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High Efficiency *Agrobacterium*-mediated Transformation of Sweet Orange (*Citrus sinensis* L. Osbeck) with Fungal Ornithine Decarboxylase Gene

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ABSTRACT

Citrus crop occupies a distinguished status in the plant world because of its significant contribution towards human nutrition, as well as due to the substantial economic contribution it offers to society in the form of foreign trades and employment. Under natural conditions, *Citrus* is subjected to many biotic and abiotic stresses throughout its life cycle, which pose critical problems leading to massive loss in productivity. *Fusarium* wilt is a devastating disease affecting *citrus* trees grafted on sour orange or lime rootstock. The conspicuous symptoms of the disease include discoloration of vascular tissue, chlorosis, wilting of the canopy, and epinasty of young leaves, which ultimately results in death of the tree.

With the advent of molecular techniques, direct genetic manipulation has offered novel opportunities for plant improvement, and plant transformation has made it feasible to modify just one or two traits, while preserving the unique background of the original cultivar.

Therefore, in order to target *Fusarium* wilt, Sweet orange (*Citrus sinensis* L Osbeck, var Nagpur) was explored for efficient transgenic plant production by means of *Agrobacterium*-mediated transformation for the introduction of fungal *ornithine decarboxylase* (*ODC*) gene, using standardized protocol. It was observed that etiolated epicotyl explants of *Citrus sinensis* cultured in BAP (6-benzylaminopurine) (1 mg/l) exhibited best morphogenic response with the transformation frequency ranging from 14% to 45.3%, which is extremely appreciable for a woody species. Transgenic shoots rooted normally in medium amended with 1 mg/l IBA (indole-3-butyric Acid). The transgenics were raised under high stringency conditions provided by the selection agent, kanamycin, at 100 mg/l to minimize occurrence of escapes. Further, molecular analysis confirmed the stable integration of transgene in the plant genome. To the best of our knowledge, this is the first report of the transformation of *Citrus* with fungal *ODC* gene in India.

Key words: 6-Benzylaminopurine, *Citrus sinensis*, epicotyl, genetic transformation, host-induced gene silencing, RNA interference, transgenics

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Introduction

Increase in world population is proceeding at an alarmingly exponential rate and there is great pressure on biological resources to ensure adequate food security for everyone, while maintaining the balance in our ecosystem. Fungal diseases and other biotic stresses are major factors responsible for the loss in productivity of nutritious fruits and vegetables which reduces average annual crop yields by more than 40% (Peng et al., 2021). The strategies of combating fungal infection include conventional breeding to produce cultivars resistant to fungal diseases and the practice of growing non-host plants along with susceptible host in alternation in the same field, to inhibit completion of pathogen life-cycle, as well as use of toxic fungicides (Hwang et al., 2012). Although plant breeders have succeeded in producing cultivars resistant to fungal diseases, the time taken in making crosses and selection of progenies displaying resistant traits makes the process difficult as new fungal races arise quickly. Fungi have an enormous impact on human welfare and they are known to destroy valuable crops as devastating pathogens. Thus, there is a need to introduce fungal resistance genes in agronomically important crops, (such as *citrus*) to close the gap between food supply and demand.

Attempts for genetic manipulation through conventional techniques in *citrus* has been a constrained task for plant breeders as this genus has several limitations

including long juvenile period, high heterozygosity, sexual incompatibility, nucellar polyembryony and large plant size, making cultivar improvement difficult (Singh and Rajam, 2009, 2010). *Citrus* is also very susceptible to various kinds of pests, viruses and fungal diseases including *citrus* wilt, which are highly devastating and results in substantial revenue losses worldwide. *Citrus* plants are affected by *Fusarium* spp. soil borne pathogens that are economically important as they affect seedlings and the nursery industry in *citrus* growing areas in the world. *Fusarium* spp. are a big cause of concern as they are responsible for various diseases of *Citrus*, including dry root rot, feeder rot, wilt and root rot. The soil-borne fungus, *Fusarium oxysporum*, is a virulent pathogen of *Citrus sinensis* (Hannachi et al., 2014). Once infection begins, the tree starts to show loss of vigor, canopy loss, chlorosis and wilting, and ultimately dies within a few years. The common symptoms of *Fusarium* wilt are wilting of sections of the canopy, chlorosis and epinasty of young leaves followed by discoloration of vascular tissue. *Fusarium* spp. are recovered from the root and trunk of affected trees (Amatulli et al., 2010; Maryani et al., 2019). *Fusarium oxysporum* was recently reported to have caused *citrus* wilt in Tunisia, that resulted in the loss of more than 50% of the crop. The disease infected 10 to 25 year old trees of *C. sinensis, C. aurantium* and *C. aurantifolia* (Hannachi et al., 2015).

