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Transgenic Trifoliate Orange (*Poncirus trifoliata* L. Raf.)

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ABSTRACT

Trifoliate orange (*Poncirus trifoliata* L. Raf.) is the only recognized species of *Poncirus*, which is closely related to *Citrus*. In Japan, trifoliate orange has been used as a rootstock for the cultivation of most citrus cultivars because many citrus cultivars are compatible with this species, which is cold-tolerant and resistant to the tristeza virus. However, some cultivars require a more diverse range of potential rootstocks. Genetic transformation of citrus genotypes by various methods has been reported, including *Agrobacterium*-mediated transformation, polyethylene glycol (PEG)-mediated transformation, and particle bombardment. *Agrobacterium*-mediated transformation using epicotyl segments has been most widely used for genetic transformation of trifoliate orange in recent years. In addition, transgenic hybrids have been obtained, including Carrizo citrange (*C. sinensis* × *P. trifoliata*) and Swingle citrumelo (*C. paradisi* × *P. trifoliata*). Introduction of foreign genes into trifoliate orange and its hybrids has resulted in diverse plant characteristics, and the suitability of these transgenic plants as rootstocks must be examined. This manuscript reviews the genetic transformation of trifoliate orange, and discusses the transformation procedures and the characteristics of the transgenic plants.

Keywords: *Agrobacterium*, citrange, citrumelo, citrus, rootstock, transformation
Abbreviations: AP1, APETALA1; **BAP**, benzylaminopurine; **CiMV**, Citrus mosaic virus; **GUS**, β-glucuronidase; **LFY**, LEAFY; **NAA**, α-naphthalene acetic acid; **pCP**, capsid polyprotein; **PEG**, polyethylene glycol

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INTRODUCTION

Trifoliate orange (*Poncirus trifoliata* L. Raf.) is used as a rootstock for the cultivation of most citrus cultivars in Japan. Many citrus cultivars are compatible with this species, and citrus trees grafted on it show good fruit quality and high yield, even though the fruits of trifoliate orange itself are inedible. Trifoliate orange is cold-tolerant and resistant to the citrus tristeza virus, but is susceptible to the citrus exocortis viroid.

Trifoliate orange shows surprisingly little variation given that it has been grown in China for thousands of years and in Japan since at least the eighth century (Swingle and Reece 1967). Trifoliate orange is a highly distinctive deciduous shrub or small tree with large, stout spines and small compound leaves with winged petioles and three leaflets. In smaller stems, the pith is discontinuous, and is present in the form of transverse plates. The flower buds are small, single, and lateral, are protected by small fleshy scales, and are formed early in the summer. The flowers have very short stalks with five white, thin, papery petals, numerous stamens with free filaments, and a compound pistil (Hodgson, 1967).

Although the species differs remarkably from *Citrus* in nearly all respects, *Poncirus* hybridizes freely with *Citrus* species. Crossing of trifoliate orange with citrus has resulted in hybrid species, including citrange (*C. sinensis* × *P. trifoliata*), citrumelo (*C. paradisi* × *P. trifoliata*), and citrandarin (*C. reticulata* × *P. trifoliata*). Some have proved to be valuable as vigorous, hardy, disease-resistant rootstocks for ordinary citrus fruit trees.

Carrizo citrange (*C. sinensis* × *P. trifoliata* L. Raf.) is currently the most extensively used citrus rootstock, especially in Spain and North America. It has been demonstrated that Carrizo citrange originated from a cross between Washington navel orange and trifoliate orange that was made in 1909 (Savage and Gardner 1965). Carrizo citrange is salt-sensitive (Castle 1987; Maas 1992) and susceptible to citrus blight (Kayim *et al*. 2004).

Swingle citrumelo (*C. paradisi* Macf. × *P. trifoliata* L. Raf.) has been an important commercial rootstock in Florida since the late 1980s. This hybrid is tolerant of blight, the citrus tristeza virus, and citrus nematodes, and has a reputation for cold tolerance. But recently, it has been shown

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that it is not suitable for every location where sour orange was previously successful (Bauer *et al.* 2005).

