GENERATION OF TRANSGENIC CUCUMBER WITH RESISTANCE TO *CUCURBIT CHLOROTIC YELLOWS VIRUS* USING POLLEN ELECTROTRANSFORMATION

Hsiu-Fung Chao[1], Yi-Fang Hsu[2], Yung-Fu Yen[2]

[1]Yichu branch of Tainan District Agricultural Research and Extension Station, 70 Muchang Hsinhua District, 71246 Tainan, Taiwan

[2]Department of Bioagricultural Science, National Chiayi University, 300, University Road, 60004 Chiayi City, Taiwan

Abstract

The Cucurbit chlorotic yellows virus (CCYV) infects many Cucurbitaceae crops, resulting in chlorotic leaves, retarded growth, and a decreased yield. Transformed cucumber plants incorporating the coat protein (CP) gene of the CCYV were generated using the transformant of CP/pCAMBIA1302 vector. The plasmid DNA was introduced into the pollen through electroporation at voltage intensity of 25 μF × 1.5kV. The electropulsed pollen was used to pollinate emasculated flowers. The transformed T1 offspring were verified using PCR and about 22% of the T1 resistant offspring exhibited CP protein expression, as verified by Western blot analysis of the GFP. In the field evaluation, the transformed plants of the T3 generation showed a high level of resistance to the CCYV. Our results showed that these transgenic cucumber plants were resistance against the CCYV, which they obtained by incorporating the CP gene through a pollen electrotransformation approach.

Key words: *Cucurbit Chlorotic Yellows Virus, pollen electrotransformation, transgenic cucumber, coat protein*

1. INTRODUCTION

Cucurbit chlorotic yellows virus (CCYV) is a newly identified virus species of the genus *Crinivirus* within the family Closteroviridae (Gyoutoku et al. 2009), and is believed to be transmitted specifically by the sweetpotato whitefly, *Bemisia tabaci*, in a semipersistent manner (Li et al. 2016). CCYV can infect 21 species of *Cucurbitaceae* and 12 other plant species. Especially, CCYV is becoming a considerable threat to melon production in both greenhouses and open-fields in Japan (Okuda et al. 2010), it has also become a major constraint for the production of cucumber, pumpkin, watermelon, bottle gourd, and melon in Taiwan (Hunag *et a*l. 2010). The CCYV disease has been found in Japan, Taiwan, China, Sudan, Lebanon, Iran and Egypt (Okuda et al. 2010; Huang et al. 2010; Gu et al. 2011; Zeng et al. 2011; Hamed et al. 2011; Abrahamian et al. 2012; Bananej et al. 2013; Mahmoud, 2015). Cucumber (*Cucumis sativus* L.) is widely grown in Taiwan, old leaves of CCYV- infected plants first show symptoms of chlorosis, then, the leaves become yellow with the veins remaining green, and finally, the plant growth is stunted, and fruit yield and quality decrease. These symptoms of infected plants depend on the host genotype, stage of plant growth, and environment.

Until now, conventional breeding of CCYV-resistant plants has not been practical because the resistant gene in the germplasm of *Cucurbitaceae* has not been identified. Nevertheless, coat protein-mediated protection (CPMP) strategies for developing virus resistance have been effective against several plant viruses, and have led to the commercial release of transgenic virus-resistant papaya, squash, and potato cultivars in the United States (Tepfer, 2002; Mendoza et al. 2008). Transgenic papaya carrying the coat protein (CP) gene of the *Papaya ringspot virus* (PRSV) is highly resistant to the Hawaiian HA strain under glasshouse and field plantation conditions (Lius et al. 1997). Moreover, transgenic tomato lines expressing the capsid protein of the tomato yellow leaf curl virus (TYLCV) have delayed the development of symptoms and enhanced recovery from the disease after whitefly-mediated inoculation (Kunik et al. 1994).

Agrobacterium-mediated DNA delivery and biolistic DNA transfer have been the most used transformation techniques for producing resistant plants (Slater et al. 2008). Using *A. tumefaciens* for

transforming monocots is not very efficient, probably because of the limited integration rates (Frame et al. 2002). In addition, this is not an effective delivery method for some cell types (Ziemienowicz et al. 2012). Biolistic transformation generates multiple integrations of truncated, duplicated, and rearranged transgenes (Travella et al. 2005), resulting in a low transgene expression or no transgene expression at all. Pollen is a natural gene transfer system, and genetically engineered pollen should be a useful tool for any flowering plant. Pollen may be an ideal transformation target (Wang et al. 2008). Matthews et al (1990) reported that the expression of a foreign gene containing the GUS gene was detected in electropulsed pollen grains of tobacco. Saunders et al (1992) addressed that pollen electrotransformation is a technology for electroporating exogenous DNA into pollen, and producing offspring that can be screened by PCR to detect foreign genes. Furthermore, seed-derived cowpea embryos expressed the GUS gene at high frequency after electroporation- mediated DNA transfer (Penza et al. 1992). Production of transformed tobacco plants by electropulsed pollen was also confirmed by GUS and chloramphenicol acetyltransferase (CAT) tests (Smith et al. 1994). Transgenic leguminous plants were obtained by electroporating exogenous DNA into intact nodal meristems (Chowrira et al. 1995). Electroporation-mediated gene transfer into intact plant tissues was demonstrated in pea, cowpea, lentil, soybean, and grain legumes (Chowrira et al. 1996). Recently, Wang et al (2008) reported that the *EPSPS* (5-enolpyruvyl- shikimate-3-phosphate synthase) gene was introduced into the *Brassica juncea* genome by sonication-assisted pollen-mediated transformation.

