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PRSV resistance in papaya (*Carica papaya* L.) through genetic engineering: A review

Swati Kumari, Mala Trivedi[1] and Maneesh Mishra*

*ICAR - Central Institute for Subtropical Horticulture, Rehmankhera, P.O. Kakori, Lucknow, India. [1]Amity Institute of Biotechnology, Amity University, Lucknow Campus, Uttar Pradesh, India. *E-mail: maneeshmishra.cish@gmail.com*

Abstract

Papaya is the first fruit crop which was not only successfully genetically engineered but also deregulated and commercialized. Pathogenic derived resistance was utilized for harnessing PRSV resistance. *Coat protein* gene from PRSV was invariably used to confer resistance against papaya ring spot virus. Microprojectile transformation has been the most preferred pathway. However, several reports are also available involving *Agrobacterium* pathway. Majority of workers found somatic embryos as the explant of choice for genetic manipulation in papaya compared to other explants. This paper highlights the global status of development of genetically engineered papaya for viral resistance.

Key words: Transgenic papaya, coat protein, papaya ring spot virus, microprojectile, *Agrobacterium*

Introduction

Papaya (*Carica papaya* L.) is grown commercially in India, USA, Brazil, Indonesia, Mexico, Philippines, Nigeria, Jamaica, China, Taiwan, Peru and Thailand (Jayavalli *et*. *al*., 2011). Papaya cultivation is hampered severely due to papaya ring spot virus which belongs to family Potyviridae (Van Regenmortel *et al*., 2000). Its genome potyviridae, is single stranded RNA of positive polarity (Prucifull *et al*., 1984). The virus spreads through aphid in non persistent manner which leads to PRSV disease eventually impairing the photosynthetic capacity of plants resulting in reduced fruit quality, yield, loss of vegetative vigor and finally fatality of plant (Van Regenmortel *et al*., 2000).

Resistance breeding has not been successful in tackling papaya ring spot virus disease. Moderate level of multigene resistance has been identified in papaya germplasm and used in resistance breeding program with little success (Conover and Litz, 1978). Genetic engineering for virus resistance in papaya has been found effective whereby transgenic plants expressing viral genome sequence resist attack by corresponding viruses. Transgenic papaya resistant to PRSV has been developed and commercialized in 1998 in Hawaii, USA by Dennis Gonsalves and his team (Gonsalves *et al.*, 2003). Later transgenic papaya was deregulated in countries like Japan and Canada (Gonsalves *et al*., 2010). Large scale planting of transgenic papaya variety Rainbow in Puna district of Hawaii not only enhanced the production of papaya but it also encouraged cultivation of non transgenic papaya due to reduced virus inoculum in Hawaii (Gonsalves *et al*., 2004).

Besides USA, many countries took a plunge in developing transgenic papaya for viral resistance such as Brazil (Junior *et al*., 2005), China (Ye *et al*., 2003; Jiang *et al*., 2005), Jamaica (Fermin *et al*., 2004), Taiwan (Bau *et al*., 2004), Indonesia (Damayanti *et al*., 2001), Malaysia (Pillai *et al*., 2001), India (Chandra *et al*., 2010), Philippines (Magdalita *et al*., 2004),Venezuela (Fermin *et al*., 2004) and Bangladesh (Azad *et al*., 2013).

This review highlights development of PRSV resistant papaya through genetic engineering globally. Transgenic papaya has been one of the most successful and safe genetically modified (GM) product amongst the horticultural crops. There is need to use this technology across papaya growing regions to combat PRSV.

Development of regeneration system

Somatic embryogenesis: A robust regeneration system is prerequisite for obtaining a transgenic plant. Somatic embryos are considered the best explants for efficient delivery of gene of interest. Several workers from USA, China, Taiwan, Brazil and India have reported somatic embryogenesis in papaya (Fitch and Manshardt, 1990; Fitch, 1993; Cai *et al*., 1999; Bhattacharya *et al*., 2002; Mishra *et al.*, 2007). *In vitro* regeneration in papaya has been critically reviewed by Chandra *et al*. (2010).

