TRADITIONAL AND TRANSGENIC STRATEGIES FOR CONTROLLING TOMATO-INFECTING BEGOMOVIRUSES*

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ABSTRACT

Viruses of to the family *Geminiviridae* are considered some of the most important pathogens in tropical and subtropical regions of the world. Members of one *Geminiviridae* genus, *Begomovirus,* have been causing severe losses, particularly in tomato (*Lycopersicon esculentum*) production in the Americas and the Caribbean. Several new begomoviruses have been reported in the region and, at least one, *Tomato yellow leaf curl virus* (TYLCV), has been brought in from the Old World via infected transplants. In addition, the recombination events that are playing an important role in *Begomovirus* diversity have increased the complexity of their control. This scenario has led to the search for control measures that go beyond traditional host genetic resistance, chemical controls and cultural practices. In this review, besides the recommended classical control measures, transgenic approaches will be discussed, as well as the mechanisms involved in their successful control of viruses.

Additional key words: whitefly-transmitted geminivirus, gene silencing, plant transformation, tomato begomovirus, geminivirus control, *Geminiviridae*.

RESUMO

Estratégias tradicionais e transgênicas para o controle de begomovirus que infetam tomateiro

Membros do gênero *Begomovirus*, família *Geminiviridae,* estão entre os vírus mais importantes que infetam plantas nas regiões tropicais e subtropicais do mundo. Nas Américas, begomovirus vêm causando danos significativos especialmente para a produção de tomates (*Lycopersicon esculentum*). Inúmeros novos vírus têm sido relatados na região, e ao menos um, o *Tomato yellow leaf curl virus* (TYLCV), foi introduzido do Velho Continente através de plântulas infetadas. Além disso, recombinação aparentemente é um fator importante contribuindo para uma maior diversidade desses vírus, dificultando seu controle. Esta situação levou à procura de medidas de controle que vão além das normalmente utilizadas como resistência genética, controle químico e cultural. Nesta revisão, além de medidas tradicionais de controle de begomovirus, são discutidas principalmente estratégias de controle alternativas, como a utilização de plantas transgênicas, assim como os mecanismos envolvidos em tais estratégias

BRIEF HISTORY AND TAXONOMY OF GEMINIVIRUSES

Symptoms now known to be associated with geminiviruses have been observed in plants grown in tropical and subtropical regions of the world since the mid-1800s (Wege *et al.*, 2000). However, it was not until the 1970s that a distinct group of single-stranded DNA (ssDNA) viruses was shown to be associated with such symptoms, and these were placed in the "Geminivirus" group (Gálvez & Castaño, 1976; Goodman 1977a; 1977b; Harrison *et al.*, 1977). Geminiviruses are characterized by the twinned, small (ca. 18-30 nm), quasi-icosahedral morphology of the virion particles and by their genomes consisting of one or two molecules of ssDNA 2.5-3.0 kb in length (Rybicki *et al.*, 2000).

During the last decade, the geminiviruses were classified into the *Geminiviridae* family (Rybicki, 1994), which contains four genera: *Mastrevirus*, *Curtovirus*, *Topocuvirus*, and *Begomovirus*, classified according to their host range, genome organization, and vector species (Palmer & Rybicki, 1998; Fauquet *et al.*, 2000).

Members of the genus *Begomovirus*, such as the type species *Bean golden yellow mosaic virus* - Puerto Rico (BGYMV) [formerly named *Bean golden mosaic virus* (BGMV-PR) by Fauquet *et al.*, 2000], are transmitted by the whitefly *Bemisia tabaci* Genn. in what is believed to be a persistent, circulative, non-propagative manner (Rybicki *et al.*, 2000), although some authors have proposed otherwise (Mehta *et al*., 1994). Begomoviruses infect dicotyledonous

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plants and most have bipartite genomes, although some, such as, *Tomato yellow leaf curl virus* (TYLCV), have a single DNA component.

As there are already some recent reviews on geminivirus genome organization and replication (Castellano *et al.*, 1999; Hanley-Bowdoin *et al.*, 1999; Faria & Zerbini, 2000; Gutierrez, 2000), as well as their interference with host cell cycle and gene expression (Gutierrez, 1998; 2000; Kong *et al.*, 2000; Settlage *et al.*, 2001), these topics will not be addressed in this review.

