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Review

# Advances in papaya biotechnology

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ABSTRACT

Papaya (*Carica papaya* L.) is an important tropical fruit crop. The fruit is consumed fresh and used in the pharmaceutical, rayon and food industries. Papaya improvement for stress tolerance and qualitative traits using conventional breeding has been difficult due to the narrow germplasm pool in the *Carica* genus and sexual incompatibility problems encountered during intergeneric hybridization with other genera in the Caricaceae family. Genetic engineering is an important tool in papaya improvement for modifying one or more traits in elite cultivars without altering existing characteristics. Advances in genetic engineering have been facilitated by concerted efforts for genome sequencing of papaya, development of papaya regeneration systems and efficient gene insertion techniques for transfer of desirable traits.

Papaya regeneration via organogenesis and somatic embryogenesis has been refined during the past 3 decades. Early efforts to optimize gene insertion protocols utilized a number of reporter and selectable marker genes, viral- and bacterial-derived regulatory sequences and functional genes for biotic and abiotic stress tolerance. Transgenic plants were routinely produced with several cultivars. One of the best success stories in the commercialization of a genetically modified fruit crop has involved the development of transgenic papaya ring spot virus (PRSV) resistant Rainbow and SunUp cultivars, which saved the Hawaiian papaya industry. Additionally, genetically modified papayas with traits for disease resistance and extended shelf life have been extensively screened in field tests.

The papaya genome sequence was published in 2008 and has opened new avenues for papaya improvement by precision breeding, which involves the use of regulatory and functional gene sequences from related genera of the Caricaceae family, and is a logical extension of conventional breeding and genetic transformation. The application of precision breeding technology for papaya can pave the way for the development of consumer and eco-friendly cultivars that would be developed in ways similar to conventional breeding while causing fewer GMO-related concerns.



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## 1. Introduction

Papaya, *Carica papaya* L. is an important tropical fruit crop. Ripe fruits are consumed fresh while unripened fruits can be used in salads or as a vegetable and as a source of papain. Papaya fruits are a rich source of vitamins and minerals, low in sodium, fat and calories, and lack starch (Hewajulige and Dhekney, 2016). Papain, a proteolytic enzyme obtained from immature fruits is used in the pharmaceuticals, leather, wool and rayon industries (Seelig, 1970). Papaya is grown on 0.43 million ha with an annual production of 11 million tons. A majority of the production occurs in Asia and the Americas (FAOSTAT, 2013).

Papaya ($2n=2x=18$) is a member of the Caricaceae family, which consists of herbaceous plants (Badillo, 1993). The crop is believed to have originated in Central America in regions ranging from Mexico to Panama. The Caricaceae was originally comprised of 31 species in the *Carica*, *Jacartia* and *Jarilla* genera from Central America and the African *Cylicomorpha* genus (Nakasone and Paull, 1998). Taxonomic revisions resulted in some species being transferred from *Carica* to the *Vasconcella* genus (Badillo et al., 2000). Consequently, the *Vasconcella* genus now consists of 21 species followed by *Jacartia* with 7 species, while papaya is now the sole species in the *Carica* genus (Badillo, 1993).

Major goals of papaya improvement programs include increased yield and productivity, resistance to biotic and abiotic stress factors and improved quality characteristics. Papaya cultivars with improved traits such as high yield and quality have been successfully developed through intensive breeding programs worldwide (Chan, 2002; Nakasone and Paull, 1998). Minisatellite and microsatellite markers have been explored to accelerate papaya genetic improvement, analyze phenotypic variation for traits of interest, and understand genetic relationships for efficient management and conservation of genetic resources (Oliveria et al., 2010; 2015a, 2015b). Papaya improvement for stress tolerance (abiotic and biotic) via hybridization with species from other genera of the Caricaceae family has been marginally successful. Limitations for transfer of useful traits are attributed to several post-zygotic incongruities including embryo abortion, poor seed viability and sterility in progeny obtained following hybridization between two genera (Horovitz and Jimenez, 1967; Manshardt and Wenslaff, 1989).

The limitations encountered in papaya improvement via conventional breeding can be overcome by biotechnological approaches such as embryo rescue and genetic engineering. Genetic engineering of papaya allows incorporation of specific traits into elite cultivars without potentially altering the existing phenotype. The process involves transfer of specific DNA sequences in cell cultures and their subsequent integration into the host genome. The prerequisites for successful gene transfer include efficient cell culture systems for plant regeneration and gene insertion techniques (Birch, 1997). Recent advances in papaya genomics along with refinement of cell culture and gene insertion protocols make genetic engineering as an important tool for studying gene function and expression in papaya and the development of improved cultivars. This chapter will review the progress in papaya cell culture, genetic engineering and genomics, and the successful application of this technology for papaya improvement.

## 2. Regeneration systems

The concept of totipotency, which is the ability of single cells to grow into entire plants, forms the basis of plant regeneration (Hansen and Wright, 1999). Plant regeneration of papaya via somatic embryogenesis, organogenesis and micropropagation is well documented (Fitch, 2005). Somatic embryogenesis and organogenesis occur through dedifferentiation and redifferentiation of explant cells. These events depend on the development of meristems from mature differentiated cells or undifferentiated callus tissues (Ziv, 1999).

