*1995).

The interaction between *Agrobacterum* sp. and plant involves a complex series of chemical signals communicated between the pathogen and the host cells. These signals include neutral and acidic sugars, phenolic compounds, opines (crown gall specific molecules synthesized by transformed plants), *Vir* (virulence) proteins and the T-DNA (Gelvin 2000). Baker *et al.* (1997) has described the chemical signalling in plant-microbe interactions. The T-DNA transfer process initiates when *Agrobacterium* perceives certain phenolic compounds from wounded plant cells (Hooykaas and Beijersbergen 1994), which serves as inducers or co-inducers of the bacterial *vir* genes. Phenolic chemicals such as acetosyringone and related compounds (Dye *et al.* 1997) are perceived *via* the *Vir*A sensory proteins (Doty *et al.*1996). Most of the induced *Vir* proteins are directly involved in T-DNA processing from the Ti plasmid and the subsequent transfer of T-DNA from the bacterium to the plant. Among them *Vir*D2 and *Vir*E2 contain plant active nuclear localization signal sequences (NLS) (Herrera-Estrella *et al.* 1990). VirD2 protein is directly involved in processing the T-DNA from the Ti plasmid. It nicks the Ti plasmid at 25-bp directly repeated sequences, called T-DNA borders that flank the T-DNA (Veluthambi *et al.* 1988). Thereafter, it strongly associates with 5' end of the resulting DNA molecule (Filichkin and Gelvin 1993) through tyrosin (Vogel and Das 1992). VirD2 contains two nuclear localization signal (NLS) sequences (Herrera-Estrella *et al.* 1990) whereas *Vir*E2 contains two separate bipartite nuclear localization signal (NLS) regions that can target linked reporter proteins to plant cell nuclei (Citovsky *et al.*1994).

Large number of plant species has been transformed by *A. tumefaciens* mediated transformation (Siemens and Schieder 1996). However, success of *Agrobacterium*–

mediated transformation depends on the plant genotype (Robinson and Firoozabady 1993), the choice of explants (Robinson and Firoozabady 1993, Jenes *et al.* 1993), the delivery system, the *Agrobacterium* strain (Gelvin and Liu 1994), the conditions of co-cultivation, the selection method and the mode of plant regeneration. *Agrobacterium* co-cultivation has been successfully used for the transformation of leaves, roots, hypocotyls, petioles, cotyledons (Zambryski 1992, Hooykaas and Beijersbergen 1994), pollen-derived embryos (Sangwan *et al.* 1993), seeds (Feldmann and Marks1987) and even whole plants (Chang *et al.* 1994).

Many plant species are still recalcitrant to *Agrobacterium* mediated transformation. This recalcitrance does not result from a lack of T-DNA transfer or nuclear targeting, rather its integration into the genome of regenerable cells appears to be limiting. In the future, it may be possible to over express endogenous genes involved in the integration process or to introduce homologous genes form other species, and thereby effect higher rates of stable transformation (Gelvin 2000).

Initially, monocotyledons were considered outside the host range of *Agrobacterium*. However, advances in understanding the biology of the infection process, availability of gene promoters suitable to monocotyledons (Wilmink *et al.* 1995) as well as selectable markers have improved transformation of monocotyledons (Smith and Hood 1995). Transgenic plants of rice (Hiei *et al.* 1994), maize (Ritchie *et al.* 1993) and sugarcane (Arencibia *et al.* 1998) have been produced *via A. tumefaciens*-mediated transformation.

4.2 *A. tumefaciens* -mediated transformation using green fluorescent protein (GFP) as the reporter gene

Marker genes are included on many transformation vectors, to allow independent verification of the transformed status of tissues growing on media containing selective antibiotics or herbicides. The most commonly used markers are, chloramphenicol acetyl transferase (CAT), β-glucuronidase (GUS), β-galactosidase, octopine synthase and nopaline synthase, Neomycin phosphotransferase-II (NPT-II) and luciferase. A specific use of a screenable marker is as a reporter gene; both in the development of transformation systems using transient expression assays to monitor success, or to test DNA sequences which may be able to regulate gene expression in stably transformed

tissues. Screenable marker systems employ a gene whose protein product is easily detectable in the cell, either because it produces a visible pigment or because it fluoresces under appropriate conditions. Visible markers rarely affect the study of a trait of interest, but they provide a powerful tool for identifying transformed cells before the gene of interest can be identified. They can also identify the tissues that have (and have not) been transformed in a multicellular organism such as a plant.

A. tumefaciens mediated delivery of foreign genes into numerous plant species has been extensively described since Horsch *et al.* (1985). To increase the transformation efficiencies, much effort has been placed on understanding molecular mechanisms of T-DNA transfer (Holford *et al.* 1992, Fullner *et al.* 1996) with the goal of manipulating and controlling the transfer process. The use of green fluorescent protein (GFP) gene from the jellyfish (*Aquorea victorea*) provides a tool for monitoring pathogen infection in time without disturbing bacterium or the host tissue. No manipulation is required for the GFP visualization, hence the integrity of cell structure and morphology of the target tissue can be maintained. Visualization doesn't kill the cells. The fluorescence can be used to study the timing of gene expression *in vivo* (Chalfie *et al.*1994). GFP provides a non-destructive method for monitoring a wide range of cellular and subcellular activities (Haseloff *et al.* 1997). GFP detection involves excitation with light of the appropriate wavelength, e.g. blue light at 395 nm.

Plants are usually transformed with relatively simple constructs, in which the gene of interest is coupled to a plant promoter of viral origin. Some promoters confer constitutive expression while others may be selected to permit tissue specific expression. The cauliflower mosaic virus (CaMV) 35S RNA promoter is often used because it directs high levels of expression in most plant tissues (Walden and Wingender 1995).

The Cauliflower Mosaic Virus (CaMV) 35S promoter is a very strong constitutive promoter causing high levels of gene expression in dicot plants and it is one of the most widely used promoters for driving the expression of genes in transgenic plants. CaMV promoters are preferred over others, as it is not greatly influenced by environmental conditions. CaMV 35S promoter activity has been detected in even the cortex and vascular cylinder of the root. CaMV promoter activity shows in cells of the leaf epidermis, mesophyll and vascular bundle (Battraw and Hall, 1990). Histochemical

staining of the anther sections from the buds shows CaMV 35S promoter activity in the vascular bundle, stomium and tapetum (Wilkinson *et al.*, 1997). CaMV 35S promoter driven GFP expression was not detected during the early stages of embryogenesis, but observed in all cell and tissue types in the hypocotyls, cotyledon, stem, leaf, petiole, root and also in the floral parts of the plant (Sunilkumar *et al.*, 2002)

4.2.1 Review of literature

Extensive work has been done on transformation of papaya. Transformation has been achieved using *A. tumefaciens* and microprojectile bombardment (Fitch *et al.*1990, 1992, 1993, Cabrera *et al.* 1995, 1996, Mahon *et al.* 1996, Zhu *et al.* 2004) or through wounding by carborundum (Cheng *et al.* 1996). Different explants have been used for transformation. These include petiole (Yang *et al.* 1996), immature zygotic embryos (Fitch *et al.*1990, Cheng *et al.*1996), leaf disc, stem, petiole (Pang and Sanford 1988), somatic embryos (Mahon *et al.* 1996), root and stem segments (Ye *et al.*1991), hypocotyl sections (Zhu *et al.* 2004) etc. Zygotic embryos were the preferred explant for developing transgenic papaya *via* particle bombardment (Fitch *et al.* 1992).

Pang and Sanford (1988) were the first to describe transformation of Sunrise Solo and Kopoho Solo varieties of papaya. These authors used leaves, stem and petiole section as explants for transformation with *A. tumefaciens* strain GV3111 harbouring the plasmid pTiB6S3, pMON200. The plasmid contained a transitionally improved chimeric NOS/NPTII/NOS gene for Kanamycin resistance as a dominant selectable marker and tumour genes (tms/tmr) conditioning hormone independent growth. Although no transgenic plants were identified, a high rate (90%) of transformed callus was obtained.

Fitch *et al.* (1990) transformed the primary and secondary embryos of a highly embryogenic culture using particle bombardment method and GUS reporter gene in addition to NPTII gene as selectable marker. The screening of the transformants for the GUS gene was essentially a destructive process.

Fitch *et al.* (1993) used *A. tumefaciens* strain C58-Z707 for transformation of hypocotyl explants from papaya cv. Kopoho. The bacterium harboured the plasmids pGA482GG or pGA482GG/cpPRV-4 with GUS as the screenable marker in addition to the Kanamycin resistant gene NPTII. Two transformed lines were identified for each construct. However,

the authors concluded that putative transgenic embryos appeared 6-9 months after co-cultivation of the hypocotyls explants with *A. tumefaciens.* Similar tissues transformed *via* microprojectile bombardment regenerated in four months (Fitch *et al.* 1990, 1992). Yang *et al.* (1996) could produce transformed lines of papaya from *A. tumefaciens* infected petioles within 8 months of co-cultivation. They used the plasmid pBII21 containing NPTII as the selective marker gene and GUS as the reporter gene. The same year, Cabrera-Ponce *et al.* (1996) used *A. rhizogenes* LBA9402 harbouring the vector pBI121 to develop transgenic papaya plants of cv. 'Yellow large'. The vector carried NPTII and the GUS genes as selectable and screenable markers respectively. Although transformed papaya lines could be obtained by *A. rhizogenes* infection, the plants derived were not normal in morphology and also exhibited poor growth. Successful transformation of papaya with the PRSV viral replicase (RP) gene *via A. tumefaciens*-mediated transformation was reported by Chen *et al.* (2001). Methods and explants used for transformation of papaya are summarized in Table 4.1.

Table 4.1 Methods and explants used for papaya transformation

Explant used	Method used for transformation	Reference
Leaf disc, stem and petiole	*Agrobacterium*	Pang and Sanford (1988)
Immature zygotic embryos, Hypocotyl, Embryogenic calli	Particle bombardment	Fitch *et al.* (1990)
Root, stem segments	Microprojectile bombardment	Ye *et al.* (1991)
Immature zygotic embryo	Microprojectile bombardment	Fitch *et al.* (1992)
Hypocotyl	*Agrobacterium*	Fitch *et al.* (1993)
Immature zygotic embryo and embryogenic callus	Particle bombardment	Cabrera Ponce *et al.* (1995)
Leaf disc	*Agrobacterium*	Cabrera Ponce *et al.* (1996)
Petioles	*Agrobacterium*	Yang *et al.* (1996)
Immature zygotic embryo	*Agrobacterium* and wounding with Carborundum	Cheng *et al.* (1996)
Immature zygotic embryo and somatic embryo	Particle bombardment	Mahon *et al.* (1996)
Immature zygotic embryo	Particle bombardment	Cai *et al.* (1999)
Roots and Hypocotyl	*Agrobacterium*	Chen *et al.* (2001)
Somatic embryos	Microprojectile bombardment	Lines *et al.* (2002)
Hypocotyl	Microprojectile bombardment	Zhu *et al.* (2004)
Immature zygotic embryos	*Agrobacterium*	Our study

In the present study Green Fluorescent Protein (GFP) gene under the control of CaMV35S P in the pBIN 35S-mgfp5-ER vector was used for the transformation of papaya. Immature zygotic embryos were selected as explants.

4.3 Materials and Methods

4.3.1 ***Explant preparation***: Immature fruits of papaya (*Carica papaya* L.) cv. HoneyDew were collected from the field. Fruits were sterilized as given in Chapter2. Immature zygotic embryos were excised from seeds and used as explants for co-cultivation with *A. tumefaciens* (Figs. 4.1A, B).

4.3.2 Bacteriological methods

The plasmid pBIN 35S-mgf5-ER was introduced into *A. tumefaciens* strain LBA4404 containing the helper Ti plasmid by freeze thaw method (See Section 2.12.3, Chapter 2). The transformed cells were grown in YEB medium (Shaw 1988) containing 250 mg/l rifampicin and 50 mg/l Kanamycin.

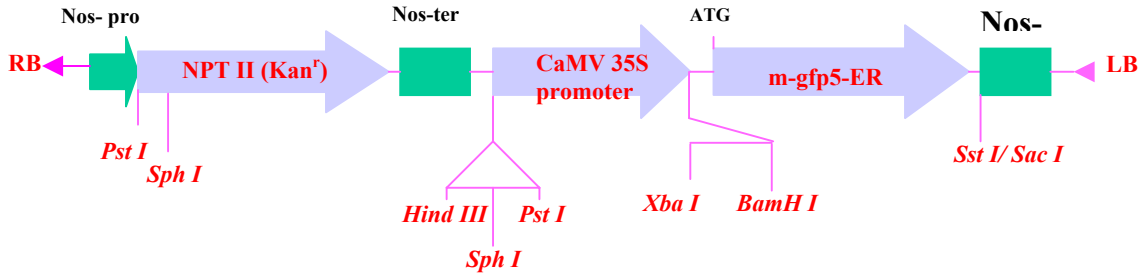


Fig. 4.2 pBINmGFP5-ER map

4.3.3 Growth media and culture conditions for *A. tumefaciens*

Growth media and culture conditions are described in Section 2.12.2.5, Chapter 2.

4.3.4 Regeneration media

Explants were cultured as described in Section 2.13, Chapter 2.

4.3.5 Determination of Kanamycin LD_{50} for papaya plants

The LD_{50} of papaya immature zygotic embryos for Kanamycin was determined as mentioned in Section 2.14, Chapter 2.

4.3.6 Co-cultivation of explants with *A. tumefaciens*

The immature zygotic embryo explants were co-cultivated with *A. tumefaciens* harbouring the pBINmGFP5-ER. The protocol is described in Section 2.15 Chapter 2.

4.3.7 Regeneration of somatic embryos and multiple shoots

The somatic embryos and multiple shoots regenerated from co-cultivated immature zygotic embryos was as described in Section 3.4.1.1 and 3.8.2 Chapter 3.

4.3.8 Rooting and hardening

Rooting and hardening procedure is described in Section 3.3.5 and 3.8.5 Chapter 3.

4.3.9 Fluorescence microscopy

Visualization of GFP fluorescence in plant tissues was achieved using a Leica Wild MPS 32 stereomicroscope (Leitz Wetzlar, Germany) fitted with G filter. The excitation wavelength was 395 nm and emission wavelength 509 nm.

4.4. DNA isolation

4.4.1 Plant DNA isolation

Genomic DNA was isolated from putative transformed papaya plants using the protocol of Lodhi *et al.* (1994) (Chapter 2 Section 2.21.1**)**.

4.4.2 Plasmid DNA isolation and preparation of probe

Plasmid DNA was isolated by the alkaline lysis method (Sambrook *et al.* 1989) (Section 2.21.2 Chapter 2). GFP gene fragment (880 bp) was excised using restriction enzymes X*ba* I and *Sac* I from the pBIN 35S mgfp5-ER plasmid gel purified (Section 2.21.2 Chapter 2) and used as hybridization probe.

4.4.3 Southern hybridization

The presence and integration of the GFP gene in the Kanamycin resistant papaya plants was analyzed by Southern blots (Southern 1975). Hybridizing bands were detected by 5 days exposure to Kodak X-MAT AR autoradiography film at -70 ^{0}C (See Chapter 2 Section 2.21.3.1). The genomic DNA from the untransformed and the putative transformed papaya. Plants were restriction digested with X*ba* I and *Sac* I, run on agarose (1%) gel and transfer to solid support (Hybond-N^{+} membrane) for Southern hybridization (Section 2.21.3.1 Chapter 2).

4.5 Results and Discussions

4.5.1 Determination of LD_{50} of papaya regenerants for Kanamycin

The LD_{50} for papaya transformants of Kanamycin, as selective pressure was determined by culturing 20 immature zygotic embryo explants in MSB medium with combination of BAP 4.44 µM: NAA 0.54 µM (designated as BN) or Picloram 4.14 µM (designated as P), supplemented with various concentrations of Kanamycin (25, 50, 75, 100, 200 mg/l).

4.5.2 Development of multiple shoots

4.5.2.1 ***Multiple shoot induction on selection medium****:*

The immature embryo explants showed green shoot initials at the apical meristimatic region of the explant (Fig. 4.2A) within two weeks of incubation. Multiple shoot formation occurred on the BN medium with Kanamycin 25 and 50 mg/l, while no response was seen on BN medium with 75, 100, 200 mg/l of Kanamycin. Explants on BN medium with 50 mg/l Kanamycin resulted in bleaching and death of the regenerants. Therefore 50 mg/l Kanamycin was used as the optimal selection pressure.

4.5.3 Development of somatic embryos

4.5.3.1 ***Somatic embryo induction on selection medium***: Somatic embryo induction from the meristimatic region of immature zygotic embryo explant within two weeks on P medium (Fig. 4.2B) with Kanamycin 25 and 50 mg/l, while no response was seen on P medium with 75, 100, 200 mg/l of Kanamycin. P medium supplemented with 50 mg/l

Kanamycin resulted bleaching and death of regenerants. Therefore 50 mg/l Kanamycin was used as the optimal selection pressure.

4.5.4 Transformation with *A. tumefaciens harbouring* pBIN 35S-mgf5-ER

The immature zygotic embryo explants infected with *A. tumefaciens* LBA4404 harbouring the binary vector pBIN 35S-mgf5-ER were inoculated on BN medium for multiple shoot induction and or on P medium for somatic embryogenesis. The media contained 50mg/l Kanamycin as the selection pressure.

4.5.5 Selection of transformed somatic embryos and multiple shoots

Eight hundred immature zygotic embryo explants were cultured on P and BN media containing *A. tumefaciens* LBA4404/ pBIN 35S-mgf5-ER and incubated for 72 h. After co-cultivation, the explants were washed with sterile distilled water, blot dry and transferred to P and BN medium containing 500 mg/l Cefotaxime and kanamycin 50 mg/l as selection pressure. One hundred and thirty-six explants inducing multiple shoots survived on BN medium supplemented with kanamycin 50 mg/l and ninety-eight explants inducing somatic embryos survived on P medium with Kanamycin 50 mg/l. The surviving somatic embryos and multiple shoots were randomly selected after eight weeks and scored for green fluorescence.

