Plant Biotechnology Journal (2007) **5**, pp. 207–220 doi: 10.1111/j.1467-7652.2006.00217.x

Review article

Engineering resistance to geminiviruses – review and perspectives

Hervé Vanderschuren[1], Martin Stupak[1], Johannes Fütterer[1], Wilhelm Gruissem[1] and Peng Zhang[1,2,*]

[1]*Institute of Plant Sciences, ETH Zurich, Universitätstrasse 2, 8092 Zurich, Switzerland*

[2]*Institute of Plant Physiology and Ecology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China*

Received 15 May 2006;
revised 21 July 2006;
accepted 24 July 2006.
**Correspondence* (fax +41-44-632 1044;
e-mail pzhang@ethz.ch)

Keywords: anti-sense, geminivirus, genetic engineering, RNA interference, viral protein, virus resistance.

Summary

Following the conceptual development of virus resistance strategies ranging from coat protein-mediated interference of virus propagation to RNA-mediated virus gene silencing, much progress has been achieved to protect plants against RNA and DNA virus infections. Geminiviruses are a major threat to world agriculture, and breeding resistant crops against these DNA viruses is one of the major challenges faced by plant virologists and biotechnologists. In this article, we review the most recent transgene-based approaches that have been developed to achieve durable geminivirus resistance. Although most of the strategies have been tested in model plant systems, they are ready to be adopted for the protection of crop plants. Furthermore, a better understanding of geminivirus gene and protein functions, as well as the native immune system which protects plants against viruses, will allow us to develop novel tools to expand our current capacity to stabilize crop production in geminivirus epidemic zones.

Introduction

Geminiviruses are rapidly becoming major plant pathogens in tropical and subtropical countries (Moffat, 1999; Boulton, 2003; Mansoor *et al*., 2003), affecting an increasing number of crops with a disastrous impact on productivity (Table 1). The recent spread of geminivirus-associated diseases has been linked to the emergence of new virus strains and the increasing abundance of viruliferous whitefly vectors, possibly as a result of enhanced insecticide resistance and global warming (Seal *et al*., 2006). Breeding for resistance to geminiviruses in plants faces many challenges, such as the availability of geminivirus resistance genes from wild relatives or cultivars, the development of dominant molecular markers linking with the pathogen resistance and the rapid introgression of resistance into susceptible cultivars. So far, variable levels of geminivirus resistance in plants have been achieved by traditional breeding or genetic engineering (Lapidot and Friedmann, 2002). Approaches to achieve any geminivirus resistance by genetic engineering require a knowledge of its genome organization, gene functions, infection process and life cycle. This information provides important clues for efficient interference with the geminivirus reproductive cycle and for the elaboration of a strategy for durable resistance in plants.

On the basis of differences in genome organization, host range and insect vectors of geminiviruses, four different genera, namely *Mastrevirus*, *Curtovirus*, *Begomovirus* and *Topocuvirus*, are recognized (Fauquet and Stanley, 2003). The leafhopper-transmitted mastreviruses are quite distinct from all other geminiviruses in terms of genomic organization and host range, and are largely confined to monocotyledonous plants. The whitefly-transmitted begomoviruses, leafhopper-transmitted curtoviruses and treehopper-transmitted topocuviruses have a comparable genomic organization. They differ only little in gene content and function, suggesting a more recent common evolutionary origin in dicotyledonous hosts (Briddon and Stanley, 2006). The genus *Begomovirus* comprises 117 of the 133 officially recognized geminiviruses (Stanley *et al*., 2005). Begomoviruses cause most of the economically important geminivirus diseases of crops (Table 1). Their single-stranded DNA (ssDNA) genome is either bipartite or monopartite (Briddon and Markham, 2001). A typical example of a bipartite begomovirus is cassava mosaic geminivirus (CMG), the causal agent of cassava mosaic disease (CMD), which is the most

Table 1 List of the economically important viral diseases caused by geminiviruses

Disease name	Virus genus	Virion structure	Host crop	Epidemic region	Yield loss	Reference
Maize streak disease	*Mastrevirus*	Monopartite	Maize	Sub-Saharan Africa	Average 20%, up to 100%	Bosque-Perez (2000)
Cassava mosaic disease	*Begomovirus*	Bipartite	Cassava	Africa, India	Overall 15–24%, up to 90%	Legg and Fauquet (2004) Patil *et al*. (2005)
Cotton leaf curl disease	*Begomovirus*	Monopartite (associated β-component)	Cotton	Pakistan	Average 30%, up to 80%	Briddon and Markham (2000)
Bean golden mosaic disease/bean golden yellow mosaic disease	*Begomovirus*	Bipartite	Bean	Florida, Central and South America	10%–100%	Blair *et al*. (1995) Faria and Maxwell (1999)
Yellow mosaic disease	*Begomovirus*	Bipartite	Grain legumes	India	10%–90%	Malathi *et al*. (2005) Rouhibakhsh and Malathi (2005) Varma and Malathi (2003)
Tomato leaf curl disease/tomato yellow leaf curl disease	*Begomovirus*	Monopartite	Tomato	Europe, Asia, Americas, Australia	20%–80%, up to 100%	Moffat (1999) Moriones and Navas-Castillo (2000)

important viral disease of cassava in Africa and the Indian subcontinent (Legg and Thresh, 2000). Because of their economical importance and the rapid spread and evolution of CMGs, in the following we discuss CMGs and CMD as an illustrative example.

Geminiviruses require insect vectors for transmission. Once delivered into plant cells, geminiviruses enter a life cycle of DNA replication, DNA accumulation and virus assembly, as well as virus spread in the host (Figure 1). Proteins encoded by begomoviruses can be classified into four groups: DNA replication-associated proteins Rep and REn, proteins with host gene regulation and silencing suppression activity (TrAP and/or AC4), the structural protein (CP) and movement proteins (MP, NSP). The first three groups are encoded by the DNA A component, and the coding sequences for movement-associated proteins are found on the DNA B component. The function and regulation of these viral proteins and their interaction with plant cell factors have been reviewed recently (e.g. Hanley-Bowdoin *et al*., 1999, 2004; Gutierrez, 2000; Gutierrez *et al*., 2004).

Any successful resistance strategy must provide protection against infection under natural conditions, i.e. against naturally occurring virus populations and under natural infection pressures. Recombination of geminiviruses in their host plants and vectors allows for rapid evolution and the emergence of new geminivirus-associated diseases. Pseudo-recombination, recombination and synergism of geminiviruses are key factors in the epidemic of geminivirus-associated diseases. For example, recombination-associated replacement of an East African cassava mosaic virus (EACMV)-like virus coat protein (CP) gene with an African cassava mosaic virus (ACMV) gene sequence was responsible for the severe epidemic of CMD in Uganda (Deng *et al*., 1997; Zhou *et al*., 1997). Field testing for virus resistance gives the opportunity to place plants under natural virus pressure. It can also ensure that the resistance is effective against the emerging virus combinations. However, virus transmission, symptom development and yield losses are highly variable in the field. The incidence of the disease depends on several parameters, such as vector population, environmental conditions, such as rainfall, cycle of the year (Legg and Thresh, 2000) and plant development stage at which virus transmission occurs. The elaboration of reliable infection tests in the field is certainly a challenge for the release of newly developed cultivars with proven and durable virus resistance.

So far, approaches to achieve geminivirus resistance by genetic engineering have been mainly tested in the model plants *Nicotiana tabacum* and *Nicotiana benthamiana* at either the cell (protoplasts) or plant level. As detailed below and illustrated in Figure 1, these experiments targeted key steps of viral propagation, such as replication, encapsidation or movement. In most cases, strategies of pathogen-derived resistance have included the expression of viral proteins or nucleic acids, sometimes in modified form, to interfere with the ordered progression through the replication cycle (Baulcombe, 1996).

Increased resistance by the expression of proteins

Viral proteins

The first improved virus resistance by the expression of a viral protein was reported for transgenic tobacco expressing the

