Chapter 6

Genetic Improvement of Tropical and Subtropical Fruit Trees via Biolistic Methods

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http://dx.doi.org/10.5772/intechopen.81373

Abstract

Biolistic is a special high-performance method for direct delivery of foreign DNA, RNA, or protein into plant cells. This method has less physiological risk on plant cell since there is no need for microbial intermediaries (*Agrobacterium* strains) and requires less additional DNA. Moreover, it can adapt for both monocotyledon and dicotyledonous plants. Recently, this method has also been successfully used to plant genome editing. Therefore, in this chapter, we discuss the application of this method for genetic improvement of some commercially important of tropical and subtropical fruit trees including banana, date palm, citrus, mango, olive, and pineapple. Also, we explain the details of biolistic protocols used for transient and stable gene expression in these fruit trees.

Keywords: gene gun, gene delivery, microprojectile bombardment, tropical fruits

1. Introduction

In the recent years, the scientists believe that the molecular methods have high potential for making gene delivery or genome editing possible in different plant species without altering their phenotypes. This capability is particularly valuable for fruit trees that have lengthy generation time and high levels of heterozygosity. In fact, the biotechnology methods now became as routine tools in biology research and plant transformation. So various methods had been introduced by the scientist for gene delivery to the plant cells (which may not be achievable by the traditional breeding methods), and subsequently they successfully regenerated without serious limitations [1–4]. Gene transferring to plant tissues can be achieved by two means: direct or indirect methods. In the direct methods, there is no need to *Agrobacterium* mediate, but the plasmids that are harboring desired DNA materials will deliver to the plant cells via physical or

chemical means [5]. However, indirect gene transformation to plant tissues is usually achieved by mediated *Agrobacterium* strains. At the present time, in the most laboratories, gene delivery to plant tissues is achieved by mainly two means including biolistic and *Agrobacterium* methods [2, 4]. However, most of the gene transformation studies on fruit trees have been mediated by *Agrobacterium* strains, and biolistic has been less frequently used [6]. Infection with *Agrobacterium* may potentially produce unpredictable effects on the plant cells when transformed with T-DNA [7, 8]. The biolistic is a more applicable method for gene transformation in a wide range of plant cells and tissues [9], even those that could not transform by other transformation methods. In this method the precipitated DNA on gold or tungsten particles is transferred directly into the plant cells and tissues. Therefore, it is possible to introduce new traits with lower risk of the GMO effect with high reproducibility and no significant damage or artifacts [8]. Further, this method can be more adapted for breeding of plant species with a high degree of heterozygosity [10].

The biolistic method was introduced for the first time by Sanford [11]. Optimization of the transformation condition is very critical for achievement of an efficient protocol with high transformation frequency [12]. This strongly depends on the construct and promoter type and optimization of the physical and biological parameters. In order to achieve the best results with the biolistic method, the following are needed:

1. Appropriate construct (the type of genes and promoter)
2. Proper tissue (eases to regeneration as well as pretreatment prior to bombardment)
3. Optimized bombardment condition (biological parameters, as well as physical parameters, should been optimized)
4. Detecting of the insertion (integrated to the genome)

The biolistic method has a potential use for breeding of several tropical and subtropical fruit trees so that different genes were transferred to these trees for different purposes. Most of these genes are selectable and scorable marker genes which were used for the establishment of the optimized transformation protocols and some other genes of interest (which are encoding the economical traits).

One of the more permissible applications of the biolistic method is using it for genome editing or CRISPR in plants [13].

In this chapter, we explain the different gene transformation procedures introduced by scientists for various economically important tropical and subtropical fruit trees by the use of the biolistic method.

2. Banana

Several limitations had been reported for the breeding of banana cultivars through traditional methods mainly including long regenerating time, polyploidy, and male sterility [14, 15]. The biolistic method was successfully used for banana transformation so that several genes were transferred to different banana tissues for different purposes. However, this method may be integrated with the *Agrobacterium* to increase the efficiency of the gene transformation.