### 2.1. Embryogenic cell culture system

The papaya embryogenic culture system involves the production of somatic embryos from a wide array of explant material. Embryogenic cultures are produced on induction medium via an indirect embryogenic pathway that involves a callus phase or a direct pathway where embryos are produced without an intervening callus phase. Somatic embryo development and maturation is observed when cultures are transferred to medium devoid of growth regulators. Embryogenic cultures are used as target tissues for inserting desired genes of interest and developing cultivars with improved traits (Fitch, 2005).

Papaya embryogenic cultures are obtained from hypocotyl, axillary bud, stem, ovule, zygotic embryo and root explants (Anandan et al., 2012; Ascencio-Cabral et al., 2008; Chen et al., 1987; Fitch and Manshardt, 1990; Fitch, 1993; Jordan and Velozo, 1997; Litz and Conover, 1980; Abreu et al., 2014; Razali and Drew, 2014). In most cases, cultures develop via indirect embryogenesis. Highly reproducible protocols for obtaining embryogenic cultures were reported by Fitch and Manshardt (1990) and Fitch (1993). Hypocotyl explants obtained from germinated seedlings produce embryogenic cultures on induction medium containing ½ strength MS salts and vitamins with 9.0 μM 2,4-D, 400 mg $L^{-1}$ glutamine, 60 g $L^{-1}$ sucrose and 7 g $L^{-1}$ TC agar (Fig. 1a). Repeated transfer of embryogenic cultures on induction medium results in the development and proliferation of proembryonic masses or PEMs (Fig. 1b; c). Embryogenic cultures can be maintained by repeated transfer of PEMs to induction medium. Alternatively, immature zygotic embryos excised from fruits obtained 90–113 days after pollination also produce embryogenic cultures on induction medium (Fig. 1d). Cultures appear 4 weeks after induction and the production of somatic embryos (SE) occurs exclusively from the embryo axis (Fig. 1e). Such cultures proliferate on induction medium (Fig. 1f) and can be maintained up to 12 weeks. Somatic embryogenesis appears to be genotype-dependent with some genotypes exhibiting greater production of somatic embryos (Fitch, 1993). Embryogenic cultures have also been induced from ovule explants cultured on White's medium modified with the addition of 60 g $L^{-1}$ sucrose, 400 mg $L^{-1}$ glutamine, and 20% (v/v) filter-sterilized coconut milk (Litz and Conover, 1982). Other studies have indicated successful production of embryogenic cultures using 2,4-D in the induction medium (Bukhori et al., 2013; Razali and Drew, 2014).

Papaya embryogenic cultures can be maintained by transfer to semi-solid or liquid induction medium at 3 weeks intervals (Fitch, 1993; Litz and Conover, 1983; Mahon et al., 1996). Embryogenic cultures in liquid induction medium proliferate by repetitive budding of PEMs. Cultures growing in liquid medium can be synchronized by sieving to retain the smallest fraction of PEM, which is subsequently transferred to fresh medium for further proliferation. Such PEM effectively produce somatic embryos when transferred to growth regulator-free medium. The proliferation rate of PEMs is higher in suspension cultures after culture synchrony is attained (Von Arnold et al., 2002). Suspension cultures exhibit greater proliferation potential and higher plant regeneration rates compared to solid medium grown cultures and may be better suited as targets for gene insertion and the production of transgenic plants (Castillo et al., 1998; Ying. et al., 1999; Lines et al., 2002; Carlos-Hilario and Christopher, 2015).

