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Coat protein-mediated resistance as an approach for controlling an Egyptian isolate of *Cucumber mosaic virus* (subgroup I)

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Abstract: *Cucumber mosaic virus* (CMV, *cucumovirus*) is the most important virus infecting cucurbit crops in Egypt and worldwide causing significant loss in yield quality and quantity. The main target of the present work was to establish a simple controlling system for an Egyptian isolate of such virus (belonging to the subgroup I) via production of tobacco transgenic plants expressing viral coat protein (CP). Coat protein gene (*cp*) was isolated and amplified using immunocapture-reverse transcriptase-polymerase chain reaction (IC-RT-PCR) and primers with add-on restriction sites for *Sma*I and *Sac*I enzymes. The genes were cloned in pBI121 vector plasmid between the CaMV 35S promoter and the nos terminator after removing the Gus gene by restriction enzymes digestion. The new construct was used for *Agrobacterium tumefaciens* transformation, which was then used for tobacco transformation. Evaluation of transformation success and CP expression degree were confirmed using indirect enzyme-linked immunosorbent assay (I-ELISA) and dot blot immuno-binding assay (DBIA). PCR and RT-PCR were used to study the integration of cp within genetic plant system and to what extent this gene was transcript. It was concluded that in spite of integration success some transformed plants can transcript the gene more than the others do. Plants resistance was tested by challenging with CMV under study and remarkable success was obtained in plants with higher gene transcription and translation degree.

Key words: *Cucumber mosaic virus*; *cucumovirus*; coat protein gene; immunocapture-RT-PCR; indirect-ELISA; dot blot immune-binding assay; transformation; *Agrobacterium*.

Abbreviations: CaMV, *Cauliflower mosaic virus*; CMV, *Cucumber mosaic virus*; CP, coat protein; *cp*, coat protein gene; DBIA, dot blot immuno-binding assay; IC-RT-PCR, immunocapture-reverse transcriptase-polymerase chain reaction; I-ELISA, indirect-enzyme linked immunosorbent assay; PVX, *Potato virus X*; PVY, *Potato virus Y*; TE, Tris-EDTA; TMV, *Tobacco mosaic virus*.

Introduction

Cucumber mosaic virus (CMV, genus: *Cucumovirus*, family: *Bromoviridae*) is geographically wide spread and has been reported in Australia, North America, New Zealand, Europe and Africa (Brunt et al. 1996; Roossinck 1999). It has four functional pieces of single-stranded RNA, packaged in icosahedral particles, about 30 nm in diameter. The virus is transmitted by numerous species of aphid in a non-persistent manner (Brunt et al. 1996).

The virus has an extremely wide host range and it induces severe symptoms. Douine et al. (1979) listed 775 plant species representing 365 genera and 85 families that are susceptible to CMV infection. In Egypt CMV infects cucurbit and solanaceous crops, banana and sugar beet causing remarkable yield loss (El-Afifi et al. 2007).

Virus disease is incurable and so searchers are focusing on controlling the infection by different means, i.e. removal of weed hosts, starting with virus-free plant material and breeding of plants resistant to virus infection (Fraser 1990).

Techniques of modern molecular biology have been used for more than a decade, mostly for elimination of virus disease in plant material (Trigiano & Gray 1996; Foster & Taylor 1998). Powell-Abel et al. (1986) first demonstrated that the plants engineered to express the *Tobacco mosaic virus* (TMV) coat protein gene (*cp*) can be resisting to corresponding degrees of viral infections. Many researches on CP-mediated protection have produced transgenic plants resistant to a multitude of different plant viruses, e.g., *Alfalfa mosaic virus* (Loesch-Fries et al. 1987), CMV (Cuozzo et al. 1988), *Potato virus X* (PVX) (Hemenway et al. 1988) and *Potato virus Y* (PVY) (Kaniewski et al. 1990). A number of viral proteins-mediated resistances was studied, such as the movement protein (Lapidot et al. 1993) and replicase (Quemada et al. 1991), but the most widely and successfully used strategy is the use of CP-mediated resistance (Grumet 1994; Higgins et al. 2004; Srivastava & Raj 2008).