IMPORTANCE OF GEMINIVIRUSES

The *Geminiviridae* family has received a great deal of attention in recent years and is becoming one of the most important and studied families of plant virus. Some reasons why so much effort has been dedicated to their study include the economic and social impact of the diseases they cause (Palmer & Rybicki, 1998; Harrison & Robinson, 1999; Morales & Anderson, 2001); their use as episomal vectors and gene silencing inducers (Hayes *et al.*, 1988; Shen & Hohn, 1995; Atkinson *et al.*, 1998; Kjemtrup *et al.*, 1998); and their contribution as models for studying intracellular and intercellular movement strategies of macromolecules (Noueiry *et al.*, 1994; Sanderfoot & Lazarowitz, 1995; 1996; Sanderfoot *et al.*, 1996; Rojas *et al*., 1998; Gutierrez, 1999; Lazarowitz, 1999; Ward & Lazarowitz, 1999).

In addition, concern for this family has increased due to the emergence of new geminiviruses through recombination or pseudorecombination among strains and/or species in various crops; the role of the recently discovered satellite-like DNA-ß components; and findings regarding the integration of begomovirus sequences into the genome of plants such as *Nicotiana* species (Bejarano *et al*., 1996; Ashby *et al*., 1997; Zhou *et al*., 1997; Navas-Castillo *et al*., 2000; Saunders *et al*., 2000; Saunders *et al.*, 2001; Harper *et al*., 2002; Mette *et al*., 2002; Ribeiro *et al*., 2002).

These findings indicate that recombination has contributed to the diversity of geminiviruses and therefore, to the emergence of new variants and species reported worldwide. In the particular case of tomato (*Lycopersicon esculentum* Mill.)-infecting begomoviruses, recombination is likely an important factor in their evolution, even in the short and medium term (Zhou *et al.*, 1997; Navas-Castillo *et al*., 2000; Saunders *et al.*, 2001; Ribeiro *et al*., 2002). In the 1970s, there were only three begomoviruses reported to infect tomatoes in the Americas. Less than 30 years later, at least 14 new begomoviruses have been found in tomatoes in the region (Polston & Anderson, 1997; Morales & Anderson, 2001), and this number could be significantly higher since at least eight new putative whitefly-transmitted geminivirus (WTG) species were reported recently to infect this solanaceous plant in Brazil alone (Faria *et al.*, 2000; Ribeiro *et al.*, 2001; 2002).

Besides the large number of viruses, the losses they cause in tomato crops are extensive and some of them, such as TYLCV, can cause total yield loss (Czosnek & Laterrot, 1997). Morales & Anderson (2001) stated that the introduction of TYLCV to the Dominican Republic was the greatest tragedy in the history of WTG affecting economically important crops in the Caribbean, and caused the collapse of the tomato industry in that country.

TRADITIONAL APPROACHES FOR CONTROLLING BEGOMOVIRUSES INFECTING TOMATOES

As mentioned above, there are several reasons why geminiviruses are studied worldwide. Their main importance, however, is related to their ability to cause significant yield losses to numerous crops. Several approaches have been used in attempts to control begomoviruses infecting tomato plants, but only a few of them have proven to be effective. There is a possibility of controlling *B. tabaci* biologically, but it has not been used for tomato production since the results are very unsatisfactory (Mason *et al.*, 2000). Additional information on the current situation, problems, and the potential use of fungi and predators or parasitoids to control whiteflies can be found in reviews by Faria & Wraight (2001) and Gerling *et al*. (2001), respectively.

Cultural practices such as roguing, intercropping, avoidance, use of barriers, crop residue disposal, among others, are recommended, but they should be combined with the use of insecticides and/or resistant varieties in order to be effective, especially in tropical areas, where tomato production occurs throughout the year (Polston & Anderson, 1997; Villas Bôas *et al.*, 1997; Faria *et al.*, 2000; Hilje *et al*., 2001). Reductions in the incidence of TYLCV (Cohen, 1982; Suwwan *et al.*, 1988) and *Tomato mottle virus* (ToMoV) (Csizinszky *et al.,* 1995) in tomatoes were observed with different levels of efficiency when yellow or orange polyethylene films and aluminum mulches were used. However, despite of some positive results, the use of mulches is not always practical and cost-effective, especially when tomatoes are grown in large areas.