To confirm the efficiency of pollen transformation, reporter genes have been transferred, and their expressions confirmed in the target plant (Matthews et al. 1990; Penza et al. 1992; Camelia et al. 1994). As expected, transformant plants incorporating the *CP* gene of the CCYV were disease-resistant, which may be useful for breeding programs. In this study, transgenic cucumber plants incorporating the *CP* gene of the CCYV were generated by pollen electrotransformation, which is a rapid and efficient gene transfer technology. This is the first reported case of resistance to CCYV by a *Cucurbitaceae* species.

2. MATERIAL AND METHODS

2.1. Materials

The inbred lines of cucumber (*Cucumis sativus* L.) 'Yichu no.3' and 'Xin no.4' were developed in the Tainan District Agricultural Research and Extension Station, Taiwan.

2.2. Virus Source and cDNA of CCYV

CCYV was maintained in infected cucumber plants in a greenhouse. Total RNA from the infected (chlorotic) cucumber leaves was isolated following the instructions of the Plant Total RNA Miniprep Purification Kit (Invitrogen U.S.A.). cDNA was synthesized using the One-Step RT-PCR Kit (GeneMark Technology Co., Ltd) as the template for RT-PCR, according to the instructions of the manufacturer.

2.3. Cloning the CCYV Coat Protein (CP) Gene

A full-length cDNA clone of *CP* was obtained by RT-PCR. The forward primer 5'-GGCCATGGGCCATATGGAGAAGACTGACAATAAAC-3' and reverse primer 5'-GGCTCGAGTTAGCTAGCTTTACTACAACCTCCCG-3' used for RT-PCR was designed based on the CCYV sequence (Okuda *et al.* 2010). PCR was performed with the following cycling conditions: 1 cycle at 94 °C for 2 min; 40 cycles at 94 °C for 30 sec., 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 7 min, and a 4 °C hold. The presence of the *CP* gene was demonstrated by PCR amplification of a 753 bp amplicon, which was then cloned into the *p*GEM-T Easy vector (Promega, U.S.A.) for sequencing.

2.4. Construction of the CCYV Coat Protein (CP) Gene into Expression Vector

To test its role on expression, the *CP* gene was constructed into an overexpressed *pCAMBIA* 1302 vector (Cambia Labs, Australia). The entire *CP* was first amplified by PCR, using the overexpressed 5' primer, which introduced an *Nco*I site in front of the starting ATG codon, and the overexpressed 3' primer, which introduced a *Bgl* II site before the stop codon. The PCR-generated amplicon was cloned into the

*p*GEM-T Easy vector to produce the *CP*/*p*GEM-T, and the DNA was transformed into *E. coli*. After digestion of the *CP*/*p*GEM-T with *Nco*I and *Bgl* I, a 753-bp fragment (including the entire *CP* ORF) was gel-purified from agarose, using the WizardR SV Gel and PCR Clean-Up System kit (Promega, USA), and ligated to a similarly digested *pCAMBIA* 1302 expression vector. The integrated *CP* gene of the plasmid *CP/pCAMBIA*1302 was verified by sequencing.

2.5. Pollen electrotransformation

Pollen was gathered from the inbred lines 'Yichu no.3' and 'Xin no.4', kept in a greenhouse, during the morning of the flowering day. Approximately 0.1 g of the collected pollen was washed 2 times with 1 mL of PGM buffer solution [0.8 M sucrose, 100 μg/mL of H_3BO_3, 300 μg/mL of $Ca(NO_3)_2$, 200 μg/mL of $MgSO_4$, 100 μg/mL of KNO_3, and 1.7 μg/mL of $MnSO_4$]. The washed pollen and purified plasmid DNA were added to a cool PGM buffer solution, at a concentration of 0.1 μg/μL and 0.5 μg/μL, respectively. Then, the mixture was electropulsed with voltage intensities of 25 μF × 0.5 kV, × 1.0 kV, × 1.5 kV, and × 2.0 kV (Gene Pulser ® II Electroporation System (BIO-RAD). The electropulsed pollen was pollinated onto the stigma of emasculated flowers, which were wrapped immediately with paper bags to prevent arbitrary cross-pollination. For measuring the uptake efficiency of plasmid DNA into the pollen, trypan red was added to a portion of the pollen suspension buffer after electropulsation, and the number of pollen grains showing trypan uptake were counted as the uptake efficiency index of plasmid DNA into the pollen.

