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Citrus Transgenics: Current Status and Prospects

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ABSTRACT

Citrus is one important fruit crop in China and worldwide. Genetic transformation with more recently cloned target genes is an effective alternative for citrus improvement. In the past years, transformation systems using several explant sources such as protoplasts, embryogenic calluses, epicotyl segments and mature tissues have been well established and optimised. Selectable marker genes i.e. *GUS* and *GFP* are extensively used in citrus. Protoplast and embryogenic callus as explants are convenient and available at any time for transformation of seedless cultivars. Epicotyl segments as explants from seed germination are only available for seedy cultivars. *Agrobacterium*-mediated transformation is the most extensively used one with a few reports on PEG or electroporation-mediated protoplast transformation. With the optimized regeneration systems, genetic transformation with target genes such as *CTV* coat protein, green fluorescent protein (*GFP*, an *in vivo* visual marker), *LFY*, *AP1*, *CiTF* (to shorten juvenility), peptide *D* and *Xa21* (for potential citrus bacterial canker resistance), *Barnase* (to induce seedless fruit) and abiotic stress related genes, was conducted and numerous transgenic plants were regenerated from many citrus cultivars. Transgenic lines containing the *GFP* gene are also being used as a visual marker in citrus somatic fusion for several purposes. The prospects of transformation for citrus improvement are discussed.

Keywords: citrus, genetic transformation, stress resistance, fruit quality, cultivar improvement

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INTRODUCTION

Citrus is one most important fruit crop worldwide, it is just behind wheat and corn, ranking third among all the agricultural crops as far as international trade value is concerned (Deng 2005). The Chinese citrus industry ranks first for acreage and the production is the second largest in the world, after Brazil.

For the citrus industry, to improve fruit quality and reduce biotic and abiotic stresses are major breeding objectives at any time. Citrus traditional breeding methods (bud sports selection, cross and other breeding channels) are a laborious task, and often hampered by long juvenility, a high degree of heterozygosity, polyembryony, self-incompatibility and abortion of reproductive organs (Grosser and Gmitter 1990). Modern biotechnologies (genetic transformation and protoplast fusion) swarmed into practice and made it possible to overcome some of these breeding difficulties. Genetic transformation is a new approach that provides an avenue for modifying horticultural traits and providing new solutions to fulfill specific needs and study the gene function without altering their phenotype. Compared with traditional breeding methods, genetic transformation has its own characteristics: rational improvement of horticultural traits, widening the available gene pool, control and better understanding of gene expression and functions in plants, control of a single character through several desirable genes, reducing the time to introgress novel genes into elite backgrounds, *inter alia*. Although genome transfer via protoplast fusion is effective to solve problems such as sexual incompatibility, polyembryony, and male or female sterility encountered in conventional sexual crossing, transferring only the desirable gene into one *Citrus* species is difficult and nearly impossible. During protoplast fusion, desirable and undesirable traits in the whole genome of donor parent are all introduced into recipient partner at the same time. Comparatively, genetic transformation is more target-oriented than genome transfer via protoplast fusion.

OVERVIEW OF TRANSFORMED CITRUS: HISTORY, EXPLANTS, AND METHODOLOGY

Transgenic research in citrus was first reported by Kobayashi and Uchimyia (1989) and the first transgenic citrus plant was obtained by Vardi *et al*. (1990). Both reports used direct DNA transfer method and protoplast regeneration sys-

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tem. Since then, transgenic technology was reported in many citrus species, by various methods and using different explants. Various genes were introduced into citrus to improve agronomic traits including enhanced bacterial, viral, pest and environmental stress resistance, extended storage life span, shortening juvenile stage and improved fruit qualities.

Attempts to improve crop plants by genetic engineering depend strongly on the availability of reliable protocols for transformation, selection and regeneration. Transformation efficiency which usually means the ratio of antibiotic-resistant or GUS- or GFP-positive lines to total numbers of explants inoculated, is also crucial for successful transformation. Optimizing the protocol is essential for genetic transformation. For example, the use of proper co-cultivation medium and conditions, preculture of explants, use of acetosyringone or feeder plates and the optimized *Agrobacterium* strains, will lead to a higher number of stably transformed cells and an increase in the final number of regenerated transgenic plants. However, it is not easy to establish a sufficiently reliable and efficient citrus transformation system. A citrus transformation protocol was explored by examining effects of different factors on the transformation efficiency and combining the best treatment for each factor (Cervera *et al*. 1998c; Costa *et al*. 2002). Carrizo citrange transformation efficiency was improved by using epicotyl explants (Yu *et al*. 2002; Kayim *et al*. 2005b). Li *et al*. (2003) used embryogenic calluses as explants and developed a new transformation system, subsequently analyzing the factors influencing transformation efficiency. Dominguez *et al*. (2002, 2004) analyzed transformation efficiency under selective or non-selective conditions. The results showed that inefficient selection could be attributed to the protection of non-transformed cells from selective agent by the surrounding transformed cells, and to the persistence of kanamycin-resistance of *Agrobacterium*.