4.5.6 Visualization of green fluorescence

Upon random selection leaf petioles from multiple shoots (Fig. 4.3A) and somatic embryos (Fig. 4.3B) showed green fluorescence. All the multiple shoots and somatic embryos survived on Kanamycin selection medium showed green fluorescence. As the development progressed GFP fluorescence was observed in the epidermis and vascular tissue of leaf petiole (Fig. 4.3A) while control tissues showed red auto-fluorescence due to presence of chlorophyll. Plant tissues containing both chlorophyll and GFP appeared yellow as reported earlier (Garabagi and Strommer 2000). GFP expression was not detected during the early stages of embryogenesis in cotton (Sunilkumar *et al.* 2002), on the contrary GFP expression was detected during early stages of embryogenesis in papaya. GFP gene expression was detected at the juvenile stages *viz*. at early stages of

somatic embryogenesis in papaya. 35S promoter driven GFP gene activity was also detected in the epidermis and the vascular tissues of the leaf petiole.

4.5.7 Rooting and hardening

Germination and development of plantlet took place within 12-16 weeks (Fig. 4.4A-C). These plantlets were transferred to pots containing soil and sand mixture (3:1) for hardening in green house. About 80-85% plantlets were survived under green house condition.

4.6 Preparation of probe

GFP gene fragment (880 bp) was excised using restriction enzymes *xba* I and *sac* I from the pBIN 35S mgfp5-ER plasmid (Fig.4.5) and used as hybridization probe (Section 2.21.2 Chapter 2).

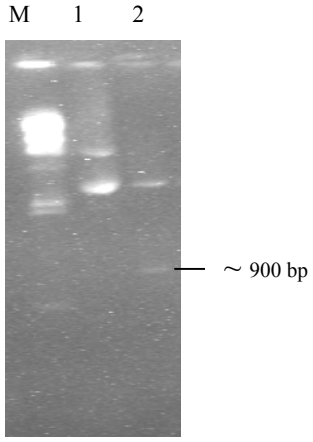


Fig. 4.5 Characterization of pBIN 35S-mgf5-ER restriction digested with *Xba* I and *Sac* I

Lane M: 1 kb marker, lane 2: Uncut pBIN 35S-mgf5-ER, Lane 3: restriction digested with *Xba* I and *Sac* I

4.7 Southern analysis

Putatively transformed papaya plants regenerated either *via* somatic embryogenesis or multiple shoot induction was restriction digested with *Xba* I/ *Sac* I and analysed to confirm the integration of 880 bp GFP gene. While no hybridization signal was observed

in the non-transformed control plants (Fig.4.6), the putative transformants showed the presence of an ~ 800 bp band.

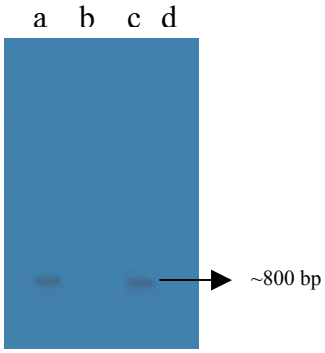


Fig.4.6. Hybridization analysis of representative untransformed and putative GFP transformed plants of papaya

a. Transformed multiple shoot tissue
b. Control multiple shoot tissue
c. Transformed somatic embryo tissue
d. Control somatic embryo tissue

4.8 Conclusion

In the present study, an attempt was made to standardize a protocol for *A. tumefaciens* mediated transformation system for papaya using GFP as the screenable marker. The immature zygotic embryo explants were co-cultivated with *A. tumefaciens* LBA4404 strain harbouring pBIN-mgfp5-ER vector. The integration of the GFP gene in somatic embryos and multiple shoots was confirmed by both fluorescence and Southern analysis.

A. tumefaciens mediated transformation has been used the tool for transformation studies. This is because of the relative ease and precision of gene transfer to intact, regenerable explants. This attribute was to confirm the versatility and usefulness of GFP as the screenable marker and tissue specific expression of 35S promoter in papaya transformation work. GFP is increasingly being used in plant biology from the cellular level to whole plant level. GFP is the first truly *in vivo* reporter system useful in whole plants (Leffel *et al.* 1997). Insertion of the GFP gene into plant viruses has allowed the direct observation of viral movement through host plants during infection (Baulcombe *et*

al. 1995, Casper and Holt 1996). Another use of GFP in whole plants is to use it as an *in vivo* screenable marker to monitor transgene spread in the environment.

One added advantage in the non-destructive means of analysis of GFP. 35S promoter driven GFP reporter gene is a useful, non-destructive, inexpensive and standard screenable reporter marker because of its easy detection without harmful effect on the living tissue. This work provides simple and rapid protocol for the genetic transformation.

CHAPTER 5

Isolation and cloning of PRSV Coat Protein gene and its Characterization

5.1 Introduction:

Viruses are sub-microscopic, obligate intracellular parasites that infect living organisms. Viruses are as ancient as life itself, leave no fossils, but their rapid replication, large population, high mutation rate, extensive genetic recombinations, gene duplications and ancient origin contribute to their variability. They are not functionally active outside of their host cells. Developing countries lack resources to control or limit damage caused by plant viruses, hence, suffers the most economic losses. The severe losses of papaya due to Papaya ringspot virus (PRSV) have lead farmers to destroy the crops and or switch over to other crops. Virus infected crops cannot be cured and may be protected using traditional cross protection methods. Using conventional methods of cross protection it is difficult to control the disease. Alternative method is to use DNA sequences from the virus and express these in papaya so as to impair virus assembly, or replicase or movement. Posttranscriptional gene silencing (PTGS) may also be used to develop PRSV resistant papaya plants. As viruses are continuously changing, exploring new sequence, space to adapt to the alterations in their hosts, so resistance-breaking strains appear.

5.1.1 Potyviridae

Potyviridae is the largest single taxonomic group of plant viruses, and the subject of more scientific research than any other taxonomic group of plant viruses except perhaps the Gemini viruses. The potyviruses, and specifically the aphid-transmitted viruses of the genus *Potyvirus,* are one of the most successful group of plant pathogens in the world. The genus has been claimed to be almost as ancient as flowering plants, and has worldwide distribution. Another intriguing aspect of emerging potyviral plant diseases is the appearance of hitherto latent virus infections.

5.1.2 *Papaya ringspot virus (PRSV)*

Papaya ringspot virus (PRSV) is a serious threat to papaya cultivation (Purcifull 1972). It has been reported from N. America (Conover 1964), Australia, Africa (Kulkarni 1970), Hawaii (Holmes *et al.* 1948), the Caribbean (Acuna and Zayas 1939), South-East Asia, China, Japan, Philippines and India (Yeh *et al.* 1992, Wang *et al.* 1994, Jain *et al.* 1998,

2004). Vigor of the plants (Figs. 5.1A, B) and fruit set are hampered due to the viral attack. The disease derives its name from the striking symptoms that develop on the fruit (Fig.5.2A) and on the leaves (Fig.5.2B). It is in the form of concentric rings and spots or c-shaped markings, dark greener than the background green fruit color. Fruit quality, particularly flavor and shape are adversely affected. The virus is transmitted by aphids.

Papaya ringspot virus (PRSV) is a member of the genus *Potyvirus* of the family *Potyviridae*, with flexuous particles of 780X12 nm and a genome consisting of single stranded RNA of positive polarity (Purcifull *et al.* 1984a,b, Shukla *et al.* 1991, Riechmann *et al.* 1992, Yeh *et al.* 1992). Each virion is made up of 2000 units of a single structured protein surrounding one molecule of single stranded RNA that is on an average 10,000 nucleotides long and of messenger polarity. Definitive potyviruses are transmitted in a non-persistent manner by many aphid species, while some of the potyviruses have fungus, mite or whitefly vectors (Purcifull *et al.* 1984, Shukla *et al.* 1991). All potyviruses examined so far are reported to induce the characteristics pinwheel or scroll shaped inclusion bodies in the cytoplasm of the infected cells (Edwardson 1974). Virions are found in all parts of the host plant. Papaya ringspot viruses are grouped into two types, PRSV-P and PRSV-W. PRSV-P type infects papaya and cucurbits, but watermelon mosaic virus 1 (PRSV-W) infects cucurbits, but not papaya. The two types are very closely related, except for the inability of the type W to infect papaya.

The nucleotide sequences of the 3'-terminal regions of papaya ringspot virus strains W and P genome were first reported by Quemada *et al.* (1990). The complete nucleotide sequence and genetic organization of papaya ringspot virus RNA reported by Yeh *et al*. (1992). The PRSV-P isolate from Thailand (PRSVthP) has also been characterized (Charoensilp *et al.* 2003). The nucleotide sequence of the Coat Protein (CP) gene and the 3' untranslated region of papaya ringspot virus W were reported by Bateson and Dale (1992) and Bateson *et al.* (1994). Thai isolate of type W has been completely sequenced (Attasart *et al.* 2002). Comparative development and impact of the transgenic papaya in Hawaii, Jamaica and Venezuela was studied by Fermin *et al.* (2005). Papaya was transformed using modified and unmodified PRSV CP gene in sense (S-CP), antisense (AS-CP), frameshift mutation (FS-CP) and sense orientation mutant (SC-CP). Papaya plants expressing the FS-CP and SC-CP were highly fertile while those expressing the S-

CP and AS-CP transgene were practically infertile (Davis and Ying 2004). PRSV Coat protein gene isolated and sequenced from various geographical locations (Bateson *et al.* 1994, Wang and Yeh 1997, Wang and Yeh 1998, Natsuaki and Bajet 2001, Bateson *et al.* 2002, Lima *et al.* 2002, Bau *et al.* 2003), show both nucleotide and amino acid variability (Roy and Jain 2002, Jain *et al.* 2004, Hema and Prasad 2004, Tripathi *et al.* 2004).

5.1.3 Characterization of PRSV

The potyvirus genome comprising of a ssRNA has a protein (VPg) covalently attached to its 5' end and a single open reading frame (ORF) coding for a large polyprotein precursor and a poly (A) tail at the 3' end. This ORF is flanked by two non-coding regions (NCR) at the 5' and the 3' ends (Hari *et al.* 1979, Hari 1981, Riechmann *et al.* 1992). The average PRSV genome length is 10000 nucleotides. It is a positive strand RNA, encoding a large polyprotein of 381 KDa (Yeh and Gonsalves 1985, Yeh *et al.* 1992). The polyprotein is processed into 8 or 9 final products by three virus-encoded proteinases (P1, HC-Pro and NIa). Like other potyviruses the proposed genetic organization of PRSV (Fig.5.3) is VPg-5' leader-P1- HC Pro-P3-CI-P5-NIa-NIb-CP-3'noncoding region-poly (A) tract (Yeh *et al.* 1992). The 63 kDa P1 protein of PRSV is the most variable protein among the potyviruses, and which is 18-34 kDa larger than those of the other potyviruses (Yeh *et al.* 1992).

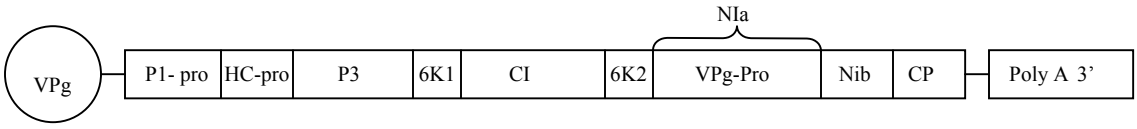


Fig.5.3 PRSV genome organization

5.1.4 Infection cycle of PRSV

Potyviruses normally enter their hosts *via* the stylet of the aphid. The acquisition of the virus by aphids may take seconds, and the loss of virus transmissibility occurs after a short time (minutes). Virus acquisition by aphids is dependent on an N-terminal amino

acid motif Asp-Ala-Gly in the CP (Shukla *et al.* 1994), as well as the N-terminal motifs Lye-Ile-Thr-Cys (Atreya *et al.* 1992, Blanc *et al.* 1998, Sasaya *et al.* 2000) and Pro-Thr-Lys (Peng *et al.* 1998) in the non-structural helper component protein (HC-Pro). HC-Pro forms a connection or 'bridge' between the virus particles and the inner surface of the aphid maxillary stylets (Blanc *et al.* 1998). The possibility that HC-Pro enables conformational changes in the CP or an aphid factor in the stylet, thereby enabling virus binding to the stylet is not excluded (Salmon and Bernardi 1995). Whatever the mechanism, upon feeding on the plant, the aphid regurgitates some saliva and by this process inoculates the plant with the virus (Martin *et al.* 1997).

As the virus enters the cell it starts to disassemble and being recognized by the host cell as an endogenous mRNA, is probably simultaneously translated in a process called "co-translational disassembly" (Shaw *et al.* 1986). By the time virus has fully disassembled, the first viral proteins have already been produced and are ready to start with the viral RNA. Some of the known functions of potyviral protein are tabulated below (Table 5.1).

Table 5.1 Known functions of *potyviral proteins*:

Protein	Possible Functions	Reference
P1	Proteinase	Carrington *et al.* 1989, Carrington *et al.* 1990
	Modulator of gene silencing	Anandalakshmi *et al.* 1998, Brigneti *et al.* 1998, Kasschau and Carrington 1998
	Replication/ Virus propagation	Atreya *et al*. 1992, Klein *et al.* 1994, Kasschau and Carrington 1995, Verchot and Carrington 1995, Kasschau *et al*. 1997, Merits *et al*. 1999, Kekararainen *et al.* 2002
	Cell-to-cell movement	Revers *et al*. 1999
HC-Pro	Proteinase	Carrington *et al.* 1989, Revers *et al*. 1999
	Aphid transmission	Atreya *et al.* 1992, Sasaya *et al.* 2000
	Seed transmission	Wang and Maule 1994
	Cell-to-cell and systemic movement	Klein *et al.* 1994, Cronin *et al*. 1995, Kasschau *et al.* 1997, Rojas *et al*. 1997
	Suppressor of gene silencing	Anandalakshmi *et al.* 1998, Brigneti *et al.* 1998, Kasschau and Carrington 1998
	Replication/virus propagation	Atreya *et al*. 1992, Klein *et al.* 1994, Kasschau and Carrington 1995, Kasschau *et al*. 1997, Kekararainen *et al.* 2002
P3	Replication/virus propagation	Atreya *et al*. 1992, Klein *et al.* 1994, Kasschau and Carrington 1995, Kasschau *et*

		al. 1997, Merits *et al*. 1999, Kekararainen *et al.* 2002
6K1	Replication/virus propagation	Riechmann *et al.* 1992, Kekararainen *et al.* 2002,
CI	RNA helicase	Lain *et al.* 1990
	Cell- to- cell movement	Rodriguez *et al*. 1997, Carrington *et al.* 1998, Roberts *et al.* 1998
	Replication/virus propagation	Lain *et al.* 1990, Eagles *et al*. 1994, Klein *et al*. 1994, Kekararainen *et al.* 2002
6K2	Long distance movement	Rajamäki and Valkonen 1999
	Replication/virus propagation	Schaad *et al.*1997a, Kekararainen *et al.,* 2002
NIa/ VPg	Binds to initiation facto eIF (iso) 4E	Wittman *et al.* 1997, Schaad *et al.* 2000
	Cell-to-cell and systemic movement	Schaad *et al.* 1997a,b
	Replication/virus propagation	Schaad *et al.* 1996 Merits *et al*. 1998, Kekararainen *et al.* 2002
NIa/ Pro	Proteinase	Dougherty *et al.* 1989
	Replication/virus propagation	Daros and Carrington 1997, Kekararainen *et al.* 2002
NIb	RNA-dependent RNA polymerase	Hong and Hunt 1996
	Replication/virus propagation	Hong and Hunt 1996, Kekararainen *et al.* 2002
CP	Encapsidation of RNA	Jagdish *et al.* 1993
	Cell-to-cell and systemic movement	Dolja *et al*. 1994, 1995, Rojas *et al*. 1997, Lopez-Moya and Pirone 1998
	Aphid transmission	Atreya *et al.* 1995
	Seed transmission	Wang and Maule 1994
	Replication/virus propagation	Haldeman *et al.* 1998, Merits *et al*. 1998, Kekararainen *et al.* 2002

After infection of the inoculated cell, the virus moves to the neighboring cells (Cell-to-cell, or short distance movement), and into the vascular tissue, where it spreads throughout the plant following the source-sink stream (systemic or long distance movement). Both HC-Pro and CP have been shown to be able to increase the size inclusion limit of plasmodesmata in mesophyll cells (Rojas *et al.* 1997). The CI is transiently located to the plasmodesmata at the infection front (Roberts *et al.* 1998).

Many movement proteins are suppressors of host cell defense responses. There is also increasing evidence that a multitude of plant-encoded mRNAs travel through phloem. Some virus proteins facilitate cell-to-cell or systemic movement of viruses by suppressing cell-to-cell communication of plant defense responses, rather than by actively mediating transport through plasmodesmata (Carrington 1999). Upon infection potyviruses can cause the complete shut down, or up-regulation of several host genes (Aranda *et al.* 1996, Escaler *et al.* 2000, Wang and Maule 1995). Viral replication is restricted to a narrow zone of cells at the infection front (Aranda *et al.* 1996, Wang and Maule 1995).