2.6. DNA Extraction and Polymerase Chain Reaction

Seeds were collected from hand-pollinated fruits, which developed from flowers pollinated with electropulsed pollen. The putative-transformed seeds were germinated at 25 ± 2 ℃, and 200 offspring (100 seedlings of each 'Yichu no.3' and 'Xin no.4') were transplanted into pots, and kept in a greenhouse for 3 weeks. Total DNA was extracted from the leaves of the putative-transformed plants, using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). RNase A-treated DNA (1 μg) was employed as a template for verifying *CP* gene integration into the genome of the transformed plants, using the polymerase chain reaction (PCR). PCR was performed with the following cycling conditions: 1 cycle at 94 °C for 5 min, 30 cycles at 94 °C for 1 min, 59 °C for 2 min, and 72 °C for 1 min, followed by 72 °C for 10 min, and a 4 °C hold. The presence of the nucleotide band from the 35 S promoter of the *CP* gene was demonstrated by PCR amplification of a 1291 bp amplicon, using the forward primer 5'-GAACTCGCCGTAAAGACTGG-3'and the reverse primer 5'-TCCTTTCAGAGCTTGGCACT-3'. PCR products were analyzed by electrophoresis in 1% agarose gel.

2.7. Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Western Blot Analysis

A. Reverse Transcription Polymerase Chain Reaction, RT-PCR

Total RNA was extracted from leaves of the PCR-positive T1 offspring, using the Plant Total RNA Extraction Kit (Invitrogen, U.S.A.). DNase A-treated RNA (2 μg) was employed as a template for cDNA synthesis, using the High Capacity cDNA Reverse Transcription Kit (P/N 4368814, ABI, U.S.A.). cDNA was used as a template for the PCR reaction, to verify the transcription of the *CP* gene in the transformed plants. PCR was performed with the following cycling conditions: 1 cycle at 94 °C for 5 min; 15 cycles at 94 °C for 1 min, 59 °C for 2 min, and 72 °C for 1 min, followed by 72 °C for 10 min, and a 4 °C hold. The amplicon of the *CP* gene was identified by PCR amplification of a 753 bp amplicon, using the forward primer 5'-GGCCATGGGCCATATGGAGAAGACTGACAATAAAC-3' and the reverse primer 5'-GGCTCGAGTTAGCTAGCTTTACTACAACCTCCCG-3'. PCR products were analyzed by electrophoresis in 1% agarose gel.

B. Western Blot Analysis of the GFP-CP Fusion Protein

For verifying the CP protein expression of the PCR- and RT-PCR-positive transformed T2 offspring, the GFP-CP fusion protein was analyzed using the Western blot. Total proteins were extracted from young leaves of T2 transgenic plants, using the Total Plant Protein Miniprep Purification Kit (GMbiolab Co, Ltd.). Thirty micrograms of protein from the transgenic plants were loaded onto a 12% polyacrylamide gel, and electrophoresed for Western blot analysis. Then, the proteins from the transformed and non- transformed plants were transferred to a Hybond TM-P membrane (Amersham

Pharmacia Biotech Inc., Piscataway, NJ, USA) and immunostained.

2.8. Evaluation of CCYV Resistance of Transgenic Plants and Progeny Selection

For the field evaluation of CCYV resistance, transgenic T2 offspring plants were inoculated using whitefly in the greenhouse. RT-PCR was performed according to Hunag et al. (2010), who obtained the 389-bp DNA amplicon corresponding to the RNA2 nucleotide 1,324 to 1,712 of the original CCYV Japan isolate (Accession No. AB523789) designed for CCYV detection. The forward primer 5'- GCC ATA ACC ATT ACG GGA GA-3' and the reverse primer 5'- CGC AGT GAA AAA CCC AAA CT-3' were used for amplification. Healthy (negative control) plants were self-pollinated to produce the T3 offsping, and the T3 offsping were also evaluated in the open field.

3. RESULTS

3.1. Cloning the CCYV Coat Protein (*CP*) Gene

In this study, RT-PCR was used to clone the CCYV coat protein (CP) gene from infected cucumber plants, which were identified by their disease symptoms. To obtain a full-length *CP* gene, the primer pair was designed based on the *CP* gene sequence of CCYV (Okuda *et al.* 2010). A PCR amplicon product was sequenced, having 753 bp (full-length cDNA of the *CP* gene), which is consistent with the HM581658, NCBI (Fig. 1).

