REVIEW

Twenty Years of Transgenic Plants Resistant to *Cucumber mosaic virus*

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Plant genetic engineering has promised researchers improved speed and flexibility with regard to the introduction of new traits into cultivated crops. A variety of approaches have been applied to produce virus-resistant transgenic plants, some of which have proven to be remarkably successful. Studies on transgenic resistance to *Cucumber mosaic virus* probably have been the most intense of any plant virus. Several effective strategies based on pathogen-derived resistance have been identified; namely, resistance mediated by the viral coat protein, the viral replicase, and post-transcriptional gene silencing. Techniques using non-pathogen-derived resistance strategies, some of which could offer broader resistance, generally have proven to be much less effective. Not only do the results obtained so far provide a useful guide to help focus on future strategies, but they also suggest that there are a number of possible mechanisms involved in conferring these resistances. Further detailed studies on the interplay between viral transgene-derived molecules and their host are needed in order to elucidate the mechanisms of resistance and pathogenicity.

Cucumber mosaic virus (CMV) is the type member of the genus *Cucumovirus*, family *Bromoviridae* (Palukaitis and Garcia-Arenal 2003; Palukaitis et al. 1992). Since its first discovery in Michigan and New York States (Doolittle 1916), CMV has been found in most countries of the world and has been identified as the causal agent of several disease epidemics. Its host range exceeds 800 plant species, making CMV one of the most important viruses for its economic impact. Because of its importance and the absence of resistance genes in the germplasm of most crops, CMV has been one of the primary targets for development of transgene-mediated resistance. In compiling a list as complete as possible of articles published in English on CMV resistance transgenes, we found more than 50 papers, which certainly exceeds by far the number of equivalent papers devoted to resistance to any other plant virus. Our aim in reviewing this information is to make apparent the particular features of CMV resistance, to clarify which resistance strategies against this virus are most promising, and to identify interesting areas for further research. There have been two recent reviews (Prins et al. 2008; Sudarshana et al. 2007) covering all the strategies for creating virus-resistant transgenic plants (VRTP); however, by focusing on CMV, the present review will be able to go into greater depth, and this will make it possible to highlight features specific to this virus.

The CMV genome is organized into three single-stranded messenger-sense genomic RNAs (RNAs 1, 2, and 3) and two major subgenomic RNAs (RNAs 4 and 4A, which serve for the expression of the 3'-proximal gene of RNAs 3 and 2, respectively). RNAs 1 and 2 code for components of the replicase complex, and RNA 2 also codes for the 2b protein, which is expressed from subgenomic RNA 4A and is involved in the suppression of gene silencing. RNA 3 encodes the 3a protein, which is essential for virus movement, and the coat protein (CP), which is expressed from subgenomic RNA 4. The CP has an important role not only in the formation of the viral particles but also in virus movement, transmission by aphid vectors, and symptom expression. In addition, some CMV isolates include a satellite RNA, which is a small, linear, noncoding, single-stranded RNA that depends entirely on the helper CMV for replication, encapsidation, and transmission but has almost no sequence similarity with the CMV genome. CMV satellite RNAs can modify CMV pathogenicity drastically, worsening or attenuating its symptoms, depending on the satellite RNA and on the host species (Palukaitis and Garcia-Arenal 2003).

Based on their biological, serological, and molecular properties, CMV isolates can be classed in two subgroups, I and II, the former being further divided into subgroups IA and IB. The nucleotide identity between subgroups I and II ranges from 69 to 77%, depending on the RNA species, and within-subgroup identities are generally greater than 95% (Palukaitis and Garcia-Arenal 2003). Because most crops are susceptible to both subgroups, one of the major challenges is to create resistance that is effective against the entire diversity of CMV strains. The importance of CMV has led many groups to work on creating transgenes that confer resistance to this virus. Most of this work has been devoted to using viral sequences in resistance transgenes; thus, this review will focus primarily on virus-derived genes.

Transgenic virus-derived resistance.

Although many viral genes have been transferred to plant genomes since the first reports of CMV-resistant transgenic plants, during the earliest years viral CP genes were the most used in plant transformation. The first report of stable transformation of a plant with a viral CP gene was in 1985, with the introduction of a gene encoding the CP of *Tobacco mosaic virus* (TMV) into tobacco (Bevan et al. 1985). This was quickly followed by the first demonstration of pathogen-derived resistance, which had been hypothesized by Sanford and Johnston (1985), in the pioneering work from the Beachy lab describing CP-mediated resistance (CPMR) to TMV (Powell-Abel et al. 1986). The following year, CPMR was reported for *Alfalfa mosaic virus* (Tumer et al. 1987b), and CMV (Cuozzo et al.

