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Recent Advances in Fruit Species Transformation

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1. Introduction

Rapid increase of human population together with global climate variability resulted in increased demand of plant based food and energy sources (Varshney et al., 2011). Fruits and nuts have essential role to enhance quality of humankind life since a diet based on cereal grains, root and tuber crops, and legumes is generally lacked a wide range of products such as fiber, vitamin, provitamins or other micronutrients and compounds exist in fruit and nut species (Heslop-Harrison, 2005). According to last FAOSTAT statistics, totally about 594.5 million t fruit crops (except melons) were produced in the world in 2009 (http://faostat.fao.org). Because an increase demand exists in global food production, many economically important fruit crops production need to be improved, however, conventional breeding is still limited due to genetic restrictions (high heterozygosity and polyploidy), long juvenile periods, self-incompatibility, resources restricted to parental genome and exposed to sexual combination (Akhond & Machray, 2009; Malnoy et al., 2010; Petri et al., 2011). Thus, there is an urgent need for the biotechnology-assisted crop improvement, which ultimately aimed to obtain novel plant traits (Petri & Burgos, 2005).

Plant genetic engineering has opened new avenues to modify crops, and provided new solutions to solve specific needs (Rao et al., 2009). Contrary to conventional plant breeding, this technology can integrate foreign DNA into different plant cells to produce transgenic plants with new desirable traits (Chilton et al., 1977; Newell, 2000). These biotechnological approaches are a great option to improve fruit genotypes with significant commercial properties such as increased biotic (resistance to disease of virus, fungi, pests and bacteria) (Ghorbel et al., 2001; Fagoaga et al., 2001; Fagoaga et al., 2006; Fagoaga et al., 2007) or abiotic (temperature, salinity, light, drought) stress tolerances (Fu et al., 2011); nutrition; yield and quality (delayed fruit ripening and longer shelf life) and to use as bioreactor to produce proteins, edible vaccines and biodegradable plastics (Khandelwal et al., 2011).

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Currently, public concerns and reduced market acceptance of transgenic crops have promoted the development of alternative marker free system technology as a research priority, to avoid the use of genes without any purpose after the transformation protocol as selectable and reporter marker genes. Typically, it is employed for the selection strategy that confers resistance to antibiotics and to herbicides (Miki & McHugh, 2004; Manimaran et al., 2011). A large proportion of European consumers considered genetically modified crops as highly potential risks for human health and the environment. European laws are restrictive and do not allow the deliberate release of plant modified organism (Directive 2001/18/EEC of the European Parliament and the Council of the European Union). Under these premises, great efforts have also been realized to develop alternative marker free technologies in fruit species. Recently, it was demonstrated in apple and in plum, that transgenic plants without marker genes can be recovered and confirmed its stability by molecular analysis (Malnoy et al., 2010; Petri et al., 2011). In 2011, for first time it was described authentically "cisgenic" plants in apple cv. Gala (Schouten et al., 2006a,b; Vanblaere et al., 2011).

Efficient regeneration systems for the generation of transgenic tissues still appear as an important bottleneck for most of the species and cultivars. In the literature, different protocols were described to transform fruit cells using various DNA delivery techniques, however the attempts generally focused on transformation via *Agrobacterium* or microprojectile bombardment. In this chapter, a detailed application of these techniques in fruit transformation is summarized together with usage of proper marker and selection systems and *in vitro* culture techniques for regeneration of the transgenic plants.

2. Techniques used to transform fruit species

Improvement of the plant characteristics by transfer of selected genes into fruit plant cells is possible mainly through two principal methods: *Agrobacterium*-mediated transformation and microprojectile bombardment (also called "biolistic" or "bioballistic"). Soil-borne Gram negative bacteria of the genus *Agrobacterium* infect a wound surface of the plants via a plasmid called Ti-plasmid containing three genetically important elements; *Agrobacterium* chromosomal virulence genes (*chv*), T-DNA (transfer DNA) and Ti plasmid virulence genes (*vir*) that constitute the T-DNA transfer machinery. Since Ti plasmid encodes mechanisms of integration of T-DNA into the host genome, it is used as a vector to transform plants.

Since direct gene transfer procedures involve intact cells and tissues as targets, in some species breaching of the cell wall is needed in order to enable entrance of DNA to cell (Petolino, 2002). This is accomplished by making some degree of cell injury or totally enzymatic degradation of the cell wall. Advantages of microprojectile bombardment can be summarized as i) transfer of multiple DNA fragments and plasmids with co-bombardment, ii) unnecessity pathogen (such as *Agrobacterium*) infection and usage of specialized vectors for DNA transfer (Veluthambi et al., 2003). Although microprojectile bombardment eliminates species-dependent and complex interaction between bacterium and host genome, stable integration is lower in this technique in comparison to *Agrobacterium*-mediated transformation (Christou, 1992). Moreover, the existence of truncated and rearranged transgene DNA can also lead transgene silencing in the transgenic plants (Pawlowski & Somers, 1996; Klein & Jones, 1999; Paszkowski & Witham, 2001). On the other hand, other important requirement for this technique is that the explants or target cells have to be

physically available for the bombardment (Hensel et al., 2011). Also, it was described that transgenic explants regenerated can be chimeric (Sanford et al., 1990). Nevertheless, application of both of the techniques for the transfer of foreign DNA results in "transient" or "stable" expression of the DNA fragment. In the following sections, recent advances in genetic transformation of fruit species via *Agrobacterium*-mediated and direct gene transfer techniques are presented.

2.1. *Agrobacterium*-mediated gene transfer

2.1.1 A complex relationship

In the decade of the 80´s, the first reports were published related to the introduction of foreign DNA in plant genome thanks to the Ti plasmid of *Agrobacterium tumefaciens* (De Block et al., 1984; Horsch et al., 1985). After more than 25 years, *Agrobacterium*-mediated gene transfer is still the most used method for fruit species transformation including apple, almond, banana, orange, grapevine, melon etc. (Table 1; Rao et al., 2009).

Plant transformation by using *Agrobacterium* has some advantages since the technique is relatively simple; transfer and integration of foreign DNA sequences with defined ends (left and right borders of T-DNA) is precise; stable transformation is high; transgene silencing is typically low and long T-DNA sequences (>150 kb) can be transferred (Veluthambi et al., 2003). However, it is still far from to be a routine transformation application in plants because of its host-range restrictions (Gelvin, 1998).

The initial drawback of *Agrobacterium*-mediated transformation method is the host-range restrictions. However, the bacterium and the target tissue can be manipulated to overcome this obstacle (Trick & Finer, 1997). These authors proposed a new approach to facilitate *Agrobacterium* penetration into plant tissues, the sonication assisted *Agrobacterium*-mediated transformation (SAAT) method. This method consists the use of ultrasounds to produce cavitations on and below the plant surfaces and into the membrane cells, wounding the tissues to enhance *Agrobacterium* infection (Trick & Finer, 1997, 1998).. Also, this method can be combined with vacuum infiltration to promote bacteria agglutination around the tissues to increase the *Agrobacterium* infection as it was demonstrated in kidney bean (Liu et al., 2005) and in woody plants as *Eucalyptus* (Villar et al., 1999; Gallego et al., 2002; Gallego et al., 2009). Today, application of these modifications solely or in combination with other approaches has made it possible to transfer foreign DNA via *Agrobacterium* even to monocots (Hiei et al., 1994; Ishida et al., 1996; Hensel et al., 2011) which were initially transformed with direct gene transfer methods since *Agrobacterium* is not a natural host. Following its first successful usage in soybean and Ohio buckeye (Trick & Finer, 1997), SAAT was also applied recently to fruit species including orange (Oliveira et al., 2009); banana (Subramanyam et al., 2011) and grapevine (Gago et al., 2011). In the last paper the developed efficient methodology that combined SAAT with vacuum infiltration allowed to obtain reporter gene expression in different newly formed organs such as stems, petioles and leaves. Expression was related to vascular tissues due to the *EgCCR* promoter of *Eucalyptus gunnii* and demonstrated that its activity is conserved and fully functional in grapevine as it was shown by *uidA* (GUS) and *gfp* reporter marker genes. Transgenic grapevine lines were verified by Southern blot analysis in five randomly chosen transgenic lines showing simple integration patterns in four lines with different band length indicating

independent transformation events into the grapevine genome. We also applied the optimized protocol to pistachio nodes to reveal out if this method of transformation and vascular-specific promoter of eucalyptus, also works in this species. Histological observations of GFP activity presence in vascular bundles and leaves (Fig. 1) together with PCR amplification of 858 bp fragment of *nptII* and 326 bp of *uidA* (Fig. 2) genes confirmed not only gene integration but also showed that SAAT in combination with vacuum infiltration and vascular specific promoter could also be used for pistachio transformation. With PCR amplification, four out of five GFP+ putative transgenic shoots showed the amplified bands of *nptII* and *uid*A genes (Fig 2).