Therefore, the present investigation aimed to establish and examine a relatively simple and inexpensive genetic engineering method for controlling CMV depending on CP-mediated protection, and to study the effect of CP mRNA transcription on plant resistance degree.

Material and methods

Virus and plasmid source

In this study, CMV (subgroup I) was obtained from Plant Pathology Department, Faculty of Agriculture, Ain Shams University, Egypt). The virus isolate was identified depending on symptomatology, differential hosts, specific monoclonal antibodies (Agdia®, USA) and electron microscopy.

The pBI121 vector plasmid was obtained from Clontech Lab. Inc., USA.

Isolation CMV cp using immunocapture-RT-PCR

Immunocapturing and cDNA synthesis were performed according to Minafera & Hadidi (1994). The following primers (Invitrogen Corp., USA) were used for isolation and amplification of CMV whole *cp*, which were designed using Primer Premier Software (PREMIER Biosoft International, USA) depending on CMV (subgroup I) *cp* sequence data collected from GeneBank (Benson et al. 2007; http://www.ncbi.nlm.nih.gov/GenBank/). Each primer has a restriction enzyme site for *Sma*I and *Sac*I to facilitate gene cloning into the pBI121.

SmaI
5'CCCGGG ATGGACAAATCTGAATCAAC3' (Sense)
SacI
5'GAGCTCTCAAACTGGGAGCACCCCAG3' (Antisense)

PCR was carried out as described by Ghosh et al. (2002). Aliquots of the resulting cDNA (5 μL each) were transferred to tubes each containing 45 μL PCR reaction mixture consisting of primers (1 μM final concentration each), *Taq* DNA polymerase (1 unit), 200 μM of each dNTPs, 5 μL 10X PCR buffer (500 μM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl, pH 8.0), completed to 45 μL with sterile deionized water. The reaction mixture was then overlaid with 50 μL sterilized mineral oil. The Thermal Cycler (PerkinElmer cetus thermal cycler, PerkinElmer Inc., USA) was programmed by an initial denaturation cycle at 94 °C for three minutes. The following 35 cycles were composed of: denaturation step at 94 °C for 1 minute, annealing step for 1 minute at 55 °C and elongation step at 72 °C for 2 minutes. The final cycle of polymerization was performed at 72 °C for 10 minutes.

For PCR product analysis 1.5% agarose gels were used. Electrophoresis was carried out in Sub-Cell DNA apparatus (Bio-Rad Lab., USA) at 80 V. The gels were photographed under UV-transilluminator using a gel documentation system; band size and DNA concentration of each lane were determined from the gel photograph using Gel-Pro Analyzer software (Media Cybernetics, USA).

Purification of PCR products

DNA fragments representing viral *cp* (with the restriction enzymes sites) were purified from agarose gel using the gel slicing and melting method of Wieslander (1979). The final pellets were washed with 1 mL of 70% ethanol, air-dried and re-suspended in 30 μL TE buffer and stored at −20 °C.

Restriction digestion and purification of PCR products

Purified PCR product was digested with *Sma*I and *Sac*I restriction enzymes as described by Sambrook et al. (1989) with some modifications. Digestion was performed in 20 μL total volume containing 1 μg PCR product and 2 μL of 10X buffer: (10 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris-HCl, pH 8.0 and 1 mM DTT), 1 unit of each enzyme and completed with sterile deionized water. The reaction was incubated at 37 °C for 2 hours, and then 20 μL of the digested product was mixed with 6 μL agarose gel loading buffer and electrophoresis was carried out on 1% agarose gel.

Purification of *cp* was done using the gel slicing and melting method Wieslander (1979). The final pellet was dissolved in 20 μL TE buffer.