Somatic embryos are preferred explant for genetic manipulation of papaya. The earliest report of successful callus culture in papaya was by De Bruijne *et al.* (1974). They were successful in induction of somatic embryos from petiole sections cultured on Murashige and Skoog (1962) media in a multistep protocol. They obtained somatic embryos but were not able to regenerate plants. Later, many other authors, Yie and Liaw (1977), Mehdi and Hogan (1979), Chen *et al.* (1987), Chen (1988 a, b) Yamamoto and Tabata (1989) have reported *in vitro* grown seedlings as their source of explant for somatic embryo induction. Green house grown seedlings were also used as source of explant for inducing somatic embryos. Fitch and Manshardt (1990) followed the protocol published by Litz and Conover (1981, 1982 and 1983) and also of Manshardt and Wenslaff (1989 a,b) and could produce somatic embryos for their transformation studies. Somatic embryos were also induced from hypocotyl tissues (Fitch, 1993). Although different explants were found to produce somatic embryos, immature zygotic embryos still remain the most preferred explant (Cai *et al.,* 1999; Bhattacharya *et al.,* 2002; Mishra *et al*., 2007, Fig.1). Protocols for somatic embryo induction in papaya have been developed for a variety of

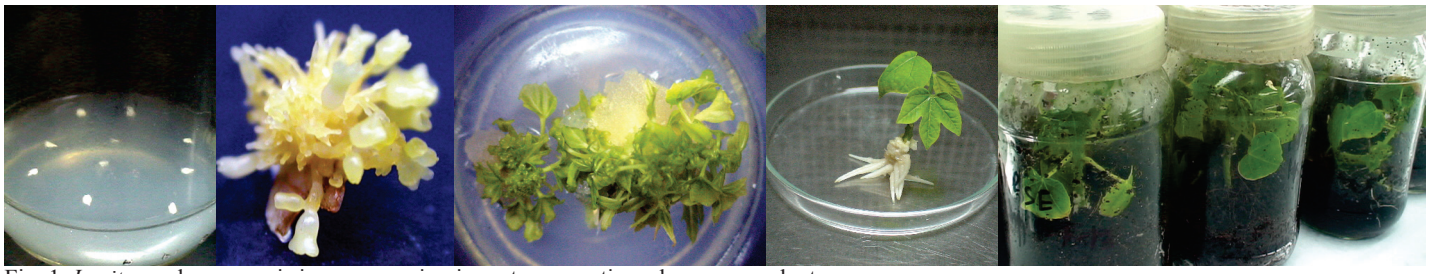

Fig. 1. *In vitro* embryogenesis in papaya using immature zygotic embryos as explant

reasons ranging from interest in methods for mass propagation to a requirement for recipient tissues for gene transfer technology (Fitch and Manshardt, 1990; Fitch, 1993).

Gene delivery system

Microprojectile: Microprojectile is one of the preferred methods of transformation in papaya (Fitch *et al*., 1992, Gonsalves *et al.*, 2004). Microprojectile or Biolistic method consists of transporting biologically active DNA in to cell by using metal particles with high velocity. Klein *et al.* (1987) found that tungsten particles could be used to introduce macromolecules such as RNA and DNA into epidermal cells of onion with subsequent transient expression of enzymes encoded by these compounds. Christou *et al.* (1988) demonstrated that the process could be used to deliver biologically active DNA into living cells which results in the recovery of stable transformants. Microprojectile offers no biological limitation to the actual DNA delivery process; therefore it can be used across the genotypes. It is a substitute for difficult tissue culture processes which is observed in *Agrobacterium* mediated transformation. Microprojectile system was refined using soybean and rice as model plant (Christou and Swain, 1990; McCabe *et al.,* 1988; Christou *et al.,* 1991) which proves that this system can work across all dicotyledonous and monocotyledonous species.

The concept of microprojectile has been described in detail by Sanford (1988). He developed the first acceleration device which accelerated tungsten particles coated with biologically active DNA to high velocities (328-656 m sec^{-1}) which were able to penetrate cell wall and membranes, and enter cells. Several recalcitrant crops were genetically engineered with ease using this technology. However, there are flip sides of this technology too. It is difficult to transform organized structures using microprojectile (shoot tips, meristems). Optimizing pressure of compressed gas is very crucial otherwise soft tissues can be killed. Sometimes transgenic plants developed through microprojectile have been reported to have multiple copy numbers. Papaya was transformed using microprojectile technique (Fitch *et al.*, 1992; Cai *et al.*, 1999).