Explants used in citrus transformation include internodes, epicotyls, cotyledon, protoplasts, and embryogenic callus. Epicotyls and internodes are now often used in citrus transformation for their low cost and ease of regeneration. Over 90% of transgenic citrus plants were obtained by using these explants (**Table 1**). But seed availability and limited season hampered its usage. For this reason, embryogenic callus and protoplasts isolated from embryogenic callus are good substitutes. Calluses of most varieties could be induced, and used for transformation at anytime (Li *et al*. 2002). Protoplasts isolated from embryogenic calluses are good explants but difficulties of protoplast culture and regeneration also limit its development. Different genotypes, explants and culture methods also affect the transformation efficiency. With plasmolysis treatment, the eight different citrus species had different transformation efficiencies (Kayim and Koc 2005).

In plant genetic transformation, juvenile explants were mostly used for their high morphogenetic potential and low contamination rates. But in transformation, the juvenile period prolonged the time of early evaluation and plant propagation. Using mature tissue as explant could overcome these problems. The mature tissue is important for early evaluation of genetically modified characteristics, for shortening the time to release transgenic varieties into market, and for maintaining the true-to-type of the variety for those species whose seedlings are from zygotic instead of nucellar embryos. The transformation technique of citrus mature tissue was exploited in the past years (Cervera *et al*. 1998a; Almeida *et al*. 2003b). Cervera *et al*. (1998a) firstly showed a reliable method for the production of mature transgenic citrus plants via *Agrobacterium*-mediated transformation. They obtained transgenic sweet orange that could flower and bear fruit in 14 months. However, the regeneration capacity and transformation rate were low when mature tissue was used, and most researchers still use juvenile explants for transformation.

Presently, production of transgenic citrus is achieved using two alternative strategies. One is to utilize the natural ability of *Agrobacterium tumefaciens* to transfer T-DNA from a plasmid vector into citrus genome (Hidaka *et al*. 1990; Moore *et al*. 1992; Bond and Roose 1998; Kayim *et al*. 2004; Carneiro *et al*. 2006; Cevik *et al*. 2006). The other is the 'direct DNA transfer', which includes methods such as polyethylene glycol (PEG) mediated protoplast transformation (Kobayashi and Uchimiya 1989; Vardi *et al*. 1990; Fleming *et al*. 2000; Guo *et al*. 2005; Omar *et al*. 2006), protoplast electroporation (Vardi *et al*. 1990; Hidaka and Omura 1993; Niedz *et al*. 2002) and microprojectile bombardment (Yao *et al*. 1996). Among these methods, *Agrobacterium tumefaciens* mediated transformation is most extensively used (**Table 1**). It was demonstrated that cells competent for transformation are located in the newly formed callus growing from the cambial ring. Conditions conducive to further development of this callus, such as treatment of explants in a medium rich with auxins, resulted in more pronounced formation of cambial callus and slower shoot regeneration process, both in *Agrobacterium*-inoculated and non-inoculated explants. Furthermore, co-cultivation in the medium rich in auxins caused a significant increase in the rate of actively dividing cells in the S-phase when cells are more prone to integrate foreign DNA (Peña *et al*. 2004b). Increasing the wounding area of explants by cutting internodes longitudinally into two halves, and optimization of inoculation density, dramatically enhanced both regeneration and transformation frequency (Yu *et al*. 2002). Adding 2,4-dichlorophenoxyacetic acid (2,4-D) into explant pretreatment medium and co-culture medium also improved transformation efficiency (Costa *et al*. 2002; Peña *et al*. 2004b).

For gene promoters being used in citrus transformation, in almost all cases, CaMV 35S or double 35S were extensively applied.

