Journal of Agricultural Engineering and Food Technology
p-ISSN: 2350-0085; e-ISSN: 2350-0263; Volume 3, Issue 2; January-March, 2016 pp. 155-159

http://www.krishisanskriti.org/Publication.html

Milestones in Standardization of *Agrobacterium*-Mediated Transformation and Development of *Citrus Sinensis* Transgenics

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Abstract—*As the world population is growing at an alarming exponential rate there is great pressure placed on biological resources to provide an adequate supply of food while maintaining the integrity of our ecosystem. Viral diseases are the major factor for the loss in productivity of nutritious fruits and vegetables which reduces average crop yields by more than 70%. Therefore, there is an urgent need to improve crop plants by introducing virus resistance genes in the agronomically important crops e.g. citrus to fill the gap between the population growth and the food production.*

Among fleshy fruits, citrus represents one of the most important commercial crops in the world and is immensely vital for human nutrition. Therefore, it needs to be modified further to keep up with the diverse needs of consumers. Genetic manipulation through conventional techniques in this genus is 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. Citrus is also very susceptible to various kinds pests and diseases, which are devastating and results in huge revenue losses worldwide. To address a multitude of problems,genetic engineering has emerged as an important tool for introducing economicaly important traits into Citrus spp. and other woody crops. As a prelude to this investigation, experiments were designed to develop efficient plant regeneration and Agrobacterium-mediated genetic transformaton systems for sweet orange. The study provided promising results and holds potential to be routinely employed for transformation of important cultivars of Citrus spp, with genes of agronomic importance.

Keywords*: NAA (naphthalene acetic acid), BAP (benzylaminopurine), Agrobacterium-mediated transformation*

1. INTRODUCTION

Citrus crop occupies a distinguished status not only because of its significant contribution towards human nutrition, but also from substantial economic contribution it offers to society in the form of foreign trades and employment opportunities. Major citrus growing countries in the world include Brazil followed by USA, Mexico, Spain, Egypt, Italy and China. India ranks eighth in citrus production worldwide. As citrus plants are cultivated in diverse ecological conditions, they are vulnerable to various types of pathogens and diseases, caused by different viruses, viroids, nematodes, fungi and bacteria, which are difficult to control [1, 2].

Improvement of citrus by conventional breeding is inhibited by barriers of genetic incompatibility, apomixes, heterozygosity and lengthy juvenile period [3]. 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. Various transformation techniques that have been used in citrus include *Agrobacterium*-mediated transformation [4-9], chemically assisted uptake of foreign DNA by protoplasts [10] and bombardment of target tissues with DNA-coated particles [11]. However, *Agrobacterium*-mediated gene transfer, is considered more efficient and precise and is being extensively used for gene transfer into higher plants [12]. The genetic transformation of Citrus has been widely studied as a tool to generate transgenic plants with enhanced tolerance to various biotic [13] and abiotic stresses [14].

This method was therefore, was employed in this study to produce sweet orange transgenics. Application of the optimized procedure may also accelerate the efficient recovery of other citrus recalcitrant genotypes through *Agrobacterium*-mediated transformation.

2. MATERIALS AND METHODS

2.1 Plant material and culture conditions

Sweet orange (*Citrus sinensis*) fruits of 'Satgudi' variety, which is a popular and extensively grown variety of Nagpur were used for the present study. The seeds were isolated from fresh fruits and surface-sterilized aseptically in a laminar flow cabinet.

The seeds were treated with 70% ethanol for 1 min and rinsed once with sterile distilled water. Thereafter, the seeds were imbibed in 2% sodium hypochlorite solution (v/v) for 15 min followed by five rinses in sterile distilled water. Seeds were thereafter blotted dry on sterile tissue paper. Both the seed coats were removed under aseptic conditions in laminar air flow. The de-coated seeds were cultured in full-strength MT medium supplemented with 3% sucrose or 3% maltose as carbon source and solidified with 0.8% agar. Seeds were germinated under dark conditions for three weeks under controlled growth conditions (26 ± 0.5 ^{o}C) and the etiolated seedlings were then transferred to light conditions (16 h photoperiod with irradiance of 40 µE mol m-2 s-1) for about 10 days. One-month-old epicotyl explants (1 cm long) arising from the polyembryonic seedlings were used for standardization of regeneration and transformation.