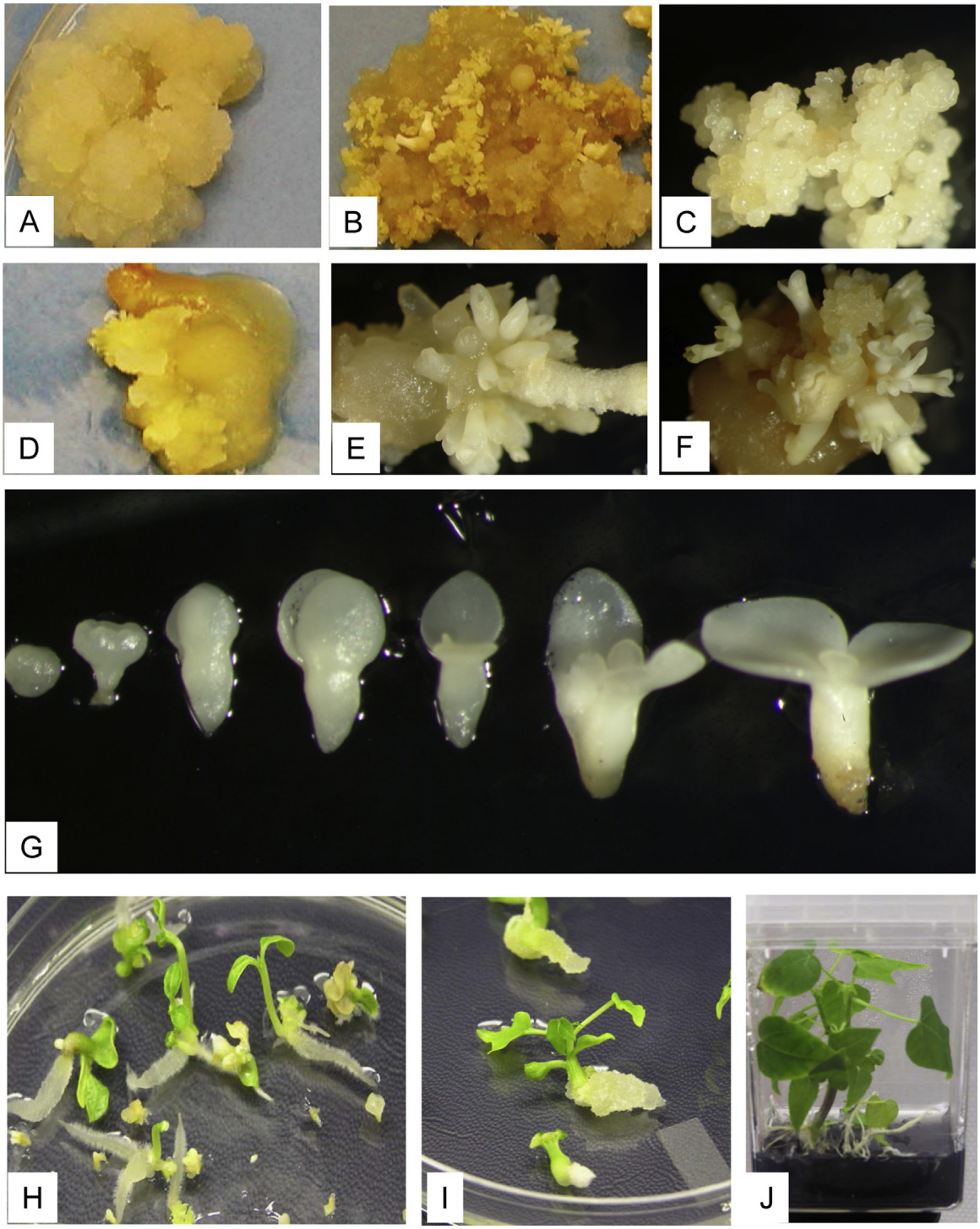


**Fig. 1.** Somatic embryogenesis and plant regeneration in *Carica papaya* L. a. Initiation of callus cultures from hypocotyl explants. b. Development and proliferation (c) of proembryonic masses (PEM) following continuous culture on induction medium. d. Initiation of embryogenic cultures from immature zygotic embryos and proliferation of somatic embryos (e; f). g. Somatic embryo development in papaya on medium without growth regulators. (h). Somatic embryo germination. Note the callus formation that frequently occurs at the base of germinated embryos (i). j. Plant regeneration from germinated somatic embryos. *Reproduced from* Dhekney et al. (2007) *with permission from Springer*.

Somatic embryo maturation occurs on induction medium devoid of growth regulators (Fitch, 1993; Litz and Conover, 1983). Development follows the classical globular, heart, torpedo and cotyledonary (early to late) stages (Fig. 1g). Asynchronous development is frequently observed and can be attributed to differing access to nutrients following subculture to fresh medium (Conger et al., 1989). Abnormal embryo development, including embryos with fused cotyledons, multiple cotyledons, misshaped and fused embryos are frequently observed. Such abnormalities occur when cell divisions in meristematic areas occur prior to differentiation of the shoot apex (Litz and Gray, 1992). The presence of plant growth regulators in the culture medium often contributes to abnormal

embryo development (Ammirato, 1987). Other factors include the absence of a seed coat in somatic embryos, which in zygotic embryos regulates respiration and gas exchange (Norstog, 1965). ABA inclusion in the maturation medium increases embryo size and improves somatic embryo development (Anandan et al., 2012; Chen et al., 1987). A significant improvement in somatic embryo maturation and the number of cotyledonary stage somatic embryos is observed when polyethylene glycol (PEG) is added to the development medium (Heringer et al., 2013). Such improvements are attributed to the ability of PEG to increase protein synthesis and induce differential expression of genes involved in carbohydrate and lipid metabolism and stress response, all of which contribute to better embryo maturation (Vale et al., 2014).

Germination of papaya somatic embryos occurs on medium containing MS macro-, micronutrients and vitamins, 100 mg $L^{-1}$ inositol, 3% sucrose and 0.5% agar (Fig. 1h). In some studies, growth regulators added to the culture medium promote germination and plant recovery (Fitch and Manshardt, 1990; Litz and Conover, 1982). Callus formation at the radical end is frequently observed during germination (Fig. 1i). Somatic embryos have the ability to conjugate auxins, which are subsequently excreted into the medium during the differentiation process resulting in callus production (Michalczuk et al., 1992). Treatments that involve addition of activated charcoal to the medium may lead to recovery of somatic embryos and plants (Fig. 1j) with normal morphology and increased germination (Buchheim et al., 1989), due to the ability of activated charcoal to adsorb auxins released from developing tissues (Ebert and Taylor, 1990).

Somatic embryos at the cotyledonary stage of development can be used in synthetic seed production (Castillo et al., 1998). In this case embryos are immersed in a 2.5% sodium alginate solution followed by transfer to calcium chloride solution to initiate encapsulation and bead formation (Fig. 2a). Such encapsulated embryos germinate normally and produce plants when transferred to germination medium (Fig. 2b; c).

