INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–344/2013/15–6–1140–1144
http://www.fspublishers.org

Full Length Article

Interference of a Synthetic Rep Protein to Develop Resistance against Cotton Leaf Curl Disease

Sumaira Yousaf[1,2*], Ghulam Rasool[1], Imran Amin[1], Shahid Mansoor[1] and Muhammad Saeed[1]
[1]*Agricultural Biotechnology Division, National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan*
[2]*Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan*
*For correspondence: sa_niab@yahoo.com

Abstract

Cotton leaf curl disease (CLCuD) associated with a complex of begomoviruses is an important agricultural issue of cotton. One of the reasons for breakdown of resistance against these viruses is gene silencing (GS) that come out as a problem while developing transgenic resistance in plants. This study is providing a strategy to manage CLCuD by controlling diverse begomoviruses and circumventing GS. A truncated Rep protein (Rep_{syn130}) of a begomovirus optimized for plant expression and avoiding virus-induced gene silencing (VIGS) was designed using bioinformatics tools and commercially synthesized. N-terminus 390 nucleotides encoding 130 amino acids of Reps of nine different begomoviruses involved in CLCuD were aligned with Rep_{syn130} and confirmed no chances of GS. Resistance evaluation of Rep_{syn130} against *Cotton leaf curl Khokhran virus* (CLCuKoV) and Cotton leaf curl Multan betasatellite (CLCuMuB) was performed by *Agrobacterium*-mediated transient assay in *Nicotiana benthamiana* plants. Agroinoculation of CLCuKoV/CLCuMuB along with Rep_{syn130} resulted in 65% resistance by visual observation of symptoms severity along with 5-6 days delayed and milder symptoms development. PCR of 20 experimental plants confirmed the presence of CLCuKoV/CLCuMuB in 90% plants however, Southern analyses showed decreased viral load. These findings showed the potential of the synthetic Rep gene for resistance against CLCuD complex. © 2013 Friends Science Publishers

Keywords: Cotton leaf curl disease; Begomoviruses; Replication-associated protein; Pathogen-derived resistance; Gene silencing

Introduction

Cotton leaf curl disease (CLCuD) is overwhelming greatly the yield of cotton in Pakistan (Akhtar *et al.*, 2013). The disease is caused by several monopartite begomoviruses associated with a betasatellite (Briddon *et al.*, 2001; Mansoor *et al.*, 2003a). Begomoviruses belong to family *Geminiviridae* and are single-stranded (ss) DNA viruses infecting dicotyledonous plants (King *et al.*, 2011). The resistance in plants against these ssDNA viruses is a major challenge especially against CLCuD, which is damaging cotton in Pakistan, India and Africa as well as now start appearing in China (Cai *et al.*, 2010; Sattar *et al.*, 2013). In Pakistan nine different species of begomoviruses have been found associated with CLCuD (Briddon, 2003; Amrao *et al.*, 2010a, b; Tahir *et al.*, 2011).

Pathogen-derived resistance (PDR) involves the addition of some part or a full viral gene into the plant, which afterward interferes with one or more important steps of the viral life cycle. Two mechanisms *viz.* RNA mediated resistance (RMR) and protein mediated resistance (PMR) operate PDR. RMR is highly sequence specific mechanism and has a narrower range potential (Dasgupta *et al.*, 2003). While PMR, based upon the expression of protein, possess a broader range potential. Viral Rep protein, due to its functional importance and manifold properties is a preferred target for PDR against geminiviruses. Full-length or truncated Reps have been explicitly used to increase resistance against several geminiviruses causing diverse diseases in plants (Hanley-Bowdoin *et al.*, 1990; Noris *et al.*, 1996; Brunetti *et al.*, 2001; Lucioli *et al.*, 2003; Antignus *et al.*, 2004). A Rep based resistance utilizing N-terminal 210 and 130 amino acid (aa) described that 130 aa were enough to act as *trans*-dominant negative inhibitor but the resistance was shown to be overcome by virus-induced gene silencing (VIGS; Lucioli *et al.*, 2003).