Transgene silencing is a problem that occurs in citrus transformation, and host genes can also be silenced affected by the homologous transgene, thus limiting the potential application of gene transformation. The structure of a transgene locus is a major influence on the level and stability of transgene expression (Kohli *et al*. 2003). Mexican lime (*Citrus aurantifolia*) plants transformed with the p25 coat protein gene of *Citrus tristeza virus* (CTV) presented more than 30% silencing among the regenerates under non-selective conditions; and in all cases, silencing affected the transgene incorporated; inverted repeats as well as direct repeats and even single integrations could also trigger gene silencing (Dominguez *et al*. 2002). Among grapefruit (*Citrus paradisi* Macf. cv. 'Duncan') plants transformed with several sequences from CTV, a great variability in titer was observed in both controls and transgenic plants, and all were apparently susceptible to the virus, although some transgenic plants averaged lower titers of virus than controls (Febres *et al*. 2003). Dominguez (2002) observed that selection impedes the regeneration of plants with silenced transgenes.

TARGET GENES INTRODUCED INTO CITRUS

Except for gene function study, different genes have been introduced into citrus, which comprise antibiotic and reporter genes, shortening the juvenile phase genes, abiotic-stress tolerance genes, disease and insect resistance genes, and fruit quality related genes.

Antibiotic and reporter genes

In any plant genetic transformation system, a marker gene is important to recover a high proportion of transgenic plants from transgenic events. Antibiotic and reporter genes were first introduced into citrus species for exploring and optimizing citrus transgenic system and methodology. Much work has been done to improve transformation methods using only marker genes. The most commonly used marker gene is the neomycin phosphotransferase gene (*npt*II), which confers resistance to the antibiotic kanamycin (Peña *et al*. 2004a). Selection efficiency of this marker

Table 1 Different genes introduced into citrus.

Variety	Explant	Method	Target genes	Reference
Antibiotic and reporter genes				
Trovita sweet orange	EC	PEG	*NPTII*	Kobayashi and Uchimiya 1989
Washington navel, Trovita	EC	Ag	*NPTII*	Hidaka *et al.* 1990
Rough lemon	P	E	*NPTII*	Vardi *et al.* 1990
Trifoliate orange	ES	Ag	*NPTII*	Kaneyoshi *et al.* 1994
Carrizo citrange and Lime	ES	Ag	*GUS*	Moore *et al.* 1992
Ohta ponkan	P	E	*GUS*	Hidaka and Omura 1993
Pineapple sweet orange	ISS	Ag	*GUS*	Peña *et al.* 1995a
Carrizo citrange	ES	Ag	*GUS*	Peña *et al.* 1995b
Page tangelo	EC	PB	*GUS*	Yao *et al.* 1996
Mexican lime	ISS	Ag	*GUS*	Peña *et al.* 1997
Washington navel orange	ES	Ag	*GUS*	Bond and Roose 1998
Pineapple	MISS	Ag	*GUS*	Cervera *et al.* 1998a
Washington navel orange, Fino	ES, ISS	Ag	*GUS*	Cervera *et al.* 1998b
Carrizo citrange	ES	Ag	*GUS*	Cervera *et al.* 1998c
Citrange, lime	ES	Ag	*GUS*	Peña *et al.* 1998
Mexican lime	ISS	Ar	*GUS*	Perez-Molphe-Balch and Ochoa-Alejo 1998
