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Agrobacterium tumefaciens Mediated Transformation of *Nicotiana tabacum* Plant with PRSV Coat Protein Gene

RAMACHANDRA ANANTAPUR, M. ROSHNI AND ANITHA PETER
Department of Plant Biotechnology, College of Agriculture, UAS, GKVK, Bengaluru-560 065
e-Mail : ramchandra.anantapur@gmail.com

ABSTRACT

Papaya ringspot virus is a major limiting factor for the cultivation of papaya in tropical and subtropical areas throughout the world. The present investigation was undertaken with the aim of incorporating coat protein gene of the virus to impart pathogen derived resistance through genetic engineering. Attempts were made to incorporate *PRSV CP* gene in the model plant *Nicotiana tabacum* through *Agrobacterium* mediated approach. The viral RNA was isolated and converted to cDNA by Reverse transcription polymerase chain reaction and amplified with *PRSV CP* gene specific primers to obtain an amplicon size of ~900bp. This amplicon was further cloned into pTZ57R/T vector, transformed into *E. coli* DH5α and sequenced to confirm its identity as CP. The CP gene was further sub-cloned into binary vector pBI121 and transformed to *N. tabacum* through *Agrobacterium* mediated approach, regenerated on selective media with 100 mg L^{-1} kanamycin and 200 mg L^{-1} cefatoxime, confirmed by polymerase chain reaction and serological approaches like Dot Blot.

Keywords : Coat protein, cDNA, Amplicon, Nicotiana

PAPAYA (*Carica papaya* L.) is an important fruit crop of tropical and subtropical regions of the world belonging to the family *Caricaceae* and has both economical and nutritional value. It is infested by an important viral disease Papaya Ring Spot Virus (PRSV) which causes ringspot on fruits and stems. The disease was reported for the first time in the island of Ohau in Hawaii (Parris, 1938), which was sap transmissible and later named as 'Wailu' disease (Lindner *et al.,* 1945) and shown to be of viral nature by Jensen (1949) from Kailu, island of Ohau in Hawaii.

The virus is ssRNA belonging to the family potyviridae and transmitted by numerous species of aphids in a non-persistent manner with limited host range (Anonymous, 2004 and Hema & Prasad, 2004). The conventional methods are not very effective in controlling the disease, as traditional genetic sources of virus resistance are rare and the resistance is frequently broken under field conditions (Lecoq *et al.,* 2004). In the 1990s, the Papaya industry on Hawaii suffered a 50 per cent decline in production due to an outbreak of the potyvirus, PRSV (Gonsalves, 1998). Virus resistance was obtained in a high-yielding papaya hybrid using the viral coat protein sequence as the transgene which led to development of Rainbow and Sunup (Gonsalves, 2004).

A similar approach has been successfully applied in US cucurbit production, although the situation has been more complicated due to the presence of several different viruses (Fuchs *et al.,* 1997 and Fuchs & Gonsalves, 2007). The results obtained repeatedly in laboratory and field studies demonstrate that transgenic strategies for virus resistance works effectively.

The genetically engineered resistance is referred as pathogen-derived resistance (PDR) (Sanford & Johnston, 1985), classified as RNA and protein mediated. RNA-silencing is a sequence specific RNA degradation or silencing mechanism, operates as a natural antiviral defense system and provides promising potential for viral resistance (Eamens *et al.,* 2008) and followed by protein mediated resistance, where the transformation cassette is designed to be translated and expressed into the plant leading to interference of viral assembly (Ritzenthaler, 2005).

Controlling plant viruses by genetic engineering, including the globally important PRSV, mainly involves coat protein (CP) gene mediated resistance *via* post-transcriptional gene silencing (PTGS). Although the CP gene of PRSV has been transferred into papaya by particle bombardment and transgenic lines with high resistance to Hawaii strains have been obtained, they are susceptible to PRSV isolates outside Hawaii. This strain-specific resistance limits the application of the transgenic lines in other areas of the world (Bau *et al*., 2002). The study involves development of a transgenic *N. tabacum* a model plant, where *PRSV CP* gene of south Indian isolate (Hosakote strain) is transferred and expressed in T_0 plants through *Agrobacterium* mediated transformation and confirmed by molecular studies.