Cloning of cp into binary vector

Vector digestion was accomplished according to Sambrook el al. (1989). One μg of pBI121 vector plasmid was digested with *Sma*I and *Sac*I to execute the *Gus* gene between 35S CMV promoter and *nos* terminator under conditions described before. The remaining linearized vector was purified from gel using the gel slicing and melting method (Wieslander 1979). For ligation 100 ng of linearized vector were combined with 20 ng of *cp* for each virus (ratio according to Sambrook et al. 1989), 2 μL of 10X ligation buffer (300 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 100 mM DTT and 10 mM ATP) and 1 μL of T_4 DNA ligase (1 unit) were added. The mixture was incubated overnight at 1 °C and stored at 4 °C until used for *Agrobacterium* transformation.

Agrobacterium transformation, minipreparation and PCR

The freeze and thaw method was carried out as described by An et al. (1988) for the transformation of *Agrobacterium tumefaciens* disarmed EHA101 strain with the new construct for CMV *cp*. The transformed *Agrobacterium* cells were evaluated by spreading on LB agar plate (containing 50 μg/mL kanamycin and 25 μg/mL streptomycin) and incubated at 28 °C for 2 days; growing *Agrobacterium* colonies were subcultured on new LB agar with the previously mentioned antibiotics.

Concerning minipreparation, DNA plasmids were extracted from transformed and untransformed (control) *Agrobacterium* cells. This was carried out according to Li et al. (1995).

PCR was performed according to Ghosh et al. (2002) using 1 μL of *Agrobacterium* minipreparations to confirm the transformation success.

Tobacco transformation and regeneration

The *Nicotiana tabacum* cv. White Burley plant materials (50 explants from the upper healthy fully-grown leaves) were placed in a 250 mL glass flask containing 50 mL of liquid MS medium free from bacteria and incubated at 25 °C for 24 hours. The flask was then inoculated with 0.5 mL of an overnight *Agrobacterium* culture (OD_{600} = 0.5) and incubated for 48 hours at 28 °C. The explants were then washed trice with sterilized water and cultured on the regeneration MS medium supplemented with 100 mg/mL kanamycin and 300 mg/mL carbincillin (Horsch et al. 1989). The tobacco regeneration was then carried out using tissue culture systems as described by Horsch et al. (1989); PCR was performed on samples from callus and plantlets to confirm the *cp* integration.

Evaluation of putative transgenic tobacco plants
Fifty of CMV-*cp* transformed tobacco plants (from the 2nd generation) were tested for the viral CP expression with CP-specific monoclonal antibodies (Agdia®, USA) using the indirect enzyme-linked immunosorbent assay (I-ELISA) and dot blot immuno-binding assay (DBIA) as described by Koenig (1981) and Hibi & Saito (1985), respectively.

DNA and RNA were extracted from tobacco plants giving different I-ELISA values and DBIA color degrees (negative results, medium and high) as described by Aldrich & Cullis (1993) and Verwoerd et al. (1989), respectively. PCR was carried out to detect the integration of viral *cp* in the plant DNA, while RT-PCR was performed on the DNase-treated RNA preparation to detect the presence and level of mRNA for CMV *cp* (after confirmation of DNA absence by PCR).

As controls, untransformed healthy tobacco plants and CMV-infected plants were used as negative and positive controls, respectively.

Challenge of transgenic tobacco with CMV
N. tabacum cv. White Burley plants transformed with CMV *cp* and giving different serological results were challenged by mechanical inoculation with CMV. Healthy untransformed plants were inoculated as a control. Plants kept in a greenhouse were monitored daily for symptoms development and severity.

Fifteen days post inoculation, virus level was tested biologically in samples obtained from transformed plants (showing different degrees of symptoms), symptomless and untransformed plants (control). Biological assay was carried out by inoculation of each sample on 25 healthy tobacco plants.

Mechanical inoculation was carried out with constant volume and dilution of the inoculum.

Results

Isolation of CMV cp using IC-RT-PCR
Successfully agarose gel electrophoresis revealed the CMV *cp* bands, with a size of about 670 bp (Fig. 1). The results confirmed that the bands were consisting of the gene fragment with the restriction sites for the desired selected restriction enzymes.