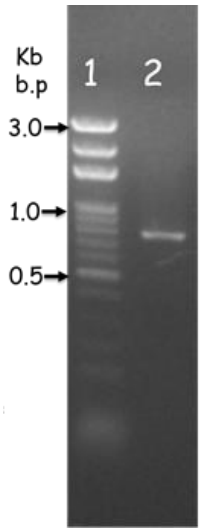

Fig. 1. Gel analysis of the CCYV CP gene from infected cucumber plants.

Lane 1: M (Marker), Lane 2: CCYV coat protein gene

3.2. Pollen Electrotransformation

Collected pollen suspended in cooled PGM buffer solution was electropulsed to facilitate the incorporation of the *CP/pCAMBIA*1302 plasmid DNA into the pollen grains. Nevertheless, electropulsation may damage the pollen, which was electropulsed under various voltage intensities. The ratios of intact pollen grains electropulsed at 25 μF × 0.5 kV, × 1.0 kV, × 1.5 kV, and × 2.0 kV were 91.4%, 85.8%, 79.1%, and 69.7%, respectively. The results showed that more pollen grains were damaged at higher electropulsing voltages. Fifteen flowers from each line were pollinated with pollen electropulsed at 25 μF × 0.5 kV, × 1.0 kV, × 1.5 kV, and × 2.0 kV; these flowers successfully developed into 9, 7, 5, and 2 fruits, respectively, containing 890, 710, 501, and 206 seeds, respectively. These results showed that the voltage intensity of 25 μF × 1.5kV damaged the pollen mildly and resulted in fruit, so it was applied in this study. In practice, 20 emasculated female flowers from each line of 'Yichu no.3' and 'Xin no.4' were pollinated with electropulsed pollen. Ten fruits of 'Yichu no.3' and 21 fruits of 'Xin no.4' were harvested, and the seeds (putatively transformed offspring) were extracted.

3.3. Confirming the Integration and Expression of the *CP* Gene in the Cucumber Genome

The leaves of putative transgenic cucumbers were sampled, and the DNA was extracted and analyzed by PCR using specific primers. An expected 1291-bp amplicon was obtained from the transgenic offspring except lane 4, but not from wild-type plants (Fig. 2). Twenty-seven out of 100 plants of 'Yichu no.3' and 29 out of 100 plants of 'Xin no.4' were expected to be transformed plants, because of the positive DNA amplicon appeared in the PCR products.

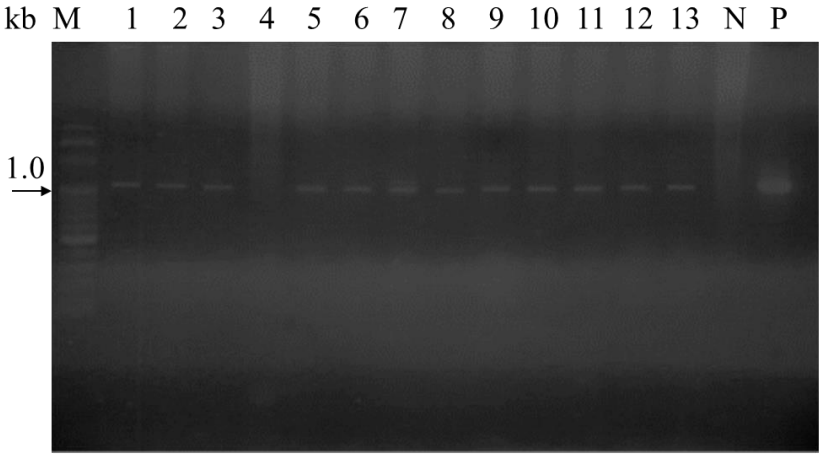

Fig. 2. Screening of transgenic cucumber plants (T1) by PCR of target gene, using leaf genomic DNA.

P: pCAMBIA 1302 plasmid, N: negative control; 1291-bp amplicon were obtained from putative transgenic plants except plant No. 4.

The purified cDNA products from the PCR-positive T2 plants were used as the templates for RT-PCR amplification with the *CP* primers. An expected 753-bp amplicon of the RT-PCR product was obtained from the transgenic lines, from plant of No. 8 (Fig. 3). Furthermore, the amplicon was sequenced for verifying the CCYV *CP* gene. The results showed that 24 out of 27 plants of 'Yichu no.3' and 25 out of 29 plants of 'Xin no.4' were confirmed to have the transcription *CP* gene. The CP protein expression of PCR- and RT-PCR-positive plants was examined by Western blot analysis of the GFP-CP fusion protein. The results showed that the GFP was expressed in the positive transgenic plants, with a GFP special band of 69 kDa and no expression in the untransformed plants (Fig. 4). The results showed that 22 out of 24 plants of 'Yichu no.3' and 23 out of 25 plants of 'Xin no.4' exhibited the presence of a GFP special band of 69 kDa.