In recent years, citrus fruits of increasing quality have been required. The quality of fruits often depends on their rootstock. In addition, areas where certain rootstocks can be planted are limited because of their tolerance of local soil conditions and resistance to various diseases and pests. In consequence, some citrus scion varieties require the availability of more diverse rootstocks.

In several countries, breeding programs have been carried out to produce new citrus rootstocks. Carrizo citrange and Swingle citrumelo are significant results of breeding programs based on traditional hybridization. Later C35 and C32 citrange rootstocks were produced from hybridization of 'Ruby' orange and trifoliate orange that was made in 1951 at the University of California Citrus Research Center (Cameron *et al.* 1954). However, it takes too long years to develop and release new rootstocks for citrus scions by means of traditional hybridization. *Citrus* and *Poncirus* trees have a long juvenile phase that delays their reproductive development, and some *Citrus* and *Poncirus* genotypes cannot easily be used as parents, because they have pollen or ovule sterility, and sometimes both. All these characteristics prevent the genetic improvement of *Citrus* and *Poncirus* by means of traditional hybridization.

In recent years, genetic transformation methods have been used in the genetic improvement of *Citrus* and *Poncirus*, and the performance of trifoliate orange and its hybrids as rootstock has been improved as a result. The rest of this section reviews the genetic transformation of trifoliate orange and its hybrids, and discusses the transformation procedures and the characteristics of the transgenic plants.

GENERAL REVIEW OF GENETIC TRANSFORMATION OF TRIFOLIATE ORANGE

Transformation of *Citrus* and *Poncirus* has been achieved by various methods, including the direct uptake of naked DNA by protoplasts (Kobayashi and Uchimiya 1989; Vardi *et al.* 1990; Hidaka and Omura 1993), *Agrobacterium*-mediated transformation of cells cultured in embryogenic suspension (Hidaka *et al.* 1990), and particle bombardment (Yao *et al.* 1996).

Since a method for the transformation of seedling stem segment explants was first reported (Moore *et al.* 1992), *Agrobacterium*-mediated transformation using juvenile explants has become the most popular method for producing transgenic *Citrus* and *Poncirus* plants. Recent transformation research using *A. tumefaciens* to modify trifoliate orange and its hybrids is summarized in **Table 1**.

PROTOCOLS FOR THE GENETIC TRANSFORMATION OF TRIFOLIATE ORANGE

Moore *et al.* (1992) produced transgenic Carrizo citrange by means of cocultivation of internodal stem segments from seedlings grown *in vitro* with *A. tumefaciens*. This technique seems to have some potential to expand the range of cultivars able to receive genes, but the observed transformation frequencies were low (≤5%).

Successful transformation of trifoliate orange using *A. tumefaciens* by a number of laboratories was conducted according to the protocol described by Kaneyoshi *et al.* (1994). Their protocol uses epicotyl segments, and is outlined below:

Plant material

Seeds of trifoliate orange were sterilized in 1% sodium hypochlorite solution containing 0.1% Tween 20 for 20 min, and then rinsed three times with sterile distilled water. The seed coat was peeled from the seeds, which were then placed on MS medium containing 5% sucrose and 0.8% agar, and incubated at 27°C in the dark. Internodal stem segments of 3-week-old seedlings were used for transformation.

Agrobacterium tumefaciens strain

Agrobacterium tumefaciens strain LBA4404 (Hoekema *et al.* 1983) was used to transform the plant material. For transforming Swingle citrumelo and Carrizo citrange, strain EHA101 and EHA105 were used (**Table 1**).

Cocultivation

Agrobacterium tumefaciens was grown overnight in 30 mL of LB medium containing 50 μg/mL kanamycin, 100 μg/mL rifampicin, and 300 μg/mL streptomycin sulfate at 28°C, and was then collected by means of centrifugation at 3500 rpm for 5 min. The resulting pellet was resuspended in hormone-free MS medium containing 100 μM acetosyringone. The density of the bacteria was adjusted to approximately 5×10^8 colony-forming units/mL. Internodal stem segments (1 cm in length) were prepared from 20-day-old seedlings and then immersed in the bacterial suspension for 15 min. The segments were then blotted dry on sterilized filter paper and placed onto a coculture medium consisting of hormone-free MS medium containing 100 μM acetosyringone.

