

# Agrobacterium Mediated Transformation of a Pure Line Variety of Hot Pepper, RCL 59M

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**Running Title:** Transformation studies of hot pepper, RCL 59M

**Abstract:** Seeds of a pure line variety chilli RCL59M were germinated on sterile media. Intact-hypocotyl explants were prepared from 12-14 days old seedlings and cocultivated with *Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pRMP9, 770bp of chilli leaf curl virus (ChLCuV) coat protein gene cloned between Hind III and Bgl II sites in the multiple cloning sites (mcs) region. After co-cultivation the explants were transferred to shoot regeneration medium (SRM) supplemented with 250 mg/l of augumentin and kanamycin (25 mg/l). The regenerating shoot buds were rooted. Molecular analysis was carried out. All PCR positive plants were transferred to pots with coco peat. PCR was followed by Southern analysis to confirm the transgenic status of the putative chilli transformants. In the present study, chilli leaf curl virus coat successful transformation was confirmed by Southern hybridization. Seven out of twelve plants tested by Southern blotting, a 0.77 kb band of coat protein gene was released confirming the transgene integration. Rooted plants transferred to soil and the transgenic plants were monitored upto T2 generation. The transgenic plants showed normal phenotype and produced fruits similar to that of non-transgenic plants.

**Keywords:** *Agrobacterium tumefaciens*, Southern blotting, transformation, binary vector

## 1. Introduction

Chilli (*Capsicum annum* L.) is a spice cum vegetable crop grown through out the world. Chilli pepper fruits are important food items worldwide as ingredients of a wide variety of dishes. Pepper is highly susceptible to fungal and viral pathogens and the losses caused by plant viruses are greater in tropics and semi-tropics, which provide ideal conditions for the perpetuation of both the viruses and their vectors (Varma et al., 2002). Gemini viruses have recently emerged as the cause of devastating diseases of important crop plants (Mansoor et al., 2003, Varma and Malathi, 2003). Chilli leaf curl virus (ChLCuV) is a Gemini virus and is transmitted by white flies, causing a yield loss up to 80-90% if virus attack is early. Genetic engineering is the major tool for developing virus resistant transgenic chilli plants. Development of reliable tissue culture regeneration systems, efficient transformation techniques, recovery and multiplication of transformants are important steps in developing transgenic chilli plants.

Although chilli belongs to the solanaceae family, whose members are easily amenable to tissue culture and transformation practices, it is considered as a highly recalcitrant crop (Manoharan et al., 1998, Li et al., 2000). The gene transfer to chilli plant has been hindered by the difficulty of regenerating whole plants from tissue culture, the difficulty in obtaining transformed pepper tissue and ultimately the difficulty in linking regeneration with transformation (Lim et al., 1999). Phillips et al., (2000) attempted to reproduce the published pepper transformation protocols reported by Lee et al., (1993), Engler et al., (1993), Zhu et al., (1996) and Manoharan et al., (1998) with chilli pepper and failed to recover transformed plants despite using sufficient numbers of explants. There are reports on engineering chilli for viral resistance as well viz., cucumber mosaic virus (CMV) coat protein gene (Zhou et al., 1994; Zhu et al., 1996) and CMV satellite RNA (Dong et al., 1992; Kim et al., 1997), but the published protocols could not be

repeated in other laboratories (Li et al., 2003). Because of the above reasons reports on successful transformation of chilli are scanty and not routine. During the last few years, transformation using *Agrobacterium tumefaciens* has been reported in sweet and chilli pepper (Li et al., 2003; Lee et al., 2004). To circumvent difficulties with cultivar dependent regeneration, shoot apics along with cotyledonary nodal region culture, which is genotype independent was originated for many crop species (Sanatambi and Sharma, 2006).

In the present paper we describe a method for *Agrobacterium* mediated transformation of a pure line variety of hot pepper, RCL 59M used as a combiner in chilli hybridization programmes.

## 2. Material and Methods

### 2.1 Plant Material

Seeds of a pure line variety chilli RCL59M was provided by Rallis India Limited Company. RCL59M one of the best combiners is being used as a male parent for the production of chilli hybrids.

### 2.2 *Agrobacterium tumefaciens* strain and plasmid

*Agrobacterium tumefaciens* strain LBA4404 containing the binary vector pRMP9 was provided by Rallis India limited. The binary vector pRMP9 is derived from base vector pGA643 in which 770bp of chilli leaf curl virus (ChLCuV) coat protein gene is cloned between Hind III and Bgl II sites in the multiple cloning sites (mcs) region.

### 2.3 Preparation of Media

Murashige and Skoog (1962) medium was used for the experiments. It was supplemented with various concentrations of IAA, BAP and AgNO<sub>3</sub> for pre -

incubation of the explants and to obtain shoots (shoot regeneration media -SRM) used in the transformation experiments.