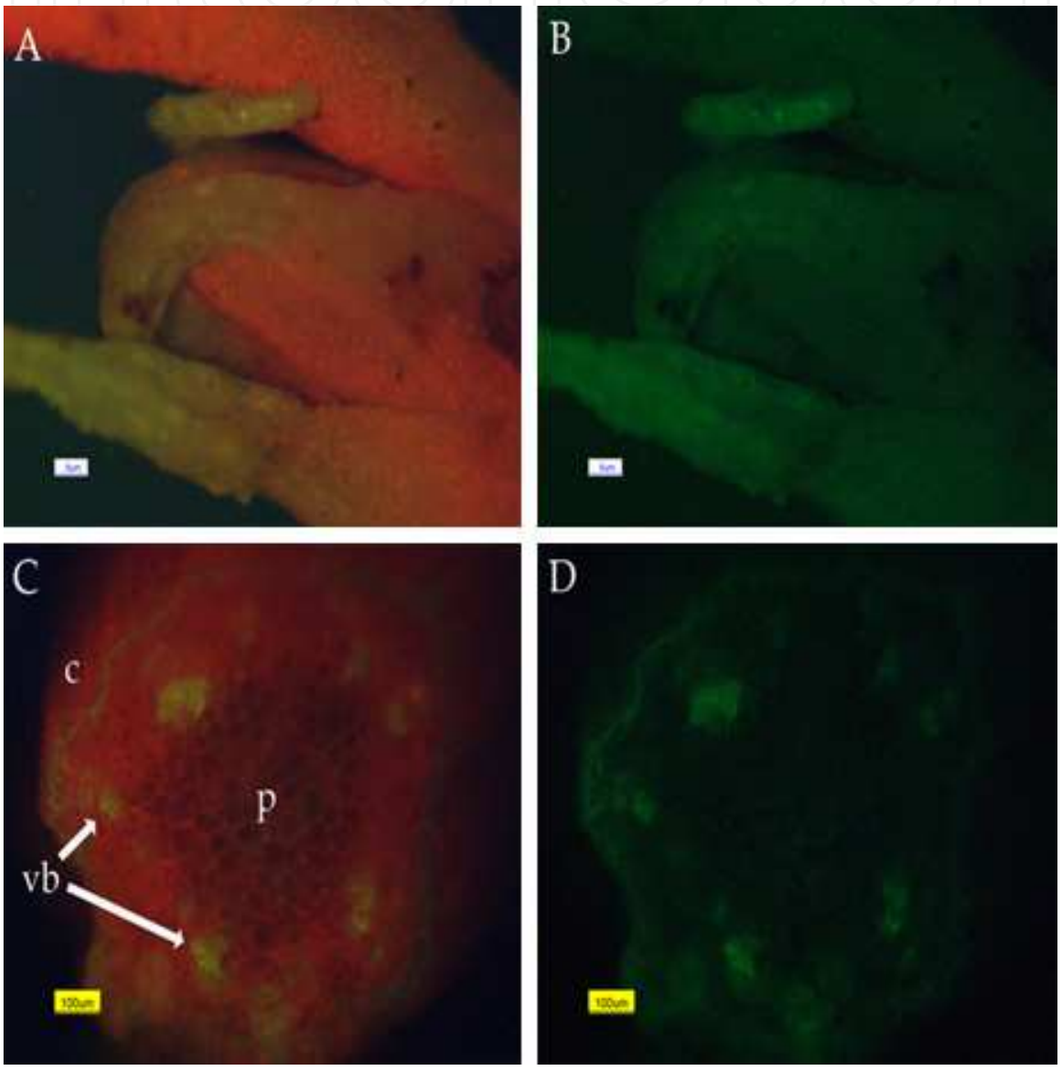

Fig. 1. Expression of GFP in pistachio transformed with *EgCCR-GFP-GUS* construct. Fluorescent images of different tissues and organs were taken 6 months after *Agrobacterium*-mediated transformation. GFP fluorescence in shoot apex (A-B, bars represent 5µm) and transverse section of the transgenic microshoot (C-D, bars represents 100µm) were carried out using a 480/40 nm exciter filters, and two-barrier filter >510 nm (wide range) and 535/550 nm (specific filter for GFP fluorescence). (Abbreviations: vascular bundles (vb), pith, p; cortex, c).

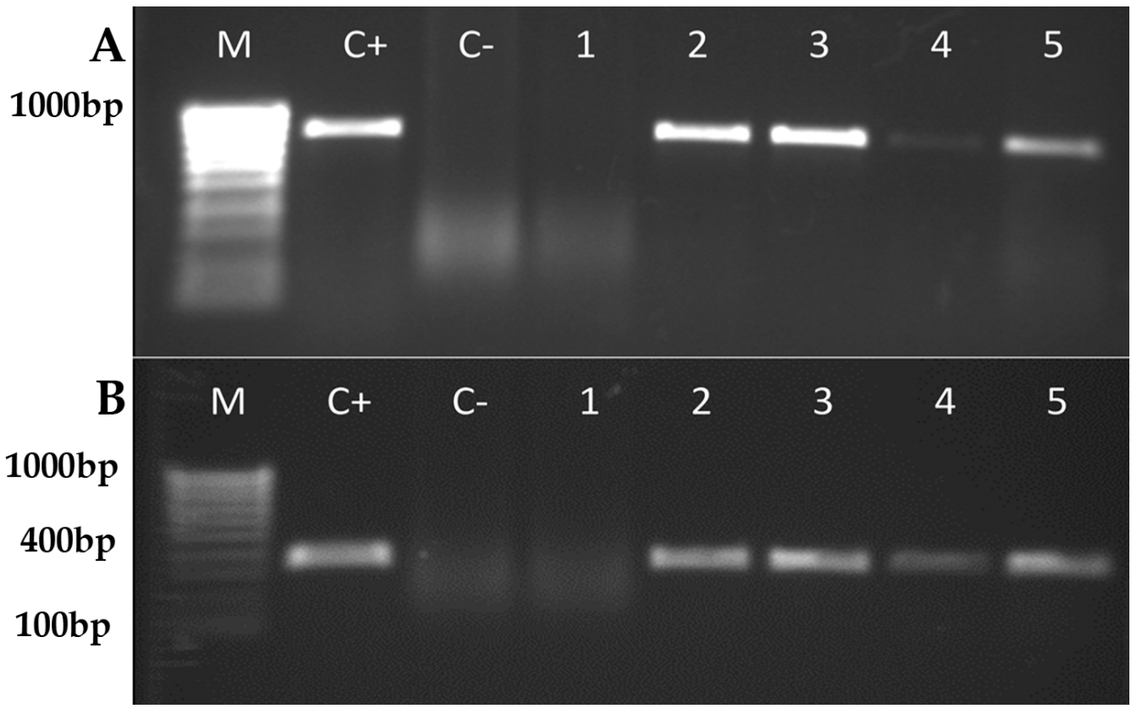

Fig. 2. Analysis of putative transgenic pistachio plants by PCR amplification using primers designed for 858 bp fragment of the *nptII* gene (A) and for a fragment of 326 bp of *uid*A gene (B). (M: DNA 1000 bp ladder, C+: positive control, C-: untransformed plant, T1-T5 putative transgenic shoots lines).

Agrobacterium-mediated transformation is highly genotype dependent for many plants (Pérez-Piñeiro, 2012) but also for fruit species. Different reports described that some cultivars were completely found to be as highly recalcitrant for transformation process whereas others are completely successful (Galun & Breiman, 1998; Petri & Burgos, 2005). This problem is widely described in different fruit species as apricot, grapevine and others (López-Pérez et al., 2008; Wang, 2011). López-Pérez and collaborators (2009) described that grapevine cultivars "Crimson Seedless" and "Sugraone" obtained different transformation efficiencies depending on the optical densities tested. Transformation of hypocotyls obtained from germination of mature seeds and nodal explants of apricot cultivars Dorada, Moniquí, Helena, Canino, Rojo Pasión and Lorna resulted in different transformation efficiencies (Wang, 2011). Some authors pointed out that one of the main goals of plant genetic engineering must be the development of genotype-independent transformation procedures, however due to this highly complex plant-pathogen interaction it will be very difficult to achieve this with the currently available technologies (Petri & Burgos, 2005).