Transgenic resistance against RNA viruses is in the farmer's field since long ago (Gonsalves, 1998). Unfortunately this success is only for RNA viruses and it has not been achieved for geminiviruses so far. Although the proof of the concept of PDR for geminiviruses was demonstrated in the early 1990's (Hanley-Bowdoin *et al.*, 1990) only recently the first transgenic plants had undergone field trials (Aragão and Faria, 2009). To overcome the problem of GS, one of the possible solutions could be the use of synthetic gene (Lucioli *et al.*, 2008). We report, using *Agrobacterium*-mediated transient assay in *N. benthamiana* plants that synthetic Rep protein could be used to develop resistance against CLCuD.

To cite this paper: Yousaf, S., G. Rasool, I. Amin, S. Mansoor and M. Saeed, 2013. Interference of a synthetic rep protein to develop resistance against cotton leaf curl disease. *Int. J. Agric. Biol.*, 15: 1140–1144

Materials and Methods

N-terminus sequences of 390 nt encoding 130 aa of Rep protein of the following begomoviruses were taken from NCBI: *Cotton leaf curl Burewala virus* (CLCuBuV: FR819707; AJ242974], *Cotton leaf curl Kokhran virus* (CLCuKoVNC_004583.1; HM468427.1], *Cotton leaf curl Multan virus* (CLCuMuV; NC_004607.1), *Cotton leaf curl Rajasthan virus* (CLCuRaV; NC_003199.1),*Cotton leaf curl Shadadpur virus* (CLCuShV; FN552001),*Cotton leaf curl Gezira virus* (CLCuGeV: HE800524), *Cotton leaf curl Alabad virus* (CLCuAlV; AJ002452), *Tomato leaf curl Bangalore virus* (ToLCBaV; HM007094) and *Papaya leaf curl virus* (PaLCuV; NC_004147). Multiple sequence alignment (MSA) was carried out using ClustalV in DNAstar.

Rep was designed via codon optimization and avoiding GS for plant expression by changing every third nt of CLCuBuV-Rep sequence (AJ242974) such that it code the same aa and there was no more than 5 consecutive nt identical to CLCuBuV-Rep. Codon optimization was carried out by considering codon usage table for *Gossypium hirsutum* [gbpln]: 557 CDS's (190383 codons) from NCBI-GenBank codon usage data base. The new sequence of the Rep gene (Rep_{syn130}) was synthesized commercially (GenScript USA Inc.). Rep_{syn130} was cloned in plant expression vector pJIT60 at the *Hind*III and *Eco*RI restriction sites to produce p35SRep under the control of the 35S *Cauliflower mosaic virus* (CaMV) promoter (Guerineau and Mullineaux, 1993) and plant transformation vector pGreen0029 at *Kpn*I and *Xho*I restriction sites (Hellens *et al*., 2000) and then transformed into *Agrobacterium tumefaciens* (LBA4404) by electroporation to produce pRep.

Resistance response of the developed construct was carried out by transient expression of Rep_{syn130} against CLCuKoV/CLCuMuB using agroinfiltration as described by Llave *et al.*(2000). Co-inoculated plants were monitored for the symptoms severity and were collected at 25 days post inoculation (dpi) when control plants developed full symptoms. Total genomic DNA was isolated from leave tissues by CTAB method (Doyle and Doyle, 1990). The presence of CLCuKoV was checked by PCR using primers CLCV1 and CLCV2 (Shahid *et al.*, 2007), while universal primers β01 and β02 (Briddon *et al.*, 2002) were used for the amplification of CLCuMuB. Virus titer was determined by Southern blot hybridization using DIG labeled full-length probes (Roche) of CLCuKoV and CLCuMuB.

Results

Viral Rep/Rep_{syn130} Sequences and Multiple Sequence Alignment (MSA)

Codon optimization of truncated CLCuBuV-Rep resulted in a new nt sequence of Rep_{syn130} encoding similar protein sequence (Fig. 1A, B and C). Rep_{syn130} possess only 64.6% nt identity to the CLCuBuV-Rep, this low identity ensured to circumvent GS by Rep_{syn130} while acting as a *trans*-dominant negative inhibitor. Paddison and associates (Paddison *et al.*, 2002) have reported that a continuous 100% homology of ~25 nt is sufficient to initiate GS without significant loss of potency. MSA of 5' end 390 nt sequences of Reps of all viruses involved in CLCuD confirmed that continuous 23 nt homology was not present, instead the maximum consecutive similarity was only 5 nt (Fig. 2). Hence the Reps of CLCuBuV, CLCuMuV, CLCuKoV, CLCuRaV and CLCuShV will not be able to silence the Rep_{syn130} using RNAi pathway. It has been illustrated that GRS may be involved in broad-spectrum resistance (Nash *et al.*, 2011). Further analysis of Rep_{syn130} showed that it contains motif I (FLTY), motif II (HLH; Argüello-Astorga and Ruiz.Medrano, 2001) motif III (YxxKD/E; Choudhury *et al.*, 2006) and GRS motif between motif II and III that are involved in viral replication, infection and initiation of rolling circle replication.

