International Journal of Innovation Sciences and Research
Vol.4, No, 8, pp.391-396, August- 2015

Available online at
http://www.ijisr.com

REVIEW ARTICLE

GENETIC TRANSFORMATION STUDIES IN *CARICA PAPAYA* L.: A BRIEF REVIEW

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Accepted 04th July, 2015; Published Online 31th August, 2015

ABSTRACT

Genetic transformation involves non sexual transfer of genes and traits they control from one organism into another. Papaya, *Carica papaya* L., is one of the major fruit crops cultivated in tropical and sub-tropical zones. Worldwide over 6.8 million tonnes (Mt) of fruit were produced in 2004 on about 389,990 Ha (FAO 2004). Although, *in vitro* techniques namely somatic embryogenesis and somaclonal variation are useful tools for genetic manipulation, genetic transformation can be used and has been used in papaya to alter superior cultivars for a specific trait. Stable transformation of papaya has been achieved through the use of various DNA transfer technologies since the initial report of Pang and Sanford (1988). A brief review of the various methods and success achieved by them in papaya is presented in the given paper.

Key Words: Genetic Transformation, Bolistics, Reporter Genes, Papaya Ringspot Virus, *CARICA Papaya*

INTRODUCTION

The essential requirements of a gene transfer system are:

- Availability of a target tissue including cells competent for plant regeneration;
- A method to introduce DNA into the targeted cells;
- A procedure to select and regenerate transformed plants at a satisfactory frequency (Birch, 1997).

Genetic transformation involves non-sexual transfer of genes and the traits they control from one organism into other. Transformation vastly increases the gene pool available and allows incremental improvement of elite genotypes without necessitating generations of back crossing to recover the original phenotype. Three main techniques used for genetic transformation are protoplast transformation, biolistics or microprojectile bombardment and *Agrobacterium*-mediated transformation (Hansen and Wright, 1999). Papaya, *Carica papaya* L., is one of the major fruit crops cultivated in tropical and sub-tropical zones. Worldwide over 6.8 million tonnes (Mt) of fruit were produced in 2004 on about 389,990 Ha (FAO 2004). This makes papaya an important plant for genomic and genetic studies. A brief review of the genetic transformation studies is as follows:

Various Methods and Reporter Genes Used in Papaya Transformation

Pang and Sanford (1988) obtained transgenic callus with the *neomycin phosphotransferase* type II (*npt*II) marker gene from oncogenic *Agrobacterium tumefaciens*-mediated transformation of 'Sunrise Solo' and 'Kapoho Solo' papaya leaf discs, stems and petioles. But they could not regenerate plantlets from these calli.

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Since then several efficient transformation and selection protocols have been developed and have resulted in transgenic plants expressing new traits including herbicide tolerance, increased the shelf-life of fruits, virus resistance, and aluminium tolerance. According to do Carmo and Souza Jr. (2003), most studies (>55%) used *Agrobacterium*- mediated trans-formation systems, 80% of those by *A. tumefaciens* (GV311, LBA4404, A136, C58-Z707) and the remaining 20% used *A. rhizogenes* (LBA9402, A4T, 8196). In addition, many transgenic papaya studies have utilized *npt*II as the marker gene of choice although Cabrera-Ponce *et al*. (1995) used the *bar* gene, which codes for phosphinothricin acetyl transferase and allows for the breakdown of phosphinothricin, or PPT, a herbicide. Given that antibiotic and herbicide resistance genes in widely grown transgenic crops may pose a risk, real or perceived, of transfer to weedy relatives or microorganisms, an alternative selection technology using phospho-mannose isomerase (PMI) was developed (Bolsen *et al*. 1999) and has been tried with papaya (Souza Jr. *et al*. 2001; Zhu *et al*. 2005). PMI converts mannose (Man) to mannose-6-phosphate. The results from these two groups were different, probably due to the different papaya cultivars used. Souza Jr. *et al*. (2001) used 'Sunrise Solo', while Zhu *et al*. (2005) used 'Kapoho Solo'. Zhu *et al*. demonstrated that embryogenic papaya calli have little or no PMI activity and cannot use Man as a carbon source. However, calli transformed with the *pmi* gene showed PMI activity and were able to use Man as efficiently as sucrose. The green fluorescent protein (GFP) from jellyfish (*Aequorea victoria*) is also becoming a popular alternative reporter gene in plant transformation. Zhu *et al*. (2004a) successfully transformed the papaya variety 'Kapoho Solo' with the GFP gene via microprojectile bombardment of embryogenic callus. A reduction in selection time (3-4 weeks as compared to the average 3 months experienced when using a geneticin [G418] selection-based medium) was demonstrated, a 5- to 8-fold increase in the number of transformants (compared to antibiotic-based selection), and a 15- to 24-fold increase in transformation throughput.

