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Original Article

Evaluation of the *Chilli veinal mottle virus* CP gene expressing transgenic *Nicotiana benthamiana* plants for disease resistance against the virus

Avaliação do gene CP do vírus Chilli veinal mottle que expressa plantas transgênicas *Nicotiana benthamiana* quanto à resistência a doenças contra o vírus

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Abstract

Vegetables are an important source of income and high-value crops for small farmers. Chilli (*Capsicum* spp.) is one of the most economically important vegetables of Pakistan and it is grown throughout the country. It is a rich source of nutrition especially vitamins A, B, C and E along with minerals as folic acid, manganese (Mn), potassium (K) and molybdenum (Mo). Chilli possesses seven times more amount of vitamin C than an orange. Vitamin A, C and beta-carotenoids are strong antioxidants to scavenge the free radicals. Chilli production is restricted due to various biotic factors. Among these viruses, *Chilli veinal mottle* virus (ChiVMV) is one of the most destructive and menacing agents that inflicts heavy and colossal losses that accounted for 50% yield loss both in quality and quantity. Pathogen-Derived Resistance (PDR) approach is considered one of the effective approaches to manage plant viruses. In this study, ChiVMV was characterized on a molecular level, the coat protein (CP) gene of the virus was stably transformed into *Nicotiana benthamiana* plants using *Agrobacterium tumefaciens*. The transgenic plants were challenged with the virus to evaluate the level of resistance of plants against the virus. It was observed that the plants expressing CP gene have partial resistance against the virus in terms of symptoms' development and virus accumulation. Translation of this technique into elite chilli varieties will be resulted to mitigate the ChiVMV in the crop as well as an economic benefit to the farmers.

Keywords: Chilli, ChiVMV, CP gene, transgenic plants, viral resistance.

Resumo

Vegetais são uma importante fonte de renda e culturas de alto valor para os pequenos agricultores. A pimenta-malagueta (*Capsicum* spp.) é uma das hortaliças mais importantes economicamente do Paquistão e é cultivada em todo o país. É uma rica fonte de nutrição, especialmente vitaminas A, B, C e E com minerais como ácido fólico, manganês (Mn), potássio (K) e molibdênio (Mo). O pimentão possui sete vezes mais vitamina C do que a laranja. Vitaminas A e C e betacarotenoides são antioxidantes fortes para eliminar os radicais livres. A produção de pimenta é restrita devido a vários fatores bióticos. Entre esses vírus, o ChiVMV é o agente mais destrutivo e ameaçador que inflige perdas pesadas e colossais que representam 50% da perda de rendimento, tanto em qualidade quanto em quantidade. A abordagem de resistência derivada de patógenos (PDR) é considerada uma das abordagens eficazes para gerenciar os vírus de plantas. Neste estudo, ChiVMV foi caracterizado em nível molecular e o gene CP do vírus foi transformado de forma estável em plantas *Nicotiana benthamiana* usando *Agrobacterium tumefaciens*. As plantas transgênicas foram desafiadas com o vírus para avaliar seu nível de resistência contra o vírus. Observou-se que as plantas que expressam o gene CP apresentam resistência parcial ao vírus em termos de desenvolvimento de sintomas e acúmulo de vírus. A tradução dessa técnica em variedades de pimenta de elite resultará na mitigação do ChiVMV na safra, bem como em benefícios econômicos para os agricultores em termos de melhor rendimento e baixo custo de produção.

Palavras-chave: Pimenta; ChiVMV, Gene CP, plantas transgênicas, resistência viral.

1. Introduction

Plant viruses are an important group of plant pathogens, which cause yield losses among economically important crops. Biotic stresses overall cause big damage to the crop production worldwide. The losses that occurred by diseases and pests in annual plants are estimated to be 20-40% (Oerke, 2006; Savary et al., 2012). Every year, fruit and

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vegetable crops are hampered by viruses that cause the losses of several billion dollars worldwide (Agrios, 2005). Plant viruses have two types of the genome and can be categorized into DNA and RNA viruses (Khan and Dijkstran, 2006). Plants are mainly hampered by RNA viruses and they share 80% of total plant viruses (Ali et al., 2004). Agricultural crops in South Asian countries including Pakistan are badly infected with many taxonomic groups of plant RNA viruses (Amin et al., 2007). Important plant viruses with RNA genome belong to potyviruses (Fauquet et al., 2005). Genus *Potyvirus* consists of 118 definite and 82 tentative species contribute more than 30% of all reported plant RNA viruses (Spetz et al., 2003; van Regenmortel et al., 2000).

Chilli is an economically valuable cash crop throughout the world (Shah et al., 2011). Chilli owing the ranges of 30-40 thousand tons per year in international trade because of its ample application and high economic value (Shah et al., 2011). The world produces an amount of almost seven million tons of chilli on an area of approximately 1.5 million hectares (Prakash and Singh, 2006). Chilli crop is hampered by a wide range of plant viruses. Results of the different experiments of artificial and natural inoculation showed that the chilli crop is infected by 82 viruses (Prakash and Singh, 2006). Of those, nine viruses belong to the genus *Potyvirus*, which include *Chilli veinal mottle virus* (ChiVMV), *Potato virus Y* (PVY), *Pepper mild mosaic virus* (PMMV), *Pepper veinal mottle virus* (PVMV), *Pepper yellow mosaic virus* (PepYMV), *Pepper mottle virus* (PepMoV), *Pepper severe mosaic virus* (PepSMV), *Tobacco etch virus* (TEV) and *Peru tomato mosaic virus* (PTV) (Tsai et al., 2008).