Duncan grapefruit	ES	Ag	*GUS*	Luth and Moore 1999
Lemon, Alemow, Cleopatra mandarin	ISS	Ag	*GUS*	Ghorbel *et al.* 2001a
Hamlin sweet orange	ES	Ag	*GUS*	Mendes *et al.* 2002
Xuegan, Carrizo citrange	ES	Ag	*GUS*	Yu *et al.* 2002
Valencia Natal and Rangpur	ES	Ag	*GUS*	Almeida *et al.* 2003a
Pera, Valencia, Hamlin, Natal oranges	MISS or leaf discs	Ag	*GUS*	Almeida *et al.* 2003b
Carrizo citrange, Mexican lime	ES, ISS	Ag	*GUS*	Dominguez *et al.* 2004
Swingle citrumelo	TES	Ag	*GUS*	Molinari *et al.* 2004a
Carrizo citrange	ES	Ag	*GUS*	Peña *et al.* 2004b
'Milam' rough lemon, 'Volkamer' lemon, Rangpur lime, 'Hamlin' sweet orange, 'Duncan' grapefruit, sour orange, 'Cleopatra' mandarin, Carrizo citrange	ES	Ag	*GUS*	Kayim and Koc 2005
Carrizo citrange	ES	Ag	*GFP*	Ghorbel *et al.* 1999
Itaborai sweet orange	P	PEG	*GFP*	Fleming *et al.* 2000
Cleopatra mandarin	EC	Ag	*GFP*	Shi *et al.* 2002
Hamlin sweet orange	P	E	*GFP*	Niedz *et al.* 2002
Carrizo citrange, Mexican lime	ES, ISS	Ag	*GFP*	Dominguez *et al.* 2004
Valencia sweet orange	P	PEG	*GFP*	Guo *et al.* 2005
'Milam' rough lemon, 'Volkamer' lemon, Rangpur lime, 'Hamlin' sweet orange, 'Duncan' grapefruit, sour orange, 'Cleopatra' mandarin and carrizo citrange	ES	Ag	*GFP*	Kayim and Koc 2005
Duncan grapefruit	ES	Ag	*GFP*	Zheng *et al.* 2006
Valencia, Hamlin, Natal and Pera oranges	ES	Ag	Phosphomannose-isomerase (*PMI*) gene	Boscariol *et al.* 2003a
Pineapple sweet orange, Carrizo citrange	ES	Ag	*ipt* gene (MAT system)	Ballester *et al.* 2007
Genes shortening the juvenile phase				
Carrizo citrange	ISS	Ag	*LEAFY*, *APETALA1*	Peña *et al.* 2001
Trifoliate orange	ES	Ag	*CiFT*	Endo *et al.* 2005
Abiotic-stress tolerance genes				
Carrizo citrange	ISS	Ag	*HAL2*	Cervera *et al.* 2000
Carrizo citrange, Poncirus trifoliata	ISS	Ag	Antisense chilling-inducible ACC synthase trifoliata gene (*CS-ACS1*)	Wong *et al.* 2001
Carrizo citrange	ES	Ag	Citrus blight-associated gene	Kayim *et al.* 2004
Carrizo citrange	ES	Ag	Pyrroline-5-carboxylate synthetase mutant gene (*p5cs*)	Molinari *et al.* 2004b
Swingle citrumelo	ES	Ag	Enzyme ^{1}-pyrroline-5-carboxylate synthetase (P5CS)	Carneiro *et al.* 2006
Troyer citrange	ES	Ag	*rol* A B C	Cirvilleri *et al.* 2003
Citrange	ES	Ag	*rol* A B C	Hu *et al.* 2006a, 2006b
Troyer citrange	ES	Ag	GA 20-oxidase gene	Fagoaga *et al.* 2007
Disease and insect resistance genes				
Jincheng, Xinhuicheng, Shatian pummelo	ES	Ag	Antibacterial peptide D gene	Chen *et al.* 1997
Ridge Pineapple, Brazilian Sour orange, Key lime, Carrizo citrange	ISS	Ag	*CP-CTV*	Gutierrez-E *et al.* 1997
Trifoliate orange	ES	Ag	*CP-CTV*	He *et al.* 1998
Mexican lime	ISS	Ag	*CP-CTV*	Dominguez *et al.* 2000
Mexican lime	ES	Ag	*CP-CTV*	Dominguez *et al*. 2000
Sour orange	ISS	Ag	*CP-CTV*	Ghorbel *et al.* 2000
Duncan grapefruit	ES	Ag	*CP-CTV*	Moore *et al.* 2000
Duncan grapefruit	ES	Ag	*RNA-dependent RNA polymerase*	Moore *et al.* 2000
Troyer citrange	ES	Ag	*CTV-RdRp*, *Basta*	Piestun *et al.* 2000
Rio Red grapefruit	ES	Ag	Untranslatable coat protein gene (uncp)-CTV	Yang *et al.* 2000