The best results of WTG cultural control in tomatoes reported to date seem to occur when ultraviolet-absorbing plastic films are used as greenhouse covers or as insect-proof nets (Antignus *et al.*, 1998; Antignus, 2000). Besides being a physical barrier for the insects, these UV-absorbing films can reduce virus incidence through the inhibition of whitefly movement, and have proven especially efficient for the control of begomoviruses. Antignus *et al.* (1996) demonstrated that TYLCV incidence in tomato plants grown under the UV-absorbing sheets was only 1% compared with approximately 80% in the uncovered control. Because of its high efficacy, the use of screens has become a standard pest management strategy for the production of tomatoes in Israel (Taylor *et al*., 2001). It is important to note, however, that besides the higher production cost, these screens alone may not sufficiently protect against TYLCV, and their use may result in increased temperature and humidity inside greenhouses

(Mason *et al.*, 2000; Moriones & Navas-Castillo, 2000).

Although the elimination of alternative hosts is often recommended for reduction of viruses in general, weeds are not normally considered to be important reservoirs of tomato-infecting begomoviruses under natural conditions (Polston & Anderson, 1997; Ucko *et al*., 1998; Moriones & Navas-Castillo, 2000). Some exceptions include the relevance of few weeds in the TYLCV epidemics in the Jordan Valley, Israel (Cohen *et al*., 1988) and the possibility of their contribution to WTG variability in the tropics. The most important aspect of removing weeds around tomato fields, however, has been considered to be the likelihood of diminishing the vector population. Whiteflies, particularly the *B. tabaci* biotype B, are polyphagous and have been reported in at least 506 species within 74 plant families of dicots and monocots (Villas Bôas *et al.*, 1997; Chatterji & Fauquet, 2000). However, Hilje *et al*. (2001) suggest that it may not be worthwhile to spend resources on weed removal for the control of the New World begomoviruses. In addition, weeds also serve an important function as reservoirs of whitefly predators, parasites and pathogens.

Regulatory measures have also been taken in a few instances. A mandatory three-month tomato-free period from June through August has been issued in the Dominican Republic and has helped to drastically reduce the incidence of TYLCV in the first half of the growing season. By the end of the season, however, high incidences of the virus can be seen in the fields, and losses can be significant if TYLCV-susceptible cultivars are planted (Polston & Anderson, 1997; Salati *et al*., 1999).

The use of resistant tomato plants is undoubtedly the best way to control begomoviruses (Polston & Anderson, 1997; Mason *et al*., 2000). A great effort has been made to obtain genetic resistance to begomoviruses, with much of it directed against TYLCV. Several groups of researchers have looked for TYLCV resistance and tolerance among wild *Lycopersicon* species and have found some promising materials within *L. chilense* Dun., *L. pimpinellifolium* (Jusl.) Mill., *L. hirsutum* Dun., *L. cheesmani* Riley, and *L. peruvianum* (L.) Mill. (Kasrawi *et al*., 1988; Zakay *et al*., 1991; Michelson *et al*., 1994; Picó *et al*.; 1998; Vidavsky & Czosnek, 1998, among others). Some accessions of tomato wild relatives exhibited good levels of resistance and tolerance to bipartite begomovirus as well, such as *Tomato yellow mosaic virus* (Piven & Uzcátegui, 1995) and the DF_1 isolate (Ferreira *et al*., 1999; Santana *et al*., 2001).

Besides the direct genetic resistance to begomoviruses, resistance to the whitefly vector has been reported in some wild *Lycopersicon* species, such as *L. hirsutum* and *L. peruvianum* (Morales, 2001). It has been associated with the large amounts of sticky substances that plants of these species exudate, entrapping the whiteflies and significantly reducing the transmission of begomoviruses (Channarayappa & Shivashankar, 1992; Morales, 2001). Unfortunately, this is not a desired trait for commercial tomato plants.

With such a broad range of tolerance and resistance in nature, only a few breeding lines and varieties have been produced (Rom *et al*., 1993; Lapidot *et al*., 1997; Mason *et al*., 2000). However, in commercial fields of most regions of the world, tomato plants are still largely susceptible to various begomoviruses (Polston & Anderson, 1997; Mason *et al*., 2000; Diaz-Plaza *et al*., 2001). In addition, it is a concern that some asymptomatic, tolerant cultivars support the replication of the virus, and can act as sources of begomovirus for susceptible crops (Lapidot *et al*., 2001).

Liu & Stansly (2000) have tested several surfactants and oils against whitefly nymphs on tomato plants. Although there were good levels of insect mortality in some cases, phytotoxicity was observed in many instances. Their effects on yields were not reported.

