Review Article

Citrus biotechnology: Achievements, limitations and future directions

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ABSTRACT

Citrus is one of the most important commercial and nutritional fruit crops in the world, hence it needs to be improved to cater to the diverse needs of consumers and crop breeders. Genetic manipulation through conventional techniques in this genus is invariably a difficult task for plant breeders as it poses various biological limitations comprising long juvenile period, high heterozygosity, sexual incompatibility, nucellar polyembryony and large plant size that greatly hinder cultivar improvement. Hence, several attempts were made to improve *Citrus* sps. by using various *in vitro* techniques. *Citrus* sps are widely known for their recalcitrance to transformation and subsequent rooting, but constant research has led to the establishment of improved protocols to ensure the production of uniformly transformed plants, albeit with relatively low efficiency, depending upon the genotype. Genetic modification through *Agrobacterium*-mediated transformation has emerged as an important tool for introducing agronomically important genes into *Citrus* sps. Somatic hybridization has been applied to overcome self and cross-incompatibility barriers and generated inter-specific and inter-generic hybrids. Encouraging results have been achieved through transgenics for resistance against viruses and bacteria, thereby augmenting the yield and quality of the fruit. Now, when major transformation and regeneration protocols have sufficiently been standardized for important cultivars, ongoing citrus research focuses mainly on incorporating such genes in citrus genotypes that can combat different biotic and abiotic stresses. This review summarizes the advances made so far in Citrus biotechnology, and suggests some future directions of research in this fruit crop. **[Physiol. Mol. Biol. Plants 2009; 15(1) : 3-22]** *E-mail : rajam.mv@gmail.com*

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INTRODUCTION

The genus Citrus, which includes few of the most important fruits worldwide, belongs to the family Rutaceae, which comprises 140 genera and 1300 species throughout the world. Citrus is the third most important fruit crop in the world after apple and banana and accounts for the production of about 100 million tons with an area of cultivation spread over a massive 7.2 million hectares (FAO, 2001). It is a long-lived perennial crop and is grown in more than 100 countries across the world (Saunt, 1990). Favourable hotspots for citrus cultivation are tropical and sub-tropical areas, falling approximately within 40^0 latitude in each side of the equator, where temperatures are predominantly warm.

Citrus cultivation is believed to have been originally started in China and South-east Asia where it has been cultivated for more than 4000 years. Brazil and the US are the leading producers of citrus in the world and produce 42 % of the world's requirement. US ranks behind Brazil in Citrus production (FAO, 2001). Other significant citrus producing countries include Spain, Italy, Egypt, Mexico and China. India ranks sixth amongst the various citrus producing countries in the world.

In citrus species, generally the plant body is in the form of large shrub or small tree reaching up to a height of 4 to 15 m. Stems are embellished with thorns and the fruit borne by citrus trees is a typical hesperidium, which is a specialized berry. Citrus fruits come in varied shapes-globose, round, oblique, ellipsoid, spheroid, pyriform, ovoid etc (Sinclair *et al*., 1984). The genus citrus is closely related with other important genera of the family Rutaceae - *Fortunella, Poncirus, Microcitrus* and *Eremocitrus*. Major economically important species of citrus are- *Citrus sinensis* (L.) Osbeck (sweet orange), *C. reticulata* Blanko (mandarin), *C. paradisi* Mac. f. (grapefruit), *C. limon* (L.) Burm. f. (lemon), *C. aurantifolia* (Christm) Swing. (lime), *C. aurantium* (L.) (sour orange) and *C. grandis* (L.) Osbeck (pummelo) and major citrus hybrids include- *Citrange* (trifoliate

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orange X sweet orange), *Citrumelo* (trifoliate orange X grapefruit), *Tangor* (sweet orange X tangerine) and *Tangelo* (tangerine X grapefruit). Sweet orange alone accounts for 75 % of the total citrus fruit production worldwide followed by mandarin, grapefruit and lemon.

Citrus is an evergreen crop and is very sensitive to temperature fluctuations and requires warm temperature for proper growth and maturation of fruit, thereby leading to quick harvest. A temperature of 20 ^{0}C at night and 35 ^{0}C during the day is required for optimum growth. Abundant sunshine of 6 to 7 h is an absolute must for their best performance. Citrus trees require a rich and fast draining sandy loam soil with a pH range of 6-7 and their growth is adversely affected by alkalinity and salinity in the soil. Citrus trees do not need high humidity, especially during blooming season and excess water is bad for their growth, as it encourages fungal and root pathogens. Cold hardiness is a major concern while raising citrus trees, as they are not generally frost hardy and are prone to be damaged by cold weather. Limes and lemons are especially sensitive to chilly weather, while hybrids of citrus like tangerines and tangors are appreciably resistant to cold and trifoliate orange is extremely cold hardy. Severe frost damages the tree and fruit, therefore the best time to plant citrus trees is when the risk of severe frost is over. Increase in cold hardiness falls in the following order from least to most cold hardy – citron < limes < lemons < grapefruit < sweet orange < tangerine and its hybrids < sour orange < kumquats < trifoliate orange and its hybrids.

Nitrogen is the key element in nutritional requirement to ensure healthy growth of trees and fruits and should be present in major quantity in soil or manure. Nitrogen deficient trees are characterized by pale and small leaves, stunted foliage with reduced flowering and fruit set. Nitrogen is followed by magnesium and calcium with traces of zinc, magnesium and copper.

Citrus fruits are known for their distinctly pleasant aroma, arising due to the terpenes present in the rind. The genus derives its commercial importance from its fruit, which is of great economic and health value can be consumed fresh or pressed to obtain juice (Talon and Gmitter Jr., 2008) Majority of citrus fruits are preferably eaten fresh - oranges, mandarins, grapefruits, clementines and tangerines. Orange and grapefruit produce very palatable juice and hence make for nutritious and popular breakfast (Duyn *et al*., 2000). Bulk of the total produce of oranges and mandarins goes into juice making. Lemons and limes can be made into lemonades and pickles, also their juices can be added to various food preparations to enhance flavor. Delicious marmalades are made out of oranges. Citrus peels too have no less importance and can be candied, used as livestock feed, in perfumeries, bakeries and in soap industry. Essential oils obtained from citrus leaves have recently been found to harbor insecticidal property. Lemon oil obtained by cold pressing of lemon peels is extensively used in furniture polish. Bergamot, a variety of sour orange is used in making perfumes and massage oils. The rind of citrus fruits is slightly bitter in taste and can be added to baked products to impart a distinct flavour.

Citrus has been utilized in more medicinal preparations than majority of other plants and finds its use in the remedy of scores of ailments ranging from toothache, diarrhea, constipation, insomnia to vomiting. Hesperidin, a biflavonoid is very effective in reducing blood pressure.

Pests and diseases of citrus

Citrus plants and fruits are very susceptible to infestation by different kinds of insects, fungi, bacteria and viruses. The foliage serves as food for some *Lepidopteran* larvae, including Emerald, Double-striped Pug, Giant Leopard Moth, citrus leaf miner, Queensland fruitfly, mites etc. Fungal diseases of citrus plants include citrus scab of lemons (*Elsinoe fawcettii*), black spot affecting orange (*Guignardia citricarpa*), brown spot (*Alternaria alternata*), black core rot, a fungal disease of mandarin (*Alternaria tenuis*), collar rot (*Phytophthora citrophthora)* etc, and common viruses of citrus are citrus yellow mosaic virus (CYMV) and satsuma dwarf virus causing dwarfism in plants. Huanglongbing (HLB), also known as citrus greening disease is a severe and pervasive bacterial disease, spread across Southeast Asia and major parts of Africa (Berg van den and van den Berg, 1999). It is transmitted by means of infected plant material and efforts have been made to control it in the afflicted areas by the use of healthy nucellar citrus seedlings (Obukosia *et al.*, 2000).

One of the most devastating diseases of citrus is the 'Tristeza' disease, caused by the citrus tristeza virus (CTV), an aphid-transmitted, single-stranded closterovirus that causes phenomenal economic damage to citrus industry. CTV is believed to have originated in China long time back and is widely spread in tropical citrus growing areas. General symptoms of the disease include either decline and death of citrus scions or stem pitting, stunting, reduced fruit yield and quality of affected plants (Bar-Joseph *et al.,* 1989; Rocha-Pena *et*

al., 1995). CTV strains that cause "quick decline", result in the death of the plant, as it is a fatal disease, whereas the strains causing stem pitting and stunting are aggressive and damaging, but the damages can be avoided by using CTV resistant rootstocks. To reduce the severity of symptoms caused by CTV, cross protection with mild CTV isolates is practiced in areas where virulent isolates are common (Costa and Muller, 1980).

CTV causes different symptoms on different species depending upon virus strain and scion-rootstock combinations. The economically important cultivars - sweet orange, mandarin, tangor, tangelo and grapefruit are particularly vulnerable to CTV when propagated on susceptible rootstocks like sour orange, pummelo and lemon. On the other hand, trifoliate orange, citranges and mandarin rootstocks are considerably resistant to the virus. Presence of infected nursery plant material and mother trees poses a grave risk for citrus propagation (Bar-Joseph *et al.,* 1989). To prevent the disease, use of certified bud stock and resistant rootstock go a long way in preventing the disease and pre-inoculation of bud-stock trees with a mild CTV prior to propagation is also important (Costa and Muller, 1980).

Conventional breeding in citrus

In nature, citrus seedlings produce trees and fruits identical to the parent tree because of nucellar polyembryony, but in general practice, conventional breeders make use of vegetative propagation by means of clonal selection. Breeding usually emphasizes on selection of genotypes obtained by spontaneous or induced mutagenesis, which is the oldest breeding method for cultivar improvement. Traditional approach of tree breeding involves the selection of trees with desirable phenotype followed by their integration into breeding programs (Kaneyoshi *et al.,* 1994).

For propagation of healthy citrus plants it is imperative that at young age scions of choice are grafted on vigorous rootstocks that are selected for disease resistance and cold hardiness to avoid very long juvenile periods and allow production of better quality fruit (Pena *et al.,* 1995a, b; Seguin and Pena, 2001). Carrizo citrange and Rangpur Lime are very popular and most widely used rootstocks nowadays due to their high vigor and vitality and tremendous efforts have been made to further improve their genotype by biotechnological methods (Moore *et al.,* 1992; Pena *et al.,* 1995a; Cervera *et al.,* 1998b; Navarro *et al*., 2004). Rangpur Lime is salt and high pH tolerant and also to CTV, therefore it is used in arid and CTV infested areas, while sour orange is tolerant to *Phytophthora* and is used in areas where *Phytophthora* root rot is common. In Spain and California citrus industry, around 90 % of the graftings are performed using citrange as a rootstock for the sake of raising commercially important fruit varieties such as sweet orange, mandarin, grapefruit etc. Use of citrange in tissue culture is all the more beneficial, as it is one of the most regenerable genotypes followed by Swingle citrumelo.

Conventional breeding in citrus has been practiced for decades and is vastly hampered by a host of unavoidable factors: large plant size, nucellar polyembryony, apomixis, high heterozygosity and pollen or ovule sterility, making controlled crosses a difficult task (Vardi *et al.,* 1975; Vardi, 1981; Martin-Trillo and Martinez-Zapater, 2002). Added to these are instances of cross or self-incompatibility that jeopardize breeding efforts even further. Majority of the species are apomictic wherein the development of embryo initiates directly from the nucellar tissue, suppressing the growth of zygotic embryo (Kultunow *et al.,* 1995). Citrus species also exhibit a very long juvenile period, which may extend from 5 to 21 years to enter the reproductive stage. Also, in nature, propagation and cultivation of citrus is limited to a particular season and favourable climatic conditions. All these factors combined together with the lack of sufficient knowledge about the pattern of inheritance of horticultural traits greatly impedes breeding efforts in citrus cultivars. A range of biotechnological techniques available such as cell and tissue culture and molecular genetics can help circumvent the problems associated with reproductive biology of citrus.