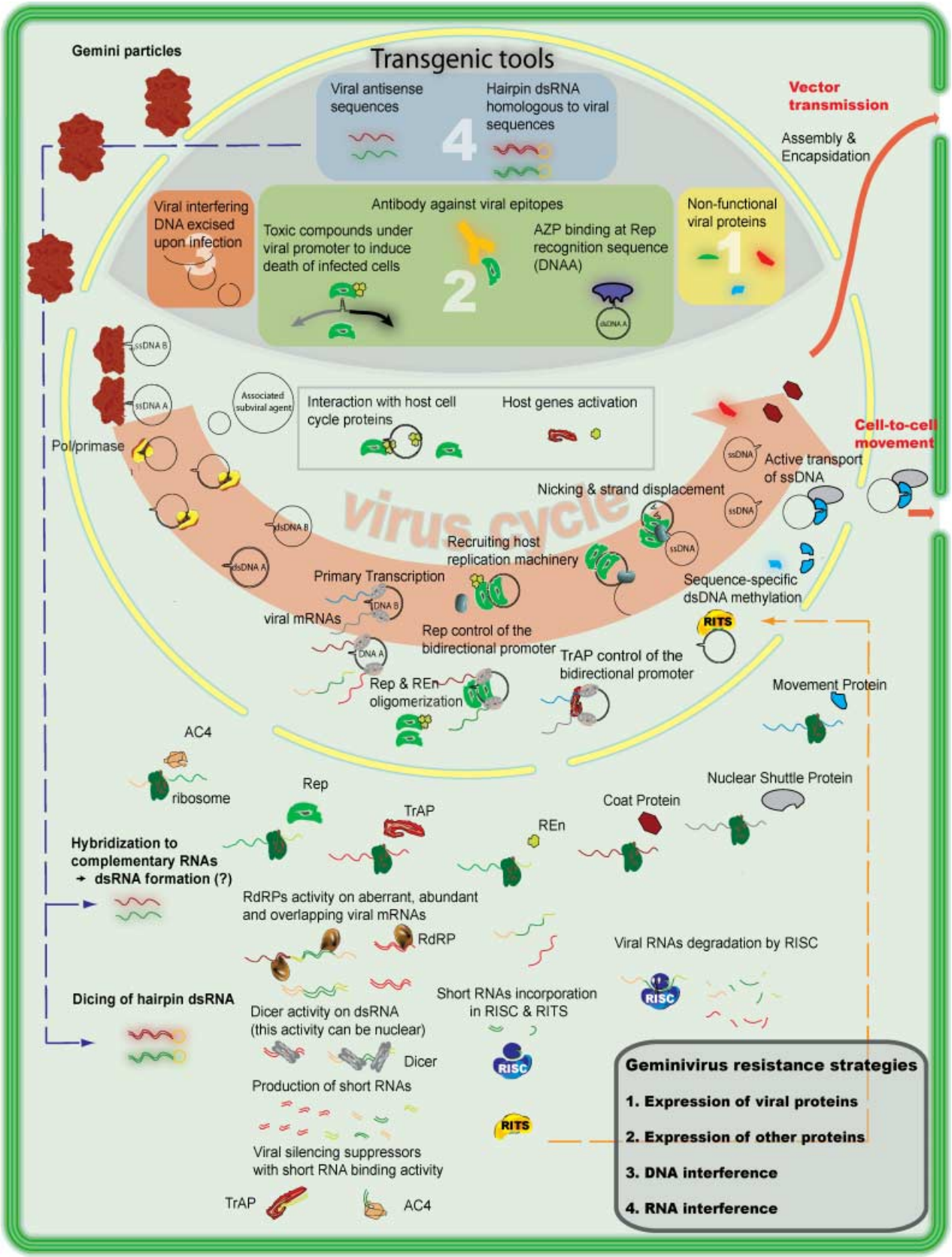

Figure 1 Four major strategies applicable to the engineering of geminivirus resistance in transgenic plants interfering with the geminivirus life cycle in plant cells. Top: four different strategies in numbered boxes. Middle: summary of the life cycle of geminviruses in plant cells. Bottom: endogenous silencing-related responses to viral infection and the potential of RNA interference to implement these pathways.

CP of tobacco mosaic virus (TMV; Abel *et al.*, 1986). Since then, CP-mediated plant virus resistance has been widely used to engineer resistance to RNA viruses. In the case of TMV, resistance was caused by interference with viral uncoating (Powell *et al.*, 1990; Beachy, 1999). In other cases, the resistance mechanisms are largely unknown. CP is required for systemic infection by monopartite geminiviruses (Briddon *et al.*, 1989; Rojas *et al.*, 2001), and tomato plants expressing the CP of the monopartite begomovirus tomato yellow leaf curl geminivirus (TYLCV) exhibited delayed symptom development that was dependent on the expression levels of transgenic CP (Kunik *et al.*, 1994). In contrast, the CP of bipartite geminiviruses is not absolutely necessary for the systematic spread of the virus, as NSP can substitute for the function of CP in transport (Ingham *et al.*, 1995; Pooma *et al.*, 1996). Therefore, it has been assumed that a CP-mediated strategy against bipartite geminiviruses will not produce a high level of resistance. Consistently, neither transgenic beans expressing the CP of bean golden mosaic virus (Azzam *et al.*, 1996) nor *N. benthamiana* plants expressing ACMV CP (Frischmuth and Stanley, 1998) displayed any resistance. Occasional resistance of CP transgenic tobacco plants to tomato mottle geminivirus (TMoV) was not correlated with CP expression (Sinisterra *et al.*, 1999). Nevertheless, geminivirus CPs may have the potential for transgenic interference as they control specific interactions with the virus vector (Briddon *et al.*, 1990; Azzam *et al.*, 1994; Hofer *et al.*, 1997; Noris *et al.*, 1998; Morin *et al.*, 2000). A CP-deficient ACMV-KE clone lost its systemic infection capacity in cassava plants and showed reduced functional interaction with its vector *Bemisia tabaci* (Liu *et al.*, 1997). Hohnle *et al.* (2001) have shown that begomovirus *Abutilon* mosaic virus CP has a minimal transmission domain followed by a region for efficient transmission by *B. tabaci*. These results suggest that the vector specificity determinants reside in the CP. Expression of a mutated non-functional CP for vector interaction could therefore potentially impede the virus spread amongst its vectors in geminivirus-infected fields.

The geminivirus Rep protein regulates transcription from the bidirectional promoter of geminivirus DNA A and mediates origin recognition and DNA cleavage/ligation to begin and end rolling circle replication (Fontes *et al.*, 1994; Heyraud-Nitschke *et al.*, 1995). It can form complexes with itself and other proteins. Rep is a key regulator in the interaction with plant cell cycle regulatory factors (Kong *et al.*, 2000; Kong and Hanley-Bowdoin, 2002). The interaction between geminivirus Rep and host retinoblastoma-related protein (pRBR) is involved in the modulation of components of the pRBR/E2F transcription regulatory network. It can eventually induce quiescent plant cells to regain the capacity to support viral DNA replication (Hanley-Bowdoin *et al.*, 2004). Rep also interacts with other cellular proteins (Morilla *et al.*, 2006) and with the geminivirus REn protein (Settlage *et al.*, 2005), which induces the cellular genes required for geminivirus DNA accumulation (Selth *et al.*, 2005). These crucial functions in the replication cycle and its multiple interactions make Rep an excellent target for interference by the expression of mutant proteins. As at least some of these functions are virus non-specific, the targeting of Rep may also provide broader resistance against different geminiviruses. Indeed, virus replication was repressed in *N. benthamiana* protoplasts expressing N-terminally truncated Rep (T-Rep) (Hong and Stanley, 1995; Brunetti *et al.*, 2001), and T-Rep transgenic plants showed a certain level of resistance (Hong and Stanley, 1996; Noris *et al.*, 1996). T-Rep expression in tomato plants also conferred resistance to the homologous virus by tightly repressing the viral Rep promoter, whereas it affected a heterologous geminivirus by the formation of dysfunctional complexes with the Rep of the heterologous virus (Lucioli *et al.*, 2003). Similar observations have been reported for the expression of the N-terminal region of tomato leaf curl New Delhi virus, encompassing the DNA binding and oligomerization domain, in *N. benthamiana* plants and protoplasts. This led to a decrease of more than 70% in DNA accumulation of the homologous virus, but also afforded a 20–50% decrease with heterologous ACMV, pepper huasteco yellow vein virus and potato yellow mosaic virus (Chatterji *et al.*, 2001). Resistance under field conditions was observed with tomatoes expressing parts of the Rep gene with the intergenic region of TYLCV (Yang *et al.*, 2004a), but, in this case, expression of the Rep protein was not necessary for resistance. It is believed that the double-stranded RNA (dsRNA) hairpin loops transcribed from the inverted repeats of the intergenic region trigger post-transcriptional gene silencing (PTGS), a gene silencing mechanism presented later in this article.

As mentioned above, a potential drawback of Rep expression in transgenic plants could be the recovery of phenotypically normal plants, because its interaction with pRBR or other plant proteins may alter the cell cycle and differentiation programmes (Kong *et al.*, 2000; Shen, 2002). To overcome this problem, a tomato golden mosaic virus (TGMV) Rep mutant that reduces viral replication by more than 95% and pRBR binding activity by up to 75% has been identified (Kong *et al.*, 2000; Arguello-Astorga *et al.*, 2004). As geminivirus REn protein can interact with Rep protein and plant factors through the formation of multimeric complexes (Selth *et al.*, 2005; Settlage *et al.*, 2005), expression of its mutated form in transgenic plants may also confer resistance to the

geminivirus. Thus, we suggest that strong and stable geminivirus resistance could be achieved by developing a dual strategy that involves the co-expression of interfering mutated Rep with reduced pRBR binding activity and REn mutants.

Viral TrAP proteins play a role in host gene activation and gene silencing suppression (Hanley-Bowdoin *et al*., 1999; Voinnet *et al*., 1999; Vanitharani *et al*., 2004; Trinks *et al*., 2005). Similarly, geminivirus AC4 protein of Sri Lankan CMV (whose coding sequence lies within the Rep sequence on DNA A) also has silencing suppressor activity (Vanitharani *et al*., 2004). Expression of TGMV and beet curly top virus (BCTV) TrAP proteins increased the susceptibility to these geminiviruses in *N. benthamiana* and tobacco plants (Sunter *et al*., 2001; Hao *et al*., 2003). Expression of the ACMV AC4 caused developmental abnormalities in *Arabidopsis* (Chellappan *et al*., 2005), which were similar to those observed after the expression of an RNA virus silencing suppressor, most probably caused by interference with the miRNA pathway (Kasschau *et al*., 2003). Interference with the viral mechanisms controlling host defence reactions may provide a useful strategy for virus resistance, but further studies of TrAP and AC4 functions and their active domains are required to develop mutation-based strategies in order to avoid interference with host plant development.