Species	Aim	Plasmid	Transgenes	References
Apple				
Malus x domestica	Method optimisation	pBIN6	*nptII, nos*	James et al., 1989
M. x domestica	Investigation of early events in transformation	pDM96.0501	*sgfp, gusA,nptII*	Maximova et al., 1998

Species	Aim	Plasmid	Transgenes	References
M. x domestica	Investigation of influence of rolA gene on shoot growth	pMRK10	*rolA, nptII*	Holefors et al., 1998
M. x domestica	Scab resistance	p35S-ThEn42, pBIN19ESR	*ech42, nptII*	Bolar et al., 2000
M. x domestica	Resistance to fireblight	pLDB15	*attE, nptII, gusA*	Ko et al., 2000
M. x domestica	Improve rooting ability	pCMB-B	*rolB, nptII, gusA*	Zhu et al., 2001
M. x domestica	Scab resistance	pBIN(Endo+Nag)	*ech42, nag70, nptII*	Faize et al., 2003
M. x domestica	Self-fertility	pGPTV-KAN	*S_3RNase, nptII*	Broothaerts et al., 2004
M. x domestica	Method optimisation Enhance rooting	pCMB-B	*rolB, nptII, gusA*	Radchuk & Korkhovoy, 2005
M. x domestica	Method optimisation	pNOV2819	*pmi, nptII, gusA*	Degenhardt et al., 2006
M. x domestica	Investigation of function of ARRO-1 in adventitious rooting	pK7GWIWG2 (II)	*ARRO-1, nptII*	Smolka et al., 2009
M. x domestica	Stability of scab resistance	pMOG402.hth.gus.intron	*Hth, nptII, gusA*	Krens et al., 2011
M. x domestica	Development of selection system	pCAMBIAVr-ERE-GUS	*VrERE, gusA*	Chevreau et al., 2011
M. x domestica	Transformation without selectable marker gene	pPin2Att.35SGUSint+.n pPin2MpNPR1.GUS−.n ptII		Malnoy et al., 2010
Almond				
Prunus dulcis	Method optimisation	pBI121mgfp-5-ER pNOV2819	*nptII pmi*	Ramesh et al., 2006
Avocado				
Persea americana Mill.	Method optimisation	pMON9749, pTiT37-SE	*nptII, gusA*	Cruz-Hernandez et al., 1998
Banana				
Musa spp.	Method optimisation (Agro + SAAT+ Vacuum infiltration)	pCAMBIA1301	*hptII, gusA*	Subramanyam et al., 2011
Musa spp.	Resistance to Fusarium wilt	pBI121-PFLP	*pflp, nptII*	Yip et al., 2011
Blueberry				
Vaccinium spp.	Method optimisation	p35SGUS-int	*gusA*	Cao et al., 1998

Species	Aim	Plasmid	Transgenes	References
Blueberry				
V. corymbosum L.	Method optimisation	pBISN1	*nptII, gusA*	Song & Sink, 2004
Grapevine				
Vitis vinifera	Method optimisation	Nr	*gusA, nptII*	Nakano et al., 1994; Gago et al., 2011
V. rootstocks	Resistance to viruses and crown gall	pBIN19 pGA482G	*mutant virE2, nptII GLRaV-3cp*	Xue et al., 1999
V. vinifera	Resistance to fungal pathogens	pBI121	*nptII rice chitinase gene*	Yamamoto et al., 2000
V. vinifera	Fungal resistance	pGJ42	*chitinase, rip, nptII*	Bornhoff et al., 2005
V. vinifera	Method optimisation	pGA643	*nptII, GFLVCP*	Maghuly et al., 2006
V. vinifera	Method optimisation	Nr	*egfp, nptII*	Dutt et al., 2007
V. vinifera	Resistance to powdery mildew	pGL2	*ricechitinase gene, hgt*	Nirala et al., 2010
V. vinifera	Method optimisation	pBin19-sgfp	*nptII, sgfp*	Pérez-López et al., 2008
V. vinifera	Method optimisation	pSGN	*nptII, egfp*	Li et al., 2006
V. vinifera	Method optimisation	pCAMBIA2301	*nptII, gusA*	Wang et al., 2005
Grapefruit				
Citrus paradisi	Resistance to Citrus tristeza virus	pGA482GG	*CP, RdRp, gusA, nptII*	Febres et al., 2003
C. paradisi	Resistance to Citrus tristeza virus	pGA482GG	*CP, gusA, nptII*	Febres et al., 2008
Kiwifruit				
Actinidia spp.	Hairy root induction	A722,C58, ICMP8302, ICMP8326, ID1576, LBA 4404, A4T	*gusA, nptII*	Atkinson et al., 1990
Actinidia spp.	Method optimisation	pLAN411, pLAN421	*gusA, nptII*	Uematsu et al., 1991
A.deliciosa	Improved rooting	pBIN19	*nptII, rol A,B,C*	Rugini et al., 1991
A. eriantha	Method optimisation	pART27-10	*gusA, nptII*	Wang et al., 2006
A. deliciosa	Manipulation of plant architecture	pBI121	*İpt*	Honda et al., 2011

Species	Aim	Plasmid	Transgenes	References
Mango				
Magnifera indica L.	Method optimisation	pTiT37-SE::pMON9749	*nptII, gusA*	Mathews et al., 1992
M.indica L.	Methodoptimisation	pGV3850::1103	*nptII*	Mathews et al., 1993
M.indica L.	Mediate ethylene biosynthesis	pBI121	*nptII, gusA antisense ACC oxidase, antisense ACC synthase*	Cruz Hernandez et al., 1997
M. indica L.	Rooting enhancement	Nr	*rol B*	Chavarri et al., 2010
Melon				
Cucumis melo	Resistance to ZYMV, TEV, PVY	FLCP core AS	*nptII, ZYMV coatpr.*	Fang & Grumet, 1993
C. melo	Salt resistance	pRS655	*nptII, gusA, hal1*	Bordas et al., 1997
C. melo	Resistance to ZYMV	pBI-ZCP3'UTR	*nptII, ZYMV coatpr.*	Wu et al., 2009
Nectarberry				
Rubus arcticus	Method optimisation	pFAJ3001	*gusA*	Kokko & Kärenlampi, 1998
Orange				
Citrus sinensis	Method optimisation (Agro + SAAT+ vacuum infiltration)	pGA482GG	*gusA, nptII*	Oliveira et al., 2009
C. sinensis	Research on expression of Mt-GFP	pBI. mgfp4.coxIV	*Mt-gfp*	Xu et al., 2011
C. sinensis	Influence of methylation on gene expression	pBIN.mgfp5-ER	*gfp, nptII*	Fan et al., 2011
C. sinensis	Modification of gibberellin levels	pBinJIT-CcGA20ox1-sense pBinJITCcGA20ox1-antisense	*nptII, CcGA20ox1 nptII, CcGA20ox1*	Fagoaga et al., 2007
C. sinensis	Resistance to fungi	pBI121.*P23*	*nptII, PR-5*	Fagoaga et al., 2001
C. aurantifolia	Resistance to virus	pBin19-sgfp	*nptII, sgfp, p23*	Fagoaga et al., 2006
Poncirus trifoliate	Enhanced salt tolerance	pBin438	*nptII, AhBADH*	Fu et al., 2011
Papaya				
Carica papaya	Resistance to PRSV	pRPTW	*PRSV replicase gene, neo*	Chen et al., 2001