Plant regeneration in citrus

With the advent of advanced transgenic techniques, it has become feasible to introduce novel characteristics in the plant genome, but for efficient plant regeneration, an optimized tissue culture system is very important as regeneration is a slow process in Citrus sps. Once the regeneration conditions are standardized, they can subsequently be used for transformation experiments successfully. Several researchers have reported regeneration in different species of Citrus using stem and epicotyl segments as explants (Sim *et al*., 1989; Duran-Vila *et al*., 1992; Goh *et al*., 1995; Perez-Molph-Balch and Ochoa-Alejo, 1997; Ghorbel *et al*., 1998; Garcia-Luis *et al*., 1999; Bordon *et al*., 2000; Moreira-Dias *et al*., 2000, 2001; Zou *et al*., 2008) and from ovule, stigma and style via somatic embryogenesis (Cariami,

2005). Perez-Molph-Balch and Ochoa-Alejo (1997) developed efficient regeneration protocol in lime and mandarin through direct organogenesis by culturing the internodal stem segments of cultivars in the presence of benzylaminopurine (BAP) and naphthalene acetic acid (NAA). Regenerated shoots rooted successfully in response to NAA or IBA in the rooting medium and 70 % rooting efficiency was obtained. Studies were carried out to standardize regeneration protocol for sweet orange, citron and lime and healthy plantlets were obtained that rooted successfully in soil, although a high concentration of BAP (3 mg/l) and NAA (10 mg/l) was used for shoot and bud proliferation and root induction respectively for sweet orange and citron (Duran-Vila *et al*., 1988). A simple protocol for regeneration of citrus was described by Kobayashi *et al.* (2003) where thin sections of mature stem segments of sweet orange were used as the starting material and highest percentage of explants regenerated in MS medium before being transferred to woody plant medium (WPM). Regenerated buds were shoot-tip grafted on *C. citrange* rootstock to develop whole plants (Navarro, 1992). Phytohormones exert a profound impact on regeneration through organogenesis of any species. Important cultivars in Brazilian citrus industry - Natal, Valencia and Rangpur lime were cultured *in vitro* in MT (Murashige and Tucker, 1969) medium in presence of different concentrations of phytohormones and 1 mg/l of BAP for bud induction followed by 1 mg/l of IBA for root formation and was found to be the best combination for bud regeneration and rooting respectively (Alemida *et al.,* 2002). We have also used MT medium supplemented with 1 mg/l BAP for regeneration of epicotyls of sweet orange and more than 300 healthy shoots have been obtained. For rooting of *in vitro* shoots, half-strength MT medium with 1 mg/l IBA was employed which has yielded a large number of rooted plantlets (unpublished results). Lime (*C. aurantifolia*) can also be regenerated using nodes of mature trees as explants, and cultured on MS (Murashige and Skoog, 1962) medium containing auxin (NAA 1 mg/l) and cytokinin (BAP 2 mg/l and Kinetin 1 mg/l) (Al-Khayri and Al-Bahrany, 2001). Best regeneration of multiple shoots was observed with 1 mg/l of BAP and 0.5 mg/l kinetin in the medium, while highest percentage of rooting was obtained with 1 mg/l of IAA. Initiation of adventitious buds takes place directly from the cambial region at the cut surface of explants. It has been shown that regeneration of shoots from the apical end of epicotyl explants inserted longitudinally in semi solid culture medium follows direct regeneration pathway, whereas shoot development at the basal end follows indirect organogenesis after callus formation (Garcia-Luis *et al.,* 1999) and regeneration of adventitious buds relies heavily on BAP and IAA in Troyer citrange. Moreira-Dias *et al.* (2000) observed that differentiation of buds took place in direct organogenic manner from the exposed surface of vertically placed explants and did not require hormone supplement, although number of adventitious buds formed was significantly increased when explants were cultured in presence of BAP. On the other hand, addition of BAP and NAA was absolutely required for indirect regeneration. According to recent research, shoot regeneration pathway is determined by the polarity of the explant and its physical contact with the culture medium and not by the orientation of the explant (Garcia-Luis *et al.,* 2006). Also, the organogenic response in epicotyl explants becomes more pronounced as their distance from the cotyledonary node increases (Costa *et al.,* 2004), suggesting the farthest epicotyls to be the best candidates for use in transformation experiments. Epicotyls have been the favorite explants for the standardization of regeneration protocols because of their good *in vitro* morphogenic response. Transfer of healthy shoots to rooting medium containing IBA gives most appreciable percentage of rooting in citrus (Almeida *et al.,* 2002).

Regeneration of citrus can also be done by culturing nodes and internodes of seedlings germinated *in vitro*. Shoot induction and rooting of such explants have been found to be profoundly affected by the concentration of hormones present in the culture medium. It was observed that both shoot and root initiation were positively influenced by hormones NAA and BAP, such that the number of roots and shoots per explant increased proportionately with increase in hormone concentration and maximum number of roots and shoots regenerated at a combination of BAP 1 mg/l and NAA 10 mg/l (Usman *et al.,* 2005). Regeneration of Citrus species from pollen has also been reported (Hidaka *et al.,* 1979).

Amount and concentration of growth hormones for the regeneration of grapefruit, sour orange and alemow have also been established (Ghorbel, 1998) and healthy cultures have been raised from internodal stem segments of aseptically grown seedlings of these cultivars. Regeneration is the first step towards transformation and once successful, it paves the way for development of genetically transformed plants with desired traits.

Somatic hybridization

Somatic cell hybridization through protoplast fusion is an effective tool for circumventing bottlenecks in citrus

like sexual incompatibility, polyembryony and pollen or ovule sterility. It offers several useful applications for the development and improvement of the cultivar. Somatic hybridization is a means to augment the genetic diversity of the gene pool of crops by combining the nuclear, chloroplast and mitochondrial genomes in a novel arrangement. Therefore, it has become an integral part of citrus variety improvement worldwide (Khan, 2007). Various limitations presented by complicated reproductive biology of citrus can be successfully overcome through somatic hybridization by generating inter-specific and inter-generic allotetraploid somatic hybrids of desired cultivars for scion as well as rootstock development (Grosser *et al*., 1988; Ohgawara *et al.,* 1994; Grosser *et al.,* 1996) that can be utilized in breeding programs. It is also possible to generate somatic hybrids between sexually incompatible species, but has little scope for their incorporation in breeding programs. Somatic hybridization is accomplished by electrofusion of protoplast and characterizing the regenerated plantlets by flow cytometry and isozyme or DNA marker analysis. Electrochemical protoplast fusion is a process that combines the merits of both somatic hybridization and chemical methods (Olivares-Fuster *et al.,* 2005).

First instance of production of citrus somatic hybrids and cybrids via electrochemical protoplast fusion was provided by Olivares-Fuster *et al.,* (2005), where protoplasts of sweet orange and Mexican lime were induced to undergo fusion in presence of polyethyleneglycol (PEG) and electric impulses of direct current and exhibited high rates of embryogenesis. Although this new technique of somatic hybridization demands sharp skill and expertise, still it scores over other fusion methods in yielding better results. More than 250 interspecific and intergeneric somatic hybrids have been produced in last two decades (Guo and Deng, 2001).

First somatic hybrid in citrus was produced between C*itrus sinensis* and *Poncirus trifoliata* and was inter-generic in nature (Ohgawara *et al.,* 1985). Also, protoplasts from embryogenic nucellar calli of sour orange and rough lemon were fused with γ-irradiated protoplasts from *Microcitrus* in a bid to produce cybrid trees with potential breeding advantages (Vardi *et al*., 1989). Since then a large number of sexually or graft incompatible hybrids have been generated.

Successful plant regeneration from embryogenic callus and protoplasts has been reported by many researchers. Protoplasts obtained from nucellar-derived embryogenic callus regenerate very efficiently under tissue culture conditions (Kochba and Spiegel-Roy, 1973; Kobayashi *et al.,* 1984; Nito and Iwamasa, 1990; Niedz, 1993) and the protoclones exhibit uniformity with respect to morphological characteristics and chromosome number (Kobayashi, 1987). Very recently, sweet orange has been regenerated in this manner (Niedz *et al.,* 2006) and the size of embryos produced from protoplasts derived from callus was found to be significantly smaller than those produced from embryogenic callus. Callus can be stimulated towards embryogenesis by replacing sucrose with other carbon sources such as glycerol (Ben-Hayyim and Neumann, 1983), galactose (Button, 1978; Kochba *et al.,* 1978) lactose (Kochba *et al.,* 1982) or maltose (Hidaka and Omura, 1989; Tomaz, 2001). Nucellus can also be cultured to raise cell lines that possess the embryogenic potential of the parent tissue and can be genetically manipulated by protoplast fusion and transformation.

Somatic hybrid between Caipira sweet orange, a blight tolerant variety and Rangpur lime, a potential drought tolerant rootstock in Brazil was developed by PEG-mediated fusion for use as a vigorous rootstock (Gloria *et al.,* 2000). Hybrid between 'Hamlin' sweet orange and Rangpur lime has also been produced in the same way (Louzada *et al.,* 1992).

The evaluation of formerly produced citrus somatic hybrids using 'Page' tangelo and 'Murcott' tangelo as parents has demonstrated that some tetraploids produce superior quality fruit with medium thickness of peel and optimum juice content, displaying a potential to be used as fresh fruit cultivar straightaway. Thereafter, some additional hybrids were made using the same parents in combination with high quality scions - 'Murcott' tangor + 'Dancy' tanegrine, 'Murcott' + LB8-8, 'Page' tangelo + 'Murcott', 'Page' + LB8-9, 'Page' + ('Clementine' X 'Satsuma'), 'Page' + 'Ortanique' tangor etc (Guo *et al.,* 2004). Besides promising good quality fruit, tetraploids evolved by somatic hybridization of elite scion varieties can serve as suitable parents for the production of seedless triploid progeny (Grosser *et al.,* 1998).

Wild relatives of citrus are unexplored germplasm reservoirs, which hold tremendous promise by possessing several elite resistance traits, for example; orange jessamine, (*Murraya paniculata*) which is a remote and wild relative of citrus, belonging to tribe *Clauseneae* is unique in exhibiting high tolerance to citrus huanglongbing (Chen and Liao, 1982) and CTV (Yoshida, 1996), has been utilized in producing somatic hybrids with 'Page' tangelo by protoplast electrofusion (Guo *et al.,* 1998). Somatic hybridization has been

successfully used in Citrus to produce plants from more than 200 parental combinations (Grosser *et al.*, 2000).

The chief application of somatic hybridization technique would be the utilization of polyembryonic and sterile cultivars to produce fertile tetraploid hybrids and in generating superior rootstocks resistant to CTV, fungi (*Phytophthora*) and other constraints like drought, salinity, alkalinity, nematodes etc. Besides these advantages, the concept of seedlessness can be realized through this technique by creation of triploids. Somatic hybridization was hailed as a revolutionary technique during the 1980s and was thought to bring about major improvement in the development of scion and rootstock cultivars. Although it is a promising approach, but unfortunately it has not materialized and the promise has yet to be realized through release and successful use of new cultivars.

Agrobacterium-mediated transformation of citrus

Improvement of citrus by conventional breeding is inhibited by barriers of genetic incompatibility, apomixes, heterozygosity and lengthy juvenile period (Soost and Cameron, 1975). Therefore, genetic transformation is a promising tool that can ensure improvement of citrus crop by enabling the introduction of desirable and commercially important traits into known genotypes without altering their existing elite genetic background. In fact, transgenic crops are being grown worldwide on an enormous scale and are spread over 100 million hectares across the world (James, 2006).

Various transformation techniques that have been used in citrus include *Agrobacterium*-mediated transformation (Cervera *et al*., 1998a; Dominguez *et al*., 2000), chemically assisted uptake of foreign DNA by protoplasts (Fleming *et al*., 2000) and bombardment of target tissues with DNA-coated particles (Yao *et al*., 1996).

Genetic engineering approach is more convenient than conventional methods of plant breeding, especially in case of woody perennial crops like citrus and *Agrobacterium*-mediated transformation is an appreciably reliable, efficient and rapid gene transfer technique to introduce genes of agronomic interest in existing cultivars in order to increase their productivity and tolerance to various stresses (Bond and Roose, 1998; Gutierrez *et al.,* 1997; Yang *et al.,* 2000; Giri *et al.*, 2004; Perez-Clemente *et al*., 2008). Genetic manipulation of plants is done with the aim of improvement of the crop but the stability of the transgenes over a long period of time and after many cycles of graft propagation in a vegetatively propagated crop like citrus is the main prerequisite, therefore the transgene should be stably expressed to validate transformation technology.

Generally two common methods used for citrus transformation are *Agrobacterium*-mediated transformation and by direct uptake of DNA by protoplasts. However the former is the most preferred and extensively used one because of comparatively high percentage of transformants and accounts for the production of over 80 % of the transgenic plants (Wang and Fang, 1998).