Ultimately, the management of begomoviruses infecting tomatoes relies heavily on the use of a combination of systemic and topical insecticides to control the vector (Polston & Anderson, 1997; Villas Bôas *et al.*, 1997; Faria *et al.*, 2000; Mason *et al.*, 2000; Ahmed *et al.*, 2001). However, although effective for some time, there are some concerns about chemical controls. In Honduras, there were outbreaks of secondary pests such as leafminers due to the repetitive use of insecticides to control whiteflies (Rafie *et al.*, 1999). Cahill *et al.* (1996) reported the development of whiteflies resistant to imidacloprid in Spain. This observation is of major concern because this active ingredient is the most important insecticide used to control whiteflies and thus, begomoviruses (Cahill *et al.*, 1996; Polston & Anderson, 1997; Ahmed *et al.*, 2001).

This scenario, associated with increasing concerns for obtaining more environmentally friendly ways to control pests and diseases, has encouraged a search for alternatives to control begomoviruses in tomatoes, especially through transgenic approaches. The strategies used by various research groups and the results obtained so far will be discussed after a brief review of the mechanisms involved in transgenic resistance against plant viruses.

MECHANISMS OF GENETIC ENGINEERING FOR PLANT VIRUS RESISTANCE

The Concept of Pathogen-Derived Resistance (PDR)

Sanford & Johnson (1985), working with bacteriophages, described the concept of pathogen-derived resistance (PDR), which can be used in plant virology. It can be defined as the transformation of plants with portions of viral genomes that can generate lines of plants resistant to the virus from which the sequence was derived. Only one year after Sanford and Johnson's publication, Powell-Abel *et al.* (1986) reported that tobacco (*Nicotiana tabacum* L.) plants transformed with the capsid protein (*CP*) gene of *Tobacco mosaic virus* (TMV) genus *Tobamovirus* were resistant to infection by TMV.

Since then, numerous papers and reviews have been published on PDR, and the concept has been proven valid for a wide variety of plants, for a number of genes or portions of

genes, and for members of virtually every genus of plant viruses.

Several researchers classify PDR based on the open reading frame (ORF) used for transformation or its product. In this review we briefly describe what we consider to be the three major groups within PDR, regardless of the viral sequence that led to resistance. It is important to note that so far these concepts have been accepted for RNA viruses, but not yet proven for DNA viruses. However, the observations suggest that the mechanisms involved in resistance are similar for both RNA and DNA viruses.

Protein-Mediated Resistance

Early experiments demonstrated that plants transformed with the *CP* gene of TMV were more resistant when high levels of the viral capsid protein were expressed, confirming the importance of the actual protein in resistance (Powell-Abel *et al*., 1986). Because of that, it was named protein-mediated resistance (PMR) or, in this particular case, coat protein-mediated resistance (CPMR). Other characteristics associated with PMR are that the resistance is normally broken down or reduced when the inoculum is nucleic acid rather than intact virions, it is often manifested as a delay in the appearance of symptoms, it is normally partial but broad-spectrum, and it is dependent on inoculum concentrations and environmental conditions (Powell-Abel *et al.*, 1986; Nejidat & Beachy, 1989; Pappu *et al.*, 1995; Baulcombe, 1996).

At least for plants transformed with the TMV *CP* gene, the formulated hypothesis to explain resistance is that it occurs by the inhibition of challenge virion disassembly in the initial infected cells (Bendahmane *et al.*, 1997). Interestingly, this agrees with the concept of cross protection, defined as the ability of one virus to inhibit or prevent infection or the manifestation of a closely related second virus (Dodds, 1982). Yet, another similarity between CPMR and cross protection is that, in both cases, unencapsidated viral RNA can overcome the resistance suggesting that the protective virus in cross protection blocks the disassembly of the challenge virus as well (Sherwood & Fulton, 1982; Register & Beachy, 1988).

Similarly to what was described for the CPMR, movement protein-mediated resistance (MPMR) has often been associated with high levels of protein production by transgenic plants, but only when dysfunctional, rather than full-length MP, is expressed (Ziegler-Graff *et al.*, 1991; Lapidot *et al.*, 1993; Beck *et al.*, 1994; Cooper *et al.*, 1995).

It is thought that the resistance obtained in plants transformed with a dysfunctional TMV *MP* gene occurs due to competition for plasmodesmatal binding sites between the mutant MP and the wild-type MP of the inoculated virus (Lapidot *et al.*, 1993). Some examples of the broad-spectrum MPMR suggest that different virus movement proteins interact with the same plasmodesmatal components (Baulcombe, 1996), likely pectin methyl esterase (Chen *et al.*, 2000). Lapidot *et al.* (1993) observed a correlation between the accumulation of a defective TMV MP and resistance to the viral infection. This is characteristic of a dominant negative mutation, defined as the ability of a mutant gene to code for a mutant product, which then inhibits the wild-type gene product in a cell, causing the cell to be deficient in the function of that gene product (Herskowitz, 1987).