1988; Tumer et al. 1987a). Since these early reports, although many resistance strategies have been explored (Fig. 1), CPMR clearly has been the predominant strategy for creating VRT (Prins et al. 2008; Sudarshana et al. 2007).

There are principally two experimentally well-established mechanisms at work to explain virus-derived resistance, one in which the effector is a protein and another in which it is the RNA transcribed from the transgene; in the latter case, resis-

Fig. 1. Timeline of different techniques used to produce transgenic plants resistant to *Cucumber mosaic virus* (CMV). Boxes on the left contain examples of the first practical applications of each technique for plant virus resistance. The black dots in each line represent reports in which the level of resistance of the transformed plants was analyzed. The articles cited are considered of particular significance in the development of the technique. TMV = *Tobacco mosaic virus*, CP = coat protein, CPMR = CP-mediated resistance, satRNA = satellite RNA, PR = pathogenesis related, PTGS = post-transcriptional gene silencing, AMCV = Artichoke mottle crinkle virus, RIP = ribosome inactivating protein.

tance is mediated by post-transcriptional gene silencing (PTGS). However, the first mechanism proposed to explain CPMR was that it acted at a protein level. This was based on the observation that, in the case of CPMR to TMV, resistance was expressed when plants were inoculated with virions but not when inoculated with viral RNA or even with partially stripped virions, suggesting a mechanism at an early stage during infection, possibly by inhibition of uncoating of virus particles (Asurmendi et al. 2007; Bendahmane et al. 2007; Osbourn et al. 1990; Register and Beachy 1988; Wu et al. 1990). However, since the first description of transgenes conferring resistance via a mechanism related to PTGS (Lindbo et al. 1993), there has been a tendency to assume that the latter is the predominant mechanism for resistance to viruses other than TMV. In this case, the transgene mRNA serves as the template for synthesis of double-stranded RNA, which is then acted upon by Dicer-like enzymes responsible for generating small interfering (si)RNA. The virus-specific siRNAs then are loaded into the effector RNA induced silencing complex (Ding and Voinnet 2007). Diagnostic features of PTGS-mediated resistance are i) the presence of siRNAs, ii) the observation of strong resistance in plants in which neither transgene mRNA nor the corresponding protein are observed, and iii) that it can be obtained with transgenes encoding untranslatable mRNAs. However, it should be noted that, in the case of tospoviruses, resistance can be conferred by both protein- and PTGS-mediated mechanisms (Pang et al. 1994). Therefore, perhaps more generally, in a given virus-resistant plant line, resistance can be due to complementary action of more than one mechanism.

CP-mediated resistance to CMV.

As mentioned above, CPMR to CMV was reported in 1987, the year following the first description of CPMR. It should be noted that CMV CP antisense genes also were tested; however, because they conferred little or no protection, this strategy has not been explored further. As for other viruses, CPMR has been used widely to create CMV resistance, with positive results reported with six plant species (tobacco, cucumber, tomato, melon, squash, and pepper) in more than 20 publications (Table 1). In the earliest work, transformation of tobacco plants with a gene encoding the CP of D-CMV (subgroup I) resulted in strong reduction in the incidence of virus infection in both inoculated and systemically infected leaves when inoculated with D-CMV (Cuozzo et al. 1988; Tumer et al. 1987a). Using a similar methodology, Quemada and associates (1991) obtained three tobacco lines expressing a CP gene from C-CMV (subgroup I). When challenged with either C-CMV, the severe subgroup I Chi-CMV, or the subgroup II WL-CMV, two lines were more resistant to the more homologous strains. Nevertheless, the fact that two lines were also moderately resistant to the more divergent subgroup II strain suggested that there was no consistent positive correlation between the level of amino acid sequence identity between the transgene and the challenging virus and the level of protection conferred. Following up on the above study, Namba and associates (1991) used the CP gene of WL-CMV (subgroup II) to obtain a higher level of resistance to all three strains tested by Quemada and associates (1991). Encouraged by these results, several groups then used the same WL-CMV CP construct in other crops, with some-

Table 1. Reports of plants transgenic for *Cucumber mosaic virus* (CMV) coat protein (CP) where resistance was analyzed

