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Transformation of Red Chilli Variety Cillibangi-2 (*Capsicum annum* L.) with cDNA of Cucumber Mosaic Virus Coat Protein Gene by Direct Uptake

Ahmad, I.B., M.N. Hanina, A.M. Mahir and A. Zaiton
Plant Biotechnology Laboratory, Faculty of Science and Technology,
University Kebangsaan Malaysia, Bangi, Selangor, Malaysia

Abstract: The 670 bp cDNA of cucumber mosaic virus coat protein (CMV-CP) gene synthesized from purified viral RNA using a cDNA synthesis kit, was amplified by PCR using primers containing *Xba*I and *Bam*HI restriction sites at the 5' and 3' ends, respectively. The cDNA fragments were cloned into pUC18, sequenced and then subcloned into plant transformation vector pBI221 to generate pBI221CP recombinant plasmid. The recombinant plasmid pBI221CP was mobilized into 12 days old chilli shoot tip explants of the variety Cillibangi-2 by a modified direct uptake method. Analysis of T1 plants derived from a T0 plant showed variability when analyzed by transient GUS assay and PCR analysis for the GUS gene and the CMV-CP cDNA. Further PCR analysis of T2 plants which were derived from eight T1 plants showed that 25 to 54% of the plants carried the CMV-CP cDNA. Repeat of the transformation method was successful in obtaining new positive plants.

Key words: *Capsicum annum*, chilli, transgenic, transformation, CMV, coat protein

Introduction

Red chilli (*Capsicum annum* L.) is a popular vegetable fruit in South East Asia and used as food additives. This crop is prone to attack to several diseases, of which cucumber mosaic virus (CMV) is the most important (Green, 1992). The virus causes various symptoms on different plants ranging from mild mosaic to severe symptoms including death of plants (Ahmad & Scott, 1985). In chilli it causes symptoms of extreme variability in forms and severity including stunted growth, mosaic, filiform leaves and fruit discoloration and malformation (Black *et al*., 1991). Although resistant genes for this virus in chilli exist (Chew, 1992), other sources of gene for resistance to the virus are also required for maintaining a larger pool of resistant genes to particular pathogens. This is very important in commercially planted chilli, since CMV is very ubiquitous and infecting many species of crop plants (Kaper & Waterworth, 1981; Palukaitis *et al*., 1992), which can serve as reservoir for infection into chilli fields.

The success of CP-mediated resistance in various plants to viruses (Abel *et al*., 1986; Cuozzo *et al*., 1988; Hull, 1994) offers new avenue for incorporating resistance to CMV in chilli. However, the effort to obtain transgenic chilli is hampered by the difficulty of regenerating transformed explants (Ahmad *et al*., 1997a; 1997b; Liu *et al*., 1990) although regeneration of non-transgenic plants for the red chillies has been reported (Nurina *et al*., 1992; Gunay & Rao, 1978; Valera-Montero & Ochoa-Alejo, 1992). This is particularly true with the variety Cillibangi-2 and its parent line, WL-1 that was not very amendable to tissue culture and transformation (Ahmad *et al*., 1997a). Although shoot-like structures were frequently obtained, the inability of these structures to elongate has been cited as the main reason for failure to obtain complete transgenic chilli plants. It is only recently that successful *Agrobacterium*-mediated transformation of red chilli being reported (Manoharan *et al*., 1998). However, since it has been known that chilli tissue culture is genotype specific (Manoharan *et al*., 1998) and that Cillibangi-2 is recalcitrant, an alternative method of transformation of this chilli variety was also studied, i.e by direct uptake method without undergoing calli stage.

Materials and Methods

Viral nucleic acid preparation and amplification: The study was carried out at the Plant Biotechnology Laboratory, University Kebangsaan Malaysia, from 1994 to 2001, beginning with virus purification and followed by nucleic acid preparation. The virus was maintained in 7-8 weeks old tobacco plants (*Nicotiana tabacum* var. White Burly) and the infected plants were used for virus purification after 8 days of inoculation. Virus purification was carried out using the method of Ahmad & Scott (1985). For RNA genome extraction, the purified virus was first disrupted with 0.1% sodium dodecyl sulfate (SDS) and its non-RNA components were extracted out with equal volume of phenol. Following 1-minute centrifugation (13,500g), the aqueous phase was further subjected to chloroform-isoamyl alcohol extraction and centrifugation. The RNA was precipitated with ethanol (-70°C overnight) and centrifuged at 13,500g and finally suspended in sterilized distilled water.