In present times genetic engineering has emerged as an important tool for introducing economically important traits into *citrus* spp. and other woody crops to fortify them against different pathogens. Thus, a program to generate *citrus* transgenics for resistance using partial fungal *ODC* gene was undertaken.

Citrus crop being highly susceptible to *Fusarium* spp., in order to incorporate host-induced gene silencing in transgenic *citrus* plants, *ODC* gene of *F. oxysporum* was targeted. The 541 bp region that is conserved among members of *Ascomycetes* was selected as it bears no homology with any of the genes present in humans, mouse and *Arabidopsis*. Further, the partial *ODC* gene of *F. oxysporum* shared high homology (approximately 97%) both at nucleotide as well as protein level with *ODC* gene sequence of *F. solani*, that causes dry root rot disease in *citrus* trees. This offers the prospect of using the same stretch of *ODC* gene sequence, conserved amongst several pathogenic fungi, to induce constitutive resistance against multiple fungal pathogens, wherein, multiple pathogens refers to those fungal pathogens which bear more than 95% ODC gene sequence similarity with that of the *F. oxysporum* ODC gene.

In order to counter infectious pathogens, plants have been genetically modified by the introduction of constructs that form self-complementary hairpin RNA to silence target gene expression efficiently (Akbar et al., 2022). To achieve stable resistance against insect pests, viruses and fungal pathogens, a recent innovation is host induced gene silencing (HIGS), which is an RNAi based strategy (Zand Karimi and Innes, 2022). RNAi refers to a sequence-specific gene silencing mechanism brought about by 21-25 nucleotide long small interfering RNA (siRNA) molecules, which act as mediators of the phenomenon (Waterhouse and Helliwell., 2003). The siRNA molecules in turn are generated from longer double stranded RNA, upon cleavage by the enzyme Dicer. These siRNA molecules get incorporated into endonuclease enabled RNA-induced silencing complex (RISC), which targets homologous RNAs for degradation in a sequence-specific manner (Hannon, 2002; Pickford and Cogoni, 2003).

The success of any genetic transformation strategy relies on careful selection of target fungal gene/s which must be key gene/s either required for growth or pathogenicity of the pathogen. Putrescine (Put), spermidine and spermine are naturally found polyamines, and are required for growth and development by all organisms (Tabor and Tabor, 1985). They occur as polycationic, small molecules in the cells. Therefore, in our transgenic approach, ornithine decarboxylase (ODC) gene involved in polyamine biosynthesis was selected as the target gene of interest. ODC gene codes for an important enzyme ornithine decarboxylase that catalyzes the conversion of ornithine to putrescine, which is the initial and rate-limiting step in polyamine biosynthesis in fungi, protozoa and animals (Galston and Kaur-sawhney, 1990). However, putrescine can also be formed from arginine by arginine decarboxylase enzyme in plants and bacteria (Zarb and Walters, 1994). In most of the fungi, put formation takes place exclusively by ODC pathway (Rajam, 1993). Therefore, ODC appears to be a suitable target gene to control fungal infection through genetic transformation.

Though RNAi is a highly gene-specific phenomenon, it can induce the "off-target" gene silencing effect on unintended genes (Mamta and Rajam, 2017; Kumar et al., 2022). Therefore, to validate if PA biosynthesis in transgenic *citrus* plants was unaffected

by the expression of fungal *ODC* sequence, the native *ODC* and *ADC* (arginine decarboxylase) gene expressions and polyamine levels (data not shown) were evaluated and found to be comparable with wild-type plants; thus, it can be inferred that fungal ODC gene did not show any off-target effects.

With the objective of imparting resistance to *Fusarium* wilt of sweet orange, the present study was designed to develop *citrus* transgenics harboring fungal *ODC* (ornithine decarboxylase) gene. *ODC* gene was selected because it plays a key role in polyamine biosynthesis pathway of all living organisms and polyamines are ubiquitous compounds, essential for proper growth and development of all organisms (Tabor and Tabor, 1985).