As with conventional cross protection (Wang *et al.* 1987, Yeh *et al.* 1988), transformation of papaya with PRSV-CP gene to produce genetically engineered virus resistance will preserve the genotype and horticultural merits of the original cultivar. There are a number of important ways in which the transgenic plants may prove to be superior to cross protected plants for example stable incorporation of the CP gene in a chromosome of the transformed plant will result in Mendelian inheritance of virus resistance in the progeny, eliminating the need to inoculate each generation with a mild protective viral strain, expression of the CP gene in every cell of the transformed plant should reduce the frequency of the breakdown in resistance such as may occur in conventionally cross protected plants due to poor systemic spread of the mild resistance. The absence of a replicating albeit mild infection in the genetically engineered plants mean that there should be no disease symptoms or yield reductions, and there will be no possibility of a mild virus escaping to infect other crops.

Several approaches have been taken to introduce virus resistance into desired crop plants. These are cross protection, generating transgenic plants which produce components of the virus that confer cross protection without causing viral disease (processes involving virus coat protein, replicase, movement protein, transmission protein, disease attenuation with satellite RNA, defective interfering RNA and antisense RNA). Coat protein (CP) mediated resistance has been the most consistent and effective of these (Beachy *et al.* 1990). Plants transformed with the CP gene of a pathogenic virus may in some cases be virtually immune to infection by the same or a closely related virus or may show delayed or reduced symptom expression. CP mediated resistance has been successful in tests

involving different groups of plant viruses (Lawson *et al.* 1990, Hoekema *et al.* 1989). Coat protein mediated protection (CPMR) is found to be very successful for viruses with single stranded RNA genome. Hence, there is reason to believe that this approach will be useful in improving virus resistance in Indian papaya varieties.

Genetically engineered Coat protein gene immunity to PRSV in Australian papaya cultivars has been reported (Lines *et al.* 2002) while Pathogen derived resistance (PDR) to control PRSV in Hawaii resulting in acquired immunity against the attacking virus has also been achieved (Ferreira *et al*. 2002).

Two transgenic cultivars of papaya, Rainbow and SunUp, resistant to PRSV in Hawaii have been commercialized (Gonsalves 1998, Manshardt 1999, Chiang *et al.* 2001). SunUp was derived from transgenic papaya line 55-1 (Tennent *et al.* 2001) using a mild mutant of PRSV-HA (Yeh and Gonsalves 1984). Rainbow is a hybrid of SunUp and the non-transgenic cultivar 'Kopoho'. It is, therefore, hemizygous for the CP gene (Mansherdt 1999). Tennant *et al.,* (1994, 2001) reported that Rainbow and the hemizygous plants of line 55-1 are resistant to these PRSV isolates from Hawaii that share at least 97% nucleotide identity to the CP transgene but are susceptible to isolates from outside Hawaii that have 89-94% nucleotide identity to the transgene. A relationship between papaya ringspot virus coat protein transgene expression levels and the age dependent resistance in transgenic papaya Rainbow and SunUp has been established (Gaskill *et al.* 2000). PRSV-resistant papayas may also be generated with the introduction of the PRSV replicase gene (Chen *et al.* 2001) or by blocking the viral movement.

The present Chapter deals with the isolation, cloning and characterization of the PRSV-CP gene from Pune, India.

5.2 Materials and Methods

Fruits (Fig.5.2A) and leaves (Fig.5.2B) of papaya cv. Honey Dew showing green islands between veins, reduced size of leaves and dark circles on the surface of fruit rind were collected and used for total RNA isolation. Details of total RNA isolation protocol are described in Section 2.19.3 in Chapter 2. The RNA was used for PCR based amplification of the PRSV-CP gene.

PCR primers were synthesized from the consensus sequence from reported protein Coat Protein (CP) gene. The optimum length of a primer depends upon its (A+T) content. The optimal length of PCR primers is 18-30 bp. Primers with melting temperatures in the range of 52-58 ^{o}C generally used. Primers with melting temperatures above 65^{o}C have a tendency for secondary annealing. The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be 40-60% to avoid non-specific annealing. Mg^{2+} ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of $MgCl_2$ has to be selected for each experiment. The concentration of each dNTP in the reaction mixture is usually 200µM. Usually 1-1.5u of *Taq* DNA Polymerase are used in 50µl of reaction mix. Usually the optimal annealing temperature is 5°C lower than the melting temperature of primer-template DNA duplex. Incubation for 0.5-2min is usually sufficient. Usually the extending step is performed at 70-75°C. The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. If the initial quantity of template DNA is higher, 25-35 cycles are usually sufficient. After the last cycle, the samples are usually incubated at 72°C for 5-15 min to fill-in the protruding ends of newly synthesized PCR products. Melting Temperature (T_m) is the temperature at which one half of the DNA duplex will dissociate and become single stranded. Annealing temperature is generally 5^{o}C lower than the estimated melting temperature.

5.3 Results and Discussions

5.3.1 Total RNA observation

Denaturing formaldehyde-formamide agarose (1%) gel electrophoresis of the total RNA samples prepared from PRSV infected papaya leaves was run in 1X MOPS/EDTA (50mM MOPS pH 7.0, 1mM EDTA pH 7.5). The gel upon visualization showed several fluorescent bands. The RNA integrity was judged by the sharpness and presence of 28S and 18S ribosomal RNA bands (Fig.5.3).

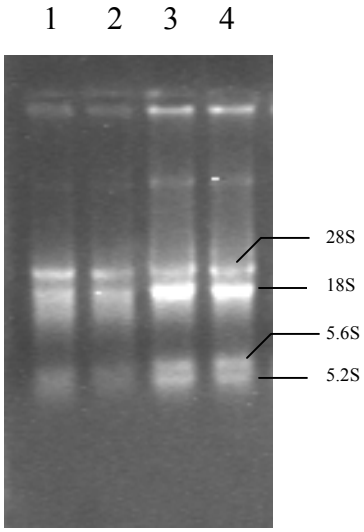


Fig.5.3. Total RNA isolation from PRSV infected leaves: a representative gel picture

Lane 1-4 total RNA isolated from PRSV infected leaves

5.3.2 RT PCR

RT-PCR methodology was adopted to generate cDNA fragments for specific sequences and to amplify them (Kawasaki 1990). In this method the first strand was synthesized by reverse transcriptase using mRNA as the template. First strand served as the template for the second strand synthesis. The two strands then undergo routine exponential amplification reaction. In the present study primers for RT PCR were designed on the basis of available sequence data on the coat protein gene of PRSV (Quemada *et al.* 1990, Slightom 1991).

The following primers were used for RT-PCR

Set 1:

Forward primers PRSV-CP-F-I: 5'd(TATGGATCCTCCAAGAATGAAGCT)3' Tm=64^{o}C

PRSV-CP-F-II: 5'd(TATGGATCCAGTCCAAAAATGAAGCTG) 3' Tm=66^{o}C

Reverse primer

PRSV-CP-R-I: 5'd(TATGGATCCTTAGTTGCGCATACC) 3' Tm=63^{o}C

Set 2:

Forward primer PRSV -FIII:

5'd(GATCCATGCTGAGAGGTACATTTCAAGAGAATGTACC
TCTCAGTAGCATTTTTTTGCTAGCG) 3' Tm=71^{o}C

Reverse primer

PRSV-RIII:

5'd(AATTCGCTAGCAAAAAAATGCTACTGAGAGGTACATTCTCTTG AAATGTAC CTCTCAGTAGCATG) 3' Tm= 70°C

5.3.4 RT-PCR Reaction

First strand was synthesized using total RNA and RT-PCR reaction was set up as given below.

First strand synthesis:

Total RNA 2 µg

Oligo-dT primers 25 ng/µl

RNase free water

Keep at 70 °C for 10 min. And then add following:

dNTPs	2 µl (0.2 mM)
$MgCl_2$	1 µl (1 mM)
Taq Pol buffer 10X	2 µl
Forward primer	1µl (8 picomoles)
Reverse primer	1 µl (8 picomoles)
RT/TAQ (10 units/µl)	0.5 µl

Water to make volume to 20 /µl.

The First strand synthesis reaction was set up as below and run for 35 cycles.

95°C

70°C / 10min \ 4 °C 5min / 42 °C /5 min / 30 min \ 4 °C ∞

Second strand synthesis:

Second strand was synthesized using first strand as template and routine PCR reaction was set as given below and run for 35 cycles.

Template from First strand synthesis (10ng)	5 µl
dNTPs	2 µl (0.2 mM)

$MgCl_2$	1 µl (1 mM)
Taq pol buffer 10X	2 µl
Forward primer	1 µl (8 picomoles)
Reverse primer	1 µl (8 picomoles)
Taq Polymerase (3 units/µl)	0.3 µl

Water to make up volume to 20 µl

The PCR reaction was set up as below and run for 35 cycles.

$$\underline{95^oC} \;/\; 5min \;|\; \underline{95^oC} \;1min\; \backslash\; \underset{30\ sec}{\underline{50^oC}} \;/\; \underline{72^oC} \;2min\; |\; \underline{72^oC} \;6\ min\; \backslash\; \underset{\infty}{\underline{4^oC}}$$

The RT-PCR amplified product using primer sets FI: RI, FII: RI, FI: FII: RI, FIII: RIII was run on 1X TAE agarose gel (1%). A ~1.0 kb fragment was observed (Fig.5.4). The amplicon was eluted from the gel (See Chapter 2) and cloned in pGEM-T Easy vector (Fig.5.5). The clone was designated as pNRSKR and was transformed into *E. coli* DH5-α cells.

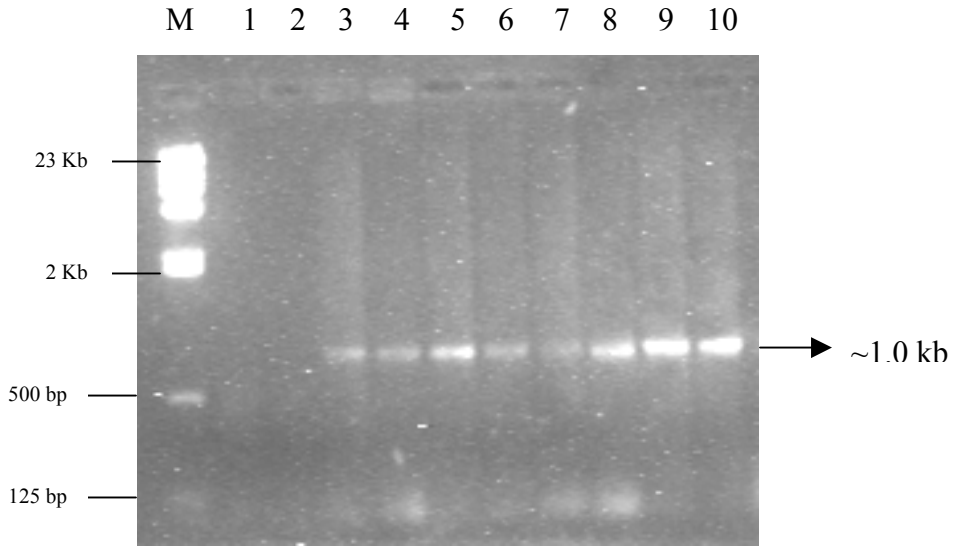


Fig.5.4. RT-PCR amplified products

Lane M: λ Hind dIII marker, Lane 1&2 uninfected plants, Lane 3-10 PRSV infected plants (Lane 3&4 primers FI: RI; Lane 5&6 primers FII: RI; Lane 7&8 primers FI: FII: RI; Lane 9&10 FIII: RIII)

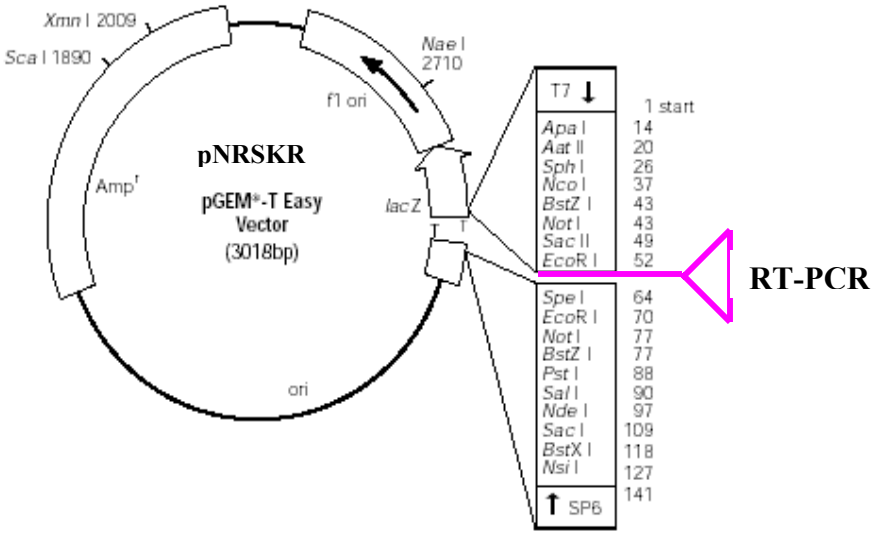


Fig.5.5 Plasmid map of pNRSKR

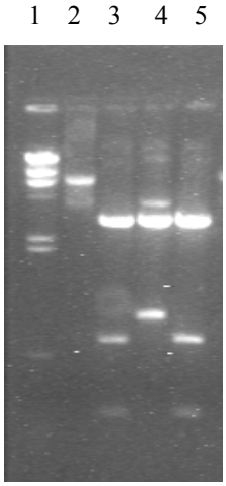


Fig.5.6 Characterization of pNRSKR

Lane 1: λ-*Hind* III marker, Lane 2: pNRSKR uncut, Lane 3 & 5: pNRSKR digested with *EcoR* I, Lane 4: *Not* I

5.3.6 Restriction mapping of pNRSKR

Plasmid DNA pNRSKR was digested with different restriction enzymes singly and in combinations to restriction map the insert (Fig.5.7 and 5.8). The restriction enzymes used were *Kpn* I, *Hinc* II, *Bam* HI, *Nsi* I, *Nco* I, *Sph* I, *EcoR* I, *Pst* I, *Sal* I, *Sac* I, *Nde* I, *Not* I,

Pvu II, *Sac* II, *Spe* I, *Xho* I (Table 5.2). The enzymes *Kpn* I, *Spe* I, *Bam* HI, *EcoR* I, *Hinc* II, *Sal* I, *Sac* I, Pvu II, *Xho* I, *Sac* II, and Spe I cut the insert once.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

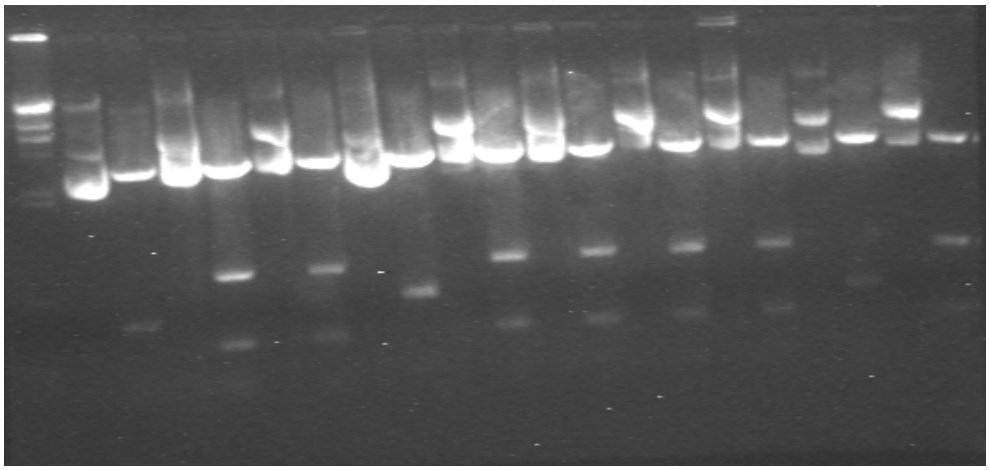


Fig.5.7. Restriction mapping of pNRSKR

Lane M: λ-*Hind* III marker, Lanes 1,3,5,7,9,11,13,15,17,19: Uncut pNRSKR, Lane 2: *Not* I, Lane 4: *EcoR* I, Lane 6: *Nco* I, Lane 8: *Nde* I, Lane 10: *Nsi* I, Lane 12: *Sph* I, Lane 14: *Spe* I, Lane 16: *Pst* I, Lane 18: *Sal* I, Lane 20: *Sac* I

Table 5.2 Summary of restriction digestions of pNRSKR

Sr.No.	Restriction sites	No. of fragments obtained	Sites in insert	Sites in vector
1	BamH *I*	1	1	0
2	EcoR *I*	3	1	2
3	Hinc *II*	1	1	0
4	Kpn *I*	1	1	0
5	Nco *I*	2	1	1
6	Nde *I*	2	1	1
7	Not *I*	2	0	2
8	Nsi *I*	1	0	1
9	Pst *I*	1	0	1
10	Pvu *II*	1	1	0
11	Sac *I*	2	1	1
12	Sac *II*	3	2	1
13	Sal *I*	1	0	1
14	Spe *I*	3	1	1
15	Sph *I*	2	1	1
16	Xho *I*	1	1	0