Engineered resistance approaches against the plant virus have extensively explored since the first experiment whereby tobacco plant transformed with the CP gene showed the resistance against *Tobacco mosaic virus* (TMV) (Powell et al., 1986; Goldbach et al., 2003). Genetic engineering enables us to produce transgenic plants that confer suitable resistance to viruses and protection to crop as compared to conventional breeding strategies (Lecoq et al., 2004). Genetic engineering approaches used the pathogen-derived transgenes, plant-derived natural R genes and even non-pathogen and non-plant-derived transgenes (Lecoq et al., 2004). The idea of pathogen-derived resistance (PDR) was suggested by Sanford and Johnston in 1985 which has started a new era of research on obtaining virus resistance crops through this approach (Sanford and Johnston, 1985). Through the PDR mechanism, transgenic plants are protected against pathogens by gene or part of gene sequences derived from a pathogen in an experimental or natural host which provides resistance against the same or related pathogen species (Campbell et al., 2002). In a variety of crops, effective transgenic resistance was developed by introducing viral genes, or part of a gene, in the target crop by genetic transformation (Campbell et al., 2002: Goldbach et al., 2003). Coat protein (CP), Replicase protein (RP) and Movement protein (MP) have been frequently usually used to induce resistance against viruses in PDR approaches. Other PDR approaches reported in the literature are the expression of untranslatable sense or anti-sense, satellite RNA and defective interfering RNA or DNA (Powell et al., 1986).

It was found that transgenic plants of tobacco and tomato plants with the coat protein gene of TMV showed resistance response or delayed infection when they were inoculated with TMV (Nelson et al., 1988). Initially, it was proposed that the translation of the viral genome is prevented by the transgene-derived CP which blocked the disassembly of the virus particle (Lindbo and Dougherty, 1992). TEV transgenic plants expressing an untranslatable form of TEV CP which was able to confer resistance to the same virus (Lindbo and Dougherty, 1992) which was based on RNA and became known as RNA-mediated resistance. The objective of the current study was to characterize ChiVMV isolated from infected plants and to evaluate the CP gene expressing *N. benthamiana* plants for virus resistance.

2. Material and Methods

2.1. Molecular characterization

Total RNA was extracted from virus-infected leaf tissues of *C. annum* (cv. Loungi) at 14 days post-inoculation (dpi) using the TRI-Reagent method (Life Technologies, Carlsbad, CA, USA, Cat No. 15596026) as per the manufacturer's instruction. For cDNA synthesis, M-MLV (Moloney Murine Leukemia Virus) first Strand cDNA Synthesis Kit (Life Technologies, Carlsbad, CA, USA) was used following the manufacturer's protocol.

2.2. Cloning of CP Gene

Purified PCR products amplified using the degenerate primer pair: Poty3 (5-TGAGGATCCTGGTG(C/T) AT(A/C)GA(A/G)AA(C/T)GG-3) and CVMV1037 Pol (5'-AGCATGGAGAGAGCGACATTAGTC-3) as the upstream primers (Hiskias, 1998) and Oligo(dT) (5´-GCGGGATCCT17-3´) as the downstream primer were ligated into the pTZ57R/T vector (Thermo Scientific, EU. InsTAclone PCR Cloning Kit, K1213). The Presence of CP cDNA in recombinant vector P01 was then confirmed through the sequencing process and checking for the absence of errors. Universal primers M13 forward (5' TGTAAAACGACGGCCA GT 3') and M13 reverse (5' CAGGAAACAGCTATGACC 3') were used for sequencing the CP gene inserted in the multiple cloning site (MCS) of the pTZ57R/T gene. ClustalW program was used to compare 22 highly matched ChiVMV sequences present in the database using the BLAST application. The Phylogenetic tree was created by the Maximum likelihood method from ClustalW aligned sequences using the MEGA version 6.0 program (Tamura et al., 2013) and bootstrap analysis was conducted in 1000 replications.

2.3. Cloning of the CP gene amplified with specific primers

Three pairs of coat protein gene-specific primers based on the obtained sequence of ChiVMV (KJ472764) were designed (Table 1) by comparing with known

gene sequence data available in the NCBI database for the amplification of CP gene with restriction sites for subsequent cloning in a binary vector. The *BglII* and *BstEII* restriction sites were put on the both ends of CP gene primer respectively.

PCR amplified fragment of the CP gene with restriction sites was directly ligated into the pGEM®-T Easy vector (Figure 1) using pGEM®-T Easy vector system I. Finally, the CP gene was cloned into the plant expression vector, pCAMBIA1301, at *BglII* and *BsteII* sites.

2.4 Agrobacterium-mediated transient assay

Agrobacterium strain LBA4404 was used for transient assay studies. pCAMBIA1301 vector harboring the ChiVMV CP gene (p1301: CP) was mobilized into LBA4404 using freezing and thawing protocol. For agro-infiltration, culture was prepared from transformed bacterial cells by transferring the single colony in 50 mL liquid LB medium containing the kanamycin antibiotic (50µg/mL). Transformed bacteria were incubated overnight with shaking at 28 °C at 250 rpm. From overnight culture, 250 µl was put into 10 ml of LB medium containing 10 mM MES buffer (pH 5.7), kanamycin (50µg/mL) and 150 µM acetosyringone and was grown at 28 °C to OD_{600} 1. The culture was spun at 4000 rpm for 15 min and then the pellet was taken and suspended in 10 mM MgCl2, 10 mM MES (pH 5.7) and 100µM acetosyringone. The suspension was left on 25°C for 3-4 h. Two leaves from each plant of *N. benthamiana* (4 to 5 weeks old plants) were selected. Leaves were slightly perforated from the lower side with a needle and the bacterial suspension was infiltrated under pressure into the leaf stab using a sterile 2ml needleless syringe.