Therefore, the *ODC* gene was introduced in plant in both sense and antisense orientation, which, upon transcription would result in the formation of double stranded hairpin RNA, that would be sufficient to silence fungal *ODC*, a vital gene of *F. oxysporum*, leading to fungal resistance in host plant (Wesley et al., 2001). .

Materials and Methods

Plant Material and Culture Conditions

Fruit of sweet orange, *Citrus sinensis* (L.) Osbeck were procured from the Central Citrus Research Institute, Nagpur for use in the present study. The seeds were isolated from fresh fruits and surface-sterilized under aseptic conditions in a laminar air flow cabinet. Freshly isolated seeds were initially 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 and thoroughly cleaned by five rinses in sterile distilled water. Seeds were blotted dry on sterile tissue paper. Both seed coats were removed under aseptic conditions under laminar air flow and cultured on full-strength MT medium (Murashige and Tucker, 1969) supplemented with 3% sucrose or 3% maltose as carbon source in 0.8% agar. Seeds were germinated under dark conditions under controlled growth conditions (26-28 ^{o}C) for three weeks, and etiolated polyembryonic seedlings were transferred to light conditions (16 h-light/ 8 h-dark photoperiod with irradiance of 40 µE mol m^{-2} s^{-1}) for 10-15 days. One-month-old epicotyl explants (1 cm long) arising from the polyembryonic seedlings were used for transformation experiments.

Transformation with Binary Plasmid Harboring Fungal ODC Gene

Transformation of sweet orange was carried out with binary vector pCAMBIA2300, having fungal *ODC* gene of *Fusarium oxysporum* and *NPT* II as selection marker gene (Fig. 1). The T-DNA in the construct carries *ODC* gene in both sense and antisense orientation, separated by an intron sequence (RNAi construct), such that the mRNA forms a hairpin structure after transcription. Both the ODC and *NPT* II genes are driven by CaMV 35S promoter. Several replicates of transformation sets were carried out with the fungal *ODC* gene construct. A highly virulent strain of *Agrobacterium tumefaciens* EHA 105 (Hood et al., 1993), which is widely employed in transformations of woody species like *citrus* was used, given the highly recalcitrant nature of the crop. For *Agrobacterium*-mediated genetic transformation, previously optimized protocol was followed (Singh and Prabhavathi, 2016). The *in vitro* grown etiolated seedlings (8 to10 cm long) were chosen as the source of epicotyl explants. One-month-old epicotyls were sectioned into about 1 cm long pieces and utilized for transformation experiments.

Agrobacterium strain EHA 105 containing binary plasmid pCAMBIA 2301 was cultured overnight in liquid YEB (yeast and beef extract) 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 placed horizontally on to co-cultivation medium (MT + 2 mg/l BAP) + acetosyringone (100 µM) + NAA (0.5 mg/l)) and cultured 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°C in dark for one month and transferred to 16 h photoperiod for further two months.

Shoot sections 1 cm long were excised from explants and kept in Petri plates 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 putative transgenic shoots had established a well-developed root

system measuring 5-6 cm, rooted shoots were transferred to culture tubes. 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) × 100.

PCR Screening of the Transgenics for Integration of Transgenes

The integration of transgenes was confirmed by PCR analysis, for which about 50-60 ng genomic DNA from putative transgenics was added to PCR reaction mix vial containing 100 nM of forward and reverse primers specific to genes of interest (fungal *ODC, NPT* II), 1 × PCR buffer, 2 mM $MgCl_2$, 100 M dNTP mix and 0.5 U of Taq polymerase and the reaction volume was made upto 25 µl with sterile distilled water. The PCR conditions followed initial denaturation at 94 °C for 5 min, followed by 30 cycles of 30 sec denaturation at 94 °C, primer annealing at designated temperature for 45 sec and 30 sec of extension at 72 °C, with a final extension at 72 °C for 10 min. Subsequently, the PCR products were analyzed on 1% agarose gel (see Table 1).