### 2.2. Papaya micropropagation

Micropropagation enables rapid production of uniform, disease-free planting material of elite papaya cultivars (Fitch, 2005). MS macro- and micronutrients combined with BA and NAA at various concentrations are most frequently used for shoot proliferation while auxins are included in the culture medium to induce rooting (Fitch et al., 2003; Drew, 1988; Mumo et al., 2013; Tetsushi et al., 2008; Roy et al., 2013; Veena et al., 2015).

Limitations encountered during papaya micropropagation include the presence of endophytic bacteria in cultures, poor response of mature explant tissues and loss of regeneration potential following long-term culture (Drew, 1988; Drew and Smith, 1986; Litz and Conover, 1982; Thomas et al., 2007). Presence of endophytic bacteria affects shoot proliferation and rooting. Frequent indexing of cultures stock assists in the identification endophytes, which can be eliminated to improve plant regeneration (Thomas et al., 2007). Other techniques including alternating culture regimes in liquid and solid medium, and eliminating sucrose in the medium following shoot proliferation to produce clean plants (Drew, 1988; Fitch et al., 2003). Modifications in culture environment such as providing ventilation to improve gas exchange in culture vessels, $CO_2$ enrichment of culture vessels, and providing illumination using red light during micropropagation may simulate photoautotrophic conditions to improve plant growth and recovery (Lai et al., 1998; Yu et al., 2000; Perez et al., 2015; Schmildt et al., 2015). Improved regeneration rates are attributed lower ethylene accumulation in culture vessels, which decreases shoot epinasty and leaf senescence, symptoms that are frequently associated with the presence of ethylene in culture vessels. Alternatively, addition of ethylene biosynthesis inhibitors such as aminoethoxyvinylglycine (AVG) and silver thiosulfate (STS) during culture also improves shoot quality and plant regeneration (Magdalita et al., 2002). Plants obtained by micropropagation exhibit uniform growth and vigor, precocious bearing and higher yields compared to seedling-derived plants (Fitch et al., 2005a, 2005b). Papaya plants obtained through in vitro culture exhibit higher levels of antioxidants compared to seedling-derived plants (Tiwari et al., 2014).

### 2.3. Protoplast culture

Protoplast culture and somatic hybridization have potential applications to overcome sexual incompatibility barriers between *Carica* and other genera for of transfer desirable traits such as improved yield and quality, disease resistance and abiotic stress tolerance (Chen, 1994).

Protoplasts isolated from papaya leaves produced callus cultures and somatic hybridization following protoplast fusion between sexually incompatible *V. cundinamarcensis* and *C. papaya* produced microcolonies, but no plant regeneration occurred (Litz and Conover, 1979; Jordan et al., 1986). Protoplasts isolated from embryogenic cultures of *C. papaya* X *V. cauliflora* could be regenerated into whole plants via direct embryogenesis (Chen and Chen, 1992).

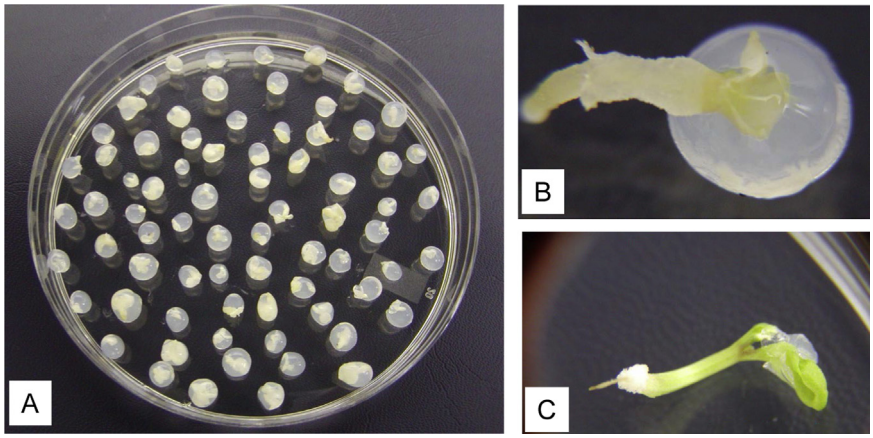


**Fig. 2.** Synthetic seed production in papaya. a. Somatic embryos at the cotyledonary stage of development are used for encapsulation. b and c. Such embryos exhibit normal germination and development when transferred to germination medium.