#### 2.4 Preparation of explants

Seeds were sterilized using 1.6 % sodium hypochlorite for 12-14 minutes, washed in distilled water and grown on ½ MS. Intact-hypocotyl explants were prepared from 12-14 days old seedlings after excising the cotyledonary leaves from the seedling and removing the meristematic region of the seedling by cutting 1-2 mm below the cotyledonary node. Explants were cultured vertically down with 3-5 mm of the apical region inserted in to the medium and the root region away from the medium.

#### 2.5 Pre-incubation of explants for transformation

Freshly excised explants were pre-cultured for 2-4 days on MS medium supplemented with 0.5 mg/l IAA, 5mg/l BAP. Agrobacterium was grown in Trypton Yeast Extract Medium with 10mg/l rifampicin and 5mg/l tetracyclin on an orbital shaker (Scigenics Biotech, Chennai, India) to an OD of 0.5 to 0.72. Acetosyringone was added at 50 µM to the culture 2-3 hours before harvesting. The bacterial pellet obtained after centrifuging the culture, was washed twice by re-suspending MS liquid medium, pH 5.6. Explants were infected with the re-suspended bacterial culture in MS liquid medium.

#### 2.6 Co-cultivation and Recovery

The explants were blotted dry on filter paper after the infection. The explants were inoculated on the same media which was used for pre incubation. The culture bottles were kept in dark for co-cultivation at 23 °C for 2-3 days. After co-cultivation the explants were transferred to shoot regeneration medium (SRM) supplemented with 250 mg/l of augumentin for recovery. The recovery period varied from 2-5 days.

#### 2.7 Selection and rooting of transformants

SRM with 250 mg/l of augumentin and kanamycin (25 mg/l) was used for selecting the regenerating shoot buds. Explants were transferred to fresh selection medium after every two weeks period. Usually the experiments were concluded by six or seven selections, when well developed shoots were obtained in some cases or all the buds died as in some other cases. Harvested shoots were put for rooting on MS medium with 250 mg/l of augumentin and 25 mg/l kanamycin.

#### 2.8 Molecular analysis of transformants

Genomic DNA was extracted from the plants that were rooted on kanamycin containing medium by a modified method of SDS-potassium-acetate method (Dellaporta et al., 1983). PCR was carried out by using forward and reverse primers designed to amplify to 675bp fragment of chilli leaf curl virus (ChLCuV) coat protein gene (CP). (Forward primer: 5' CGC CGC CGT CTC AAC TTC 3' and reverse primer: 5' AGC ATA CAC AGG GTT AGA GGC 3'). The PCR was done by using 200ng of plant DNA, 25nM of each

primer, 250 µM of dNTP mixture, 10X PCR buffer having 1.5mm MgCl<sub>2</sub> in 50 µl reaction volume. PCR was done for 30 cycles each cycle consisted 94 °C for 1min, 62 °C for 1 min and 72 °C for 2 min for 30 cycles. The amplification product of PCR was checked in 1.0% agarose gel. For southern blotting of T0 plants, 6-8 µg of genomic DNA was restriction digested with Hind III and Bgl II at 37 °C for overnight, to release the CP (coat protein) gene integrated into the plant genome. Whereas DNA from T1 plants was digested with Bgl II to cut the coat protein gene at one end to determine the number of copy numbers of transgene. PCR amplified fragment of CP gene from pRMP9 plasmid was cloned in to pTZ57R (Fermentas life sciences, USA) for preparing the probe. Hybridization was carried out as per the instructions of AlkPhos-non-radioactive label and chemiluminiscent detection kit (Geneimages- Amersham).

#### 2.9 Acclimatization of transformants and advancing the generation

All PCR positive plants were transferred to pots with coco peat. Each plant was covered with a transparent plastic cup during day time. After 8 days plantlets were transferred to bigger pots filled with soil. These plants (T0 generation) were carried to the next generation (T1 generation) by selfing. T1 seeds were germinated on ½MS with augumentin (250 mg/l) and 30mg/l kanamycin. The length of the root was taken as main criteria to score the progeny to distinguish the seedlings carrying the transgene from the null segregant that have not received the transgene.

### 3. Results

A total of 22 PCR positive plants were recovered from 394 intact-hypocotyl explants, giving a transformation frequency of 5.58 %. A representative picture of PCR is given in the Figure.5 a, where 675 bp of amplification product from CP (coat protein gene) of ChLCuV (chilli leaf curl virus) is seen in the putative transformants. Seven of the twelve putative transgenic plants analyzed showed positive result in southern blot analysis where 0.77kb insert of coat protein gene was released (lane no. 83, 98 & 112 in Figure 5b).

Acclimatization of transgenic plants was achieved without any difficulties. All the plants survived at both the stages of transfer to the pots i.e., from the bottles to pots containing coco peat and from pots containing coco peat to pots containing soil.