2.2. Standardization of *Agrobacterium*-mediated transformation of *Citrus sinensis*

The previously optimized regeneration protocol was subsequently used for standardizing *Agrobacterium*- mediated genetic transformation of sweet orange. Transformation experiments for sweet orange were carried out using binary vector pCAMBIA 2301, harboring *GUS* reporter gene and *NPT*-II plant selection marker gene. Both the *GUS* gene and *NPT*-II gene are driven by the constitutive CaMV35S promoter **(Fig.1.)**. A highly virulent strain EHA 105 [16], which is widely employed in transformations of woody species like citrus was used, given the highly recalcitrant nature of the crop.

Different variables affecting transformation efficiency of citrus e.g. bacterial density, infection time, co-cultivation period and co-cultivation medium were thoroughly studied for optimization of transformation protocol (data not included).

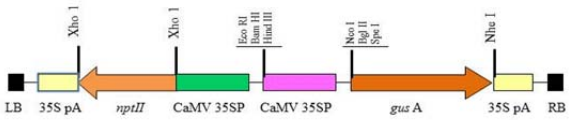

Fig. 1: T-DNA map of pCAMBIA 2301 with GUS and *NPT*-II genes

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To determine the most suitable bacterial density for transformation, epicotyl explants were infected with five different densities of bacterial suspension – 0.2, 0.4, 0.6, 0.8 and 1.0 O.D. (A_{600}). The infection time of explants with bacteria also varied from 5, 10, 15 and 20 min. Similarly co-cultivation medium was supplemented with various concentrations of NAA and BAP.

For optimization of *Agrobacterium*-mediated transformation protocol for *C. sinensis,* one-month-old etiolated epicotyl explants were collected from axenically raised polyemryonic seedlings and employed in the experiments. *Agrobacterium* strain EHA 105 containing binary plasmid pCAMBIA 2301 was cultured overnight in liquid YEB medium containing antibiotics kanamycin (50 mg/l) and rifampicin (50 mg/l) and grown to the O.D. (A_{600}) of 0.6. The suspension was spun at 4500 rpm for 10 min at room temperature and cells were pelleted down. The pellet was resuspended in liquid MT medium containing acetosyringone (100 µm) and was used to infect the epicotyl explants for 15 min. The infected explants were blotted dry on a sterile filter paper and transferred on to co-cultivation medium (MT + 2 mg/l BAP) + acetosyringone (100 µM) + NAA (0.5 mg/l)) and placed in dark for three days. The co-cultivated explants were then cultured in selection medium (SM) comprising MT + 1 mg/l BAP, containing 100 mg/l kanamycin and 500 mg/l cefotaxime) at 26±1^{0}C in dark for one month and transferred to 16 h photoperiod for further two months.

Small shoots appeared from the cut ends of responsive explants after a period of two months. After repeated subcultures, one cm long shoots were excised and kept in petriplates on rooting medium consisting of half-strength MT medium supplemented with 1 mg/l IBA along with 50 mg/l kanamycin and 250 mg/l cefotaxime for about two months. When putatively transgenic shoots had established a well-developed root system, measuring 5-6 cm, the rooted shoots were transferred to culture tubes, in the same medium, without IBA. Transformation frequency in these studies was calculated in the following manner:

Transformation frequency = (number of co-cultivated explants responding for shoot regeneration on selection medium/ total number of explants co-cultivated) X 100

For hardening, well-grown plantlets with fully expanded leaves were washed under tap water to remove traces of agar and planted in plastic pots containing autoclaved soil instead of vermiculite, as citrus grows best under natural conditions.

2.3. Molecular analyses of transgenics

Genomic DNA isolation and PCR

Genomic DNA was isolated from newly formed citrus leaves by CTAB method as described by Doyle and Doyle (1990). 1ml of DNA isolation buffer, was prepared fresh, and pre-heated in sterile Eppendorf tubes at 65°C. The leaf tissue (100 mg) was ground to a fine powder in liquid N_2 and transferred to Eppendorf tubes containing the pre-heated isolation buffer. The tubes were thereafter incubated at 65°C for 60 min. The proteins were removed by treating the lysate twice with equal volume of freshly prepared chloroform: isoamyl alcohol (24:1) (v/v).

The aqueous layer containing the DNA was precipitated using 0.6-0.8 volume of isopropanol at 13,000 rpm. The pellet was

washed in 70% ethanol, air-dried and dissolved in sterile double distilled water. DNA was then quantified by nanodrop and quality was checked by running on 0.8% agarose gel. The isolated genomic DNA was stored at -20°C to be used for further molecular analysis.