Table 1 (Cont.)

Variety	Explant	Method	Target genes	Reference
Pineapple sweet orange	ISS	Ag	Tomato pathogenesis I sweet orange related protein *PR-5*	Fagoaga *et al.* 2001
Mexican lime	ISS	Ag	*P23-CTV*	Ghorbel *et al.* 2001b
Trifoliate orange	ES	Ag	*CP-CTV*	He *et al.* 2001
Mexican lime	ISS	Ag	*P25-CTV*	Dominguez *et al.* 2002a, 2002b
Hamlin sweet orange	ES	Ag	Attacin A	Boscariol *et al.* 2003b, 2006
Duncan grapefruit	ES	Ag	*CP-CTV*	Febres *et al.* 2003
Hamlin sweet orange	P	PEG	Xa21	Guo and Grosser 2004
Jincheng sweet orange	ES	Ag	Hepatitis A capsid protein fusion gene	Hu *et al.* 2004
Trifoliate orange	ES	Ag	*CP-CMV*	Iwanami *et al.* 2004
Ponkan mandarin	Nucelli	Ag	*Bacillus thuringiensis* (Btt)	Rhim *et al.* 2004
Lime, Sevillano, Sweet and Sour orange and Trifoliate orange	ISS	Ag	*P23-CTV*	Fagoaga *et al.* 2005, 2006
Grapefruit 'Rio Red', 'Ruby Red', 'Duncan' and sweet orange 'Hamlin'	ES	Ag	Bovine lysozyme gene and spinach defensin gene	Gonzalez *et al.* 2005
Duncan grapefruit, Hamlin sweet orange, Carrizo citrange	ES	Ag	*CP- CPsV*	Kayim *et al.* 2005
Hamlin sweet orange	P	PEG	*Xa21*	Omar and Grosser 2005; Omar *et al.* 2006
Rangpur lime	ES	Ag	*Bacterio-opsin* (*bO*)	Azevedo *et al.* 2006
Duncan grapefruit	ES	Ag	*CTV-RdRp*	Cevik *et al.* 2006
Shatianyou pummelo	ES	Ag	*ShivaA*	Han *et al.* 2006
Ruby red, Rio red or Duncan	ES	Ag	Candidate CTV resistance gene	Rai *et al.* 2006
Femminello lemon	ES	Ag	Chit42	Gentile *et al.* 2007
Fruit quality related genes				
Trifoliate orange	ES	Ag	*hEGF*	Kobayashi *et al.* 1996
Duncan grapefruit	ES	Ag	Carotenoid biosynthetic genes	Costa *et al.* 2002
Ponkan mandarin	EC	Ag	pTA29-barnase gene	Li *et al.* 2002
Valencia orange	EC	Ag	pTA29-barnase gene	Li *et al.* 2003a
Anliucheng	EC	Ag	pTA29-barnase gene	Li *et al.* 2003b
Valencia sweet orange	P	PEG	Pectin methylesterase gene (*PME*)	Guo *et al.* 2005

Note: Ar, *Agrobacterium rhizogenes*-mediated transformation; At, *Agrobacterium tumufaciens*-mediated transformation; *Btt*, Modified Δ-endotoxin gene of *Bacillus thuringiensis*; C, cotyledon; *CiFT*, othologue gene of *FT* (*FLOWERING LOCUS T*) in citrus; CP, coat protein; CTV, *Citrus tristeza virus*; EC, embryogenic callus; E, electroporation; ES, epicotyl segments; *GFP*, green fluorescent protein gene; *GUS*, β-glucuronidase; *HAL2*, halotolerance gene; ISS, internodal stem segments; MISS, mature internodal stem segments; *NPTII*, neomycin phosphotransferase II; P, protoplast; PB: particle bombardment; PEG: polyethylene glycol; *RdRp*, RNA dependent RNA polymerase (*FLOWERING LOCUS T*); TES, thin epicotyl segments

gene is highly variable among citrus genotypes, especially in citrange transformation (Cervera *et al.* 1998a). Nevertheless, a low transformation efficiency was obtained in the cases of mature sweet orange (Cervera *et al.* 1998b) and sour orange (Ghorbel *et al.* 2000). In order to improve mature explant transformation efficiency, the *ipt* gene was used in citrus transformation with the easy visual detection of the gene phenotype (Ballester *et al.* 2007). Besides the *npt*II gene, hygromycin phosphotransferase (*hpt*) gene and phosphinothricin acetytransferase gene (*bar*), are also being used in citrus transformation.

β-glucuronidase gene (*uid*A or *GUS*) was the first reporter gene used in citrus transformation (Moore *et al.* 1992). The reason for the extensive use of *uid*A gene was the stable GUS assay and high sensitivity and amenability. And it can assay gene expression by qualitative (histochemical assay) and quantitative (fluorometric or spectrophotometric assay) detection. Compared with the *uid*A gene, green fluorescent protein (GFP) has a number of desirable traits as the reporter gene. Direct visualization of gene expression in individual cells is therefore possible without cell lysis and subsequent biochemical analysis, and tissue distortion caused by fixation, staining and section could be avoided (Chiu *et al.* 1996). By spatial visualization and revealing temporal patterns of gene expression *in vivo*, GFP facilitated citrus transformation (Ghorbel *et al.* 1999; Fleming *et al.* 2000; Shi *et al.* 2002; Niedz *et al.* 2002; Guo *et al.* 2005; Liu *et al.* 2006). At the same time, GFP could also be used as a quantitative reporter of gene expression. It was suggested that the use of *gfp* could significantly reduce labor, cost and time in citrus transformation system (Ghorbel *et al.* 1999; Liu *et al.* 2006; Duan *et al.* 2007).