Transient Expression of Rep_{syn130} by Agroinfiltration

Transient expression of Rep_{syn130} showed resistance response against CLCuKoV/CLCuMuB in *N. benthamiana*. 100% infection efficiency of CLCuKoV/CLCuMuB was observed as all control plants showed typical symptoms of downward leaf curling, vein swelling and darkening along with enations on newly emerging leaves at 21dpi (Fig. 3A) as compared to healthy *N. benthamiana* (Fig.3B). Only 10% plants co-inoculated with CLCuKoV/CLCuMuB and Rep_{syn130} showed sever symptoms while a 5-6 days delay along with milder symptoms was found in 30% plants and other 60% plants remained asymptomatic (Fig.3C). Rep_{syn130} showed reproducible resistance response (60 to 75%) in three independent experiments (Table 1). The asymptomatic plants were kept under observation till senescence and no symptoms were observed.

Molecular analyses of systemic leave of the plants by PCR resulted in the amplification of both CLCuKoV and CLCuMuB in all plants co-inoculated with CLCuKoV/CLCuMuB and pRep showing the presence of virus in the plant (data not shown). However, a considerable decrease in the titer of CLCuKoV and CLCuMuB DNA was observed by Southern blot hybridization as compared to positive control plants (Fig. 3).

Discussion

Inhibitions based on dominant negative interference has already been reported for homologous viruses (Lucioli *et al.*, 2003, 2008) however, in present study we have checked the ability of Rep_{syn130} for resistance against a heterologous CLCuKoV, which had 74.8% similarity to the CLCuBuV-Rep and its associated betasatellite (CLCuMuB). Although

Table 1: Resistance evaluation of Rep_{syn130} in *N. benthamiana* plants co-inoculated with *Cotton leaf curl Khokhran virus* (CLCuKoV)/Cotton leaf curl Multan betasatellite (CLCuMuB) and Rep_{syn130}

Inocula for Agroinfiltration	Experiment No.	Total No. of plants	Plants Infected at 21-25 dpi	% Symptomatic Plants	Symptoms severity			% Resistance	Molecular Analyses		
					Sever	Mild	Latent period (days)		PCR	Southern Analysis for CLCuKoV	Southern Analysis for CLCuMuB
N. benthamiana (non-inoculated)	in all experiments	10	0	0	NA	NA	NA	NA	_	_ _ _	_ _ _
N. benthamiana +	I	10	10	100	10	-	-	-	+ 10/10	+++	+++
CLCuKoV/	II	10	10	100	10	-	-	-	+10/10	+++	+++
CLCuMuB	III	20	20	100	20	-	-	-	+20/20	+++	+++
N. benthamiana +	I	10	4	40	1	3	5-6	60	+10/10	+	+
CLCuKoV/ CLCuMuB	II	10	4	40	1	3	5-6	60	+10/10	+	+
$+pRep_{syn130}$	III	20	5	25	1	5	5-6	75	+20/20	+	+

*Virus presence by PCR; + present, - absent. Virus titer by Southern analysis; ++++ strong signals, ++ weak signals, + very weak signals, -- No signals