Species	Aim	Plasmid	Transgenes	References
Pear				
Pyrus communis	Alter growth habit	pGA-GUSGF	*rolC, gusA, nptII*	Bell et al., 1999
P. communis	Method optimisation	pPZP pME504	*gusA, nptII*	Yancheva et al., 2006
P. communis	Method optimisation	PBISPG	*nptII, gusA*	Sun et al., 2011
Peanut				
Arachis hypogaea	Production of edible vaccines for *Helicobacter pylori*	pBI121.Oleosin-UreB	*ureB, nptII*	Yang et al., 2011
A. hypogaea	Improvement of salt and drought resistance	pGNFA-(pAHC17)	*AtNHX1*	Asif et al., 2011
A. hypogaea	Production of vaccines for Peste des petits ruminants (PPR)	pBI121	*Hn*	Khandelwal et al., 2011
Plum				
Prunus armeniaca	Method optimisation	pBIN19-sgfp	*nptII, gfp*	Petri et al., 2004
P. armeniaca	Method optimisation	pBIN19-sgfp, p35SGUSint	*nptII, gfp/nptII, gusA*	Petri et al., 2008
P. domestica	Transformation of marker free plants	pCAMBIAgfp94(35S) / pGA482GGi ihpRNAE10´	*nptII, gfp, gusA, ppv-cp*	Petri et al., 2011
P. domestica	New selection system with hygromycin	pC1381, pC1301, pC2301	*gusA, hpt, nptII*	Tian et al., 2009
P. domestica	Control of PPV infection	pGA482GG	*nptII, gusA, PRVcp*	Scorza et al., 1995
P. salicina	Method optimisation	pCAMBIA2202	*nptII, gfp*	Urtubia et al., 2008
Pomegranate				
Punica granatum	Method optimisation	pBIN19-sgfp	*nptII, gfp*	Terakami et al., 2007
Strawberry				
Fragaria spp.	Method optimisation	pBI121	*nptII, gusA*	Barcelo et al., 1998
Fragaria x ananassa Duch.	Modulation of fruit softening	pBI121	antisense of *endo-β-1,4-glucanase*	Lee & Kim, 2011
White mulberry				
Morus alba	Method optimisation	pBI121	*nptII, gusA*	Agarwal & Kanwar, 2007

Table 1. Some important reports on genetic transformation of fruit species via *A. tumefaciens* or *A. rhizogenes*.

Some abbreviations: *AtNHX1*: a vacuolar type Na^+/H^+ antiporter gene; *gfp:* green fluorescent protein coding gene; *hal1:* yeast salt tolerance gene; *hpt*: hygromycin phosphotransferase coding gene; *ipt*: isopentyl transferase gene; *neo*: neomycin phosphorate transferase coding gene; *nos*, nopaline synthase coding gene; *nptII*, neomycin phosphtransferase II coding gene; *pmi*: phosphomannose isomerase coding gene; *ppv*: Plum pox virus; *prsv*: papaya ringspot virus; *pvy*: potato virus Y; *tev*: tobacco etch virus; *gusA (uidA)*: β-glucuronidase coding gene; *UreB*: antigen gene; *zymv*: zucchini yellow mosaic virus.

2.2 Direct gene transfer

Direct gene transfer techniques include microprojectile bombardment, microinjection, electroporation, and usage of whiskers. Among them, microprojectile bombardment is an alternative technique of *Agrobacterium*-mediated transformation since its physical nature overcomes biological barriers and enables naked DNA delivery directly into host genome or alternatively into mitochondria and chloroplasts. In this technique, plasmid or linearized DNA-coated metal microparticles (gold or tungsten) at high velocity is bombarded to intact cells or tissues (Sanford et al. 1987; Klein et al. 1987; Sanford, 1988). Furthermore, biological projectiles such as bacteria (i.e., *E. coli, Agrobacterium*), yeast and phage associated with tungsten can also be used in microprojectile bombardment (Bidney, 1999; Kikkert et al. 1999).

Microprojectile bombardment was developed in the 1980s for transformation of plants which were recalcitrant to *Agrobacterium*–mediated transformation (Paszkowski et al., 1984) such as agronomically important cereals. Following the development of the first particle delivery system (Sanford et al. 1987; Sanford 1988), different effective devices such as PDS-1000/He, Biolistic® particle delivery system; particle inflow gun; electrical discharge particle acceleration; ACCELL™ technology and microtargeting bombardment device were also evolved to improve transformation capacity. Among them, PDS-1000/He, Biolistic® particle delivery system (BIO-RAD), which is a modified version of Sanford's system, is the most used system for biolistic transformation due to its efficient and relatively simple application and acquisition of reproducible results between laboratories (Taylor & Fauquet, 2002). Particle inflow gun can be an alternative to other biolistic systems due to its very low cost and it was used successfully in banana transformation (Becker et al., 2000). Electrical discharge particle acceleration, ACCELL™ technology uses high voltage electrical discharge into a droplet water to generate shock waves and project microprojectiles to different cell layers of target tissues (McCabe & Christou, 1993). Microtargeting bombardment device was designed for shoot meristem transformations (Sautter, 1993) but it is not widely used for plant transformation. All of the microprojectile bombardment systems are not depend on any plant cell type but target cells which will be bombarded need to be physically accessible (Hensel et al., 2011).

Particle bombardment were carried out not only to optimize plant transformation but also to transfer gene constructs encoding for various antimicrobial peptides or proteins for fungal resistance against to *Fusarium oxysporum* f. sp. cubense and *Mycospaerella fijiensis* or preharvest and postharvest diseases *Verticillium theobromae* or *Trachysphaera fructigen* (i.e., Remy et al., 2000; Sagi et al., 1998; Tripathi, 2003), virus (i.e., Fitch et al., 1992; Tennant et al., 1994; Gonsalves et al., 1994; Scorza et al., 1996), pest (i.e., Serres et al., 1992) and herbicide tolerance (i.e., Zeldin et al., 2002). This technique has been applied to transformation of various fruit species including banana, cranberries, citrus, grapevine, melon, papaya and peanut (Table 2).

Species	Aim	Transfer system	Plasmid	Transgenes	References
Apple					
Malus x domestica	Method optimisation	PEG-mediated	pKR10	*Gfp*	Maddumage et al., 2002
Banana					
Musa spp.	Method optimisation	Particle bombardment	pUbi-BtintORF1 pBT6.3-Ubi-NPT pUbi-BTutORF5 pBT6.3-Ubi-NPT pUGR73 pDHkan	*nptII, gusA, BBTV*	Becker et al., 2000
Musa spp.	Tolerance to Sigatoka leaf spot	Particle bombardment	pYC39	*ThEn-42, StSy, Cu, Zn-SOD*	Vishnevetsky et al., 2011
Musa spp.	Resistance to virus	Particle bombardment	pAB6, pAHC17,pH1	*gusA, bar,ubi, BBTV-G-cp*	Ismail et al., 2011
Cranberry					
Vaccinim macrocarpon	Method optimisation & Pest control	Particle bombardment	pTvBTGUS	*nptII, gusA, Bt*	Serres et al., 1992
V. macrocarpon	Herbicide resistance	Particle bombardment	pUC19	*bar, aphII*	Zeldin et al., 2002
Grapevine					
Vitis vinifera	Method optimisation	Biolistic	pBI426	*nptII, gusA*	Hebert et al., 1993
V. vinifera	Method optimisation	Particle bombardment & Agro	pGA482GG	*nptII, gusA TomRSV-CP*	Scorza et al., 1996
V. vinifera	Method optimisation	Biolistic	pSAN237	*nptII, magainin,PGL*	Vidal et al., 2003
V. vinifera	Comparison of minimal cassette with standard circular plasmids	Biolistic	pSAN168, pSAN237	*Magainin, nptII*	Vidal et al., 2006
Kiwifruit					
Actinidia spp.	Method optimisation	PEG 4000	pDW2	*Cat*	Oliveira et al., 1991
Actinidia spp.	Method optimisation	Electroporation	pB1121, pTi35SGUS	*gusA, nptII*	Oliveira et. al., 1994
A. deliciosa	Method optimisation	PEG 4000	p35SGUS	*gusA*	Raquel & Oliveira, 1996

Species	Aim	Transfer system	Plasmid	Transgenes	References
Melon					
Cucumis melo	Protection against	Particle bombardment İnfection & Agro	pGA4822GG/ CP	*nptII, gusA, CMV-WLCP*	Gonsalves et al., 1994
Papaya					
Carica papaya	PRV resistance	Particle bombardment	pGA482GG	*PRV, nptII*	Fitch et al., 1992
C. papaya	PRV resistance	Particle bombardment	pGA482GG	*nptII, gusA, cpPRVHA*	Tennant et al., 1994
C. papaya	Control of PRSV	Particle bombardment	pGA482GG	*cpPRSV-pHA5, nptII, gusA*	Cai et al., 1999
C. papaya	Method optimisation	Particle bombardment	pCAMBIA1303 pML202	*hpt, nptII, mgfp5'*	Zhu et al., 2004
C. papaya	Use of PMI/Man	Particle bombardment	pNOV3610	*Pmi*	Zhu et al., 2005

Table 2. Some important reports on genetic transformation of fruit species via direct gene transfer.