Material and Methods

RNA Isolation and cDNA Synthesis of *PRSV* CP Gene

Field infected PRSV plant leaf sample was collected and used for the isolation of total RNA using Lithium chloride method (LiCl) (Yang *et al*., 2008). The isolated RNA was used as template for the synthesis of cDNA by MMuLV-RT enzyme (Fermentas). The synthesized cDNA was amplified by sense and antisense coat protein gene specific primers (CP gene specific FP: CCATGGCCAAAAATGAAGCTGTGGATAC and CP gene specific RP : AGATCTATACCCAGGAGA GAGTGCATGT).

Plasmid Constructs

The PCR product was purified and cloned in pTZ57RT cloning vector (InsTAclone™ PCR cloning kit, Fermentas, USA), pTZ57RT+*PRSV* CP gene was transformed into competent *E. coli* DH5α cells following standard protocols (Sambrook and Russell, 2001). The positive recombinant clones were identified by blue-white screening, confirmed by gene specific PCR amplification using gene specific primers and by restriction analysis. For PCR confirmation of clones, the template DNA from plasmid was isolated following the alkaline lysis protocol of Brimbion and Dolly (1979) and restriction analysis was done using BamHI-HF and SacI-HF restriction enzymes. The PRSV CP gene cloned into pTZ57R/T was sequenced using M13 forward and reverse primers. The sequence was analyzed using BLAST algorithm (https://www.ncbi.nlm.nih.gov/). Confirmed clone of *PRSV* CP gene was sub-cloned into a binary vector pBI121, using the same restriction enzymes, BamHI-HF and SacI-HF where *GUS* gene was removed from pB121 and *PRSV CP* gene was cloned in between right and left borders of T-DNA region, driven by CaMV35s promoter, with a selectable *neomycin/kanamycin* gene and nos terminator sequence (Fig. 1). The construct of pBI121+*PRSV CP* gene was transformed into competent *E. coli* DH5α cells. The transformants were confirmed by colony PCR, plasmid PCR amplification and restriction analysis was done using the same restriction enzymes mentioned above.

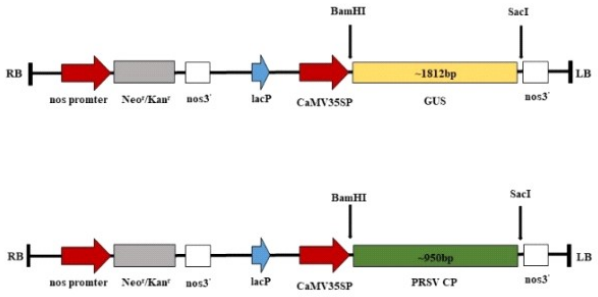

Fig. 1: The construct of pBI121+*PRSV CP*

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The confirmed clones were further transferred in to *Agrobacterium tumefaciens* strain LBA4404 by freeze thaw method (Jyothishwaran *et al*., 2007). Transformed Agrobacterium were confirmed by colony PCR and plasmid PCR with gene specific primers. The positive colonies of transformed *Agrobacterium* with the construct of pBI121+*PRSV* CP gene were used for co-cultivation of *N. tabacum*, which were grown in YEP medium with rifamycin 100mgL^{-1} and 50mgL^{-1} kanamycin at 28^{0}C for 48 hours. Bacterial cells were pelleted at a speed of 3500 rpm for 10 min and re-suspended in half MS solution plant culture medium at a density of 10^9 cells per ml (OD560=1) with 100mM of acetosyrinzone. This infection solution

was used for co-cultivation of tobacco explants (leaf discs of 1 cm^2), which were tap dried and kept in dark for 48 hours (Jyothishwaran *et al*., 2007).

The co-cultivated explants from dark were transferred to a fresh MS medium with 2 mgL^{-1} Kinetin, 1 mgL^{-1} IAA for multiple shoot regeneration. Explants with multiple shoots were subjected to selection media containing 100 mgL^{-1} kanamycin for transformed plant confirmation and 200 mgL^{-1} cefatoxime to kill the excess growth of *Agrobacterium.* Further they were transformed into rooting media consisting of half strength MS media. Rooted plants were hardened at *in vitro* condition for about 15 days and later transferred to greenhouse conditions.