2.5. Development of Transgenic N. benthamiana Plants

Transgenic *N. benthamiana* plants were developed using the leaf disc method through agrobacterium-mediated transformation protocol. Briefly, leaf

Table 1. List of primer pairs used for the CP gene amplification from p01 the clone.

S.NO.	Primer Sequence	Primer Name
1	GAAGATCTAAGCAGGAGAGTATTGATGC	CPF1-14
2	GGGTGACCGAACGCCCAACAGATTATG	CPR1-15
3	GAAGATCTTCATCAAGCAGGAGAGAG	CPF2-16
4	GGGTGACCTCATACTCCCCGAACG	CPR2-17
5	ATAGATCTAGCATGGAGAGAGCGACATTA	ChiCP1037-18

Restriction sites within the primer sequence are underlined.

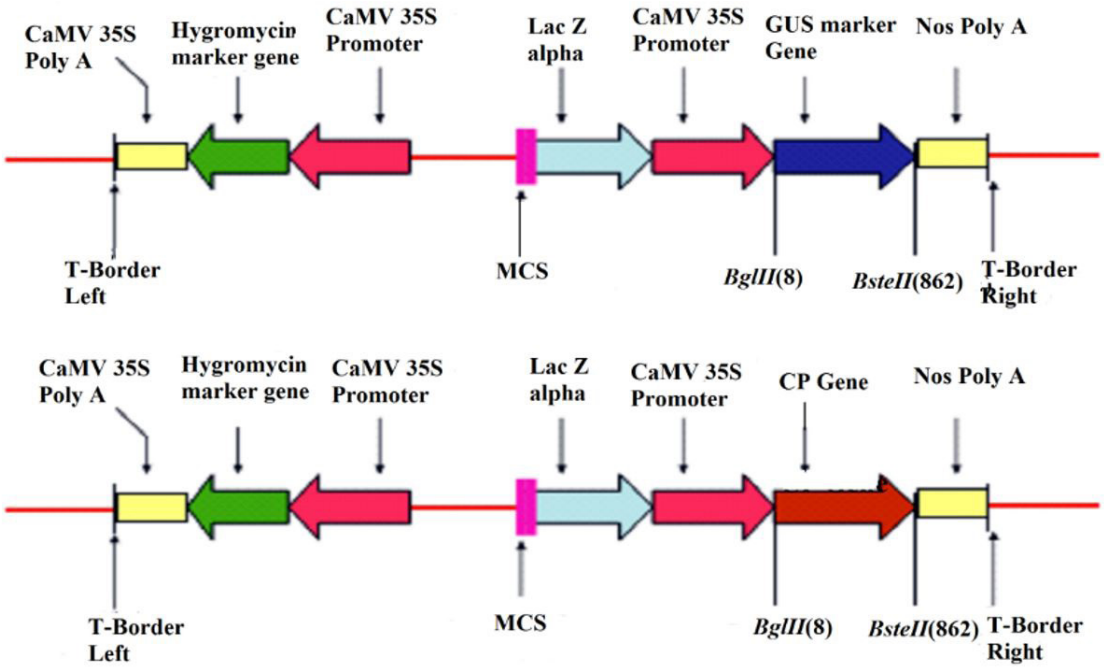

Figure 1. Schematic representation of p03 (T-DNA of p1301-CP of ChiVMV ATIPK) plasmid.

discs (8cm) were prepared from younger leaves of *N. benthamiana* plants. leaf discs were dipped in LBA4404 culture (OD600=0.4) harboring p1301CP for 30 minutes and then they put on MS co-culture media for 2-3 days at 230 °C in dark. After 3 days, the leaf discs were washed with cefotaxime (250-500 mg/L) and placed on MS selection media. After 3-4 weeks, the emerging shoots from the callus were shifted to rooting medium supplemented with antibiotic; thereafter, hardening was done after 3-4 weeks. Seeds of T0 plants were collected for further generations.

2.6. Confirmation of the transgenic plants

From the transformed plants, genomic DNA and RNA were isolated at the 3-4 leaf stage and subjected to PCR and RT-PCR amplification, respectively. DNA and RNA were extracted from the leaves of transgenic plants, agro-infiltrated (three plants) as well as from the untransformed *N. benthamiana* plants. Approximately 1kb CP gene fragment was amplified from 9 putative *N. benthamiana* plants through PCR using specific primers. The hpt gene was determined by PCR amplification with hpt specific forward and reverse primers from transgenic plants. RT-PCR was also done using specific primers for the CP gene. The PCR product was run on 1% agarose gel for observing the band intensity.

2.7. Virus resistance assay

To infect the transgenic plants, the pure sap of ChiVMV-ATIPK (plants were maintained as infection source in a greenhouse) was made by crushing the leaves of a systemically infected plant in 0.1M phosphate buffer solution. Sap was passed through muslin cloth and mixed with carborundum powder (600 mesh, 1mg/ml). The mixture was mechanically inoculated to the understudied transgenic and non-transgenic control plants by softly rubbing the leaves. The plants were analyzed for ChiVMV titer through DAS-ELISA (Clark and Adams, 1977) after 2 weeks of inoculation. A commercially available ELISA detection kit (LOEWE biochemical, USA, Cat. No. 07185S/100) was used for the detection of ChiVMV.