The B component of bipartite geminiviruses encodes two proteins, NSP and MP, which are required for cell-to-cell and long-distance movement, respectively (Noueiry *et al*., 1994). Partially defective or incompatible MPs have been shown to disrupt the systemic spread of RNA viruses (Heinlein, 2002). In geminiviruses, TGMV MP had a deleterious effect on systemic infection of ACMV DNA A in *N. benthamiana* plants (Von Arnim and Stanley, 1992). Tobacco plants expressing a mutated version of TMoV MP also showed resistance to TMoV and cabbage leaf curl geminivirus (CaLCuV) infection (Duan *et al*., 1997). Redirection of squash leaf curl geminivirus (SqLCV) NSP from the nucleus to the cell periphery by MP has been demonstrated in protoplasts and yeast (Sanderfoot and Lazarowitz, 1995, 1996; Sanderfoot *et al*., 1996; Hehnle *et al*., 2004), and the physical interaction has been extended to viral DNA by electron microscopy studies (Hehnle *et al*., 2004). Non-functional MPs may compete for NSP interaction or oligomerization (Frischmuth *et al*., 2004), and this could explain the resistance previously observed in mutated MP-expressing plants. Although the B DNA component of the bipartite geminiviruses is not required for virus replication, strategies based on B component proteins may still have some effect on the pool size of viral DNA produced in the host by freezing the mobilization of geminiviruses.

Expression of non-viral proteins with antiviral effects

Plants often resist viruses by mounting a hypersensitive reaction to induce cell death at the initial site of infection. To engineer virus-inducible cell death mechanisms to confine ACMV to the primary infection site, attempts have been made to express cell death-inducing agents controlled by the ACMV DNA A virion strand promoter, which is transactivated by ACMV TrAP on infection (Figure 1; Haley *et al*., 1992; Hong *et al*., 1997). Expression of dianthin, a ribosome-inactivating protein, under this promoter in transgenic *N. benthamiana* plants reduced the susceptibility to infection by ACMV isolates originating from widely separated locations (Hong *et al*., 1996). As homologous and heterologous TrAP proteins (TGMV, ACMV, Texas pepper geminivirus and SqLCV) are able to activate virion strand expression of the TGMV promoter (Sunter *et al*., 1994), this strategy may confer resistance to a broad spectrum of geminiviruses. However, this approach would only be of agronomic usefulness if residual transgene expression in the absence of infection did not cause any detrimental effects on plant performance. To counteract the unwanted activities of cell death-inducing agents in the absence of infection, we have taken advantage of further regulatory properties of ACMV proteins on the DNA A promoter, which is not only activated by about 15-fold by the TrAP protein (AC2) in the virion sense direction, but is also repressed two- to fivefold by the Rep protein (AC1) in the complementary strand orientation (Frey *et al*., 2001). The barnase and barstar coding sequences from *Bacillus amyloliquefacien* were cloned under the control of the sense and complementary promoters from ACMV, respectively (Zhang *et al*., 2003). Theoretically, in the absence of geminivirus infection, the barnase and barstar transgenes should be expressed at similar levels and no active RNase is produced. During infection, the Rep (AC1) and TrAP (AC2) proteins increase the barnase/barstar ratio, leading to the production of active RNase, local cell death and, finally, inhibition of virus spread. The required precision of promoter regulation is difficult to achieve in transgenic cassava, but may be possible in other plants, for which larger numbers of transformants can be generated. As an alternative, virus-induced host promoters, such as the AC2-induced promoters in *Arabidopsis* (Trinks *et al*., 2005), may be used to engineer virus-induced hypersensitivity, provided that they have a very low basal expression during plant development.

The expression of antibodies against viral proteins, such as the RNA-dependent RNA polymerase (RdRP) or CP, has provided resistance to *Nicotiana* plants against RNA viruses (Zimmermann *et al*., 1998; Boonrod *et al*., 2004). Antibodies

against geminivirus viral proteins may be efficient factors for the impairment of key functions of these proteins when they target their active sites (Figure 1). The respective epitopes are highly conserved between virus families, and therefore this may be a promising technology for broad-spectrum resistance. For instance, antibodies directed against active sites of geminivirus Rep protein could interfere with the functions of Rep, which generally acts at low concentration for viral DNA replication and transcription (Hanley-Bowdoin *et al*., 1999). Another highly conserved sequence motif is a 38-amino-acid region of NSP, which is involved in the interaction between CaLCuV NSP and an *Arabidopsis* nuclear acetyltransferase (AtNSI). Inhibition of AtNSI activity by NSP interaction contributes greatly to virus infection and pathogenicity (Carvalho and Lazarowitz, 2004; Carvalho *et al*., 2006). Expression of a single-chain antibody targeting the 38-amino-acid conserved region of NSP should impede this interaction, required for the nuclear export of the viral ssDNA–CP complex, leading to broad-spectrum geminivirus resistance.

The replication of geminiviruses requires controlled DNA–protein interactions in the small intergenic region. The sequence-specific dsDNA binding activities of geminivirus Rep have a role in origin recognition and transcriptional repression, whereas the ssDNA binding activity of Rep is involved in DNA cleavage (Hanley-Bowdoin *et al*., 1999). Sera and Uranga (2002) have produced artificial zinc finger proteins (AZPs) with a high affinity and selectivity for the Rep dsDNA binding site in the viral replication origins of TGMV and beet severe curly top virus (BSCTV). Expression of a six-finger AZP with a nuclear localization signal (NLS) under the control of a *Cestrum* yellow leaf curling virus promoter in *Arabidopsis thaliana* also produced transgenic lines with reduced or no replication of BSCTV (Sera, 2005). This approach is likely to generate stable resistance as the virus needs to evade the AZP effect by simultaneously mutating both the Rep sequence and the origin of replication.

All other known interactions of DNA with geminiviral proteins, such as TrAP/C2, CP, MP and NSP, are sequence nonspecific (Sung and Coutts, 1996; Rojas *et al*., 1998; Kirthi and Savithri, 2003; Van Wezel *et al*., 2003; Hehnle *et al*., 2004), and thus may not provide specific targets for the application of AZPs. However, non-specific DNA binding activities may also be used for resistance engineering. Padidam *et al*. (1999) reported that the expression of the *Escherichia coli* G5 protein, which binds to ssDNA, competed with CP and affected geminivirus movement. Moreover, the expression of proteins with non-specific DNA binding activity will not have deleterious effects on host plant development.

Several proteins involved in the RNA silencing pathway have important roles in protecting plants against viruses (Lecellier and Voinnet, 2004). Their characterization is now allowing virologists to interpret the susceptibility to viruses of certain cultivars by the absence and/or deficiency of certain key components of the RNA silencing pathway. The expression of endogenous RdRP genes in many plants is greatly stimulated during virus infection. In *N. tabacum*, a virus-inducible host RdRP gene has been identified, which displays anti-RNA virus function. This RdRP is also induced by salicylic acid (SA), which stimulates generalized antiviral and antimicrobial defence responses (Xie *et al*., 2001). The extreme susceptibility of *N. benthamiana* to viruses has been associated with a lack of an active SA- and virus-induced RdRP. Expression of an RdRP orthologue from *Medicago trunculata* restored resistance to tobamoviruses in *N. benthamiana* (Yang *et al*., 2004b). Geminiviruses have different replication intermediates from RNA viruses, and it is unclear what kind of role plant endogenous RdRPs may play in the antiviral defence system, and how they interact with their targeted sequences. Increased expression of an RdRP (*SGS2/SDE1*) in *Arabidopsis* resulted in reduced viral symptoms on CaLCuV infection (Muangsan *et al*., 2004). Investigation of the related orthologues for the virus response and silencing pathways in geminivirus-susceptible species may provide an interesting tool for understanding the variable levels of virus susceptibility observed amongst different cultivars.

DNA interference

ACMV-infected plants contain, in addition to the two genomic DNAs, small amounts of DNA of approximately twice and half the genomic DNA length (Figure 1; Stanley and Townsend, 1985). The function of these DNA forms is still unclear, but ACMV multiplication in *N. benthamiana* host plants has been negatively correlated to the concentration of so-called defective interfering (DI) DNA B (half of the DNA B size). *N. benthamiana* plants transformed with a tandem repeat of subgenomic defective ACMV DNA B showed reduced symptoms compared with untransformed plants on ACMV infection (Stanley *et al*., 1990). This phenomenon was virus specific because no resistance phenotype could be observed when the transgenic plants were challenged with other geminiviruses (BCTV and TGMV). This specificity was further confirmed in transgenic *N. benthamiana* plants with a DI-based BCTV (Logan strain) resistance, which were not resistant to other strains (Stenger, 1994). However, no other investigation of the mechanism of such interference, or of the DI molecules associated with the different cassava

geminivirus species, has been reported. Whether the integration of several DI sequences isolated from the different cassava geminiviruses in cassava could protect against the infection by these viruses is still unknown. The function of other subviral agents of geminiviruses (reviewed by Briddon and Stanley, 2006) during infection has not yet been elucidated. Integration of *Ageratum* yellow vein disease (AYVD) DNA-β into the *N. benthamiana* genome produced severe developmental abnormalities in transgenic plants (Saunders *et al*., 2004). This may be a result of open reading frames (ORFs) in the subviral agent coding for viral proteins, such as βC1 from TYLCV DNA-β, which acts as a silencing suppressor (Cui *et al*., 2005). Nevertheless, their potential negative effect on virus accumulation may provide a novel strategy for most geminivirus diseases, provided that they do not trigger deleterious effects when integrated in the host genome. The appropriate mutagenesis of the subviral components could expand the interference technology to all geminivirus species whose replication level is affected by subviral components.