A successful protocol was studied very recently in banana cv. Williams apical meristems with microprojectile bombardment of a new construct pRHA2 plasmid containing *bar* and coat protein of banana bunchy top nanovirus (*BBTV-cp)* genes that encoded the viral coat protein by using Biolistic™ PDS-1000/He system, 650 psi helium pressures and 5 µg DNA/shot for acquisition of virus resistance (Ismail et al., 2011). After bombardment, 62% of apical meristems were survived on the selective medium and 80% of explants produced shoots in the following first subculture and all shoots were rooted (Ismail et al., 2011). In addition to those disease-based studies, others were also carried out in order to develop efficient transformation protocols via biolistic transformation (Sagi et al., 1995; Becker et al., 2000). Among them, Sagi and co-workers (1995) reported the transformation of embryogenic cell suspensions of cooking banana 'Bluggoe' (ABB genome) and plantain 'Three Hand Planty' (AAB genome) via particle bombardment. Then, Cavendish banana cv. Grand Nain embryogenic suspension cells were co-bombarded with the plasmid containing *npt*II selectable marker gene under the control of *BBTV* promoter or the cauliflower mozaic virus (*CaMV*) 35S promoter, the β-glucuronidase (*gus*A) reporter gene and *BBTV* genes under the control of the maize polyubiquitin promoter by using particle inflow gun and stably integration was obtained in all of the tested transformed plants (Becker et al., 2000). Very recently, microprojectile bombardment was also applied to induce tolerance to Sigatoka leaf spot caused by *Mycosphaerella fijiensis* in banana by transferring endochitinase gene of *ThEn-42* from *Trichoderma harzianum* together with the grape stilbene synthase gene (*StSy*) under the control of 35 S promoter and the inducible PR-10 promoter, respectively (Vishenevetsky et al., 2011). Moreover, in order to improve scavenging of free radicals generated during fungal attack, the superoxide dismutase gene (*Cu, Zn-SOD*) of tomato was also included to this gene cassette under the control of ubiquitin promoter. Both PCR and Southern blot analysis confirmed the stable integration of the transgenes and 4-year field trial showed that several transgenic banana lines had improved tolerance not only to Sigatoka but also other fungus such as *Botrytis cinerea.* Gene transfer via microprojectile

bombardment was also carried out in American cranberry (*Vaccinium macrocarpon*) firstly to increase productivity by transferring *Bacillus thuringiensis* subsp. Kurstaki crystal protein gene (*Bt*) for pest resistance (Serres et al., 1992), and latter on, by *bar* gene to confer tolerance to the phosphinothricin-based herbicide glufosinade (Zeldin et al., 2002). Although preliminary bioassays for efficiency of the *Bt* gene against an important lepidopteran demonstrated no consistently effective control in former, stable transmission and expression of herbicide tolerance was observed in both inbred and outcrossed progeny of cranberry trans clone in latter.

In tangelo (*Citrus reticulata* Blanco × *C. paradisi* Macf.) cv. Page embryogenic suspension cells were bombarded with tungsten coated plasmid containing *gusA* and *nptII* genes (Yao et al., 1996). Following to bombardment, 600 transient and 15 stable transformants were obtained and integration of the interest genes confirmed by PCR and Southern blot analyses. A large of kanamycin-resistant embryogenic calli showed also GUS activity. In another study, Kayim and associates (1996) bombarded tungsten-coated plasmid (pBI221.2) containing the *gusA* gene into lemon cv. Kütdiken nucellar cells by biolistic device and expression of the *gusA* gene was histochemically confirmed.

Feronia limonia L. is important fruit tree because of its edible fruits. It is suitable for cultivation in semi-arid tropics and also can be used for reforestation and wasteland reclamation projects (Sing et al., 1992; Purohit et al., 2007). *Feronia limonia* L. hypocotyl segments were also bombarded with tungsten-coated plasmid pBI121 having *gusA* reporter gene driven by *CaMV35S* promoter and *nptII* as a selective marker under control of *nos* promoter using Biolistic™ PDS-1000/He particle delivery system at different rupture disc pressures (1100 and 1350 psi) and target distances (6 and 9 cm) (Purohit et al., 2007). This study revealed that 1100 psi/6 cm and 1350 psi/9 cm were the optimal bombardment condition with supplying a maximum 90% of GUS transient expression.

In grapevine, the initial transformation studies via microprojectile bombardment were performed for method optimization with transferring *nptII* and *gus* genes as selective and reporter marker genes, respectively (Hebert et al., 1993; Kikkert et al., 1996; Scorza et al., 1996). Later, Vidal and co-workers (2003) studied the efficiency of biolistic cotransformation in grapevine for multiple gene transfer of *nptII* and antimicrobial genes (*magainin* and *peptidyl-glycine-leucine*). The stable transformation was confirmed by *gus* gene expression, followed by PCR and Southern blot analyses of *nptII* and antimicrobial genes showed. Three years later, same research group (Vidal et al., 2006) reported the efficient biolistic transformation of grapevine by using minimal gene cassettes, which are linear DNA fragments lacking the vector backbone sequence.

Papaya is economically important and preferred another fruit species because of its nutritional and medicinal properties grown in tropical and subtropical regions (Tripathi et al., 2011). Papaya ringspot virus (PRSV) is major limiting factor in papaya production in Hawaii (Gonsalves, 1998; Fuchs & Gonsalves, 2007). First PRSV resistant papaya plants (cv. SunUp) were obtained by PDS/1000-He particle bombardment device of cv. Sunset with the transformation vector pGA482GG/cpPRV4 containing the *prsv* coat protein (*CP*) gene (Fitch et al., 1992). The PRSV resistant papaya has been commercialized, reached to end user and improved papaya is now under production in Hawaii (Tripathi et al., 2008). This study was followed by other reports mainly on improvement of PRSV tolerance in papaya via

microprojectile bombardment-based transformation (Tennant et al., 1994; Cai et al., 1999; Guzman-Gonzalez et al., 2006). The deployment of transgenic papayas has showed that virus CP protein supplies durable and stable resistance to homologous strains of PRSV (Fermin et al., 2010). Moreover, no ecological influence of transgenic papayas on adjacent non-transgenic papaya trees, microbial flora and beneficial insects was evident (Sakuanrungsirikul et al., 2005). However, political and social factors have negatively affected the technology in Thailand (Davidson, 2008).

Although there are various wild peanut species having disease resistance traits, hybridization between wild and cultivars is difficult due to self-incompatibility, low frequency of hybrid seed production and linkage drag (Stalker & Simpson, 1995) and because of that genetic transformation is a practical tool to improve disease resistant cultivars. Singsit and associates (1997) transformed peanut somatic embryos with gold-coated plasmid constructs containing both *Bacillus thuringiensis cryIA*(c) and *hph* genes driven by *CaMV35S* promoters by PDS 1000 He biolistic device for resistance lepidopteran insect larvae of lesser cornstalk borer. The embryogenic cell lines showed hygromycin resistance and integration of *hph* and *Bt* genes were confirmed by PCR and/or Southern blot analyses in regenerated plants and a progeny. 18% CryIA(c) protein of total soluble protein was detected by ELISA immunoassay in the hygromycin resistant plants. Production of peanut stripe virus (PStV) resistant peanut is another attempt for biotechnologists since the virus negatively affects seed quality and yield in Asia and China (Higgins et al., 1999). Somatic embryos of peanut cv. Gajah and cv. NC-7 were transformed by co-bombardment of *hph* gene and one of two forms of the *PStV* coat protein genes and both of the transgenic plants showed high level resistance to the homologous virus isolate (Higgins et al., 2004). Transfer of anti-apoptotic genes originated from mammals, nematods or virus into plants is another approach for enhancement of plant resistance against to biotic and abiotic stresses (Chu et al., 2008a). With this aim, peanut cv. Georgia Green embryogenic callus was bombarded with anti-apoptotic *Bcl-xL* gene by microprojectile bombardment. Although Bcl-xL protein was detected in four transgenic lines, just one transgenic line (25-4-2a-19) had stable protein expression and showed tolerance to 5μM paraquat (commercial herbicide) (Chu et al., 2008a). Around 0.6% of total population in USA is affected of IgE-mediated allergic reaction following to peanut consumption (Sicherer et al., 2003). To produce hypoallergenic peanut, peanut cv. Georgia Green embryogenic cultures were also transformed via microprojectile bombardment and silenced peanut allergens (Ara h 2 and Ara h 6) by RNA interference. Expression of these allergens was not decreased effectively but, binding of IgE to the two allergens, significantly declined (Chu et al., 2008b).