Isolated genomic DNA was subsequently utilized for PCR reactions using primers specific to GUS, or *NPT* II genes to determine transgene integration in putative transgenic lines. Approximately 200 ng of genomic DNA from untransformed control and putative transgenic citrus lines were taken and mixed with 100 nM of forward and reverse primers (*GUS*, or *NPT* II genes), 1X PCR buffer, 2 mM $MgCl_2$, 100 µM dNTP mix and 0.5 U of Taq polymerase (BIOTOOLS, India) and the volume was made up to 25 µl. The samples were denatured initially at 94 ^oC for 5 min, followed by 30 cycles of 1 min denaturation at 94 ^oC, 1 min of primer annealing at suitable temperature (55 ^oC for *GUS*, 60 ^oC for *CP* and 50 ^oC for *NPT* II) and 2 min of extension at 72 ^oC, with a final extension of 72 ^oC for 5 min. The PCR products were analyzed on a 1 % agarose gel.

3. RESULTS AND DISCUSSION

Citrus is a long lived and slow growing perennial crop [17], which constantly exposed to many diseases, pests and extreme weather patterns, which greatly reduce fruit quality and production.

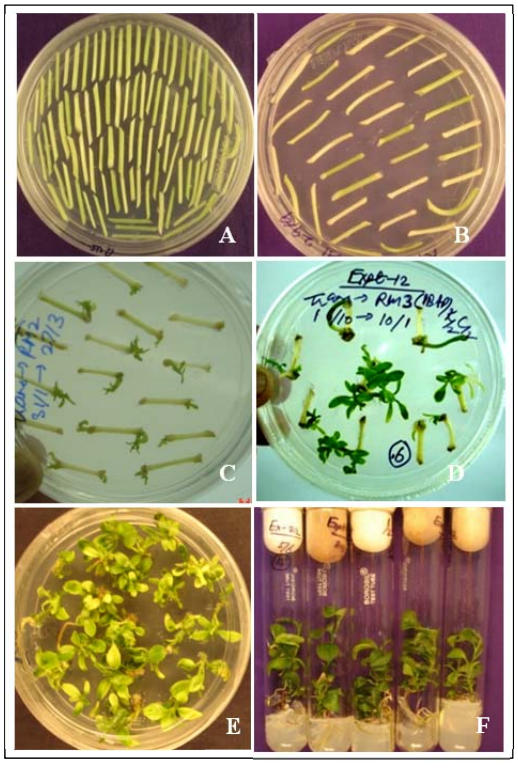

Fig. 2. Step-wise representation of the developed *Agrobacterium*-mediated transformation protocol for *Citrus sinensis* (sweet orange)

Hence, to successfully introgress target genes of agronomical interest a reliable and reproducible regeneration and transformation systems is absolutely necessary.

In order to develop an efficient and reproducible *Agrobacterium*-mediated transformation protocol for *Citrus sinensis*, several important factors that play crucial role in bringing about genetic transformation and subsequent regeneration were investigated thoroughly.

A – 1 cm long epicotyl segments cultured in co-cultivation medium comprising MT medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA, **B** – Co-cultivated explants placed in selection medium consisting of shoot regeneration medium (MT medium+ 1 mg/l BAP), fortified with kanamycin 100 mg/l and cefotaxime 500 mg/l, **C** – Initiation of shoot induction, **D** - Shoot proliferation in shoot regeneration medium, **E** – Well differentiated shoots placed in rooting medium in half-strength MT medium supplemented with 1 mg/l IBA and kanamycin 50 mg/l along with cefotaxime 250 mg/l, **F –** Shoots responding for rooting transferred to test tubes in the same medium, without hormones

For the aim of optimization of *Agrobacterium*-mediated transformation protocol, the vector pCAMBIA 2301 was utilized, which harbors *GUS* as reporter and *NPT* II selection marker gene, both driven by the constitutive CaMV 35S promoter. For transformation studies one-

month-old etiolated epicotyl explants that were collected from axenically raised polyemryonic seedlings were employed for optimization of transformation protocol. The explants infected with over-night grown *Agrobacterium* (A_{600} 0.6-0.8) for 15 min and co-cultured for 3 days in dark condition. After a 3-day co cultivation period, the epicotyl explants were placed on selection medium supplemented with 1 mg/l BAP and kanamycin 100 mg/l and bacteriostatic agent cefotaxime 500 mg/l for shoot regeneration. Although, in citrus transformations, some researchers have used less

stringent selection pressure of kanamycin i.e. 50 mg/l [18] we decided to opt for a higher dosage to minimize the occurrence of escapes. Small shoots appeared from the cut ends of explants after a period of two months. In our transformation system, *in vitro* shoots arose directly from the wounded epicotyl segments without callus phase, which increases the probability of producing morphologically similar plants than the systems incorporating the callusing phase.