Table 1

Gene	Primers	Annealing Temperature
ODC (*F. oxysporum*)	F.P: 5'GTC CTC GAG CAA GAT GGC CAA GCT CTA CC 3'	76 °C
	RP: 5' CTG ATT TAA ATT CTG GTG ATC GAA GAC GAT G 3'	72.5 °C
NPT II	F.P: 5' ACC TTG TCA CTG AAG GGG GAA GG 3'	55 °C
	R.P: 5' GTC GTC CCT CCG TTT GTT ACT AGT 3'	56 °C

Results

In the present study, we report a genetic transformation strategy that allows efficient production of *citrus* transgenics harbouring *ODC* gene sequences of 541 bp in sense and antisense orientation within hairpin construct to impart fungus resistance to transgenic plants. About 1 cm long etiolated epicotyl explants from polyembryonic seedlings were used for transformation.

Appreciable results were obtained in genetic transformations of *Citrus sinensis* with *ODC* gene of *F. oxysporum*, both in terms of transformation frequency and number of shoots per explant (Table 2). The overall transformation frequency ranged between 14.7 to 45.3 % (Table 2) and none of the transformation sets cultured resulted in complete absence of regeneration, which is a common feature observed with constructs used for transforming a recalcitrant system like *citrus*. Percentage of rooting of well-developed shoots was between 26.6 to 46.6% in these experiments (Table 2) which is appreciably higher than the values reported in literature. Similarly, number of shoots achieved per explant was also higher i.e., two shoots per explant had formed in three transformation sets out of several experimental set ups.

We maintained a stringent selection pressure of 100 mg/l kanamycin throughout the regeneration period of putatively transgenic shoots, followed by reduction of antibiotic concentration to half after the formation of roots, which probably had increased the fidelity of our protocol. *Citrus sinensis* transgenics developed with the fungal *ODC* RNAi construct were completely normal and healthy throughout their growth and similar in appearance like untransformed control plants and no morphological abnormalities were observed in any of the transgenics. The putative *ODC* transgenics had developed a well-branched root system (Fig. 2). This suggests that introduction of fungal *ODC* had minimal deleterious effect on the *citrus* transcriptome. However, one observable anomaly displayed by the transgenic plants was their extremely slow growth in comparison with control plants; this is a normal feature observed in the studies with transgenic plants (Bevan, 1984).

The putative *C. sinensis* transgenics developed with *ODC* construct were analyzed by PCR, with primers specific to *ODC* and *NPT* II genes respectively, to verify transgene integration. PCR analysis of the genomic DNA obtained from these transformants revealed an amplicon of 541 bp, as expected for *ODC* gene (Fig. 3) and an amplicon of 750 bp, as expected for *NPT* II gene (Fig. 4). Genomic DNA from untransformed control plant did not show any amplification specific for these two genes. Based upon PCR amplification, about 70% of the transformed shoots raised with *ODC* construct were positive for the integration of fungal *ODC* gene (Fig. 3). All transgenics obtained appeared to be normal and healthy under prevalent culture conditions (Fig. 2).

Table 2. *Agrobacterium*-mediated genetic transformation of sweet orange with binary vector pCAMBIA 2300, harbouring *ODC* gene of *Fusarium oxysporum*

Experiment No.	No. of explants co-cultivated	No. of explants with shoots	No. of shoots per explant	Transformation frequency (%)*	Rooting frequency (%)
1	86	39	2.22	45.3	35.8
2	104	37	1.0	35.5	43.2
3	110	26	2.04	23.6	38.4
4	102	15	1.0	14.7	26.6
5	92	23	1.06	25.0	34.7
6	95	30	1.01	31.5	33.3
7	82	15	2.07	18.2	46.6
8	96	30	1.07	31.2	26.6

*Transformation frequency was calculated on the basis of number of explants exhibiting shoots on selection medium.

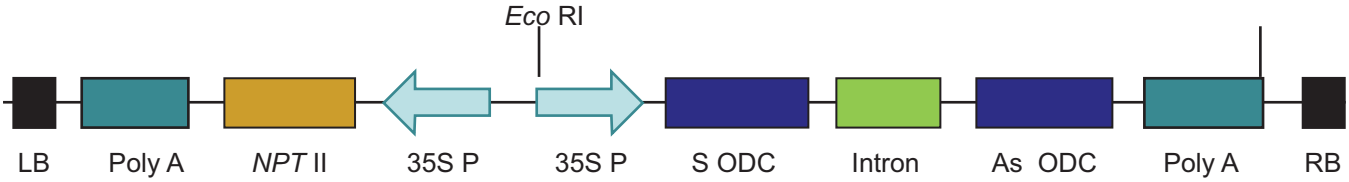

Figure 1. T-DNA map of binary vector pCAMBIA 2300 harboring sense and antisense fungal ODC gene. (Source: Singh et al., 2020)