As part of the process in citrus transformation, selectable markers are used to select transgenic cells, from which intact transgenic plants could be regenerated (Ghorbel *et al.* 1999). However, once transformation is accomplished the presence of the marker gene becomes not only unnecessary and even undesirable but also generating environmental and consumer concerns. Several strategies have been proposed to produce transgenic plants free from selectable marker genes, but little was done on citrus. An attractive alternative is to use the safe selectable marker genes or produce marker-free transgenic plants. In order to avoid the use of antibiotics or herbicides in genetic transformation, the reporter gene GFP as a safe marker was first used (Ghorbel *et al.* 1999; Dominguez *et al.* 2002b). With its extensive use as a reporter gene, the GFP gene has a potential application in citrus transgenics and other fields, and several new GFP plasmids for citrus transformation were constructed (Chen *et al.* 2007). Boscariol *et al.* (2003a) and Ballester *et al.* (2007) used a positive selection system to recover transgenic citrus. The MAT system (multi-auto-transformation), which combines the *ipt* gene for positive selection with the recombinase system R/RS for removal of marker genes from transgenic cells, was also used in citrus transformation (Ballester *et al.* 2007). And it was found the *ipt* gene was an efficient positive selection marker, which was successfully removed from 65% of sweet orange transformants.

Shortening the juvenile phase

Citrus cultivars have a long juvenile period, and it delays their reproductive development by between 6 and 20 years, depending on the species. Flower regulating genes like *LEAFY* (*LFY*) and *APETALA1* (*AP1*) from *Arabidopsis* were used to shorten citrus juvenility and promote precocious flowering. Peña *et al.* (2001) demonstrated that constitutive expression of these two genes significantly shor-

tened the juvenile phase of citrus trees. Both types of transgenic trees flowered in consecutive years under environmental control. And plants harboring one of the two genes flowered and produced fruits within one year after seed germination. The accelerated juvenile period was heritable in crosses with non-transformed plants. Recently, Endo *et al*. (2005) reported transgenic trifoliate orange (*Poncirus trifoliata* L. Raf.) showed very early flowering and fruiting (i.e. 12 weeks after transfer to the greenhouse) when overexpressing *Citrus FT* (*CiFT*, homolog of *Arabidopsis FLOWERING LOCUS T*). The results not only modified citrus genotypes but also provided an available tool to test gene function within a short time.

Abiotic-stress tolerance

Tolerance or resistance to stresses such as cold, drought and salinity is one major target for citrus breeding. Citrus-growing regions include the Mediterranean, subtropical, semitropical, and tropic zones. So the environment is a restricting factor of citrus production. There are several successful reports with modified stress tolerance by transformation such as drought and salt tolerance of Carrizo citrange (Cervera *et al*. 2000a; Wong *et al*. 2001). But research on citrus cold-tolerance is rare.

Rootstock improvement is one major objective to increase abiotic-stress tolerance. The potential CTV resistant genes (the following text) and the tolerance to the salinity gene *HAL2* (Cervera *et al*. 2000a) have been introduced into Carrizo citrange and the transgenic plants expressed some resistance. Dwarfness is another target for rootstock improvement. *RolA*, *B*, and *C* genes from *Agrobacterium rhizogenes* single or combined together were transformed into citrus (Cirvilleri *et al*. 2003; Hu *et al*. 2006). Transgenic plants showed evident dwarfing characteristics, and by propagation of *rolA*, *B*, *C* transgenic citrus plants (Hu *et al*. 2006), transgenic dwarfing rootstock could be released in the future. Dwarfing transgenic 'Troyer' citrange lines were recently recovered by overexpressing the GA 20-oxidase gene (Fagoaga *et al*. 2007).

Disease and insect resistance genes

Disease and insect can cause severe losses by substantially reducing yield, affecting fruit quality, and shortening the lifespan of infected plants. Genetic transformation allows the insertion of specific disease and insect resistance traits directly into desirable elite varieties. CTV and citrus bacterial canker (CBC) are two most devastating diseases of citrus worldwide (Bar-Joseph *et al*. 1989). Traditional breeding could not solve the resistance problem. Genetic transformation provides an efficient strategy to develop new types of citrus resistant to the diseases. Presently, one major objective of citrus transformation is to produce transgenic plants resistant to CTV mediated by pathogen-derived genes. Though the coat protein genes of CTV have been introduced into citrus (Gutierrez-E *et al*. 1997; Dominguez *et al*. 2000, 2002a, 2002b; Febres *et al*. 2003), no consistent resistance characters had been expressed. At the same time most Mexican lime lines expressing the p23 gene failed to develop resistance to CTV (Ghorbel *et al*. 2001b; Fagoaga *et al*. 2005). It was reported that transgenic citrus expressing a translatable p23 gene are showing symptoms similar to CTV-induced symptoms. In order to alleviate the limitations, candidate resistance genes (i.e. candidate *R* gene, CTV-Rdrp) have been introduced into citrus varieties and expressed some resistance in limiting the virus infection (Cevik *et al*. 2006; Rai 2006). In addition, Fagoaga *et al*. (2006) revealed that some propagated silenced lines were immune in the p23 transgenic Mexican limes. And the phenotype of propagations from the same transgenic line indicates that factors other than genetic background of transgenic plants, i.e. environmental conditions or developmental stage, play a key role in PTGS-mediated resistance.