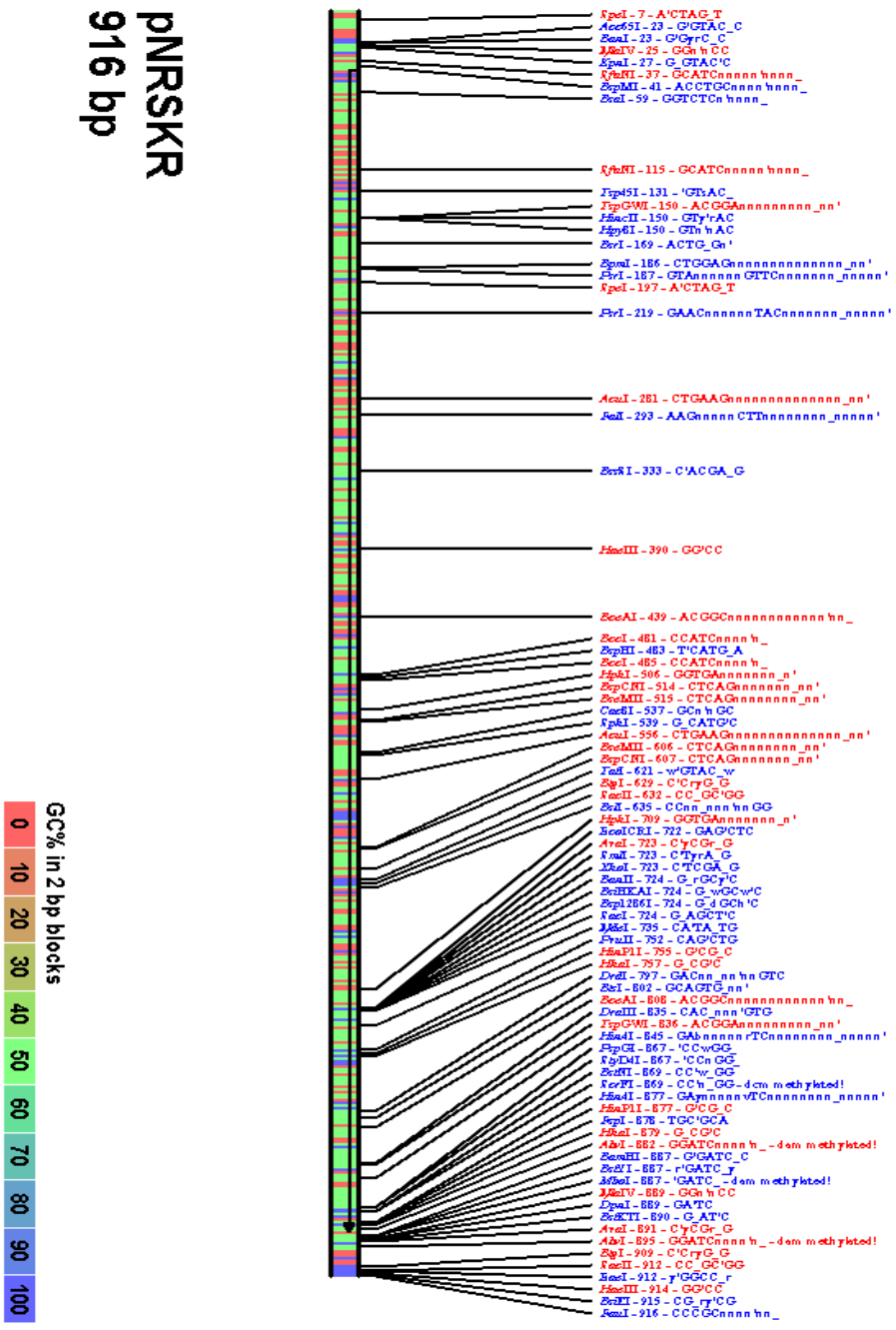


Fig. 5.8 Restriction map of pNRSKR

5.3.7 DNA Sequencing

The ~1.0 kb insert pNRSKR was sequenced bidirectionally by Beckman Coulter CEQ™ 8000 Genetic Analysis System using CEQ™ DTCS Quick Start Kit Dye Terminator Cycle sequencing kit provided by the manufacturer**.** Open Reading Frame (ORF) and restriction analysis was done using pDRAW32 version 1.1.61**.** Sequence comparisons and alignments were performed with the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information). Protein parameters were determined by using software ExPasy (http://au.expasy.org/).

Sequence analysis

The insert was determined to be 858bp in size (Fig. 5.7). This was deposited with NCBI GenBank and is available under the accession No. **DQ192587**.

```
  1 TCCAAGAATG AAGCTATGGA TGCAGGTCTC AATGATAAGC TGAAAGAGAA
 51 GGAAAAAGAA GATAAAGAGA AAGAAAAAGA AAAGAAAGAA AAGAAGGATG
101 CTAGTGACGG AAGTGATGTG TCAACTAGCA CAAAAACTGG AGAGAGAGAT
151 AGAGATGTCA ATGCTGGAAC TAGTGGTACA TTCACAGTTC CAAGAATTAA
201 GTCATTTACT GATAAAATGA TTCTGCCCAG AATTAAGGGA AAAGTTATCC
251 TTAATTTGAA TCATCTTCTT CAGTATAATC CACAGCAAAT TGACATCTCA
301 AACACTCGTG CCACACAATC ACAGTTTGAG AAGTGGTATG AGGGAGTGAG
351 GAATGATTAT GGCCTTACTG ATAATGAAAT GCAAGTGATG TTAAACGGCT
401 TAATGGTTTG GTGTATTGAA AATGGTACAT CCCCAGACAT ATCTGGTGTC
451 TGGGTCATGA TGGATGGTGA AACTCAGGTC GATTATCCAA TTAAACCGTT
501 AATTGAGCAT GCAACTCCTT CATTTAGGCA AATCATGGCT CACTTCAGTA
551 ACGCGGCAGA AGCATATATC GCAAAACGAA ATGCAACTGA GAAGTACATG
601 CCGCGGTATG GAATCAAGAG AAATTTGACT GACATTAGCC TCGCTAGATA
651 TGCTTTCGAT TTCTATGAGG TGAATTCAAA AACACCTGAT AGAGCTCGAG
701 AGGCTCATAT GCAGATGAAA GCAGCTGCGC TGCGGAACAC AAATCGTAGA
751 ATGTTTGGAA TGGACGGCAG TGTCAGTAAC AAGGAAGAAA ACACGGAGAG
801 ACACACAGTG GAAGATGTCA ATAGAGACAT GCACTCTCTC CTGGGTATGC
851 GCAAC
```

Fig. 5.8 Nucleotide sequence of pNRSKR

5'3' Frame 1

```
SKNEAMDAGLNDKLKEKEKEDKEKEKEKKEKKDASD
GSDVSTSTKTGERDRDVNAGTSGTFTVPRIKSFTDK
MILPRIKGKVILNLNHLLQYNPQQIDISNTRATQSQ
FEKWYEGVRNDYGLTDNEMQVMLNGLMVWCIENGTS
PDISGVWVMMDGETQVDYPIKPLIEHATPSFRQIMA
HFSNAAEAYIAKRNATEKYMPRYGIKRNLTDISLAR
YAFDFYEVNSKTPDRAREAHMQMKAAALRNTNRRMF
GMDGSVSNKEENTERHTVEDVNRDMHSLLGMRN
```