Biolistic method for transforming papaya (*C. papaya* L.) was developed which targeted a thin layer of embryogenic tissue. The key factors in this protocol include : 1) spreading of young somatic embryo tissue that arose directly from excised immature zygotic embryos, followed by another spreading of the actively growing embryogenic tissue three days before biolistic transformation, 2) removal of kanamycin selection from all subsequent steps after kanamycin resistant clusters been isolated from induction media containing kanamycin, 3) transfer of embryos with finger-like extensions to maturation medium and 4) transferring explants from germination to the root development medium only after the explants had elongating root initials, with at least two true green leaves and about 0.5 to 1.0 cm long. A total of 83 transgenic papaya lines expressing the non translatable *coat protein* gene of papaya ring spot virus (PRSV) were obtained from somatic embryo clusters that originated from 63 immature zygotic embryos. The transformation efficiency was very high: 100% of the bombarded plates produced transgenic plants (Cai, *et al*.,1999).Christou (1992) identified three critical variables *viz*., physical, environmental and biological, which require careful optimization.

Physical parameters: Microprojectile has to be chemically inert, of high mass, so that it can generate sufficient momentum to penetrate the papaya tissue. It should be able to form organometallic complex with DNA, dissociate with coated DNA once it reaches to target cell and penetrates to desirable depth in the tissue. Additives such as spermidine and calcium chloride which are positively charged can be used for coating the DNA with metal. Palladium, rhodium, platinum, iridium *etc*. and possibly some third generation metals can be used instead of gold and tungsten particles which are the most commonly used metals for microprojectile transformation of papaya.

Environmental: Temperature, humidity and photoperiod of donor papaya plant have to be considered before bombardment as some papaya tissues require specific light regime for healing after bombardment while some may require specific temperature.

Biological: Selection and nature of papaya explant is taken into consideration for microprojectile transformation. Pre and post bombardment culture conditions has to be optimized for different plant species (Christou, 1992).

***Agrobacterium*:** *A. tumefaciens* is a soil plant pathogenic bacterium which is being used to deliver gene in plant cells and subsequently transgenic plants can be developed. *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Binns *et al.*, 1995). *Agrobacterium*-mediated transformation has several advantages over direct transformation methods. This method usually gives single copy number of transgene, leading to reduced problems with transgene co-suppression and instability. Moreover, it is a single-cell transformation system and does not produce mosaic plants, which are more frequent when direct transformation is used. *Agrobacterium*-mediated gene transfer into monocotyledonous plants was not possible until reproducible and efficient methodologies were established on rice (Hiei *et al*., 1994), banana, corn (Ishida *et al*., 1996), wheat (Cheng *et al*., 1997), strawberry (James *et al*., 1990), grapes (Bouamama *et al*., 2000).

Genetic transformation of papaya using microprojectile method has been very useful in papaya. Several groups attempted

successful gene delivery through *Agrobacterium*. Thus, like numerous other dicotyledonous plant species, papaya can be transformed with *A. tumefactions* and regenerated into phenotypically normal appearing plants that express foreign genes. Pang and Sanford (1988) transformed papaya with leaf disks co-cultivated with *A. tumefaciens* strain, GV 3111.

Yeh and Gonsalves (1994) developed a plant-expressible PRSV-*cp* gene construct from a Taiwanese PRSV strain. Transgenic plants expressing ß-glucuronidase (*gus*) were regenerated following co-cultivation of petiole explants with *A. tumefaciens*. Petioles from mature zygotic embryos were induced to form multiple shoots at the axillary nodes after treatment with 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 6- benzylaminopurine (Yang and Ye, 1992). Transgenic papaya plants were regenerated from embryogenic cultures that were co-cultivated with a disarmed C-58 strain of *A tumefaciens*. In addition, the plant expressible *coat protein* (*cp*) gene of papaya ring spot virus (PRSV) is flanked by the *npt-II* and *gus* genes in þGA 482GG/CþPRV-4. Cheng *et al*. (1996) reported that generation of transgenic papaya (*C. papaya* L.) has been hampered by the low rates of transformation achieved by conventional *Agrobacterium* infection or microprojectile bombardment. They described an efficient *Agrobacterium*-mediated transformation method based on wounding of cultured embryogenic tissues with carborundum in liquid phase (Yeh and Gonsalves, 1994).