The *cp* fragment was purified from gels and subjected to digestion with *Sma*I and *Sac*I enzymes to create the cohesive ends that facilitate ligation.

Cloning of cp into the pBI121 plasmid
The pBI121 plasmids were digested with *Sma*I and *Sac*I to execute the *Gus* gene. The agarose gel electrophoresis (Fig. 2) revealed the digestion products: bands for *Gus* gene (about 1.88 kbp), linearized vector ready for ligation (about 12.87 kbp) and bands for the intact plasmid (without digestion) as a control.

The opened vectors were ligated to CMV *cp* to create the new construct named L100 (Fig. 3).

Agrobacterium transformation, minipreparation and PCR
After *Agrobacterium* transformation with the new constructs L100, successfully transformed cells (which can resist kanamycin) grew to form colonies, while the untransformed ones failed to grow in the presence of kanamycin antibiotic.

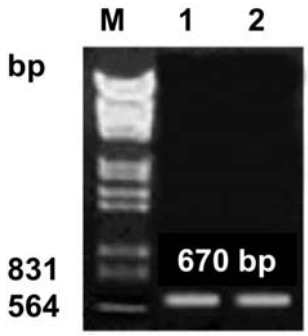

Fig. 1. Isolation of CMV *cp* using IC-RT-PCR. Lanes 1 and 2: PCR product; lane M: Lambda DNA/*Eco*RI+*Hind*III marker (Promega, USA).

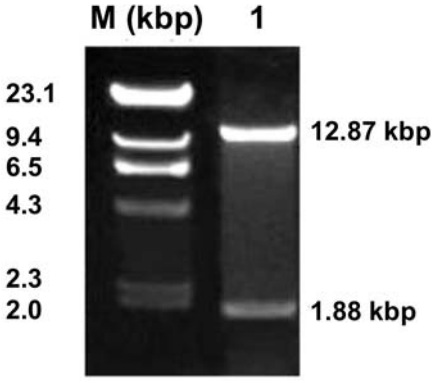

Fig. 2. Restriction digestion products of pBI121 (digested with *Sma*I and *Sac*I). Lane 1: digestion products; lane M: Lambda DNA/*Hind* III marker (Promega, USA).

PCR of the minipreparations analyzed on agarose gel (obtained from transformed and untransformed bacteria) gave the expected bands representing viral *cp* gene (about 657 bp), while the untransformed bacteria gave negative results.

Evaluation of putative transgenic tobacco plants
Tobacco transformation success was evaluated within the 2nd generation plants by testing for the viral CP expression using I-ELISA. According to I-ELISA results, the fifty studied plants were divided into three categories: (i) twenty plants gave negative results (range of 0.051–0.106); (ii) twelve plants gave positive results (range of 0.32–0.433); and (iii) eighteen plants gave positive results ranged from 0.511 to 0.600, representing the highest rate of expression. As a control healthy tobacco I-ELISA values ranged from 0.034 to 0.059, while the CMV-infected values ranged from 0.890–0.941.

I-ELISA results were confirmed by DBIA, performed on five plants from each category (Fig. 4).

PCR and RT-PCR were performed to confirm the CMV *cp* integration and transcription, respectively (Fig. 5). The PCR was carried out for the three plant categories and results indicated that category I gave