Fifty seeds obtained from each of T0 transgenic plants (number T1 and T2) were inoculated on ½MS + augumentin (250 mg/l) and 30mg/l kanamycin. The root system was reduced in 29.4% of the progeny from plant T1 and 11.1% of the progeny of plant T2. For plant number T1, the ratio of the progeny with normal root system to reduced root system was 2.4:1 which is nearly 3:1 that corresponds to Mendelian fashion of segregation of a single copy of transgene integrated into the plant genome. This segregation pattern matched with the results obtained from southern blot (Fig. 5c). Whereas for plant T2 the ratio of the progeny with normal root system to reduced root system was 6.5:1 which indicates that there are more than one copy of transgene

integrated into the chilli genome. This also corresponded with observed copy number of two in the southern blot (Fig. 5c).

The transgenic plants showed normal phenotype and produced fruits similar to that of non-transgenic plants (Fig. 6).

#### 4. Discussion

We have been successful in obtaining transformants in the chilli variety RCL-59M using intact-hypocotyl explants. In contrast, Szász et al., 1995 failed to obtain the transformants in chilli using intact hypocotyl explants. Szász et al., 1995 discussing the reasons for not obtaining the transformants suggest that, after first selection when the explants were brought to the erect position there was no selection because of which the non-transformed shoot primordia over grew and suppressed the growth of transformed ones. In the present study the intact-hypocotyl explants were kept in the selection medium for 90 to 100 days and hence selection pressure was persistent throughout bud formation. Further only the first formed vigorous shoots were taken for rooting on kanamycin and subsequently for molecular analysis.

#### Kanamycin Selection for transformation

Various concentrations of kanamycin viz., 50mg/l (Zhu et al., 1996, Manoharan et al., 1998, Li et al., 2003), 100 mg/l (Lee et al., 1993), 200 mg/l (Liu et al., 1990) have been used for Chilli transformation. In some studies however, kanamycin was found to be not effective in selecting the chilli transformants (Engler et al., 1993, Ochoa -Alejo and Ramírez-Malagón 2001). In the present study the 25mg/l kanamycin was found to be suitable for selection of the transformants. 25mg/l of kanamycin was also found to be sufficient to suppress root formation from non-transformed shoots for visual differentiation of transformants from non-transformants. The same concentration was also successfully used by Li et al., (2003). Where as in case of other reports Lee et al., (1993) used 50mg/l kanamycin; Zhu et al., (1996) used 50 mg/l kanamycin; Kim et al., (1997) used 200 mg/l kanamycin for rooting of putative transformants.

#### Molecular analysis of transformants

Molecular analysis was carried either by PCR alone (Ahmad et al., 2002; Li et al., 2003) or PCR followed by Southern analysis (Lee et al., 1993; Manoharan et al., 1998; Shivegowda et al., 2002; Lee et al., 2004) to confirm the transgenic status of the putative chilli transformants. In the present study, chilli leaf curl virus coat protein gene (675bp) was amplified in 22 transformants from different experiments. Confirmation of transformed nature of transgenics was done by Southern blotting of PCR positive transformants. In seven out of twelve plants tested by Southern blotting, a 0.77 kb band of coat protein gene was released confirming the transgene integration.

Southern blotting was done for the progeny of the two of the transgenic plants to check the copy number of the coat protein gene integrated into the genome. T1 progeny of the line 83 showed single copy of gene integration, where as

progeny of the plant 98 showed two copies of the transgene integrated into chilli genome. The copy number of analysis by southern blotting confirmed the transgenic lines containing single copy or two copies of transgene integration matched with the ratios of the seedlings with reduced root system to the normal system in kanamycin germination test.

#### Efficiency of Transformation

The earlier reports on chilli transformation showed low transformation frequencies such as 0.3% (Engler et al., 1993), 4% (Lee et al., 1993 and Kim et al., 1997), 2% (Manoharan et al., 1998), 0.8% (Kim et al., 2001), 0.06% (Ochoa-Alejo and Ramírez-Malagón, 2001) and 5 % (Cai et al., 2003). In our study also we could achieve a low transformation frequency of only 2.1% in experiment T10, and 6.6% in T25 and T36 experiments. But, Li et al., (2003) reported 40.8% of transformation frequency in their study, which is the highest among the reported transformation frequencies in chilli. The reasons for high transformation frequency in the report by Li et al., (2003) could be because the genotypes they have used were of bell pepper.

In conclusion we are successful in establishing a protocol for obtaining the transgenics of a pure line chilli variety RCL-59M. After checking these transgenic plants for the resistant against Chilli leaf curl virus (ChLCuV), the resistant lines will be used in the hybrid seed program to develop Viral resistant chilli hybrid seeds.

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