Increased resistance by RNA interference

RNA interference (RNAi) in eukaryotes is a sequence-specific gene silencing mechanism that may have evolved initially as a defence against viruses and transposons through transcriptional gene silencing (TGS) and PTGS. RNAi was inadvertently evoked in many of the previous successful attempts to engineer plant virus resistance by introducing virus-derived sequences into plants. As the mechanism of RNAi is now better understood, it has been developed into a widely used technique for studying gene function and for engineering virus resistance (Tenllado *et al*., 2004). RNAi is triggered by the expression of 'artificial' dsRNAs homologous to viral sequences, which can be most easily achieved by fusion of the respective regions in sense and anti-sense orientation within one transcript (Figure 1). Several vectors have been developed for the efficient expression and processing of such hairpin dsRNA in plants (Wesley *et al*., 2001; Miki and Shimamoto, 2004). Linking the sense and anti-sense sequences by an intron, which is eventually spliced, resulted in the most efficient silencing in plants (Smith *et al*., 2000; Wesley *et al*., 2001). The dsRNA region is processed into small interfering RNAs (siRNAs), which guide silencing complexes to target regions on RNA or DNA. In plants, the predominant action of silencing complexes on RNA seems to be cleavage (Meister and Tuschl, 2004), leading to PTGS, whereas targeted DNA regions often become methylated (Mette *et al*., 2000; Sijen *et al*., 2001; Cao *et al*., 2003; Zilberman *et al*., 2003) and transcriptionally silenced (TGS). RNA viruses can only be affected by PTGS, whereas, for geminiviruses, both silencing mechanisms may be applicable, as reviewed by Vanitharani *et al*. (2005).

Targeting viral promoters with dsRNAs

TGS of a transgene can be induced by infection with a virus carrying a sequence homologous to the promoter of the transgene. Silencing can be mediated by cauliflower mosaic virus, a pararetrovirus with a DNA and an RNA phase (Al-Kaff *et al*., 2000; Covey and Al-Kaff, 2000), and by potato virus X, an RNA virus (Jones *et al*., 1999). In both cases, TGS was correlated with increased methylation of the transgene promoter in the infected plants (Jones *et al*., 1999; Al-Kaff *et al*., 2000). Similarly, methylation of a tomato leaf curl virus (TLCV)-derived transgene promoter and consequent transgene silencing have been observed on TLCV infection (Seemanpillai *et al*., 2003), strongly suggesting that virus-derived siRNAs are also generated during geminivirus infection. Such siRNAs have been detected for transcribed regions of the genome (see below), but also for the theoretically non-transcribed intergenic region, which harbours the viral promoters and replication origin (Akbergenov *et al*., 2006). Double small RNA-directed methylation of geminivirus bidirectional promoters may down-regulate the transcription of viral genes, resulting in inefficient virus replication. Recovery of *Vigna mungo* yellow mosaic virus-infected *Vigna mungo* plants has indeed been reported after bombardment with DNA constructs expressing dsRNAs homologous to the bidirectional viral promoter (Pooggin *et al*., 2003). We have also observed that the replication of ACMV DNA A can be impaired in leaf discs from transgenic cassava plants expressing dsRNA homologous to the DNA A promoter (H. Vanderschuren *et al*., unpublished data). Triggering TGS of geminivius promoters by pre-expression or induced expression of specific dsRNAs may therefore constitute a promising strategy to interfere with virus replication. As a result of the low level of homology of the promoter sequences between different species of geminivirus, it is expected that this type of resistance will be strain specific.

dsRNAs homologous to viral coding sequences

Vectors for virus-induced gene silencing (VIGS), i.e. silencing of an endogenous gene following viral infection with a virus carrying the targeted sequence, were first developed with RNA viruses (Kumagai *et al*., 1995; Ruiz *et al*., 1998). VIGS is a manifestation of an RNA-mediated defence mechanism against virus infection that is related to PTGS. VIGS vectors have also been developed with the geminiviruses TGMV, CaLCuV and ACMV (Kjemtrup *et al*., 1998; Turnage *et al*.,

2002; Fofana *et al*., 2004), indicating that silencing is also triggered by DNA viruses. siRNA accumulation in tomato plants infected with the monopartite geminivirus TYLCV (Lucioli *et al*., 2003) and in cassava plants infected with the bipartite geminivirus ACMV (Chellappan *et al*., 2004b) reflects the role of the RNAi pathway as a natural defence mechanism against these DNA viruses. In cassava, the appearance of siRNA against a region within the Rep coding sequence was correlated with the recovery of infected plants from infection (Chellappan *et al*., 2004b). These findings, together with the emerging roles of geminiviral proteins as modulators of the plant gene silencing machinery, strongly suggest that the effectiveness of the plant RNAi response is a major determinant of viral proliferation. Manipulation of this balance by pre-expression of dsRNAs or by interference with the viral counteraction should constitute a promising approach to increase plant resistance.

Any hairpin dsRNAs homologous to viral coding sequences may enter both known RNAi pathways (Baulcombe, 2004). On the one hand, they may act in TGS complexes as sequence-specific mediators for the methylation of homologous viral DNA sequences in the nucleus. On the other hand, they may serve as mediators for sequence-specific PTGS, i.e. degradation of viral transcripts and/or inhibition of translation. As described above for the intergenic region, siRNA-directed methylation may also affect coding regions and thereby cause reduced transcription. Methylation can spread from the primary dsRNA-targeted genomic DNA sequence in the 5′ and 3′ directions (Vaistij *et al*., 2002; Van Houdt *et al*., 2003). Therefore, any initial effect of a specific hairpin dsRNA may extend further to non-targeted sequences.

The transcription of methylated coding sequences may be reduced but is usually not completely abolished. PTGS can provide more efficient interference because target RNAs will be largely degraded. Therefore, it is believed that an RNAi approach against geminivirus coding sequences should work as efficiently as against RNA viruses. The RNAi strategy has no limitation in terms of targeted sequences. As the Rep gene is strictly required for replication (Hanley-Bowdoin *et al*., 1999), it has been considered the most promising RNAi target. The correlation between the natural accumulation of siRNAs homologous to the Rep C-terminal sequence and the recovery of ACMV-infected plants (Chellappan *et al*., 2004b) suggests that resistance may be achieved by interfering with the 3′ end of the Rep sequence. This region, which overlaps TrAP, provides double targeting to the two crucial viral genes by PTGS (Haley *et al*., 1992). Vanitharani *et al*. (2003) observed a strong decrease in Rep mRNA accumulation and reduced viral replication in tobacco BY2 protoplasts transiently expressing the siRNAs homologous to ACMV *Rep*. In addition, the accumulated siRNAs in cassava plants recovering from infection by ACMV-CM were derived from this genomic region (Chellappan *et al*., 2004b). As it is a highly conserved region, targeting this specific region may offer broad-spectrum resistance. For example, the highest homology of Rep sequences between ACMV and EACMV lies in this region, and an siRNA correlated resistance, accidentally induced by an ACMV *Rep* transgene, was found to be active against both ACMV and EACMV (Chellappan *et al*., 2004a).

This approach can certainly be expanded to any viral coding sequences, provided that the decrease in viral mRNA has a sufficient effect on the virus life cycle. Fagoaga *et al*. (2006) have engineered *Citrus tristeza* virus (ssRNA virus) resistance based on the PTGS of p23, a viral silencing suppressor. As mentioned above, several geminivirus proteins with silencing suppressor activities have been identified, and should be considered as potential candidates for RNAi-based resistance.

Viral silencing suppressors also regulate the level and activity of some miRNAs in plants (Kasschau *et al*., 2003; Chen *et al*., 2004; Chellappan *et al*., 2005). It is unclear so far, however, whether this is an accidental by-product of interference with the gene silencing machinery or whether plant viruses target miRNAs, which could be a natural response to virus infection. Such a mechanism has recently been reported for Epstein–Barr virus, a human DNA virus that influences the miRNA pathways to regulate host and viral genes in response to infection (Pfeffer *et al*., 2004). Exploring the miRNA pathway via the expression of miRNA precursors homologous to viral sequences may also offer possibilities to repress geminivirus gene expression in plants.

So far, RNAi against geminivirus infections has been accidentally induced in transgenic plants engineered for protein expression, and the activity of specifically designed siRNA expression constructs has been tested in transient systems (Pooggin *et al*., 2003). The characterization of short RNAs produced by natural infection and transgene expression (Akbergenov *et al*., 2006) should provide key information to optimize RNAi approaches. Moreover, characterization of the native genes required to trigger and maintain silencing processes on DNA virus infection in the geminivirus model system CaLCuV-infected *Arabidopsis* (Muangsan *et al*., 2004) will provide new opportunities for engineering improved geminivirus resistance.