Reference	Donor virus strain (subgroup) transgene	Host species	Challenging virus strain (subgroup)	Inoculum ^a	Degree of resistance (%) ^b
Tumer et al. 1987a	D (I)	Tobacco	D (I)	Virions 25 µg/ml	40 to 90
Cuozzo et al. 1988	D (I)	Tobacco	C (I)	Virions 25 µg/ml	0 to 100
Namba et al. 1991	WL (II)	Tobacco	C Chi (I) WL (II)	Sap	100 65 to 85 75 to 85
Quemada et al. 1991	C (I)	Tobacco	C Chi (I) WL (II)	Sap or aphids	100 85 55
Gonsalves et al. 1992	C (I)	Cucumber	Cat (I), NI	Sap, NI	86 to 100
Yie et al. 1992	O (I)	Tobacco	B	Virions 25 µg/ml	40
Nakajima et al. 1993	O (I)	Tobacco	O, Y (I), TAV ^c	Virions 1 to 50 µg/ml	0 to 100
Okuno et al. 1993a,b	Y (I)	Tobacco	Y, O (I), Pepo, CS, FT	Virions, RNA 1 to 100 µg/ml	0 to 100
Gonsalves et al. 1994	WL (II)	Melon	Fny (I)	Sap	Delay
Xue et al. 1994	WL (II)	Tomato	Chi (I) WL (II)	Sap	80 to 100 91 to 100
Provvidenti and Gonsalves 1995	WL (II)	Tomato	9 strains (I) 3 strains (II)	Sap	100
Tricoli et al. 1995	C (I)	Squash	C (I)	Sap	92 to 100
Gielen et al. 1996	ZU (I)	Tomato	CMV-I17F (I), CMV-ARN5 (I), CMV-A (II), NI	Sap NI	50 to 100 45 to 100
Fuchs and Provvidenti 1996	WL (II)	Tomato	Fny (I)	Sap, NI	100
Rizos et al. 1996	CP91/367, SB91/366 (I)	Tobacco	CP91/367, SB91/366 (I) LU91/166 (II)	Sap	0 to 66 37 to 83
Singh et al. 1998	C (I)	Tobacco	3 strains (I) 5 strains (II)	Sap Graft	0 to 80 0 to 80
Kaniewski et al. 1999	D, 22 (I), PG (II)	Tomato	22 (I), PG (II)	Virions 100 µg/ml or aphids	70 to 100
Tomassoli et al. 1999	D, 22 (I), PG (II)	Tomato	22 (I), NI	NI	0 to 100
Jacquemond et al. 2001	R (II)	Tobacco	R (II)	Virions 5 to 10 µg/ml, aphids, RNA 5 to 10 µg/ml	Recovery
Shin et al. 2002a	Kor	Pepper	Kor	ND	10 to 90

^a NI = natural infection in the field and ND = no data.

^b Percentage of the number of noninfected plants over total number of inoculated plants.

^c In the original paper, *Tomato aspermy virus* (TAV) is reported as *Chrysanthemum mild mottle cucumovirus* (CMMV).

what varying degrees of success. For example, the WL-CMV CP construct conferred a broad resistance to a large number of CMV subgroup I and II strains in tomato (Fuchs et al. 1996; Provvidenti and Gonsalves 1995; Xue et al. 1994) and cucumber (Gonsalves et al. 1992), but only weak protection in melon (Gonsalves et al. 1994). In squash, on the other hand, the efficacy of the C-CMV CP gene was demonstrated by the creation of the triply resistant line CZW-3, in which expression of the three corresponding CP genes confers resistance not only to CMV but also to *Zucchini yellow mosaic virus* and *Watermelon mosaic virus* (Quemada 1998; Tricoli et al. 1995). In 1996, line CZW-3 became the second VRTP to be deregulated for commercial use in the United States. In the ensuing years, other transgene constructs designed to induce CPMR to CMV have been introduced into a number of plant species (Table 1), some of which have been further tested in both the laboratory and the field with the aim of assessing levels of resistance (Fuchs et al. 1996; Gielen et al. 1996; Jacquemond et al. 2001; Tricoli et al. 1995), the fitness of the virus-resistant crop plants (Fuchs et al. 2004b; Gonsalves et al. 1992; Tomassoli et al. 1999), the risks that could be mediated by gene flow to wild populations (Fuchs et al. 2004a), potential impacts of messenger or viral RNA recombination (Pierrugues et al. 2007; Turturo et al. 2008), and food safety (Chen et al. 2003).