The pioneering attempt to produce citrus transgenics was made as early as in the 1980s (Kobayashi and Uchimiya, 1989), involving direct uptake of DNA by protoplasts, but the very first authentic reports of successful transformation and production of citrus transgenics via *Agrobacterium* were that of trifoliate orange (*Poncirus trifoliata*). Epicotyls of citrus seedlings were used as transforming material with *GUS* and *NPT* II as reporter and marker genes respectively. Over 100 transgenic plants were obtained in all the experiments collectively and a transformation efficiency of 25 % was achieved in three months (Kaneyoshi *et al.,* 1994).

Citrus transformation has been successfully performed on many species and hybrids, including *Carrizo citrange* (Moore *et al.,* 1992), *Poncirus trifoliata* (Kaneyoshi *et al.,* 1994), Washington naval orange (Bond and Roose, 1998), Mexican lime (Pena *et al.,* 1997), sour orange (Gutierrez *et al.,* 1997), Pineapple sweet orange (Pena *et al.,* 1995b; Bond and Roose, 1998; Ballester *et al*., 2007), swingle citrumelo, which is a very popular rootstock for commercial citrus production in the US and Brazil (Molinari *et al*., 2004) and *Citrus reticulata* (Khawala *et al*., 2006). Sweet orange and tobacco transgenics with transgene driven by citrus PAL (phenylalanine ammonia-lyase) promoter have also been produced (Azevedo *et al*., 2006). Citrus transformation can be performed on a number of explants ranging from seeds, epicotyls (Kaneyoshi *et al.,* 1994; Almeida *et al.,* 2002), embryogenic cells (Yao *et al.,* 1996), nodal and internodal stem segments (Moore *et al.,* 1992), embryogenic cells (Yao *et al.,* 1996), callus (Hidaka *et al.,* 1990) to protoplast (Fleming *et al.,* 2000). However, the most favoured explant is invariably the epicotyl of *in vitro* germinated seedling, as it is the most responsive explant (Moore *et al.,* 1992) and therefore is most widely used in transformation experiments nowadays. For preparation of epicotyls as explants, seeds of the respective cultivar are peeled and

surface-sterilized with 0.5 % sodium hypochlorite solution containing 0.1 % Tween 20 and placed on full- or half-strength MS or MT medium having a pH of 5.7. Seeds are cultured in the dark for an initial 2 weeks and then transferred to a photoperiod of 16-h for one to three weeks (Cervera *et al.,* 1998b). Upon germination, 1 cm long epicotyls are harvested and used in transformation experiments.

Duncan grapefruit was transformed via *Agrobacterium* for the first time using epicotyls of nucellar seedlings as explants (Luth and Moore, 1999) and 25 transgenics were recovered after PCR and histochemical staining and Southern hybridization confirmed the integration of transgenes *GUS* and *NPT* II in the genome. Majority of the regenerants were however chimeras, where the transgenic tissue is composed of a mosaic of transgenic and non-transgenic sectors. As different species exhibit varying levels of compatibility towards tissue culture media and transformation protocols, the latter can be optimized accordingly for a better output. Transgenic grapefruit plants were produced in a similar manner using epicotyls as explants and kanamycin as selection agent (Yang *et al.,* 2000). Epicotyl explants have also been employed for *Agrobacterium*-mediated transformation of citrange (Cervera *et al.,* 1998c) and sweet orange (Yu *et al.,* 2002). There have also been attempts to transform suspension cultures raised from different parts of seed and flower by *Agrobacterium* to obtain viable and healthy plants. Embryogenic callus was inoculated with *Agrobacterium* and the resulting embryoids exhibited clear resistance to the selection agent (Hidaka *et al.,* 1990). Though transformation efficiency in this protocol was well below the expectations, (0.5 %) it offers a simple and reliable alternative to transform and regenerate commercially important Citrus sps. Interestingly, the co-cultivates that were kept still on the table gave rise to transformed colonies, while those kept on orbital shaker did not, suggesting that there are specific sites on the host cell walls which facilitate attachment of *Agrobacterium* and process of transformation. Interestingly when transgenics were raised *in vitro* in the absence of antibiotic selection, the frequency of plants regenerated with silenced transgenes is much higher than otherwise (Dominguez *et al.,* 2002). The actual reason behind the phenomenon is unknown but it reveals the fact that the rate of gene silencing is greatly underestimated when it is calculated on the basis of regenerants obtained under selective conditions.

Besides *A. tumefaciens*, *A. rhizogenes* was also used for citrus transformation. For example, internodal stem segments of sour orange (*C. aurantium*) were transformed using *A. rhizogenes* and the transformation efficiency came to an appreciable 91 % for regeneration of roots (Chavez-Vela *et al*., 2002). Genetic transformation system for pomelo (*Citrus grandis*) has also been optimized using *A. rhizogenes* (Xiao-hong *et al*., 2006).

After confirmation of the transgenic nature of the target plant, the most important step further is the grafting of transgenic scions or shoot tips onto vigorous and healthy rootstock to ensure hardening and speedy growth of the scion and quick recovery of transgenic plants. For micrografting, rootstock seedlings are decapitated, leaving 1-1.5 cm of epicotyls, roots are shortened to 4-5 cm and cotyledons are removed. Thereafter the regenerated transgenic shoot, as small as 0.2 cm to 1 cm is placed onto the cut surface or inserted into a V-shaped incision made in apical end of the decapitated epicotyl, so that the vascular rings of both the scion and the rootstock remain in contact. Thereafter the plants are transferred to potted soil. Most commonly used rootstocks are citrange, rough lemon, Rangpur lime and sour orange. Without grafting, in most species, newly produced transgenic shoots being weak and fragile, are liable to grow very poorly or die. Thus, grafting being a necessary process in citriculture, the improvement of rootstock cultivars through genetic engineering becomes mandatory for optimum output of citrus crops.

The biggest stumbling block on the way to citrus transformation is the recalcitrance to *Agrobacterium*-mediated transformation exhibited by majority of citrus species (Spolaore *et al.,* 2001). One possible reason behind this phenomenon could be the fact that Citrus species are not the natural hosts of *Agrobacterium* and their mutual interaction has not evolved to the optimum level so as to bring about efficient communication between them. Besides, genetic transformation comprises two different and independent events: integration of foreign nucleic acid in the plant genome and regeneration of complete plants from the transformed cells. Transformation and regeneration potential of the cells are not necessarily of the same competence, which is one of the reasons for recalcitrance towards transformation of many plant species. After transformation, further growth and regeneration of the explants pose their own problems: high frequency of occurrence of escapes and chimaeras, delay and difficulty in rooting of transgenic shoots. Chimaeras can be successfully eliminated from the transgenic population by repeated subculture of transgenic shoots in a medium containing gradually increasing

concentration of selection agent during various stages of shoot development (Mathews *et al.,* 1998). Prevalence of chimaeras has also been reported in soyabean (Christou *et al.,* 1990), rice (Christou and Ford, 1995), tobacco (Schmulling and Schell, 1993), cabbage (Berthomieu *et al.,* 1994) etc. The use of healthy explant material, appropriate selection marker and reporter genes, strain of *Agrobacterium*, establishment of suitable co-cultivation conditions and composition of culture media go a long way in ensuring rapid production of transgenic plants in good numbers. Keeping in view the recalcitrance of Citrus species towards transformation, it is advisable to use super-virulent strains of *Agrobacterium* such as A281, which is known to bring about successful transformation even in the less amenable genotypes (Cervera *et al.,* 1998a). A non-oncogenic derivative of A281 is EHA105, which is widely used in Citrus transformations nowadays. A correlation between super virulence of a strain and an increased transformation ability has been suggested, and the possible reason for super virulence could be the over activation of *vir* genes (Ghorbel *et al.,* 2000).

Factors affecting *Agrobacterium*-mediated transformation

Substantial yield of healthy transgenics is the outcome of cumulative effect of several factors- pre-culture of explants, infection time of explants with *Agrobacterium* culture and its density, period of co-cultivation of explants, use of acetosyringone, feeder plates during co-cultivation, presence of auxins in co-cultivation medium, co-cultivation in the dark and concentration of selection agent in culture medium (Cervera *et al.,* 1998c). For woody plants the standardization of tissue culture conditions is a difficult task (Giri *et al*., 2004), as there are no general protocols suitable for all genotypes. Moore *et al.* (1992) performed genetic transformation on citrange for the first time, but faced problems in rooting of transgenic shoots that led to very low transformation efficiency. Further experiments were carried out with improved protocols but rooting problems still persisted (Gutierrez *et al.,* 1997) until an efficient gene delivery system was described for *Poncirus trifoliata* (Kaneyoshi *et al.,* 1994) that gave an appreciably high transformation frequency of 25.5-43.1 %. This method, however, when applied to citrange did not give good results, probably due to the genotypic differences. Since then several modifications have been made to existing gene transfer protocols (Pena *et al.,* 1995a) to address the factors affecting transformation and regeneration of citrus plants. On similar lines, in a recent study (Rodriguez *et al*., 2008), it was observed that the same hormomonal treatment extended to two closely related sweet orange genotypes *viz*. Pineapple and Navelina elicited opposite response for transgenic shoot regeneration.

Pre-culturing of explants on co-cultivation medium rich in auxins prior to *Agrobacterium* inoculation has been shown to increase transformation efficiency in many woody plants such as plum (Mante *et al.,* 1991), apricot (Laimer *et al.,* 1992) and *Arabidopsis* (Sangwan *et al.,* 1992) by increasing the number of competent cells at cut ends for transformation, but in case of citrus the reverse happened to be true as the transformation efficiency dropped to half after pre-culture treatment (Cervera *et al.,*1998c; Costa *et al.,* 2002).

Period of incubation of explants with *Agrobacterium* is the first step that takes the plant tissue towards transformation. An infection time of 20 min has been found to be suitable for most cultivars including Natal and Valencia sweet orange and Rangpur lime (Almeida *et al.,* 2003). Duration of co-cultivation of the explants with *Agrobacterium* is also crucial towards bringing about transformation. Various co-cultivation periods have been worked out with different species ranging from 15 min to 5 days (Cervera *et al.,* 1998) and the most suitable one has been found to be 3 days, in the absence of light, whereas for apple, the best co-cultivation period has been worked out to be 4 days (Seong *et al*., 2008) and 2 days for kiwifruit (Janssen and Gardner, 1993). In citrus, although transformation frequency increased beyond 3-day time period, reaching to a maximum at 5 days, it promoted an overgrowth of *Agrobacterium*, thereby decreasing the actual transformation frequency drastically. Co-cultivation period of one day or less proved too inadequate for transformation. Hence, in routine experiments a co-cultivation time of 3 days is practiced. Co-cultivation of explants in a medium rich in auxins is known to stimulate the cells to shift towards dedifferentiation involving cell division and callus induction, making them more competent for transformation (Cervera *et al.,* 1998b). Cutting the epicotyl explants longitudinally into two halves to increase the exposed surface area for infection elevates regeneration frequency in both infected and uninfected explants (Yu *et al.,* 2002), but in some cases it also promotes an overgrowth of *Agrobacterium*, eventually resulting in decreased yield of viable transformants (Pena *et al.,* 2004).

Acetosyringone, a phenolic compound secreted by the wounded plant tissues also plays an important role as transformation enhancer in case of woody and recalcitrant species by bringing about induction of *vir*

genes during co-cultivation (Kumar and Rajam, 2005). It is generally used at a concentration of 100 μM in co-cultivation medium in citrus (Mendes *et al.,* 2002). Its positive role as a stimulator in facilitating infection by *Agrobacterium* has been established in the transformation of many woody plants such as apple (James *et al.,* 1993) and kiwifruit (Janssen and Gardner, 1993) and crop plants (Kumar and Rajam, 2005).

Selection agent present in the culture medium also has an important role in confirming the transgenic nature of the plant and deciding their survival and regeneration. Usually genes conferring resistance to antibiotics or herbicides are employed as selection markers. Normally a concentration of 100 mg/l of kanamycin in culture medium works fairly well for selection of transformants in Citrus species. Concentrations of 200 mg/l and 50 mg/l have also been tested but the former reduced the number of regenerants drastically and the latter allowed the growth of a large number of escapes (Cervera *et al.,* 1998c). The prevalence of escapes and chimeras is a major setback in citrus transformation (Moore *et al.,* 1992; Pena *et al.,* 1995a) and it has been pointed that kanamycin at 100 mg/l is not a very trustworthy indicator of transformation (Moore *et al.,* 1992; Pena *et al.,* 1995a) as untransformed cells still grow into escapes on it because they are shielded from the selection agent by the peripheral transformed cells of the explant (Ghorbel *et al.,* 1999). However, the presence of escapes can not entirely be attributed to the neighboring transformed cells as the consistent presence of kanamycin resistant *Agrobacterium* at the exposed surfaces of the explants continues to detoxify the antibiotic (Birch, 1997) in the surrounding untransformed cells and promotes the growth of escapes. Neverthless, 100 mg/l kanamycin is still used in Citrus transformations because it gives maximum number of transformed shoots. Selection can be further improved by the application of liquid medium overlay containing the selection agent, on top of the shoot elongation medium. It has been found to be sufficiently effective in preventing the regeneration of escape shoots (Yang *et al.,* 2000).