Embryogenic cells initiated from different tissues including immature male flowers [16], immature embryos [17], male inflorescence, and buds [18] were reported with high potential to gene transformation in banana and plantain. A protocol optimized for transient and stable transformation of the *uid*A gene in banana cells using a special gene gun is illustrated by [14]. The tungsten particles coated with various plasmids harboring *uid*A gene including pEmuGN (with Emu promoter), pBI-364, pBI-426, pBI-505 (with 35S promoter), and pAHC27 (with Ubi promoter) were delivered into banana cells and then comprised among them based on the level of transient expression after assayed with X-Gluc and MUG. The highest transient transformation was obtained with the pAHC27 plasmid. Also, the stable transformation was achieved after the bombardment of banana cells with pWRG1515 plasmid harboring *uid*A gene along with *hph* as a selectable marker gene (conferring resistance to hygromycin) and cultured on the medium contain 50 mg/L hygromycin.

Stable transformation of the Cavendish banana (*Musa* spp. AAA group) cv. Grand Nain was also reported by [16], using *uid*A and potential virus-resistance (BBTV) genes along with *npt*II gene as selectable marker gene using various plasmids (**Table 1**).

In other experiment [18], researchers transferred to the *Musa* spp. (AAB group) cv. Maçã the three plasmid constructions harboring *uid*A gene including pBI426 (70S promoter), pFF19 (70S promoter), and pCAMBIA1303 (35S promoter). The plasmids had been precipitated on the tungsten particles using 20 μL of spermidine and 50 μL of $CaCl_2$ and then accelerated to penetrate callus tissue located at 9 cm from stopping screen using 1100 psi helium pressure force. The transient expression was observed for all constructs, but the best result was obtained for pBI426 due to achievement of the highest regenerated plant after 3 months.

For obtaining a successful transformation through the biolistic method, it is important to reduce physical stress entered on target tissues promoted by bombardment shock waves. Bombarded tissues may reduce their regeneration potential especially in the case of embryogenic callus and immature tissues. Therefore, such sensitive tissues should bombard with lower helium pressures and target distance. In most studies on gene transformation of banana by biolistic methods, it had been found that best results were obtained in 1100–1350 psi helium pressure and 6–9 cm target distance (**Table 1**).

Another strategy for increasing the transformation frequency in biolistic method is the integrating biolistic with *Agrobacterium*-mediated transformation especially with monocotyledons plants. It has been found that the infection of the Gongjiao (*Musa acuminata* L. AA group, cv. Mas) floral apices with *Agrobacterium tumefaciens* (AGL1 contains pCAS04) suspension for 30 min after bombardment thrice with pCAS04 plasmid coated on the 0.6 μm gold particles under 1300 psi helium pressure force was increased transformation frequency 1.6- and 3.3-fold higher than that of gene gun and *Agrobacterium* methods, respectively [15].

2.1. Plant-based vaccine

Hepatitis B virus (*HBV*) is a worldwide disease causing chronic and acute infections in the human liver. Therefore, needful to produce a vaccine for this disease is very important. On the other hand, production of vaccines required a high cost whereas may not be possible to secure the large segment of the population in the world. An attempt was made by [19], to transfer the *HBsAg* gene, coding hepatitis B surface antigen to banana cv. Williams to make