In conclusion, CPMR has been shown to be a useful tool in the development of new CMV-resistant plants, although different qualities of resistance were observed depending on the donor strain, the challenging strains, and the plant species. Indeed, considering the number of publications, it is remarkable that it is still impossible to predict which kind of CP gene construct, from which subgroup, will be most effective. As mentioned above, the C-CMV CP gene gave quite different levels of resistance in different host plants. When very similar CP transgenes were created from nearly identical subgroup I donor strains, in some cases, high levels of resistance were observed in tobacco, as with C- or D-CMV, whereas, in others, there was absolutely no resistance, such as in the tobacco lines expressing high levels of CP of I17F-CMV (Jacquemond et al. 2001). The range of resistances obtained also can vary from complete susceptibility to extreme resistance, even in lines obtained with the same transgene (Table 1). Therefore, in order to have a clearer picture of the effectiveness of any transgene in conferring resistance, it is essential to screen a large number of lines. In an attempt to bring some clarity to this rather confusing situation, in the following section we will discuss the current state of knowledge regarding the mechanisms of CPMR to CMV.

Mechanisms of CPMR to CMV.

Regarding CMV, there are no cases described of resistance in the absence of transgene mRNA or CP (Cuozzo et al. 1988; Jacquemond et al. 2001; Namba et al. 1991; Okuno et al. 1993a and b; Quemada et al. 1991; Tricoli et al. 1995; Tumer et al. 1987a). Also, in many but not all cases, the most resistant lines were those that accumulated the highest levels of CP, which would be expected for protein-mediated resistance (Kaniewski et al. 1999; Okuno et al. 1993a; Shin et al. 2002b). In addition, considering the level of CP gene sequence identity between subgroups I and II (approximately 70%), it is unlikely that PTGS could be involved in resistance to subgroup II strains observed in plants expressing a CP gene from a subgroup I strain, and vice versa (Namba et al. 1991; Provvidenti and Gonsalves 1995; Quemada et al. 1991; Xue et al. 1994).

Two groups have clearly demonstrated cases of CPMR to CMV that was not mediated by PTGS. Jacquemond and associates (2001) described plants expressing the CP of R-CMV (subgroup II), which displayed resistance by recovery. If resistance in these plants was due to PTGS, the transgene-derived

viral RNAs also would have been targeted for degradation along with the RNA of the challenging virus. However, they showed that the level of the transgene mRNA remained constant or increased in the inoculated resistant plants, which is clearly incompatible with a PTGS-based mechanism (Jacquemond et al. 2001). Another interesting study was reported by Okuno and associates (1993a and b). They showed that plants expressing a Y-CMV CP gene were resistant to Y-CMV infection when challenged with either virions or viral RNAs, whereas protoplasts were resistant to virions but susceptible to RNA inoculation (Okuno et al. 1993a and b). Thus, this situation is different from the protein-mediated resistance in plants expressing a TMV CP gene, which are resistant to inoculation by virions but susceptible when inoculated with RNA or partially stripped virions (Register and Beachy 1988). Significantly, the resistance described by Okuno and associates (1993b) was also temperature sensitive, being markedly reduced above 30°C, which coincided with a reduction in transgene-derived CP levels but not in RNA levels, as also has been shown for TMV (Nejdat and Beachy 1989). The TMV results suggest that the CP interferes with the very earliest steps of infection (i.e., the initial decapsulation) whereas, with CMV, the results of Okuno and associates (1993a and b) suggest a blockage at a somewhat later step in the infection cycle. They proposed that the transgene-derived CP blocked interaction between the viral RNA and the viral CP, thereby inhibiting its transit through the plant (Okuno et al. 1993a and b).

Such observations concur well with the CMV CP's essential role in cell-to-cell and systemic CMV movement (Canto et al. 1997; Kaplan and Palukaitis 1998; Taliansky and Garcia-Arenal 1995). However, interference with CMV movement does not take into account the observations with protoplasts (Okuno et al. 1993a and b) which, as mentioned above, were resistant to virions but susceptible to RNA inoculation; this suggests at least some contribution related to decapsulation, as has been described for TMV. In fact, there also is evidence that interference with movement also contributes—albeit in a minor way—to CPMR to TMV (Wisniewski et al. 1990). Therefore, the apparent differences in mechanism between CPMR to TMV and to CMV may, in fact, reflect differences in the relative contributions of the two mechanisms. For TMV, it has been shown clearly that interference with decapsulation depends on rather subtle differences in protein interactions (Bendahmane et al. 2007); therefore, this could well explain the strain-specific differences in CPMR to CMV. Similarly, considering that movement of CMV requires interactions between the CP, viral movement protein, and host proteins, it is to be expected that, in addition, this type of CPMR would be highly sensitive to differences in the sequence of the pertinent host proteins.