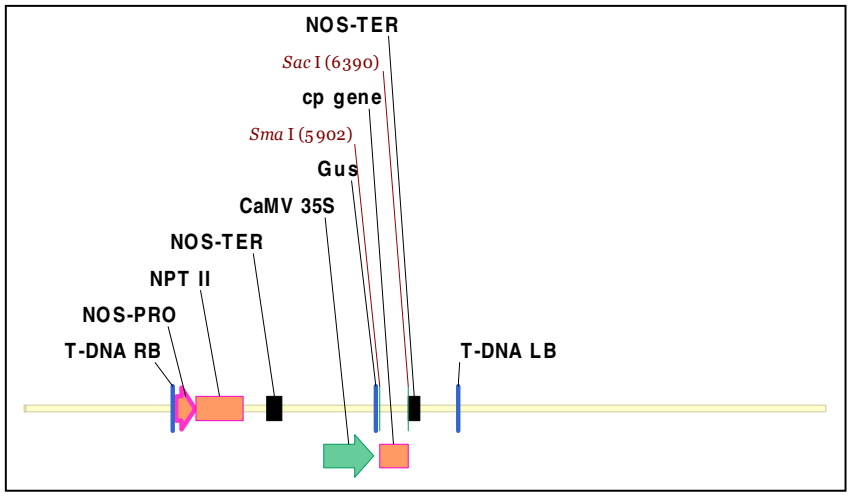

Fig. 3. Schematic drawings of L100 (T-DNA of pBI121–CMV*cp*)

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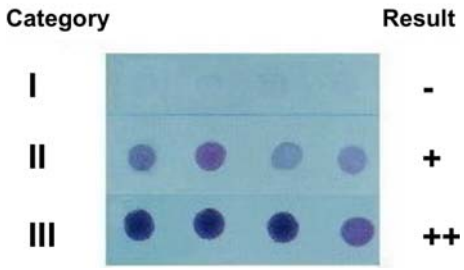

Fig. 4. DBIA for the confirmation of CMV CP expression success in putative tobacco plants: category I gave negative results, category II gave mild positive reaction, and category III gave high degree of positive reaction.

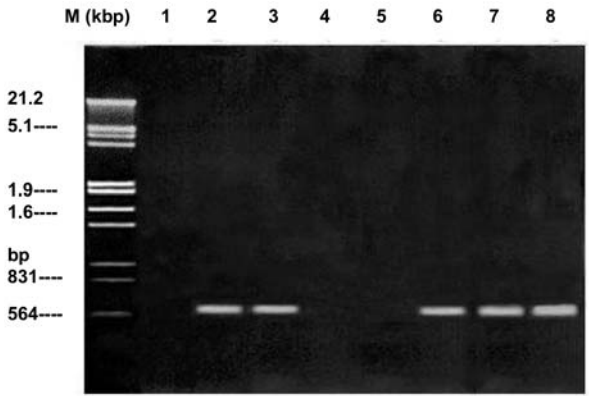

Fig. 5. PCR and RT-PCR for CMV *cp* tobacco transformation evaluation. PCR for plant category I (lane 1), category II (lane 2), category III (lane 3) and untransformed healthy plants (lane 4). RT-PCR of plant category I (lane 5), category II (lane 6), category III (lane 7) and of virus infected plants (lane 8). Lane M: DNA ladder (Promega, USA).

negative results, while category II and III gave the expected bands size representing the *cp* gene. The same bands intensity proved that the integration was successful and with the same degree in categories II and III. PCR gave negative results with untransformed healthy tobacco plants. On the other hand, RT-PCR results were in accordance with those of I-ELISA and DBIA and PCR results. Plants of the third category gave bands with higher intensity confirming the higher mRNA transcription, therefore gave higher CP formation level within plant cells.

Evaluation of transgenic tobacco resistance to CMV infection

After challenging plants with CMV, transformed plants gave negative I-ELISA results (category I) revealing severe symptoms, while plants giving higher I-ELISA values (category III) produced mild or no symptoms (Fig. 6). The mentioned results confirmed the relation between viral protein expression level within transformed plant cells and its resistance to the challenging viral infection.

Fig. 6. Tobacco transformed plants showing different symptoms degrees after challenging with CMV. (A) Category I gave severe symptoms, (B) category II gave moderate symptoms, while (C) category III gave mild symptoms.

Discussion

Since the first demonstration of TMV CP-mediated tobacco transformation (Powell-Abel et al. 1986), a decade of research on CP protection has produced transgenic plants resistance to a multitude of different plant viruses, e.g., *Alfalfa mosaic virus* (Loesch-Fries et al. 1987), CMV (Cuozzo et al. 1988), PVX (Hemenway et al. 1988), and PVY (Kaniewski et al. 1990).