Plant name	Explant type	Plasmid (s)	Reporter gene(s)/ promoter (s)	Selectable gene (s)/ promoter (s)	Helium pressure	Particle size (μm)/ type	Target distance (cm)	Osmoti-cum	Transfor-mation efficiency	Reference
Musa spp. (ABB group) Bluggoe	Embryogenic suspension cells	pBI364, pBI426, pBI505, pEmuGN, pAHC27, pWRG1515	*uid*A/35S, Emu, Ubi	Hygromycin (*hph*)	4.5 bar	Tungsten	4		30%	[14]
Musa spp. (AAB group) Maçã	Immature male flowers	pBI426, pFF19, pCAMBIA1303	*uid*-A/neo/70S; *uid*-A/70S; *uid*-A/35S	Hygromycin	1100 psi	Tungsten	9		Best result was obtained with *uid*-A/*neo*/*70S*	[18]
Musa spp. (AAB group) Grand Nain	Immature male flower	pBT6.3-Ubi-NPT, pUbi-BTintORF1, pUbi-BTutORF5, pUGR73, pDHkan	BBTV intO1/Ubi pro, BBTV utO5/ Ubi pro, *uid*A/*Ubi* pro	*npt*II/BT6.3 pro, *npt* II/ CaMV 35S pro	550 KPa	1.0/gold	7.5		11%	[16]
Musa acuminata cv. Mas (AA)	Immature male flower	pCAMBIA-1301	*gus*/CaMV 35S	—	1100–1350 psi	1.0/gold	6		—	[17]
Musa acuminata L. (AA group, cv. Mas) Gongjiao	Floral apices	pCAS04	*uid*A, *npt*II/Ubi pro, actin pro	—	1300 psi	0.6/gold	4		9.8%	[15]
Musa sapientum cv. Rastali (AAA)	Bud	pBI333-EN4-RCC2, pMRC1301, pROKLa-Eg, pGEM.Ubi1-sgfps65T (GFP)	*npt*II/nopaline synthase gene, *gus*A and chitinase/rice actin 1, *npt*II/nopaline synthase gene (*nos*) promoter, soybean β-1,3-endoglucanse/ CaMV 35S, *gfp*/maize polyubiquitin 1 (Ubi1)		1100 psi		9		4–7.5%	[20]

Plant name	Explant type	Plasmid (s)	Reporter gene(s)/ promoter (s)	Selectable gene (s)/ promoter (s)	Helium pressure	Particle size (μm)/ type	Target distance (cm)	Osmoti-cum	Transfor-mation efficiency	Reference
Citrus reticulata Blanco × *Citrus paradisi* Macf. cultivar Page	Embryogenic cells from suspension cultures		*gus*	*npt*II		Tungsten		0.3 M sorbitol + 0.3 M mannitol		[26]
Carrizo citrange (*Citrus sinensis* (L.) Osbeck × *Poncirus Trifoliate (L.) Raf.)*, sweet orange *(Citrus sinensis* (L.) Osbeck*)* cv. Pera	Thin epicotyl sections	pE2113-GUS	*uid*A/CaMV 35S	*npt*II/NOS promoter	1550 psi	Tungsten M-25 (1.7)	6	0.2 M sorbitol + 0.2 M mannitol		[24]
Citrus macrophylla (C-mac)	Second and third newest leaves	CTV CP-CP interacting BiFC plasmids	*gfp*/35S	—	260–280 psi	0.6/gold	—	—		[8]
Olea europaea L. cv Canino	Somatic embryogenesis	pZ085 and pCGUδ0	*gus*/Ubi (sunflower)		580 kPa	Tungsten or gold				[44]
Olea europaea cv. "Picual"	Embryogenic callus	pCGUΔ1	*gus*/Ubi (sunflower)	*npt*II	900 psi	1.0/gold	6	0.2 mannitol	72.7%	[41]
Ananas comosus "Phuket" and "Pattavia"	Leaves of micropropagated shoots	AHC25	*gus*/maize Ubi	*bar*/maize ubiquitin promoter	1350 psi	Gold	7	0.2 M mannitol	66.7–86.4%	[29]
Ananas comosus L. cv. "Smooth Cayenne"	Callus	pDH-kan^R, pBS420, pART7.35S. GUS, pBS247. SCSV4.GUS, pGEM-Ubi-GFP	*gus*/35S or SCSV4, *gfp*/maize Ubi-1 and *ppo* gene (isolated from pineapple, for control of blackheart) under the control of 35S or maize Ubi-1	*npt*II/35S or SCSV4	1000 kPa	1.0/gold	18	—	0.21–1.5%	[32]