Replicase-mediated resistance to CMV.

In 1990, Zaitlin's group demonstrated in serendipitous fashion that the TMV gene encoding the putative 54-kDa protein when transferred into tobacco conferred resistance to TMV (Golemboski et al. 1990). The resistance was overcome neither by high inoculum loads nor when challenged with RNA. Two years later, the same lab showed that a similar approach could be developed for CMV. In this case, the RNA 2 transgene was engineered so that the 2a gene (of subgroup IA Fny-CMV) was truncated by some 30% by internally deleting a region surrounding the highly conserved GDD motif (Anderson et al. 1992) (Table 2). In the resulting resistant transgenic tobacco lines, both the defective RNA transcript (Anderson et al. 1992) and protein were detectable (Carr et al., 1994). The resistance obtained was durable, even when challenged with high inoculum doses of either virion or RNA, but was overcome by three

subgroup IB strains and four subgroup II strains when inoculated either mechanically or by aphids (Zaitlin et al. 1994). This same construct also was shown to be effective in conferring resistance in tomato against both subgroup IA and IB strains (Gal-On et al. 1998). In another study, plants transgenic for RNA 1 of Fny-CMV were able to complement accumulation of RNAs 2 and 3 and were susceptible to virus infection. Yet the R₃ generation progeny of one line that showed variable susceptibility was found to be resistant, with a marked decrease in the levels of viral RNA 1 in the inoculated leaf (Canto and Palukaitis 1998; Gal-On et al. 1998).

Mechanisms of replicase-mediated resistance.

Unlike for TMV replicase-mediated resistance, where it was shown that expression of the 54-kDa protein was necessary to induce resistance (Carr et al. 1992), for CMV replicase-mediated resistance, the question of whether the effector is a protein or nucleic acid has not been answered so conclusively. Most of the work carried out to determine the mechanism has been on the truncated CMV 2a protein (Anderson et al. 1992). When these plants were inoculated with high virus concentrations, local chlorotic lesions were observed in the inoculated leaves of highly resistant lines which, upon analysis, were shown to contain very low levels of the infecting viral replicase, CP, and RNA (Carr et al. 1994). There was no systemic infection, because the virus was blocked at the bundle sheath or vascular parenchyma and companion cell interface (Wintermantel et al. 1997). In microinjection assays, the movement component to the resistance was shown to be due to an inability of the viral

movement protein to both potentiate trafficking of viral RNA and increase the size exclusion limit of plasmodesmata (Nguyen et al. 1996). Contrary to these results, a study using a viral construct tagged with green fluorescent protein found no such restriction in virus cell-to-cell movement (Canto and Palukaitis 1999). Analysis of systemic viral movement showed that transgenic resistant scions grafted onto nontransgenic rootstocks infected with Fny-CMV did become infected, but with much reduced levels of viral RNA (Wintermantel et al. 1997), comparable with those found in transgenic protoplasts (Carr et al. 1994). This is in contrast to grafting experiments using the same transgene construct in tomato, where virions were unable to move into the transgenic scion (Gal-On et al. 1998). In protoplasts, accumulation of viral RNAs was up to 70-fold less than in nontransgenic plants, pointing to a resistance mechanism that worked primarily at the cellular level by suppressing virus replication but, to lesser extent, by impeding systemic movement (Carr et al. 1994). By using a pseudorecombinant between Fny-CMV and a subgroup I strains that overcame resistance, K-CMV (Zaitlin et al. 1994), the breakdown of resistance was shown to map to RNA 2, of which there is 92.4% identity between the two strains (Hellwald and Palukaitis 1994, 1995). Chimeric RNA 2 constructs generated between Fny- and K-CMV, and subsequently modified to alter nucleotide but not amino acid sequences, indicated that RNA is the target for the resistance mechanism (Hellwald and Palukaitis 1995). Nevertheless, attempts to identify the elicitor of resistance tend to point to a protein rather than RNA model; Wintermantel and Zaitlin (2000) found only 1.6% resistant