Transformants with silenced marker genes are often grouped together with escapes due to their inability to grow on selection medium, a fact that explains high frequency of occurrence of escapes, which are actually transgenics. Transformation frequency is often underestimated when deduced on the basis of marker and reporter gene expression as transgenics exhibiting low or nil activity are mistakenly grouped together with escapes and constitute 25 % of the plants considered escapes (Dominguez *et al.,* 2004).

Post-cultivation of explants under dark conditions after co-cultivation has also been known to elevate the yield of transformed shoots for many species including citrus (Pena *et al.,* 1995a) by stimulating the formation of callus at the cut ends of explants, which leads to an increase in transformation events. Best results were obtained when etiolation was performed for two to four weeks post co-cultivation. Maximum number of shoots were obtained when explants were directly transferred to light after co-cultivation with *Agrobacterium*, but majority of them were found to be escapes. In many studies it has been observed that transgenic cells in citrus epicotyl and internodal stem segments were situated in the callus tissue that originated from the cambium, suggesting that treatments promoting the proliferation of such callus could elevate transformation frequency (Cervera *et al.,* 1998a; Ghorbel *et al.,* 1999).

Further, inclusion of feeder plates during co-cultivation has been found to exert a positive influence on transformation and regeneration (Cervera *et al.,* 1998c) as well as in other species such as grapevine (Mullins *et al.,* 1990) and kiwifruit (Janssen and Gardner, 1993), possibly by permitting *vir*- activating compounds through them into the explants (Horsch *et al.,* 1985; Fillatti *et al.,* 1987a, b). After successful transformation, the rooting of putative transgenic shoots is no less problematic, as the young shoots are small and weak and compared to synthetic media, they tend to root better in soil (Moore *et al.,* 1992). It should be noted that tissue culture techniques in citrus are highly genotype-dependent and none of the techniques is entirely applicable to all genotypes under all conditions (Gutierrez *et al*., 1997).

Most of the literature reported on citrus transformation has emphasized the use of juvenile tissue as the starting material for transformation experiments (Moore *et al.,* 1992; Bond and Roose, 1998; Ali and Mirza, 2006), as young tissue is more receptive towards infection from *Agrobacterium*, but invariably exhibits juvenile characteristics upon regeneration and, for fruit traits, demands a patient wait for years for the analysis of desired incorporated characteristics in the plant. As juvenile phase in woody species like Citrus ranges between 6 to 20 years (Pena *et al*., 2001), it would be very revolutionary if the juvenile phase could be bypassed to enable quick analysis of horticultural traits in mature plants in less time and reduced costs. This can be achieved by directly transforming mature tissues of the adult plants. For this purpose, adult buds of the target plant were first invigorated by grafting them on juvenile rootstocks, stem segments from the adult tissue

were used as explants for transformation by suitably virulent *Agrobacterium* strain and cultured on synthetic media. Post-transformation, the regenerated shoots were again shoot-tip grafted onto suitable rootstocks and adult transformed plants were obtained that flower and set fruits in about 14 months, curtailing the entire process by several years (Cervera *et al.,* 1998b).

Sometimes, phenotypic variations among the Citrus transgenics are observed at low frequency. Transgenic tetraploids, as determined by molecular analysis are occasionally found within transgenic population, which could either originate from tetraploid maternal nucellar tissue or could form as a result of polyploidization during tissue culture, but the most probable course of their origin seems to be the maternal or source tissue from which the explant is derived. This information can also be supported by the fact that in Citrus, tetraploids arise naturally from tetraploid maternal tissue. There are reports of regeneration of transgenic polyploids in other plant species also, for example, potato (Imai *et al.,* 1993), *Petunia* (Tagu *et al.,* 1990), *Arabidopsis* (Scheid *et al.,* 1996), etc. In general, the event of polyploidy is attributed to source plant material employed in transformation.

The pattern and level of transgene expression is a contribution of several factors- transgene copy number (Hobbs *et al.,* 1990; Matzke and Matzke 1994; Jorgensen *et al.,* 1996), the location of the integrated transgenes in the genomic context or position effect (Peach and Velten 1991; Iglesias *et al.,* 1997), configuration of the transgenes in terms of truncation and rearrangement (Hobbs *et al.,* 1993) and environmental conditions (Meyer *et al.,* 1992).

It is common knowledge that transgenes present in single copy exhibit high levels of expression as compared to multiple copy T-DNA insertions. This phenomenon is known as post-transcriptional gene silencing (PTGS) and involves sequence-specific degradation of transgene mRNA, triggered by the over production of the latter due to transcription through multiple transgene copies. Excess production of transgene mRNA is toxic to the cell and thus is recognized and destroyed by the cellular machinery. However, no definite correlation between transgene copy number and expression level has been established, still single copy transgenics are always preferable over multiple copy ones (Pena *et al.,* 1995; Cervera *et al.,* 1998b).

Transformation by particle bombardment

Particle bombardment has been proved to be a promising technique to introduce novel characteristics into plants that are otherwise recalcitrant or less responsive to *Agrobacterium*-mediated transformation and can give rise to a large number of stably transformed plants. This technique is particularly useful where *Agrobacterium*-mediated genetic transformation fails due to host-bacterial incompatibility or problems in regeneration. Particle bombardment has a simplified protocol in terms of plasmid construction and overall transformation process and eliminates the need for complex plant-bacterial interactions (Gray and Finer, 1993).

Particle bombardment has been successfully performed on nucellar-derived embryogenic cells raised from suspension cultures of tangelo (*C. reticulata* Blanco x *C. paradisi* Macf.) and over 600 transient and 15 stably transformed lines were obtained per bombardment experiment (Yao *et al.,* 1996). The resulting calli grew well on kanamycin-containing selection medium and showed *GUS* activity, but could not regenerate into plants. Treatment of cells with osmoticum sorbitol (0.3 M) and mannitol (0.3 M) enhanced transformation efficiency in both transient and stable transformation experiments (Sanford *et al.,* 1993). Pre-conditioning of target tissue on high osmotic medium is important as it protects the explant from leakage and collapsing during the experiment. Pre-treatment of explants on osmotic medium has also proved to be useful in elevating the transformation efficiency in case of tangelo (Yao *et al.,* 1996), rice (Nandadeva *et al.,* 1999) and wheat (Altpeter *et al.,* 1996).

Transformations by this method can also be performed on thin epicotyl segments from germinated citrus seedlings (Bespalhok *et al.,* 2001). A transformation efficiency of 93 % was obtained under transient expression system when thin epicotyl sections of *C. citrange* were bombarded with tungsten particles (Bespalhok *et al.,* 2003). It was also found that incubation of the explants on culture medium prior to bombardment enhanced their receptivity towards transformation (Seki *et al.,* 1991).

Transient expression system in citrus

Promoter function and gene expression can be studied either in permanent or transient systems. Transient expression systems are designed for short-term studies of gene function and regulation (Barandiaran *et al.,* 1998, Ferrer *et al.,* 2000) and are advantageous over other protocols in being rapid, inexpensive and uncomplicated procedures. This type of protocol is particularly beneficial and uncomplicated, as it does not

require expensive apparatus or tedious and time-consuming methods for assessment of the activity of transgenes (Tucker *et al.,* 2002; Hoffmann *et al.,* 2004). A significant, yet simple method for transient expression of genes in fleshy fruits via *Agrobacterium*-mediated transformation was proposed by Spolaore *et al.* (2001), wherein they transformed intact ripe and fleshy fruits of apple, strawberry, orange, tomato and peach by directly injecting them with a syringe containing *Agrobacterium* suspension. The plasmid vector carried by *Agrobacterium* was furnished with *GUS* and *luciferase* as reporter genes. The *GUS* gene was interrupted by plant-derived *GUS* intron so as to enable its splicing and subsequent expression only in eukaryotic or plant tissues for convenient deduction of transformation efficiency. *GUS* activity in injected tissues was measured both qualitatively by histochemical staining with X-gluc as well as quantitatively by fluorimetric assay. In a similar study carried out on whole fruits of rough lemon (*C. jambhiri* Lush), a major rootstock in citrus cultivation, (Ahmed and Mirza, 2005) an incubation period of 48 h with *Agrobacterium* was found to be the best and immature fruits proved to be most suitable for the experiment and the transformed seeds germinated normally on culture medium.

Fleshy and juicy fruits are attractive targets to apply such techniques aimed at genetic improvement of the crop. Being a transient gene expression system, it offers an additional advantage of studying promoter strength and function in a short duration of time as regeneration of transformed cells into plants is not required (Tucker *et al*., 2002; Hoffmann *et al.,* 2004). Moreover, this system is especially valuable in the study of species that are recalcitrant to transformation or that bear fruit after a long duration of time post transformation. However, in transient systems, the expression of reporter genes might be very low or absent due to gene silencing or failure of transformation event. Therefore, it is advisable to use two reporter genes at a time to analyze promoter function accurately. In woody species such as Citrus (which is appreciably recalcitrant to transformation and has a long juvenile phase), transient expression studies for quick analysis of promoter function and gene regulation are particularly valuable (Ghorbel *et al.,* 1999).

Giant leaps towards genetic engineering in Citrus sps

Table 1 presents a comprehensive list of important Citrus crop species that have been genetically transformed with genes of agronomic value.

Excessively long juvenile period of citrus plants is one of the key factors that delay their reproduction and genetic improvement, but now it is possible to accelerate flowering by transforming juvenile tissue with constitutively expressing *LEAFY (LFY*) or *APETALA1* (*AP1*) genes taken from *Arabidopsis*. Transgenics produced normal flowers and fruits within 14 months and did not display any abnormality (Pena *et al.,* 2001).

Transgenic key lime plants harboring genes for reduced seed set have been obtained via *Agrobacterim*-mediated transformation (Koltunow *et al.,* 2000). As seedless citrus varieties enjoy consumer preference and higher market value, limes with small-sized seeds is a significant step forward. Moreover, with a shorter juvenile period of 2-3 years (Saunt, 1990) key lime can be an ideal test plant for analyzing genes aimed at crop improvement. Similarly, Ponkan mandarin, (C. *reticulata* Blanko) was also transformed to introduce the trait of seedlessness in it by means of a chimeric *ribonuclease* gene (*barnase*) under the control of tapetum-specific promoter (TA29) through *Agrobacterium*-mediated transformation of embryogenic callus. *Bar* gene was employed as the selectable marker and a total of 43 transgenics were recovered. Ponkan mandarin is a very palatable and juicy fruit but its highly seedy trait is a major disadvantage. As the transgenics were juveniles, they would require several years to develop fruits and determine the success of the procedure. Further, marker-free transgenic Carrizo citrange and sweet orange plants have been produced by employing inducible recombination and site-specific excision of the marker gene, with a view to avoid apprehensions regarding the possible risks posed to human health by the presence of these genes or the protein products derived from them (Ballester *et al*., 2008).

As citrus plants are cultivated in diverse ecological conditions, naturally they are subjected to various types of pathogens, CTV being most detrimental viral disease. In some plants virus resistance has been genetically engineered by transforming them with an untranslatable version of viral coat protein (Baulcomb, 1996) to inhibit viral replication. The coat protein gene of this virus has been completely sequenced (Sekiya *et al*., 1991). An insecticidal gene, derived from snowdrop lily (*Galanthus nivalis*) and a CTV untranslatable coat protein sequence have been introduced into a commercially important RioRed variety of grapefruit for developing resistance against CTV and aphids that spread the virus (Yang *et al.,* 2000).