Anti-sense RNA

The suppression of gene expression by anti-sense RNA (asRNA) sequences was used before the discovery of the

mechanisms of gene silencing. Day *et al*. (1991) successfully used asRNA technology to engineer geminivirus resistance in tobacco plants. TGMV replication was reduced in transgenic plants expressing a *Rep* asRNA sequence, and one transgenic line showed more than 90% symptomless plants after infection. Expression of an asRNA sequence covering the entire TGMV *Rep*, *TrAP* and *REn* also repressed BCTV replication in tobacco (Bejarano and Lichtenstein, 1994), but failed to control the less related ACMV. *Rep* asRNA-mediated resistance was also engineered against the monopartite TYLCV in *N. benthamiana* (Bendahmane and Gronenborn, 1997) and tomato (Yang *et al*., 2004a). We have successfully produced transgenic cassava plants resistant to ACMV infection by expressing ACMV genes of *Rep*, *TrAP* and *REn* in anti-sense orientation. By linking the viral asRNA to the selectable marker RNA, we have avoided the production of transgenic plants in which the asRNA gene is post-transcriptionally silenced. Both *in vitro* transient assays and infection experiments confirmed the reduction of ACMV replication and the increased resistance to ACMV infection. Our results demonstrate that resistance to ACMV infection of cassava can be achieved with high efficacy by expressing asRNAs against viral mRNAs encoding essential non-structural proteins (Zhang *et al*., 2005). Asad *et al*. (2003) achieved cotton leaf curl virus resistance in tobacco with a similar anti-sense approach against *Rep*, *REn* and *TrAP*. It is still unclear whether or how asRNA molecules enter the RNAi pathway to contribute to geminivirus resistance in transgenic plants. We found no siRNAs in asRNA transgenic cassava plants prior to infection, suggesting that resistance is achieved by sense–anti-sense interactions after infection and not by the constitutive production of siRNAs from the transgene. The anti-sense approach may therefore be a better option than the expression of hairpin dsRNA, which may be more prone to develop transgene silencing (TGS) over generations, because the short RNAs generated from the hairpin transcript (or from other types of transgenes) can direct site-specific transgene methylation (Aufsatz *et al*., 2002; Zilberman *et al*., 2004). TGS may become more severe with increasing numbers of cell cycles (Fojtova *et al*., 2003) and lead to the inactivation of the transgene and subsequent loss of protection against virus infection. The dynamics of siRNA production are also very likely to play a key role in virus resistance. Further analysis of asRNA-mediated geminivirus resistance would certainly provide useful information to optimize the RNAi approach by entering the siRNA pathway at different points and by providing better control of siRNA production dynamics.

The hairpin dsRNA and asRNA technologies offer the possibility to engineer easily multitarget-based resistance. Indeed, scattered regions of the viral genome can be targeted by plants expressing fused viral asRNA or hairpin dsRNA sequences. Considering that geminivirus DNA A is highly recombinogenic (Briddon and Stanley, 2006), a multitarget approach against coding sequences of both sense strand and virion strand is more likely to produce stable and durable virus resistance.

Conclusion

Multiple approaches to the engineering of resistance to geminiviruses have been exploited in plants (Figure 1), in most cases using model plant systems. If such technologies can be applicable to crops, they will certainly bring significant benefit to crop breeders and producers. The elevated level of resistance to geminivirus diseases in crop plants must be durable under agronomic growth conditions. Indeed, the extrapolation of these results to other species may be difficult with regard to the resistance level and its stability in host species. In most cases, transgenic plants have been tested for virus resistance under glasshouse conditions and over one or two generations. The engineered virus resistance may appear variable under natural field conditions and agricultural practices over several generations. Therefore, more extensive field tests and resistance stability evaluations are required before improved lines can be released for cultivation.

In addition to pathogen-derived resistance in transgenic plants, the enhancement of native antiviral mechanisms may allow the engineering of virus resistance without affecting plant development. As discussed above, constitutive expression of mutated geminivirus proteins often produces phenotypical abnormalities in transgenic plants. Therefore, the mutation-based approach needs to consider how to minimize the potential interactions between the mutated viral protein and host proteins. In this context, mutant candidates that compete for viral replication or transmission without the ability to interfere with host factors are our first choice. Further characterization of the key domains in these viral proteins will provide more reliable information for the development of novel antiviral strategies.

The availability of transformation techniques in many major crops (e.g. tomato, bean, cotton and cassava) and the strategic development of geminivirus resistance will allow us to employ different strategies to increase geminivirus resistance in these crops within a reasonable time. For example, transgenic cassava lines resistant to CMG infection have been reported in two laboratories using different resistance mechanisms (Chellappan *et al*., 2004a; Zhang *et al*., 2005). Resistance has been demonstrated in these transgenic plants under

glasshouse conditions and needs to be confirmed under natural infection pressures.

From a technical point of view, it is also important to focus on an understanding of the mechanisms on which engineered resistance is based. This information will allow efficient and reproducible transfer of resistance from model plants to crop plants. In addition, a better evaluation of engineered resistance can be designed via appropriate measurements, such as short RNA load in PTGS-based resistance. The recent loss of virus resistance observed in transgenic cassava lines (Donald Danforth Plant Science Center, 2006) advocates for further studies and for the characterization of virus resistance mechanisms. Eventually, this knowledge will also contribute to a better evaluation of the resistance sustainability and stability.

Genetic engineering remains an alternative and rapid method to transfer resistance genes to traditional cultivars, bypassing the long procedure of introgression and the appearance of undesired traits usually associated with it. When traditional breeding can offer cultivars with a medium level of resistance, genetic engineering could provide additional tools to implement virus resistance. In this respect, genetic engineering and traditional breeding are complementary. Development of the genetic engineering tool and ongoing studies on geminiviruses will help to achieve stable resistance in geminivirus-susceptible crops.

References

Abel, P.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T. and Beachy, R.N. (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science*, **232**, 738–743.

Akbergenov, R., Si-Ammour, A., Blevins, T., Amin, I., Kutter, C., Vanderschuren, H., Zhang, P., Gruissem, W., Meins, F. Jr, Hohn, T. and Pooggin, M.M. (2006) Molecular characterization of geminivirus-derived small RNAs in different plant species. *Nucleic Acids Res*. **34**, 462–471.

Al-Kaff, N.S., Kreike, M.M., Covey, S.N., Pitcher, R., Page, A.M. and Dale, P.J. (2000) Plants rendered herbicide-susceptible by cauliflower mosaic virus-elicited suppression of a 35S promoter-regulated transgene. *Nat. Biotechnol*. **18**, 995–999.

Arguello-Astorga, G., Lopez-Ochoa, L., Kong, L.J., Orozco, B.M., Settlage, S.B. and Hanley-Bowdoin, L. (2004) A novel motif in geminivirus replication proteins interacts with the plant retinoblastoma-related protein. *J. Virol*. **78**, 4817–4826.

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

Aufsatz, W., Mette, M.F., van der Winden, J., Matzke, A.J. and Matzke, M. (2002) RNA-directed DNA methylation in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA*, **99**, 16 499–16 506.

Azzam, O., Frazer, J., de la Rosa, D., Beaver, J.S., Ahlquist, P. and Maxwell, D.P. (1994) Whitefly transmission and efficient ssDNA accumulation of bean golden mosaic geminivirus require functional coat protein. *Virology*, **204**, 289–296.

Azzam, O., Diaz, O., Beaver, J.S., Gilbertson, R.L., Russell, D.R. and Maxwell, D.P. (1996) Transgenic beans with the bean golden mosaic geminivirus coat protein gene are susceptible to virus infection. *Ann. Rep. Bean Improv. Cooperat*. **39**, 276–277.

Baulcombe, D. (1996) Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell*, **8**, 1833–1844.

Baulcombe, D. (2004) RNA silencing in plants. *Nature*, **431**, 356–363.

Beachy, R.N. (1999) Coat-protein-mediated resistance to tobacco mosaic virus: discovery mechanisms and exploitation. *Philos. Trans. R. Soc. B: Biol. Sci*. **354**, 659–664.

Bejarano, E.R. and Lichtenstein, C.P. (1994) Expression of TGMV antisense RNA in transgenic tobacco inhibits replication of BCTV but not ACMV geminiviruses. *Plant Mol. Biol*. **24**, 241–248.

Bendahmane, M. and Gronenborn, B. (1997) Engineering resistance against tomato yellow leaf curl virus (TYLCV) using antisense RNA. *Plant Mol. Biol*. **33**, 351–357.

Blair, M.W., Bassett, M.J., Abouzid, A.M., Hiebert, E., Polston, J.E., Mcmillan, R.T., Graves, W. and Lamberts, M. (1995) Occurrence of bean golden mosaic-virus in Florida. *Plant Dis*. **79**, 529–533.

Boonrod, K., Galetzka, D., Nagy, P.D., Conrad, U. and Krczal, G. (2004) Single-chain antibodies against a plant viral RNA-dependent RNA polymerase confer virus resistance. *Nat. Biotechnol*. **22**, 856–862.

Bosque-Perez, N.A. (2000) Eight decades of maize streak virus research. *Virus Res*. **71**, 107–121.

Boulton, M.I. (2003) Geminiviruses: major threats to world agriculture. *Ann. Appl. Biol*. **142**, 143.

Briddon, R.W. and Markham, P.G. (2000) Cotton leaf curl virus disease. *Virus Res*. **71**, 151–159.

Briddon, R.W. and Markham, P.G. (2001) Complementation of bipartite begomovirus movement functions by topocuviruses and curtoviruses. *Arch. Virol*. **146**, 1811–1819.