Table 2. Reports of plants transgenic for *Cucumber mosaic virus* (CMV) replicase component (either RNA 1, RNA 2, or both)

Reference	Donor virus strain (subgroup) transgene	Host species	Challenging virus strain (subgroup)	Inoculum	Degree of resistance (%) ^a
Anderson et al. 1992	Fny (IA) RNA2 with truncated 2a ^b	Tobacco	Fny (IA)	Virions 500 µg/ml RNA 50 µg/ml	0 to 56 64
Zaitlin et al. 1994	Fny (IA) RNA2 with truncated 2a	Tobacco	11 strains (IA)	Sap Virions ≤ 500 µg/ml RNA 50 µg/ml ^c	100 100 100
	Fny (IA) RNA2 with truncated 2a	Tobacco	3 strains (IB)	Sap Virions 200 µg/ml RNA 200 µg/ml	0 to 100 90 to 100 100
	Fny (IA) RNA2 with truncated 2a	Tobacco	4 strains (II)	Sap Virions ≤ 100 µg/ml	0 0
	Fny (IA) RNA2 with truncated 2a	Tobacco	PSV, TAV ^d	Sap	0
Hellwald and Palukaitis 1994	Fny (IA) RNA2 with truncated 2a	Tobacco	K (IB)	RNA	0
	Fny (IA) RNA2 with truncated 2a	Tobacco	Fny (IA)	RNA	100
	Fny (IA) RNA2 with truncated 2a	Tobacco	F1K2F3 ^e	RNA	0 to 13
Suzuki et al. 1996	Y(IA) RNA1	Tobacco	Y(I)	Virions ≤ 5 µg/ml RNA 0.5 µg/ml RNA 200 µg/ml	0 14 0
	Y(IA) RNA2	Tobacco	Y(I)	Virions ≤ 5 µg/ml RNA 0.5 µg/ml RNA 200 µg/ml	0 14 0
	Y(IA) RNAs 1 and 2	Tobacco	Y(I)	Virions ≤ 5 µg/ml RNA ≤ 200 µg/ml	0 to 40 0 to 77
	Fny (IA) RNA2 with truncated 2a	Tomato	Fny (IA), K (IB)	Virions ≤ 1 mg/ml, aphid	100
Gal-On et al. 1998	Fny (IA) RNA2 with truncated 2a	Tomato	LS (II)	Virions ≤ 1 mg/ml Aphid	7 to 67 27 to 71
	Fny (IA) RNA1	Tobacco	Fny (IA)	Virions 500 µg/ml	50
Canto and Palukaitis 1998	Fny (IA) RNA1	Tobacco	F1F2M3 ^f	Sap	0 to 100
	Fny (IA) RNA2 with truncated 2a	Tobacco	Fny (IA)	Sap, virions 50 µg/ml	ND
	Fny (IA) RNA2 with restored 2a	Tobacco	Fny (IA)	Sap, virions 50 µg/ml	ND
	Fny (IA) RNA2 with nontranslatable 2a	Tobacco	Fny (IA)	Sap, virions 50 µg/ml	ND

^a Percentage of the number of noninfected plants over total number of inoculated plants. ND = no data.

^b Data shown for original lines 1-2 and 1-8 and subsequent generations (Anderson et al. 1992).

^c Some strains were inoculated as either sap or virions or RNA.

^d *Peanut stunt virus* and *Tomato aspermy virus*.

^e Pseudorecombinant containing RNAs 1 and 3 from Fny-CMV (I) and RNA2 from K-CMV (I).

^f Pseudorecombinant containing RNAs 1 and 2 from Fny-CMV (I) and RNA3 from M-CMV (II).

lines with plants transformed with a noncoding CMV RNA 2 construct, compared with 6.6 and 11.9% in plants transformed with constructs containing truncated or full-length gene products, respectively.