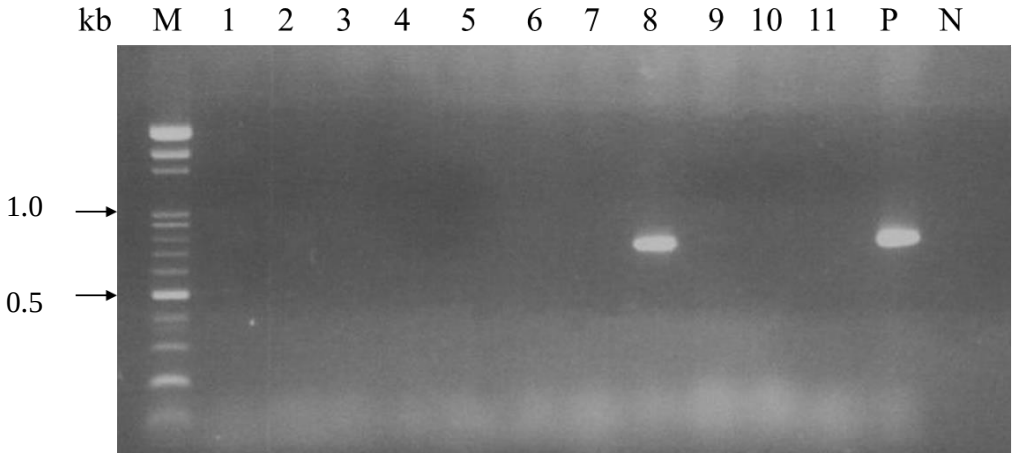

Fig. 3. Detection of CCYV CP gene expression in T2 transgenic cucumber plants by RT-PCR, using leaf total RNA. Plant No. 8: CCYV CP gene expression, P: positive control (*pCAMBIA 1302* plasmid), N: negative control

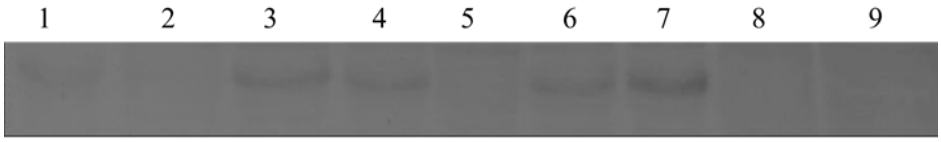

Fig. 4. Western blot analysis of leaf GFP protein in WT and transgenic cucumber plants (T2).
Lane 1-7: transgenic plants and GFP protein expression except No. 2 and No. 5.
Lane 8-9: WT (wild type).

3.4. Assay of CCYV-Resistant T3 Transgenic Plants

Some of the inoculated T3 transformed plants exhibiting no visual chlorosis of leaves were CCYV-resistant, whereas all the untransformed plants exhibiting symptom of leaf chlorosis were CCYV-susceptible (Fig. 5). These results indicated that the transgenic plants exhibited high resistance to CCYV compared with the untransformed plants. Furthermore, the transformed CCYV-resistant plants had no the RNA2 nucleotides 1,324 to 1,712 of the original CCYV isolated in Japan. An expected 389-bp amplicon was obtained from susceptible T3 transgenic lines, but not from resistant plants (Fig.6). Fifteen out of 22 T2 seedlings from ‘Yichu no.3’ and 18 out of 23 seedlings from ‘Xin no.4’, having the expected DNA fragment, were amplified. The transformed plants of the T3 generation from ‘Yichu no.3’and ‘Xin no.4’ exhibited a high level of resistance (95%) to CCYV in the open field.

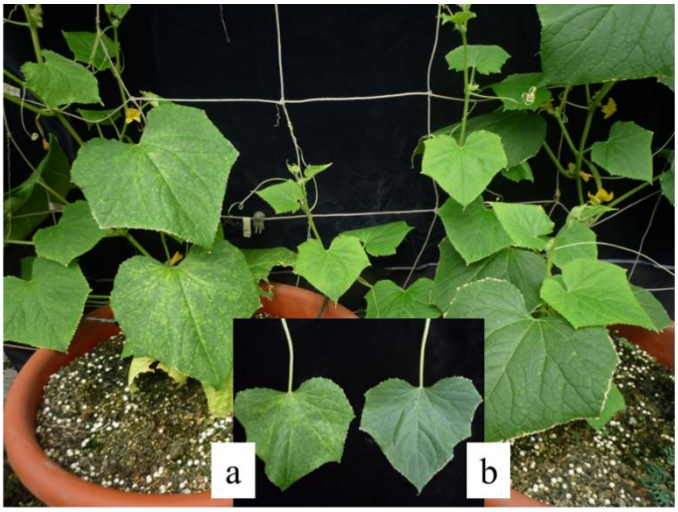

Fig. 5. CCYV-resistant transgenic T3 plants exhibiting no visual chlorosis of leaves.