It is important to note, however, that in many cases plants transformed with viral *CP* or *MP* genes (Lindbo & Dougherty, 1992a; Van der Vlugt *et al.*, 1992; Sijen *et al.*, 1995, 1996; Prins *et al.*, 1997; Sinisterra *et al.*, 1999) do not produce the corresponding viral protein, and yet are resistant to the challenge homologous virus. These examples are normally associated with a different class of PDR, the RNA-mediated resistance.

RNA-Mediated Resistance and Post-Transcriptional Gene Silencing

According to Prins & Goldbach (1996), the RNA-mediated resistance (RMR) approach arose as an unexpected spin off from the concept of PDR. In contrast to what was expected, the resistance observed in several transgenic lines, especially those transformed with the replicase gene (*Rep*) had no direct correlation with the levels of protein produced (Anderson *et al.*, 1992; Audy *et al.*, 1994; Baulcombe, 1994). In addition, in the early 1990s, several research groups reported that plants transformed with untranslatable sequences of viruses were resistant to their challenge homologous viruses (Lindbo & Dougherty, 1992a, b; Van der Vlugt *et al.*, 1992).

Lindbo *et al.* (1993) demonstrated through run-on analysis that tissues that exhibited a typical "recovery phenotype" (tissues that initially exhibited symptoms, but whose symptoms would disappear with time) had high levels of transcription of the transgenes in the nucleus and very low levels in the cytoplasm. These observations led them to propose the presence of an RNA-degradation mechanism, which would be activated by the presence of high levels of a specific transcript. Lindbo *et al.* (1993) also demonstrated that extensive methylation of the transgene sequence was likely associated with induction of the specific cytoplasmic RNA degradation mechanism. These observations were typical of gene silencing, which had been recently described in plants and was getting a great deal of attention (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). Since the degradation occurred after transcription took place, it was named post-transcription gene silencing (PTGS).

In addition to the recovery phenotype (that may or may not occur) and the failure to detect the product of the transgene due to mRNA degradation, there are other features normally associated with gene silencing. The RMR is often complete or almost complete (high levels of resistance or immunity), regardless of the inoculum concentration or environmental conditions, but it is specific to the virus from which the sequence was derived (low-spectrum resistance) (Lomonossoff, 1995).

In contrast to what was originally thought, gene silencing (GS) is not only induced by transgenes, but also by viruses carrying sequences with homology to host transgenes

or endogenous genes (Lindbo *et al.*, 1993; English *et al.*, 1996). Since 1997, it has been noticed that GS has striking similarities with natural plant defense mechanisms against viruses and that plant virus infections in the absence of any known homology to host genes could also induce GS (Covey *et al.*, 1997; Ratcliff *et al.*, 1997; Al-Kaff *et al.*, 1998; Covey & Al-Kaff, 2000).

In 1998, four groups of researchers demonstrated, virtually at the same time, that PTGS is indeed a plant defense mechanism by showing that some viruses have a counter-defensive strategy involving the suppression of GS (Anandalakshmi *et al.*, 1998; Beclin *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau & Carrington, 1998). This discovery provided some explanation to the phenomenon that had been heavily associated with transgenic plants, but that in reality, is normally targeted against naturally invading nucleic acids, particularly viruses and transposable elements (Smyth, 1999; Waterhouse *et al*., 2001).

The phenomenon of PTGS appears to be quite common in nature. With the exception of baker's yeast (Aravind *et al*., 2000), apparently all eukaryotes have mechanisms similar to gene silencing, often called RNA interference (RNAi). This demonstrates that organisms such as fungi (Cogoni *et al.*, 1996; Cogoni & Macino, 1997; Faugeron, 2000), protozoa (Ngo *et al.*, 1998), a variety of animals (Fire *et al.*, 1998; Lohmann *et al.*, 1999; Sánchez-Alvarado & Newmark, 1999; Wargelius *et al.*, 1999; Cogoni & Macino, 2000; Ketting & Plasterk, 2000; Wianny & Zernicka-Goetz, 2000; Elbashir *et al*., 2001) and plants have possibly a common ancestral origin (Cogoni & Macino, 2000, Fagard *et al.*, 2000; Zamore *et al.*, 2000; Hammond *et al*., 2001a; Zamore, 2002).