Confirmation Studies

The T_0 plants were confirmed for integration of the *PRSV CP* gene by molecular PCR amplification using gene specific primers Every PCR reactions were performed in eppendrof tubes containing 20 µl of reaction solution having, 10X reaction buffer, 2 mM dNTPs, 100 ng of DNA, 10pmol/µl of forward and reverse primers each and 3U/il of TaqDNA polymerase, Fermentas. The PCR amplification of DNA was performed using a thermal cycler (Bio-Rad), under the given conditions of temperature: 94 °C for 3 min followed by 35 cycles of 94 °C for 45 seconds, 60°C for 1:30 min, 72 °C for 1 min and final extension reaction at 72°C for 30 min. Amplified DNA fragments were then analyzed by electrophoresis using agarose gel 1 per cent and visualized through ethidium bromide staining (Sambrook *et al*., 1989) under ultraviolet light.

The expression of the gene was confirmed by serological technique, Indirect Dot Immuno-Binding Assay (DIBA) which was conventionally performed on nitrocellulose membrane with antibodies specific to PRSV CP. The DNA isolated from transformed and non-transformed plants was denatured by heating on a temperature 95 °C for five min. and cooled rapidly on ice. Dots of DNA were made with the help of a micropipette on nylon membrane. The membrane was allowed to dry at 37 °C overnight and then hybridized with the antibodies specific to PRSV CP followed by washing. Secondary antibody with the conjugated enzyme was hybridized and substrate was added to detect the color.

Results and Discussion

Isolation of *PRSV CP* Gene

RNA isolated from PRSV infected leaf samples was subjected to RT-PCR for the synthesis of cDNA, which was used as template for PCR amplification of *PRSV CP* gene specific primers. The obtained amplicon was run on 1 per cent agarose gel and the size obtained was approximately 950bp (Fig. 2). Various study report suggests several sizes of *PRSV CP* gene and it has a conserved region of ~534 bp (Byadgi, 2008 and Krubphachaya *et al*., 2007). The PCR amplification of cDNA using the PRSV-CP gene specific primers deduced from the blast search, resulted in a ~900bp fragment (Quemada *et al*., 1990; Wang and Yeh, 1992 and Silva-Rosales *et al*., 2000). Kunklikar and Byadgi (2004) reported that the PRSV isolate Type-P from India showed 715 bp long nucleotide sequence.

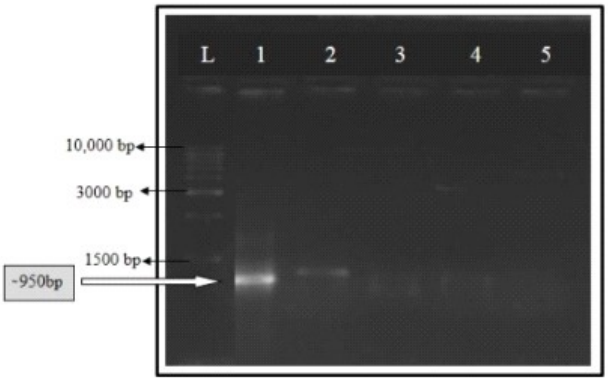

Fig. 2: Amplification of PRSV coat protein gene from the infected papaya leaf samples

Yap *et al*., (2009) reported that the CP gene obtained from transformed papaya revealed a size of 800bp. Hema and Prasad (2004), reported molecular characterization of the coat protein (CP) gene of a South Indian strain (INP-UAS) of PRSV-P and revealed an open reading frame of 849 bp. Size differences resulted from the differences in the number of lysine and glutamate (KE) repeats in the amino terminal region (Jain *et al*., 2004). NCBI database information on PRSV CP gene also supports this,

which probably is due to the fact that various groups have reported partial sequence of the CP gene. Genome structure of PRSV reveals that the CP gene exist polycistrionically with the NIb gene of PRSV and it may be difficult to separate the two and hence difference in the reported size of PRSV could exist.