Synthesis of first strand CMV-CP cDNA was carried out by using cDNA Synthesis System (Gibco BRL) with specific primers based on RNA-3 sequence of CMV-D (Quemada *et al*., 1989). The forward primer, with specific site for *Xba*I, was 5'TCTAGAATGGACAAATCTG3' and the reverse primer, with specific site for *Bam*HI was 5'GGATCCTCAGACTGGGAGCGC3'. The cDNA was then amplified by PCR as described by Doherty *et al*. (1989) using the protocol of 94°C (90 second), 55°C (2 minutes) and 72°C (3 minutes). The PCR was terminated by incubation at 72°C for 10 minutes.

Cloning and subcloning of CMV-CP cDNA: The PCR product and pUC18-cloning vector were digested separately with *Bam*HI and *Xba*I and then incubated for 15 minutes at 70°C to inactivate the enzymes. The vector and the PCR product were ligated using 1U T4 DNA ligase at 15°C for 16 hours (Sambrook *et al*., 1989). The ligation mixture was then used for transformation of *E. coli* JM109 competent cells (Sambrook *et al*., 1989) by the heat shock method (42°C, 60 seconds) (Hanahan, 1983). Recombinant plasmids from selected colonies were extracted (Birnboim, 1983) and analyzed by restriction enzyme digestion and agarose gel electrophoresis. A recombinant plasmid was selected, column-purified (Promega) and sequenced (automated).

Subcloning into pBI221 (Clontech) plant transformation vector was carried out as follows: Recombinant pUC18 and pBI221 were individually digested overnight with *BamH*I and *Xba*I and then electrophoresed to obtain CMV-CP cDNA and pBI221 fragments with sticky ends. These fragments were then electroeluted (Yang *et al*., 1979) from agarose gel and used in ligation experiments involving CMV-CP cDNA for coat protein and pBI221. These ligation mixtures were then transformed into *E. coli* JM109 and spread onto LBA plates fortified with ampicillin. White colonies growing on the plates were subcultured and then analyzed for the presence of recombinant plasmids.

Mobilization into chilli explants: Mobilization of pBI221 recombinant plasmid into 12 days old chilli explants was carried out by Carborundum-heat shock method as described below. The cotyledons were removed and the shoot tip was carefully sliced off so as to just injure the meristematic region. The top end of the hypocotyls was cut at about 10 mm length and placed into a microtube containing 1.67% suspension of Carborundum (400

mesh) in sterile water (Cheng *et al.*, 1996). The microtube was mixed thoroughly for 1 minute and then washed three times with Murashige & Skoog (1962) culture media (MS) containing 0.5M mannitol. The washed explants were placed into micro tubes containing 500µl MS media containing 0.5M mannitol, 20% PEG 6000 and 100µg/ml recombinant plasmid. The mixture was agitated in a Microtube Mixture MT-300 for 1 hour at room temperature, and after incubation on ice for a further 10 minutes, the explants were given a heat-shock treatment of 42°C for 90 seconds. The explants were washed three times with MS media containing 0.5M mannitol before being cultured onto MS agar supplemented with 5.0 mg/l BAP and incubated at 25°C with 20-Watt light intensity. Explants with shoots were let to form roots in MS media containing 0.1mg/l NAA. Rooted explants were transferred to soil to form complete plants.

Analysis for transformation: Transient GUS assay was performed according to the method described by Jefferson (1987) and Kosugi *et al.* (1990). Thirty milligrams of leaf samples were placed into microtube containing 600µl extraction buffer (0.05M Sodium, 0.01M EDTA with 0.1% Triton-X and 0.01M mercaptoethanol), ground, homogenized and then centrifuged (10, 000 rpm) for 5 minutes. The supernatant (50µl) was then added into a prewarmed (37°C, 30 minutes) microtube containing 30µl of 2mM MUG (4-methylumbelliferyl-B-D-glucoronide), 220µl extraction buffer and was mixed thoroughly. Enzymatic reaction was carried out at 37°C for 2 hours before being stopped with 0.2M Na_2CO_3. The presence of fluorescence was measured using a fluorometer. PCR analysis was carried out to determine the presence of GUS gene and CMV-CP cDNA in the transformed plants. Leaf samples were taken from one month-old plants and whole plant DNA was extracted according to the method described by Dasgupta *et al.* (1996). For each plant, 0.125 g of leaf samples were homogenized with 1.0 ml TE buffer (0.01M Tris-HCl-0.0001M EDTA), pH 8.0 and then centrifuged (12,000 rpm) for 10 min. The supernatant (400 µl) was then added with 100µl solution I (0.025M Tris-HCl-0.01M EDTA, pH 8.0), then with 200µl solution II (0.2M NaOH with 1% SDS) and finally with 150µl of solution III (5M potassium acetate, pH 4.8). After centrifugation, the aqueous solution was transferred to new tubes and the DNA was precipitated with 100% ethanol (at -20°C, overnight) and centrifuged at 14,000 rpm for 10 min. Finally the DNA was washed with 70% ethanol and left to dry by vacuum before suspended in sterilized distilled water.