Apart from microprojectile bombardment, electroporation (Oliviera et al., 1994) and PEG-mediated transformation were also carried out in apple (Maddumage et al., 2002) and kiwifruit (Raquel & Oliveira, 1996) in order to optimize transformation protocol by transferring *gusA*, *gfp* and/or *nptII*.

3. Markers and selection of transformants

3.1 Reporter genes

Reporter genes or non-selectable marker genes are commonly used components of the plasmid constructs allowing the verification of transformation and the detection of the

putative transformed cells. In many fruit transformation studies, histochemical analyses of transformed cells are visualized by using β-glucuronidase (GUS) expression as a reporter gene (Jefferson, 1987; Table 1). This enzyme is encoded by *E. coli uidA* (*gusA*) gene and histochemical localization of the gene expression is detected in subcellular levels (Daniell et al., 1991). High levels of GUS is not toxic for plant and the enzyme is very stable in cells, however, the assay is destructive to plants (Miki & McHugh, 2004). *gusA* generally co-transformed with other selective marker genes to enable the selection of transformants. The gene *gfp* encodes for the protein green fluorescent protein (GFP) (Chalfie et al., 1994). This is one of the mostly used reporter marker gene in fruit transformation protocols for monitoring transformed cells *in vivo* and in real time just by application of UV-light for the excitation of the fluorescent protein. GFP has not any cytotoxic effect on transformed plant cells (Stewart, 2001; Manimaran et al., 2011). *In vivo* detection may permit the manual selection of transformed tissues with focusing in the areas where the signal is more brightly. Fusion of GFP with other proteins of interest provides precise visualizing of intracellular localization and transport in transformed plant (Miki & McHugh, 2004; Manimaran et al., 2011). In some fruit species, it is reported that chlorophyll red autofluorescence can mask GFP expression making the detection really difficult or even impossible in species as apricot, peach and plum (Billinton & Knight, 2001; Padilla et al., 2006; Petri et al., 2008; Petri et al., 2011). However, it was described as an efficient reporter gene in other woody fruit plants, such as citrus (Ghorbel et al., 1999) and peach (Pérez-Clemente et al., 2004). These contrary results confirm the highly variability of the reporter *gfp* gene which is described by Hraška and co-workers (2008). Other reporter gene, luciferase (*luc*) (Gould & Subramani, 1988) also let the monitorization of the transgene putative cells in living tissues, however, it is not so widely employed as the *gfp* (van Leeuwen et al., 2000; Miki & McHugh, 2004).

3.2 Selection systems, a critical step

Selection of transformed regenerants is a critical step in any transformation procedure (Burgos & Petri, 2005). Selection systems can be classified as positive or negative, and conditional or non-conditional. Positive selection systems are those that promote the growth of transformed cells and tissues, by the contrary, negative selection systems are those that promote the death of the transgenic cells. Both systems can be conditioned by an external substrate to perform their activity. Currently, negative selection systems are used in combination with positive selection systems to eliminate transformed cells with incorrect molecular programmed excision of the T-DNA (Schaart et al., 2004; Vamblaere et al., 2011). Typically, in positive conditional selection systems the selectable marker gene encodes for an enzyme conferring resistance to some specific toxic substrate that enable the growth of the transformed cell, tissues and inhibiting or killing non transformed tissues (more information in the comprehensive review of Miki & McHugh, 2004). In the literature approximately 50 selection marker genes are described for genetic plant transformation, however, just only three genes of positive conditional selection system (*nptII* and *hpt*, resistance to the antibiotics kanamycin and hygromycin, respectively, and *bar* gene encoding resistance to herbicide phosphinothricin) are commonly employed in more than 90% of the papers (Miki & McHugh, 2004). These three selectable genes are also the most used ones to transform fruit species as it can be seen in Table 1 and Table 2. *Escherichia coli nptII* gene (also known *neo*) encoded protein (neomycin phosphotransferase, NPTII) inactives aminoglycoside antibiotics such as kanamycin, neomycin, geneticin (G418), and

paramomycin that inhibit protein translation in the transformed cells (Padilla & Burgos, 2010). Hygromycin B is another aminoglycoside antibiotic that inhibits protein synthesis with a broad spectrum activity against prokaryotes and eukaryotes and especially it is very toxic in plants. *Escherichia coli hpt* (*aphIV*, *hph*) gene codes for the hygromycin phosphotransferase to detoxify hygromycin B by phosphorylation via an ATP-dependent phosphorylation of a 7''-hydroxyl group and it is generally used as another selection marker gene when *nptII* was not effective in plant transformation studies (Twyman et al., 2002; Miki & McHugh, 2004).

Similar to antibiotics, herbicides have different specific target sites in plants. The resistance can be achieved by various mechanisms such as usage of natural isozyme or generation of enzyme mutagenesis or detoxification of the herbicides by metabolic processes. Phosphinothricin (PPT; ammonium glufosinate) is an active component of commercial herbicides formulations and analogous to glutamate, the substrate of glutamate synthase. In plants, this enzyme catalyzes the conversion of glutamate to glutamine by removing ammonia assimilation from the cell. Inhibition of the enzyme results in ammonia accumulation and disruption of chloroplast and finally cell death due to photosynthesis inhibition (Lindsey, 1992; OECD, 1999). In plant transformation studies, as herbicide resistance selection marker gene, *pat* from *S. viridochromogenes* (Wohlleben et al., 1988) and *bar* gene from *S. hygroscopicus* (bialophos resistance; Thompson et al., 1987) encoding the enzyme phosphinothricin N-acetyltransferase (PAT) are extensively used for resistance to PPT. PAT converts PPT to a non-herbicidal acetylated form by transferring the acetyl group from acetyl CoA to the free amino group of PPT (Miki & McHugh, 2004).

Currently, an alternative to these highly employed "toxic" approaches conditional positive selection markers based on the promotion of a metabolic advantage to transformed cells are used. Some authors mentioned that this kind of selection can improve considerably the selection of the transformants, since others such as antibiotics generally cause considerable necrosis (produced by the death of non-transformed cells) that often inhibits regeneration from adjacent tissues (Petri & Burgos, 2005). Previously, results obtained with this approach demonstrated higher yields than when the toxic selective agents were employed, and seems to be broadly applicable to crop plants (Miki & McHugh, 2004). Some of the most widely used are the *AtTPS1*/glucose (Leyman et al., 2006); *galT*/galactose (Joersbo et al., 2003); xylose isomerase (Haldrup et al., 1998); D-aminoacid/*dao1* (Alonso et al., 1998) and the *pmi*/mannose (Joersbo et al., 1998). Probably, one of the most used one in fruit species is the gene *pmi* that encodes the enzyme phosphomannose isomerase (EC 5.3.1.8) that catalyzes the reversible interconversion of mannose 6-phosphate and fructose 6-phosphate. This enzyme is present in bacteria as *E. coli* and also, in humans, however it is not present in plants, as exception of soybean and other legumes. Using a media with mannose as the unique carbon source, only transformed cells can grow and develop. Glycolysis is inhibited due to the accumulation of mannose-6-phosphate converted from mannose by hexokinase with preventing cell growth and development in non-transformed cells (Miki & McHugh, 2004). Sensitivity to the toxic effect of mannose-6-phosphate is different between species, and can be avoided by combining with other sugars such as sucrose, maltose and fructose (Joersbo et al., 1999). Diverse fruit trees were selected with this system, alone or in combination with sucrose, i.e., 12 g/L mannose and 5 g/L sucrose in orange (Ballester et al., 2008); 30 g/L mannose without any sugar more in papaya (Zhu et al., 2005); 2,5 g/L

mannose and 5 g/L sucrose in almond (Ramesh et al., 2006) or 1-10 g/L mannose and 5-30 g/L in apple (Degenhardt et al., 2006). In *Citrus sinensis*, the best results were obtained when 13 g/L mannose as unique source of carbon was added into the selection media. Mannose combined with other sugars promoted reduction in transformation efficiencies and escapes (Boscariol et al., 2003). Apricot cv. Helena and Canino required the lower combination of mannose with sucrose (1,25 g/L mannose and 20 g/L sucrose) in comparison with other woody fruit trees to obtain the most effective selection procedure. Moreover, safety assessments were revealed that there is no any adverse effect of the enzyme on mammalian allergenicity and toxicity (Reed et al., 2001).