Amplicon of PRSV CP gene was cloned into pTZ57R/T vector and the ligated product was transformed into *E.coli* DH5α cells by heat shock method. After incubation at 37^{0}C overnight, transformed colonies were observed. Transformed colonies were observed and selection was done based on blue-white screening (Fig. 3a). CP gene of PRSV- Hyderabad strain was cloned in to pTZ57R vector of 2.88kb size with T overhang. The vector was transformed into *E. coli* strain DH5α, multiplied and transformation finally confirmed (Kunkalikar, 2003). Hema and Prasad, (2004) amplified the PRSV CP gene, gel-eluted it by using the freeze-thaw method, cloned into pTZ57R and using an InstT/A PCR cloning kit transformed into *E. coli* strain XL1-Blue MRF' cells.

Cloning of PRSV CP Gene in Binary Vector

The plasmid DNA (pTZ57RT+*PRSV CP* gene) from the transformed *E. coli* DH5α cell was isolated and the pTZ57R/T plasmid containing CP gene was confirmed by using the CP gene specific primers. The PCR amplification showed the presence of ~ 950 bp *PRSV-CP* gene amplicon when it was run on 1 per cent agarose gel and by restriction analysis 950 bp *PRSV-CP* gene was released (Fig. 3b and 3c).

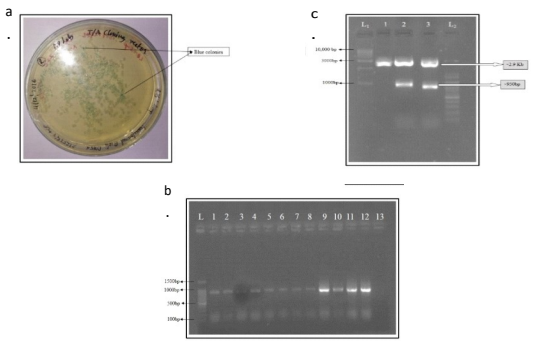

Fig. 3: (a). Transfromed *E. coli* DH5α cells with pTZ57RT + *PRSV CP* gene, (b). Colony PCR for confirmation of recombinant clones and (c). Restriction analysis of the positive transformed colonies

The plasmid DNA isolated from the transformed *E.coli* DH5α cell was sequenced using M13 primers. The sequences obtained from the automated sequencing was analyzed using NCBI-BLAST to find possible matches with the papaya ring spot virus coat protein gene reported throughout the world in order to confirm that it was *PRSV CP* gene.

According to the sequencing results, the gene was inserted in the proper orientation in into pTZ57R/T vector. Therefore, the restriction enzymes BamHI and SacI were used to cleave the insert from the pTZ57RT+*PRSV CP* gene construct, simultaneously binary vector pBI121 was also digested with same enzymes. The release of *PRSV CP* gene and cleaved pBI121 were eluted and ligated to develop a construct of pBI121+*PRSV CP* gene, which was transformed into *E. coli* DH5α cells by heat shock method. After incubation at 37^{0}C overnight, transformed colonies were observed (Fig. 4a). Yepes *et al*. (1996) cloned the sense coat protein gene of tomato ringspot nepovirus into pBI121 binary vector.

A binary vector containing the coat protein gene under the control of a 35S promoter, was constructed and transformed into somatic embryos of papaya cultivar Khak Dum by micro projectile bombardment (Kertbundit *et al*., 2007). Chon-Seng *et al*. (2007) cloned and sequenced a 927bp CP gene of PRSV. Subsequently the gene was sub-cloned into bacterial

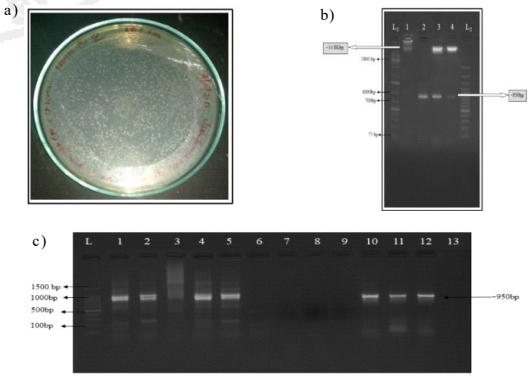

Fig. 4: (a). Transfromed *E. coli* DH5α cells with pBI121 + *PRSV CP* gene, (b). Colony PCR for confirmation of recombinant clones (L1- 1kb ladder; Lane 1, 2, 4, 5, 10, 11 and 12 positive transformed colonies) and (c). Restriction analysis of the positive transformed colonies (L1- 1kb ladder, L2- 100bp ladder, Lane 1 is isolated pBI121 + PRSV CP construct; Lane 2- PRSV CP; Lane 3 and 4- restriction digestion)

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expression vector pRSET, to form pRSET:PRSVCP and expressed in *E. coli* BL21(DE3) strain.