Citrus canker, caused by the bacterial pathogen *Xanthomonas axonopodi* pv. *citri*, is also a serious disease of most commercial citrus cultivars and some citrus relatives. The potential antibacterial peptide *D* gene (Chen *et al*. 1997), the rice *Xa21* gene (Guo *et al*. 2004; Omar and Grosser 2005) and the *Attacin A* gene (Boscariol *et al*. 2003b, 2006) were introduced into citrus varieties and transgenic plants are being evaluated for canker resistance. Besides these, citrus transformation with resistance genes such as bovine lysozyme gene, spinach defensin gene (Gonzalez *et al*. 2005), tomato pathogenesis I sweet orange related protein *PR-5* (Fagoaga *et al*. 2001), and *CP-CMV* (Iwanami *et al*. 2004) were also reported, and transgenic plants with these genes expressed resistance to some extent.

In addition, insects often destroy citrus fruit tree on leaf, stem, root, flower and fruit, and lead to the decrease of both fruit quality and production. A modified Δ-endotoxin gene of B. *thuringiensis* subsp. *tenebrionis* (*Btt*), encoding a coleopteran-specific toxin, was utilized to transform 'Ponkan' mandarin. Hybridization experiments demonstrated that the transgenic citrus contained and expressed the toxin protein gene (Rhim *et al*. 2004). Transgenic lemon plants expressing a *Trichoderma harzianum* chitinase gene showed enhanced resistance to *Phoma tracheiphila* and *Botrytis cinerea* (Gentile *et al*. 2007).

Fruit improvement

The variety trend of fresh citrus fruit is easy peeling, seedless, strong flavour and aroma (Deng 2005). Genetically modified citrus for such a target have been reported. Genes to modify fruit traits include carotenoid biosynthetic genes controlling fruit and juice color, Barnase gene for fruit seedlessness (Koltunow *et al*. 2000; Li *et al*. 2002, 2003a, 2003b), and a juice quality related pectin methylesterase gene (Guo *et al*. 2005).

Most genes transformed into citrus expressed stably. Cervera *et al*. (2000b) exploited the stability of gene expression and phenotype through analyzing citrus transgenic population. Transgene integration and expression revealed that: (1) a significant negative correlation was found between copy number and GUS activity; (2) rearrangements of the T-DNA inserts did not imply low expressing levels; and (3) stability of integration and expression of the transgenes was confirmed for all transformants growing under natural environmental conditions. All these results confirmed that transformation technology could modify citrus traits, improve heredity and promote field production. Currently, main problems faced by citrus transformation are not to obtain transgenic citrus, but how to properly utilize it. The effect of storage and processing on plasmid, yeast and plant genomic DNA stability in juice from genetically modified oranges was recently reported (Weiss *et al*. 2007).

Great progress has been made in citrus genetic engineering. Though many genes have been introduced into citrus and expressed stably, few of them were cloned from citrus. Currently, some genes had been cloned from citrus and transformed into citrus, e.g. the ten candidate *Ctv* resistance genes (*R*-1, *R*-2,…, *R*-10) cloned from *Poncirus trifoliata*, were transformed into 'Ruby Red', 'Rio Red' and 'Duncan', three CTV susceptible grapefruit varieties (Rai 2006). Whereas many genes cloned from citrus have not been utilized efficiently, such as the cold related genes (Jia *et al*. 2004; Lang *et al*. 2005), MADS-box cDNAs (Endo *et al*. 2006), the Miraculin-like proteins (Tsukuda *et al*. 2006), the phytoene synthase geens (DQ235260, DQ109038, AB114664, AB114648, AY204550, etc.), phytoene desaturase (EF193860, DQ235261, AB114657, etc.), acid invertase (AB074886, AB074885, etc.), ACC synthase (AJ276295, AJ011095, AJ012696, etc.). Efficient utilization of these genes cloned from citrus will greatly expedite citrus improvement by genetic engineering.