Table 1. Studies on genetic transformation of Carica papaya L. by *Agrobacterium* and particle bombardments methods

S. No.	Explant Used	Method Used	Reference
1.	Leaf stem and petiole	*Agrobacterium*	Pang and Sanford (1988)
2.	Immature zygotic embryo, hypocotyls, embryogenic calli	Particle bombardment method	Fitch *et al*. (1990)
3.	Somatic embryos	*Agrobacterium*	Ye *et al*. (1990)
4.	Immature zygotic embryo, hypocotyls, embryogenic calli	Particle bombardment method	Fitch *et al*. (1992)
5.	Hypocotyl	*Agrobacterium*	Fitch *et al*. (1993)
6.	Immature zygotic embryo	Particle bombardment method	Carbrera Ponce *et al*. (1995)
7.	Leaf	*Agrobacterium*	Carbrera 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 method	Mahon *et al*. (1996)

Development of PRSV Resistance Lines

Fitch *et al*. (1993) were the first to successfully transform and regenerate transgenic papaya plants. Transgenic papaya plants were regenerated from microprojectile bombarded immature *in vitro* 'Sunrise Solo' and 'Kapoho Solo' papaya zygotic embryos, hypocotyl sections, or somatic embryos derived from both embryos or hypocotyls that were cultured on medium containing 2,4-D (Fitch and Manshardt 1990). The transgenes included *npt*II, β-glucuronidase (GUS) and coat protein (*cp*) of a mild strain of PRSV (PRSV HA 5-1). The latter gene codes for the viral capsid protein used for packaging the viral RNA, assisting the movement of the virus *in planta* and interaction with insect vectors. The objective of the study was to develop resistance to PRSV. By the late 1990s, the first transgenic line designated as line 55-1 was used to develop PRSV-resistant transgenic cultivars 'Rainbow' and 'SunUp'. In 1998, two PRSV resistant papaya cultivars, 'SunUp' and 'Rainbow', were released to growers in Hawaii (Fitch *et al*. 1992; Manshardt 1998).

The transgenic papayas have offered durable resistance to PRSV and have controlled the virus in Hawaii (Ferreira *et al*. 2002). According to figures out of the USDA's statistical service, 'Rainbow' makes up 47% of the Big Island's 779 papaya hectares. 'Rainbow' is a yellow- flesh F1 hybrid of a cross between the transgenic cultivar 'SunUp' and nontransgenic cv. 'Kapoho Solo' (Manshardt 1998; Gonsalves 2002) which is the preferred nontransgenic cultivar in Hawaii. 'SunUp' is homozygous for the single *cp* gene insert of the mild strain PRSV HA 5-1 (Manshardt 1998) and was derived from the red-flesh transgenic papaya line 55-1 (Fitch *et al*. 1992).

Initial greenhouse studies of transgenic line 55-1, hemizygous for the *cp*, showed that although the plants were resistant to Hawaiian virus isolates, they were susceptible to PRSV isolates from 11 geographical regions, including Bahamas, Florida, Mexico, Jamaica, Brazil, and Thailand (Tennant *et al*. 1994). Later work showed that the resistance of line 55-1 is RNA-mediated and dependent on the dosage of the *cp* gene, *cp* sequence homology of the challenge virus, and plant development stage (Tennant *et al*. 2001).