The plasmid DNA (pBI121+*PRSV CP* gene) from the transformed *E.coli* DH5α cell was isolated and the pBI121 plasmid containing CP gene was confirmed by using the CP gene specific primers. The PCR amplification showed the presence of ~ 950 bp *PRSV-CP* gene amplicon when it was run on 1 per cent agarose gel and by restriction analysis 950 bp *PRSV-CP* gene was released (Fig. 4b and 4c).

Transformation of *Agrobacterium* with Recombinant pBI121 Vector

The plasmid DNA (pBI121+*PRSV CP* gene) was isolated from *E. coli* DH5α cells and was confirmed with CP gene specific primers. Then it was mobilized to *Agrobacterium* strain LBA4404. The transformants from *Agrobacterium* strain LBA4404 were streaked onto YEP media containing 50mg L^{-1} kanamycin and 100mg L^{-1} rifamycin, after incubation for 48 hours at 28 ^{0}C transformed colonies were observed (Fig. 5a).

The transformed *Agrobacterium* colonies were confirmed by performing colony PCR using *PRSV CP* gene specific primers which showed ~ 950bp CP gene from the pBI121 vector backbone on 1 per cent agarose gel (Fig. 5b). This confirmed the recombinant nature of the transformed colonies. The sense coat protein gene of tomato ringspot nepovirus was cloned into binary vector and transformed into *Agrobacterium* (Yepes *et al*., 1996).

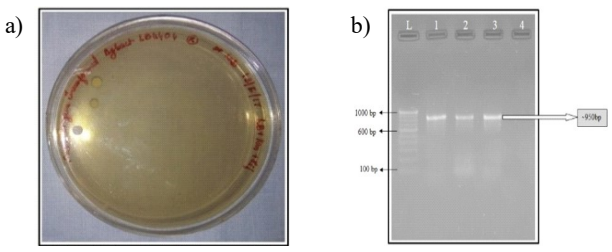

Fig. 5 : (a) Transformed *Agrobacterium* cells with pBI121 + *PRSV CP* gene and (b) Colony PCR for confirmation of recombinant clones

Transformation of pBI121+*PRSV CP* Gene Construct into *N. tabacum*

N. tabacum leaf discs were co-cultivated with the confirmed *Agrobacterium* colonies with pBI121+*PRSV CP* gene construct and were incubated for 48 hours in dark conditions for co-cultivation. *N. benthamiana* and *N. tabacum* transformed by *Agrobacterium* mediated transformation (Yepes *et al*., 1996). The co-cultivated explants were washed with cefataxime, tap dried and sub-cultured in shooting media containing 100mgL^{-1} kanamycin for transformed plant confirmation and 200mgL^{-1} cefatoxime to kill the bacteria. Transformed explants survived and response like curling of disc end was observed after 7-10days. Multiple shoots were observed after 20-25 days of inoculation and they were transformed to rooting media (Fig 6).

Fig. 6: Regeneration of *N. tabacum* on selection media after *Agrobacterium* mediated transformation, shoot emergence, multiple shooting and transferred to rooting media

PCR and Dot Blot Analysis/ Molecular confirmation of Transgenic Plants

Before transferring to the rooting media the DNA was isolated from the transformed shoots, which was used for PCR amplification with *PRSV CP* gene specific primers (Fig. 7a). Among the T_0 plants in two, there was amplification and the band size of ~ 950 bp was observed on 1 per cent agarose gel. Further to confirm the gene expression, protein dot blot analysis was conducted using PRSV CP antibody at different