The expressible *coat protein* (*cp*) gene of a Taiwan strain of papaya ringspot virus (PRSV) was constructed in a Ti binary vector þBGCP, which contained the *npt-II* gene as a selection marker. Genetic transformation of papaya cv. Shahi by infecting mature zygotic embryos with *A. tumefaciens* in Bangladesh was successfully done (Azad and Rabbani, 2005; Azad *et al*., 2013). Mishra *et al*. (2007) successfully achieved *Agrobacterium* mediated shoot tip transformation in papaya cv. Pusa Delicious. Carbenicillin and cefotaxime, two antibiotics commonly used for excluding *A. tumefaciens* during plant transformation, were tested for their bacteriostatic effects as well as their effects on plant regeneration in adventitious root explants of papaya following co-culture with *Agrobacterium*. A washing step with sterilized distilled water two days after co-culture enhanced the bacteria-suppressing effect of antibiotics. Proliferation of *Agrobacterium* was completely suppressed in the medium containing 125 mgL^{-1} carbenicillin or cefotaxime. Callus fresh weight increase was apparently enhanced in the media with higher concentrations of carbenicillin (250-500 mgL^{-1}), but was extremely inhibited in media with the same concentrations of cefotaxime. Higher percentage of somatic embryos was found in the medium with 125 mgL^{-1} carbenicillin or 250 mgL^{-1} cefotaxime; however, larger numbers of somatic embryos from the individual callus were obtained in the medium with 125 mgL^{-1} carbenicillin than the medium with 250 mgL^{-1} cefotaxime. Percentage of abnormal somatic embryos was lower in the medium with lower concentrations of carbenicillin 125-250 mgL^{-1} (Yu *et al*., 2001).

Pathogenic derived resistance: Sanford and Johnston (1985) gave the concept of pathogen-derived resistance (PDR) which paved the way for control of PRSV. Pathogen-derived resistance is a phenomenon whereby transgenic plants containing genes or sequences of a pathogen are protected against adverse effects of the same or related pathogens. Coat protein mediated production is based on the phenomenon of cross-protection. Cross protection is the term used for the phenomenon that a plant, when first inoculated with a mild strain of given virus, becomes protected against the infection with a second, more severe strain of the same virus with which it has been infected (Fermin *et al*., 2010). Roger Beechy's group demonstrated the application of this technology (Abel *et al*., 1986) for developing resistant crops against several viruses.

Yeh *et al*. (2003) reported that the *coat protein* (*cp*) gene mediated transgenic resistance is the most promising approach for protecting papaya against the devastating effects of Papaya ring spot viruses (PRSV). Viral *cp* gene imparting resistance against virus is known as coat protein mediated resistance (CPMR). Accumulation of the *cp* gene in transgenic crops has been proven to counter resistance to infection and/or disease development by the virus from which the *cp* gene was derived and by related viruses (Rosales *et al*., 2000). There is little or no genetic resistance to PRSV and PaLCuV in papaya germplasm. Large collections of papaya germplasm and cultivars representing the world's major production have been screened, but resistance has not been found.

Pathogen derived resistance (PDR) *via* coat protein (cp) has proved to be effective tool in combating plant viruses. The particular gene-silencing strategies have been shown to be effective. However, for viruses in which the *cp* is part of a polyprotein (*e.g*. potyviruses and comoviruses), the *cp* ORF must be artificially provided with an extra AUG start codon. Because of the genetic structure of most plant (RNA) viruses, which encode their most abundant structural protein (*cp*) at the 3-terminal part of the genome, clones of these genes were the first available for genetic studies. Introduction of the *cp* gene into the plants is mostly done by *Agrobacterium*-mediated gene transfer. Resistance in all cases recorded as a significant delay in, or an escape from, disease symptom development. Indeed, *cp*-mediated resistance is reported for several viruses, as described in numerous reviews dealing with this subject (Kavangh, and Spillane, 1995).