3. Results

3.1. Isolation and confirmation of virus

Leaf samples of infected chilli plants with characteristic symptoms of ChiVMV like mosaic, mottling, leaf distortion, vein banding, vein clearing and reduced sized leaves (Figure 2), were subjected to ChiVMV specific ELISA. DAS-ELISA results confirmed the presence of ChiVMV in the collected leaf samples, by using virus specific polyclonal antibodies and only 40% samples out of 35 reacted positively with antibodies (Table 2).

The ChiVMV isolate used in this study was named ATIPK (chilli isolate). ATIPK was biologically purified from *Chenopodium quinoa* plants after passing through three lesion transfers in separate plants. Biologically purified ChiVMV isolate ATIPK was further propagated on tobacco *(N. tabacum)* and chilli (cv. Loungi) plants for virus propagation and as a continuous source of virus for molecular studies. Infective sap was mechanically transmitted to the propagative host species. Investigated tobacco and chilli plants showed typical symptoms of mosaic and mottling on leaves after 10-14 dpi (Figure 3a and b).

3.2. Molecular Characterization and Cloning of the CP Gene

cDNA was used as template for amplification of CP gene fragment. Initially, degenerate primer pair poty3 (forward) and oligo (dT) (reverse) were used for molecular detection of ChiVMV from infected leaf samples. Fragments of approximately 1.2 kbp were amplified from each ChiVMV positive sample (Figure 4). The size of pTZ57R/T is 2.8 kb without the insert and extended to 3.9 kb after insertion of cDNA of CP gene. Ligation of the CP gene was confirmed in the vector using restriction digestion with EcoR1 and Pst1 enzymes. Finally, the clone was sequenced to confirm the sequence and orientation of the CP gene in the vector.

3.3. Phylogenetic analysis

A total of 22 best-matched nucleotide sequences from different geographical locations were retrieved from the database and aligned (Table 3). Nucleotide sequence

Figure 2. Symptoms of ChiVMV on chilli leaves collected from Islamabad. (a) Shows mottling and severe vein clearing and distortion. (b) Shows reduced leaf size with mottling and distortion.

Table 2. Reaction of different host species against ChiVMV isolate ATIPK after mechanical inoculation.

Sr. No.	Host Plant	Plant Family	No. of Plants tested	Symptoms	ELISA OD Value
1	Capsicum annum *cv.* Sanum *and cv.* Loungi	Solanaceae	5	Severe mottling, mosaic, vein clearing and vein banding	2.98
2	*Nicotiana tabacum*	Solanaceae	5	Severe mottling, mosaic and leaf curling	3.01
3	*Datura metal*	Solanaceae	5	Mottling, vein clearing and rat tailed	2.99
4	*Solanum lycopersicum*	Solanaceae	5	Mottling, mosaic and leaf curling	3.1
5	*Solanum nigrum*	Solanaceae	5	Mottling and mosaic	2.71
6	*Chenopodium amarinticular*	Chenopodiaceae	5	Nil	-ve
7	*Cucumis stavis*	Cucurbitaceae	5	Nil	-ve
8	*Vigna mungo*	Fabaceae	5	Nil	-ve
9	*Vigna radiata*	Fabaceae	5	Nil	-ve
10	*Spinacia oleracea*	Amarantheaceae	5	Nil	-ve

Figure 3. Symptoms development on propagative host plants after mechanical inoculation with ChiVMV isolate ATIPK. (a) *N. tabacum* showing mosaic, mottling and vein clearing (b) *C. annum* (cv. Loungi) displays the symptoms of mottling, mosaic, leaf deformation and vein clearing.

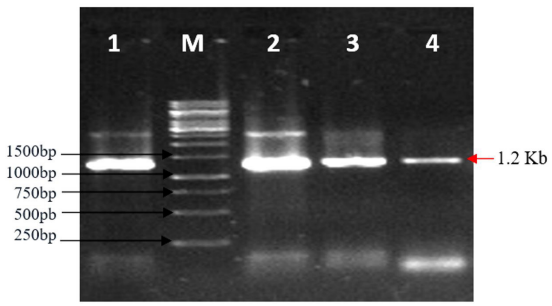

Figure 4. RT-PCR amplification of the CP gene with CVMV1037/ oligo(dT). Lane 1, 2, 3 and 4 contains 1.2kb amplified product of NIb and CP gene.

analysis revealed about the sequence of isolate ATIPK (KJ472764) consists of 96 bases of NIb region and 864 nucleotides of CP gene as well as 23 bases from 3' UTR. Sequence (KJ472764) was aligned with available data by deleting the 96 bases of NIb region.

Comparative CP sequence analysis of nucleotides revealed that nucleotide identity ranges from 88.4%-86.8%. The highest nucleotide identity was 88.4% with an isolate from Thailand (DQ854956) and lowest nucleotide identity was 86.8% with an isolate identified in India (DQ854964). While amino acid identity ranged from 91.8%-89.3%. The highest value of amino acid identity (92.7%) was observed with 3 isolates from India (EF213679, EF213681 and EF213703) and the lowest amino acid identity was 89.3% for an isolate from Indonesia (AB703256).