a: susceptible plant, b: transgenic resistant plant

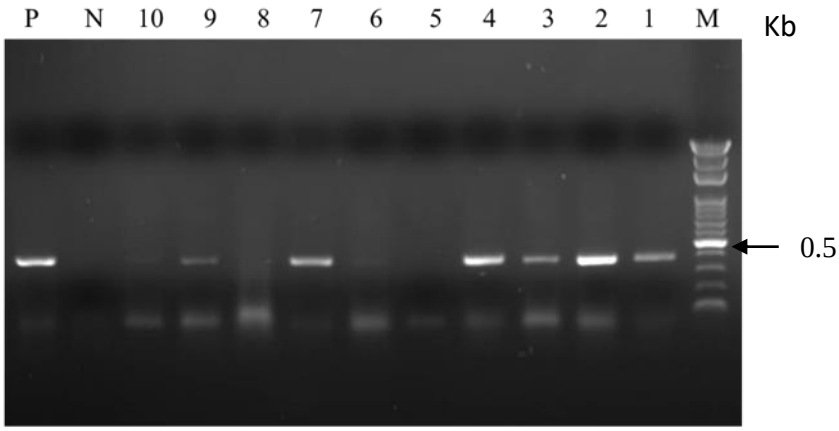

Fig. 6. The transformed CCYV-resistant plants of No. 5 and No. 8 had no 389-bp amplicon of the RNA2 nucleotides of CCYV. Lane 1-10: PCR-positive transformed T3 plants, P: positive (CP/pCAMBIA1302 vector), N: negative control.

4. DISCUSSION

CCYV viruses infect over 21 species of *Cucurbitaceae* crops, resulting in stunted growth and poor fruit production. CCYV is a newly characterized *Crinivirus* species responsible for a worldwide viral disease that strictly threatens the production of *Cucurbitaceae* crops, because CCYV is whitefly-transmitted virus. In this paper, we used the RT-PCR protocol to target the *CP* gene of the CCYV for cloning and subsequent production of a construction against it, which was used for pollen electrotransformation. Finally, we obtained a CCYV-resistant transgenic cucumber.

Smith et al (1994) reported that there are several gene transfer procedures that have been used successfully in plants; these procedures include electroporation, electrofusion, *Agrobacterium*-mediated gene transfer, particle gun, microinjection, and liposome-mediated DNA transfer. Presently, two major transformative techniques are used: *Agrobacterium*-mediated DNA delivery and biolistic DNA transfer (Slater et al. 2008). However, these techniques require time-consuming, labor-intensive, and expensive procedures because of the constraints of in vitro tissue culture, regeneration, and associated problems. Eapen (2011) developed a simple and rapid transformative protocol for transgenic plants, which minimized or eliminated the need for tissue culture and regenerative steps, and directly generated

transgenic seeds. Pollen electrotransformation is relatively cheap, and plant breeders could use it for developing novel genotypes with ease (Wang et al. 2008). In our study, the transgenic cucumber plants incorporating the *CP* gene were efficiently and rapidly generated using pollen as a vector. The T1 offspring included a 27% of transgenic plants, which were generated within 3 months, so it is possible to avoid the tedious tissue culture procedures of the *Agrobacterium*- mediated and bombardment approaches. Thus, the proposed approach is easy to apply in conventional breeding programs.

Our results inferred that the *CP* gene of the CCYV could be delivered into pollen by electroporation. Eapen (2011) reported that pollen grains are covered by a plasma membrane and a hard in tine and examine, so the foreign DNA fragment should be delivered across these barriers and also withstand chemical stress. The mechanism of delivery consists in moving ions of pollen suspension according to their charge during electropulsion; then, pathways of electropulsed pollen are formed across the pollen wall, allowing the foreign DNA fragment to enter. Andreason & Evans (1988) reported that the intensity of the electrical field is a critical factor for successful electroporation, because a low electropulse voltage maintains the normal shape of pollen grains but has low efficiency, whereas a high electropulse voltage damages the pollen grains but is highly efficient. This result indicated that a foreign DNA fragment could penetrate into pollen by electroporation with a mild electropulse voltage of 25 μF × 1.5 kV, which was recommended because the transformed efficiency of the T1 offspring was 27% of transgenic plants.