Briddon, R.W. and Stanley, J. (2006) Subviral agents associated with plant single-stranded DNA viruses. *Virology*, **344**, 198–210.

Briddon, R.W., Watts, J., Markham, P.G. and Stanley, J. (1989) The coat protein of beet curly top virus is essential for infectivity. *Virology*, **172**, 628–363.

Briddon, R.W., Pinner, M.S., Stanley, J. and Markham, P.G. (1990) Geminivirus coat protein gene replacement alters insect specificity. *Virology*, **177**, 85–94.

Brunetti, A., Tavazza, R., Noris, E., Lucioli, A., Accotto, G.P. and Tavazza, M. (2001) Transgenically expressed T-Rep of tomato yellow leaf curl Sardinia virus acts as a trans-dominant-negative mutant, inhibiting viral transcription and replication. *J. Virol*. **75**, 10 573–10 581.

Cao, X., Aufsatz, W., Zilberman, D., Mette, M.F., Huang, M.S., Matzke, M. and Jacobsen, S.E. (2003) Role of the DRM and CMT3 methyltransferases in RNA-directed DNA methylation. *Curr. Biol*. **13**, 2212–2217.

Carvalho, M.F. and Lazarowitz, S.G. (2004) Interaction of the movement protein NSP and the *Arabidopsis* acetyltransferase AtNSI is necessary for cabbage leaf curl geminivirus infection and pathogenicity. *J. Virol*. **78**, 11 161–11 171.

Carvalho, M.F., Turgeon, R. and Lazarowitz, S.G. (2006) The geminivirus nuclear shuttle protein NSP inhibits the activity of AtNSI, a

vascular-expressed *Arabidopsis* acetyltransferase regulated with the sink-to-source transition. *Plant Physiol*. **140**, 1317–1330.

Chatterji, A., Beachy, R.N. and Fauquet, C.M. (2001) Expression of the oligomerization domain of the replication-associated protein (Rep) of tomato leaf curl New Delhi virus interferes with DNA accumulation of heterologous geminiviruses. *J. Biol. Chem*. **276**, 25 631–25 638.

Chellappan, P., Masona, M.V., Vanitharani, R., Taylor, N.J. and Fauquet, C.M. (2004a) Broad spectrum resistance to ssDNA viruses associated with transgene-induced gene silencing in cassava. *Plant Mol. Biol*. **56**, 601–611.

Chellappan, P., Vanitharani, R. and Fauquet, C.M. (2004a) Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *J. Virol*. **78**, 7465–7477.

Chellappan, P., Vanitharani, R. and Fauquet, C.M. (2005) MicroRNA-binding viral protein interferes with *Arabidopsis* development. *Proc. Natl. Acad. Sci. USA*, **102**, 10 381–10 386.

Chen, J., Li, W.X., Xie, D., Peng, J.R. and Ding, S.W. (2004) Viral virulence protein suppresses RNA silencing-mediated defense but upregulates the role of microRNA in host gene expression. *Plant Cell*, **16**, 1302–1313.

Covey, S.N. and Al-Kaff, N.S. (2000) Plant DNA viruses and gene silencing. *Plant Mol. Biol*. **43**, 307–322.

Cui, X., Li, G., Wang, D., Hu, D. and Zhou, X. (2005) A begomovirus DNA beta-encoded protein binds DNA, functions as a suppressor of RNA silencing, and targets the cell nucleus. *J. Virol*. **79**, 10 764–10 775.

Day, A.G., Bejarano, E.R., Buck, K.W., Burrell, M. and Lichtenstein, C.P. (1991) Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus. *Proc. Natl. Acad. Sci. USA*, **88**, 6721–6725.

Deng, D., Otim-Nape, W.G., Sangare, A., Ogwal, S., Beachy, R.N. and Fauquet, C.M. (1997) Presence of a new virus closely related to East African cassava mosaic geminivirus. *Afr. J. Root Tuber Crops*, **2**, 23–38.

Donald Danforth Plant Science Center (2006) *Danforth Center Cassava Update 5-26-2006. Press Release 2006.* URL http://www.danforthcenter.org/newsmedia/NewsDetail.asp?nid=119 [accessed on 26 May 2006].

Duan, Y.P., Powell, C.A., Webb, S.E., Purcifull, D.E. and Hiebert, E. (1997) Geminivirus resistance in transgenic tobacco expressing mutated BC1 protein. *Mol. Plant Microb. Interact*. **10**, 617–623.

Fagoaga, C., Lopez, C., de Mendoza, A.H., Moreno, P., Navarro, L., Flores, R. and Pena, L. (2006) Post-transcriptional gene silencing of the p23 silencing suppressor of citrus tristeza virus confers resistance to the virus in transgenic Mexican lime. *Plant Mol. Biol*. **60**, 153–165.

Faria, J.C. and Maxwell, D.P. (1999) Variability in geminivirus isolates associated with Phaseolus spp in Brazil. *Phytopathology*, **89**, 262–268.

Fauquet, C.M. and Stanley, J. (2003) Geminivirus classification and nomenclature: progress and problems. *Ann. Appl. Biol*. **25**, 165–189.

Fofana, I.B., Sangare, A., Collier, R., Taylor, C. and Fauquet, C.M. (2004) A geminivirus-induced gene silencing system for gene function validation in cassava. *Plant Mol. Biol*. **56**, 613–624.

Fojtova, M., Van Houdt, H., Depicker, A. and Kovarik, A. (2003) Epigenetic switch from posttranscriptional to transcriptional silencing is correlated with promoter hypermethylation. *Plant Physiol*. **133**, 1240–1250.

Fontes, E.P., Gladfelter, H.J., Schaffer, R.L., Petty, I.T. and Hanley-Bowdoin, L. (1994) Geminivirus replication origins have a modular organization. *Plant Cell*, **6**, 405–416.

Frey, P.M., Scharer-Hernandez, N.G., Futterer, J., Potrykus, I. and Puonti-Kaerlas, J. (2001) Simultaneous analysis of the bidirectional African cassava mosaic virus promoter activity using two different luciferase genes. *Virus Genes*, **22**, 231–242.

Frischmuth, S. and Stanley, J. (1998) Recombination between viral DNA and the transgenic coat protein gene of African cassava mosaic geminivirus. *J. Gen. Virol*. **79**, 1265–1271.

Frischmuth, S., Kleinow, T., Aberle, H.J., Wege, C., Hulser, D. and Jeske, H. (2004) Yeast two-hybrid systems confirm the membrane association and oligomerization of BC1 but do not detect an interaction of the movement proteins BC1 and BV1 of Abutilon mosaic geminivirus. *Arch. Virol*. **149**, 2349–2364.

Gutierrez, C. (2000) DNA replication and cell cycle in plants: learning from geminiviruses. *EMBO J*. **19**, 792–799.

Gutierrez, C., Ramirez-Parra, E., Mar Castellano, M., Sanz-Burgos, A.P., Luque, A. and Missich, R. (2004) Geminivirus DNA replication and cell cycle interactions. *Vet. Microbiol*. **98**, 111–119.

Haley, A., Zhan, X., Richardson, K., Head, K. and Morris, B. (1992) Regulation of the activities of African cassava mosaic virus promoters by the AC1, AC2, and AC3 gene products. *Virology*, **188**, 905–909.

Hanley-Bowdoin, L., Settlage, S.B., Orozco, B.M., Nagar, S. and Robertson, D. (1999) Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. *Crit. Rev. Plant Sci*. **18**, 71–106.

Hanley-Bowdoin, L., Settlage, S.B. and Robertson, D. (2004) Reprogramming plant gene expression: a prerequisite to geminivirus DNA replication. *Mol. Plant Pathol*. **5**, 149–156.

Hao, L., Wang, H., Sunter, G. and Bisaro, D.M. (2003) Geminivirus AL2 and L2 proteins interact with and inactivate SNF1 kinase. *Plant Cell*, **15**, 1034–1048.

Hehnle, S., Wege, C. and Jeske, H. (2004) Interaction of DNA with the movement proteins of geminiviruses revisited. *J. Virol*. **78**, 7698–7706.

Heinlein, M. (2002) The spread of tobacco mosaic virus infection: insights into the cellular mechanism of RNA transport. *Cell. Mol. Life Sci*. **59**, 58–82.

Heyraud-Nitschke, F., Schumacher, S., Laufs, J., Schaefer, S., Schell, J. and Gronenborn, B. (1995) Determination of the origin, cleavage and joining domain of geminivirus Rep proteins. *Nucleic Acids Res*. **23**, 910–916.

Hofer, P., Bedford, I.D., Markham, P.G., Jeske, H. and Frischmuth, T. (1997) Coat protein gene replacement results in whitefly transmission of an insect nontransmissible geminivirus isolate. *Virology*, **236**, 288–295.

Hohnle, M., Hofer, P., Bedford, I.D., Briddon, R.W., Markham, P.G. and Frischmuth, T. (2001) Exchange of three amino acids in the coat protein results in efficient whitefly transmission of a nontransmissible Abutilon mosaic virus isolate. *Virology*, **290**, 164–171.

Hong, Y. and Stanley, J. (1995) Regulation of African cassava mosaic virus complementary-sense gene expression by N-terminal sequences of the replication-associated protein AC1. *J. Gen. Virol*. **76**, 2415–2422.