The CP nucleotides sequence of ChiVMV isolates ATIPK had substitutions of 68 bp at different positions of the CP gene from world isolates. The N-terminal region of CP gene exhibited more diversity in nucleotides bases substitutions as compared to 3' region. Substitutions were present mostly in first 200 nucleotides out of the total 864 bases of CP gene.

The phylogenetic analysis based on CP gene nucleotide sequences of ChiVMV isolates ATIPK showed that the Pakistani isolate was clustered with an isolate (HQ218936) from China (Figure 5).

Table 3. Twenty-two isolates of ChiVMV used in phylogenetic study.

Country	Isolate name	Accession number	Reference
China	YN75	HQ218936	(Ding et al., 2011)
Indonesia	Cikabayan2	DQ854960	(Tsai et al., 2008)
Taiwan	ChiVMV-VN/C3	DQ925442	(Ha et al., 2008)
Thailand	SKh5	DQ854959	(Tsai et al., 2008)
Thailand	K37	DQ854956	(Tsai et al., 2008)
Thailand	CM1	DQ854953	(Tsai et al., 2008)
Thailand	BP	DQ854954	(Tsai et al., 2008)
Thailand	SRt8	DQ854958	(Tsai et al., 2008)
Veitnam	ChiVMV-VN/C5	DQ925444	(Ha et al., 2008)
India	S7	EF213700	Unpublished
India	Be21	EF213684	Unpublished
India	CHL40	EF213681	Unpublished
India	CH34	EF213679	Unpublished
India	DCV3	DQ854965	Unpublished
India	Be22	EF213685	Unpublished
India	PM1	EF213703	Unpublished
India	BCV1	DQ854962	Unpublished
India	BeCV1	DQ854963	(Tsai et al., 2008)
India	CCV3	DQ854964	(Tsai et al., 2008)
Indonesia	2	AB703256	Unpublished
India	D10	EF221615	Unpublished
Indonesia	Pataruman	DQ854961	(Tsai et al., 2008)

The maximum-likelihood tree based on nucleotide sequence CP gene fell into two divergent clades (I and II). Clade II contained two isolates, one from Pakistan (KJ472764) and other from China (HQ218936) while the rest of the 22 isolates belonged to India, Taiwan, Thailand and Indonesia fell in clade II. Phylogenetic tree was supported by a strong bootstrap value (1000). Lower correspondence was seen between the ChiVMV-ATIPK and other Asian isolates. The phylogenetic analysis showed that the ChiVMV isolate from Pakistan formed a distinct clade with Chinese isolate that reflecting its close geographical relatedness with Chinese isolate as compare to Indian isolates.

3.4. Agrobacterium-mediated transient assay

The plasmid pCAMBIA1301: CP was used for agro-infiltration transient assay into *N. benthamiana* plants. Genomic DNA was isolated at 3-4 leaf stage from the leaves of 9 transgenic plants, agro-infiltrated (3 plants) and run on 1% agarose gel (Figure 6).

3.5. Confirmation of Transgenic N. benthamiana Plants

DNA and RNA were extracted from the leaves of transgenic (9 plants), agro-infiltrated (3plants) as well as from control (untransformed) *N. benthamiana* plants. Approximately 1kbp CP gene fragment was obtained from 9 putative *N. benthamiana* plants. PCR was performed to confirm the presence of the transgene ChiVMV-CP; whereas no band was observed for non-transgenic control plants. Band intensity was almost the same for all the tested plants which were indicated that the integration was the same and successful. The analysis of gene integration in transgene through PCR is a general practice that has been followed by a number of scientists (Missiou et al., 2004; Bazzini et al., 2006). The hpt gene was determined by PCR amplification with hpt specific forward and reverse primers from transgenic plants. The 700 bp amplified fragments of hpt gene from p03 represent the presence of the transgene in transgenic plants (Figure 7).

RT-PCR gave the same results according to the PCR but the band intensity was different for putative transgenic plants (Figure 8a and b). Different intensity levels indicated the amount of mRNA transcription in cells of each transgenic plant. The detectable amount of RNA was present in the tested plants, suggesting that the resistance is conferring through the mechanism of post-transcriptional gene silencing (Ghosh et al., 2002). Mechanically inoculated transgenic and control (non-transgenic) plants were evaluated by DAS-ELISA test before and after infection, in order to evaluate the virus titer level in transgenic and control plants. Before infection, all the transgenic lines gave a low level of signal, almost negative ELISA values.

The ELISA readings confirmed the phenotypic symptoms and showed virus accumulation was in a detectable amount in systemically infected leaves of these transgenic and control plants at 15 dpi. The transgenic plants of *N. benthamiana* had shown various levels of resistance against ChiVMV isolate ATIPK. ELISA values of the inoculated non-transgenic plants indicated that these plants possessed a high amount of ChiVMV titer as compare to transformed plants (Figure 9). Transgenic line T3, T4, and T7 did not show any symptoms during the entire life cycle resulted in negative or less than an average ELISA values (<0.25) were regarded as “Resistant” transgenic lines on