Even though 'Rainbow' (RB), a transgenic papaya cultivar hemizygous for PRSV *cp* gene, exhibited early plant susceptibility (>70% of plants infected) to mechanical inoculation with crude preparations of PRSV isolates from Hawaii, RB plants become highly resistant by approximately 9 weeks after seeding in the greenhouse (2.5% of plants infected) and by 13 weeks in the field (<16% of plants infected) (Gaskill *et al*. 2002).

In contrast 'SunUp', a transgenic papaya cultivar homozygous for the *cp* gene, exhibited complete resistance against all isolates of PRSV from Hawaii, but is susceptible to isolates from outside of Hawaii. Among 18 virus isolates collected in Taiwan, four (5-19, CY4, TD2, and DL1) were able to breakdown the transgenic resistance of papaya lines carrying the *cp* gene of PRSV and caused symptoms on non-transformed papaya plants different from those induced by the strain YK (Chen *et al*. 2002); the DL1 isolate was further identified as *Papaya leaf distortion mosaic virus*. Resistance against PRSV through a *cp* gene of mild PRSV was also shown to be transmitted to non-transgenic 'Solo' plants through conventional crossing between a female transgenic R0 and a non-transgenic plant (Tennant *et al*. 1995).

Mode-rate genetic resistance to PRSV in papaya germplasm has been used in other conventional breeding programs in Florida, Jamaica and Hawaii to create PRSV tolerant cultivars (Manshardt *et al*. 1995; Turner *et al*. 2004). Thus, the hemizygous 'Rainbow' is resistant to Hawaiian isolates, but susceptible to isolates from outside of Hawaii whereas homozygous 'SunUp' is resistant to isolates from outside of Hawaii, with the exception of the Thailand isolate. The resistance of another Hawaiian transgenic line, line 63-1, was recently tested against PRSV from various locations (Tennant *et al*. 2005). Line 63-1 originated from the same transformation experiment that resulted in line 55-1 from which the transgenic commercial cultivars, 'Rainbow' and 'SunUp', were derived.

ELISA and PCR tests provided evidence that there are at least two segregating *cp* loci in line 63-1. Souza Jr. *et al*. (2005) further demonstrated that line 63-1 has two sites of transgene insertion (designated locus S and locus L) and that both the *cp* and the *npt*II genes are present in both loci. Unlike line 55-1, a significant percentage of inoculated transgenic plants were susceptible to some isolates from Hawaii and others were resistant to Hawaiian and non-Hawaiian isolates. Line 63-1, therefore, presents Hawaii with PRSV-resistant transgenic germplasm that could be used as a source of transgenes for resistance to PRSV isolates within and outside of Hawaii. Souza *et al*. (2005) also provided evidence that the number of resistant plants in a 63-1-derived population is directly correlated with the number of plants with multiple transgene copies (Souza *et al*. 2005).

Other countries, Brazil, Jamaica, Venezuela, Thailand, Australia (Lines *et al*. 2002), Taiwan (Bau *et al*. 2003), and recently with Bangladesh and the east African countries of Tanzania, Uganda, and Kenya, have since used the technology and the *cp* gene from their region to develop their own transgenic varieties.

The transgenic papayas are at various stages of development and evaluation. For example, translatable and untranslatable versions of the *cp* gene of PRSV collected in the State of Bahia, Brazil, were engineered for expression in papaya varieties, 'Sunrise Solo' and 'Sunset Solo' (Souza *et al*. 2005). The genes were transferred to somatic embryo cultures derived from immature zygotic embryos via microprojectile bombardment. Fifty four transgenic lines, 26 containing translatable and 28 containing untranslatable gene versions, were regenerated. Greenhouse evaluation of the resistance of the regenerated transgenic plants was conducted with PRSV from Brazil, Hawaii and Thailand. The plants showed mono-, double- and even triple-resistance against the viruses from the three countries. However, the transgenic papayas have been subjected to very limited field evaluation in Brazil. Fermin *et al*. (2004) used *Agrobacterium* to transform local Venezuelan varieties of papaya with the *cp* gene from two PRSV isolates, ElVigía (VE) and Lagunillas (LA), Merida. They found that transgenic plants were effectively protected against both homologous (VE and LA) and heterologous isolates from Hawaii and Thailand.