Hong, Y. and Stanley, J. (1996) Virus resistance in *Nicotiana benthamiana* conferred by African cassava mosaic virus replication-associated

protein (AC1) transgene. *Mol. Plant–Microbe Interact*. **9**, 219–225.

Hong, Y., Saunders, K., Hartley, M.R. and Stanley, J. (1996) Resistance to geminivirus infection by virus-induced expression of dianthin in transgenic plants. *Virology*, **220**, 119–127.

Hong, Y., Saunders, K. and Stanley, J. (1997) Transactivation of dianthin transgene expression by African cassava mosaic virus AC2. *Virology*, **228**, 383–387.

Ingham, D.J., Pascal, E. and Lazarowitz, S.G. (1995) Both bipartite geminivirus movement proteins define viral host range, but only BL1 determines viral pathogenicity. *Virology*, **207**, 191–204.

Jones, L., Hamilton, A.J., Voinnet, O., Thomas, C.L., Maule, A.J. and Baulcombe, D.C. (1999) RNA–DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell*, **11**, 2291–2301.

Kasschau, K.D., Xie, Z., Allen, E., Lave, C., Chapman, E.J., Krizan, K.A. and Carrington, J.C. (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. *Dev. Cell*, **4**, 205–217.

Kirthi, N. and Savithri, H.S. (2003) A conserved zinc finger motif in the coat protein of tomato leaf curl Bangalore virus is responsible for binding to ssDNA. *Arch. Virol*. **148**, 2369–2380.

Kjemtrup, S., Sampson, K.S., Peele, C.G., Nguyen, L.V., Conkling, M.A., Thompson, W.F. and Robertson, D. (1998) Gene silencing from plant DNA carried by a geminivirus. *Plant J*. **14**, 91–100.

Kong, L.J. and Hanley-Bowdoin, L. (2002) A geminivirus replication protein interacts with a protein kinase and a motor protein that display different expression patterns during plant development and infection. *Plant Cell*, **14**, 1817–1832.

Kong, L.J., Orozco, B.M., Roe, J.L., Nagar, S., Ou, S., Feiler, H.S., Durfee, T., Miller, A.B., Gruissem, W., Robertson, D. and Hanley-Bowdoin, L. (2000) A geminivirus replication protein interacts with the retinoblastoma protein through a novel domain to determine symptoms and tissue specificity of infection in plants. *EMBO J*. **19**, 3485–3495.

Kumagai, M.H., Donson, J., Dellacioppa, G., Harvey, D., Hanley, K. and Grill, L.K. (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proc. Natl. Acad. Sci. USA*, **92**, 1679–1683.

Kunik, T., Salomon, R., Zamir, D., Navot, N., Zeidan, M., Michelson, I., Gafni, Y. and Czosnek, H. (1994) Transgenic tomato plants expressing the tomato yellow leaf curl virus capsid protein are resistant to the virus. *Biotechnology*, **12**, 500–504.

Lapidot, M. and Friedmann, M. (2002) Breeding for resistance to whitefly-transmitted geminiviruses. *Ann. Appl. Biol*. **140**, 109–127.

Lecellier, C.H. and Voinnet, O. (2004) RNA silencing: no mercy for viruses? *Immunol. Rev*. **198**, 285–303.

Legg, J.P. and Fauquet, C.M. (2004) Cassava mosaic geminiviruses in Africa. *Plant Mol. Biol*. **56**, 585–599.

Legg, J.P. and Thresh, J.M. (2000) Cassava mosaic virus disease in East Africa: a dynamic disease in a changing environment. *Virus Res*. **71**, 135–149.

Liu, S.J., Bedford, I.D., Briddon, R.W. and Markham, P.G. (1997) Efficient whitefly transmission of African cassava mosaic geminivirus requires sequences from both genomic components. *J. Gen. Virol*. **78**, 1791–1794.

Lucioli, A., Noris, E., Brunetti, A., Tavazza, R., Ruzza, V., Castillo, A.G., Bejarano, E.R., Accotto, G.P. and Tavazza, M. (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.

Malathi, V.G., Surendranath, B., Naghma, A. and Roy, A. (2005) Adaptation to new hosts shown by the cloned components of mungbean yellow mosaic India virus causing cowpea golden mosaic in northern India. *Can. J. Plant Pathol*. **27**, 439–447.

Mansoor, S., Briddon, R.W., Zafar, Y. and Stanley, J. (2003) Geminivirus disease complexes: an emerging threat. *Trends Plant Sci*. **8**, 128–134.

Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature*, **431**, 343–349.

Mette, M.F., Aufsatz, W., van der Winden, J., Matzke, M.A. and Matzke, A.J. (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J*. **19**, 5194–5201.

Miki, D. and Shimamoto, K. (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant Cell Physiol*. **45**, 490–495.

Moffat, A.S. (1999) Geminiviruses emerge as serious crop threat. *Science*, **286**, 1835.

Morilla, G., Castillo, A.G., Preiss, W., Jeske, H. and Bejarano, E.T. (2006) A versatile transreplication-based system to identify cellular proteins involved in geminivirus replication. *J. Virol*. **80**, 3624–3633.

Morin, S., Ghanim, M., Sobol, I. and Czosnek, H. (2000) The GroEL protein of the whitefly Bemisia tabaci interacts with the coat protein of transmissible and nontransmissible begomoviruses in the yeast two-hybrid system. *Virology*, **276**, 404–416.

Moriones, E. and Navas-Castillo, J. (2000) Tomato yellow leaf curl virus, an emerging virus complex causing epidemics worldwide. *Virus Res*. **71**, 123–134.

Muangsan, N., Beclin, C., Vaucheret, H. and Robertson, D. (2004) Geminivirus VIGS of endogenous genes requires SGS2/SDE1 and SGS3 and defines a new branch in the genetic pathway for silencing in plants. *Plant J*. **38**, 1004–1014.

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

Noris, E., Vaira, A.M., Caciagli, P., Masenga, V., Gronenborn, B. and Accotto, G.P. (1998) Amino acids in the capsid protein of tomato yellow leaf curl virus that are crucial for systemic infection, particle formation, and insect transmission. *J. Virol*. **72**, 10 050–10 057.

Noueiry, A.O., Lucas, W.J. and Gilbertson, R.L. (1994) Two proteins of a plant DNA virus coordinate nuclear and plasmodesmal transport. *Cell*, **76**, 925–932.

Padidam, M., Beachy, R.N. and Fauquet, C.M. (1999) A phage single-stranded DNA (ssDNA) binding protein complements ssDNA accumulation of a geminivirus and interferes with viral movement. *J. Virol*. **73**, 1609–1616.

Patil, B.L., Rajasubramaniam, S., Bagchi, C. and Dasgupta, I. (2005) Both Indian cassava mosaic virus and Sri Lankan cassava mosaic virus are found in India and exhibit high variability as assessed by PCR-RFLP. *Arch. Virol*. **150**, 389–397.

Pfeffer, S., Zavolan, M., Grasser, F.A., Chien, M., Russo, J.J., Ju, J., John, B., Enright, A.J., Marks, D., Sander, C. and Tuschl, T. (2004) Identification of virus-encoded microRNAs. *Science*, **304**, 734–736.

Pooggin, M., Shivaprasad, P.V., Veluthambi, K. and Hohn, T. (2003) RNAi targeting of DNA virus in plants. *Nat. Biotechnol*. **21**, 131–132.