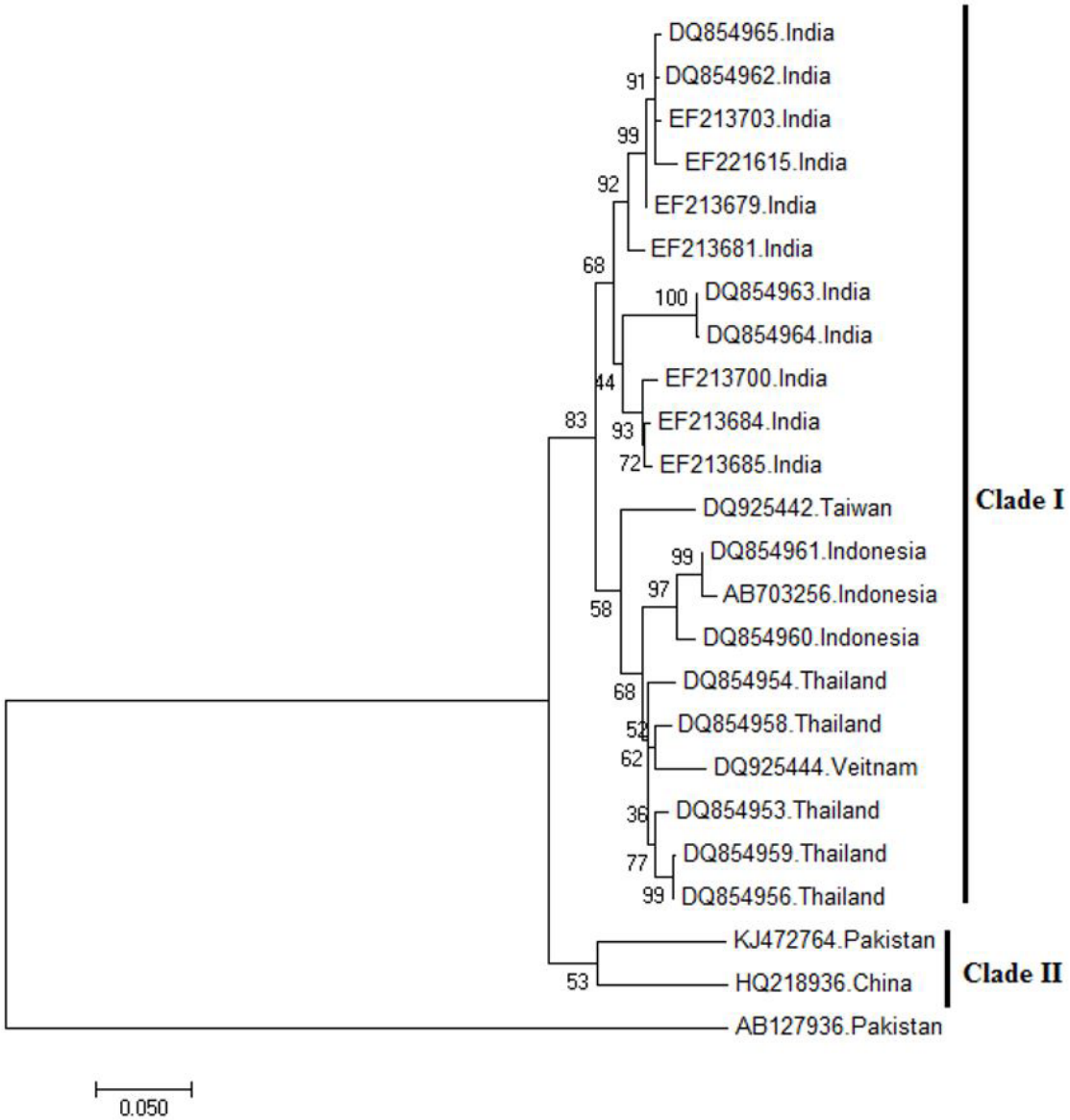

Figure 5. Maximum likelihood tree of ChiVMV isolate ATIPK on nucleotide sequence of CP gene with 22 ChiVMV isolates from the world. The bootstrap analysis was conducted in 1000 replications. ZYMV (AB127936) sequence is used as out-group.

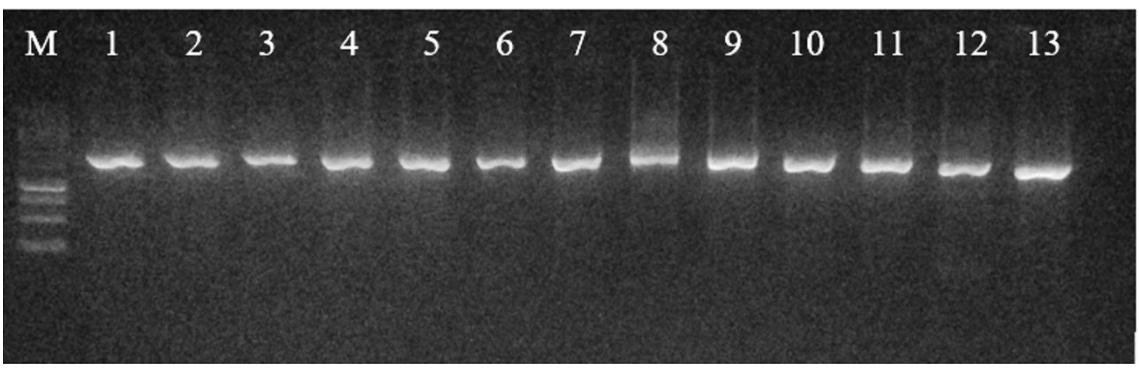

Figure 6. DNA bands from T0 transgenic and Agro-infilterated plants. Lane 1-10, transgenic plants. Lane 11-13, agro-infilterated plants.