Field evaluations were initiated but activists destroyed all transgenic plants before useful data was collected (Fermin *et al*. 2004). In Jamaica, the transgenic papayas were developed by microprojectile bombardment of somatic embryogenic materials (Cai *et al*. 1999). Transgenic papayas, containing translatable (CPT) or nontranslatable coat protein (CPNT) gene constructs, were evaluated over two generations for field resistance to PRSV in a commercial papaya growing area in Jamaica (Tennant *et al*. 2005). Trees with acceptable horticultural characteristics exhibited a range in resistance phenotypes. Reactions of R0CPT transgenic lines ranged from asymptomatic, mild or severe leaf and fruit symptoms, or all three phenotypes in one line or between different lines. Trees of most CPNT lines exhibited severe responses to infection and some also showed mild reactions. R1 offspring showed phenotypes previously observed with parental R0 trees, however, phenotypes not previously observed or a lower incidence of the phenotype was also obtained. It was concluded that the transgenic lines appear to possess virus disease resistance against PRSV that can be manipulated in subsequent generations for the development of a product with acceptable commercial performance.

However, local deregulation efforts have stalled research and the development of a transgenic product. In Thailand, the transgenic papaya has been field trialed extensively. Three lines were selected for their horticultural characteristics and resistance. These lines, derived from 'Khaknuan' papaya variety, yielded fruit 70 times that of the nontransgenic Khaknuan'. Safety assessments have shown no impact on the surrounding ecology and there were no differences in the nutritional composition of the transgenic fruit compared to the nontransgenic fruit (Sakuanrungsirikul *et al*. 2005). While the processes for deregulating the transgenic papaya are well under way, public acceptance of genetically modified products appears to be keeping the project from reaching the ultimate goal of deregulation and commercialization of the transgenic papayas. The efficacy of other genes in the control of PRSV is being investigated. In other studies with transgenic lines against PRSV in Hawaii, lines containing a nontranslatable *cp* version of the mild strain of PRSV conferred varying degrees of resistance (Cai *et al*. 1999; Gonsalves 1998).

Twenty-two lines of 77, conferred complete resistance against the homologous isolate and 23 lines showed 47% resistance. When inoculated with PRSV isolates from other regions of Hawaii, moderate levels ranging from 11 to 26% were obtained. Chen *et al*. (2001) reported the first successful PRSV-resistant 'Tai-nong-2' papaya through replicase mediated resistance, i.e. using the RP or viral replicase gene with *A*. *tumefaciens* as vector. The RP fragment that was used showed a 82.8%, 91.83% and 95.07% sequence similarity to the sequences of PRSV strains HA5-1 from Hawaii (Quemada *et al*. 1990), Sm from mainland China (Liu *et al*. 1994) and YK from Taiwan (Wang *et al*. 1994), respectively.