Grapefruit is highly susceptible to CTV. In an effort to produce CTV resistant citrus transgenics, Duncan grapefruit was transformed with coat protein gene and

Table 1. Major crop species for which genetic transformation system has been applied to produce transgenic citrus plants having genes of agronomic interest

Common name	Scientific name	Gene Introduced	Reference
Kiwifruit and Trifoliate orange	*Actinidia chinensis* *Poncirus trifoliata*	Gene encoding human epidermal growth factor (*hEGF*)	Kobayashi *et al.,* 1996
Sour orange	*C. aurantium*	Coat protein gene of CTV	Gutierrez *et al.,* 1997
Trifoliate orange	*Poncirus trifoliata*	*rolC* gene	Kaneyoshi *et al.,* 1999
Sour orange	*C. aurantium*	Coat protein gene of CTV	Ghorbel *et al.,* 2000
West Indian lime	*C. aurantifolia*	Genes for decreasedseed set	Kultunow *et al.,* 2000
Grapefruit	*C. paradisi*	Coat protein gene of CTV	Moore *et al.,* 2000
Carrizo citrange	*C. sinensis* × *P. trifoliata*	*HAL2* gene	Cervera *et al.,* 2000
Troyer citrange	*C. sinensis* × *P. trifoliata*	Truncated version of CTV and *Bar* gene	Piestun *et al.,* 2000
Mexican Lime	*C. aurantifolia*	Coat protein gene of CTV	Dominguez *et al.,* 2000
Carrizo citrange	*C. sinensis* × *P. trifoliata*	*LEAFY* and *APETALA1*	Pena *et al.,* 2001
Grapefruit	*C. paradisi*	Carotenoid Biosynthetic genes	Costa *et al.,* 2002
Ponkan mandarin	*Citrus reticulata. Blanco*	Chimeric *ribonuclease gene*	Li *et al.,* 2002
Troyer citrange	*C. sinensis* × *P. trifoliata*	*rolABC* genes	Gentile *et al.,* 2002
Grapefruit	*C. paradisi*	CTV genes	Febres *et al.,* 2003
Carrizo citrange	*C. sinensis* × *P. trifoliata*	Citrus blight-associated gene	Kayim *et al.,* 2004
Trifoliate orange	*Poncirus trifoliata*	Capsid polyprotein gene (pCP)	Iwanami and Tokurou, 2004
Trifoliate orange	*Poncirus trifoliata*	Citrus *FT (CiFT*)	Endo *et al.,* 2005
Valencia orange	*C. sinensis*	Pectin methylesterasegene	Guo *et al.,* 2005
Rangpur Lime	*C. limonia*	*bO* (bacterio-opsin)	Azevedo *et al*., 2006

a portion of the gene sequence encoding the RNA-dependent RNA polymerase (RdRp) from Florida CTV strain T36 (Moore *et al.,* 2000). Regenerated buds were grafted as scions on to T36 infected mandarin rootstock, but their response against CTV remains to be seen. Recently, Duncan grapefruit has also been transformed with RNA-dependent RNA polymerase genes from CTV and several transgenics were obtained (Cevik *et al*., 2006). In a major step forward to prevent virus infection, several grapefruit varieties were transformed with candidate sequences derived from a single dominant gene *Ctv,* present in trifoliate orange, that naturally confers broad spectrum resistance against CTV and the transgenics are under consistent monitoring (Rai *et al*., 2006). However, till so far, the efforts extended towards imparting lasting pathogen-derived resistance to citrus crops against CTV have met with little success and demand for novel and revised approach to reach the aim (Batuman *et al*., 2008). Carrizo citrange transgenics were made carrying citrus-blight associated gene in both sense and antisense orientation through *Agrobacterium*-mediated transformation (Kayim *et al.,* 2004). Citrus blight is a tremendously devastating disease of citrus, and citrange is particularly susceptible to it. Evaluation of transgenes and their effect on citrus blight is still a few years away, as blight symptoms are never evident

on trees younger than four years and usually require about 5-15 years to be observed, depending upon the rootstock (Castle *et al*., 1993). In a recent study, Singh *et al*. (2008) have produced ICRSV *(*Indian citrus ringspot virus) free young kinnow mandarin (*Citrus nobilis* Lour X *C. deliciosa* Tenora) plants by micrografting the shoot apices of virus infected plant onto rough lemon rootstock and achieved an appreciable success rate of 20 %.

The very first attempt to transform Key lime and sour orange with coat protein gene of CTV to produce agriculturally important citrus transgenics was made using internodal stem segments of *in vitro* grown seedlings as explants (Gutierrez *et al.,* 1997). As Mexican lime is predominantly sensitive to CTV, it makes a good model plant to study coat-protein mediated resistance against the virus. The objective of the study was to develop plants that exhibited resistance to potentially disastrous tristeza disease, but the recovery of transgenics was very poor due to rooting problems, needing further research and improvement. Later similar experiments were carried out, that suggested that no obvious correlation exists between coat-protein expression and copy number or integration pattern of the transgenes (Dominguez *et al.,* 2000).

Citrus crop, being grown in a wide variety of soil conditions has to combat various kinds of abiotic stresses, salinity being the most prominent one. Hence, it has been successfully transformed with *HAL*2 gene from yeast to impart the valuable trait of salinity tolerance to it (Cervera *et al.,* 2000).

Successful production of C. *paradisi* transgenics containing carotenoid biosynthetic genes: phytoene synthase, phytoene desaturase or lycopene-β-cyclase has been reported (Costa *et al.,* 2002). These multigene transgenics have primarily been raised to supplement human nutrition, as carotenoids are precursors of vitamin A and antioxidants.

In order to enhance fruit juice quality of commercially important fruit Valencia orange, protoplasts isolated from embryogenic suspension cultures were successfully transformed by the use of PEG (Guo *et al.,* 2005). Valencia, a highly marketable variety, is grown mainly for its juice, which is degraded due to the effect of thermostable pectin methylesterase (TSPME). Gene *CsPME4*, responsible for TSPME activity was down-regulated by the over-expression of *CsPME4* using *GFP* as a selection marker. Use of *GFP* eliminates the need for antibiotic selection marker, which is highly desirable from the commercial point of view and being non-destructive it is easy to select the transformed cells. However, only one proembryo could be generated and grown into plant by *in vitro* grafting. Recently, 'Bingtang' sweet orange transgenic plants were developed with *GFP* gene (Duan *et al*., 2007).

CONCLUSIONS AND FUTURE DIRECTIONS

After isolation of a useful transformant, it can be conveniently propagated vegetatively to provide unlimited number of desired transgenic lines. The common setbacks faced in citriculture work were that of low transformation efficiency, problems in rooting and high percentage of escapes and chimeras, which in part could be due to different physiological responses of different genotypes to the culture techniques. Therefore, it becomes necessary that a well-established, reliable and tested tissue culture system for transformation and regeneration is available to properly investigate gene function and make progress towards genetic improvement of the crops. Besides, in view of the fact that the chloroplast genome of *Citrus sinensis* has been completely sequenced, it will facilitate the introduction of traits that are governed by the chloroplast genome. Further, chloroplast genetic engineering offers distinct advantages over nuclear transformation, for e.g. increase in transgene expression and containment of the transgene (Bausher *et al*., 2006).

As present day citriculture relies heavily upon limited number of rootstocks, improvement in their quality and number is the need of the hour and can to some extent be achieved through somatic hybridization by raising allotetraploid hybrids of available rootstocks that combine desirable characteristics of different species (Grosser *et al.,* 1998) and by producing wide somatic hybrids that enrich citrus germplasm for future use. Somatic hybridization is an alternative method to circumvent sexual and graft incompatibility to a large extent. Some of the intergeneric somatic hybrids produced for this purpose involve species of Citrus, Fortunella, Feronia, Microcitrus, Poncirus, etc.

Seedlessness is one of the best attributes to look for when it comes to citrus fruits. An efficient strategy to generate seedless scion cultivars in citrus is by production of triploids from interploid crosses. Somatic hybrid parents can also be utilized in production of triploids that mature late in the season and enter the market at an uncompetitive time. It would be desirable if more competent and suitable protocols were developed

for transforming mature tissues of commercially important scion cultivars and rootstocks, so that evaluation of horticultural traits becomes more easy and speedy.

Citrus species are vulnerable to various types of fungi, bacteria, viruses and climatic conditions during cultivation like other woody perennials. Several Citrus species have been genetically engineered in this direction to enable them to counter various biotic and abiotic stresses. Presently, production of transgenic plants resistant to viruses and bacteria is underway by the expression of coat protein and pathogen-related proteins, respectively, but adequate levels of resistance is yet to be achieved (Olivares-Fuster *et al*., 2003; Ananthakrishnan *et al*., 2007). In contrast, salinity tolerance has been achieved by introduction of *HAL*2 gene. Likewise, early flowering and dwarfing was also achieved by the introduction of *APETALA1* or *LEAFY*, and *rolABC* genes, respectively. Citrus transgenics carrying *Xa21* gene for resistance against bacterial canker and barnase gene, to produce seedless fruit have been produced and are being evaluated (Guo *et al*., 2006). In most of the woody crops including Citrus, the frequency of *Agrobacterium*-mediated transformation is very low, which hinder the routine production of transgenics. Genetic engineering has enabled the production of commercial cultivars lacking one or two desirable genes by incorporation of specific gene controlling that character, which is impossible by conventional breeding. Therefore, the present emphasis lies in thorough understanding of transformation procedures by evaluating and optimizing the critical factors that decide the fate of transformation *viz*. genotype of plant, type of explant, *Agrobacterium* strain, period of infection and co-cultivation, growth medium of transgenics, etc to produce novel transgenics.

Cryopreservation of agronomically important cultivars is the most promising answer to biological and climatic hazards. Embryogenic callus cultures have been demonstrated to survive repeated cryopreservation treatments with routinely used dimethylsulfoxide (DMSO) (Kobayashi *et al.,* 1990; Aguilar *et al.,* 1993). Cryopreserved cultures of various tissues including recalcitrant seeds, ovules, embryos, callus, etc can successfully be used in future after months of storage at low temperature.

Biotechnology offers promising solutions to many of the difficult challenges and impediments to citrus breeding that result from citrus biology and reproduction and, through transformation and regeneration, can expedite the improvement of citriculture worldwide (Poupin and Arce-Johnson, 2005).

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REFERENCES

Aguilar ME, Engelmann F and Michaux-Ferrière N (1993). Cryopreservation of cell suspensions of *Citrus deliciosa* Tan. an histological study. *Cryo-Letters,* 14: 217-228.

Ahmad M and Mirza B (2005). An efficient protocol for transient transformation of intact fruit and transgene expression in *Citrus. Plant Mol. Biol. Rep.,* 23: 419a-419k.

Al-khayri JM and Al-bahrany AM (2001). *In vitro* micropropagation of *Citrus aurantifolia* (lime). *Curr. Sci*., 18: 1242-1246.

Ali S and Mirza B (2006). Micropropagation of rough lemon (*Citrus jambhiri* Lush.): Effect of explant type and hormone concentration. *Acta Bot. Croat*. 65, 137–146.

Almeida WAB de, Mourão Filho F de AA, Mendes BMJ and Rodriguez APM (2002). *In vitro* organogenesis optimization and plantlet regeneration in *Citrus sinensis* and *C. limonia*. *Sci. Agr*., 59: 35-40.

Almeida WAB de, Mourão Filho F de AA, Mendes BMJ, Pavan A and Rodriguez APM (2003). *Agrobacterium*-mediated transformation of *Citrus sinensis* and *C. limonia* epicotyl segments. *Sci. Agr*., 60: 23-29.

Altpeter F, Vasil V, Srivastava V, Stoger E and Vasil IK (1996). Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. *Plant Cell Rep*., 16: 12-17.

Ananthakrishnan G, Orbovic V, Pasquali G, Calovic M and Grosser JW (2007). Transfer of citrus tristeza virus (CTV)-derived resistance candidate sequences to four grapefruit cultivars through *Agrobacterium*-mediated transformation. *In Vitro Cell. Dev. Biol. Plant*, 43: 593-601.

Azevedo FA, Mourão Filho F, Schinor EH, Paoli LG de, Mendes BMJ, Harakava R, Gabriel DW and Lee RF (2006). *GUS* gene expression driven by a citrus promoter in transgenic tobacco and 'Valencia' sweet orange. *Pesq. Agropec. Bras*., 41: 1623-1628.

Azevedo FA, Mourão Filho F, Mendes BMJ, Almeida WAB, Schinor EH, Pio R, Barbosa JM, Gonzalez SG, Carrer H and Lam E (2006). Genetic transformation of Rangpur

lime (*Citrus limonia* Osbeck) with the *bO* (bacterio-opsin) gene and its initial evaluation for *Phytophthora nicotianae* resistance. *Plant Mol. Biol. Rep*. 24: 185-196.

Ballester A, Cervera M and Pena L (2007). Efficient production of transgenic citrus plants using isopentenyl transferase positive selection and removal of the marker gene by site-specific recombination. *Plant Cell Rep*., 26: 39-45.