Other selective strategies were developed as positive non-conditional systems, or in other words, using selectable marker genes that "promote" plant regeneration. Currently, there is more information about the genetic and biochemical control of organogenesis than embryogenesis for plant regeneration. Because of this, commonly genes related with cytokinins synthesis are employed for shoot organogenesis. More efforts are required to discover molecular mechanisms of embryogenesis to use these strategies in species highly dependent on embryogenesis regeneration to develop transgenic plants. Genes as *cki1* or the most employed isopentenyl transferase *ipt* gene encoding the enzyme IPT, catalyze the synthesis of isopentyl-adenosine-5-monophosphate, which is the first step in cytokinin biosynthesis (Miki & McHugh, 2004). This gene modify the endogenous balance between cytokinins and auxins, stimulating cell division and differentiation of the cells that promote an altered morphology, development and physiology of transgenic plants (Sundar & Sakthivel, 2008). Some authors observed that the *ipt* gene improved transformation efficiency in apricot leaf explants in comparison with the selection through *nptII* (López-Noguera et al., 2009).

3.3 A differential transgene expression: Constitutive versus specific promoters

Currently, an important debate is carrying out about the risks of the "unpredictable" behavior and recombinogenic potential of constitutive promoters (Gittins et al., 2003) and to avoid the public concerns about the risks of ubiquitous transgene expression in crops.

Commonly, most of the fruit species have been transformed with plasmidic constructions harbouring constitutive or ubiquitous promoters, as the Cauliflower Mosaic virus 35S (*CaMV35S*). In this sense, different authors described that constitutive expression may be harmful for the host plant, causing sterility, retarded development, abnormal morphology, yield penalty, altered grain composition or transgene silencing (Cai et al., 2007 and references therein) and its expression level is dependent on the cell type, the developmental stage and on the perception of environmental triggers (Hensel et al., 2011). Moreover, under constitutive promoters reporter and selectable marker, and genes of interest are expressed continuously in all tissues without any temporal control. In this sense, specific-promoters appear as an alternative approach to avoid the undesirable side effects of constitutive promoters and to target transgene expression in a spatial or temporal specific way (Gago et al., 2011; Hensel et al., 2011).

Recently, vascular specific promoter *EgCCR* from *Eucaliptus gunnii* was checked in pistachio in this study as mentioned above as well as other fruit species such as kiwifruit and grapevine (Paradela et al., 2006; Gago et al., 2011) and results demonstrated that this promoter is conserved and fully functional in these species. Vascular promoters can drive

resistance to biotic or abiotic stresses related with vascular tissues. Specific promoters could be useful to synchronize transgene activity spatially and/or temporally to control with more accuracy the pathogenic process (Gago et al., 2011).

3.4 Alternative transformation systems: Transgenics without marker genes

A highly desirable approach to promote public acceptation for future commercialization of transgenic plants and products is focused on the elimination of marker genes from transformed plants or the direct production of marker-free transgenics (Kraus, 2010). These newly and promising approaches are highly dependent on previously established highly efficient regeneration protocols that may be based on organogenesis or embryogenesis (Petri et al., 2011). There are various technologies such as homologous recombination, co-transformation, site-specific recombination (Cre/loxP site specific recombination system, R/RS system, FLP/FRT system etc) or marker elimination by transposons to remove selective marker genes (Hao et al., 2011; Manimaran et al., 2011). However, there are still few marker-free fruit species transformation protocols.

Strawberry leaf explants were transformed with site-specific recombinase for the precise elimination of undesired DNA sequences and a bifunctional selectable marker gene used for the initial positive selection of transgenic tissue and subsequent negative selection for fully marker-free plants (Schaart et al., 2004).

MAT (multi-auto-transformation) (Ebinuma et al., 1997) combined with the *Agrobacterium* oncogene *ipt* gene, for positive selection with the recombinase system R/RS for removal of marker genes acting as "molecular scissors" after transformation were used as alternative approach in citrus plants (Ballester et al., 2007; 2008). Also, in apricot (López-Noguera et al., 2009) a similar strategy was used. Regeneration of apricot transgenic shoots was significantly improved to non-transformed plants (regenerated in non-selective media). Moreover, it was significantly higher in comparison with previous published data using resistance to kanamycin mediated by *nptII* gene. The lack of *ipt* differential phenotype promoted difficulties to assess the excision of the marker genes, that require periodic assays. Complete excision of marker genes ranged from 5 to 12 months, however, only 41% of the regenerated transgenic shoots R-mediated recombination occurs correctly. In *Citrus sp.*, it was also reported that anomalous excision of marker genes promoting failures in the expression of the reporter genes (Ballester et al., 2007, 2008).

Apple (Malnoy et al., 2010) and pineapple sweet orange (Ballester et al., 2010) transformation using ''clean'' binary vector including only the transgene of interest were carried out to create marker-free transformants. Very recently, melon (*C. melo* L. cv Hetao) was transformed with a marker-free and vector-free antisense 1-aminocyclopropane-1-carboxylic acid oxidase construct via the pollen-tube pathway and transgenic lines are choosen by PCR without using any selectable marker agent (Hao et al., 2011).

In plum (*Prunus domestica*), transformation was carried out without reporter or selectable marker genes using a high-throughput transformation system (Petri et al., 2011). Previously, authors checked the efficiency of the regeneration of transformed shoots using conventional constructs harbouring reporter marker such as *gusA* and *gfp,* and *nptII* gene. Transformation efficiency varied from 5.7-17.7%. Using a marker free construct, the intron-hairpin-RNA (ihpRNA) harbouring the Plum Pox Virus coat protein (*ppv-cp*) gene, these authors

regenerated five transgenic lines confirmed by Southern blot. It is important to take into account that this kind of free marker strategy is widely dependent on highly yields in regeneration systems.

3.5 Cisgenesis, the P-DNA technology and multigene transformation

Other relevant advance in fruit species transformation was the proposal made by Schouten and coworkers (2006), the "cisgenesis". This term means the use of recombinant DNA technology to introduce genes from crossable donors plants, isolated from within the existing genome or sexually compatible relative species for centuries therefore, unlikely to alter the gene pool of the recipient species. Cisgenesis includes all the genetic events of the T-DNA as introns, flanking regions, promoters, and terminators (Vanblaere et al., 2011).

This methodology proposes to transfer the own plant DNAs, the P-DNAs. The use of this technology requires the construction of whole plant derived vector from the target species. Within the target species genome, it must be a DNA fragment with two T-DNA border-like sequences oriented as direct repeats ideally about 1-2 kb apart with suitable restriction sites for cloning of a desirable gene.

In the last years, different works were considered to step towards introducing regulatory elements and genes of interest from crossable donor plants, however with some foreign elements as marker genes in species as melon and apples (Benjamin et al., 2009; Joshi, 2010; Szankowski et al., 2009). Up to 2011 there is no any report of real "cisgenesis" plantlets, in agreement with Schouten et al. (2006) definition of the topic. In 2011, Vanblaere and coworkers developed apple cv. Gala cisgenic plants by expressing the apple scab resistance gene *HcrVf2* encoding resistance to apple scab. Marker-free system was employed for the development of three cisgenic lines containing one insert of the P-DNA after removing by recombination with using chemical induction. These lines were not observed different from non-transformed cv. Gala plants.

Cisgenic plants are essentially the same as the traditionally bred varieties, and they might be easier to commercialise than the "problematic" transgenic plants (Schouten et al., 2006; Rommens et al., 2007). Critical opinions to these proposals also were clearly exposed, the uncontrolled P-DNA integration into the plant target genome can cause mutations or affect to the expression of other native genes, altering the behaviour of that cisgenic plants in an unpredictable manner (Schubert & Willims, 2006; Akhond & Machray, 2009). Recently, interesting approaches are being proposed for genome editing using ZFNs (Zinc finger nucleases) that can promote induction of double-strand breaks at specific genomic sites and promote the replacement of native DNA with foreign T-DNA (Weinthal et al., 2010).

The multigen transfer (MGT) methodology consist in introducing more than one gene at once. Commonly, most of the transgenic plants are generated by introducing just one single gene of interest, but now MGT are being developed to obtain more ambitious phenotypes as the complete import of metabolic pathways, whole protein complex and the development of transgenic fruit species with various new traits simultaneously transferred (Naqvi et al., 2009). In this sense, this technology would be highly desirable for commercial fruit species cultivars to obtain new traits related with large fruit size, high coloration of the fruit epidermis, flesh firmness and virus resistance (Petri et al., 2011) at the same time without the need of several rounds of introgressive backcrossing.