Studies on *cp*-mediated protection have revealed that *Cp*-mediated protection works at the protein level by the expression of *cp*. In most of the cases, there is no protection against viral RNA inoculation with an exception of PVX. The protection is not absolute; it can be overcome by (very) high virus inoculums concentrations. For TMV/tobacco the resistance level is approximately 10^4, which suggests transgenic *cp* plants were diseased at an inoculum concentration 10,000 times higher than needed for infection of control plant. Protection is rather specific; it works for the corresponding virus or very close relatives that have more than 60 percent amino acid sequence homology in their *cp*s. *Cp*-mediated resistance is a genuine form of resistance, not tolerance. Resistance segregates as a conventional, single and dominant resistance gene and it works under greenhouse and field conditions.

Global status of development of PRSV resistant transgenic papaya: With the development of concept of pathogenic derived resistance to combat plant viruses effectively, lot of research was diverted towards developing PRSV resistant papaya using coat protein gene. PRSV resistant transgenic papaya was first developed in USA in 1998 due to concerted efforts of Dennis Gonsalves and his team. They transformed Sunrise, Sunset and Kapoho variety of papaya with HA5-1 cp gene which resulted in half of progenies into transgenic lines. The transgenic plants had one insert of *npt-II* gene and probably *cp* gene. The Hawaiian group developed somatic embryogenesis system in papaya

(Fitch and Manshardt, 1990; Fitch, 1993; Cai *et al*., 1999). They bombarded embryonic clumps with tungsten particle coated with HA5-1 *cp* gene using gene gun (Fermin *et al*., 2010). The bombarded embryos were selected under kanamycin and resistant embryos were developed into transgenic plants. The transgenic plants were rigorously tested against PRSV. Tennant *et al*. (1994) tested the resistance of R1 plants of line 55-1 against 3 PRSV isolates from Hawaii and 13 isolates from different part of the world. Tennant *et al*. (2005) found that R1 plants were completely resistant to PRSV Hawaii isolates. The transgenic papaya was commercialized in Hawaii in 1998. In next 12 years after its commercialization, almost 80% of Hawaiian papaya was genetically modified. This led to increase in planting of non transgenic papaya probably due to reduced inoculum of PRSV in Hawaii (Gonsalves *et al*., 2004). The transgenic papaya variety was named as Rainbow. Later, the genetically engineered (GE) papaya was deregulated for Canada and Japan (Gonsalves *et al*., 2010). The transgenic research started in Brazil way back in 1992. Translatable and nontranslatable versions of the *coat protein* (*cp*) gene of a Papaya ringspot virus (PRSV) isolate collected in the state of Bahia, Brazil, were engineered for expression in Sunrise and Sunset Solo varieties of papaya. Fifty-four transgenic lines, 26 translatable and 28 nontranslatable gene versions, were regenerated, with a transformation efficiency of 2.7%. Inoculation of cloned R0 plants with PRSV BR, PRSV HA or PRSV TH; Brazilian, Hawaiian and Thai isolates, respectively, revealed lines with mono-, double-, and triple-resistance (Junior *et al.*, 2005).

China successfully developed transgenic papaya which is being cultivated over 3500 h area by 2007 after the start of transgenic research at Huazhong Agricultural University (Jiang *et al*., 2005), Zhongshan University (Ye *et al*., 2003), Sun Yat-sen University and South China Agricultural University. Jiang *et al*. (2005) transformed papaya (cv. Sunset) using *A. tumefaciens* strain LBA4404 carrying the binary plasmid pGA482G containing the *cp* and *npt-II* genes. Ye *et al*. (2003) reported the field test of two transgenic papaya T1 lines with a replicase mutant gene derived from a strain of PRSV.