http://dx.doi.org/10.5772/intechopen.81373

Plant name	Explant type	Plasmid (s)	Reporter gene(s)/ promoter (s)	Selectable gene (s)/ promoter (s)	Helium pressure	Particle size (μm)/ type	Target distance (cm)	Osmoti-cum	Transfor-mation efficiency	Reference
Mangifera indica "Carabao" and "Kensington Pride"	Nucellar proembryonic masses	pBI426, pBINgfp-Ser	*gus*, *gfp* /CaMV 35S	*npt*II/*CaMV* 35S	125 psi	7/tungsten	15	0.2 M mannitol	1101 foci per microgram of DNA	[46]
Phoenix dactylifera L. "Estamaran"	Emberyogenic callus	pAct1-D	*gus*/5′ region of the rice actin 1		1100 psi	1.6/gold	9	0.4 M mannitol	1383 GUS blue spots/ bombard-ment	[39]
Phoenix dactylifera L. "Estamaran"	Somatic embryos	pAct1-D	*gus*/5′ region of the rice actin 1		1350 psi	0.6/gold	6	0.4 M mannitol	6–12 blue spots/ bombard-ment	[39]
Phoenix dactylifera L. "Siwy"	Emberyogenic callus	pBC4	Cholesterol oxidase gene/35S, *gus*/35S	Kan resistance/35S	1300 psi	Tungsten	9	0.2 M mannitol		[40]

Table 1. Description of gene transformation to some economically important tropical and subtropical fruit trees through biolistic method.

an alternative plant-based oral vaccine. After the bombardment of the banana meristems with pBHsAg plasmid vector harboring *bar* gene (inactivates phosphinothricin) as a selectable marker and *HBsAg* gene, they had detected the expression of antigen in banana which may have a potential to use it for security against this disease.

2.2. Disease resistance

Fusarium wilt race 1 is one of the limitation factors in banana production, caused by *Fusarium oxysporum* cubense f. sp. Due to cell wall of these fungi mainly made from chitin and β-1, 3-glucan, therefore, presence of chitinase and β-1,3-glucanase in banana tissues can increase the level of resistance to this disease. This was achieved by banana gene transformation with chitinase and β-1,3-glucanase genes using biolistic method [20]. They also transferred reporter genes *gfp* and *uid*A along with the chitinase and β-1,3-glucanase genes to detection of the transformation occurrence and subsequent expression in buds of Rastali cultivar (*Musa* spp. AAB group).

Black Leaf Streak Disease (BLSD) is another worldwide banana disease caused by *Mycosphaerella fijiensis*. The fungi induce streaks on the banana leaves which may lead to reduced fertility and may destroy the whole trees. On the other hand, infested plants usually produce a high level of free radicals, causing more challenges. In a research with goal to increase the level of banana tolerance to the BLSD reported by [21], they transferred two genes, including endochitinase (*ThEn-42*) and grape stilbene synthase (*StSy*) antifungal genes (with synergistic effect) together with chloroplastic (*chl*) Cu, Zn superoxide dismutase gene (*Cu*, *Zu-SOD*) (scavenging of free radical) to embryogenic callus of Cavendish banana (*Musa* spp. AAA group) cv. Grand Nain. After 4 years, the infection of the transgenic banana with these three genes was significantly reduced without any decrease in yield.

3. Citrus

The citrus breeding by traditional methods has some limitations including lengthy period of juvenility (8–10 years), polyembryony, incompatibility, parthenocarpy [22, 23], and high heterozygosity [24]. Molecular methods and gene transformation could be an alternative for breeding of the citruses and rapid regeneration with less time consumption. Currently, gene delivery into the epicotyl segments by *Agrobacterium*-mediated transformation is the most widely used method for gene transformation of the citruses. However, this approach has several drawbacks including the high number of chimeric or non-transformed plants due to the requirement for larger explant and gradient concentrations of the selective agent to the explant [24] and low regeneration frequency of stably transformed cells and recalcitrant of some citrus genotypes to *Agrobacterium* infection [23]. On the other hand, the biolistic method provides several advantages over *Agrobacterium*-mediated transformation such as high transformation efficiency, simplicity of the plasmid constructs which allows for the integration of larger inserts, the co-transformation of more than one construct, and less biological damage to the explant [23–25].