4. *In vitro* culture techniques for the recovery of transgenic plants

Plants are complex, diverse organisms and have adapted evolutionarily to almost every ecological niche on the planet. Development of successful transformation protocol depends on a reliable and highly efficient regeneration system. Explant types are highly variable since it depends on the selected organogenetic process optimized for each species. Commonly, the genetic transformation protocols of fruit species employed explants such as ovules, anthers, seedlings, zygotic and somatic embryos, cotyledons, epicotyles, hypocotyles, leaf pieces, roots, meristems (Fagoaga et al., 2007; Lopez- Perez et al., 2008; Petri et al., 2008; Husaini, 2010; Malnoy et al., 2010; Bosselut et al., 2011; Petri et al., 2011; Gago et al., 2011). Typically, it is recommended that those tissues have high and active cell division to enhance the regeneration of the transgenic lines (Mante et al., 1991; Schuerman & Dandekar, 1993; Wang, 2011). Ideally, fruit species transformation must be done with somatic tissues such as leaves and roots to transform varieties already well known and accepted in the market by the consumers. Recently, some authors also proposed the possibility of the use of transgenic seedlings to develop new fruit varieties through subsequent cross-breeding. These transgenic seedlings can add new traits impossible to obtain in the species genome-pool (Petri et al., 2011).

Organogenesis was the strategy selected in different species to develop most of the known and efficient regeneration protocols for fruit species, concretely for fruit trees (Petri et al., 2011). Almond (Costa et al., 2006); apple (Smolka et al. 2009; Lau & Korban, 2010; Vanblaere et al., 2011); banana (Subramanyam et al., 2011); fig (Yancheva et al., 2005); kiwifruit (Tian et al., 2011); peach (Padilla et al., 2006); strawberry (Mercado et al., 2010); peanut (Asif et al., 2011); watermelon (Huang et al., 2011) and pear (Sun et al., 2011) are some examples of transformed cultivars for some fruit species that the transformed tissues were regenerated via organogenesis. Since organogenesis protocols are developed for many different fruit species, it is easier to adapt the regeneration system into genetic transformation methods (Frary & Eck, 2005). However, some risks also are assumed in using this regeneration system. Generally, it is considered that the origin of the new adventitious shoots is based on the involvement of few cells (George et al., 2008), enhancing the risks of chimera development.

Somatic embryogenesis that leads the formation of an embryo from somatic cells is another procedure to regenerate fruit transformants such as banana (Vishnevetsky et al., 2011); papaya (Zhu et al., 2001); grapevine (Nirala et al., 2010) and mango (Chavarri et al., 2010). Regeneration from transformed embryos can be achieved via direct germination or shoot organogenesis and the method is useful for large-scale and rapid propagation of transformants. In grapevine most of the approaches are being performed by using embryogenic cultures from different tissues such as zygotic embryos, leaves, ovaries and anther filaments to provide cells amenable to gene insertion and regeneration (Mezzetti et al., 2002; Dutt et al., 2007; López-Noguera et al., 2009). However, these techniques are highly genotype dependent and for many cultivars they have been difficult to obtain successful results (Dutt et al., 2007). Moreover, it is considered that anther filaments, as commonly employed in grapevine for embryogenic calli, are laborious, cultivar-dependent, depend on availability of immature flowers and may affect strongly the phenotype of the regenerated plantlets (Mezzetti et al., 2002). However, it is really interesting to take into account that regeneration from somatic embryos and secondary somatic embryos are currently assumed that they are derivatives of single cell origin.

In the decade of the 90´s some unsuccessful efforts were reported to transform meristems from micropropagated shoot tips due to high explant mortality and uncontrolled *Agrobacterium* overgrowth after coculture stages (Ye et al., 1994; Druart et al., 1998; Scorza et al., 1995). Mezzetti and co-workers (2002) described in grapevine the development of meristematic bulk tissues, a highly aggregate of meristematic cells produced after three months in increased concentrations of BA (N^6-benzyladenine) and the removal of the apical meristem. After 90 days, under the previous conditions, these highly regenerative tissues produced easily adventitious shoots and can be transformed by *Agrobacterium*, being able to regenerate several transgenic lines. Other interesting approach was the genetic transformation of shoot apical meristems, previously subjected to a dark growth stage after wounding for transformation. Authors reported that 1% of shoot tips produced stable transgenic lines after weeks (Dutt et al., 2007). Ismail and co-workers (2011) transformed successfully banana apical meristems via microprojectile bombardment and regenerated 80.3% percent of the transformed meristematic tissues.

4.1 The chimeric question: Are my transgenic plants genetically uniform?

This is one of the most exciting questions that plant biotechnology researchers ask to themselves after all the long extensive, intensive and difficult labour needed to transform most of the fruit species. Some of the transformed regenerants can be chimeras, a mix of transformed and non transformed cells in the tissues, in other words, non genetically uniform organisms (Hanke et al., 2007). Recently, Petri and collaborators (2011) described that most of the known and efficient regeneration methods for fruit trees are based on organogenesis, where new adventitious shoot formation is originated from a determined number of cells. So, it comes hard to detect non chimeric and stable transgenic lines without using a selectable marker gene. Very recently, different authors using marker free technology as alternative systems or with genetically programmed marker excision reported the appearance of chimeric transformants in apple, strawberry, lime, citrus or plum (Domínguez et al., 2004; Schaart et al., 2004; Ballester et al., 2007; Malnoy et al., 2011; Petri et al., 2011).

Strawberry is highly sensitive to kanamycin selection, and it was described that selection of transgenic regenerants in these sensitive tissues can be associated with chimeric shoots containing transgenic and non-transgenic sections (Husaini, 2010). It was observed that increasing antibiotic concentration gradually avoid chimerisms in strawberry (Mathews et al., 1998; Husaini et al., 2010). Even under this strictly methodology some authors pointed out the inactivation events on the selection agent must be performed through the transformed cells, so, non transformed cells can develop and grown (Petri & Burgos, 2005; Wang, 2011). A useful methodology was also proposed for the quick and low-cost identification of chimeras by Faize and collaborators (2010) in tobacco and in apricot based in quantitative real-time PCR even in early developmental stages, and also let to monitor their dissociation.

5. Future perspectives and concluding remarks

Currently, most of the fruit genetic transformation protocols integrated the new genes randomly and in unpredictable copy numbers influencing negatively its expression. Also public concerns and reduced market acceptance of transgenic crops have promoted the

development of alternative marker free technologies in fruit species. For those reasons development of protocols to obtain transgenic fruits without marker genes and the use of the own plant DNA resources, such as "cisgenic" fruit plants, are the big challenges. ZFNs have also been succesfuly used to drive the replacement of native DNA sequences with foreign DNA molecules and to mediate the integration of the targeted transgene into native genome sequences.

Most of the fruit transgenic plants are generated by introducing just one single new character (gene of interest), however, some authors proposed that multigene transfer technology (MGT) needs to be developed to obtain new traits related at the same time. The combination of multiple traits can be a highly interesting approach as it could be applied to achieve resistance to several biotic or abiotic stresses and traits related to fruit quality such as large fruit size, high coloration of the fruit epidermis, increase flesh firmness to improve ripening without the need of several rounds of introgressive backcrossing.

The future of fruit genetic transformation is required of genotype-independent protocols, accuracy molecular tools to drive the T-DNA insertion and its expression, and efficiency and highly-yield selection and regeneration in vitro culture methodology. But *Agrobacterium*-mediated transformation procedure is a high non linear complex biological process, and its complexity can be understood with the composition of many different and interacting elements governed by non-deterministic rules and influenced by external factors. In this sense, the emergent technology dedicated to meta-analysis can be really useful to increase our understanding of fruit genetic transformation, making possible to identify relationships among several factors and extracting useful information generating understable and reusable knowledge (Gago et al., 2011; Gallego et al., 2011; Perez-Pineiro et al., 2012) Under these perspectives, modeling any fruit transformation procedure (*Agrobacterium*-mediated, biolistics, electroporation etc.) including the genetic engineering, *in vitro* plant tissue culture and regeneration stages will be improved for the next years.

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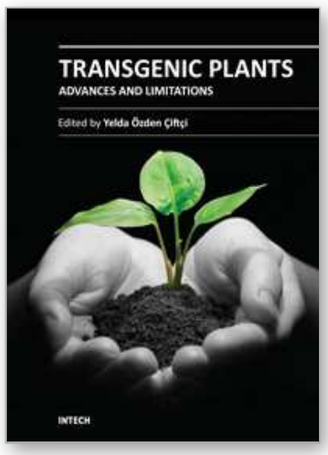

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