In this review, the mechanisms involved in PTGS as well as the role of double-stranded (ds) RNA transcripts and small (s) RNA in GS will not be discussed since several reviews covering these aspects have already been written (Wassenegger & Pélissier, 1998; Waterhouse *et al.*, 1998; Bass, 2000; Marathe *et al.*, 2000; Hammond *et al.*, 2001b; Hutvágner *et al.*, 2000; Zamore *et al.*, 2000; Li & Ding, 2001; Miller *et al*., 2001; Zamore, 2002).

Interestingly, it has been recently proposed that common processing machinery generates sRNAs that mediate both RNAi and endogenous gene regulation involved in development (Elbashir *et al*., 2001; Grishok *et al*., 2001; Hutvágner *et al*., 2001; Zamore, 2002). This suggests that PTGS might not only be a defense mechanism against viruses and transposable elements, but also might be part of a developmental regulation system (Hutvágner & Zamore, 2002).

RNA- and Protein-Mediated Resistance

Some researchers have found yet more complex results in their studies on transgenic resistance. Pang *et al.* (1994) reported that the mechanisms involved in the *CP* gene-mediated resistance against tospoviruses were variable. When the resistance was against closely related isolates, it was RNA-mediated, but when it was against more distantly related tospoviruses, it was protein-mediated. Wintermantel & Zaitlin (2000) suggested that, in tobacco plants transformed with the *Cucumber mosaic virus* (CMV) family *Bromoviridae*, genus *Cucumovirus* replicase gene, the resistance obtained is likely a result of a complex mechanism involving both transgene mRNA and its expressed protein.

Recently, Goregaoker *et al.* (2000) demonstrated that in TMV replicase-mediated resistance, both RNA and protein are involved in protecting against the challenge virus. Interestingly, over the years many authors have reported divergent conclusions regarding the mechanism involved in the resistance of plants transformed with the TMV *RdRp* gene. In some cases, the resistance seemed to be mediated by the RNA transcripts (Tenllado *et al.*, 1996; Marano & Baulcombe, 1998), while in others, it seemed to be protein-mediated (Carr *et al.*, 1992; Donson *et al.*, 1993).

Finally, Goregaoker *et al.* (2000) propose that the protection conferred by segments of the TMV *RdRp* gene expressed from a heterologous viral vector can be credited to the *RdRp* mRNA and also to the protein expression from segments of the polymerase (POL) domain, the latter conferring greater delays in the accumulation of challenge TMV when compared to the RNA-mediated mechanism. The authors further propose that both mechanisms possibly work cooperatively, with the protein-mediated mechanism functioning to slow down wild-type virus replication to a level that allows the RNA-mediated mechanism to be more effective (Goregaoker *et al.*, 2000).

Nonpathogen-Derived Approaches

Although most of the transgenic resistance to viruses has been obtained by PDR, there are some cases where it can be achieved through nonpathogen-derived approaches. The ribosome-inactivating proteins (RIPs), such as dianthin extracted from *Dianthus caryophyllus* L., have natural control effects against several plant and animal viruses. Although their use for genetic engineering has not been reported for any tomato-infecting begomovirus so far, the dianthin gene, under the control of the *African cassava mosaic virus* (ACMV) family *Geminiviridae*, genus *Begomovirus* promoter, has been used successfully to confer resistance in *N. benthamiana* Domin against this WTG (Hong *et al*., 1996; Hong & Stanley, 1996).

To our knowledge, other nonpathogen-derived strategies such as the expression of antiviral proteins (i.e. ribozymes), ribonucleases, and antibodies, have not yet been reported as alternative methods to control begomoviruses, and will not be addressed in this review.

TRANSGENIC APPROACHES USED FOR CONTROLLING BEGOMOVIRUSES INFECTING TOMATO PLANTS

As previously mentioned, most of what is known about PDR derives from plants transformed with RNA viral sequences, but an increasing number of papers have been

published on transgenic resistance for DNA viruses as well.

Of the 104 begomoviruses characterized to date, approximately 30 are reported as pathogens of tomato (Fauquet *et al*., 2000). However, transgenic approaches have been used so far in attempts to control only a few of them, mainly ToMoV, TYLCV, *Tomato yellow leaf curl Sardinia virus* (TYLCSV), *Tomato golden mosaic virus* (TGMV), and *Pepper huasteco virus* (PHV). Most of the effort has been directed toward the control of TYLCV and TYLCSV, which are considered the most important tomato-infecting begomoviruses in several countries. However, most of those studies have been carried out on *N. tabacum* or *N. benthamiana* plants, and only a few transgenic tomato lines have been produced that are resistant to WTGs.