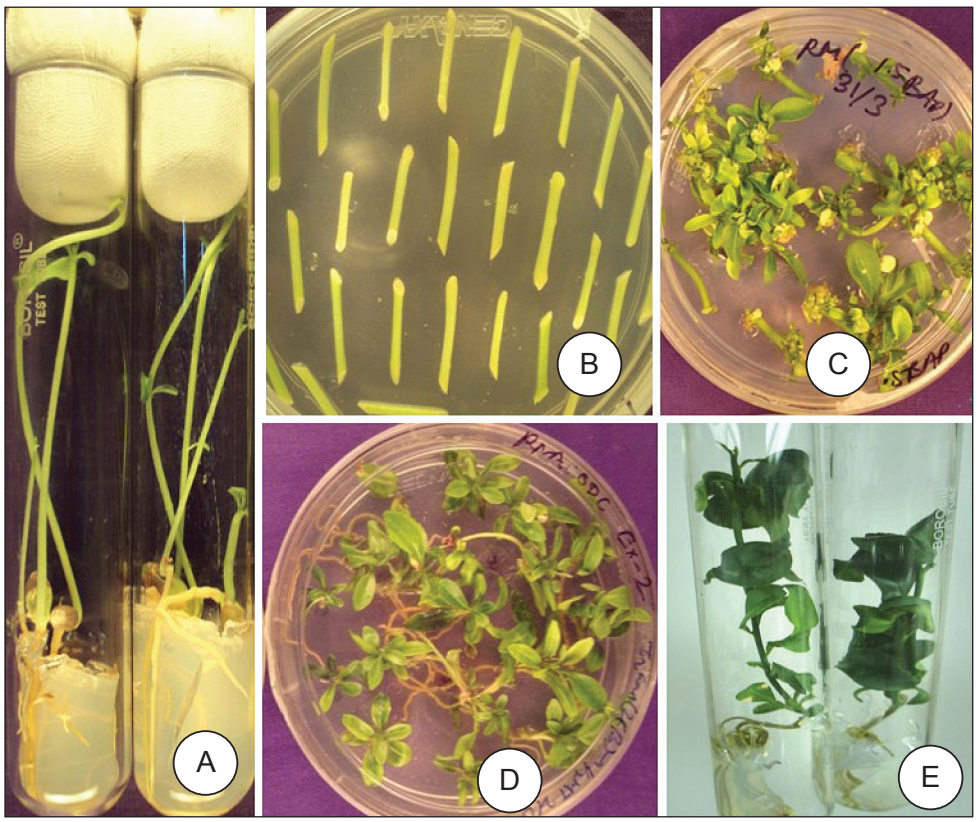

Figure 2. *Agrobacterium*-mediated transformation sweet orange with binary vector pCAMBIA 2300, carrying fungal ODC gene (A) One month old polyembryonic seedlings used for explant collection; (B) ~1 cm long etiolated epicotyl segments cultured in selection medium consisting of shoot regeneration medium (MT medium+ 1 mg/l BAP), fortified with kanamycin 100 mg/l and cefotaxime 500 mg/l; (C) Proliferation of shoots in the same medium; (D) Well grown 1.5 cm long shoots placed in rooting medium for root induction, consisting of half-strength MT medium supplemented with kanamycin 50 mg/l and cefotaxime 250mg/l; (E) Well-rooted shoots transferred to test tubes for elongation.