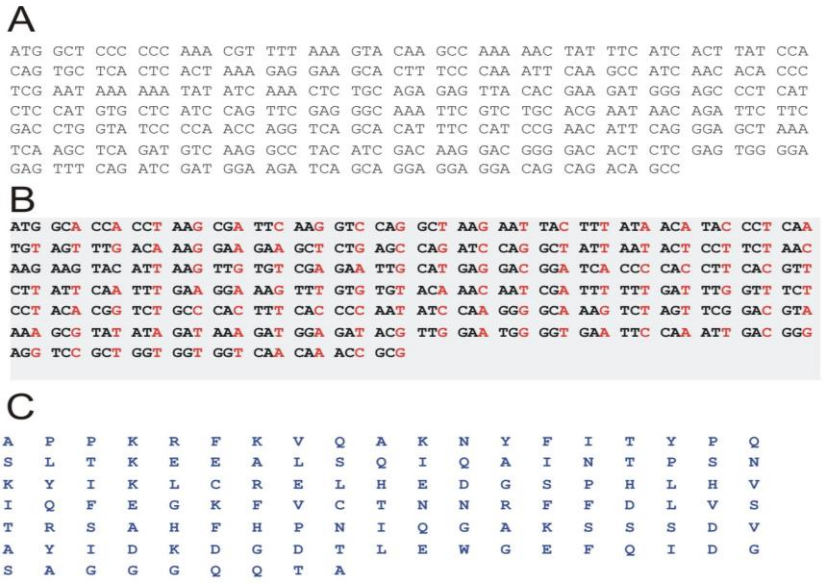

Fig. 1: (A) N-terminus 390 nucleotide sequence of CLCuBuV-Rep (**B**) codon optimized designed and synthesized nucleotide sequence of synthetic Rep (Rep_{syn130}) (**C**) protein sequence of CLCuBuV-Rep and Rep_{syn130}

Fig. 2: Multiple sequence alignment of Rep_{syn130} with N-terminus 390 nucleotide sequence of Rep of nine viruses (CLCuBuV, CLCuKoV, CLCuMuV, CLCuRaV, CLCuShV, CLCuAlV, CLCuGeV, ToLCBaV and PaLCuV) involved in cotton leaf curl disease

the present study is providing the results of transiently expressing Rep_{syn130} in *N. benthamiana*, where the protein expression is confined to the inoculated patch only, yet the transient expression system is a highly adaptable method in plants to check the expression of genes in plant without developing transgenic plants by tissue culture method (Orzaez *et al.*, 2006) and has widely used to check the effect of many transgenes (Llave *et al.*, 2000; Voinnet *et al.*, 2003). Recently Kaliappan *et al.* (2012) have reported the role of a host protein interacting with geminiviral Rep protein by a transient assay. In this study we have used higher inoculum dose by taking equal V/V ratios of pRep and CLCuKoV/CLCuMuB, while co-inoculating the *N. benthamiana* plants and found it as an effective strategy for evaluating the resistance of plants against begomoviruses. This is to mention here that variation in the symptoms could be attributed to this possibility that plants were infiltrated with viral and Rep_{syn130} in equal ratio and it is possible that some of the initially cells in infiltrated leaves only received viral construct and developed disease symptoms.

The results of PCR and Southern hybridization indicated that although the virus is present in these plants but its level has been decreased to a considerable level when it is co-inoculated with pRep. Moreover, in the systemic leave the level of both CLCuKoV and CLCuMuB was negligible with comparison to control and the decreased level was unable to produce a symptomatic infection. Thus Rep_{syn130} has been acting as *trans*-dominant negative inhibitor of CLCuKoV/CLCuMuB. Here it is observed that the expression of Rep_{syn130} had inhibited but not completely abolished the transcription of attacking viral Rep. The reduced amount of Rep and Rep_{syn130} would be competing for iteron binding to initiate plus-strand viral replication. Since we have analyzed iterons and iteron related domains (IRD) for cotton infecting begomoviruses and found minor variation (results not shown). The combined action of both Reps possibly reduced the replication rate by forming large amount of dysfunctional Rep_{syn130}/Rep complexes.