Several viral sequences have been used in attempts to obtain plants resistant to tomato-infecting begomoviruses, with results that vary from immunity to complete susceptibility. The *CP* genes of TYLCV and ToMoV were used, respectively, by Kunik *et al.* (1994) to transform tomato and by Sinisterra *et al.* (1999) to transform tobacco plants. Although in the first case the authors used the full-length of the *CP* gene and in the latter, a truncated *CP* gene (with a deletion of 30 nucleotides at the 5'end), both studies reported resistance to challenge TYLCV and ToMoV, respectively. However, while the resistance reported by Kunik *et al*. (1994) was expressed as a delay in symptoms, recovery phenotype, and was associated with high levels of expressed CP protein, Sinisterra *et al*. (1999) observed higher levels of resistance and suggested that it was mediated by the RNA transcripts. Unfortunately, Sinisterra *et al.* (1999) noticed that the progeny from plants that were resistant in the R_1 generation were susceptible to ToMoV in the R_2, and Kunik *et al*. (1994) did not seem to have carried out the experiments to further generations.

Resistance was also observed in tobacco plants transformed with antisense sequences of the *CP* gene plus the 5' portion of the transcription activator (*TrAP*) and replication enhancer (*REn*) genes of TGMV (Bejarano & Lichtenstein, 1994). In this case, transgenic plants were asymptomatic after inoculation with the challenge virus, probably due to a drastic impairment in its replication (Bejarano & Lichtenstein, 1994).

The cell-to-cell movement and the nuclear shuttle protein genes (*MP* and *NSP*, respectively) also have been used to confer resistance to begomoviruses. The ToMoV and *Bean dwarf mosaic virus* (BDMV) *MP* and *NSP* genes have been used to transform tobacco and tomato by Duan *et al.* (1997) and Hou *et al.* (2000), respectively. Some resistance to ToMoV was obtained for constructs containing the *MP* sequence in the first case, and for *NSP* and *MP* constructs in the latter study. However, even though it appears that the expression of the protein is involved in the resistance, this was not clearly demonstrated. Besides, the resistance obtained by Hou *et al*. (2000) was expressed as only a delay in the appearance of the ToMoV symptoms.

A recent report demonstrated that tobacco plants transformed with ToMoV *MP* gene behaved biologically as if the resistance was RNA-mediated (recovery phenotype, high levels of narrow-range resistance, independent of levels of inoculum, and even after challenge with viral DNA through biolistic inoculation), but exhibited some characteristics at the molecular level that are typical of protein-mediated resistance (low, but detectable levels of *MP* mRNA and protein after challenge with ToMoV) (Freitas-Astúa, 2001; Freitas-Astúa *et al.*, 2001a). The use of antisense sequences of ToMoV *NSP* and *MP* did not confer resistance to tobacco plants (Duan *et al*., 1997; Freitas-Astúa *et al*., unpublished data).

Another gene often used for obtaining transgenic resistance to tomato-infecting begomoviruses is the replication-associated (*Rep*) gene. Noris *et al.* (1996) were the first to demonstrate that the expression of a truncated TYLCSV *Rep*, encoding the first 210 amino acids of the Rep protein and potentially co-expressing the C4 protein, could confer high levels of resistance in *N. benthamiana* plants. However, resistance was overcome with time. This truncated gene was also used to transform tomato plants (Brunetti *et al.*, 1997). Transformed plants that expressed high levels of the truncated TYLCSV Rep protein were resistant to TYLCSV infection, whereas those tomato lines in which the protein was not expressed (lines containing the antisense *Rep* or both sense and antisense *Rep* gene) were susceptible to TYLCSV. However, resistant plants exhibited an undesired, altered phenotype, and the resistance did not seem to be effective against a different begomovirus, *Tomato leaf curl - Australia virus* (Brunetti *et al*., 1997). Further studies of the same research group demonstrated that *N. benthamiana* plants expressing the truncated *Rep* of TYLCSV were resistant to the homologous virus, but susceptible to the related TYLCV Murcia strain (TYLCV-ES[1]). According to the authors, the truncated *Rep* acts as a trans-dominant-negative mutant inhibiting transcription and replication of TYLCSV, but not of TYLCV-ES[1] (Brunetti *et al*., 2001).