Transgenic-based forms of disease resistance have become useful means of plant protection. Based on the pathogen-derived resistant (PDR) concept, Sanford & Johnston (1985) proposed that various transgenic approaches based on viral genes and sequences could be applied to many plant species. The first report of a stable transformation of a plant with a viral *CP* gene was the introduction of the CP encoding gene of TMV into tobacco (Bevan et al. 1985). Abel et al (1986) reported the development of CP-mediated resistance (CPMR) to TMV; CPMR to AMV (Tumer et al. 1987) and CMV (Cuozzo et al. 1988) were also reported. Bendahmane et al (2007) proposed that the degree of regulation of replication by CP aggregates determines the relative strength of CPMR. Therefore, CPMR has clearly been the predominant strategy for creating VRTP (virus-resistant transgenic plants) (Prins et al. 2008; Sudarshana et al. 2007). In this study, the integration of the *CP* gene into the genome of the transformed cucumber was verified using PCR-based DNA analysis. The expression of the incorporated *CP* gene varied among the transformed plants sampled from the field. Field evaluation across multiple growing seasons and generations demonstrated that transgenic cucumber plants expressing the *CP* gene were resistant to CCYV, which these plants did not appear visually leaf chlorosis symptom. The correlation between *CP* gene expression and CCYV resistance through the T1-T3 generation was confirmed, suggesting that the virus resistance of the transgenic plants resulted from the incorporation and expression of the *CP* gene of the CCYV.

CONCLUSIONS

1. Genetic transformation can improve agricultural traits considerably faster than conventional breeding.
2. This experiment demonstrated that transferring the CP gene of the CCYV might result in plant resistance to the CCYV disease.
3. The results of the transgenic cucumber plants generated indicated that the *CP* gene could be successfully transferred by electroporation, using pollen as a vector, which is a relatively simple procedure because tissue culture and regeneration steps are not necessary.

REFERENCES

Abel PP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT & Beachy RN 1986, 'Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene'. Science, vol. 232, pp. 738-43

Abrahamian PE, Sobh H & Abou-Jawdah Y 2012, 'First Report of Cucurbit chlorotic yellows virus on Cucumber in Lebanon'. Plant Disease vol. 96, pp. 1704-1704

Andreason GL & Evans GA 1988, 'Introduction and expression of DNA molecules in eukaryotic cells by electroporation'. Biotechniques, vol.6, pp. 650-60

Bananej K, Menzel W, Kianfar N, Vahdat A & Winter S 2013, 'First Report of Cucurbit chlorotic yellows virus Infecting Cucumber, Melon, and Squash in Iran'. Plant Disease, vol. 97 pp. 1005-1005

Bendahmane M, Chen I, Asurmendi S, Bazzini AA, Szecsi J & Beachy RN 2007, 'Coat protein-mediated resistance to TMV infection of Nicotiana tabacum involves multiple modes of interference by coat protein'. Virology, vol. 366, pp. 107-16

Bevan MW, Mason SE & Goelet P 1985, 'Expression of tobacco mosaic virus coat protein by a cauliflower mosaic virus promoter in plants transformed by Agrobacterium'. The EMBO Journal, vol. 4, pp. 1921-6

Chowrira GM, Akella V, Fuerst PE & Lurquin PF 1996, 'Transgenic grain legumes obtained by in planta electroporation-mediated gene transfer'. Molecular biotechnology, vol. 5, pp. 85-96

Chowrira GM, Akella V, & Lurquin PF 1995, 'Electroporation-mediated gene transfer into intact nodal meristems in planta. Generating transgenic plants without in vitro tissue culture'. Molecular biotechnology, vol. 3, pp. 17-23

Cuozzo M, O'Connell KM, Kaniewski W, Fang R-X, Chua N-H & Tumer NE 1988, 'Viral Protection in Transgenic Tobacco Plants Expressing the Cucumber Mosaic Virus Coat Protein or its Antisense RNA'. Nat Biotech, vol. 6, pp. 549-557

Eapen S 2011, 'Pollen grains as a target for introduction of foreign genes into plants: an assessment'. Physiology and molecular biology of plants : an international journal of functional plant biology, vol. 17 pp. 1-8

Frame BR, Shou H, Chikwamba RK, Zhang Z, Xiang C, Fonger TM, Pegg SE, Li B, Nettleton DS, Pei D & Wang K 2002, 'Agrobacterium tumefaciens-Mediated Transformation of Maize Embryos Using a Standard Binary Vector System'. Plant physiology, vol. 129, pp. 13-22

Gu QS, Liu YH, Wang YH, Huangfu WG, Gu HF, Xu L, Song FM & Brown JK 2010, 'First Report of Cucurbit chlorotic yellows virus in Cucumber, Melon, and Watermelon in China'. Plant Disease, vol. 95, pp. 73

Gyoutoku Y, Okazaki S, Furuta A, Etoh T, Mizobe M, Kuno K, Hayashida S & Okuda M 2009, 'Chlorotic yellows disease of melon caused by Cucurbit chlorotic yellows virus, a new crinivirus'. Japanese Journal of Phytopathology, vol. 75, pp. 109-111

Hamed K, Menzel W, Dafalla G, Gadelseed AMA & Winter S 2011, 'First Report of Cucurbit chlorotic yellows virus Infecting Muskmelon and Cucumber in Sudan'. Plant Disease, vol. 95, pp. 1321