Fig. 5.9 Deduced Amino acid sequence of pNRSKR

Sequence analysis of the pNRSKR, 858bp by BLAST analysis revealed similarity with the CP gene of PRSV-P isolate. pNRSKR with BLAST and ClustalW Multiple alignment (Fig 5.10) showed 88-94% nucleotide sequence similarity with hundred and ten references (Genbank accessions) of PRSV-P strain. The gene showed 93%-97% homology with the virulent PRSV-P strains from Indian (GenBank Acc No. AY238884: 2004; GenBank Acc No. AY238880: 2004; GenBank Acc No. AY458618: 2004; GenBank Acc No. AY687386: 2004). It showed only 88% homology with one Australian isolate reported by Bateson *et al.,* (GenBank Acc No. AF506860: 2002). Other Asian and Australian and American isolated from Brazil, Mexico and United States showed 87-92% homology. The sequence similarity with the PRSV-W was 90% (GenBank Acc No. AY010722: 2002) and 91% (GenBank Acc No. AY027810: 2002). The sequence similarity was 90% with the Hawaii isolate (papaya ringspot virus PRSV-HA, Hawaii isolate GenBank Acc No. S46722).

```
AY238880        ----------------------------TCCAAAACTGAAGCGGTGGATGCAGGTCTCAA
AY238884        ----------------------------TCCAAAACTGAAGCGGTTGATGCAGGTCTCAA
AY458618        ----------------------------TCCAAAACTGAAGCGGTTGATGCAGGTCTCAA
pNRSKR          GAATTCACTAGTGATTATTCGGGGTACCTCCAAGAATGAAGCTATGGATGCAGGTCTCAA
DQ077175        ----------------------------TCCAAGACCGAAGCGGTGGATGCGGGTCTCAA
AY687386        ----------------------------TCCAAGAATGAAGCTGTGGATGCTGGTCTCAA
D00595          ---------------------------AGTCCAAGAATGAAGCTGTGGATGCTGGTTTGAA
NC_001785       ----------------------------TCCAAGAATGAAGCTGTGGATGCTGGTTTGAA
AY238881        ----------------------------TCCAAAAATGAAGCCGTGGAAGCTGGCTTGAA
AY491011        ----------------------------TCAAAGGTTGATGCTGTAGATGCGGGCTTAAA
AY238883        ----------------------------TCGAAGGCTGAGGCTGTGGATGCGGGCTTAAA
AY458620        ----------------------------TCGAAGGCTGAGGCTGTGGATGCGGGCTTAAA
                                            ** **    ** **  * ** ** **  * **

AY238880        TGATAAGCTGAAAGAGAGGGAAAAAGAAAAAGATAAAGAGAAAGAAAAA---GAAAAGAA
AY238884        TGATAAGTTGAAAGAGAAAGAAAAAGAAAAAGATAAAGAGAAAGAAAAA---GAAAAGAA
AY458618        TGATAAGCTGAGAGAGAAAGAAAAAGAAAAAGATAAAGAGAAAGAAAAA---GAAAAGAA
pNRSKR          TGATAAGCTGAAAGAGAAGGAAAAAGA---AGATAAAGAGAAAGAAAAA---GAAAAGAA
DQ077175        TGATAAGCTGAAGGAGAAAGAAAAAGAAAAAGATAAAGAGAGAGAAAAA---GAAAAGAA
AY687386        TGATAAGCTGAAGGAGAAAGAAAAAGAAAGAGATAAAGAGAGAGAAAAA---GAAAAGAA
D00595          TGAAAAACTCAAAGAGAAGGAAAATCAGAAAGAAAAAGAAAAAGAAAAACAAAAAGAGAA
NC_001785       TGAAAAACTCAAAGAGAAGGAAAAACAGAAAGAAAAAGAAAAAGAAAAACAAAAAGAGAA
AY238881        TGAAAAGCTCAAAGAAAAAGAAAAACAGAAAGAAAAAGAAAAAGAA------AAAGGAAA
AY491011        TGATAAGCTCAAAGAGAAAGAA---CAGAAAGAGAAGGAGAAAAAG------AAAGAAAA
AY238883        TGATAAGCTCAAAGAGAAAGAA---CAGAAAGAGAAAGAGAAAAAG------AAAGAAAA
AY458620        TGATAAGCTCAAAGAGAAAGAA---CAGAGAGAGAAAGAGAAAAAG------AAAGAAAA
                *** **  * *  ** *  ***    *   *** ** ** * * *        **   **

AY238880        AGACAAGAAGGATGCTAGTGACGGAGGTGATGTGTCAACTAGCACAAAAACTGGAGAGAG
AY238884        AGACAAAAAGGATGCTAGTGACGGAG---ATGTGTCAACTAGCACAAAAACTGGAGAGAG
AY458618        AGACAAGAAGGATGCTAGTGACGGAG---ATGTGTCAACTAGCACAAAAACTGGAGAGAG
pNRSKR          AGAAAAGAAGGATGCTAGTGACGGAAGTGATGTGTCAACTAGCACAAAAACTGGAGAGAG
DQ077175        AGACAAGAAGGATGCTGGTGACGGAGGTTATGTGTCAACTAGCACAAAAACTGGAGAGAG
AY687386        AGACAAGAAGGATGCTAGTGACGGAGGTTATGTGTCAACTAGCACAAAAACTGGAGAGAG
D00595          AGAAAAAGACGGTGCTAGTGACGGAAATGATGTGTCAACTAGCACAAAAACTGGAGAGAG
NC_001785       AGAAAAAGACGGTGCTAGTGACGGAAATGATGTGTCAACTAGCACAAAAACTGGAGAGAG
AY238881        AGAAAAAGATGATGCTAGTGACGGAAATGATGTGTCGACTAGCACAAAAACTGGAGAGAG
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AY458620        AGAAAAAGATGAAGCTAGTGGCGTAGATGATGTGTCAACTAGCACGAAAACTGGAGAGAG
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AY238880        AGATAGAGATGTCAATGCTGGAACTAGTGGTACATTTACAGTTCCAAGGATCAAGTCATT
AY238884        AGATAGAGATGTCAATGCTGGAACTAGTGGTACATTCACAGTTCCAAGGATCAAGTCATT
AY458618        AGATAGAGATGTCAATGCTGGAACTAGTGGTACATTCACAGTTCCAAGGATCAAGTCTTT
pNRSKR          AGATAGAGATGTCAATGCTGGAACTAGTGGTACATTCACAGTTCCAAGAATTAAGTCATT
DQ077175        AGATAGAGATGTCAATGCTGGGACTAGTGGTACATTCACAGTTCCAAGAATCAAGTCATT
AY687386        AGATAGAGATGTCAATGCTGGGACTAGTGGTACATTCACAGTTCCAAGAATCAAGTCATT
D00595          AGATAGAGATGTCAATGTTGGGACCAGTGGAACTTTCACTGTTCCGAGAATTAAATCATT
NC_001785       AGATAGAGATGTCAATGTTGGGACCAGTGGAACTTTCACTGTTCCGAGAATTAAATCATT
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AY491011        AGATAGAGATGTTAACGCTGGAACCAGTGGAACTTTTACAGTTCCAAGAATAAAGTCATT
AY238883        AGATAGAGATGTTAACGCTGGAACCAGTGGAACTTTTACAGTTCCAAGGATAAAGTCATT
AY458620        AGATAGAGATGTTAACGCTGGAACTAGTGGAACTTTTACAGTCCCAAGGATAAAATCGTT
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AY238884        TACTGATAAAATGATTTTGCCCAGAATTAAGGGAAAAGTTATCCTTAATTTGAATCATCT
AY458618        TACTGATAAAATGATTTTGCCCAGAATTAAAGGAAAAGTTATCCTTAATTTGAATCATCT
pNRSKR          TACTGATAAAATGATTCTGCCCAGAATTAAGGGAAAAGTTATCCTTAATTTGAATCATCT
DQ077175        TACTGATAAAATGATTTTGCCCAGAATTAAGGGGAAAGCTGTCCTTAATTTGAATCATCT
AY687386        TACTGATAAAATGATTTTGCCCAGAATTAAGGGGAAAGCTGTCCTTAATTTGAATCATCT
D00595          TACTGATAAGATGGTTCTACCGAGAATTAAGGGGAAGACTGTCCTTAATTTAAATCATCT
NC_001785       TACTGATAAGATGGTTCTACCGAGAATTAAGGGGAAGACTGTCCTTAATTTAAATCATCT
AY238881        TACTGACAAGATGATTTTACCGAGAATTAAGGGAAAGACTGTCCTTAATTTAAATCATCT
AY491011        TACTGACAAGATGGTTCTACCAAAAATTAAAGGAAAAACTGTCCTTAATTTGAATCATCT
AY238883        TACTGACAAGATGGTTCTACCAAGAATTAAGGGAAAAACTGTCCTTAATTTAAATCATCT
AY458620        TACTGACAAGATGGTTCTACCAAGAATTAAGGGAAAAACTGTCCTTAATTTGGATCATCT
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AY238884        TCTTCAGTATAATCCACAGCAAATTGACATCTCAAACACTCGTGCCACACAATCACAGTT
AY458618        TCTTCAGTATAATCCACAGCAAATCGACATCTCAAACACTCGTGCCACACAATCACAGTT
pNRSKR          TCTTCAGTATAATCCACAGCAAATTGACATCTCAAACACTCGTGCCACACAATCACAGTT
DQ077175        TCTTCAGTATAATCCACAGCAAATTGACATCTCAAACACTCGTGCCACACAATCGCAGTT
AY687386        TCTTCAGTATAATCCACAGCAAATTGACATCTCAAACACTCGTGCCACACAATCACAGTT
D00595          TCTTCAGTACAATCCGCAACAAATTGACATTTCTAACACTCGTGCCACTCATTCACAATT
NC_001785       TCTTCAGTACAATCCGCAACAAATTGACATTTCTAACACTCGTGCCACTCATTCACAATT
AY238881        CCTTCAGTATAATCCGCAACAAATTGACATTTCTAATACTCGTGCCACTCAATCACAATT
AY491011        TCTTCAGTATAATCCACAGCAAATTGACATCTCTAACACTCGTGCCACCCAGTCTCAGTT
AY238883        TCTTCAGTATAATCCACAGCAAATTGACATCTCTAACACTCGTGCCACCCAGTCTCAGTT
AY458620        TCTTCAGTATAATCCACAGCAAATTGACATCTCAAACACTCGTGCTACCCAATCTCAGTT
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AY458618        TGAGAAGTGGTATGAGGGAGTGAAGAATGACTATGGCCTTAATGATAATGAAATGCAAGT
pNRSKR          TGAGAAGTGGTATGAGGGAGTGAGGAATGATTATGGCCTTACTGATAATGAAATGCAAGT
DQ077175        TGAGAAGTGGTATGAAGGAGTGAGTAATGACTATGGCCTTAGTGATAATGAAATGCAAGT
AY687386        TGAGAAGTGGTATGAGGGAGTGAGGAATGACTATGTCCTTAGTGATAATGAAATGCAAGT
D00595          TGAGAAGTGGTATGAGGGAGTGAGGAATGATTATGGCCTTAATGATAATGAAATGCAAGT
NC_001785       TGAGAAGTGGTATGAGGGAGTGAGGAATGATTATGGCCTTAATGATAATGAAATGCAAGT
AY238881        TGAGAAGTGGTACGAGGGAGTGAGGAATGATTATGGCCTTAATGATACTGAAATGCAAGT
AY491011        TGAGAGATGGTACGAGGGGGTGAGGAATGATTATGGCCTTGACGATAATGAGATGCAAGT
AY238883        CGAGAGATGGTACGAGGGGGTGAGGAATGATTATGGTCTTAATGATAACGAAATGCAAGT
AY458620        TGAGAGGTGGTACGAGGGGGTGAGGGATGATTATGGTCTTAACGACAGTGAAATGCAAGT
                 ****  ***** ** ** ****   **** ****  ***   ** *  ** ********

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AY238884        GATGTTAAACGGCTTAATGGTTTGGTGTATCGAAAATGGTACATCCCCAGACATATCTGG
AY458618        GATGTTAAACGGCTTAATGGTTTGGTGTATCGAAAATGGTACATCCCCAGACATATCTGG
pNRSKR          GATGTTAAACGGCTTAATGGTTTGGTGTATTGAAAATGGTACATCCCCAGACATATCTGG
DQ077175        GATGTTGAATGGCTTAATGGTTTGGTGTATCGAAAATGGTACATCCCCAGACATATCTGG
AY687386        GATGTTAAATGGCTTAATGGTTTGGTGTATCGAAAATGGTACATCTCCAGACATATCTGG
D00595          GATGCTAAATGGTTTGATGGTTTGGTGTATCGAGAATGGTACATCTCCAGACATATCTGG
NC_001785       GATGCTAAATGGTTTGATGGTTTGGTGTATCGAGAATGGTACATCTCCAGACATATCTGG
AY238881        GATGTTAAATGGCTTAATGGTTTGGTGTATTGAGAATGGTACATCTCCAGACATGTCTGG
AY491011        GATGTTAAATGGCTTGATGGTGTGGTGTATTGAAAATGGTACATCTCCGGACATATCTGG
AY238883        GATGTTAAATGGCTTGATGGTATGGTGCATTGAAAATGGGACATCTCCGGACATATCTGG
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AY458620        GATGTTAAATGGTTTGATGGTATGGTGTATTGAAAATGGTACATCTCCAGACATATCTGG
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AY238880        TGTCTGGGTCATGATGGATGGTGAAACTCAGGTCGATTATCCAATTAAACCGTTAATTGA
AY238884        TGTCTGGGTCATGATGGATGGTGAAACTCAGGTCGATTATCCAATTAAACCGTTAATTGA
AY458618        TGTCTGGGTTATGATGGATGGTGAAACTCAGGTCGATTATCCAATTAAACCGTTAATTGA
pNRSKR          TGTCTGGGTCATGATGGATGGTGAAACTCAGGTCGATTATCCAATTAAACCGTTAATTGA
DQ077175        TGTCTGGGTTATGATGGATGGTGAGACTCAGGTCGATTACCCAATTAAACCATTAATTGA
AY687386        TGTCTGGGTCATGATGGATGGTGAGACTCAGGTCGATTACCCAATTAAACCATTAATTGA
D00595          TGTCTGGGTTATGATGGATGGGGAAACCCAAGTTGATTATCCAATCAAGCCTTTGATTGA
NC_001785       TGTCTGGGTTATGATGGATGGGGAAACCCAAGTTGATTATCCAATCAAGCCTTTGATTGA
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AY491011        TGTCTGGGTAATGATGGATGGCGACACTCAAGTCGACTATCCAATCAAGCCTTTGATTGA
AY238883        TGTCTGGGTAATGATGGATGGCGAAACTCAAATCGAATATCCGATCAAGCCTTTGATTGA
AY458620        TGTCTGGGTAATGATGGATGGCGACAATCAAGTCGACTATCCGATCAAGCCTTTGATTGA
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AY238880        GCATGCAAATCCTTCATTTAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAAGCATA
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AY458618        GCATGCAAATCCTTCATTTAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAAGCATA
pNRSKR          GCATGCAACTCCTTCATTTAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAAGCATA
DQ077175        ACATGCAACTCCTTCATTTAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAAGCATA
AY687386        ACATGCAACTCCTTCATTTAGGCAAATCATGGCTCACTTCAGTAACGCGGCAGAAGCATA
D00595          GCATGCTACTCCGTCATTTAGGCAAATTATGGCTCACTTTAGTAACGCGGCAGAAGCATA
NC_001785       GCATGCTACTCCGTCATTTAGGCAAATTATGGCTCACTTTAGTAACGCGGCAGAAGCATA
AY238881        GCACGCTACTCCGTCATTTAGGCAAATTATGGCTCACTTCAGTAACGCGGCAGAAGCATA
AY491011        ACATGCAACTCCTTCGTTTAGGCAGATCATGGCTCACTTCAGCAACGCGGCAGAAGCATA
AY238883        ACATGCAACTCCTTCCTTTAGGCAGATCATGGCTCACTTCAGCAACGCGGCAGAAGCATA
AY458620        ACATGCAACTCCTTCATTTAGGCAGATCATGGCTCACTTCAGTAACGCGGCAGAAGCATA
                 ** ** * *** ** ******** ** ************ ** *****************

AY238880        TATCGCAAAACGAAATGCAACTGAGAGGTACATGCCGCGTTATGGAATCAAGAGGAATTT
AY238884        TATCGCAAAACGAAATGCAACTGAGAGGTACATGCCGCGTTATGGAATCAAGAGGAATTT
AY458618        TATCGCAAAACGAAATGCAACTGAGAGATACATGCCGCGTTATGGAATCAAGAGAAATTT
pNRSKR          TATCGCAAAACGAAATGCAACTGAGAAGTACATGCCGCGGTATGGAATCAAGAGAAATTT
DQ077175        TATCGCGAAGCGAAATGCAACTGAGAGATACATGCCGCGGTATGGAATCAAGAGAAATTT
AY687386        TATCGCGAAGCGAAATGCAACTGAGAGATACATGCCGCGGTATGGAATCAAGAGAAATTT
D00595          CATTGCGAAGAGAAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGAAATTT
NC_001785       CATTGCGAAGAGAAATGCTACTGAGAGGTACATGCCGCGGTATGGAATCAAGAGAAATTT
AY238881        CATTGCAAAGAGAAATGCTACTGAGAGATACATGCCGCGGTATGGAATCAAGAGAAATTT
AY491011        CATCGCAAAGAGAAACGCAACTGAAAAGTACATGCCGCGTTATGGAATCAAGAGAAATTT
AY238883        CATTGCAAAGAGAAACGCAACTGAGAAGTACATGCCGCGATATGGAATCAAGAGAAATTT
AY458620        CATTGCAAAGAGAAATGCTACTGAGAGATACATGCCGCGGTATGGAATCAAGAGAAATTT
                 ** ** **  **** ** ***** *  *********** ************** *****

AY238880        GACTGACATTAGCCTCGCCAGATATGCTTTTGATTTCTATGAGGTGAATTCAAAAACACC
AY238884        GACTGACATTAGCCTCGCCAGATATGCTTTTGATTTCTATGAGGTGAATTCAAAAACACC
AY458618        GACTGACATTAGCCTCGCTAGATATGCTTTCGATTTCTATGAGGTGAATTCAAAAACACC
pNRSKR          GACTGACATTAGCCTCGCTAGATATGCTTTCGATTTCTATGAGGTGAATTCAAAAACACC
DQ077175        GACTGACATTAGCCTCGCTAGATATGCTTTCGATTTCTATGAGGTGAATTCAAAAACACC
AY687386        GACTGACATTAGCCTCGCTAGATATGCTTTCGATTTCTATGAGGTGAACTCAAAAACACC
D00595          GACTGACATTAGCCTCGCTAGATACGCTTTCGACTTCTATGAGGTGAATTCGAAAACACC
NC_001785       GACTGACATTAGCCTCGCTAGATACGCTTTCGACTTCTATGAGGTGAATTCGAAAACACC
AY238881        GACTGACATTAGCCTCGCTAGATACGCTTTCGATTTCTATGAGGTGAATTCGAAAACACC
AY491011        GACTGACACTAGCCTCGCTAGATATGCATTCGATTTCTATGAGGTGAATTCAAAAACGCC
AY238883        GACTGACACTAGCCTCGCTAGATATGCTTTCGATTTCTATGAGGTGAATTCCAAAACGCC
AY458620        GACTGACATTAGCCTCGCTAGATACGCTTTCGATTTCTATGAGGTGAATTCGAAAACACC
                ******** ********* ***** ** ** ** ************** ** ***** **

AY238880        TGATAGAGCTCGAGAGGCTCATATGCAGATGAAAGCAGCTGCACTGCGAAACACAAATCG
AY238884        TGATAGAGCTCGAGAGGCTCATATGCAGATGAAAGCAGCTGCACTGCGAAACACAAATCG
AY458618        TGATAGAGCTCGAGAGGCTCACATGCAGATGAAAGCAGCTGCACTGCGAAACACAAATCG
pNRSKR          TGATAGAGCTCGAGAGGCTCATATGCAGATGAAAGCAGCTGCGCTGCGGAACACAAATCG
DQ077175        TGATAGAGCTCGGGAGGCTCATATGCAGATGAAGGCAGCTGCACTGCGGAACACAAGTCG
AY687386        TGATAGAGCTCGAGAGGCTCATATGCAGATGAAGGCAGCTGCACTGCGGAACACAAATCG
D00595          TGATAGGGCTCGCGAAGCTCACATGCAGATGAAGGCTGCAGCGCTGCGAAACACCAGTCG
NC_001785       TGATAGGGCTCGCGAAGCTCACATGCAGATGAAGGCTGCAGCGCTGCGAAACACCAGTCG
AY238881        TGATAGGGCTCGCGAAGCTCACATGCAGATGAAAGCTGCGGCATTGCGAAACACTAATCG
AY491011        TGATAGGGCTCGCGAAGCTCACATGCAGATGAAAGCTGCGGCATTGCGAAACACTAATCG
AY238883        TGATAGAGCTCGCGAAGCTCACATGCAGATGAAAGCTGCAGCGCTACGCAACGCTACTCG
AY458620        TGATAGGGCTCGCGAAGCTCACATGCAGATGAAAGCTGCGGCATTGCGAAACACTAATCG
```

```
                ****** ***** ** ***** ********** ** ** **  * ** *** * * ***

AY238880        TAGAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACAC
AY238884        TAGAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACAC
AY458618        TAGAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAGGAAAACACGGAGAGACACAC
pNRSKR          TAGAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACAC
DQ077175        CAGAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACAC
AY687386        CAGAATGTTTGGAATGGACGGCAGTGTCAGTAACAAGGAAGAAAACACGGAGAGACACAC
D00595_         CAAAATGTTTGGTATGGACGGCAGTGTTAGTAACAAGGAAGAAAACACGGAGAGACACAC
NC_001785       CAGAATGTTTGGTATGGACGGCAGTGTTAGTAACAAGGAAGAAAACACGGAGAGACACAC
AY238881        CAGAATGTTTGGTATGGACGGCAGTGTCAGTAACAAGGAAGAGAACACGGAGAGACACAC
AY491011        CAGAATGTTTGGCATGGACGGCAGTGTCAGTAACAAGGAAGAGAACACGGAGAGACACAC
AY238883        TAGAATGTTTGGCATGGATGGTAGTGTCAGTAACAAGGAAGAAAACACGGAAAGACACAC
AY458620        CAGAATGTTTGGCATGGACGGCAGTGTCAGTAACAAGGAAGAGAACACGGAGAGACACAC
                 * ********* ***** ** ***** ********** ** ******** ********

AY238880        AGTGGAAGATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAAC-----------
AY238884        AGTGGAAGATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAAC-----------
AY458618        AGTAGAAGATGTCAATAGAGACATGCGCTCTCTCCTGGGTATGCGCAAC-----------
pNRSKR          AGTGGAAGATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAACTAAGGATCCCG
DQ077175        AGTGGAAGATGTCAATAGAGACATGCGCTCTCTCCTGGGTATGCGCAAC-----------
AY687386        AGTGGAAGATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAAC-----------
D00595          AGTGGAAGATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAAC-----------
NC_001785       AGTGGAAGATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAAC-----------
AY238881        AGTAGAAGATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAAC-----------
AY491011        AGTAGAAGATGTCAATAGAGACATGCGCTCTCTCCTGGGTATGCGCAAC-----------
AY238883        AGTGGAAGATGTCAATAGAGACATGCACTCTCTCCTGGGTATGCGCAAC-----------
AY458620        AGTAGAAGATGTCAATAGAGACATGCGCTCTCTCCTGGGTATGCGCAAC-----------
                *** ********************** **********************
```

Fig.5.10. ClustalW nucleotide multiple sequence Alignment: pNRSKR

Amino acid sequence analysis of the pNRSKR by BLAST analysis revealed amino acid sequence similarity with the CP gene of PRSV-P isolates reported worldwide. pNRSKR with BLAST and ClustalW Multiple alignment (Fig 5.11) showed 92-96% amino acid sequence similarity with hundred and ten references (Genbank accessions) of PRSV-P strain. The gene showed 94%-96% homology with the virulent PRSV-P strains from Indian (GenBank Acc No. AAO59522: 2003; GenBank Acc No. AAO59526: 2003; GenBank Acc No. AAR88138: 2004; GenBank Acc No. AAO93506: 2004). The sequence similarity was 92% with the Chinese PRSV-P isolate (GenBank Acc No. AAF70461 and 93% with Taiwanian PRSV-P isolate (GenBank Acc No. CAA65886). It showed only 94% homology with one Australian isolate reported by Bateson *et al.,* (GenBank Acc No. AAB21856: 1992). Other Asian and Australian and American isolated from Brazil, Mexico and United States showed 92-94% homology.

```
AY238884        --------------------SKTEAVDAGLNDKLKEKEKEKDKEKEK---EKKDKKDASD
AY458618        --------------------SKTEAVDAGLNDKLREKEKEKDKEKEK---EKKDKKDASD
AY238880        --------------------SKTEAVDAGLNDKLKEREKEKDKEKEK---EKKDKKDASD
AY687386        --------------------SKNEAVDAGLNDKLKEKEKERDKEREK---EKKDKKDASD
pNRSKR          --------------------SKNEAMDAGLNDKLKEKEKE-DKEKEK---EKKEKKDASD
```

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D00595          --------------------SKNEAVDAGLNEKLKEKENQKEKEKEK--QKEKEKDGASD
NC_001785       --------------------SKNEAVDAGLNEKLKEKEKQKEKEKEK--QKEKEKDGASD
AF506868        ----------------------------GLNEKLKEKEKQKEKEKEKDKQKDKDNDGASG
AY094984        SRSTDDYQLVCSSNTHVFHQSKNEAMDAGLNEKLKEKEKQKEKEREK--QKEKEKDDASD
                                           ***:**:*:*:: :**:**   :.*::..**.

AY238884        G-DVSTSTKTGERDRDVNAGTSGTFTVPRIKSFTDKMILPRIKGKVILNLNHLLQYNPQQ
AY458618        G-DVSTSTKTGERDRDVNAGTSGTFTVPRIKSFTDKMILPRIKGKVILNLNHLLQYNPQQ
AY238880        GGDVSTSTKTGERDRDVNAGTSGTFTVPRIKSFTDKMILPRIKGKVILNLNHLLQYNPQQ
AY687386        GGYVSTSTKTGERDRDVNAGTSGTFTVPRIKSFTDKMILPRIKGKAVLNLNHLLQYNPQQ
pNRSKR          GSDVSTSTKTGERDRDVNAGTSGTFTVPRIKSFTDKMILPRIKGKVILNLNHLLQYNPQQ
D00595          GNDVSTSTKTGERDRDVNVGTSGTFTVPRIKSFTDKMVLPRIKGKTVLNLNHLLQYNPQQ
NC_001785       GNDVSTSTKTGERDRDVNVGTSGTFTVPRIKSFTDKMVLPRIKGKTVLNLNHLLQYNPQQ
AF506868        GNDVSTSTKTGERDRDVNAGTSGTFTVPRIKSFTDKMILPRIKGKSILNLNHLLQYNPKQ
AY094984        GNDVSTSTKTGERDRDVNVGTSGTFTVPRTKSFTDKMILPRIKGKTVLNLNHLLQYNPQQ
                *  ***************.********** *******:******* :***********:*

AY238884        IDISNTRATQSQFEKWYEGVKNDYGLNDNEMQVMLNGLMVWCIENGTSPDISGVWVMMDG
AY458618        IDISNTRATQSQFEKWYEGVKNDYGLNDNEMQVMLNGLMVWCIENGTSPDISGVWVMMDG
AY238880        IDISNTRATQSQFEKWYEGVKNDYGLNDNEMQVMLNGLMVWCIENGTSPDISGVWVMMDG
AY687386        IDISNTRATQSQFEKWYEGVRNDYVLSDNEMQVMLNGLMVWCIENGTSPDISGVWVMMDG
pNRSKR          IDISNTRATQSQFEKWYEGVRNDYGLTDNEMQVMLNGLMVWCIENGTSPDISGVWVMMDG
D00595          IDISNTRATHSQFEKWYEGVRNDYGLNDNEMQVMLNGLMVWCIENGTSPDISGVWVMMDG
NC_001785       IDISNTRATHSQFEKWYEGVRNDYGLNDNEMQVMLNGLMVWCIENGTSPDISGVWVMMDG
AF506868        IDISNTRATQSQFEKWYEGVRNDYGLSDNEMQVMLNGLMVWCIENGTSPDISGVWVMMDG
AY094984        IDISNTRATQSQFEKWYEGVRNDYGLNDNEMQIMLNGLMVWCIENGTSPDISGVWVMMDG
                *********:**********:*** *.*****:**************************

AY238884        ETQVDYPIKPLIEHANPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLAR
AY458618        ETQVDYPIKPLIEHANPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLAR
AY238880        ETQVDYPIKPLIEHANPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLAR
AY687386        ETQVDYPIKPLIEHATPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLAR
pNRSKR          ETQVDYPIKPLIEHATPSFRQIMAHFSNAAEAYIAKRNATEKYMPRYGIKRNLTDISLAR
D00595          ETQVDYPIKPLIEHATPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLAR
NC_001785       ETQVDYPIKPLIEHATPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLAR
AF506868        ETQVDYPIKPLIEHATPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLAR
AY094984        ETQVDYPIKPLIEHATPSFRQIMAHFSNAAEAYIAKRNATERYMPRYGIKRNLTDISLAR
                ***************.*************************:******************

AY238884        YAFDFYEVNSKTPDRAREAHMQMKAAALRNTNRRMFGMDGSVSNKEENTERHTVEDVNRD
AY458618        YAFDFYEVNSKTPDRAREAHMQMKAAALRNTNRRMFGMDGSVSNKEENTERHTVEDVNRD
AY238880        YAFDFYEVNSKTPDRAREAHMQMKAAALRNTNRRMFGMDGSVSNKEENTERHTVEDVNRD
AY687386        YAFDFYEVNSKTPDRAREAHMQMKAAALRNTNRRMFGMDGSVSNKEENTERHTVEDVNRD
pNRSKR          YAFDFYEVNSKTPDRAREAHMQMKAAALRNTNRRMFGMDGSVSNKEENTERHTVEDVNRD
D00595          YAFDFYEVNSKTPDRAREAHMQMKAAALRNTSRKMFGMDGSVSNKEENTERHTVEDVNRD
NC_001785       YAFDFYEVNSKTPDRAREAHMQMKAAALRNTSRRMFGMDGSVSNKEENTERHTVEDVNRD
AF506868        YAFDFYEVNSKTPDRAREAHMQMKAAALRNTSRRMFGMDGSVSNKEENTERHTVEDVNRD
AY094984        YAFDFYEVNSKTPDRAREAHMQMKAAALRNTNRRMFGMDGSVSNKEENTERHTVEDVNRD
                ******************************.*:************************