The Rep_{syn130} used in this study encompasses all the essential domains of Rep. Its multifunctional property related with the oligomerization nature (Orozco *et al.*, 2000) is found sufficient for its dominant inhibition function as 1-130 aa of Rep protein is the binding domain, specifically motif I and II (Argüello-Astorga and Ruiz.Medrano, 2001; Campos-Olivas *et al.*, 2002; Nash *et al.*, 2011). This inhibition might be due to the presence of the conserved IRD that possess DNA-binding domain (Argüello-Astorga and Ruiz.Medrano, 2001), binding with viral DNA by electrostatic forces of interactions between positively charged aa and negatively charged DNA (Campos-Olivas *et al.*, 2002), oligomerization and *trans*-dominant negative inhibition by Rep_{syn130}. Together with this study and the earlier report of (Lucioli *et al.*, 2008) it is inferred that such strategies may provide the resistance against begomoviruses. Due to RNAi and RNA silencing suppressor activity of virus genes *e.g.*, (A)C2, (A)C4, (A)V2 and βC1 as a counter defense strategy by geminiviruses (Bisaro, 2006; Amin *et al.*, 2011) RMR could be concealed and has a narrower range potential. An RNAi based resistance against CLCuMuV was achieved (Asad *et al.*, 2003) but later on resistance break down was also reported (Mansoor *et al.*, 2003b). Recently first geminiviral resistant common bean plants using RMR have been reported in the field (Aragão and Faria, 2009) but this should also be kept in mind that no diversity in bean viruses have been found so far (Nahid *et al.*, 2008). Present study is providing PMR against DNA viruses. Thus we can achieve resistance against most common viruses associated with CLCuD in Pakistan (CLCuBuV, CLCuKoV, CLCuMuV, CLCuRaV and CLCuShV) via the expression of Rep_{syn130}. However, further detailed analysis of transgenic plants may confirm and validate our findings.

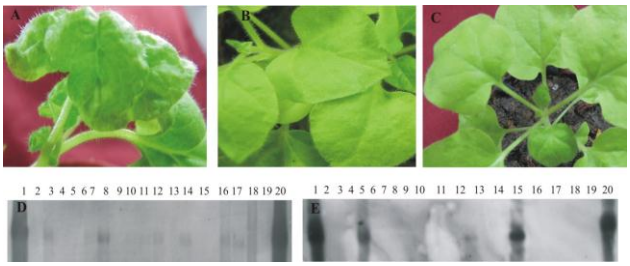

Fig. 3: (A) Control plants inoculated with infectious clones of CLCuKoV/CLCuMuB **(B)** Non-inoculated healthy *N. benthamiana* **(C)** Plants co-inoculated with infectious clones of CLCuKoV/CLCuMuB +$pRep_{syn130}$ **(D)** Southern blot showing CLCuKoV-DNA inhibition by transiently expressed Rep_{syn130}. Lane 1 and 20 are positive control plants showing higher level of CLCuKoV-DNA. Lane 2 and 19 are control plants of healthy *N. benthamiana*. Lanes 3-18 are showing level of CLCuKoV-DNA in plants co-inoculated with CLCuKoV/CLCuMuB and $pRep_{syn130.}$ **(E)** Southern blot showing CLCuMuB-DNA inhibition by transiently expressed Rep_{syn130} along with CLCuKoV/CLCuMuB. Lane 1 and 20 are positive control plants showing higher level of CLCuMuB-DNA. Lane 2 and 19 are negative control plants. Lanes 3-18 are showing level of CLCuMuB-DNA in plants co-inoculated with CLCuKoV/CLCuMuB and $pRep_{syn130.}$ Titer of CLCuKoV and CLCuMuB was determined on the same plant. DNA from plant leave tissues was extracted at 25 dpi and approximately 10μg genomic DNA was loaded in each well

Acknowledgements

This work was conducted by a financial support of Higher Education Commission (HEC), Government of Pakistan, under the "Indigenous PhD Fellowship program".

References

Akhtar, K.P., R. Ullah, I.A. Khan, M. Saeed, N. Sarwar and S. Mansoor, 2013. First Symptomatic Evidence of Infection of Gossypium arboreum with Cotton Leaf Curl Burewala Virus through Grafting. *Int. J. Agric. Biol.*, 15: 157–160

Amin, I., K. Hussain, R. Akbergenov, J.S. Yadav, J. Qazi, S. Mansoor, T. Hohn, C.M. Fauquet and R.W. Briddon, 2011. Suppressors of RNA silencing encoded by the components of the cotton leaf curl begomovirus-betasatellite complex. *Mol. Plant Microbe Interact.*, 24: 973–983

Amrao, L., S. Akhter, M.N. Tahir, I. Amin, R.W. Briddon and S. Mansoor, 2010a. Cotton leaf curl disease in Sindh province of Pakistan is associated with recombinant begomovirus components. *Virus Res.*, 153: 161–165

Amrao, L., I. Amin, M.S. Shahid, R.W. Briddon and S. Mansoor, 2010b. Cotton leaf curl disease in resistant cotton is associated with a single begomovirus that lacks an intact transcriptional activator protein. *Virus Res.*, 152: 153–163