Five sets of primers were used for PCR detection of the GUS, CP or both the genes. The GUS-F1/GUS-R1 primer combination was used for the detection of GUS gene sequence, while the pBI-F1/pBI-R1 and CP-F1/CP-R1 primer combinations were for the detection of CP. The CP-F1/GUS-R1 and pBI-F1/GUS-R1 primer combinations were for the detection of GUS-CP sequence. An additional primer combination, CPI-F1/CPI-R1, was based on sequence within the CP gene used for the detection of CMV-CP cDNA in T1 plants. The sequences of the primers, complementary sequences on the gene cassette and expected product after PCR are given in Fig. 1. The protocols for the six sets of primers, also respectively, were as follows: 95°C (1min)-58°C (1min)-72°C (2 min), 95°C (1 min)-58°C (1min)-72°C (2 min), 95°C (1 min)-58°C (1min)-72°C (2 min), 95°C (1 min)-58°C (1min)-72°C (2 min), 95°C (1 min)-58°C (1min)-72°C (2 min), 95°C (1 min)-58°C (1min)-72°C (2 min).

Results

RNA preparations and amplification: The successful extraction of CMV RNA from purified virus preparation was evident from the existence of 4 RNA molecules when run in SDS-PAGE. The molecular weights of the RNA molecules were $1.12x10^6$d (RNA 1) and $1.11x10^6$d (RNA 2), $7.48x10^5$d (RNA 3) and $3.74x10^5$d (RNA 4). The cDNA synthesis and subsequent PCR using the viral RNA preparation as the template produced a single band of 670 bp when analyzed by agarose gel electrophoresis. The size of the PCR product obtained was as expected from the data of Quemada *et al.* (1989).

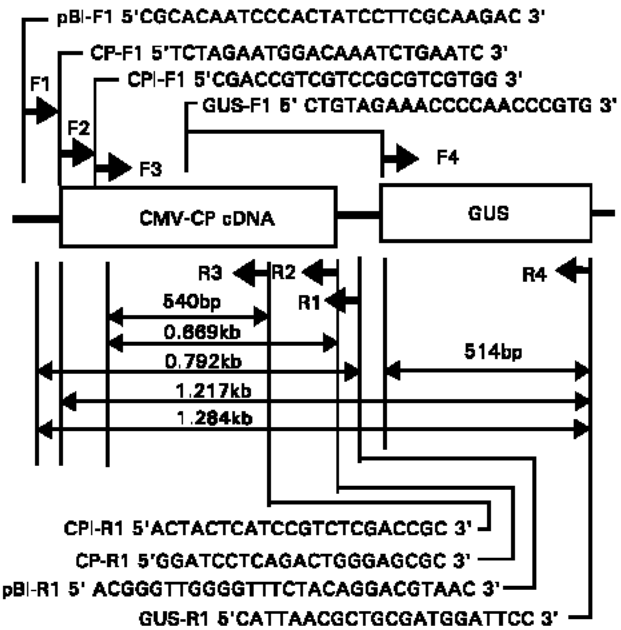

Fig. 1: Schematic presentation of the sequences of the primers, their complementary sequences on the gene cassette, and expected product size after PCR. Letters "F" and "R" signify forward and reversed primers, respectively.

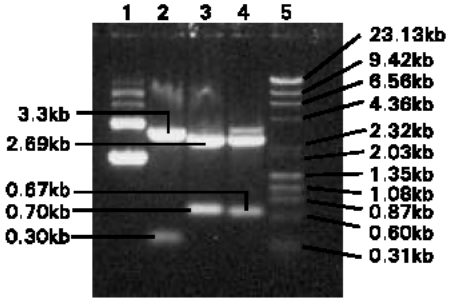

Fig. 2: Agarose profile of undigested (lane 1) and digested (lanes 2-4) pUC18 recombinant plasmid. Digestion was made with *Hind*III (lane 2), *Xba*I/*Eco*RI (lane 3) and *Xba*I/*Bam*HI (lane 4). Lane 5 contains mixture of λ DNA *Hind*III and ϕ X174 RF DNA *Hae*III molecular size markers. Fragments with sizes of 0.67 and 0.70kb contain cDNA CP-CMV gene sequence.