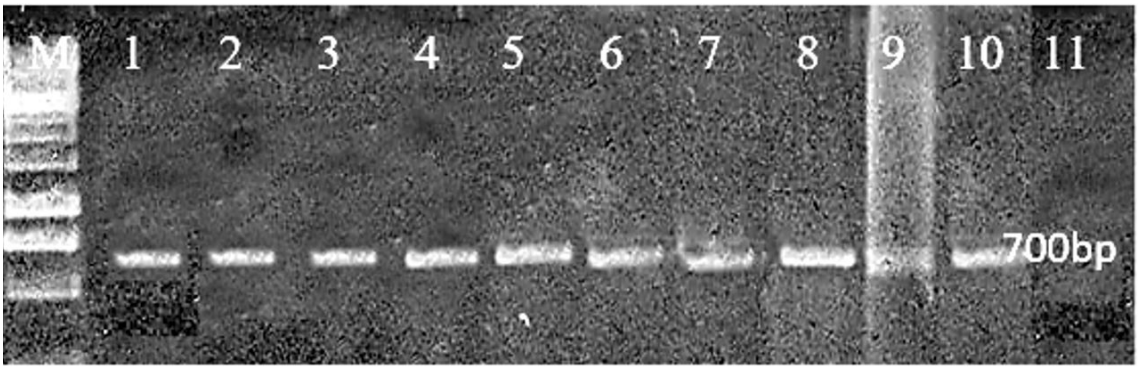

Figure 7. PCR Products of the hpt gene from T0 transgenic plants. Lane 1-9 are transgenic. Lane 10, +ve control. Lane 11, control (untransformed) plant.

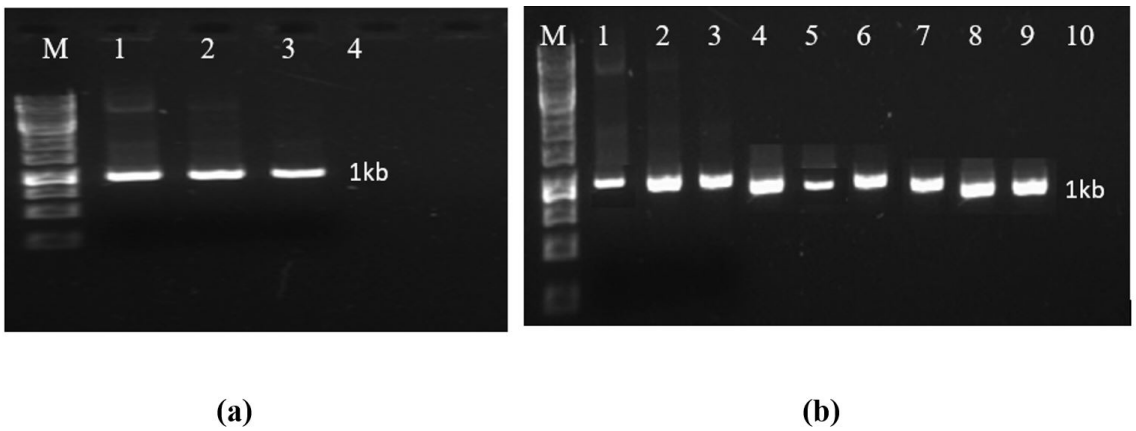

Figure 8. PCR products from transgenic plants. (a) Lane 1-3, represent PCR product from agro-infilterated plants. Lane 4, control plant. (b) Lane 1-9, RT- PCR product from transgenic plants represent the presence of CP gene transcription. Lane 10, represents untransformed control sample.

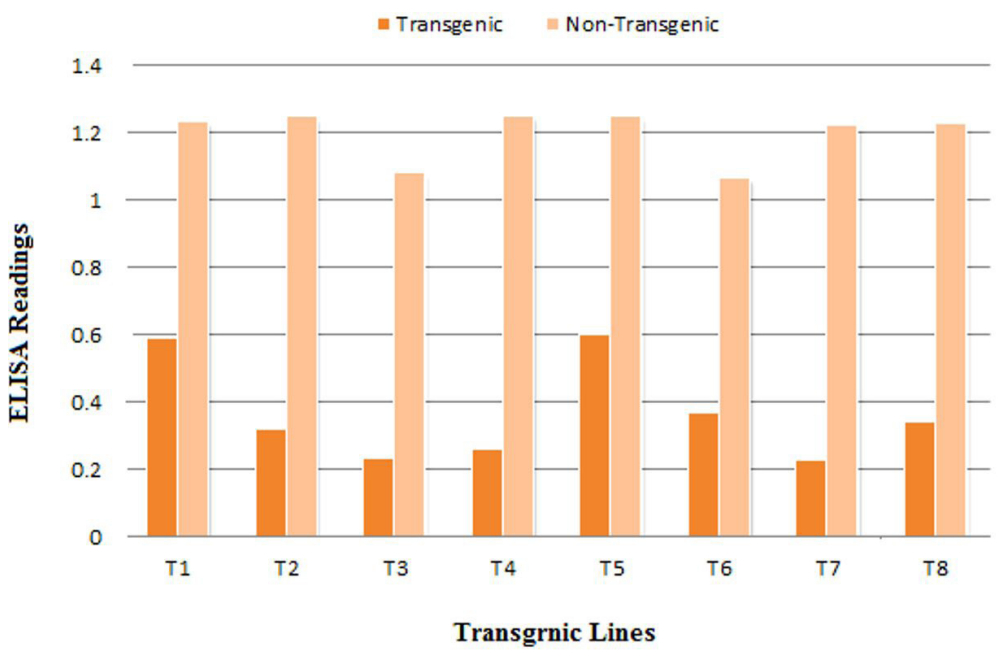

Figure 9. ELISA plate readings (O.D at 405_{nm}) of leaf samples of transgenic lines (T1-T8) and control plants after 15 dpi.