Antignus, Y., R. Vunsh, O. Lachman, M. Pearlsman, L. Maslenin, U. Hananya and A. Rosner, 2004. Truncated Rep gene originated from *Tomato yellow leaf curl virus*-Israel [Mild] confers strain-specific resistance in transgenic tomato. *Annl. Appl. Biol.*, 144: 39–44

Aragão, F.J.L. and J.C. Faria, 2009. First transgenic geminivirus-resistant plant in the field. *Nat. Biotechnol.*,27: 1086–1088

Argüello-Astorga, G.R. and R. Ruiz.Medrano, 2001. An iteron-related domain is associated to motif 1 in the replication proteins of geminiviruses: identification of potential interacting amino acid-base pairs by a comparartive approach. *Arch. Virol*., 146: 1465–1485

Asad, S., W.A.A. Haris, A. Bashir, Y. Zafar, K.A. Malik, M.N. Malik and C.P. Lichtenstein, 2003. Transgenic tobacco expressing geminiviral RNAs are resistant to the serious viral pathogen causing cotton leaf curl disease. *Arch. Virol.*, 148: 2341–2352

Bisaro, D.M., 2006. Silencing suppression by geminivirus proteins. *Virology*, 344: 158–168

Briddon, R.W., 2003. Cotton leaf curl disease, a multicomponent begomovirus complex. *Mol. Plant Pathol.*, 4: 427–434

Briddon, R.W., S. Bull, S. Mansoor, I. Amin and P.G. Markham, 2002. Universal primers for the PCR-mediated amplification of DNA β; a satellite associated with some monopartite begomoviruses. *Mol. Biotechnol.*, 20: 315–318

Briddon, R.W., S. Mansoor, I.D. Bedford, M.S. Pinner, K. Saunders, J. Stanley, Y. Zafar, K.A. Malik and P.G. Markham, 2001. Identification of DNA components required for induction of cotton leaf curl disease. *Virology*, 285: 234–243

Brunetti, A., R. Tavazza, E. Noris, A. Lucioli, G.P. Accotto and M. Tavazza, 2001. Transgenically expressed T-Rep of tomato yellow leaf curl Sardinia virus acts as a *trans*-dominant-negative mutant, inhibiting viral transcrition and replication. *J. Virol.*, 75: 10573–10581

Cai, J., K. Xie, L. Lin, B. Qin, B. Chen, J. Meng and Y. Liu, 2010. Cotton leaf curl Multan virus newly reported to be associated with cotton leaf curl disease in China. *Plant Pathol.*, 59: 794–795

Campos-Olivas, R., J.M. Louis, D. Cleroy, B. Gronenborn and A.M. Gronenborn, 2002. The structure of a replication initiator unites diverse aspects of nucleic acid metabolism. *Proc. Nat. Acad. Sci. USA*, 99: 10310–10315

Choudhury, N.R., P.S. Malik, D.K. Singh, M.N. Islam, K. Kaliappan and S.K. Mukherjee, 2006. The oligomeric Rep protein of Mungbean yellow mosaic India virus (MYMIV) is a likely replicative helicase. *Nucl. Acids Res.*,34: 6362–6377

Dasgupta, I., V.G. Malathi and S.K. Mukherjee, 2003. Genetic engineering for virus resistance. *Curr. Sci.*, 84: 341–354

Doyle, J.J. and J.L. Doyle, 1990. Isolation of plant DNA from fresh tissue. *Focus*, 12: 13–15

Gonsalves, D., S. Ferreira, R. Manshardt, M. Fitch and J. Slightom, 1998. Transgenic virus resistant papaya: New hope for control of papaya ringspot virus in Hawaii. *APSnet Feature, American Pythopathological Society. DOI:10.1094/PHP-2000-0621-01-RV*

Guerineau, F. and P. Mullineaux, 1993. Plant transformation and expression vectors. *In:* "*Plant Molecular Biology Labfax*", pp: 121–147. Croy, R.R.D. (ed.). Bios Scientific Publishers, Oxford

Hanley-Bowdoin, L., J.S. Elmer and S.G. Rogers, 1990. Expression of functional replication protein from tomato golden mosaic virus in transgenic tobacco plants. *Proc.Nat. Acad. Sci. USA*, 87: 1446–1450