Cloning of CMV-CP and subcloning into transformation vectors: The PCR product was successfully ligated into pUC18 vector. Following transformation into *E. coli* JM109 and screening of white colonies, a colony containing a recombinant plasmid was selected. The CMV-CP sequence was confirmed by restriction enzymes analysis (Fig. 2) and sequencing (Quemada *et al.*, 1989). Colonies of *E. coli* containing recombinant pBI221 were subcultured in LB ampicillin and subjected to plasmid purification. RE diagnostics on the purified plasmid showed that recombinant plasmids of pBI221 were obtained. The plasmid showed the presence of 670 bp fragment when cut with *Xba*I (situated at 5'-end of the cDNA fragment) and *Bam*HI (situated either at 3'- end

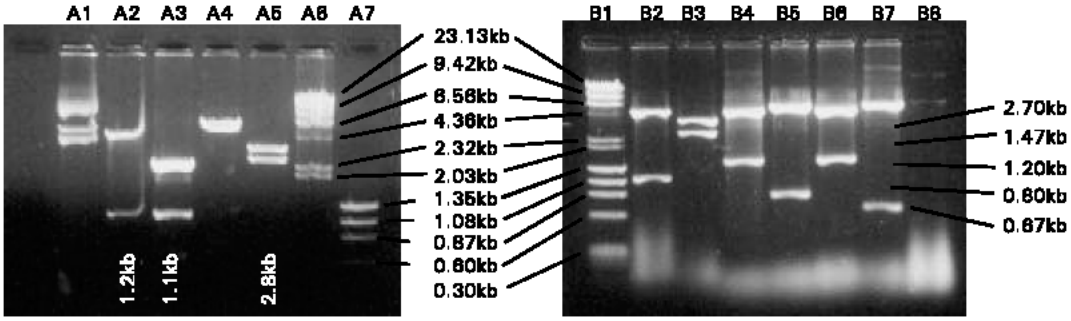

Fig. 3: Profile of recombinant pBI221 plasmid after digestion with restriction enzymes: Undigested control (Lanes A1; B8) and digested (Lanes A2-A5; Lanes B2-B7) with: *Hin*dIII (lanes A2 & B2; insert in 1.2 kb fragments); *Eco*RI/*Hin*dIII (lane A3; 1.1kb fragment); *Sa*lI (lane A4; single site; 6.37 kb); *Sal*I/*Eco*RI (lane A5; 2.8 kb fragment); *Pst*I/*Eco*RI (lane B3; 2.7 kb fragment); *Pst*I/*Bam*HI (lane B4; 1.47 fragment); *Pst*I/*Xba*I (lane B5; 0.80 kb fragment); *Pst*I/*Sma*I (lane B6; 1.47kb fragment); *Xba*I/*Bam*HI (lane B7, 0.67 kb insert); Molecular weight markers are in lane A6 (λ DNA *Hin*dIII), lane A7 ϕ X174 RF DNA *Hae*III, and lane B1 (mixture of λ DNA *Hin*dIII and ϕ X174 RF DNA *Hae*III).