Table 1 Summary of studies conducted on transformation of *Poncirus* and its hybrids.

Species	Vector	Inserted gene	*Agrobacterium* strain	Reference
Trifoliate orange	pBI121, PBI101-O12-p1	β-glucuronidase gene	LBA4404	Kaneyoshi *et al.* 1994
	pBE121	Human epidermal growth factor gene	LBA4404	Kobayashi *et al.* 1996
	pBY09, Bin19-O12	*rolC* gene	LBA4404	Kaneyoshi and Kobayashi 1999
	pBCiCP	Gene for capsid polyprotein of citrus mosaic virus	LBA4404	Iwanami *et al.* 2004
	pCGN1547	Flowering-time (FT) gene	LBA4404	Endo *et al.* 2005
	pBI121	Rice chitinase gene	LBA4404	Mitani *et al.* 2006
Swingle citrumelo	pMON9793	GUS gene	EHA101	Moore *et al.* 1992
	pBI121	GUS gene	EHA105	Molinari *et al.* 2004
Carrizo citrange	pGA472, pMON9793	GUS gene	EHA101	Moore *et al.* 1992
	p35SGUSINT	GUS gene	EHA105	Peña *et al.* 1995
	pMON9793, pGA482GG, pGA482GG-CTVCP	GUS gene, gene for coat protein of citrus tristesa virus	EHA101	Gutiérrez-E *et al.* 1997
	pBI121, pBI121.HAL2	Halotolerance gene	EHA105	Cervera *et al.* 2000
	pROKII	LFY gene, AP1 gene	EHA105	Peña *et al.* 2001
	pGA482GG	GUS gene	EHA101	Yu *et al.* 2002
	pGA482GGWp12, pC2301Wαp12	Gene for 11.8-kDa blight-associated protein (p12)	EHA101	Kayim *et al.* 2004
	pBI-P5CSF129A	Δ^1-pyrroline-5-carboxylate synthetase mutant gene	EHA105	Molinari *et al.* 2004

Selection and plant regeneration

After cocultivation for 3 days, the segments were transferred to a selection medium (MS medium supplemented with 5 mg/L 6-benzylaminopurine (BAP), 0.1 mg/L α-naphthalene acetic acid (NAA), 100 µg/mL kanamycin, and 500 µg/mL Claforan®. The cultures were maintained at 26°C under a 16-h photoperiod (25 $\mu E/m^2 \cdot s$) for 3-4 weeks. Segments with adventitious shoots were then transferred onto MS medium supplemented with 0.5 mg/L BAP, 200 µg/mL kanamycin, and 500 µg/mL Claforan to allow further shoot development. For rooting, the shoots that developed were excised and then cultured on MS medium supplemented with 0.5 mg/L NAA.

With this protocol, a transformation efficiency of 25.5% to 43.1% was obtained, and more than 100 transformed plants were obtained within 2 to 3 months.

Peña *et al.* (1995) also reported a transformation protocol with high efficiency for Carrizo citrange. In this protocol, shoot tip grafting was shown to be a rapid and efficient method for producing regenerated transgenic plants.

A protocol for efficient transformation of Swingle citrumelo via *A. tumefaciens*, using thin transversal epicotyl sections as explants, was reported (Molinari *et al*. 2004). This approach, combined with the application of increasing selection pressure (25 and 50 mg/L kanamycin) reduced the occurrence of escapes. With this procedure, 23 shoots were β-glucuronidase (GUS)-positive from 268 inoculated explants, giving a transformation efficiency of 8.6%, with fewer than 10% escapes.