Ballester A, Cervera M and Pena L (2008). Evaluation of selection strategies alternative to *npt*II in genetic transformation of citrus. *Plant Cell Rep*., 27: 1005-1015.

Barandiaran X, Pietro AD and Martín J (1998). Biolistic transfer and expression of a *uidA* reporter gene in different tissues of *Allium sativum* L. *Plant Cell Rep*., 17: 737-741.

Bar-Joseph M, Marcus R and Lee RF (1989). The continuous challenge of citrus tristeza virus control. *Annu. Rev. Phytopathol*., 27: 291-316.

Batuman O, Mawassi M, Dawson WO and Joseph M Bar (2008). Transgenic pathogen derived resistance is difficult to obtain for citrus tristeza virus and probably also for other closteroviridae. *Indian J. Virol.*, 19. (Abstract)

Baulcombe DC (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell* 8: 1833–1844.

Bausher MG, Singh ND, Lee SB, Jansen RK and Daniell H (2006). The complete chloroplast genome sequence of *Citrus sinensis* (L.) Osbeck var 'Ridge Pineapple: organization and phylogenetic relationships to other angiosperms. *BMC Plant Biol.*, 6: 1-11.

Ben-Hayyim G and Neumann H (1983). Stimulatory effect of glycerol on growth and somatic embryogenesis in *Citrus* callus cultures. *Z. Pflanzenphysiol*., 110: 331–333.

Berg van den M and van den Berg M (1999). Measures to reduce *Citrus psylla* and the spreading of the greening disease. *Neltropica Bull.,* 20: 5-6.

Berthomieu P, Beclin C, Charlot F, Dore C and Jouanin L (1994). Routine transformation of rapid cycling cabbage (*Brassica oleracea*) molecular evidence for regeneration of chimeras. *Plant Sci*., 96: 223-235.

Bespalhok FJC, Kobayashi AK, Luiz FPP, Hissano Z and Vieira LGE (2001). *In vitro* adventitious shoot regeneration from sweet orange (*Citrus sinensis*) using thin epicotyl sections. *Crop Breed. Appl. Biotech*., 1: 27-34.

Bespalhok FJC, Kobayashi AK, Pereira LFP, Galvao RM and Vieira LGE (2003). Transient gene gxpression of â-glucuronidase in citrus thin epicotyl transversal sections using particle bombardment. *Brazilian Arc. Biol. Technol.,* 46: 1-6.

Birch RG (1997). Plant transformation: problems and strategies for practical application. *Ann. Rev. Plant Physiol. Plant Mol. Biol.,* 48: 297–326.

Bond JE and Roose ML (1998). *Agrobacterium*-mediated transformation of the commercially important citrus cultivar Washington navel orange. *Plant Cell Rep*., 18: 229–234.

Bordon Y, Guardiola JL and Garcia-Luis A (2000). Genotype affects the morphogenic response in vitro of epicotyl segments of *Citrus* rootstocks. *Ann. Bot*., 86: 159–166.

Button J (1978). The effect of some carbohydrates on the growth and organization of Citrus ovular callus. *Z. Pflanzenphysiol*., 88: 61–68.

Cariami F (2005). Somatic embryogenesis protocol: *Citrus*. *Protocol for Somatic Embryogenesis in Woody Plants* (Jain SM and Gupta PK, Eds.), Springer, The Netherlands, pp. 321-343.

Castle WS, Tucker DPH, Krezdorn AH and Youtsey CO (1993). *Rootstocks for Florida Citrus*: rootstock selection, the first step to success. 2nd ed. Gainesville: University of Florida, 1993, p92.

Cervera M, López MM, Navarro L and Peña L (1998a).Virulence and supervirulence of *Agrobacterium tumefaciens* in woody fruit plants. *Physiol. Mol. Plant. Pathol*., 52: 67–78.

Cervera M, Juárez J, Navarro A, Pina JA, Duran-Vila N, Navarro L and Peña L (1998b). Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage. *Transgenic Res.,* 7: 51–59.

Cervera M, Pina JA, Juarez J, Navarro L and Peña L (1998c). *Agrobacterium*-mediated transformation of citrange: factors affecting transformation and regeneration. *Plant Cell Rep.,* 18: 271–278.

Cervera M, Ortega C, Navarro A, Navarro L and Peña L (2000). Generation of transgenic citrus plants with the tolerance-to-salinity gene *HAL2* from yeast. *J. Hortic. Sci. Biotechnol.,* 75: 26–30.

Cervera M, Juarez J, Navarro L and Peña L (2004). Genetic transformation of mature citrus plants. *Meth. Mol. Biol.*, 286: 177-187.

Cevik B, Lee RF and Niblett CL (2006). Genetic transformation of *Citrus paradisi* with antisense and untranslatable RNA-dependent RNA polymerase genes of citrus tristeza closterovirus. *Turk. J. Agric*. 30: 173-182.

Chavez-Vela NA, Chavez-Ortiz LI and Pérez-Molphe-Balch E (2003). Genetic transformation of sour orange using *Agrobacterium rhizogenes*. *Agrociencia,* 37: 629-639.

Chen XY and Liao CQ (1982). Observation of biological characteristics of *Citrus psyllid, Diaphorina citri* and its relationship with citrus huanglongbing (in Chinese). *China Citrus,* 4: 14-15.

Christou P (1990). Morphological description of transgenic soybean chimeras created by the delivery, integration and expression of foreign DNA using electric discharge particle acceleration. *Ann. Bot*., 66: 379-386.

Christou P and Ford TL (1995). Recovery of chimeric rice plants from dry seed using electric discharge particle acceleration. *Ann. Bot*., 75: 449-454.

Costa AS and Müller GW (1980). Tristeza control by cross protection: a US–Brazil cooperative success. *Plant Dis.,* 64: 538–541.

Costa MGC, Otoni WC and Moore GC (2002). An evaluation of factors affecting the effciency of *Agrobacterium*-mediated transformation of *Citrus paradisi* (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. *Plant Cell Rep.,* 21: 365-373.

Domínguez A, Guerri J, Cambra M, Navarro L, Moreno P and Peña L (2000). Efficient production of citrus transgenic plants expressing the coat protein gene of Citrus Tristeza Virus. *Plant Cell Rep*., 19: 427–433.

Domínguez A, Fagoaga C, Navarro L, Moreno P and Peña L (2002). Regeneration of transgenic citrus plants under non selective conditions results in high frequency recovery of plants with silenced transgenes. *Mol. Genet. Genom*., 267: 544–556.

Domínguez A, Cervera M, Pérez RM, Romero J, Fagoaga C, Cubero J, López MM, Juárez JA, Navarro L and Peña L (2004). Characterisation of regenerants obtained under selective conditions after *Agrobacterium*-mediated transformation of citrus explants reveals production of silenced and chimeric plants at unexpected high frequencies. *Mol. Breed*., 14: 171–183.

Duan Y, Liu X, Fan J, Li D, Wu R and Guo W (2007). Multiple shoot induction from seedling epicotyls and transgenic citrus plant regeneration containing the green fluorescent protein gene. *Bot. Studies,* 48: 165-171.

Duran-Vila N, Ortega V and Navarro L (1988). Morphogenesis and tissue cultures of three citrus species. *Plant Cell Tiss. Org. Cult*., 16: 123-133.

Duran-Vila N, Gogorcena Y, Ortega V, Ortiz J and Navarro L (1992). Morphogenesis and tissue culture of sweet orange (*Citrus sinensis* (L.) Osb.): effect of temperature and photosynthetic radiation. *Plant Cell Tiss. Org. Cult*., 29: 11–18.

Endo T, Shimada T, Fuji H, Kobayashi Y, Araki T and Omura M (2005). Ectopic expression of an FT homolog from Citrus confers an early flowering phenotype on trifoliate orange (*Poncirus trifoliata* L. Raf.). *Trans. Res.,* 14: 703–712.

FAO (2001). http.//apps.fao.org/lim500/nph-wrap.pl.

Febres VJ, Niblett CL, Lee RF and Moore GA (2003). Characterization of grapefruit plants (*Citrus paradisi* Macf.) transformed with citrus tristeza closterovirus genes. *Plant Cell Rep*., 21: 421–428.

Ferrer E, Linares C and González JM (2000). Efficient transient expression of the β̃glucuronidase reporter gene in garlic (*Allium sativum* L.). *Agronomie,* 20: 869-874.

Fillatti JJ, Kiser J, Rose R and Comai L (1987). Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. *Biotechnology,* 5: 726-730.

Fillatti JJ, Sellmer J, McCown B, Haissig B and Comai L (1987). *Agrobacterium*-mediated transformation and regeneration of *Populus*. *Mol. Gen. Genet.,* 206: 192-199.

Fleming GH, Olivares-Fuster O, Del-Bosco S and Grosser JW (2000). An alternative method for the genetic transformation of sweet orange. *In Vitro Cell Dev. Biol. Plant.,* 36: 450–455.

Garcý´ a-Luis A, Bordo´ n Y, Moreira-Dias JM, Molina RV and Guardiola JL (1999). Explant orientation and polarity determine the morphogenic response of epicotyl segments of Troyer citrange. *Ann. Bot*., 84: 715–723.

Garcý´ a-Luis A, Molina RV, Varona V, Castello S and Guardiola JL (2006). The influence of explant orientation and contact with the medium on the pathway of shoot regeneration in vitro in epicotyl cuttings of Troyer citrange. *Plant Cell Tiss. Org. Cult*., 85: 137–144.

Gentile A, Monticelli S and Damiano C (2002). Adventitious shoot regeneration in peach [*Prunus persica* (L.) Batsch]. *Plant Cell Rep.,* 20: 1011–1016.

Ghorbel R, Navarro L and Dura´ n-Vila N (1998). Morphogenesis and regeneration of whole plants of grapefruit (*Citrus paradisi*), sour orange (*C. aurantium*) and alemow (*C. macrophylla*). *J. Hort. Sci. Biotechnol*., 73: 323–327.

Ghorbel R, Juárez J, Navarro L and Peña L (1999). Green fluorescent protein as a screenable marker to increase the efficiency of generating transgenic woody fruit plants. *Theor. Appl. Genet*., 99: 350–358.

Ghorbel R, Domínguez A, Navarro L and Peña L (2000). High efficiency genetic transformation of sour orange *Citrus aurantium* L. and production of transgenic trees containing the coat protein gene of Citrus Tristeza Virus. *Tree Physiol*., 20: 1183-1189.

Gill MIS, Singh Z and Agrez V (2004). Factors affecting *Agrobacterium*-mediated transformation in fruit and nut crops- An overview. *Food, Agric. Environ*., 2: 327-347.

Giri CC, Shyamkumar B and Anjaneyulu C (2004). Progress in tissue culture, genetic transformation and applications in biotechnology to trees: an overview. *Trees,* 18: 115-135.

Gloria FJM, Mourao Filho, Camargo LEA and Mendes MEJ (2000). Caipira sweet orange + Rangpur Lime: A potential somatic hybrid to be used as rootstock in the Brazilian citrus industry. *Genet. Mol. Biol.,* 23: 661-669.

Goh CJ, Sim GE, Morales CL and Loh CS (1995). Plantlet regeneration through different morphogenic pathways in pommelo tissue culture. *Plant Cell Tiss. Org. Cult*., 43: 301–303.

Gray DJ and Finer JJ (1993). Development and operation of five particle guns for introduction of DNA into plant cells. *Plant Cell Tiss. Org. Cult*., 33: 219.

Grosser JW, Gmitter FG Jr and Chandler JL (1988). Intergeneric somatic hybrid plants of *Citrus sinensis* cv. Hamlin and *Poncirus trifoliata* cv. Flying Dragon. *Plant Cell Rep*., 7: 5-8.

Grosser JW, Garnsey SM and Halliday C (1996). Assay of sour orange somatic hybrid rootstocks for quick decline disease caused by citrus tristeza virus. *Proc. Int. Soc. Citricult.,* 1: 353-356.

Grosser JW, Jiang J, Louzada ES, Chandler JL and Gmitter FG Jr (1998). Somatic hybridization, an integral component of citrus cultivar improvement: II. Rootstock improvement. *HortSci.,* 33: 1060-1061.

Grosser JW, Ollitrault P and Olivares-Fuster O (2000). Somatic hybridization in citrus: An effective tool to facilitate variety improvement. *In Vitro Cell. Dev. Biol. - Plant*, 36: 434-449.

Guo WW and Deng XX (1998). Somatic hybrid plantlets regeneration between Citrus and its wild relative, via protoplast fusion. *Plant Cell Rep.,* 18: 297-300.