Table 4. Evaluation of resistance level in putative transgenic plants

Transgenic Lines	Resistance Level (RL)	Symptoms	ELISA Readings	+ ive control plants		- ive control plants	
				Resistance level	Symptoms	Resistance level	Symptoms
T3, T4, T7	Resistance	No Symptoms	0.232, 0.245, 0.225	Susceptible	Mosaic, mottling, leaf curling and leaf size reduction	Resistance	Healthy
T2, T6, T8	Moderate Resistance	Symptomless infection or delayed symptoms	0.322, 0.371, 0.340	Susceptible	Mosaic, mottling and foliar distortion	Resistance	Healthy
T1, T5	Moderately susceptible	Mild mosaic and mottling	0.593, 0.600	Susceptible	Mosaic, mottling, leaf curling and leaf size reduction	Resistance	Healthy

Positive control plants: Untransformed *N. benthamiana* plants challenged with ChiVMV isolate ATIPK

the basis of symptoms and ELISA readings. Transgenic lines T2, T6 and T8 had the ability to give moderate resistance to the viral attack and no symptoms were developed on the leaves during 15 dpi while symptoms were appeared on 20 dpi and vanished on the lateral developmental stages and can be called as delayed symptoms. These three lines (T2, T6 and T8) were referred as "Moderate resistant" transgenic lines on the basis of symptoms and ELISA readings (0.32- 0.37) that were less than two times of average ELISA value (<0.25). Whereas T1 and T5 displayed the mild mosaic and mottling symptoms and showed the high ELISA values (0.59-0.60) i.e. three times or less than three times of average ELISA value (<0.25), were referred as "Moderately susceptible". On the other hand, control plants showed high viral accumulation as an indicator of highly susceptible behavior after inoculation with infective sap of ChiVMV isolate ATIPK (Table 4 and Figure 10).

Negative control plants: Untransformed *N. benthamiana* plants challenged with distilled water

DAS ELISA value for each transgenic line is the average value of five replicates

Figure 10. Symptoms development in transgenic plants after challenging with ChiVMV isolate ATIPK at15dpi. A) Resistance transgenic lines. B) Moderately resistance transgenic lines. C) Moderately susceptible transgenic lines.

4. Discussion

The present study has revealed the molecular identification and biological purification of ChiVMV infecting chilli crop in Pakistan. This study highlighted the phylogenetic position of ChiVMV isolate ATIPK (KJ472764) among potyviruses found elsewhere in the world. ChiVMV isolate ATIPK from Pakistan clade up separately with Chinese isolate which showed its distinct phylogenetic position. The use of CP gene of Pakistani ChiVMV isolate ATIPK enabled us to produce the transgenic model plants that expressed the resistance against understudied virus. Results of molecular characterization and resistance evaluation studies confirmed that resistance has been generated through the post-transcriptional gene silencing (PTGS) mechanism.

Pathogen derived resistance (PDR) approach becomes the method of choice because it attained effective resistance against many plant viruses. It seems that there is no single mechanism that explains all example of PDR. Several models have been developed to explain the PTGS mechanism in the cell. Overall, the mechanism of protein-mediated resistance is connected to inhibit a specific step in the virus infection cycle, depending upon the viral gene used for the transformation. Most of the example of PDR to potyviruses is, however, RNA mediated, being connected to PTGS of a transgene.

The mechanism of observed variable resistance operates in transformants of our experiments may be due to many reasons: (i) it is generally recognized that not all siRNA species are equally effective against a given mRNA and

due to extensive positional effects along the mRNA, some siRNA shows limited efficiency, for example, secondary structure (Overhoff et al., 2005); (ii) the accumulation of siRNAs was known to inhibit by temperature variation and both virus and transgene-triggered RNA silencing become attenuated (Szittya et al., 2003); (iii) all the plants may not be homogenous in their transgenic content, and may not represent the same behavior or the resistance in some lines may be in a fragile balance and overcome sometimes by a virus due to variation in inoculum doze; (iv) one possible mechanism of low resistance in some lines is that ribosome scanning or shunting of the sense RNA prevents proper folding of the RNA into perfect dsRNA and thereby suppresses effective dicer function (Bucher and Prins, 2006); (v) resistance is often associated with a low steady-state transgene expression level and a high transgene copy number, while susceptibility correlates with a high steady-state transcription level and low transgene copy (Fitchen and Beachy, 1993; Lindbo and Dougherty, 1992; Baulcombe, 1996; Goodwin et al., 1996). Viruses transmit through the vectors and CP plays a central role in transmission; an additional advantage of the CP mediated resistance is that it confers hindrance to vector inoculation.

5. Conclusions

ChiVMV was characterized for the first time on the nucleic acid basis in Pakistan and it was observed that most of the cultivating chilli genotypes in the farmer fields are vulnerable to this menacing threat. The role of CP gene in imparting resistance against ChiVMV was defined and now it could be utilized to develop effective control measures against ChiVMV. Further studies are also required to estimate and compare the yield losses in transgenic and non-transgenic chilli plants and resistance level would be challenged under field conditions. The role of other genes of ChiVMV in conferring resistance will be checked. There is also a need to develop full-length infectious clone of ChiVMV and to determine genetic recombination of Pakistani ChiVMV isolate ATIPK.

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