CHARACTERISTICS OF TRANSFORMED PLANTS

Improving rootstock performance

Trifoliate orange has been used as a rootstock in Japan because it produces a compact tree that facilitates harvesting, pruning, and other cultural practices. In such situations, a rootstock that consistently imposes dwarfism on the scion is desired. It has been demonstrated in some plant species that dwarf plants can be produced using the soil bacterium *Agrobacterium rhizogenes* or a DNA fragment of its Ri plasmid. Transgenic trifoliate orange plants possessing the *rolC* gene, which is open reading frame 12 of the Ri plasmid, have been produced (Kaneyoshi and Kobayashi 1999). Transformed plants carrying the *rolC* gene along under the control of the cauliflower mosaic virus 35S RNA promoter were dwarfed, and the the dwarfed plants were only 22.6 to 40.0% of the height of normal plants. Almost all of the transformed plants possessed better rooting abilities than the control plants.

Carrizo citrange is very sensitive to salt stress, which restricts its use in salty soils. The *HAL2* halotolerance gene, which encodes a salt-sensitive 3'(2'),5'-biphosphate nucleotidase required for sulfate assimilation, has been isolated from *Saccharomyces cerevisiae* (Murguía *et al.* 1994; Serrano and Gaxiola 1994). It was introduced into Carrizo citrange for improving the behavior under the salt stress, but the result of experiments using HAL2 transgenic rootstocks has not been shown yet (Cervera *et al.* 2000).

Citrus blight one of the most devastating diseases of citrus, is a problem wherever citrus is grown in hot and humid areas. The cause of citrus blight is not known, but p12, an 11.8-kDa blight-associated protein (Derrick *et al.* 1990), has been detected in trees with citrus blight through the use of monoclonal antibody techniques. The p12 protein is similar in size and sequence to a hypothetical protein and homologous to a blight-associated protein homolog gene in *Arabidopsis* (Derrick and Timmer 2000). Carrizo citrange is a popular rootstock that is very susceptible to citrus blight. Transformed Carrizo citrange plants with the p12 gene in sense and antisense orientations were produced (Kayim *et al.* 2004). In that study, plasmolysis using 8% sucrose or 10% maltose was effective, and produced a stable transformation efficiency for the explants. The transgenic plants from this study will be budded with sweet orange and used to determine whether there are any effects of p12 on citrus blight or on rootstock performance. These evaluations will take several years, as symptoms of citrus blight do not occur on trees that are not producing a significant amount of fruit.

The integration and expression of the coat protein gene of citrus tristeza virus in Carrizo citrange has been reported (Gutiérrez-E *et al.* 1997). Factors affecting transformation frequencies were evaluated in an effort to reduce the number of escape shoots that were produced. In Carrizo citrange, both the use of a liquid medium/kanamycin overlay and the horizontal placement of stem segments increased the efficiency of kanamycin selection. The concentration of benzyladenine used in the regeneration/selection medium was inversely related to the number of shoots that regenerated and their subsequent ability to root. Transgenic Carrizo citrange plants that appeared to be solidly transformed maintained gene expression for up to 5 years and protein expression for up to 4 years.

Citrus mosaic virus (CiMV) is a serious production constraint for citrus growers in Japan. The infected trees grow poorly and often develop ringspot symptoms on the fruit, which drastically reduces its commercial value. Trifoliate orange was transformed with a binary vector containing the capsid polyprotein (pCP) gene of CiMV via *A. tumefaciens* LBA4404 (Iwanami *et al.* 2004). Transgenic lines were screened for their tolerance to CiMV by means of mechanical inoculation. Infection was monitored for 30, 60, 90, and 120 days after inoculation by means of reverse transcriptase – polymerase chain reaction. Transgenic line 24 had the lowest infection rate (7.1%) 60 days after inoculation, in contrast to the much higher infection rate in nontransgenic plants (65.1%). The response of other lines to inoculation ranged from susceptibility similar to that of nontransgenic plants to moderate tolerance.