Pooma, W., Gillette, W.K., Jeffrey, J.L. and Petty, I.T. (1996) Host

and viral factors determine the dispensability of coat protein for bipartite geminivirus systemic movement. *Virology*, **218**, 264–268.
Powell, P.A., Sanders, P.R., Tumer, N., Fraley, R.T. and Beachy, R.N. (1990) Protection against tobacco mosaic virus infection in transgenic plants requires accumulation of coat protein rather than coat protein RNA sequences. *Virology*, **175**, 124–130.
Rojas, M.R., Noueiry, A.O., Lucas, W.J. and Gilbertson, R.L. (1998) Bean DWARF mosaic geminivirus movement proteins recognize DNA in a form- and size-specific manner. *Cell*, **95**, 105–113.
Rojas, M.R., Jiang, H., Salati, R., Xoconostle-Cazares, B., Sudarshana, M.R., Lucas, W.J. and Gilbertson, R.L. (2001) Functional analysis of proteins involved in movement of the monopartite begomovirus, tomato yellow leaf curl virus. *Virology*, **291**, 110–125.
Rouhibakhsh, A. and Malathi, V.G. (2005) Severe leaf curl disease of cowpea – a new disease of cowpea in northern India caused by mungbean yellow mosaic India virus and a satellite DNA beta. *Plant Pathol*. **54**, 259.
Ruiz, M.T., Voinnet, O. and Baulcombe, D.C. (1998) Initiation and maintenance of virus-induced gene silencing. *Plant Cell*, **10**, 937–946.
Sanderfoot, A.A. and Lazarowitz, S.G. (1995) Cooperation in viral movement: the geminivirus BL1 movement protein interacts with BR1 and redirects it from the nucleus to the cell periphery. *Plant Cell*, **7**, 1185–1194.
Sanderfoot, A.A. and Lazarowitz, S.G. (1996) Getting it together in plant virus movement: cooperative interactions between bipartite geminivirus movement proteins. *Trends Cell Biol*. **6**, 353–358.
Sanderfoot, A.A., Ingham, D.J. and Lazarowitz, S.G. (1996) A viral movement protein as a nuclear shuttle. The geminivirus BR1 movement protein contains domains essential for interaction with BL1 and nuclear localization. *Plant Physiol*. **110**, 23–33.
Saunders, K., Norman, A., Gucciardo, S. and Stanley, J. (2004) The DNA beta satellite component associated with ageratum yellow vein disease encodes an essential pathogenicity protein (betaC1). *Virology*, **324**, 37–47.
Seal, S.E., vandenBosch, F. and Jeger, M.J. (2006) Factors influencing begomovirus evolution and their increasing global significance: implications for sustainable control. *Crit. Rev. Plant Sci*. **25**, 23–46.
Seemanpillai, M., Dry, I., Randles, J. and Rezaian, A. (2003) Transcriptional silencing of geminiviral promoter-driven transgenes following homologous virus infection. *Mol. Plant–Microbe Interact*. **16**, 429–438.
Selth, L.A., Dogra, S.C., Rasheed, M.S., Healy, H., Randles, J.W. and Rezaian, M.A. (2005) A NAC domain protein interacts with tomato leaf curl virus replication accessory protein and enhances viral replication. *Plant Cell*, **17**, 311–325.
Sera, T. (2005) Inhibition of virus DNA replication by artificial zinc finger proteins. *J. Virol*. **79**, 2614–2619.
Sera, T. and Uranga, C. (2002) Rational design of artificial zinc-finger proteins using a nondegenerate recognition code table. *Biochemistry*, **41**, 7074–7081.
Settlage, S.B., See, R.G. and Hanley-Bowdoin, L. (2005) Geminivirus C3 protein: replication enhancement and protein interactions. *J. Virol*. **79**, 9885–9895.
Shen, W.H. (2002) The plant E2F-Rb pathway and epigenetic control. *Trends Plant Sci*. **7**, 505–511.
Sijen, T., Vijn, I., Rebocho, A., van Blokland, R., Roelofs, D., Mol, J.N. and Kooter, J.M. (2001) Transcriptional and posttranscriptional gene silencing are mechanistically related. *Curr. Biol*. **11**, 436–440.
Sinisterra, X.H., Polston, N.J.E., Abouzid, A.M. and Hiebert, E. (1999) Tobacco plants transformed with a modified coat protein of tomato mottle begomovirus show resistance to virus infection. *Phytopathology*, **89**, 701–706.
Smith, N.A., Singh, S.P., Wang, M.B., Stoutjesdijk, P.A., Green, A.G. and Waterhouse, P.M. (2000) Gene expression – total silencing by intron-spliced hairpin RNAs. *Nature*, **407**, 319–320.
Stanley, J. and Townsend, R. (1985) Characterisation of DNA forms associated with cassava latent virus infection. *Nucleic Acids Res*. **13**, 2189–2206.
Stanley, J., Frischmuth, T. and Ellwood, S. (1990) Defective viral DNA ameliorates symptoms of geminivirus infection in transgenic plants. *Proc. Natl. Acad. Sci. USA*, **87**, 6291–6295.
Stanley, J., Saunders, K., Briddon, R.W., Brown, J.K., Fauquet, C.M., Harrison, B.D., Rybicki, E.P. and Stenger, D.C. (2005) Geminiviridae. In *VIIIth Report of the International Committee on Taxonomy of Viruses-Virustaxonomy* (Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U. and Ball, L.A., eds), pp. 301–326. London: Elsevier/Academic Press.
Stenger, D.C. (1994) Strain-specific mobilization and amplification of a transgenic defective-interfering DNA of the geminivirus beet curly top virus. *Virology*, **203**, 397–402.
Sung, Y.K. and Coutts, R.H. (1996) Potato yellow mosaic geminivirus AC2 protein is a sequence non-specific DNA binding protein. *FEBS Lett*. **383**, 51–54.
Sunter, G., Stenger, D.C. and Bisaro, D.M. (1994) Heterologous complementation by geminivirus AL2 and AL3 genes. *Virology*, **203**, 203–210.
Sunter, G., Sunter, J.L. and Bisaro, D.M. (2001) Plants expressing tomato golden mosaic virus AL2 or beet curly top virus L2 transgenes show enhanced susceptibility to infection by DNA and RNA viruses. *Virology*, **285**, 59–70.
Tenllado, F., Llave, C. and Diaz-Ruiz, J.R. (2004) RNA interference as a new biotechnological tool for the control of virus diseases in plants. *Virus Res*. **102**, 85–96.
Trinks, D., Rajeswaran, R., Shivaprasad, P.V., Akbergenov, R., Oakeley, E.J., Veluthambi, K., Hohn, T. and Pooggin, M.M. (2005) Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. *J. Virol*. **79**, 2517–2527.
Turnage, M.A., Muangsan, N., Peele, C.G. and Robertson, D. (2002) Geminivirus-based vectors for gene silencing in *Arabidopsis*. *Plant J*. **30**, 107–114.
Vaistij, F.E., Jones, L. and Baulcombe, D.C. (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell*, **14**, 857–867.
Van Houdt, H., Bleys, A. and Depicker, A. (2003) RNA target sequences promote spreading of RNA silencing. *Plant Physiol*. **131**, 245–253.
Vanitharani, R., Chellappan, P. and Fauquet, C.M. (2003) Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. *Proc. Natl. Acad. Sci. USA*, **100**, 9632–9636.
Vanitharani, R., Chellappan, P., Pita, J.S. and Fauquet, C.M. (2004) Differential roles of AC2 and AC4 of cassava geminiviruses in mediating synergism and suppression of posttranscriptional gene silencing. *J. Virol*. **78**, 9487–9498.

Vanitharani, R., Chellappan, P. and Fauquet, C.M. (2005) Geminiviruses and RNA silencing. *Trends Plant Sci*. **10**, 144–151.

Van Wezel, R., Liu, H., Wu, Z., Stanley, J. and Hong, Y. (2003) Contribution of the zinc finger to zinc and DNA binding by a suppressor of posttranscriptional gene silencing. *J. Virol*. **77**, 696–700.

Varma, A. and Malathi, V.G. (2003) Emerging geminivirus problems: a serious threat to crop production. *Ann. Appl. Biol*. **142**, 145–164.

Voinnet, O., Pinto, Y.M. and Baulcombe, D.C. (1999) Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. *Proc. Natl. Acad. Sci. USA*, **96**, 14 147–14 152.

Von Arnim, A. and Stanley, J. (1992) Inhibition of African cassava mosaic virus systemic infection by a movement protein from the related geminivirus tomato golden mosaic virus. *Virology*, **187**, 555–564.

Wesley, S.V., Helliwell, C.A., Smith, N.A., Wang, M.B., Rouse, D.T., Liu, Q., Gooding, P.S., Singh, S.P., Abbott, D., Stoutjesdijk, P.A., Robinson, S.P., Gleave, A.P., Green, A.G. and Waterhouse, P.M. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J*. **27**, 581–590.

Xie, Z., Fan, B., Chen, C. and Chen, Z. (2001) An important role of an inducible RNA-dependent RNA polymerase in plant antiviral defense. *Proc. Natl. Acad. Sci. USA*, **98**, 6516–6521.

Yang, S.J., Carter, S.A., Cole, A.B., Cheng, N.H. and Nelson, R.S. (2004b) A natural variant of a host RNA-dependent RNA polymerase is associated with increased susceptibility to viruses by *Nicotiana benthamiana*. *Proc. Natl. Acad. Sci. USA*, **101**, 6297–6302.

Yang, Y., Sherwood, T.A., Patte, C.P., Hiebert, E. and Polston, J.E. (2004a) Use of tomato yellow leaf curl virus (TYLCV) Rep gene sequences to engineer TYLCV resistance in tomato. *Phytopathology*, **94**, 490–496.

Zhang, P., Fütterer, J., Frey, P., Potrykus, I., Puonti-Kaerlas, J. and Gruissem, W. (2003) Engineering virus-induced ACMV resistance by mimicking a hypersensitive reaction in transgenic cassava plants. In: *Plant Biotechnology 2002 and Beyond* (Vasil, I.K., ed.), pp. 143–146. Town?: Kluwer Academic.

Zhang, P., Vanderschuren, H., Futterer, J. and Gruissem, W. (2005) Resistance to cassava mosaic disease in transgenic cassava expressing antisense RNAs targeting virus replication genes. *Plant Biotechnol. J*. **3**, 385–397.

Zhou, X., Liu, Y., Calvert, L., Munoz, C., Otim-Nape, G.W., Robinson, D.J. and Harrison, B.D. (1997) Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. *J. Gen. Virol*. **78**, 2101–2111.

Zilberman, D., Cao, X. and Jacobsen, S.E. (2003) ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science*, **299**, 716–719.

Zilberman, D., Cao, X., Johansen, L.K., Xie, Z., Carrington, J.C. and Jacobsen, S.E. (2004) Role of *Arabidopsis* ARGONAUTE4 in RNA-directed DNA methylation triggered by inverted repeats. *Curr. Biol*. **14**, 1214–1220.

Zimmermann, S., Schillberg, S., Liao, Y.C. and Fisher, R. (1998) Intracellular expression of TMV-specific single-chain Fv fragments leads to improved virus resistance in shape *Nicotiana tabacum*. *Mol. Breed*. **4**, 369–379.