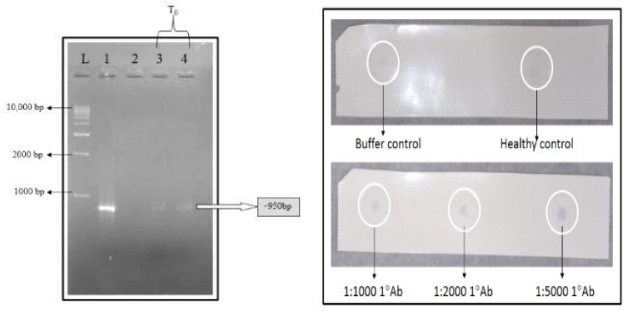

Fig. 7: (a). Colony PCR for confirmation of transgenic *N. tabacum* plants and (b). Indirect Dot Immuno-Binding Assay for confirmation of protein expression

concentrations, there was no color development in buffer control and healthy controls. Color was observed in transgenic plants, which confirmed the expression of protein in the transformed T_0 plants. The plants will be further advanced to T_1 generation and bioassay studies will be done.

References

Bau, H. J., Cheng, Y. H., Yu, T. A., Yang, J. S. and Yeh, S. D., 2003, Broad-spectrum resistance to different geographic strains of papaya ringspot virus in coat protein gene transgenic papaya. *Phytopathol.*, **93** (1) : 112 - 120.

Brimbion, H. C. and Dolly, J., 1979, A rapid alkaline lysis procedure for screening recombinant plasmid DNA. *Nucl. Acid Res.,* **7** : 1515 - 1523.

Byadgi, A. S., 2008, Characterization, diagnosis and management of plant virus. *Horticultural crops*, pp. 125 - 144.

Chon-seng, T., Poh, H. W. A. T., Ching Ang, O. and Mat, D. H., 2007, Cloning and expression of papaya ring spot virus (PRSV) coat protein gene in bacteria. *J. Hort. Sci. Biotech.,* **3** : 234 - 241.

Eamens, A., Wang, M. B., Smith, N. A. and Waterhouse, P. M., 2008, RNA silencing in plants: yesterday, today, and tomorrow. *Pl. physiol.*, **147** (2) : 456 - 468.

Fuchs, M. and Gonsalves, D., 2007, Safety of virus-resistant transgenic plants two decades after their introduction: Lessons from realistic field risk assessment studies. *Annu. Rev. Phytopathol.*, **45** : 173 - 202.

Fuchs, M., Mcferson, J. R., Tricoli, D. M., Mcmaster, J. R., Deng, R. Z., Boeshore, M. L and Gonsalves, D., 1997, Cantaloupe line CZW-30 containing coat protein genes of cucumber mosaic virus, zucchini yellow mosaic virus, and watermelon mosaic virus-2 is resistant to these three viruses in the field. *Mol. Breed.*, **3** (4) : 279 - 290.

Gonsalves, D., 1998, Control of papaya ringspot virus in papaya : A case study. *Annu. Rev. Phytopathol.*, **36** : 415 - 37.

Gonsalves, C., Lee, D. R. and Gonsalves, D., 2004, Transgenic virus-resistant papaya : The Hawaiian 'Rainbow' was rapidly adopted by farmers and is of major importance in Hawaii today. *APSnet Features*.

Hema, M. V. and Prasad, D. T., 2004, Comparison of the coat protein of a South Indian strain of PRSV with other strains from different geographical locations. *J. Plant Pathol.*, **86** : 35 - 42.

Jain, R. K., Sharma, J., Sivakumar, A. S., Sharma, P. K., Byadgi, A. S., Verma, A. K. and Varma, A., 2004, Variability in the coat protein gene of papaya ringspot virus isolates from multiple locations in India. *Arch. Virol.*, **149** : 2435 - 2442.

Jensen, D. D., 1949, Papaya virus disease with special reference to papaya ring spot. *Phytopathol.*, **39 :** 191 - 210.

Jyothishwaran, G. D., Kotresha, T., Selvaraj, S. M., Srideshikan, P. K., Rajvanshi and Jayabaskaran, C., 2007, A modified freeze-thaw method for efficient transformation of *Agrobacterium tumefaciens*. *Curr. Sci*., **93** (6) : 770 - 772.