The most successful and widely used strategy for viral protein derived resistances was the use of CP-mediated resistance (Grumet 1994; El-Afifi et al. 2005) compared with other strategies, i.e. movement protein (Lapidot et al. 1993) and replicase (Quemada et al. 1991).

The present investigation aimed to establish a simple method for controlling plant viruses depending on molecular techniques by producing tobacco plants cv. White Burley (as a model) transformed with CMV *cp* via pBI121 plasmid and *A. tumefaciens*.

The *cp* was isolated and amplified using IC-RT-PCR with the aid of primers having add-on restriction sites for *Sma*I and *Sac*I enzymes. Viral gene and linearized pBI121 (after removing *Gus* gene by *Sma*I and *Sac*I digestion) were ligated with T_4 ligase. Work with the same trend has been performed before for *cp* of PVY (Johnson 2001) and PVY and TMV (El-Afifi et al. 2005).

The new construct was transferred into *A. tumefaciens* using freeze and thaw method. Although the transformation frequency using such method is low (approximately 10^3 per μg DNA), the method was reliable, simple and successfully used by some investigators (Holsters et al. 1978; An et al. 1988; Zibata 2001; El-Afifi et al. 2005).

Tobacco 2nd generation transformed plants were evaluated using I-ELISA and DBIA to confirm viral CP expression. Results obtained were negative with some plants, while others gave different positive I-ELISA values revealing degrees of viral protein expression. Many investigators used ELISA and DBIA for transformation evaluation and obtained comparable results (Powell-Abel et al. 1986; Pfitzner & Pfitzner 1992; Bendahmane et al. 1997; El-Afifi et al. 2005).

Plants giving positive I-ELISA values gave positive and approximately similar results with PCR; this confirmed the integration success of viral gene within tobacco genetic system and with the same degree. Concerning RT-PCR (after confirmation of DNA absence by DNase treatment and PCR), plants gave higher I-ELISA values producing bands with high intensity, revealing that the rate of mRNA transcription was more than that for plants with lower I-ELISA values.

When transformed tobacco plants were challenged with the virus of gene origin, plants gave higher I-ELISA values and mRNA transcription showed no or mild symptoms confirming viral infection blockage. On the other hand, plants with low degrees of I-ELISA and RT-PCR gave moderate or severe symptoms. The results obtained proved the relation between mRNA transcription and viral protein expression with the protection levels of viral infections.

Wintermantel & Zaitlin (2000) examined transgenic tobacco plants exhibiting a range of resistance levels for transgene copy number, mRNA and protein levels for CMV replicase gene. They found that resistant lines had consistently more steady-state transgene mRNA than susceptible lines.

Srivastava & Raj (2008) studied the CP-mediated resistance against an Indian isolate of the CMV subgroup IB in transgenic *Nicotiana benthamiana* plants transformed through *A. tumefaciens*. The transgenic lines exhibiting complete resistance remained symptomless and showed reduced or no virus accumulation after virus challenge. Moreover, higher degrees of resistance were not related with CMV CP expression level (Srivastava & Raj 2008).

The underlying mechanisms involved in CP-mediated resistance are not well understood although inhibition of virion assembly and reduced spread of the virus appear to play central roles (Chapman et al. 1992; Taschner et al. 1994; Clark et al. 1995). Nejidat & Beachy (1990) indicated that it was unknown why some CP afford high levels of protection and why others provide broader or lower levels of resistance.

The CP strategy directed to achieving disease resistance has many specific advantages: (i) the cloned cDNA copies of *cp* genes can be obtained relatively easily; (ii) the strategy can generally be extended to further plant species to obtain resistance against other viruses; and (iii) the analysis of transgenic plants that block one or more stages of the infection process is simplified because the block is conferred by a single known gene, in comparison with naturally occurring forms of resistance.

Since CP plays a major role in vector transmission, CP-mediated resistance confers additional advantage of resistance to vector inoculation in a majority of cases. For example, potato (expressing PVX and PVY CP) and tobacco, tomato and cucumber (expressing CMV CP) were found to be highly resistant to aphid transmissions (Lawson et al. 1990).

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