Huang LH, Tseng HH, Li JT, & Chen TC 2010, 'First Report of Cucurbit chlorotic yellows virus Infecting Cucurbits in Taiwan'. Plant Disease, vol. 94, pp. 1168

Kunik T, Salomon R, Zamir D, Navot N, Zeidan M, Michelson I, Gafni Y & Czosnek H 1994, 'Transgenic tomato plants expressing the tomato yellow leaf curl virus capsid protein are resistant to the virus'. Bio/technology (Nature Publishing Company), vol. 12, pp. 500-4

Li J, Liang X, Wang X, Shi Y, Gu Q, Kuo Y-W, Falk BW & Yan F 2016, 'Direct evidence for the semipersistent transmission of Cucurbit chlorotic yellows virus by a whitefly vector'. Scientific Reports, vol. 6, pp. 36604

Lius S, Manshardt RM, Fitch MMM, Slightom JL, Sanford JC & Gonsalves D 1997, 'Pathogen-derived resistance provides papaya with effective protection against papaya ringspot virus'. Molecular Breeding, vol. 3 pp. 161-8

Mahmoud AA 2015, 'Serological and Molecular Characterization of Cucurbit chlorotic yellows virus Affecting Cucumber Plants in Egypt'. International Journal of Virology, vol. 11, pp. 1-11

Matthews BF, Abdul-Baki AA & Saunders JA 1990, 'Expression of a foreign gene in electroporated pollen grains of tobacco'. Sexual Plant Reproduction, vol. 3, pp. 147-151

Okuda M, Okazaki S, Yamasaki S, Okuda S & Sugiyama M 2010, 'Host range and complete genome sequence of Cucurbit chlorotic yellows virus, a new member of the genus Crinivirus'. Phytopathology, vol. 100, pp. 560-6

Penza R, Akella V & Lurquin PF 1992, 'Transient expression and histological localization of a gus chimeric gene after direct transfer to mature cowpea embryos'. Biotechniques, vol.13, pp. 576-80

Prins M, Laimer M, Noris E, Schubert J, Wassenegger M & Tepfer M 2008, 'Strategies for antiviral resistance in transgenic plants'. Molecular plant pathology, vol. 9, pp. 73-83

Sanford JC & Johnston SA 1985, 'The concept of parasite-derived resistance—Deriving resistance genes from the parasite's own genome'. Journal of Theoretical Biology, vol. 113, pp. 395-405

Saunders JA, Lin CH, Hou BH, Cheng J, Tsengwa N, Lin JJ, Smith CR, McIntosh MS & Van Wert S 1995, 'Rapid optimization of electroporation conditions for plant cells, protoplasts, and pollen'. Molecular biotechnology, vol. 3, pp. 181-90

Slater A, Scotter NW & Fowler MR 2008, 'Techniques for plant transformation. In Plant Biotechnology: The Genetic Manipulation of Plants'. Oxford University Press, Oxford, pp. 54–76.

Smith CR, Saunders JA, Van Wert S, Cheng J & Matthews BF 1994, 'Expression of GUS and CAT activities using electrotransformed pollen'. Plant Science, vol. 104, pp. 49-58

Sudarshana MR, Roy G & Falk BW 2007, 'Methods for engineering resistance to plant viruses'. Methods in molecular biology (Clifton, NJ), vol. 354, pp. 183-95

Tecson Mendoza EM, A CL & Botella JR 2008, 'Recent advances in the development of transgenic papaya technology'. Biotechnology annual review, vol. 14, pp. 423-62

Tepfer M 2002, 'Risk assessment of virus-resistant transgenic plants'. Annual review of phytopathology, vol. 40, pp. 467-91

Travella S, Ross SM, Harden J, Everett C, Snape JW & Harwood WA 2005, 'A comparison of transgenic barley lines produced by particle bombardment and Agrobacterium-mediated techniques'. Plant cell reports, vol. 23, pp. 780-9

Tumer NE, O'Connell KM, Nelson RS, Sanders PR, Beachy RN, Fraley RT& Shah DM 1987, 'Expression of alfalfa mosaic virus coat protein gene confers cross-protection in transgenic tobacco and tomato plants'. The EMBO Journal, vol. 6, pp. 1181-88

Wang J, Li Y & Liang C 2008, 'Recovery of transgenic plants by pollen-mediated transformation in Brassica juncea'. Transgenic research, vol. 17, pp. 417-24

Zeng R, Dai FM, Chen WJ & Lu JP 2011, 'First Report of Cucurbit chlorotic yellows virus Infecting Melon in China'. Plant Disease, vol. 95, pp. 354

Ziemienowicz A, Shim YS, Matsuoka A, Eudes F & Kovalchuk I 2012, 'A novel method of transgene delivery into triticale plants using the Agrobacterium transferred DNA-derived nano-complex'. Plant physiology, vol. 158, pp. 1503-13