Guo WW and Deng XX (2001). Wide somatic hybrids of *Citrus* with its related genera and their potential in genetic improvement. *Euphytica,* 118: 175-183.

Guo WW, Prasad D, Cheng YJ, Serrano P, Deng XX and Grosser JW (2004). Targeted cybridization in citrus: transfer of Satsuma cytoplasm to seedy cultivars for potential seedlessness. *Plant Cell Rep*., 22: 752–758.

Guo WW, Duan YX, Olivares-Fuster O, Wu ZC, Arias CR, Burns JK and Grosser JW (2005). Protoplast transformation and regeneration of transgenic Valencia sweet orange plants containing a juice quality related pectin methylesterase gene. *Plant Cell Rep*., 24: 482-486.

Guo WW, Duan YX, Li DL, Liu X, Tan B, Cai XD, Grosser JW and Deng XX (2006). Citrus genetic transformation with interest target genes and further uses of transgenic lines in somatic fusion. *Acta Hort*. 773: XXVII International Horticultural Congress.

Gutiérrez MA, Luth DE and Moore GA (1997). Factors affecting *Agrobacterium*-mediated transformation in *Citrus* and production of sour orange (*Citrus aurantium* L.) plants expressing the coat protein gene of citrus tristeza virus. *Plant Cell Rep*., 16: 745–753.

Hara M, Fujinaga M and Kuboi T (2004). Radical scavenging activity and oxidative modification of citrus dehydrin. *Plant Physiol. Biotechnol*., 42: 657-662.

Hidaka T, Yamada Y and Shichijo T (1979). *In vitro* differentiation of haploid plants by anther culture in *Poncirus trifoliata* (L.) Raf. *Japan J. Breed*., 29: 248-254.

Hidaka T and Omura M (1989). Control of embryogenesis in Citrus cell culture: regeneration from protoplasts and attempts to callus bank. *Bull. Fruit Tree Res. Stn. B*., 16: 1–17.

Hidaka T, Omura M, Ugaki M, Tomiyama M, Kato A, Ohshima M and Motoyoshi F (1990). *Agrobacterium*-mediated transformation and regeneration of *Citrus* spp. from suspension cells. *Japan J. Breed*., 40: 199–207.

Hobbs SLA, Kpodar P and DeLong CMO (1990). The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. *Plant Mol. Biol.,* 15: 851–864.

Hobbs SLA, Warkentin TD and Delong CMO (1993). Transgene copy number can be positively or negatively associated with transgene expression. *Plant Mol. Biol.,* 21: 17-26.

Hoffmann M, Bedhomme M, Carthy EM, Gambonnet B, Moran RG, Rébeillé F and Ravane S (2004). Identification and functional characterization of a chloroplastic transporter for vitamin B9 in *Arabidopsis*. Proceedings of 13th International Workshop on Plant Membrane Biology, Montpellier – France.

Horsch RB, Fry JE, Hoffmann NL, Eighholtz D, Rogers SG and Fraley RT (1985). Transferring genes into plants. *Science,* 227: 1229-1231.

Iglesias VA, Moscone EA, Papp I, Neuhuber F, Michalowski S, Phelan T, Spiker S, Matzke M and Matzke AJM (1997). Molecular and cytogenetic analyses of stably and unstably expressed transgene loci in tobacco. *Plant Cell,* 9: 1251–1264.

Imai T, Aida R and Ishige T (1993). High frequency of tetraploidy in *Agrobacterium*-mediated transformants regenerated from tuber discs of diploid potato lines. *Plant Cell Rep*., 12: 299–302.

Iwanami T and Shimizu T (2004). Tolerance to Citrus mosaic virus in transgenic trifoliate orange lines harboring capsid polyprotein gene. *Plant Dis*., 88: 865-868.

James DJ, Uratsu S, Cheng J, Negri P, Viss P and Dandekar AM (1993). Acetosyringone and osmoprotectants like betaine or proline synergistically enhance *Agrobacterium*-mediated transformation of apple. *Plant Cell Rep*., 12: 559–563.

James C (2006). Global status of commercialized biotech/GM crops, ISAAA Brief No. 35, ISAAA, Ithaca.

Janssen BJ and Gardner RC (1993). The use of transient *GUS* expression to develop an *Agrobacterium*-mediated gene transfer system for kiwifruit. *Plant Cell Rep*., 1: 28–31.

Jorgensen RA, Cluster PD, English J, Que Q and Napoli C (1996). Chalcone synthase co-suppression phenotypes in petunia flowers: Comparison of sense vs antisense constructs and single copy vs complex T-DNA sequences. *Plant Mol Biol*., 31: 957–973.

Kaneyoshi J, Kobayashi S, Nakamura Y, Shigemoto N and Doi Y (1994). A simple and efficient gene transfer system of trifoliate orange (*Poncirus trifoliata* Raf.). *Plant Cell Rep*., 13: 541–545.

Kayim M, Ceccardi TL, Berretta MJG, Barthe GA and Derrick KS (2004). Introduction of a citrus blight-associated gene into Carrizo citrange [*Citrus sinensis* (L.) Osbc. *Poncirus trifoliata* (L.) Raf.] by *Agrobacterium*-mediated transformation. *Plant Cell Rep*., 23: 377–385.

Khan IA (2007). Citrus genetics, breeding and biotechnology. CABI International, Wallingford, UK.

Khawale RN, Singh SK, Garg G, Baranwal VK and Ajirlo SA (2006). *Agrobacterium*-mediated genetic transformation of Nagpur mandarin (*Citrus reticulate Blanco). Curr. Sci*., 91: 1700-1705.

Kobayashi S, Ikeda I and Nakatani M (1984). Induction of nucellar callus from orange (*Citrus sinensis* L. Osb.) ovules, and uniformity of regenerated plants. *Bull. Fruit Tree Res. Stn*; Series E, Akitsu, 5: 43-54.

Kobayashi S (1987). Uniformity of plants regenerated from orange (*Citrus sinensis* Osb.) protoplasts. *Theor. Appl. Genet*. 74: 10-14.

Kobayashi S, Sakai A and Oiyama I (1990). Cryopreservation in liquid nitrogen of cultured navel orange (*Citrus sinensis* Osb.) nucellar cells and subsequent plant regeneration: *Plant Cell Tiss. Org. Cult.,* 23: 15–20.

Kobayashi S, Nakamura Y, Kaneyoshi J, Higo H and Higo K (1996). Transformation of kiwifruit (*Actinidia chinensis*) and trifoliate orange (*Poncirus trifoliata*) with a synthetic gene encoding the human epidermal growth factor (hEGF). J. *Jpn. Soc. Hort. Sci*., 64: 763-769.

Kobayashi AK, Bespalhok JC, Pereira LFP and Vieira LGE (2003). Plant regeneration of sweet orange (*Citrus sinensis*) from thin sections of mature stem segments. *Plant Cell Tiss. Org. Cult.,* 74: 99–102.

Kochba J and Spiegel-Roy P (1973). Effect of culture media on embryoid formation from ovular callus of 'Shamouti' orange (*Citrus sinensis*). *Z. Pflanzenzuchtg.,* 69: 156–162.

Kochba JP, Spiegel-Roy P, Neumann H and Saad S (1978). Stimulation of embryogenesis in Citrus ovular callus by ABA, ethephon, CCC and alar and its suppression by GA3. *Z. Pflanzenphysiol*., 89: 427-432.

Kochba JP, Ben-Hayyim G, Spiegel-Ro YP, Neumann H and Saad S (1982). Selection of stable salt-tolerant callus cell lines and embryos in *C. sinensis* and *C. aurantium*. *Z Pflanzenphysiol*., 106: 111-118.

Koltunow AM, Soltys K, Nito N and McClure S (1995). Anther, ovule, seed, and nucellar embryo development in *Citrus sinensis* cv Valencia. *Can. J. Bot.,* 73: 1567–1582.

Koltunow AM, Brennan P and Protopsaltis S (1998). Regeneration of West Indian limes (*Citrus aurantifolia*) containing genes for decreased seed set. *Acta Hortic.,* 535: 81-92.

Kumar SV and Rajam MV (2005). Enhanced induction of *vir* genes results in the improvement of *Agrobacterium*-mediated transformation of eggplant. *J. Plant Biochem. Biotechnol*., 14: 89-94.

Laimer da Câmara Machado M, Câmara Machado A da, Hanzer V, Weiss H, Regner F, Steinkellner H, Mattanovich D, Plail R, Knapp E, Kalthoff B and Katinger H (1992). Regeneration of transgenic plants of *Prunus armeniaca* containing the coat protein gene of plum pox virus. *Plant Cell Rep.,* 11: 25–29.

Li DD, Shi W and Deng XX (2002). *Agrobacterium*-mediated transformation of embryogenic calluses of Ponkan mandarin and the regeneration of plants containing the chimeric ribonuclease gene. *Plant Cell Rep*., 21:153-156.

Louzada ES, Grosser JW, Gmitter FG, Nielsen B, Chandler JL, Deng XX and Tusa N (1992). Eight new somatic hybrid citrus rootstocks with potential for improved disease resistance. *HortSci.,* 27: 1033–1036.

Luth D and Moore G (1999). Transgenic grapefruit plants obtained by *Agrobacterium tumefaciens*-mediated transformation. *Plant Cell*, 57: 219-222.

Mante S, Morgens PH, Scorza R, Cordts JM and Callahan AM (1991). *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.) hypocotyl slices and regeneration of transgenic plants. *Biotechnology,* 9: 853–857.

Martin-Trillo M and Martinez-Zapater JM (2002). Growing up fast: manipulating the generation time of trees. *Curr. Opin. Biotechnol*., 13: 151–155.

Mathews H, Dewey V, Wagoner W and Bestwick RK (1998). Molecular and cellular evidence of chimaeric tissues in primary transgenics and elimination of chimaerism through improved selection protocols. *Transgenic Res.,* 7: 123-129.

Matzke AJM, Neuhuber F, Park Y-D, Ambros PF and Matzke MA (1994). Homology dependent gene silencing in transgenic plants: epistatic silencing loci contain multiple copies of methylated transgenes. *Mol. Gen. Genet*., 244: 219–229.

Mendes BMJ, Boscariol RL, Mourão Filho FAA and Almeida WAB (2002). *Agrobacterium*-mediated transformation of citrus Hamlin cultivar (*Citrus sinensis* L. Osbeck) epicotyl segments. *Pesquisa Agropecuária Brasileira*, 37: 955-961.

Meyer P, Linn F, Heidmann I, Meyer HZA, Niedenhof I and Saedler H (1992). Endogenous and environmental factors influence 35S promoter methylation of a maize A1 gene construct in transgenic petunia and its colour phenotype. *Mol. Gen. Genet*., 231: 345–352.

Molinari HBC, Bespalhok JC, Kobayashi AK, Pereira LFP and Vieira LGP (2004). *Agrobacterium tumefaciens*-mediated transformation of Swingle citrumelo (*Citrus paradisi* Macf. X *Poncirus trifoliata* L. Raf.) using thin epicotyl sections. *Sci. Hort*. 99: 379-385.

Moore GA, Jacono CC, Neidigh JL, Lawrence SD and Cline K (1992). *Agrobacterium*-mediated transformation of Citrus stem explants and regeneration of transgenic plants. *Plant Cell Rep*., 11: 238–242

Moore GA, Febres VJ, Niblett CL, McCaffery Luth D and Garnsey SM (2000). *Agrobacterium*-mediated transformation of grapefruit (*Citrus paradisi* macf.) with genes from citrus tristeza virus. *Acta Hort.,*. 535: 237-243.

Moreira-Dias JM, Molina RV, Bordon Y, Guardiola JL and Garcýa-Luis A (2000). Direct and indirect shoot organogenic pathways in epicotyl cuttings of Troyer citrange differ in hormone requirements and in their response to light. *Ann. Bot*., 85: 103–110.

Moreira-Dias JM, Molina RV, Guardiola JL and Garcia-Luis A (2001). Daylength and photon flux density influence the growth regulator effects on morphogenesis in epicotyl segments of Troyer citrange. *Sci. Hort*., 87: 275–290.

Mullins MG, Tang FCA and Facciotti D (1990). *Agrobacterium*-mediated genetic transformation of grapevines: transgenic plants of *Vitis rupestris* Scheele and buds of *Vitis vinifera* L. *Biotechnology,* 8: 1041–1045.

Murashige T and Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant,* 15: 473–479.

Murashige T and Tucker DPH (1969). Growth factor requirements of Citrus tissue culture. *Proc. First Intern. Citrus Symp*., 3: 1155–1161.