## 3. Papaya cryopreservation

Cryopreservation is used for medium and long-term storage of elite papaya germplasm and conservation of genetic diversity. Cryopreservation of papaya embryogenic cultures, shoot tips and seeds has been successfully demonstrated (Ashmore et al., 2001; Lu and Takagi, 2000). For cryopreservation of tropical plant species such as papaya the vitrification technique, which consists of treating cultures with a solution that causes water content of cells to transition from a liquid to a non-crystalline viscous phase thereby preventing cellular damage during cryopreservation, is frequently used. Higher survival rates are observed with vitrification compared to the slow cooling techniques (Reinhoud et al., 1995). Vitrification is most frequently used for cryopreservation of papaya shoot tips (Tsai et al., 2009; Wang et al., 2005). The protocol consists of a loading step involving a loading solution (2 M glycerol and 0.4 M sucrose) and a dehydration step using a vitrification solution. Plant vitrification solutions (PVS) containing glycerol, ethylene glycol and DMSO at varying concentrations (PVS-2, PVS-3) are used for long-term storage of shoot tips in liquid nitrogen. Following removal from liquid nitrogen, shoot tips are rapidly thawed in a water bath at 37–40 °C for 2–3 min, and then transferred to regeneration medium. Shoot proliferation is observed following culture at ambient temperature with plant regeneration rates ranging from 35–60% across various genera and cultivars (Ashmore et al., 2007). A similar technique is used for cryopreservation of papaya embryogenic cultures (Dhekney et al., 2003; 2004). Maintenance of genetic fidelity and clonal homozygosity are important prerequisites for successful cryopreservation (Ashmore et al., 2011). Assessment of plant performance after cryopreservation reveals no morphological differences in flowering and fruiting characteristics between control plants and plants obtained following cryopreservation (Kaity et al., 2009; Ashmore et al., 2011). In other studies, plants regenerated from cryopreserved shoot tips and seeds exhibit reduced growth compared to control plants (Azimi et al., 2005). Molecular marker analyses of plants obtained following cryopreservation reveals varying levels of modification in genomic DNA and methylation patterns (Kaity et al., 2008), which may be attributed to the stress imposed on cultures during the process of cryopreservation and subsequent regrowth.

## 4. Genetic engineering of papaya

Genetic engineering of papaya has been successful for overcoming limitations encountered in conventional breeding. Introduction of foreign genes into papaya is possible using particle bombardment (biolistic techniques) and *Agrobacterium*-mediated transformation (Fitch et al., 1990; 1993). The β-glucuronidase (GUS) gene (Jefferson et al., 1987) and the Green Fluorescent Protein (GFP) gene (Stewart, 2001) are commonly used as reporter genes in papaya transformation to screen transgenic tissues from non-transformed ones (Fitch et al., 1993; Zhu et al., 2004a). The GUS gene isolated from *E. coli* produces a β-glucuronidase enzyme that reacts with a colorless substrate 5- Bromo 4- Chloro- 3-indolyl- β glucuronic acid (X-Gluc) to produce an intense blue color. Transgenic cells expressing GUS will produce a blue color in the presence of the substrate thereby facilitating transgene detection. The GFP gene, isolated from the Pacific jellyfish *Aequoria victoria,* is commonly used as a reporter gene. Plant cells expressing GFP produce a bright green fluorescence, which can be observed under a stereomicroscope equipped for epi-fluorescence illumination. The use of GFP provides the added benefit of non-destructive screening identification of transformed tissues. The neomycin phosphotransferase II (npt II) that confers resistance to kanamycin antibiotic is the most widely used selectable marker in papaya transformation (Dhekney et al., 2007). Transgenic cells expressing nptII selectively grow on culture medium containing kanamycin, while inhibiting the growth of non-transformed cells. Particle bombardment protocols consist of coating gold or tungsten microcarriers/particles with plasmid DNA containing the gene/s of interest along with a reporter and selectable marker gene. The processed microcarriers are then bombarded onto embryogenic cultures. Following bombardment, cultures are transferred to induction medium containing a selective agent for promoting growth and proliferation of transgenic cells. Transformed embryogenic cultures produce somatic embryos following transfer to hormone-free development medium. Regenerated transgenic plants are screened for transgene presence and copy number using PCR and Southern blot hybridization (Fitch et al., 1993). *Agrobacterium*-mediated transformation involves co-cultivation of embryogenic cultures with disarmed *Agrobacterium tumefaciens* containing the vector with genes of interest. Acetosyringone, a phenolic compound, is frequently added to the bacterial suspension to enhance the virulence of *vir* genes and improve transformation efficiency (Ying. et al., 1999). Following co-cultivation for 24–72 h in darkness, embryogenic cultures are washed in liquid induction medium containing carbenicillin and cefotaxime antibiotics to inhibit bacterial growth. Cultures are then transferred to induction medium containing carbenicillin, cefotaxime and a selective agent (e.g. kanamycin). Transgenic plants are regenerated from embryogenic cultures as described above.

Alternative selection systems such as phospho-mannose isomerase (PMI)/mannose (Man) have also been demonstrated to work efficiently for papaya transformation (Zhu et al., 2005). Papaya embryogenic cultures do not produce the PMI enzyme under normal condition and are unable to survive in culture medium containing mannose (instead of sucrose) as a carbon source. Embryogenic cultures transformed with a *pmi* gene constitutively express the PMI enzyme and are able to utilize mannose added to the culture medium. The efficiency of transgenic plant recovery in this system may be higher than that using an antibiotic selection or with a reporter gene (Zhu et al., 2005).