Kertbundit, S., Pongtanom, N., Ruanjan, P., Chantasingh, D., Tanwanchai, A., Panyim, S. and Juøíèek, M., 2007, Resistance of transgenic papaya plants to papaya ring spot virus. *Biol. Plant.*, **51** (2) : 333 - 339. LECOQ, H., MOURY, B., DESBIEZ, C.,

Krubphachaya, P., Juricek, M. and Kertbundit, S., 2007, Induction of RNA-mediated resistance to *papaya ring spot* virus type W. *J. Biochem. Mol. Biol.,* **40** : 404 - 411.

KUNKALIKAR, S. AND BYDAGI, A. S., 2004, Molecular characterization, cloning of coat protein gene, Epidemiology and management of *Papaya Ring Spot Virus*. *Indian Phytopathol*., **57** (3) : 330 - 340.

KUNKALIKAR, S., 2003, Molecular characterization, cloning of coat protein gene, epidemiology and management of papaya ring spot virus, *Ph.D. Thesis*, University of Agricultural Sciences, Dharwad, p.109.

LECOQ, H., MOURY, B., DESBIEZ, C., PALLOIX, A. AND PITRAT, M., 2004, Durable virus resistance in plants through conventional approaches: a challenge. *Virus Res*., **100** (1) : 31 - 39.

LINDNER, R. C., JENSON, D. D. AND IKEDA, W., 1945, Ring spot new papaya plunderer. *Hawaii Farm and Home*, **8** : 10 - 12.

PARRIS, G. K., 1938, A new disease of papaya in Hawaii. *Proc. American Soc. Hort. Sci*., **36** : 263 - 265.

QUEMADA, H., L HOSTIS, B., GONSALVES, D., REARDON, I. M., HEINRIKSON, R., HIEBERT, E. L., SIEU, L. C. AND SLIGHTOM, J. L., 1990, The nucleotide sequences of the 32 -terminal regions of papaya ringspot virus strains W and P. *J. Gen. Virol*., **71** : 203 - 210.

RITZENTHALER, C., 2005, Resistance to plant viruses: old issue, new answers. *Curr. Opin. Biotechnol.*, **16** (2) : 118 - 122.

SAMBROOK, J. AND RUSSELL, D. W., 2001, Molecular cloning: A laboratory manual. *Cold Spring Harbor Laboratory Press*, ISBN-10 0-87969-577-3, pp. 132 - 150.

SANFORD, J. AND JOHNSTON, S., 1985, The concept of pathogen derived resistance : Deriving resistance genes from the parasite's own genome. *J. Theor. Biol.,* **113** : 395 - 405.

SILVA-ROSALES, L., BECERRA-LEOR, N., RUIZ-CASTRO, S., TELIZ-ORTIZ, D. AND NOA-CARRAZANA, J. C., 2000, Coat protein sequence comparisons of three Mexican isolates of papaya ringspot virus with other geographical isolates reveal a close relationship to American and Australian isolates. *Arch. Virol*., **145** : 835 - 843.

WANG, J. J. AND YEH, S. D., 1998, Characterization of the papaya ringspot virus W type isolates collected from different areas of Taiwan by host reactions, immune diffusion tests and RT PCR. *FAO Plant Prot. Bull.,* **40** (4) : 383 - 395.

YANG, G., ZHOU, R., TANG, T. AND SHI, S., 2008, Simple and efficient isolation of high-quality total RNA from *Hibiscus tiliaceus*, a mangrove associate and its relatives. *Prep. Biochem. Biotech.*, **38** : 257 - 264.

YAP, K., DUANGJIT, J. AND PANYIM, S., 2009, N-terminal of *Papaya ringspot virus* Type-W (PRSV-W) helper component proteinase (HC-Pro) is essential for PRSV systemic infection in zucchini Yun. *Virus genes.*, **38** (3) : 461 - 467.

YEPES, L. M., MARC, F., JERRY, L. S. AND DENNIS, G., 1996, Sense and antisense coat protein gene constructs confer higher levels of resistance to tomato ringspot nepovirus in transgenic *Nicotiana* species. *Phytopathol*., **86** (4) : 417 - 424.

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