Day *et al*. (1991) reported the production of resistant tobacco plants expressing an antisense sequence of the TGMV *Rep* gene in the R_1 generation; however, the studies did not continue on further generations. Bendahmane & Gronenborn (1997) demonstrated that the use of the full-length antisense *Rep* conferred moderate resistance to TYLCSV in *N. benthamiana*, and this resistance was inherited in the R_2 generation as well. Interestingly, in both cases the level of homology between the antisense RNA and the challenge virus sequence specified the level of resistance obtained.

Recently, Franco *et al.* (2001) have shown resistance of *N. benthamiana* to TYLCSV by a double mechanism involving antisense RNA of TYLCSV *Rep* gene and extrachromosomal molecules; however, the plants were not protected against TYLCV, which is a more severe virus.

As it can be clearly seen, most studies on transgenic plants expressing the *Rep* gene or its antisense RNA were done on *N. benthamiana*, a known permissive host. There are a few exceptions to that, though. Polston & Hiebert (personal communication) used the full length ToMoV *Rep*

gene to obtain tomato plants resistant to ToMoV. Stout *et al.* (1997), based on the work by Hanson *et al*. (1995), who demonstrated that the NTP-binding domain of BGYMV is required for replication, mutated this motif in ToMoV, transformed tomato plants with such construct, and showed that it interferes with ToMoV replication.

Even though some tomato lines are resistant to ToMoV, until recently there were no reports of transgenic tomato plants satisfactorily resistant to TYLCV, a virus that is considered the most important begomovirus infecting tomatoes, both for its wide geographical distribution and for the severe losses it can cause. Only a short time ago, a construct consisting of 2/5 of the TYLCV *Rep* gene was demonstrated to confer high levels of resistance and often immunity to TYLCV in both tobacco and tomato, probably through the mechanism of PTGS (Freitas-Astúa *et al.*, 2001b; Polston *et al.*, 2001). The relevance of these studies is based on the fact that several lines of transformed tomato and tobacco plants were immune to TYLCV in the R_1 and R_2 generations, and that similar responses were observed in two different hosts, in independent transformations. These results suggest that the 2/5 TYLCV *Rep* construct is a strong gene silencing inducer (Polston, personal communication).

However, since numerous viruses can infect tomatoes, often in mixed infections, it is imperative that in some regions of the world the resistant plants exhibit broad-spectrum resistance. For that reason, new strategies are leading towards gene pyramiding or crossing of material already resistant to one virus with lines resistant to other viruses, or the use of negative dominant mutants that can confer good levels of resistance not only to the virus from which the sequence was derived, but also to related viruses (such as recombinants and variants or even other begomovirus species).

Diaz-Plaza *et al.* (2001) reported that tobacco plants expressing mutated PHV *MP* gene were resistant to the homologous virus and also exhibited some resistance to *Texas pepper virus* (TPV) family *Geminiviridae*, genus *Begomovirus*, probably through negative dominance. The authors have transformed tomatoes with the same mutated PHV *MP* gene, but the plants have not yet been tested (Diaz-Plaza & Rivera-Bustamante, personal communication). However, it is expected that the same construct would provide similar broad-spectrum resistance in another solanaceous host, such as tomato plants.

Chatterji *et al*. (2001) have recently shown that the transient expression of the *Tomato leaf curl New Delhi virus* (ToLCNDV) family *Geminiviridae*, genus *Begomovirus*, Rep protein, mutated at the oligomerization and DNA binding domains, inhibits viral DNA accumulation in tobacco protoplasts and in *N. benthamiana* plants. Interestingly, *in vivo* experiments of co-bombardment of this construct with infectious clones of ACMV, PHV, or *Potato yellow mosaic virus* (PYMV) in *N. benthamiana* suggest that the mutated protein might interfere, at different levels, not only with the homologous ToLCNDV, but with these other begomoviruses as well (Chatterji *et al*., 2001). These results are also promising, and transgenic tobacco and tomato plants are being tested for resistance to begomoviruses (Chatterji *et al*., 2001).

Finally, a new possibility for broad-spectrum resistance for begomoviruses relies on a recent study done by Argüello-Astorga & Ruiz-Medrano (2001). The authors found similarities among iterons (high affinity binding sites for the Rep protein, functioning as origin recognition sites) of more than 100 geminiviruses and proposed that the common specificities of the *Rep*-iteron interactions might be used in developing *Rep*-based virus resistance to a range of geminiviruses with similar interaction specificities.

FINAL REMARKS

Only time will tell if strategies chosen today will actively help control tomato-infecting begomoviruses or if new approaches will need to be pursued. However, although no begomovirus-resistant transgenic tomato plants are yet available to growers, some of these lines are very promising and might in the near future be cultivated or used in breeding programs.

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