AY238884        MHSLLGMRN
AY458618        MRSLLGMRN
AY238880        MHSLLGMRN
AY687386        MHSLLGMRN
pNRSKR          MHSLLGMRN
D00595          MHSLLGMRN
NC_001785       MHSLLGMRN
AF506868        MHSLLGMRN
AY094984        MHSLLGMRN
                *:*******
```

Fig.5.11. ClustalW amino acid Multiple sequence Alignment: pNRSKR

5.4 Cloning of PRSV-CP gene in pCAMBIA MCS11

The pNRSKR Plasmid was restriction digested with *BamH* I and *Kpn* I and the 858 bp PRSV-CP insert gel purified. pCAMBIA MCS11 was restriction digested with *Bgl* II and

Kpn I and ligated with gel purified PRSV-CP fragment. The clone was characterized, sequenced and designated as pCAMBIA- PRSV-CP.

5.5 Mobilization of pCAMBIA- PRSV-CP into A. *tumefaciens*

The plasmid pCAMBIA-PRSV-CP (Fig. 5.12) was isolated and mobilized into *A. tumefaciens* GV2260 by freeze-thaw method (An *et al.* 1988). Detailed plasmid mobilization protocol is given in Chapter 2.

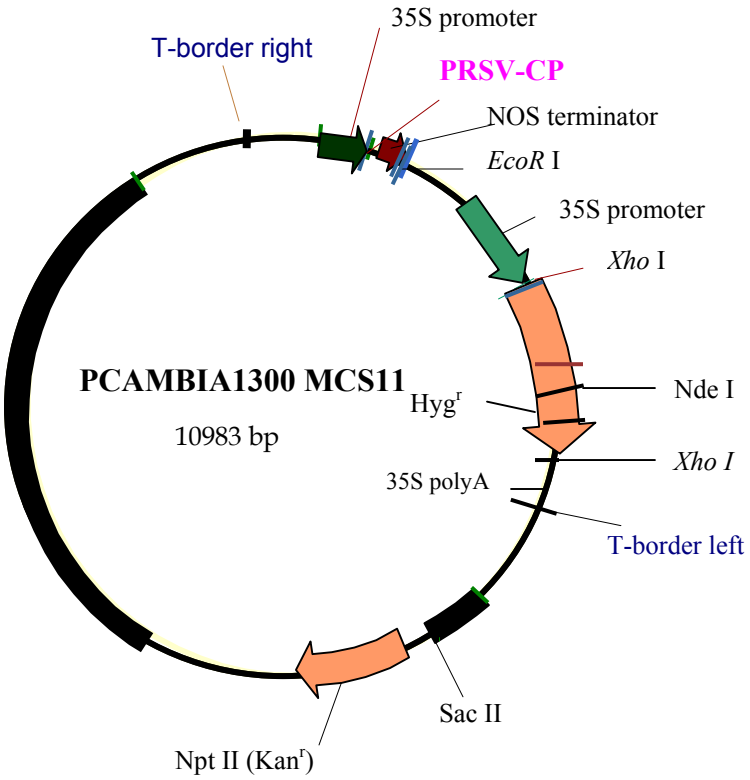

Fig.5.12 pCAMBIA-PRSV-CP construct map

5.6 Transformation into papaya

A. tumefaciens harbouring pCAMBIA-PRSV-CP construct (Fig.5.12) was used to transform immature zygotic embryos of papaya. Detailed transformation protocol is given in Chapter 2. pCAMBIA-PRSV-CP construct transformed plants were survived on hygromycin (2mg/l) selection medium (Fig.5.13).

Reverse transcription polymerase chain reaction (RT-PCR) was able to develop a sensitive test for PRSV infected leaves and ring spots on fruit rind of papaya. RT-PCR amplified products confirmed specificity of amplification. RT-PCR has the advantage of allowing the study of genetic variation between geographically distributed isolates. Nucleotide and amino acid sequences were used to evaluate the degree of variation between geographical distributions of isolates from reported PRSV-P strain sequences. Earlier studies demonstrated the usefulness of PCR as a tool (Henson and French 1993). Langeveld *et al.* (1991) detected potyvirus in a combined RT-PCR using degenerate oligonucleotide primers, which were designed from conserved amino acid sequences. RT-PCR with virus-specific primers has been widely used to detect potyviruses, including plum pox virus (Wetzel *et al.* 1991), Dasheen mosaic virus (Pappu *et al.* 1993). Papaya ringspot virus in naturally infected papaya plants was detected by reverse transcription polymerase chain reaction (Sharma *et al.* 2004, Jain *et al*. 2004).

5.7 Conclusion

Using primer two sets of primers FI: RI, FII: RI, FI: FII: RI, FIII: RIII (Set 1: PRSV-CP-F-I: 5'd(TATGGATCCTCCAAGAATGAAGCT) 3' PRSV-CP-F-II: 5'd(TATGGATCCAG TCCAAAAATGAAGCTG) 3' Reverse primer PRSV-CP-R-I: 5'd (TATGGATCCTTAGTTGC GCATACC) 3' Set 2: Forward primer PRSV -FIII: 5'd(GATCCATGCTGAGAGGTACATTT CAAGAGAATGTACCTCTCAGTAGCATTTTTTTGCTAGCG) 3' Reverse primer: PRSV-RIII: 5'd(AATTCGCTAGCAAAAAAATGCTACTGAGAGGTACATTCTCTTGAAATGT ACCTCTCAGTAGCATG) 3' ~1.0 kb amplicon was generated. The amplicon was determined to be 858bp in size (Fig. 5.7). The single ORF coded for 285 amino acids (Fig.5.8) of an approximate molecular mass of 31.35 KDa. The sequence was deposited with NCBI GenBank and is available under the accession No. **DQ192587**. Multiple sequence analysis of the amplicon with BLAST and CLUSTAL showed a 87-97% nucleotide sequence similarity (Fig.5.10 and Fig.5.11) with other reported PRSV-P strain. Comparative sequence analysis of the PRSV isolates from different countries at nucleotide and amino acid level revealed that there was substantial sequence diversity with the geographical distribution of the isolates. The isolates from India shared 89-97% nucleotide similarity, while other Asian and the isolates from Australia and America

(Brazil, Mexico and United States) shared 87-92% nucleotide similarity. Multiple sequence analysis of the amplicon with BLAST and CLUSTAL showed a 92-96% amino acid sequence similarity (Fig.5.11) with worldwide reported PRSV-P strain.

CHAPTER 6

Short interference RNA (PRSV-CP) and gene silencing

6.1 Introduction

In plants, gene silencing serves as a component of the defence mechanism. The process leads to the degradation of homologous mRNAs. RNA silencing does not affect the transcription of a gene locus, but only cause sequence-specific degradation of the target mRNAs consequently it is also known as Posttranscriptional gene silencing (PTGS). The RNA degradation process is achieved through sequence-specific nucleotide interaction induced by double-stranded RNA.

RNA interference is a new biotechnological tool for the control of virus diseases in plants (Tenllado *et al.* 2004). It is an efficient mechanism and an essential component of the defense system targeted against viral infection (Voinnet 2002). RNA silencing is triggered by double-stranded RNA (dsRNA), which may be naturally derived from the transcription of inverted-repeat loci or replicating exogenous RNAs by the host, or the viral encoded RNA dependent RNA polymerase (RDRP) (Dalmay *et al.* 2000, Mourrain *et al.* 2000, Sijen *et al.* 2001).

Gene silencing in plants can be transcriptional, taking place in the nucleus, or post-transcriptional (RNA silencing), taking place in the cytoplasm. Recent evidence, however, suggests that they are different phenomena of the same system (Tang and Zamore 2004, Lu *et al.* 2004, Wesley *et al.* 2004, Gilmore *et al.* 2004, Ainley *et al.* 2004, Kusaba M. 2004, Yu and Kumar 2003, Pal-Bhadra *et al.* 2002, Finnegan *et al.*2001, Waterhouse *et al.* 2001, Jones *et al.* 1999, Voinnet *et al.* 1999). Although exact mechanism involved in RNA silencing is yet to be elucidated, double stranded RNA (dsRNA) has an important role to play (Lu *et al.* 2004, Wensley *et al.* 2004, Tenllado and Diaz-Ruiz 2001, Fire *et al.* 1998). Small interfering RNA (SiRNA) is invariably associated with RNA silencing (Waterhouse *et al*. 2001). Crucial roles have also been shown for cellular RNA dependent RNA polymerases (RdRps), RNA helicases and ribonuclease III (RNaseIII)-like molecules (Bernstein *et al*. 2001, Dalmay *et al.* 2001, Elbashir *et al.* 2001, Xie *et al.* 2001). The RNA silencing system recognizes and specifically degrades RNA it perceives as foreign or unusual and sends a systemic signal, which includes RNA silencing to homologous RNA in distal parts of the plant. Systemic translocation of RNA exerts non-cell-autonomous control over plant development and defence (Yoo *et al.* 2004). There are three pathways for silencing specific genes in plants

where silencing signals can be amplified, transmitted between cells and self-regulated by feedback mechanisms (Baulcombe 2004). Aspects and applications for RNA interference in transcriptional regulation have been described by Karkare *et al.* (2004).

Salient features of RNA interference are:

- high degree of specific gene silencing with less effort,
- highly potent and effective (only a few double stranded RNA molecules per cell are required for effective interference),
- silencing can be introduced in different developmental stages,
- systemic silencing,
- avoids problems with abnormalities caused by knocked out gene in early stages (which could mask desired observations) and
- silencing effects passed through generations.

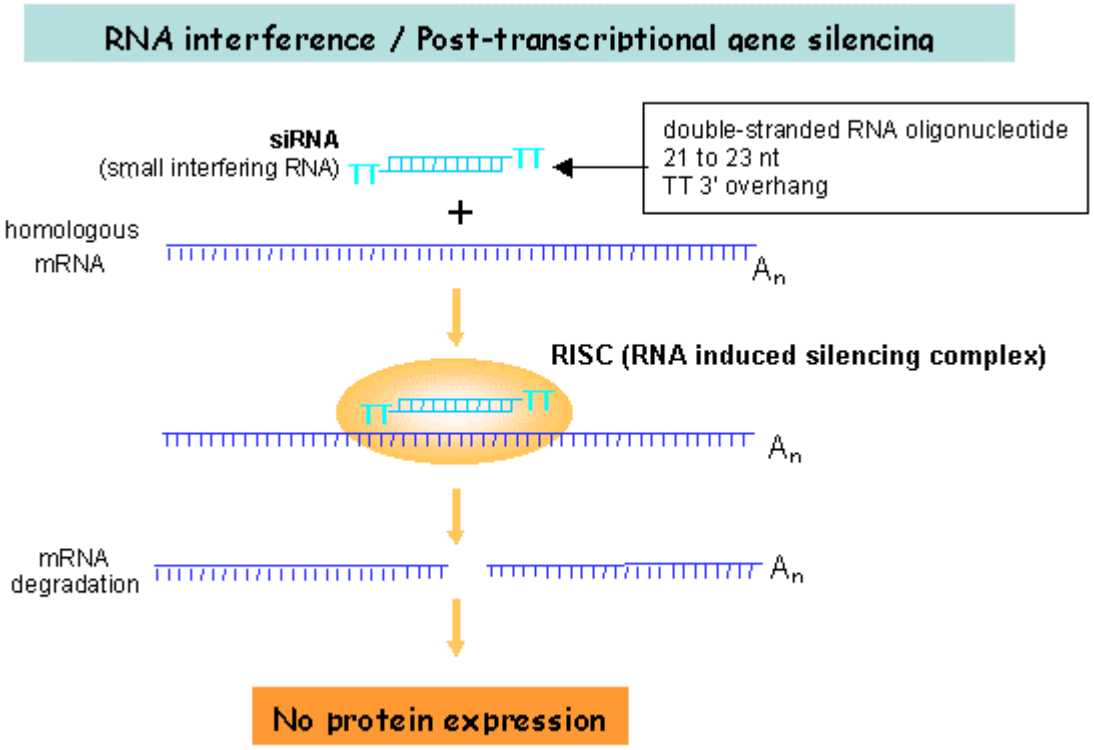

Fig. 6.1 How RNA silencing may function

A putative model for the mechanisms of RNA silencing in plants, suggests that RNA silencing is triggered by double stranded RNA (dsRNA). The dsRNA can originate from replicating RNA, or DNA viruses (Voinnet 2001) or the large stem loop structure in the pre-genomic RNA leader of caulimovirus (Khane and Dijkstra 2002). Single copy gene can be silenced, probably through the production of RNA that the cell somehow recognizes as being aberrant and replicates into a dsRNA form. The dsRNA is recognized by a Dicer-like nuclease (Bernstein *et al.* 2001, Knight and Bass 2001) and cleaved into 21-22 nucleotides long (two helical turns), small interfering RNAs (siRNA) with 3' overhangs of 2-3 nucleotides (Elbashir *et al.* 2001). Following the initial cleavage into siRNA, first the siRNA may serve as templates for a host RdRp complex which uses complementary single stranded RNA (ssRNA) and possibly dsRNA as a template to create more dsRNA which can be degraded into new siRNA in a cycle of 'degradative PCR' (Lipardi *et al.* 2001, Nishikura 2001, Sijen *et al.* 2001). The siRNAs are mobilized into a multimeric RNase complex (RISC, RNA Induced Silencing Complex, Hammond *et al.* 2000), that is guided to the target RNA (ssRNA and possibly dsRNA) by base pairing of the siRNA. RISC cleaves the target RNA into siRNAs, at a position approximately in the middle of the guide sequence (Elbashir *et al.* 2001). The RISC is composed siRNA (Hammond *et al.* 2000), a protein similar to initiation factor eIF2C (Hammond *et al.* 2001) with additional factors, including an RNA helicase (Nykänen *et al*. 2001), Dicer-like nuclease (Hammond *et al.* 2001). A systemic signal is also produced which can confer the specific RNA silencing to distal parts of the plant. HC-Pro of potyvirus eliminates the small RNAs but not the mobile signal (Mallory *et al.* 2001). The 25 kDa movement protein (p25) of *potato virus X* (PVX) can suppress RNA silencing induced by a (sense) transgene but not RNA silencing induced by a virus (Voinnet *et al*. 2000). The 2b protein of cucumoviruses suppresses the initiation of silencing and cannot reverse silencing in already silenced tissue (Brigneti *et al.* 1998), Nuclear localization is required for the activity of CMV 2b protein (Lucy *et al.* 2000).

Plant viruses are inducers and targets of RNA silencing, which poses a potent defence against them in all plants. Many viruses still manage to infect their host plants successfully. Plant viruses may have developed mechanisms to counter the effects of RNA silencing by encoding suppressors of RNA silencing. Several viral suppressors have

been identified among the virus-encoded proteins, namely HC-Pro of potyviruses (Anandalaxmi *et al.* 1998, Brigneti *et al.* 1998, Kasschau & Carrington, 1998), 2b of cucumoviruses (Brigneti *et al.* 1998), P1 of sobemoviruses (Voinnet *et al.* 1999), P19 of tombusviruses (Voinnet *et al.* 1999, Ye *et al.* 2003, Vargason *et al.* 2003). The 25 kDa movement protein (p25) of *potato virus X* (PVX) genus *potexvirus* prevents the spread of the gene-silencing signal (Voinnet *et al.* 2000). All these proteins have been identified as viral pathogenicity determinants with the exception of AC2, important in viral long distance movement, suggesting a link between long distance movement and RNA silencing. The viral suppressors of RNA silencing can, in turn, be the targets of other host resistance mechanisms (Li *et al.* 1999). For several synergistic viral diseases between a potyvirus and an unrelated virus, the silencing suppressing properties of HC-Pro are enough to explain the enhanced accumulation of the non-potyviral component of the synergism (Anandalaxmi *et al.* 1998, Brigneti *et al.* 1998, Kasschau and Carrington 1998, Savenkov and Valkonen 2001, Shi *et al.* 1997).

As most viruses require insect vectors to spread to new host, resistance against the virus vector may also provide, indirectly, resistance to virus. Plant recovery can be the result of RNA silencing (Ratcliff *et al*. 1997) and the ability of plants to recover is then probably dictated by a balance between the RNA silencing properties of a virus and the ability of virus encoded factors to suppress RNA silencing.

6.2 Genetic diversity

Most of the breeders germplasm collections are small and not necessarily representative of total diversity within the species. A survey of morphological variation in papaya for economically important characters e.g. fruit size and quality factors, will reveal striking differences, reflecting the cumulative effect of human selection. The basic genetic relationships between different accessions may be better-determined surveying variation in an array of characters that have not been subjected to human selection. Molecular polymorphisms in isozymes and DNA are useful for this purpose (Tanksley 1983). Germplasm collection, including wild materials, is needed to resolve the questions of the origin of domesticated papayas and the genetic diversity within the species.

6.3. Chemical synthesis for CPsi:

There are several methods for preparing siRNA, such as chemical synthesis, in vitro transcription, siRNA expression vectors and PCR expression cassettes. Irrespective of method one uses, the first step in designing a siRNA is to choose the siRNA target site. The best results have been obtained with double-stranded siRNA made of 19-21-nucleotides bearing a 2-deoxyribonucleotide 3' overhang at each end.

The siRNA linked to the RISC complex, recognizes its mRNA target on a specific and complementary way. The specific down regulation is directly correlated to the uniqueness of the sequence of the interest in the genome but also to particular criteria and the guide lines listed below for choosing siRNA:

- position into the mRNA sequence,
- the sequence has to be selected into the coding region of the RNA,
- the GC ratio has to be as close as possible to 50%,
- when multiple possibilities are available select the one just above 50%,
- avoid sequences containing three or more G or C in a row,
- avoid regions within 50-100 nucleotides of the AUG or Stop codon,
- select target sequences that start with two adenosines,
- avoid maximum of complex secondary structures, hairpin or loop structures are competing with the stabilized siRNA form and reduce its efficiency significantly, and
- avoid stretches of more than four T's or A's in the target sequence as 4-6 nucleotide poly (T) tract acts as a termination signal for RNA pol III.

For papaya using these guidelines we chemically synthesized siRNA for the PRSV Coat Protein (CP) gene with flanking *EcoR* I and *BamH* I compatible sequences.

Cpsi sequence:

5'(d) GATCCATGCTACTGAGAGGTACATTTCAAGAGAATGTACCTCTCAGTAGCATTTTTTTGCTAGCG 3'
3'(d) GTACGATGACTCTCCATGTAAAGTTCTCTTACATGGAGAGTCATCGTAAAAAAACGATCGCTTAA 5'

6.4 Results and Discussion

6.4.1 Cloning strategy for CPsi

6.4.1.1 Bacterial cell lines

E. coli DH5α, JM109 and *E. coli* XL1-blue (Stratagene) were used as the host cell lines.

6.4.1.2 Preparation of competent cells

Competent cells preparation was detailed described in Chapter 2.

6.4.1.3 Transformation of *E.coli* JM109 or DH5-α cells

pBS KS+ plasmid DNA was restriction digested with *EcoR* I and *BamH* I. The gel purified vector was ligated with CPsi. Competent *E. coli* JM109 cells were mixed with 1:4 dilution of ligation reaction for transformation. Detailed transformation protocol is described in Section 2.19.9.3 in chapter 2.

6.4.1.4 Screening for recombinants

Positive colonies were picked after screening by blue/white selection (disruption of *lacZ* gene). Each isolated colony was grown in 10ml LB-agar amp^{50} overnight. Plasmid DNA was isolated by the alkaline lysis method as given in Section 2.21.2 in Chapter 2, (Sambrook *et al.* 1989). Plasmid map of pNRO5 is given in Fig. (6.2). The DNA was characterized by restriction digesting with enzymes *EcoR* I and *BamH* I (Fig.6.3).

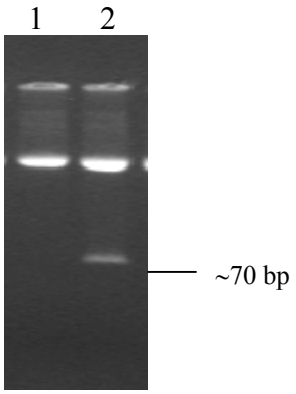

Fig.6.2 Characterization of pNRO5 with *Eco*R I and *Bam*H I

Lane 1: pNRO5 uncut, Lane 2: pNRO5 restriction digested with *Eco*R I and *Bam*H I

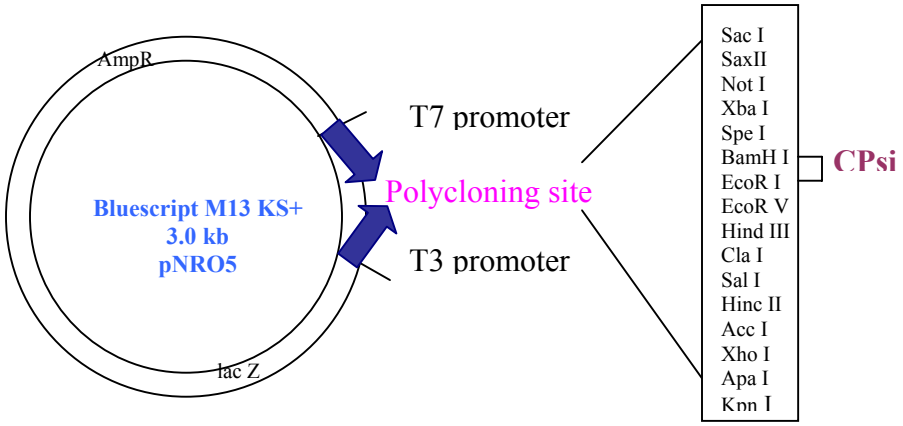