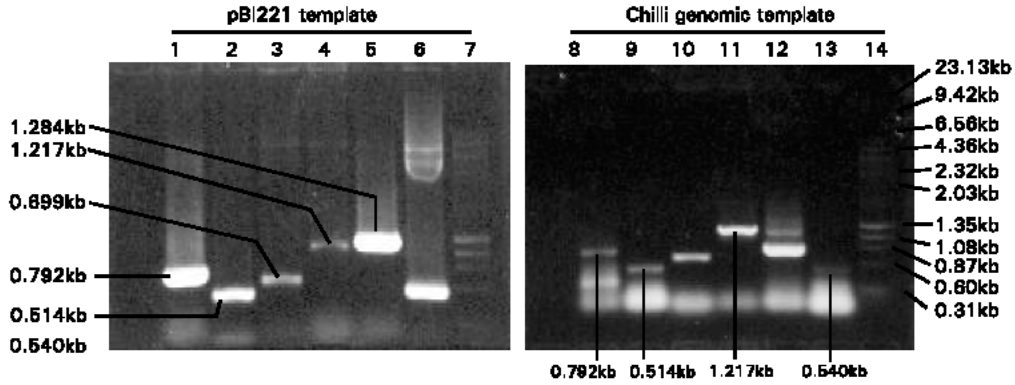

Fig. 4: The bands generated from PCR using primer sets as in Table 1 and Fig. 1 and pBI221 recombinant plasmid (lanes 1-6) or transgenic chilli genomic DNA (lanes 8-13) as the templates. The primer sets used were: Set-1 (pBI-F1/pBI-R1, 0.792 kb) in lanes 1 & 8; Set-2 (GUS-F1/GUS-R1, 0.514 kb) in lanes 2 & 9; Set-3 (CP-F1/CP-R1, 0.669 kb) in lanes 3 & 10; Set-4 (CP-F1/GUS-R1, 1.217 kb) in lanes 4 & 11, Set-5 (pBI-F1/GUS-R1, 1.284 kb) in lanes 5 & 12; Set-6 (CPI-F1 / CPI-R1, 0.540 kb) in lanes 6 &13. Lane 7 & 14 contain a mixture of λ DNA *Hin*dIII and ϕ X174 RF DNA *Hae*III markers.

Fig. 5: The T1 transgenic chilli plant produced fruits as non-transgenic plants.

of the cDNA fragment). Further proof was obtained from the presence of fragment when the recombinant plasmids were cut with six combinations of restriction enzymes, which cut the DNA either in the CMV-CP sequence or in the vector (Fig. 3). Besides, single digestion with *Hin*dIII (situated in CMV-CP sequence and vector) showed the presence of two fragments (5.2 and 1.1 kb). A fragment of 6.37 kb was obtained when the recombinant plasmids were cut with *Sa*lI (situated in CMV-CP sequence).

Table 1: The detection of CMV-CP specific cDNA in T1 chilli plants when analyzed by PCR using the six primer sets

	Primer sets (1-6) and expected sizes of PCR products[a]					
T1 Plant	1	2	3	4	5	6[b]
15	+	+	+	+	+	+ + +
19	+	-	-	-	-	+ + +
23	+	+	+	+	+	+ + +
24	+	+	+	-	+	- + +
25	+	+	+	-	+	- + +
29	+	+	+	-	-	- + +
31	+	+	+	+	+	- + +
37	+	+	+	-	+	+ + +
I	+	+	+	+	+	- - +
J	+	+	+	+	+	+ + +
L	+	+	+	-	+	+ + +
M	+	+	+	+	+	- + +
O	+	+	+	+	+	- - +
P	+	+	+	-	+	- + +
Q	+	+	-	-	0.8 kb	- - +
16	-	+	+	-	0.8 kb	+ + +

[a]Primer sets: 1. PBI-F1/pBI-R1 (0.792 kb); 2. GUS-F1/GUS-R1 (0.514 kb); 3. CP-F1/CP-R1 (0.669 kb); 4. CP-F1/GUS-R1(1.217 kb) 5. PBI-F1/GUS-R1 (1.284 kb) 6. CPI-F1/CPI-R1 (0.540 kb).

[b]PCR analysis of similar plants in using three different automated thermocyclers.

Table 2: Percentage of T2 plants showing the presence of specific CMV-CP gene when analyzed by PCR using a primer sequence within the CP gene

T1 plants[a] from which T2 plants were derived	The number of T2 plants tested	The number of T2 tested positive	Percentage of T2 tested positive
15	40	21	52.5
19	37	20	54.2
23	38	14	36.8
24	43	15	34.9
25	43	16	37.1
29	47	12	25.5
31	44	13	29.5
37	46	16	34.8

[a]Identification codes of T1 plants from which T2 plants were derived from T1 plants

Mobilization of pBI221 into plantlets: Of the 40 explants treated with pBI221 plasmids using Carborundum-heat shock method, 22 plantlets were produced. Upon generation into complete plants they were transferred into the greenhouse. A T0 plant which was positive for transient GUS assay, was let to fruit. Similar procedure carried out on explants but without recombinant plasmid generated 23 plants. The seeds of the fruits from the putatively transgenic T0 plant were pooled and randomly planted to generate T1 plants, which showed positive results when analyzed by PCR using the six primer sets (Table 1; Fig. 1). Of the 16 T1 plants tested with PCR, only five plants (15, 23, 31, J and M) were shown to produce the expected bands with all the six primer sets used (Table 1; Fig. 4). The others were positive for 4-6 primer sets. Subsequently, seeds derived from eight T1 plants were randomly selected and planted. Following PCR analysis, amplification of the expected CMV-CP fragment was successful in 25 - 54% of T2 plants that have been derived from each T1 plant (Table 2). The transgenic plants produced fruits (Fig. 5) normally adding to the contention that the transformation study was successful. When this transformation procedure was repeated, several new transgenic plants were obtained (results not shown).