FURTHER USES OF TRANSGENIC CITRUS IN PROTOPLAST FUSION

Transgenic citrus not only holds direct potential for cultivar improvement, could also be used in further study by biotechnological approaches. For instance, transgenic gfp cell lines could be used as a visual marker in somatic fusion (Olivares-Fuster *et al*. 2002). By fusion of embryogenic callus protoplasts with gfp transgenic mesophyll protoplasts, and facilitated by gfp expression and visualization in hybrid cells, somatic hybrid vigor or regeneration advantage was revealed and evidenced (Guo and Grosser 2005). Currently, using the fusion model of transgenic gfp embryogenic callus protoplasts + mesophyll protoplasts (Cai *et al*. 2006), studies on mechanism for cybrid regeneration via symmetric fusion, are being conducted. Furthermore, cybrids produced by this fusion model will have no gfp contamination and hold commercial potential if they are proved seedless. Hopefully this study will further facilitate targeted cybridization to produce more cybrids between male sterile Satsuma and seedy citrus cultivars for potential seedlessness.

SUMMARY AND CONCLUSIONS

Citrus is one most extensively growing fruit crop worldwide. Genetic transformation has become an attractive method for improving citrus species by modifying one or two traits without changing the cultivar integrity. Since the first report of transgenic citrus in late 1980s, great progress was achieved, including the establishment of efficient transformation system, and successive regeneration of genetically transformed citrus plants. And there are many *in vitro* protocols such as callus and cell suspension cultures, organogenesis induction, and protoplast manipulation that are available for transformation. Transgenic citrus plants were obtained by direct DNA transfer into protoplasts, particle bombardment of nucellar embryogenic cell suspensions and co-cultivation of internodes or epicotyl segments with *Agrobacterium*. The most widely used method of gene transfer in citrus is the *Agrobacterium*-mediated transformation of epicotyl segments. Using this system, transgenic plants of citrus species and relatives were obtained, including sweet oranges, sour oranges, limes, grapefruit, and Carrizo citrange rootstock. As for the transgenes, more and more agronomic genes were introduced into citrus plants. These include the citrus tristeza virus (CTV) resistant genes (CP-CTV, p23, *R* genes, etc.), citrus mosaic virus (CiMV) coat protein, the halotolerance gene *HAL2* originally isolated from yeast that confers tolerance to salinity, Arabidopsis *LEAFY* and *APETALA1* genes and citrus *FT* gene that promote early flower initiation, carotenoid biosynthetic genes from fruits of *C. paradisi* cv. 'Flame' that control fruit and juice color, and *CS-ACS1* gene from *Citrus sinensis* that controls the ethylene biosynthesis, *Xa21* providing broad spectrum *Xanthomonas* resistance in rice having potential citrus canker disease resistance. And recently, chimaeric ribonuclease gene (*barnase*) for seedlessness and a juice quality related pectin methylesterase gene (*PME*) from Valencia orange was introduced into sweet orange by Li *et al.* (2003) and Guo *et al.* (2005), respectively.

There are four transformation strategies worthy to note. First, mature tissue transformation (Cervera *et al*. 1998a, 2005). The reliable method for producing mature transgenic citrus plants via *Agrobacterium* was proceeded with *C. sinensis* L. Osbeck cv. 'Pineapple', 'Hamlin', 'Pera', 'Valencia' and 'Natal', though only the former two cultivars succeeded. Second, thin epicotyl section transformation for Carrizo citrange (*C. sinensis* × *Poncirus trifoliata*), and Swingle citrumelo (*C. paradisi* × *P. trifoliata*) was provided (Monlinari *et al*. 2004a). The third is rootstock improvement. Modified rootstocks by genetic transformation will have more advantages than on the scion, since rootstocks play an important role of a tree in its tolerance to stresses, and less concern of GMO safety is imposed on rootstocks than on scions. The fourth is to obtain safe selectable marker or marker-free transgenic citrus. The public concern about GMO safety, especially the negative attitude to the edible fresh fruits, hindered citrus transgenic research. Marker-free strategy is important for citrus transgenics.

Though great achievements have been made, there are still some problems to be addressed in citrus transformation. First, basic research including gene cloning and genetic mapping are poor for citrus. The weak genetic background hindered citrus transgenic research, and most work was based on experience or skills. Second, establishment of multiple gene transformation system is needed. Most characteristics were controlled by interaction of multi-genes, thus one or two modified genes could not result in enough transgenic traits for commercial use. Third, utilization of transgenic plants in citrus production is limited. Many transgenic citrus plants were obtained, but there has no commercial use yet, even for rootstock improvement.

Based on the present situation, it could be predicted that, improvement of citrus rootstocks tolerant to biotic and abiotic stresses via genetic transformation will result in better achievement out of all traits and could be utilized in the near future.

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