As mentioned above, trifoliate orange is tolerant to cold and resistant to the tristeza virus. However, resistance to white root rot (*Rosellinia necatrix* Prillieux) is also required. Chitinase is present in the cell wall of many species of plants and its activity increases after infection by fungal, bacterial, or viral pathogenes. Transgenic trifoliate orange plants possessing the rice chitinase gene *RCC2* (Nishizawa *et al.* 1993, 1999) have been produced (Mitani *et al.* 2006). Chitinase activity in the transformed plants was higher than that in the non-transformed plants, as demonstrated by means of activity staining. Although the resistance of these transformed plants to phytopathogens was not examined, it is reasonable to predict that high expression of the rice chitinase gene in the transformed plants will enhance their resistance. The introduced gene was not detected in *C. reticulata* scions when these transformed plants were used as a rootstock.

Promoting early flowering

Citrus trees have a long juvenile phase that delays their reproductive development. To accelerate flowering, Carrizo citrange plants have been transformed so as to constitutively express the Arabidopsis *LEAFY* (*LFY*) or *APETALA1* (*AP1*) genes, which promote flower initiation in *Arabidopsis* (Peña *et al.* 2001). Both types of transgenic plants produced fertile flowers and fruits as early as the first year, notably through a mechanism involving an appreciable shortening of their juvenile phase, and the expression of *AP1* was as efficient as that of *LFY* in initiating flowers. Both types of transgenic plants flowered in consecutive years under environmental control. In addition, zygotic and nucellar-derived transgenic seedlings had a very short juvenile phase and flowered in their first spring, demonstrating stable inheritance of this trait. These results open new possibilities for domestication, genetic improvement, and experimental research in citrus and other woody species.

FLOWERING LOCUS T (*FT*) is one of the flowering-time genes in *Arabidopsis* and has been characterized as a floral pathway integrator (Araki 2001). An *FT* homolog

(*CiFT*) was found in the expressed sequence tag catalog of a cDNA library from the fruit of satsuma mandarin (*C. unshiu* Marc.) (Hisada *et al.* 1997), and its overexpression was previously shown to introduce an early flowering phenotype in *Arabidopsis* (Kobayashi *et al.* 1999). Transgenic trifoliate orange plants in which *CiFT* was expressed constitutively have been produced (Endo *et al.* 2005). They started to flower as early as 12 weeks after transfer to a greenhouse, whereas wild-type plants usually have a long juvenile period (several years). Most of the transgenic flowers developed on leafy inflorescences, apparently in place of thorns; however, wild-type adult trifoliate orange usually develops solitary flowers in the axils of leaves. All of the transgenic lines accumulated *CiFT* mRNA in their shoots. The transgenic lines showed variation in phenotypic characteristics such as time to first flowering and tree shape. In F_1 progeny obtained by crossing 'Kiyomi' tangor (*C. unshiu* × *sinensis*) with the pollen of one transgenic line, extremely early flowering (immediately after germination) was observed. These results suggest that constitutive expression of *CiFT* could reduce the generation time in trifoliate orange.

CONCLUSION

As shown above, many kinds of agronomically interesting genes from different sources have been transferred into trifoliate orange. The transgenic plants obtained in these studies seem to acquire important characteristics and should be evaluated for practical use.

Increased resistance to potato virus X was observed in tobacco scions grafted onto transgenic rootstocks expressing the gene that encodes pokeweed antiviral protein, though expression of this gene in the scions was not detected (Smirnov *et al*. 1997). This result suggests that the introduction of a foreign gene into the rootstock might have some influence on the scion, though the foreign gene itself and its expression product are not transmitted to the scion. Recently, the transmission of post-transcriptional gene silencing has been examined using grafting, and a number of silenced rootstocks can efficiently trigger silencing in grafted scions (Sonoda and Nishiguchi 2000; Guo and Ding 2002; Mallory *et al*. 2003). When suitable transgenic rootstocks are available, it will probably be much easier to get them approved and accepted by consumers than is the case for scions in which the gene is expressed in the fruit (Kayim *et al.* 2004; Mitani *et al.* 2006). In any case, further analysis of fruits borne on the scions grafted onto transformed rootstocks should be carried out for citrus production.

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