Since the early reports on the transformation of papaya, a number of laboratories have modified the protocols and reported success with different explants, *Agrobacterium* species or strains, and selection systems. Cabrera-Ponce *et al*. (1995) established a particle bombardment protocol for 'Maradol' zygotic embryos and embryogenic callus derived from immature zygotic embryos. Ye *et al*. (1991), Fitch *et al*. (1993; in 'Kapoho Solo') and Yang *et al*. (1996) obtained transgenic 'Sunrise Solo' papaya after transformation of somatic embryos or the petioles of *in vitro* propagated multishoots, respectively, using *Agrobacterium*. Using cross sections of papaya petioles, Yang *et al*. (1996) introduced the *npt*II and *uid*A genes, used as a selection marker and reporter gene, respectively, into 'Sunrise Solo' papaya following *A*. *tumefaciens*-mediated transformation. Cabrera-Ponce *et al*. (1996) used *A*. *rhizogenes*. Cheng *et al*. (1996) inserted the PRSV YK *cp* gene using *A*. *tumefaciens*. Cabrera- Ponce *et al*. (1996) could induce hairy roots in Yellow-large hermaphrodite type *C*. *papaya* after infection with *A*. *rhizogenes*, and then induced somatic embryos from the hairy roots. Cheng *et al*. (1996) found the inclusion of carborundum to be important in the effective *Agrobacterium*-mediated transformation of 'Tainung №2' papaya embryogenic tissues with the *cp* gene of PRSV, which tended to reduce, or eliminate the high frequency of abnormalities, and reduce the regeneration time after transformation experienced by Yang *et al*. (1996). Carbenicillin and cefotaxime, two antibiotics used to suppress *Agrobacterium* growth, were shown to stimulate the number of somatic embryos at 125 mg/L for the former and 250 mg/L for the latter (Yu *et al*. 2001). Yu *et al*. (2003) found that *npt*II-transformed papaya root explants (using three PRSV-*cp* transgenic lines, 16-0-1, 17-0-5 and 18-0-9; Bau *et al*. 2003) were strongly inhibited by kanamycin, and authors recommended the use of geneticin at 125-25 mg/L.

Developing Resistance to *Phytopthora palmivora*

Zhu *et al*. (2004a) successfully transformed 'Kapoho Solo' with GFP and the stilbene synthase gene, *Vst*1, from *Vitis vinifera*. Increased resistance to *P*. *palmivora,* the main cause of root, stem and fruit rot diseases, was demonstrated. In another study, the *Dahlia merckii* defensin gene, *DmAMP*1, was used in the transformation of papaya (Zhu *et al*. 2007). Bioassays with extracts of total leaf proteins and leaf discs from transgenic papaya revealed inhibited the growth of *Phytophthora*. Similarly, transgenic plants in the greenhouse exhibited increased resistance against *P*. *palmivora* following inoculation. A reduction in the growth of *P*. *palmivora* at infection sites was observed. Murad *et al*. (2007) reviewed the use of defensins in transgenic plants.

Developing Resistance to carmine spider mites

Tolerance to carmine spider mites (*Tetranychus cinnabarinus* Boisd.) has been recently introduced in transgenic papaya varieties. McCafferty *et al*. (2006) reported on the development of papaya plants transgenic for the tobacco hornworm (*Manduca sexta*) chitinase protein for improved tolerance to spider mites. Transfer of the gene to embryogenic calli derived from the hypocotyls of the papaya cultivar 'Kapoho Solo' was done by microprojectile bombardment. Subsequent insect bioassays showed that plants expressing the chitinase gene had significantly lower populations of spider mites. Tolerance was also observed under field conditions and exposure to natural mite populations.

Developing Resistance to Aluminum Toxicity

The technology of genetic engineering has not only been applied to developing resistance to biotic factors but also abiotic environmental factors that result in poor crop productivity and soil fertility. Poor crop productivity and soil fertility in acid soils are mainly due to aluminum toxicity. Aluminum has a clear toxic effect on roots by disturbing plant metabolism and decreasing mineral nutrition and water absorption. The potential role of organic acid release,for example citric acid, in Al-tolerance was originally proposed in the early 1990s. Citric acid chelates Al3+. The strategy of producing transgenic plants with an increased capacity to secrete citric aicd was appealing since papaya production in the tropics is affected by acid soils. A citrate synthase gene (*CSb*) from *Pseudomonas aeruginosa* was mcloned and biolistics used to successfully transform papaya (de la Fuente *et al*. 1997). Transgenic plants that could root and grow on aluminium concentrations up to 300 mM were regenerated. Non-transformed controls did not root on 50 mM Al3+ or less.