Reports suggest that Indonesia initiated research on transgenic papaya and *cp* gene was introduced using particle bombardment into two Indonesian varieties of papaya, namely Bangkok and Burung (Damayanti *et al*., 2001). Malaysia took up transgenic program in papaya targeting immature zygotic embryos derived from the papaya variety Eksotika, transformed with *cp* gene using *Agrobacterium*-mediated transformation. A total of 87 transgenic lines were generated from all constructs and tested in the field (Pillai *et al*., 2001). Thailand initiated transgenic research in papaya as early as 1986. Papaya varieties Khakdum and Khaknuan were transformed with *cp* gene of PRSV isolate from North Thailand (Sakuanrungsirikul *et al*., 2005). Transgenic papaya was also developed in Philippines using *cp* gene isolated from its local strain of PRSV. All the R_0 transgenic lines had moderate to high susceptibility to PRSV (Magdalita *et al*., 2004). In 1992, Venezuela started its transgenic program in papaya. *Cp* gene was isolated from two local Venezuelan isolates (LA and EV) of PRSV (Fermin, 1996). Local papaya variety Thailand Roja was transformed using *Agrobacterium*-mediated transformation (Fermin *et al*., 2004). Nhan *et al*. (2001) reported genetic transformation of five papaya varieties in Vietnam. Taiwan, surprisingly took a lead in papaya transgenic research and papaya variety Tainung No. 2 was transformed with *cpYK* gene using *Agrobacterium*-mediated system (Bau *et al*., 2004).

Bangladesh has established the transformation protocol for papaya transformation *via Agrobacterium*-mediated transformation. Genetic transformation of papaya cv. Shahi by infecting mature zygotic embryos with *A. tumefaciens* was successfully done (Azad and Rabbani, 2005; Azad *et al*., 2013). India entered relatively late in the fray. Three groups started research in transgenic papaya *viz*., Central Institute for Subtropical Horticulture, Lucknow; Indian Institute for Horticultural Research, Bengaluru and Tamil Nadu Agriculture University, Coimbatore. Recently, Chandra *et al.* (2010) developed genetic transformation of papaya using shoot tip as explant in papaya cv. Pusa Delicious. A dual gene construct consisting truncated *cp* gene of PRSV and truncated *rep* gene of PaLCuV has been introgressed in papaya using *Agrobacterium*-mediated transformation.

Biosafety issues concerning GE papaya: Even after 17 years of release of transgenic papaya, there is no report on its adverse effect on environmental biosafety issues. The emergence of undesirable virus isolate due to transgenic papaya has not been observed so far. Recombination refers to the exchange of genetic materials between two RNA molecules during virus replication. A recombinant virus has potentially negative effects on the environment such as increasing pathogenicity, expanding host range and changing the vector (Azad *et al*., 2014). The Thai transgenic papaya lines containing PRSV-P *cp* gene were tested for possibility of infection by PRSV-W super infecting strain under containment (Warin *et al*., 2007). However, ELISA results revealed complete absence of PRSV-W. It was clearly found that transencapsidation in the GE papaya lines did not occur during artificial PRSV-W conditions (Mendoza *et al*., 2008).

The impact of GE papaya on microbial population has also been studied. Widmer (2007) found differences in soil microbial characters in transgenic and non transgenic plants. Nevertheless, Phironrit *et al*. (2007) did not observe any distinct differences in microbial communities between transgenic and non transgenic microbes. Pollen flow remains a major issue particularly with highly cross pollinated crop like papaya. Despite that, Fuchs and Gonsalves (2007) found low gene flow in transgenic papaya. Manshardt (2002) studied gene flow in Hawaii and found that transgenic seeds were found in 7% of non transgenic hermaphrodite and 43% of female plants among non transgenic trees. However, no transgenic seeds were obtained from PRSV infected non transgenic papaya plants 400 m away from transgenic plants. This clearly shows that gene flow has not been conflicting issues in transgenic papaya. Transgenic papaya has been found at par with non transgenic papaya with regard to food safety parameters. Fully mature papaya fruit estimated to have 1.3 to 3.5 ppm BITC (benzyl isothiocyanate) content in non transgenic whereas that of transgenic papaya was 1.7-1.8 ppm. It was found that BITC content in transgenic and non transgenic were similar.

The *cp* of PRSV is a dominant viral gene and has been a preferred choice for GM developers in twelve countries. The second preferred viral gene encodes the nuclear-inclusion protein-b (*Nib*) which contains conserved motif characteristics of RNA-dependent RNA polymerase of positive strand RNA virus to its adjacent *cp* gene, has also been used by several researchers. PRSV resistant GM

papaya can significantly boost papaya industry and help them in mitigitating problem of PRSV disease across the globe.

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