Evaluating the transient expression of a gene can provide valuable information in association with various properties of its produced protein, such as subcellular localization and intra−/intercellular trafficking, stability and degradation, expression levels, and interactions with other proteins [8]. In order to initiate a procedure for transient and stable transformation of the *uid*A/*npt*II genes to embryogenic cell suspension of citrus Tangelo (*Citrus reticulata* Blanco × *C. paradisi* Macf.) cultivar "Page," the researchers [26] used biolistic transformation method (**Table 1**).

Also, in other research [24], the *uid*A/*npt*II genes to thin epicotyl sections of the Carrizo citrange (*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.) and sweet orange (*Citrus sinensis* (L.) Osbeck) cv. Pera were successfully delivered (**Table 1**). Recently the Carrizo immature epicotyl with another reporter gene *gfp* and also *npt*II gene as selectable marker through biolistic transformation are transferred [27].

Most reports on citrus gene transformation by biolistic were carried out on the transformation and expression detection of the selectable and scorable marker genes. However, result reported by [8] showed that the bombardment of the young leaves of the *Citrus macrophylla* (C-mac) with pSAT4-cEYFP-C1(B) harboring CPC^{TV}-GFP using Bio-Rad Helios gene-gun could have been causing the express of CP in the cytoplasm and nuclei of the epidermal cells.

4. Pineapple

The first report on the using of the biolistic method for gene transformation of pineapple was published by [28]. They introduced an efficient system for transformation of protocorm-like bodies with *gus*/*npt*II genes and then confirmed the gene insertions at one to three loci by Southern hybridization. After that, the published results [29–31] indicated that the pineapple cv. Phuket to herbicide Basta® X (with glufosinate ammonium as the active component) can be resisted by transforming with *bar* gene using biolistic transformation method. The transgenic plants showed herbicide tolerance when they were sprayed with herbicide (with twice the routine dose which used in the field) and remained green and healthy after 7 months, whereas the non-transformed plants became necrotic and died after 21 days. The stable integration of the *bar* gene in to the genome of the transformed plants was confirmed with PCR, RT-PCR, and Southern analyses after 380 days.

One of the physiological disorders which limited the industry of the pineapple in different area productions in the world such as Australia is the internal browning or blackheart. This disorder causes severe loss when appearing at conditions with day/night temperatures below 25/20°C with low light during fruit development and also during storage and shipment [32, 33]. To control the internal browning by the molecular breeding methods, an effort was made by [32] in order to obtain a transgene resistant to blackheart through biolistic method. The leaf callus of Smooth Cayenne cultivar was bombarded with gold particles coated with pART7 plasmid harboring PINPPO1 gene (pineapple polyphenol oxidase gene) which could successfully attain resistant plants to blackheart with an efficiency of 0.21–1.5% based on the PCR and Southern blot analysis (**Table 1**). Recent studies demonstrated that low temperature (5°C) could reduce blackheart through upregulated *AcGA2ox* gene and reduce GA_4 levels

http://dx.doi.org/10.5772/intechopen.81373

compared to the higher temperature (20°C) [33]. Also, the Del Monte Foods company introduces a red-fleshed pineapple "Rosé" by overexpression/suppression of some genes related to lycopene accumulation [34].