Fig. 6.3 Plasmid map of pNRO5

6.4.2 Sequence analysis

The clone pNRO5 which released the ~70 bp insert (CPsi) was selected for further work. pNRO5 was sequenced by Beckman Coulter CEQ™ 8000 Genetic Analysis System using CEQ™ DTCS Quick Start Kit Dye Terminator Cycle sequencing kit provided by the manufacturer.

Sequence of pNRO5

AGACTCACTATGGGCGCCTGGATTCCACGCGGGGCGGCGCCTAGAACTTGTGGATCCATGCTA
CTGAGAGGTACATTTCAAGAGAATGTACCTCTCAGTAGCATTTTTTGCTAGCGAATTCGATAT
CAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCAGCTTTGTTCCCTTTAGTGA
GGGTTAATTTCGAGCTTGGCGTAAT

6.4.3 Construction of vector cassette pPRSV-CPsi-GFP

pNRO5 was restriction digested with *Not* I and *EcoR* I to release the Cpsi insert which was isolated and gel purified. Plasmid of pKOH122 (Fig. 6.4) was restriction digested with *Not* I and *EcoR* I, the vector plasmid was gel purified and ligated to Cpsi insert from the resultant clone was designated as pNRO6.

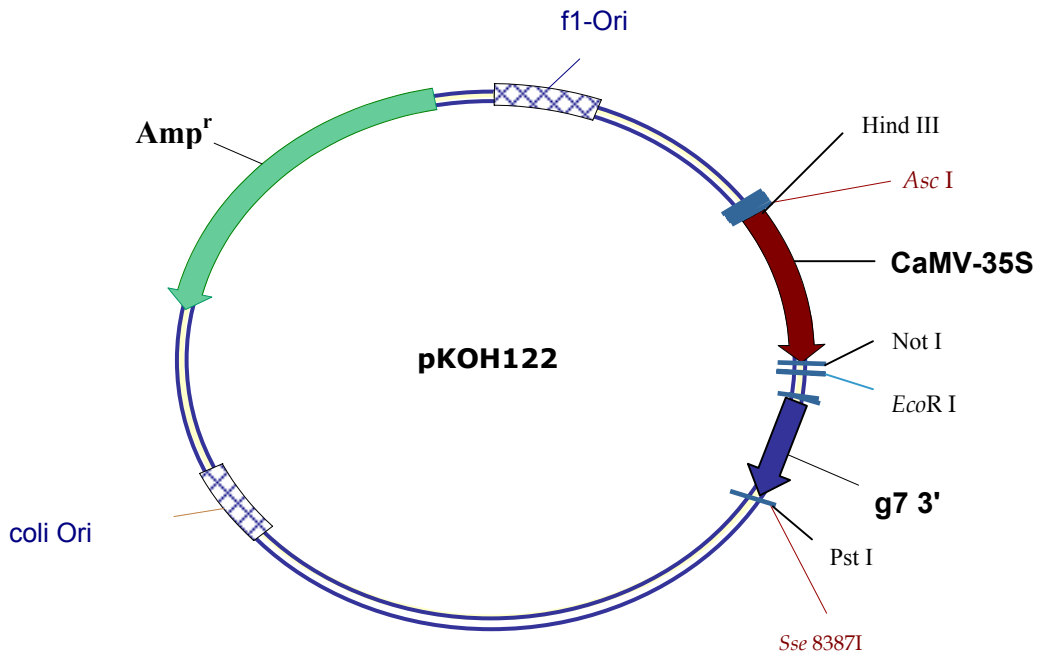

Fig. 6.4 Plasmid map of pKOH122 vector

This plasmid was digested with *Not* I and *BamH* I and purified. pCR 2.1-TOPO vector (Fig. 6.5) was restriction digested with *Not* I and *BamH* I to release GFP fragment. The GFP fragment was gel purified and ligated into pNRO6 between *Not* I and *BamH* I and transformed. The positive clones were characterized by restriction digestion with *Not* I and *BamH* I (Fig.6.6 and designated as pNRO6-F.

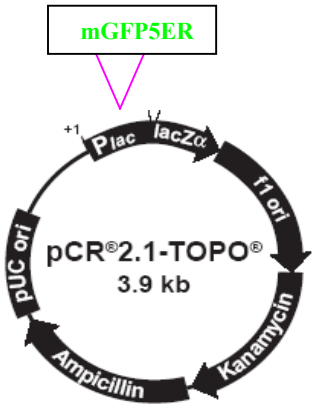

Fig. 6.5 Plasmid map of pCR 2.1-TOPO vector

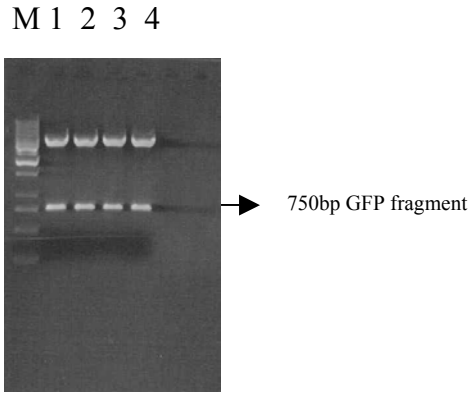

Fig.6.6 Characterization of clones in pCR 2.1-TOPO Vector

Lane M: λ-Hind III marker, Lane 1,2,3,4: Four clones showing 750bp GFP fragment restriction digested with *Not* I and *BamH* I

6.4.5 Cloning of pNRO6-F in binary vector pKOH200

The plasmid of pNRO6-F (35S P+GFP+CPsi+g7 T) was isolated and ligated into the binary vector pKOH200 (Fig. 6.8) between *Asc* I and Sse_{8387}I and transformed. The positive clone was characterized by restriction digestion with *Asc* I and Sse_{8387}I and designated as pKOH200-pPRSV-CPsi-GFP (Fig.6.7). Plasmid map of the construct

pKOH200-pPRSV-CPsi-GFP is given in Figs. (6.10and 6.11). pNRO6 without GFP fragment was designated as pKOH200-pPRSV-Cpsi. Procedure for the whole cassette preparation is given as flow chart in Fig. (6.9).

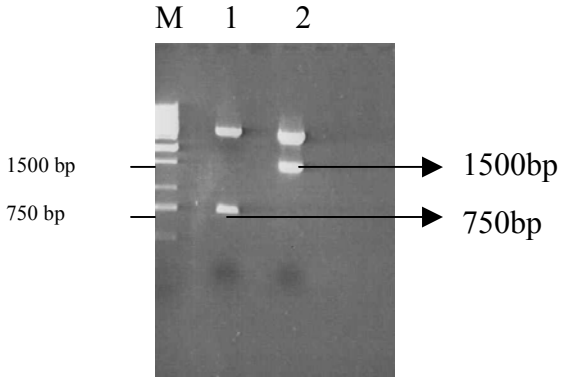

Fig.6.7 Characterization of pKOH200-pPRSV-CPsi-GFP cassette

Lane M: 1 kb marker, Lane 2: pKOH200-pPRSV-CPsi cassette Lane 2: pKOH200-pPRSV-CPsi-GFP cassette restriction digested with *Asc* I and *Sse8387* I

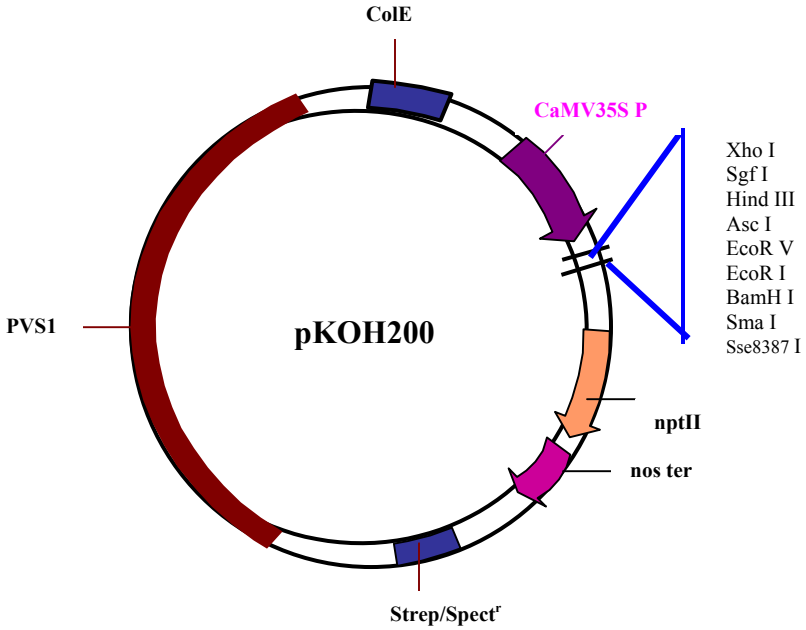

Fig. 6.8 Plasmid map of pKOH200

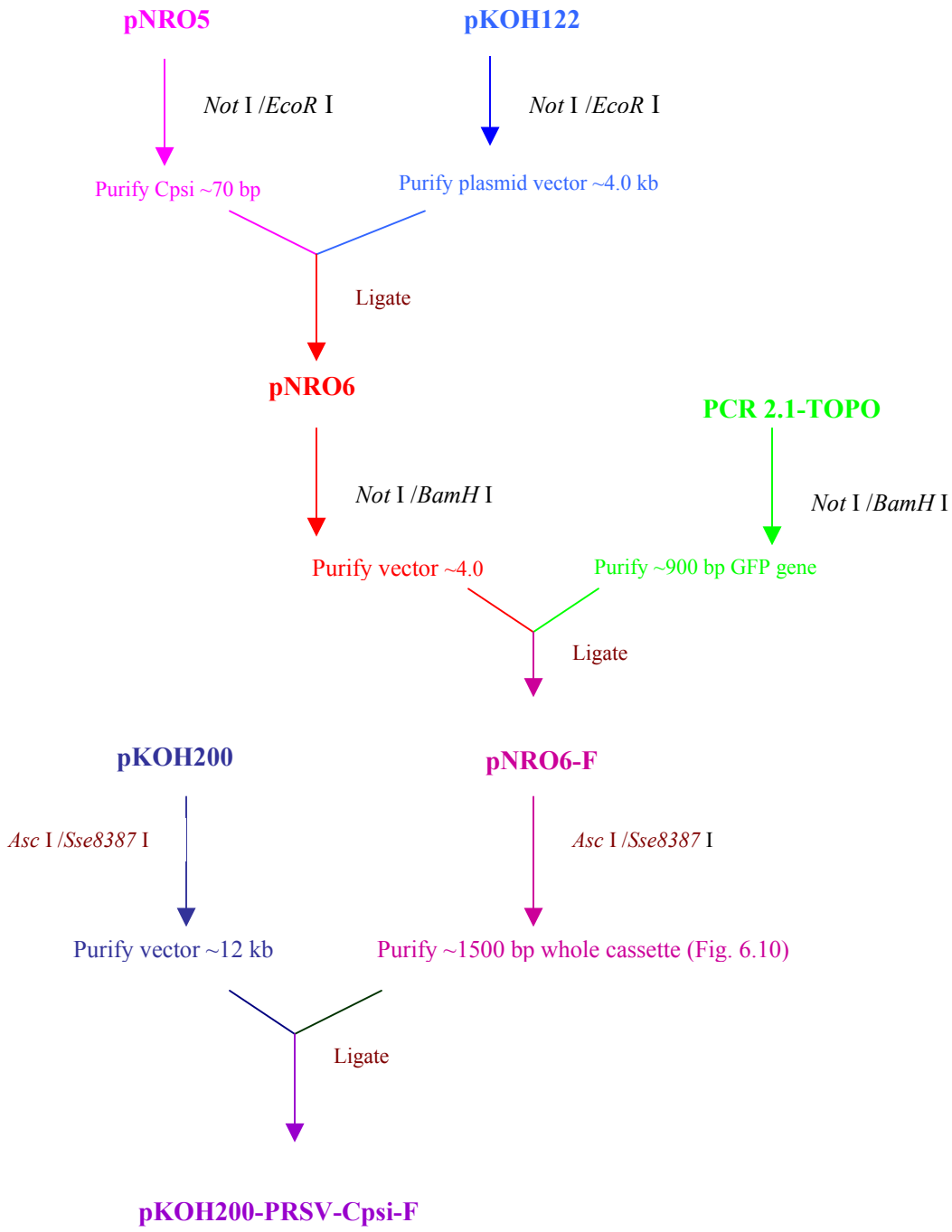

Fig. 6.9 Flow chart for the preparation of pPRSV-CPsi-GFP

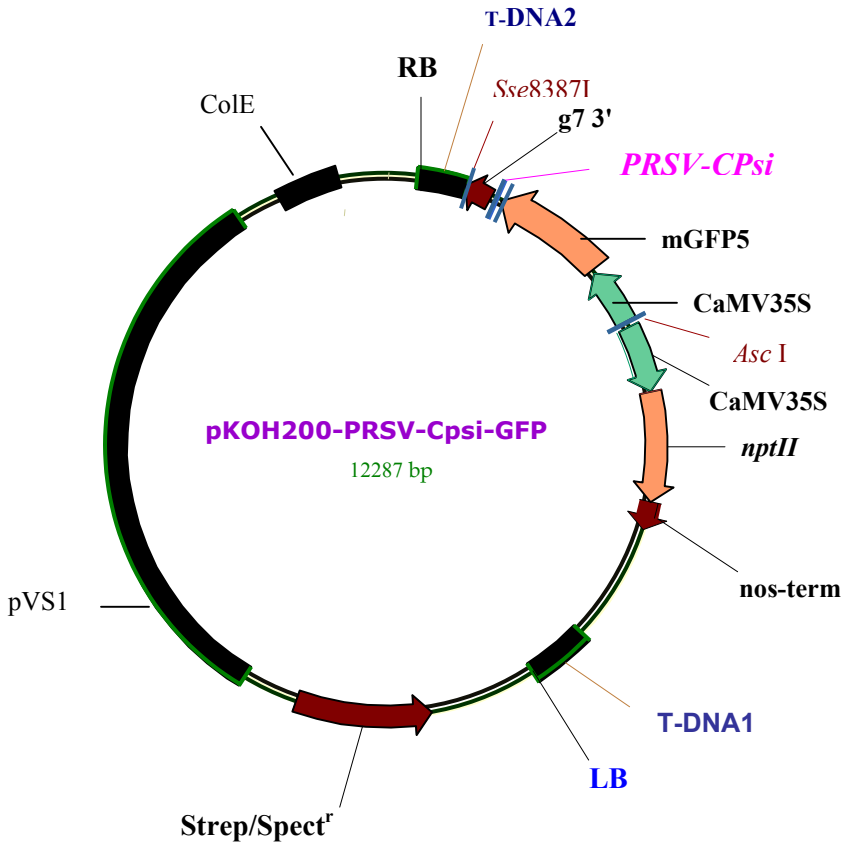

Fig. 6.10 Plasmid map of pKOH200-pPRSV-CPsi-GFP

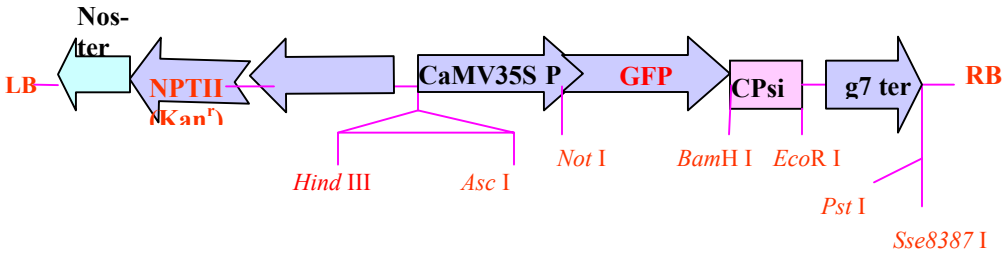

Fig.6.11 Linear construct pPRSV-CPsi-GFP

6.4.5 Sequence of pKOH200-pPRSV-CPsi-GFP

gggcctcttcgctattacgccagctggcgaaaggggatgtgctgcaaggcgattaagttgggtaacgccagggttttcccagt
cacgacgttgtaaaacgacggccagtgagcgcgcgtaatacgactcactatagggcgaattgggtacgttaattaagacgtcgg
cgccgcgatcgcgtaagcttagatctggcgcgcct**caacatggtggagcacgacactctcgtctactccaagaatatcaaa**
gatacagtctcagaagaccaaagggctattgagacttttcaacaaagggtaatatcgggaaacctcctcggattccattg
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actagtggatcc**atgctactgagaggtacatttcaagagaatgtacctctcagtagcatttttttgctagc**gaattcggccgg
cc**ttgtaccagctgatataatcagttattgaaatatttctgaatttaaacttgcatcaataaatttatgtttttgcttggactat**
aatacctgacttgttattttatcaataaatatttaaactatatttctttcaagatgggaattaacatctacaaattgccttttct
tatcgaccatgtacgtaagcgcttacgtttttggtggaccctgcaggataagctccagcttttgttccctttagtgagggttaatt
gcgcgcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacgagccggaagc
ataaagtgtaaagcctggggtgcct

35S promoter, GFP, CPsi, g7 terminator

6.4.6 Mobilization of pKOH-pPRSV-CPsi-GFP in *A. tumefaciens*:

The pKOH-CP-si (35S P+CP-si+g7 T) and pKOH-CPsi-F (35S P+GFP+CP-si+g7 T) vectors were mobilized into *A. tumefaciens* LBA4404 by freeze-thaw method (An *et al.* 1988). Detailed Plasmid mobilization protocol is given in Section 2.12.3 in Chapter 2. The pKOH-CPsi and pKOH-CPsi-F are isolated and characterized by restriction digestion *Asc* I and *Sse8387* I (Fig.6.12).

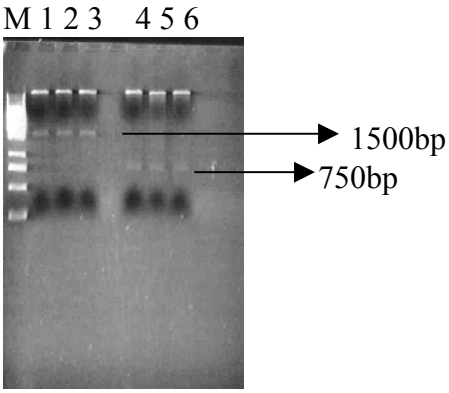

Fig.6.12 Mobilization of pKOH200-pPRSV-CPsi-GFP cassette in *A. tumefaciens*

Lane M: 1 kb marker, Lane1, 2,3: pPRSV-CPsi-GFP cassette, Lane 4,5,6: pPRSV-CPsi cassette

6.4.7 Transformation into papaya

A. tumefaciens harbouring pKOH200-pPRSV-CPsi-GFP construct was used to transform immature zygotic embryos of papaya. Detailed transformation protocol is given in Chapter 2. The transformed plants were then randomly selected for checking fluorescence. These selected plants were then used for the Southern blotting to confirm the transformation event.

6.4.8 Southern analysis

The presence and integration of the CPsi in the Kanamycin resistant plants (Fig.6.12) was analyzed by Southern hybridization blots (Southern 1975). For this 15 μg of DNA isolated from the putatively transformed plants was digested with restriction endonuclease *Eco*R I (10 units/μg of DNA), separated through a 1% agarose gel prepared in 1X TAE and transferred (Sambrook *et al.* 1989) to Hybond N^+ membrane (Amersham) by vacuum transfer. Negative controls consisted of DNA isolated from untransformed control plants. The 70bp fragment of CPsi was labelled with ^{32}[α-dATP] and used for hybridization performed according to Sambrook *et al.* 1989 in 6X SSPE, 5X Denhardt's solution, 0.4 % SDS solution at 55^0C before washing membranes with 2x SSPE, 0.5% SDS solution at 55^0C. DNA bands were detected after 5 days exposure to Kodak X-MAT AR autoradiography film at -70^0C. Randomly selected transformed GFP visualized plants after Southern analysis confirmed presence of CPsi (Fig.6.14).

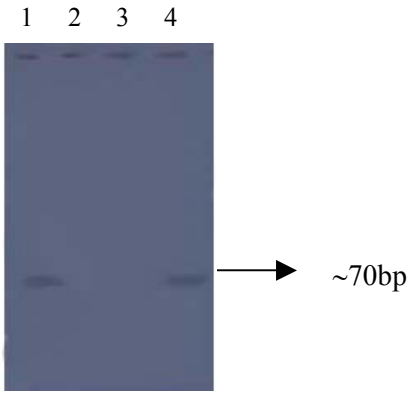

Fig. 6.14 Southern analysis of CPsi transformed plants

1.Positive Control
2. Control plant gDNA
3. Negative control
4. CP-siRNA transformed plant gDNA

The helper component proteinase (HC-Pro) is a key protein encoded by plant viruses of the genus Potyvirus. HC-Pro is involved in different steps of the viral cycle, aphid transmission, replication, and virus cell-to-cell and systemic movement and is a suppressor of post-transcriptional gene silencing. From the observations we conclude that HC-pro suppresses RNA mediated silencing, as growth of the *in vitro* grown plants was slow as compared to control plants. Expression of the HC-Pro coding sequence alone was sufficient to suppress silencing (Anandalakshmi *et al*. 1998). Candidate suppressors like 2b and HC-Pro viral proteins that blocks Post transcriptional gene silencing in plants (Voinnet *et al*. 1999, Llave *et al*. 2000, Savenkov and Valkonen 2002, Pruss *et al*. 2004). P1/HC-Pro polyprotein encoded by tobacco etch virus functions as a suppressor of PTGS of plant viruses (Kasschau and Carrington 1998).

HC-Pro does not suppress TGS induced by promoter dsRNA (Mette *et al*. 2001). Moreover, the amount of promoter small RNAs is elevated 5-fold in the presence of HC-Pro, and target promoter methylation is slightly increased without a concomitant rise in the level of promoter dsRNA. The promoter dsRNA, which is not polyadenylated, failed to trigger substantial degradation of polyadenylated, single-stranded promoter RNA. The differential effects of HC-Pro on small RNA accumulation associated with dsRNA-

mediated TGS and at least some cases of PTGS suggest that dsRNA processing can occur by alternative pathways, and they support the idea that RNA directed DNA methylation is triggered by small RNAs (Mette *et al*. 2001).

6.5 Conclusions

The availability of the complete genomic sequence of PRSV enabled us to select for CPsi to be tested for coat protein (CP) gene, even though possess RNA silencing suppressing protein, HC-Pro.

From the observations we conclude that HC-pro suppresses RNA mediated silencing, as growth of the *in vitro* grown plants was slow as compared to control plants. As natural resistance to PRSV in papaya seems to be of limited use, it is legitimate to attempt alternative strategies for obtaining virus resistance through biotechnological means.

CONCLUSIONS

I. SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN PAPAYA

In the present study the potential of immature and mature zygotic embryo explants of papaya to regenerate somatic embryos as influenced by various physiochemical agents was assessed. Among the phytohormones tested Picloram was found to elicit the best somatic embryogenesis response from both immature and mature zygotic embryo explants. Phytohormones 2,4-D, Dicamba, 2,4,5-T and combinations of Zeatin: 2,4,5-T also induced somatic embryogenesis, however, the degrees of response varied with the concentration of the phytohormone used. Picloram at 4.14 μM induced somatic embryogenesis from the maximum number of explants. About 97±6% immature zygotic embryo of cv. Honey Dew and 93±6% in cv. Washington responded with formation of somatic embryos. The response from mature zygotic embryos was 30±0% in cv. Honey Dew and 27±6% in cv. Washington. Maximum conversion of globular staged embryo to cotyledonary stage embryo was also obtained in presence of Picloram (4.14 μM). Maturation of these embryos was achieved (96.67%) in media supplemented with Spermidine (1 μM).

Increase of boric acid concentration from 6.2 mg/l to 62 mg/l resulted in significantly higher somatic embryogenesis response both in presence of Picloram or 2,4-D. Embryogenesis in the form of rosette structure was observed from the apical meristematic regions of the explant. It existed for a short span and then switched over to the cotyledonary stage embryos. Formation of embryos was continuous even after 6-8 months of repeated culture. Cotyledonary structures obtained were morphologically normal and developed shoot and root poles.

The use of ethylene antagonists in the medium ensured 10-12 fold enhancement in embryo maturation. Where as only 8.3% of the regenerated somatic embryos matured on the basal MSB medium, the respective percent embryo maturation was 96.67% in the presence of Spermidine (1.0 μM), 76.67% in the presence of Putrescine (0.5 μM), 90.0% in the presence of ABA (0.1 μM) and 86.67% in the presence of $AgNO_3$ (0.5 μM). Maturation of somatic embryos was adversely affected by higher (10 μM) concentration of ethylene antagonists. The mature cotyledonary embryos were transferred to modified MS medium for conversion into plantlets. The normal cotyledonary embryos germinated and grew into whole plantlets.

High concentrations of a phytohormone (Picloram 30-60 μM, 2,4.5-T 20-40 μM, 2,4-D 20-40 μM and Dicamba 60-135 μM) and its prolonged presence in the culture medium affected somatic embryo morphology and the development of normal plantlets. .

The present investigations offer an opportunity to achieve plant regeneration from immature and mature zygotic embryo of papaya. Somatic embryo induction response was genotype independent and could be obtained from both the immature and mature zygotic embryo explants. These regeneration protocols are amenable for genetic transformation in papaya *via A. tumefaciens* and or particle bombardment.

II. INDUCTION OF MULTIPLE SHOOTS AND PLANT REGENERATION IN PAPAYA

The present protocol describes a rapid, efficient and reproducible method for the development of papaya plants *via* multiple shoot regeneration using immature zygotic embryo explant from papaya cvs. Honey Dew, Washington and Co-2.

Maximum induction of multiple shoots (~14 shoots per explant) was achieved from immature zygotic embryo explants cultured in MBG medium supplemented either with TDZ (2.2 μM) or BAP: NAA (4.44:0.54 μM). Among all the basal media tested, MBG medium was found to be the most suitable for multiple shoot induction. Elongation of the shoots was obtained in MBG medium supplemented with GA_3 (5.7 μM). The best rooting response of the *in vitro* regenerated shoots was achieved on half strength MBG basal medium supplemented with 14.7 μM IBA.

Survival of tissue culture raised plants was 76% after hardening under greenhouse conditions. Use of immature zygotic embryo explant for multiple shoot development offers the additional advantages of maximum number of regeneration and survival of plantlets.

Agrobacterium mediated transformation using green fluorescent protein (GFP) as Screenable marker

In the present study, an attempt was made to standardize a protocol for *A. tumefaciens* mediated transformation system for papaya using GFP as the screenable marker. The immature zygotic embryo explants were co-cultivated with *A. tumefaciens* LBA4404

strain harbouring pBIN-mgfp5-ER vector. The integration of the GFP gene in somatic embryos and multiple shoots was confirmed by both fluorescence and Southern analysis. One added advantage in the non-destructive means of analysis of GFP was its easy detection without harmful effect on the living tissue. 35S promoter driven GFP reporter gene is a useful, non-destructive, inexpensive and standard screenable reporter marker. This work provides simple and rapid protocol for the genetic transformation.

Isolation and cloning of PRSV Coat Protein gene and its Characterization

Using primer two sets of primers FI: RI, FII: RI, FI: FII: RI, FIII: RIII (Set 1: PRSV-CP-F-I: 5'd(TATGGATCCTCCAAGAATGAAGCT) 3' PRSV-CP-F-II: 5'd(TATGGATCCAGTCCAAAAATGAAGCTG) 3' Reverse primer PRSV-CP-R-I: 5'd(TATGGATCCTTAGTTGCGCATACC) 3' Set 2: Forward primer PRSV -FIII: 5'd(GATCCATGCTGAGAGGTACATTTCAAGAGAATGTACCTCTCAGTAGCATTTTTTTGCTAGCG)3' Reverse primer: PRSV-RIII: 5'd(AATTCGCTAGCAAAAAAATGCTACTGAGAGGTACATTCTCTTGAAATGTACCTCTCAGTAGCATG) 3' ~1.0 kb amplicon was generated. The amplicon was determined to be 858bp in size. The single ORF coded for 285 amino acids of an approximate molecular mass of 31.35 KDa. The sequence was deposited with NCBI GenBank and is available under the accession No. **DQ192587**. Multiple sequence analysis of the amplicon with BLAST and CLUSTAL showed 87-97% nucleotide sequence similarity with other reported PRSV-P strain. Comparative sequence analysis of the PRSV isolates from different countries at nucleotide and amino acid level revealed that there was substantial sequence diversity with the geographical distribution of the isolates. The isolates from India shared 89-97% nucleotide similarity, while other Asian and the isolates from Australia and America (Brazil, Mexico and United States) shared 87-92% nucleotide similarity. Multiple sequence analysis showed a 92-96% amino acid sequence similarity with worldwide reported PRSV-P strain.

Short interference RNA (PRSV-CP) and gene silencing

The availability of the complete PRSV coat protein sequence of the Indian isolate enabled us to select for CPsi to be tested for coat protein (CP) gene.

From the observations we conclude that the introduction of the PRSV Cpsi sequence into the papaya plants occurred at some critical locus in the plant genome since the growth of the *in vitro* grown plants was slow as compared to control plants and the plants failed to develop roots..

As natural resistance to PRSV in papaya seems to be of limited use, it is legitimate to attempt alternative strategies for obtaining virus resistance through biotechnological means.

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