For woody plants like sweet orange, the standardization of tissue culture conditions is a difficult task as there are no general protocols suitable for all genotypes. It has been widely reported in literature that in citrus, different species or even different cultivars of same species are transformed with different efficiency [19, 20] (Pena *et al.*, 1997; Cervera *et al.*, 1998). Low rate of regeneration of transformed tissue is the main problem in citrus transformations. Despite several efforts and abundant literature, citrus transformation continues to be a difficult and recalcitrant system.

Table 1: Transformation of *C. sinensis* explants with *Agrobacterium tumefaciens* strain EHA 105 + pCAMBIA 2301

Experiment No.	No. of Explants Co-cultivated	Transformation frequency (%)*	Explants With Shoots ( )*	No. of Shoots per Explant	Rooting frequency
1.	221	84	1.02	38.0	38.10
2.	164	(32)	1.01	48.1	35.44
3.	72	79	1.00	44.4	40.62
4.	102	(28)	2.00	37.2	36.84
5.	122	32	1.05	45.9	48.21
6.	178	(13)	2.01	23.0	35.71
7.	120	38	1.07	43.3	30.77
8.	100	(14)	1.02	48.0	29.17
9.	135	56	1.01	37.0	32.0
10.	89	(27)	1.00	46.0	36.5
		42			
		(15)			
		52			
		(16)			
		48			
		(14)			
		50			
		(16)			
		41			
		(15)			

*Based on kanamycin resistant phenotype of regenerating explant

*Data in the parenthesis represent the number of shoots responding for rooting

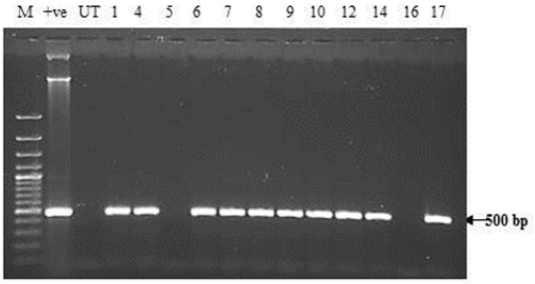

Fig. 3: PCR analysis of putative transgenics of *C. sinensis* with *Gus* specific gene primers

Lane 1- 100 bp ladder; **Lane 2**- plasmid DNA; **Lane 3**- DNA from untransformed control; **Lanes 4 to 15**- DNA from putative transgenics

In our experiments, it was observed that at an O.D. less than 0.6 and infection time of less than 15 minutes was too small to bring about successful transformation and majority of the explants died later in selection medium. Inclusion of NAA along with BAP at 2 mg/l in co-cultivation medium resulted in elevated regeneration efficiency for putatively transgenic shoots (fig. 2.).

After repeated cub-culturing for further two months in the stringent selection medium, when *de novo* shoots became ~ 1 cm long, and well-differentiated, they were excised from explants and cultured on rooting medium, consisting of half-strength MT medium supplemented with 1 mg/l IBA for about four months with subculture once in a month. At this stage, the strength of antibiotics was reduced to half *i*.e. kanamycin and cefotaxime were added at a concentration 50 mg/l and 250 mg/l respectively (fig.2.)

The entire exercise of standardization of *Agrobacterium*-mediated transformation protocol for sweet orange yielded extremely encouraging results and based upon the number of responsive shoots on selection medium, overall transformation frequency ranged from 38 to 48% (table 1.). The putative sweet orange transgenics, developed with GUS construct were analyzed by PCR, with primers specific to GUS gene, to verify transgene integration. PCR analysis of the genomic DNA obtained from these transformants revealed an amplicon of 500 bp, as expected for GUS gene (Fig. 3). Genomic DNA from untransformed control plant did not show any amplification specific for GUS gene. Based upon PCR amplification, about 50% of the transformed shoots raised with *GUS* construct were positive for the integration of the transgene.

There was no observable phenotypic difference between the transgenic and control plants, but transgenic shoots exhibited comparatively slower growth pattern than the control, which is a usual phenomenon in transformation studies.

Overall, the entire study involving optimization of *Agrobacterium*-mediated transformation and production of citrus transgenics harboring GUS and *NPT* II produced extremely convincing results. The protocols developed during the work are entirely reliable and reproducible and can be efficiently utilized for routine genetic transformation experiments and can be exploited to introgress other target genes from heterologous sources that would impart genetic superiority to the citrus crop.

4. ACKNOWLEDGEMENTS

This work was generously supported by a grant from the DBT (Department of Biotechnology).

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