5. Date palm

One most important challenge face to genetical improvement of date palm through gene transformation and genome editing methods is difficult to regenerate in vitro due to lack of an efficient procedure for raped embryogenic callus induction. However, numerous successful protocols have been developed for regeneration of palm dates in in vitro conditions [35]. At present, shoot tips and immature inflorescence are mostly used for callus induction; however, several months and high levels of auxins (such as 2,4-D with100 mg/L concentration) are necessary that may induce epigenetic variation. Among the different tissues of date palm, the embryogenic callus and somatic embryos had more competencies to gene transformation [36]. Fortunately, the first report on date palm gene transformation had been done with biolistic method [37]. In this study embryogenic callus and somatic embryos of Kabkab cultivar were bombarded with gold particle coated with plasmid DNA construct carrying *gus* gene in different helium pressures (900, 1100, and 1350 psi) and target distances (6, 9, and 12 cm). The results indicated that highest *gus* expression in embryogenic callus was achieved when bombarded with 1100 psi/6 cm (helium pressures/target distance), whereas in somatic embryos, it was obtained in 1350 psi/9 cm. Date palm embryogenic callus exhibits the highest potential of transient expression (1383 *gus* blue spot per bombardment); however, somatic embryos present very lower potential of transient expression (9 ± 3 *gus* blue spot per bombardment). But they were more competent for attainment stable transformation [36, 38]. Unfortunately, the regeneration potential of embryogenic callus was dramatically decreased after bombardment due to shock wave. Recently, we introduce an efficient and optimized protocol for stable transformation of date palm Estamaran (Sayer) cultivar through biolistic transformation method [39]. Also, [40] developed a procedure for delivering the insecticidal cholesterol oxidase (*ChoA*) gene to embryogenic callus of Siwy cultivar through particle bombardment (**Table 1**). They transferred ChoA gene along with *gus* marker gene under control of 35S promoter and confirmed the insertions by *gus* assay, ELISA, and PCR.

6. Olive

Gene transformation to olive cultivars is considered as a difficult task due to recalcitrant nature of their tissues to regeneration process in vitro condition; however, it stays the most promising technique in respect to conventional and unconventional and even some biotechnological methods such as protoplast and somaclonal variation techniques. Classical methods of the olive breeding are more time-consuming, with very low efficient, due to lengthy seedling juvenile phase, alternation bearing, low fruitfulness, and low seed germinability [41–43].

The same as the other tropical fruit trees, the most of olive gene transformation studies were conducted using *Agrobacterium*-mediated transformation. However, there are few reports on

the gene transformation by means of biolistic method in which most of them were down to optimization of scorable and selectable marker genes. Successfully transferred *gus* gene under the control of sunflower ubiquitin promoter in to small somatic embryos of Canino olive cultivar by biolistic method was reported by [44]. Afterward [45] bombarded the embryogenic tissues of Picual cultivar with three different plasmid constructs harboring *gus* gene under control of 35S, 35S with enhancer and sunflower ubiquitin promoters, and found that the ubiquitin promoter could significantly enhance the *gus* gene expression in olive.

More recently, [41] introduced an optimized protocol for transformation of olive cv. Picual embryogenic callus with *gus* gene under the control of sunflower ubiquitin promoter and *npt*II selective gene (**Table 1**) and achieved 72.7% transformation efficiency for embryogenic calli.

7. Mango

The result of [46] study reported an optimized protocol for transient and stable transformation of mango "Carabao" and "Kensington Pride" by biolistic method. They successfully optimized different bombardment parameters (**Table 1**), whereas more than thousand foci were observed per each nucellar proembryonic masses bombarded with a μg plasmid DNA. Afterwards [47], genetically transformed somatic embryos of the three mango varieties Haden, Madame Francis, and Kent with pCAMBIA 3201 construct harboring *gus* and *bar* genes by particle bombardment. After 3 months, only 4% embryos of Kent variety survived, while the other varieties did not survive. They confirmed integration of *gus* and *bar* genes by means of *gus* assay and PCR.

8. Conclusion

Gene transfer to tropical fruit trees via biolistic method can lower GMO risk. Therefore, it is recommended to use this method to gene transformation and particular genome editing via CRISPR technique. So plants can be genetically modified with low risk for humans and the environment.

Acknowledgements

The authors thank the IntechOpen Editorial Board for this publication and also would thank Mr. Muhammad Sarwar Khan for the invitation to write this chapter. There was no financial support for this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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