Nandadeva YL, Lupi YL, Lupi CG, Meyer CS, Devi PS, Potrykus I and Bilang R (1999). Microprojectile-mediated transient and integrative transformation of rice embryogenic suspension cells: effects of osmotic cell

conditioning and of the physiological configuration of plasmid DNA. *Plant Cell Rep.,* 18: 500-504.

Navarro L (1992). Citrus shoot tip grafting *in vitro*. In: Bajaj, YPS (Ed.), *Biotechnology in Agriculture and Forestry*, Vol.18. Springer, Berlin Heidelberg, pp. 327–338.

Navarro L, Olivares-Fuster O, Juarez J, Aleza P, Peña JA, Ballester-Olmos JF, Cervera M, Fagoaga C, Duran-Vila N and Peña L (2004). Applications of biotechnology to citrus improvement in Spain. *Acta Hort.*, 632: 221-234.

Niedz RP (1993). Culturing embryogenic protoplasts of 'Hamlin' sweet orange in calcium alginate beads. *Plant Cell Tiss. Org. Cult*., 34: 19–25.

Niedz RP (2006). Regeneration of somatic embryos from sweet orange (*C. sinensis*) protoplasts using semi-permeable membranes. *Plant Cell Tiss. Org. Cult*., 84: 353–357.

Nito N and Iwamasa M (1990). *In Vitro* plantlet formation from juice vesicle callus of Satsuma (*Citrus unshiu* Marc.). *Plant Cell Tiss. Org. Cult*., 20: 137-140.

Obukosia SD and Waithaka K (2000). Nucellar embryo culture of *Citrus sinensis* L. and *Citrus limon* L. *African Crop Sci. J*., 8: 109-116.

Ohgawara T, Kobayashi S, Ohgawara E, Uchimiya H and Ishii S (1985). Somatic hybrid plants obtained by protoplast fusion between *Citrus sinensis* and *Poncirus trifoliata*. *Theor. Appl. Genet*., 71: 1–4.

Ohgawara T, Uchimya H, Ishii S and Kobayashi S (1994). Somatic hybridization between *Citrus sinensis* and *Poncirus trifoliata*. In: Bajaj, YPS (Ed.) *Biotechnology in Agriculture and Forestry,*Vol. 27, Springer: Berlin, Heidelber, pp. 439–454.

Olivares-Fuster O, Fleming GH, Albiach-Marti MR, Gowda S, Dawson WO and Grosser JW (2003). Citrus tristeza virus (CTV) resistance in transgenic citrus based on virus challenge of protoplasts. *In Vitro Cell Dev. Biol. - Plant*, 39: 567-572.

Olivares-Fuster O, Duran-Vila N and Navarro L (2005). Electrochemical protoplast fusion in citrus. *Plant Cell Rep*., 24: 112–119.

Peach C and Velten J (1991). Transgene expression variability (position effect) of CAT and *GUS* reporter genes driven by linked divergent T-DNA promoters. *Plant Mol. Biol*., 17: 49–60.

Peña L, Cervera M, Juárez J, Ortega C, Pina JA, Durán-Vila N and Navarro L (1995a). High efficiency *Agrobacterium*-mediated transformation and regeneration of citrus. *Plant Sci*., 104: 183– 191.

Peña L, Cervera M, Juárez J, Navarro A, Pina JA, Durán-Vila N and Navarro L (1995b). *Agrobacterium*-mediated transformation of sweet orange and regeneration of transgenic plants. *Plant Cell Rep*., 14: 616–619.

Peña L, Cervera M, Juárez J, Navarro A, Pina JA and Navarro L (1997). Genetic transformation of lime (*Citrus aurantifolia* Swing.): factors affecting transformation and regeneration. *Plant Cell Rep*., 16: 731–737.

Peña L, Martin-Trillo M, Juarez J, Pina JA, Navarro L and Martínez-Zapater M (2001). Constitutive expression of *Arabidopsis LEAFY* or *APETALA1* genes in citrus reduces their generation time. *Nat. Biotechnol*., 19: 263–267.

Penä L, Perez RM, Cervera M, Juârez JA and Navarro L (2004). Early events in *Agrobacterium*-mediated genetic transformation of Citrus explants. *Ann. Bot.,* 94: 67-74.

Pérez-Clemente RM, Montoliu A, López P, López-Climent MF, Arbona V and Gómez-Cadenas A (2008). *In vitro* tissue culture approaches for the study of salt stress in citrus. *Biosaline Agriculture and High Salinity Tolerance,* Springer: Birkhauser Basel, pp. 37-42.

Pérez-Molphe-Balch E and Ochoa-Alejo N (1997). *In vitro* plant regeneration of Mexican lime and mandarin by direct organogenesis. *HortSci*., 32: 931-934.

Piestun D, Batuman O, Che X, Gofman R, Filatov V, Zypman S, Gafny R, Bar Joseph M, Goren R and Goldschmidt EE (2000). Trunacted version of the citrus tristeza virus (CTV) replicase and basta resistance genes incorporated in transgenic Troyer citrange. *Acta Hort.,* 535: 223-230.

Poupin MJ and Arce-Johnson P (2005). Transgenic trees for a new era. *In Vitro Cell Dev. Biol. - Plant,* 41: 91-101.

Rai M (2006). Refinement of the Citrus tristeza virus resistance gene (*Ctv*) positional map in *Poncirus trifoliata* and generation of transgenic grapefruit (*Citrus paradisi*) plant lines with candidate resistance genes in this region. *Plant Mol. Biol*. 61: 399-414.

Ray R and Walheim L (1980). Citrus. Los Angeles. Price Stern Sloan, Inc.

Rocha-Peña MA, Lastra R, Niblett CL, Ochoa-Corona FM, Garnsey SM and Yokomi RK (1995). Citrus tristeza virus and its aphid vector *Toxoptera citricida*. *Plant Dis*., 79: 437-445.

Rodriguez A, Cervera M, Peris JE and Pena L (2008). The same treatment for transgenic shoot regeneration elicits the opposite effect in mature explants from two closely related sweet orange (*Citrus sinensis* (L.) Osb.) genotypes. *Plant Cell Tiss. Org. Cult*., 93: 97-106.

Sanford JC, Smith FD and Russell AJ (1993). Optimizing the biolistic process for different biological applications. *Meth. Enzymol.,* 217: 483-509.

Sangwan RS, Bourgeois Y, Brown S, Vasseur G and Sangwan-Norreel B (1992). Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. *Planta,* 188: 439–456.

Saunt J 1990. Citrus varieties of the world. Sinclair International Ltd. pp.126.

Scheid OM, Jakovleva L, Afsar K and Maluszynska J (1996). A change of ploidy can modify epigenetic silencing. *Proc. Natl. Acad. Sci., USA,* 93: 7114–7119.

Schmulling T and Schell J (1993). Transgenic tobacco plants regenerated from leaf disks can be periclinal chimeras. *Plant Mol. Biol*., 21: 705-708.

Seguin A and Pena L (2001). Recent advances in the genetic transformation of citrus. *Trends Biotechnol.*, 19: 500-506.

Seki M, Komeda Y, Iida A, Yamada Y and Morikawa H (1991). Transient expression of β-glucuronidase in

Arabidopsis thaliana leaves and roots and *Brassica napus* stems using a pneumatic particle gun. *Plant Mol. Biol*., 17: 259-263.

Sekiya ME, Lawrence SD, McCaffery M and Cline K (1991). Molecular cloning and nucleotide sequencing of the coat protein gene of citrus tristexa virus. *J. Gen. Virol.*, 72: 1013-1020.

Seong ES and Song KJ (2008). Factors affecting the early gene transfer step in the development of transgenic 'Fuji' apple plants. *Plant Growth Regul*. 54: 89-95.

Sim GE, Goh CJ and Loh CS (1989). Micropropagation of *Citrus mitis* Blanco— Multiple bud formation from shoot and root explants in the presence of 6-Benzylaminopurine. *Plant Sci*., 59: 203–210.

Sinclair and Walter B (1984). The biochemistry and the physiology of the lemon and other citrus fruits. ANR Publications, Oakland.

Singh B, Sharma S, Rani G, Hallan V, Zaidi AA, Virk GS and Nagpal A (2008). In vitro micrografting for the production of Indian citrus ringspot virus (ICRSV)-free plants of kinnow mandarin (*Citrus nobilis* Lour X *C. deliciosa* Tenora). *Plant Biotechnol. Rep*., 2: 137-143.

Soost RK and Cameron JW (1975). Citrus. In: *Advances in Fruit Breeding*. (Janick, J. and Moore, J.N., eds.). Purdue University Press, West Lafayette, pp. 229-241.

Spolaore S, Trainotti L and Casadoro G (2001). A simple protocol for transient gene expression in ripe fleshy fruit mediated by *Agrobacterium*. *J. Exp. Bot.,* 52: 845-850.

Tagu D, Bergounioux C, Perennes C and Gadal P (1990). Inheritance of two foreign genes co-introduced into *Petunia hybrida* by direct gene transfer. *Plant Cell Tiss. Org. Cult.,* 21: 259–266.

Talon M and Gmitter Jr GF (2008). Citrus Genomics. *Intern. J. Plant Genomics*, 2008: 1-17.

Tomaz ML, Mendes BMJ, Mourao FDA, Demetrio CGB, Jansakul N and Rodriguez APM (2001). Somatic embryogenesis in Citrus spp.: carbohydrate stimulation and histodifferentiation. *In Vitro Cell Dev. Biol.- Plant,* 37: 446–452.

Tucker ML, Whitelaw CA, Lyssenko NN and Nath P (2002). Functional analysis of regulatory elements in the gene promoter for an abscission-specific cellulase from bean and isolation, expression, and binding affinity of three TGA-type basic leucine zippe transcription factors. *Plant Physiol*., 130: 1487-1496.

Usman M, Muhammad S and Fatima B (2005). *In vitro* multiple shoot induction from nodal explants of citrus cultivars. *J. Cent. Eur. Agric.,* 6: 435-442.

Van Duyn MAS and Pivonka E (2000). Overview of the health benefits of fruit and vegetable consumption for the dietetics professional. USDA National Nutrient Database for Standard Reference Release 17, 2005: Selected literature. *J. Amer. Diet. Assoc.,* 100: 1511-1521.

Vardi A, Spiegel-Roy P and Galun E (1975). Citrus cell culture: isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. *Plant Sci. Lett.*, 4: 231-236.

Vardi A (1981). Protoplast derived from different citrus species and cultivars. *Intern. Soc. Citricult. Proc.*, 1: 149-152.

Vardi A, Arzee-Gonen P, Frydman-Shani A, Bleichman S and Galun E (1989). Protoplast-fusion-mediated transfer of organelles from *Microcitrus* into *Citrus* and regeneration of novel alloplasmic trees. *Theor. Appl. Genet*., 78: 741-747.

Wang GL and Fang HJ (1998). Plant genetic engineering: principle and technique (in Chinese), Science Press, Beijing.

Xiang C and Roose ML (1988). Frequency and characteristics of nucellar and zygotic seedlings in 12 citrus rootstocks. *Sci. Hort.,* 37: 47-59.

Xiao-hong Y, Zhong-hail S and Rui-jian T (2006). Optimizing culture system of Ri T-DNA transformed roots for *Citrus grandis* cv. Changshou Shatian You. *Agric. Sci. China* 5*:* 90-97.

Yang ZN, Ingelbrecht IL, Louzada E, Skaria M and Mirkov TE (2000). *Agrobacterium*-mediated transformation of the commercially important grapefruit cultivar Rio Red (*Citrus paradisi* Macf.). *Plant Cell Rep*., 19: 1203-1211.

Yao JL, Wu JH, Gleave AP and Morris BAM (1996). Transformation of citrus embryogenic cells using particle bombardment and production of transgenic embryos. *Plant Sci*., 113: 175-183.

Yoshida T (1996). Graft compatibility of citrus with plants in the Aurantioideae and their susceptibility to citrus tristeza virus. *Plant Dis.,* 80: 414-417.

Yu C, Huang S, Chen C, Deng Z, Ling P and Gmitter FG (2002). Factors affecting *Agrobacterium*-mediated transformation and regeneration of sweet orange and citrange. *Plant Cell Tiss. Org. Cult.,* 71: 147-155.

Zou X, Li D, Luo X, Luo K and Pei Y (2008). An improved procedure for *Agrobacterium*-mediated transformation of trifoliate orange (*Poncirus trifoliata* L. Raf.) via indirect organogenesis. *In Vitro Cell Dev. Biol. Plant,* 44: 169-177.