Techniques to improve transformation efficiency include wounding embryogenic cultures with carborundum or tungsten prior to co-cultivation (Cheng et al., 1996; Ying. et al., 1999). Somatic embryos are wounded by vortexing with 600 mesh carborundum or tungsten M-15 in either distilled water or liquid medium. Embryogenic cultures are then co-cultivated with *Agrobacterium* and transferred to induction medium containing antibiotics for selection and proliferation of transgenic cells. Transfer of transgenic cells to development medium produce somatic embryos, which are germinated to obtain transgenic plants. Limitations encountered in *Agrobacterium*-mediated transformation include difficulties in controlling bacterial growth following co-cultivation, poor efficiency of transgene insertion and integration in embryogenic cultures and transgenic plant recovery. Techniques such as wounding with carborundum can produce abnormal growth and poor transgenic plant recovery (Carlos-Hilario and Christopher, 2015). *Agrobacterium* regrowth following co-cultivation despite using high levels of antibiotics in the culture medium can inhibit subsequent embryo development and plant recovery. Such limitations can be overcome by the use of well-developed suspension cultures in thin layers, co-cultivation with extremely low bacterial cell densities and decreasing the time of co-cultivation from 72 h to 24 h (Carlos-Hilario and Christopher, 2015). Transgenic plants have also been obtained from other explant sources including leaf discs, petioles of in vitro cultured shoots and shoot tips of in vitro germinated seedlings (Pang and Sanford, 1988; Yang et al., 1996; Chandra et al., 2010).

Papaya genetic engineering has been successfully exploited to

improve biotic and abiotic stress tolerance, qualitative traits and produce plant-derived vaccines. A major success story in the application of genetic engineering technology for improved disease resistance is the development of improved papaya cultivars with papaya ring spot virus (PRSV) resistance (Gonsalves, 2014). This application is based on the concept of pathogen-derived resistance (PDR), which involves engineering specific DNA sequences from the target pathogen into the host plant. The transgenic protein produced in such plants disrupts the host-pathogen interaction when attacked by the pathogen, and subsequently imparts disease resistance to the plants (Gottula and Fuchs, 2009; Sanford and Johnston, 1985). Other examples of PDR include using mild virus strains to cross-protect host plants against more virulent strains that cause severe damage (Scholthof et al., 1993).

The concept of PDR was effectively exploited to develop PRSV resistant papaya. PRSV was first reported in Hawaii in the 1940's and posed a major threat to the papaya industry by 1992 (Gonsalves, 2004). Concerted research to manage PRSV resulted in the development of transgenic plants that were produced by particle bombardment of papaya embryogenic cultures with the coat protein gene of a mutant mild PRSV strain (Fitch et al., 1992). Extensive greenhouse and field trials were conducted to confirm resistance of transgenic plants to PRSV. These results combined with breeding efforts to introgress resistant genes in other commercial cultivars resulted in two cultivars 'Sun-Up' and 'Rainbow' being released for commercial production after deregulation by the USDA in 1997 (Fitch et al., 1992; Lius et al., 1997; Manshardt, 1998; Gonsalves, 2004). Resistance of the two cultivars to PRSV appears to be dependent on the dosage of the coat protein transgene, developmental stage of plants and strain specific recognition (Tennant et al., 2001). While 'Rainbow' plants that are hemizygous for the coat protein transgene were resistant to the Hawaiian isolate but susceptible to other isolates, 'SunUp' papaya, which is homozygous for the coat protein transgene exhibited broad spectrum resistance against several isolates in addition to the HA isolate. Further screening of papaya lines obtained from the original transformation experiments identified an additional line with resistance to non-Hawaiian PRSV isolates in addition to the Hawaiian isolates (Tennant et al., 2005).

Following adoption of transgenic papaya, production in Hawaii was restored to pre-infection levels, thus averting a disaster in the papaya industry (Gonsalves, 2004). Transgenic papaya was shipped to Canada, and more recently to Japan following exhaustive testing of the product (Wieczorek, 2014). Efforts are currently ongoing to deregulate Hawaiian papaya in China for export purposes (Gonsalves, 2014). The research team of Dennis Gonsalves, Maureen Fitch, Richard Manshardt and Jerry Slightom received the Alexander von Humboldt award in 2002 for saving the Hawaiian papaya industry. A comparison of nutritional composition of transgenic and non-transgenic papaya revealed no significant differences in nutrients or allergenic compounds, thereby deeming the product safe for human consumption (Tripathi et al., 2011; Roberts et al., 2008). Field studies on transgene pollen flow have determined pollen drift from transgenic to non-transgenic papaya as being inefficient and negligible thereby allaying fears of pollen-mediated transgene flow to conventional papaya orchards (Manshardt et al., 2007; Gonsalves et al., 2012).

In addition, efficient techniques for screening the presence of transgenic seeds by PCR have been developed to avoid transgene contamination in orchards where non-transgenic papaya is grown (Matsumoto et al., 2010; Nageswara-Rao et al., 2013). Such efforts point to the successful co-existence of GM and non-GM papaya industries in Hawaii. Efforts have also been made to raise public awareness in Hawaii regarding the prevalence of PRSV (http://cms.ctahr.hawaii.edu/pic-a-papaya/Home.aspx).

Other groups have developed transgenic papaya with resistance to local PRSV strains i.e., Australia (Lines et al., 2002); double resistance to PRSV and papaya leaf-distortion mosaic virus (PLDMV) was developed in Taiwan using partial DNA fragments coding for the coat protein of PRSV and PLDMV (Kung et al., 2009). A modified protocol to obtain virus resistant plants consisted of using roots from in vitro-grown plants as explants to produce embryogenic cultures (Kung et al., 2010). Subsequent transformation and regeneration procedures were as described above. Transgenic papaya lines with resistance to other strains of PRSV have also been developed in Asia, the US Virgin islands, South America and the Caribbean, and efforts to deregulate them are ongoing (Gonsalves, 2004; Zimmerman et al., 2007).