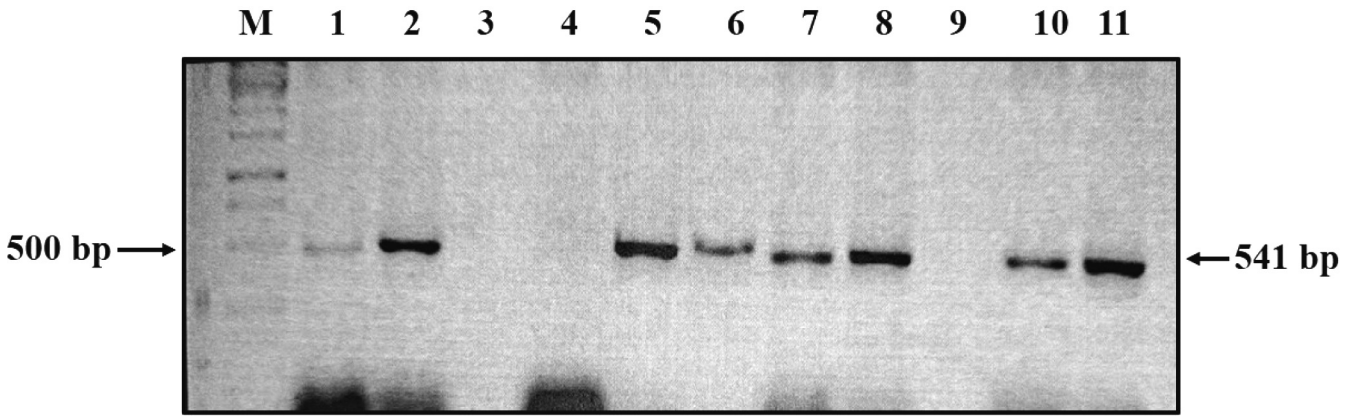

Figure 3. PCR Analysis of putative ODC *Citrus sinensis* transformants using primers specific to fungal ODC gene. Lane 1- 1 kb DNA ladder; Lane 2 to 12- DNA from putative transgenics

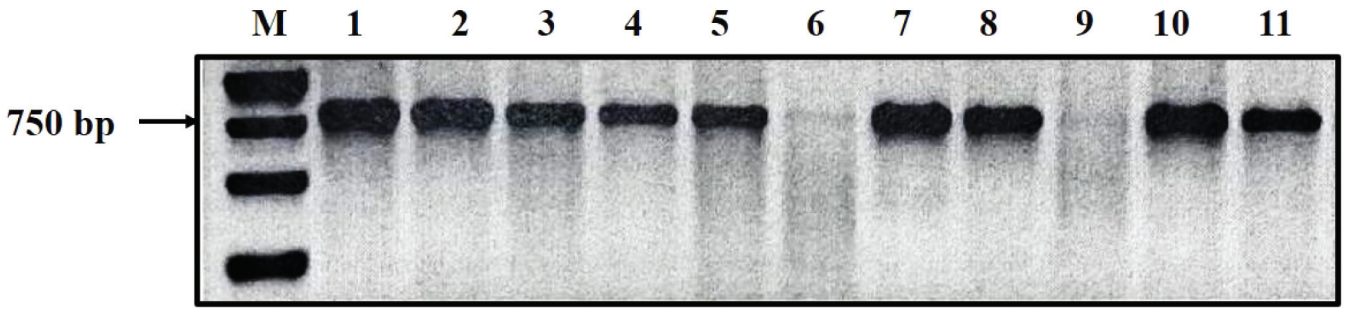

Figure 4. PCR Analysis of putative ODC *Citrus sinensis* transformants with primers specific to NPT II gene. Lane 1- 1 kb DNA ladder; Lane 2 to 12- DNA from putative transgenics.

Discussion

Fungal diseases are the most devastating amongst all plant diseases and contribute majorly to crop losses worldwide. *Fusarium oxysporum* being a highly virulent pathogen of *citrus*, causing vascular wilt disease which affects the vascular system of plants, resulting in wilting symptoms and eventually leading to plant death. A promising method to control the disease has been proposed in the current study- the concept of host induced gene silencing (HIGS), which has been effectively tested to achieve resistance in plants against insect pests, nematodes, viral diseases and fungal pathogens (Singh et al., 2020). The overall transformation efficiency obtained in our studies is an appreciable achievement, considering the extensively observed and reported highly recalcitrant nature of sweet orange in this context.

The present study extends the approach of host-induced gene silencing of invading fungi for protecting sweet orange grafted on sour orange rootstock, against few of the most devastating fungal diseases with the likely involvement of post-transcriptional gene silencing (PTGS) mechanism through RNAi. Majority of fungi depend upon ODC pathway for the formation of polyamines, which is an essential requirement for fungal growth and development. Therefore, ODC gene was selected as the target fungal gene to be silenced by RNAi. This antifungal strategy involves uptake of plant-derived siRNAs by the invading fungus (Smith et al., 2000), thereby resulting in post transcriptional silencing of homologous transcripts of *ODC* gene in the pathogen, leading to disease resistance in transgenic plants.

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