Discussion

Cloning and subcloning of a plant virus gene is an important step in a programme to produce transgenic plant exhibiting artificial resistance to a particular virus. There are several important requirements for the successful application of this approach of inducing resistance in plants. Firstly, the gene selected must be involved in the replication or transport of the viral materials in the plants. Second, the cDNA must be able to express the mRNA and protein molecules, which in turn is able to interfere with the multiplication of the infecting virus (Hull, 1994). The third and most critical step is the ability to generate complete plants after the transformation process. The main aim of transformation methods is to incorporate the DNA into plant genome and let the plant to grow into complete plants (Draper & Scott, 1991). In red chilli this is the most difficult step to overcome since tissue culture of red chilli is inconsistent, recalcitrant and genotype specific (Liu *et al.*, 1990; Manoharan *et al.*, 1998).

Transformation using Carborundum-heat shock method was carried out to circumvent the problem of regeneration in chilli. This method depends on the occurrence of imbibition of DNA molecules through the cell of explants, which are deprived of water for some time (Senaratna *et al.*, 1991). The success of this method in producing transgenic chilli was postulated to be due to several factors such as the exposure of meristematic cells, presence of PEG and the small size of recombinant plasmids. The exposure of the meristematic cells is afforded by cutting the tip of explant's meristematic tissue and Carborundum treatment. Carborundum is said to produce tiny wounds at cell walls (Cheng *et al.*, 1996) but small enough to allow recovery and proliferation. To ensure efficient entry of DNA into the cells the smaller pBI221 recombinant molecule was used. The movement into the explant cells was aided by the presence of PEG which changes the permeability of the plasma membranes thereby allowing the macro molecules to pass into the cytoplasm (Songstad *et al.*, 1995). In addition, the use of pBI221 was advantageous in that this plasmid does not transfer antibiotic resistant gene into the plants.

The successful transformation of the explants was shown by the use of PCR analysis using several pairs of primers, some of which were internal to the CMV-CP cDNA and were not used for cloning the gene fragment. The various primer sets were also designed to produce fragments of different sizes, namely GUS gene (0.514 kb), cDNA-CP insert (0.669 kb by using CP-F1/CP-R1 primers or 0.792 kb by using pBI-F1/pBI-R1 primers or 0.540 kb by using CPI-F1/CPI-R1), and cDNA-CP insert with GUS gene (1.217 kb by using CP-F1/GUS-R1 primers or 1.284 kb by using pBI-F1/GUS-R1). The ability to generate amplicons of various sizes with different primer sets reduces the risk of false positives.

The results shown in Table 2 not only proved the successful incorporation of the CMV-CP gene in T1 plants but also the ability of the gene to be inherited into the T2 generation. The ability of being inherited indicated that the proportion of cells in the chimeric plants carrying the CMV-CP cDNA was probably high. This was probably facilitated by the exposure of meristematic tissues to the recombinant plasmid molecules during heat-shock treatment. Transformed meristematic cells actively divided to form transformed tissues and later formed plant organs. With further selection from the T2, T3 and later generations, increase in the positive percentage is expected. Selection of transgenic plants in later generations will be done not only based on the presence of cDNA, but also on the resistant reaction of the plants towards inoculation challenge with CMV isolates.

The tests were repeated twice or three times on different PCR machines situated in different locations at the Plant Biotechnology Laboratory, in order to ascertain the repeatability of the detection of CMV-CP cDNA by PCR analysis. The use of internal primers, which are not used in the original experiment to obtain the original cDNA fragment, was taken as an alternative to Southern blotting in the effort to detect the viral gene sequence in the plant genome. However, the present study did not show integration of the cDNA into the genome nor did it show its expression. Currently, detection of CMV-CP cDNA by southern blotting is being carried out. Nevertheless there is indication that it is an expressed since transient GUS gene activity was detected in the early screening for positive plants. One important aspect about this transformation technique is its repeatability. The present study therefore develops an alternative method for transforming recalcitrant species such as red chillies.

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