Transgene insertion sites in papaya 'Rainbow' and 'SunUp' were characterized using Southern blot hybridization (Suzuki et al., 2008). Insertion events were analyzed using probes that spanned the entire plasmid construct. Functional transgene sequences coding for the PRSV coat protein, the npt II and GUS genes were located on a single 9.8 kb insert while two non-functional inserts containing fragments of the npt II gene and plasmid backbone were also detected. These inserts were also detected in subsequent progeny of the parents indicating stable inheritance of transgenes. In other studies, transgene integration was detected at multiple loci in the genome of transgenic plants (Fan et al., 2009).

Efforts are currently being made to develop transgenic papaya with fungal disease resistance. Transgenic plants producing resveratrol were recovered following co-transformation of embryogenic cultures with a construct containing a grapevine stilbene synthase gene (Vst1) under the control of its own pathogen inducible promoter plus a construct containing a hygromycin resistance (hpt) selectable marker gene (Zhu et al., 2004b). The presence of the Vst1 gene, the precursor of resveratrol, was confirmed in transgenic plants by Southern blot hybridization. Inoculation of transgenic plants with a suspension of *Phytopththora palmivora* spores induced the production of resveratrol and plants exhibited enhanced fungal resistance compared to non-transformed controls. Transgenic plants constitutively expressing a *Dahlia merckii* antimicrobial peptide gene (*DmAmp1)* exhibited reduced hyphal growth and enhanced resistance to *P. palmivora* (Zhu et al., 2007). Papaya cultivars have also been engineered for enhanced insect resistance. Transgenic papaya plants constitutively expressing either a chitinase gene isolated from *Manduca sexta* larvae or a *Galanthus nivalis*-derived lectin gene were obtained by particle bombardment of 'Kopoho' embryogenic cultures (McCafferty et al., 2006, 2008). In both cases, transgenic plants exhibited improved tolerance to carmine spider mites, (*Tetranychus cinnabarinus*) in greenhouse and field tests.

Efforts have also been directed towards genetic improvement of papaya for abiotic stress tolerance. The presence of cold inducible transcription factors in the *Carica* and *Vasconcella* genome were probed using degenerate primers for C repeat Binding Factor (CBF) genes from several plant species (Dhekney et al., 2005; 2007). CBF transcription factors are master switches for induction of abiotic stress tolerance pathways in several plants species (Thomashow, 1999). Transgenic 'Sunrise Solo' plants containing *Arabidopsis* CBF genes were produced *via Agrobacterium*-mediated transformation of 'Sunrise Solo' embryogenic cultures (Dhekney et al., 2007). Southern blot analysis demonstrated the presence and stable integration of transgenes in the genome of transgenic plants.

Improved post-harvest shelf life of ripe papaya fruits has been demonstrated using genetic engineering technology. Fruit ripening in papaya is accompanied by a sudden burst in ethylene biosynthesis, which triggers downstream genes responsible for fruit softening (Lelie'vre et al., 1997). Long-distance transport and marketing of papaya is limited by its poor shelf-life and chilling damage when stored at low temperature (Chen and Paull, 1986).

Other factors including physical damage of fruit during storage and transit, and storage rot can cause high losses (Paull et al., 1997). Application of ethylene inhibitors such as 1-methylcyclopropane (1-MCP) to mature fruit suppresses ethylene biosynthesis, improve firmness, and delays ripening during storage thereby improving post-harvest life (Manenoi et al., 2007). Transgenic strategies for improving post-harvest shelf life of papaya target either genes involved in ethylene biosynthesis during fruit ripening, or the receptors that perceive ethylene and trigger expression of fruit ripening- and softening-related genes (Stearns and Glick, 2003). Transgenic papaya exhibiting decreased ethylene production and altered ripening were produced using a co-suppression strategy (Lopez-Gomez et al., 2009). The cDNA sequence for a papaya 1 aminocyclopropane-1-carboxilic acid (ACC) gene was obtained from ripe 'Maradol' fruits and placed along with the bar gene under the control of a CaMV 35S promoter. Embryogenic cultures were transformed using biolistic bombardment and transgenic plants were regenerated on culture medium containing phosphinothricin. Transcript analysis using northern blot hybridization revealed a drastic reduction in ACC oxidase mRNA as a result of co-suppression and a subsequent reduction in ethylene biosynthesis in transgenic fruits compared to the controls. Similar efforts are underway in Australia, Malaysia and the Philippines for controlling fruit ripening and extending shelf life (Botella et al., 2005). Other strategies for improving post-harvest shelf life including using RNA interference (RNAi) and blocking ethylene receptors are currently being investigated (Sekeli et al., 2014).

The use of papaya for production of plant-based vaccines has been explored. Embryogenic cultures were co-transformed with codon optimized gene sequences for a procine-cysticercosis vaccine peptide along with the GUS and hygromycin gene, for selection of transgenic cells (Cruz-Hernandez et al., 2007). Transgenic plants obtained following transformation expressed the synthetic peptides for the vaccine. Mice fed with plant extracts containing the transgenic peptides exhibited complete protection and enhanced immunity against cysticercosis. Efforts have also been made to produce a plant-based vaccine against tuberculosis using transgenic papaya plants (Zhang et al., 2003). Other applications of genetic engineering technology involve transgene marker assisted selection for sex determination of papaya seedlings (Le and Manshardt, 2010). In this study, embryogenic cultures were transformed with a fluorescent yellow protein (Eyfp) and a selectable bar gene conferring glufosinate resistance. Transgenic plants with insertions close to sex linked genes are currently being screened to develop assays for rapid sex identification of seedlings.