Development of Increased Shelf Life

Projects aimed at improving the postharvest qualities of papaya by increasing the shelf-life of the fruit have been initiated and possibly have potential in reducing one of the industry's principal problems in fruit exportation. The strategy adopted to delay fruit ripening in papaya involved the suppression or inhibition of the key enzyme, ACC synthase (*ACS 1* and *ACS 2*) in ethylene production during the ripening process (Neupane *et al*. 1998) or ACC oxidase genes (*CP-ACO1* and *CP-ACO2*; Burns *et al*. 2007; Sew *et al*. 2007). Field evaluation of transgenic papayas was reported by Muda *et al*. (2003). Research is also being conducted on manipuating fruit softening. The gene of fruit cell wall enzyme β-galactosidase has been cloned and used to transform papaya (Umi *et al*. 2005).

Developing cold tolerance in papaya

Dhekney *et al.* (2007) studied the potential for introducing cold tolerance into papaya by transformation with C repeat binding factor (CBF) genes. The C repeat binding factor (CBF) gene family is known to be associated with the cold acclimation pathway in *Arabidopsis thaliana.* Embryogenic papaya cultures were induced from hypocotyls of "Sunrise Solo" zygotic embryos on semisolid medium. The CBF1/CBF3 genes along with the neomycin phosphotransferases (NPT 2) genes were placed under the control of the CaMV 35S promoter and introduced into binary vector pGA 643. Embryogenic cultures were transformed with *Agrobacterium* strain GV3101 harbouring pGA 643. After selection of transformed embryogenic cultures for resistance to 300mg/lit kanamycin, somatic embryo development was initiated and transgenic plants were regenerated. The presence of the CBF transplant in regenerated plants was confirmed by southern blot hybridization. The papaya and the related cold tolerant *Vasconcella* genome were probed for the presence of cold inducible sequences using polymerase chain reaction (PCR). Possible cold inducible sequences were present in the *Vasconcella* genome but were absent in the *Carica* genome.

Developing papaya as edible vaccine

More recently, the use of transgenic papaya as an antigen-delivery system for subunit vaccines has been explored.Transgenic papayas that carry the epitopes KETc1, KETc12, and GK-1, three promising candidates for designing a vaccine against *Taenia solium* cysticercosis, were developed (Hernández *et al*. 2007). Cysticercosis the most common parasitic infection of the central nervous system world-wide is caused by the pork tapeworm, *Taenia solium*. Infection occurs when tape worm larvae enter the body and form cysticerci (cysts). Nineteen different transgenic papaya clones expressing synthetic peptides were found to confer resistance against cysticercosis. Complete protection against cysticercosi was induced with the soluble extract of the clones that expressed higher levels of transcripts of up to 90% of immunized mice. The results indicate that transgenic papayas may be a new antigen-delivery system for subunit vaccines (Hernández *et al*. 2007). The results are significant as there is an urgent need for affordable and reliable vaccines in developing countires. Costs associated with the production, maintenance and delivery of traditional vaccines are often very high resulting in limited distribution of vaccines in these countries. Theoretically, the expression of recombinant proteins in transgenic plants offers inexpensive vaccines that could be produced directly "on site".

Conclusion

Papaya continues to increase in importance as a fruit crop. Several of the limitations in tissue culture can now be overcome using some of the novel techniques in *in vitro* culture outlined in this review. Genetic transformation is now well established and improved virus detection techniques and integrated pest control programs should allow the modern breeder to find novel solutions to any remaining problems in the propagation and production of papaya. One such example is the heavy use of pesticides in papaya culture (Hernández-Hernández *et al*. 2007). A transgenic approach could be used to introduce traits to provide resistance to important pests. However, despite all these possibilities, several factors (cultural, governmental, ecological, and environmental) need to be considered when introducing transgenic papaya varieties into new cultivation area.

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