Hellens, R.P., E.A. Edwards, N.R. Leyland, S. Bean and P.M. Mullineaux, 2000. pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. *Plant Mole. Biol.*, 42: 819–832

Kaliappan, K., N.R. Choudhury, G. Suyal and S.K. Mukherjee, 2012. A novel role for RAD54: this host protein modulates geminiviral DNA replication. *The FASEB J.*, 26: 1142–1160

King, A.M.Q., E. Lefkowitz, M.J. Adams and E.B. Carstens, 2011. "*Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*". Elsevier

Llave, C., K. Kasschau and J.C. Carrington, 2000. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proc. Nati. Acad. Sci. USA*, 97: 13401–13406

Lucioli, A., E. Noris, A. Brunetti, R. Tavazza, V. Ruzza, A.G. Castillo, E.R. Bejarano, G.P. Accotto and M. Tavazza, 2003. *Tomato yellow leaf curl Sardinia virus* Rep-derived resistance to homologous and heterologous geminiviruses occurs by different mechanisms and is overcome if virus-mediated transgene silencing is activated. *J. Virol.*, 77: 6785–6798

Lucioli, A., D.E. Sallustio, D. Barboni, A. Beradni, V. Papacchioli, R. Tavazza and M. Tavazza, 2008. A cautionary note on pathogen derived sequences. *Nat. Biotechnol.*,26: 617–619

Mansoor, S., R.W. Briddon, S.E. Bull, I.D. Bedford, A. Bashir, M. Hussain, Saeed, M.Y. Zafar, K.A. Malik, C. Fauquet and P.G. Markham, 2003a. Cotton leaf curl disease is associated with multiple monopartite begomoviruses supported by single DNA. *Arch. Virol.*, 148: 1969–1986

Mansoor, S., I. Amin, S. Iram, M. Hussain, Y. Zafar, K.A. Malik and R.W. Briddon, 2003b. The breakdown of resistance in cotton to cotton leaf curl disease in Pakistan. *New Dis. Rep.*, 52: 784

Nahid, N., I. Amin, S. Mansoor, E.P. Rybicki, E. van der Walt and R.W. Briddon, 2008. Two dicot-infecting mastreviruses (family Geminiviridae) occur in Pakistan. *Arch. Virol.*,153: 1441–1451

Nash, T.E., M.B. Dallas, M.I. Reyes, G.K. Buhrman, J. Ascencio-Ibanez and L. Hanley-Bowdoin, 2011. Functional analysis of a novel motif conserved across geminivirus Rep proteins. *Sci. Signal*, 85: 1182

Noris, E., G.P. Accotto, R. Tavazza, A. Brunetti, S. Crespi and M. Tavazza, 1996. Resistance to tomato yellow leaf curl geminivirus in *Nicotiana benthamiana* plants transformed with a truncated viral C1 gene. *Virology*, 224: 130–138

Orozco, B.M., L.J. Kong, L.A. Batts, S. Elledge and L. Hanley-Bowdoin, 2000. The multifunctional character of a geminivirus replication protein is reflected by its complex oligomerization properties. *J. Biol. Chem.*, 275: 6114–6122

Orzaez, D., S. Mirabel, W.H. Wieland and A. Granell, 2006. Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit. *Plant Physiol.*, 140: 3–11

Paddison, P.J., A.A. Caudy, E. Bernstein, G.J. Hannon and D.S. Conklin, 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Develop.*, 16: 948–958

Sattar, M.N., A. Kvarnheden, M. Saeed and R.W. Briddon, 2013. Cotton leaf curl disease – an emerging threat to cotton production worldwide. *J. Gen. Virol.*, 94: 695-710

Shahid, M.S., S. Mansoor and R.W. Briddon, 2007. Complete nucleotide sequences of cotton leaf curl Rajasthan virus and its associated DNA beta molecule infecting tomato. *Arch. Virol.*, 152: 2131–2134

Tahir, M.N., I. Amin, R.W. Briddon and S. Mansoor, 2011. The Merging of Two Dynasties—Identification of an African Cotton Leaf Curl Disease-Associated Begomovirus with Cotton in Pakistan. *PLoS One*, 6: e20366

Voinnet, O., S. Rivas, P. Mestre and D. Baulcombe, 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J.*, 33: 949–956

(Received 13 March 2013; Accepted 27 June 2013)