## 5. Papaya genomics

*Carica papaya* is a useful model fruit species for genome analyses for several reasons. Papaya has a short juvenile period, exhibits prolific flowering and fruiting, and each fruit produces several seeds. Additionally, efficient in vitro propagation and genetic engineering techniques enable the study of a number of vegetative and reproductive processes using genome sequence information (Yu et al., 2009).

The Hawaii papaya genome project was initiated in 2004 to determine DNA sequence information for increased understanding of higher biology and utilizing it to improve productivity, quality, and disease resistance. A high density genetic map using microsatellite markers from bacterial artificial chromosome (BAC) end sequences and genome sequences was created by Chen et al., (2007). A large number of markers were mapped to 12 linkage groups that included 9 major and 3 minor groups. This map was used to integrate genetic and physical maps and align the draft papaya sequence to papaya chromosomes (Ming et al., 2008; Yu et al., 2009). A comprehensive saturated map was then created by enriching it with amplified fragment linked polymorphism (AFLP) markers distributed within 14 linkage groups (Blas et al., 2009). Complementary DNA (cDNA) libraries were constructed from various plant tissues including roots, leaves, seed, callus, flowers of different sex types and developmental stages, and fruit at various stages of ripening to develop a large number expressed sequence tags (EST). This information was utilized for the draft sequence of the papaya genome (Ming et al., 2008).

The papaya genome was sequenced using genomic DNA of transgenic 'SunUp' papaya (Ming et. al. 2008). There are nine pairs of chromosomes and a genome size of 372 Mbp (Ming et al., 2008; Chagne, 2015). The genome size is larger than the *Arabidopsis* genome, but contains fewer genes than those in many other species. Greater than 50% of the genome consists of long terminal repeat retrotransposon sequences (Wei and Wing, 2008). A detailed comparative analysis of the papaya genome has been provided by VanBuren and Ming (2014) and Yu (2014). Papaya has fewer carbohydrate active enzyme genes involved in biosynthesis and transferases than *Arabidopsis*. Other genes encoding sugar accumulation, phytohormone synthesis, polyphenol oxidase and polyphenol peroxidase are also present in lower numbers. Papaya expansins are part of a large superfamily containing 19 genes. Genes responsible for ethylene biosynthesis in papaya are highly homologous with those present in *Arabidopsis*. Single genes for major enzymes involved in production of volatiles have been observed. A higher number of MADS box transcription factors have been observed in papaya compared to other plants. Several genes from the MADS box family are involved in the regulation of flowering and development of specific floral organs. Papaya is affected by several diseases, which adversely affect yield and fruit quality. The papaya genome contains diverse groups of nucleotide binding site (NBS) genes in smaller numbers. Such gene families are responsible for imparting disease resistance in plants (Porter et al., 2009). Papaya NBS genes belong to the Toll/interleukin-1 receptor (TIR) and non-TIR subclasses, and exhibit high homology with their *Vitis* counterparts. Alternative splicing of papaya NBS and other disease resistance-associated genes appears to compensate for the low numbers observed in the species.

Sexual reproduction and the genetics of sex control in papaya have been extensively studied. Three flower types, male, female and hermaphrodite have been observed in papaya. A number of RAPD and SCAR markers have been developed for predicting sex of young papaya seedlings (Deputy et al., 2002; Parasnis et al., 2000; Urasaki et al., 2002). BAC libraries have been constructed from the genomes of male and female papaya plants (Gschwend et al., 2011). Sex determination in papaya is controlled by a pair of sex chromosomes, XY, exhibiting typical characteristics of chromosomal rearrangement (Liu et al., 2004). An investigation of the chromosomal location of the hermaphroditic- and male-specific region revealed it to be approximately 8.0 Mb in length (Yu et al., 2008; Wang et al., 2012) and located near the centromeric region. The region is characterized by a high density of retrotransposons and sequence duplications that exhibit suppressed recombination. Sequences and expression analyses of genomic regions involved in sex determination revealed significant differences in expression of a short vegetative phase (SVP)-like gene in male and hermaphrodite flowers due to a transposon insertion in the hermaphrodite-derived gene sequence (Ueno et al., 2015). Analysis of the hermaphrodite-specific regions reveals low diversity of nucleotides and has been attributed to human domestication of the species (VanBuren et al., 2015).

## 6. Conclusions

The significant progress made in papaya cell culture and plant

regeneration, genomics and biotechnology has improved our understanding of growth and development of this important fruit crop. With the availability of genomic sequence information and refined genetic engineering protocols, functional analysis of genes responsible for important traits including yield, quality and disease resistance can be studied using forward and reverse genetics strategies. In addition, knowledge of the genome can enable improvement of papaya via precision breeding that would involve the sole use of papaya genes and genetic elements for the development of genetic constructs and their insertion into elite cultivars. Improved cultivars developed in this way would be similar to those obtained by conventional breeding, would be more consumer and eco-friendly and cause fewer GMO-related concerns and consequently improved consumer acceptance.

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