An additional mechanism has been proposed for tobacco plants transgenic for full-length RNAs 1 and 2 of Y-CMV, whereby an excess in the levels of both RNAs causes a reallocation of CP away from RNAs 3 and 4 and results in the latter's failure to be effectively encapsidated (Suzuki et al. 1996). Curiously, in the same study, plants containing single transgenes of RNA 1 or RNA 2 were as susceptible to infection as nontransgenic plants. In R₃ plants transgenic for RNA 1, resistance was obtained whereby no systemic viral infection occurred. However, in these same plants, it was found that noninoculated leaves not only could complement the replication of RNAs 2 and 3 but also were susceptible to virus infection; results which exclude a systemically acquired PTGS-mediated resistance mechanism (Canto and Palukaitis 1998). Further analysis of the potential mechanisms of resistance showed, similar to that for RNA 2, specific homologous suppression of accumulation of viral RNA at the cellular level

and a restriction in virus long-distance movement (Canto and Palukaitis 2001). Therefore, it appears that, despite the absence of data regarding siRNAs, although PTGS may play a secondary role, there is no clear evidence that it is the primary mechanism of CMV replicase-mediated resistance. In particular, in the plants expressing a modified 2a protein, it is more probable that resistance is conferred essentially by the protein acting as a dominant negative mutant.

Resistance to CMV mediated by PTGS.

It had been shown by Wang and associates (2000) that enhanced PTGS-mediated resistance could be obtained with an inverted-repeat transgene encoding a hairpin RNA. In the first clear demonstration of PTGS-mediated resistance to CMV, Kalantidis and associates (2002) transformed tobacco with a construct containing an inverted-repeat of a 747-bp fragment of the CP of CMV and a 1,445-bp spacer, in both a sense and antisense orientation. Their results showed that lines with two or more transgenes were more likely to become resistant, whereas levels of siRNAs, which increased with increasing temperature (25 to 32°C), correlated positively to levels of

Table 3. Reports of transgenic plants produced for *Cucumber mosaic virus* (CMV) resistance using alternative strategies

Strategy, reference	Transgene (donor)	Host species	Challenging pathogen (virus subgroup) ^a	CMV inoculum	CMV degree of resistance (%) ^b
<i>RNA satellites</i>					
Harrison et al. 1987	Sat-I17N	Tobacco	CMV-K (I)	Sap	80 to 95 ^c
Jacquemond et al. 1988	Sat-I17N	Tobacco	CMV-I17F (I)	Sap	70 to 74 ^d
Yie et al. 1992	Sat-RNA1	Tobacco	CMV-B (I)	Virions 25–50 µg/ml	0 to 20
	Sat-RNA1 + CP (CMV-O)				70 to 75
Peña et al. 1994	Sat-S	Tobacco	CMV-1	Virions 50 µg/ml	0 to 75
Kim et al. 1997	Sat-I17N	Pepper	CMV-Y (I), CMV-Kor (I)	Virions 10 µg/ml	70 to 80 ^e
Stommel et al. 1998	Sat-S	Tomato	CMV-1	Virions 20 µg/ml	40 to 84 ^f
<i>Antisense RNAs</i>					
Tumer et al. 1987a	(CMV-D)	Tobacco	CMV-D (I)	Virions 1 µg/ml	60
				Virions 15 µg/ml	0
Ali Rezaian et al. 1988	(CMV-Q)	Tobacco	CMV-Q (II)	Virions 0.5–50 µg/ml	0
Cuozzo et al. 1988	(CMV-D)	Tobacco	CMV-C (I)	Virions 1 µg/ml	≈70
				Virions 5–25 µg/ml	0
<i>RIP</i>					
Lodge et al. 1993	PAP (<i>Phytolacca americana</i>)	Tobacco, potato	CMV, PVY, PVX	Virions 50 µg/ml	67 ^g
Krishnan et al. 2002	TCS (<i>Trichosanthes kirilowii</i>)	Tobacco	CMV, TMV	Sap	DS ^g
<i>Ribonucleases</i>					
Watanabe et al. 1995	<i>pac1</i> (Yeast)	Tobacco	CMV-Y (I), TMV, PVY	Virions 10 µg/ml	DS ^g
Ogawa et al. 1996	2-5Aase + RNaseL	Tobacco	CMV-Y (I)	Virions 15 µg/ml	ND
Honda et al. 2003	2-5Aase + RNaseL	Tobacco	CMV-Y (I)	Virions 100 µg/ml	ND
<i>Enhancement of HR/SAR</i>					
Ogawa et al. 1996	2-5Aase + RNaseL	Tobacco	CMV-Y (I)	Virions 15 µg/ml	ND
Shin et al. 2002b	<i>Tsi1</i> (Tobacco)	Pepper	CMV-Y, PMMV, 1 bacteria, 1 oomycete	Sap	22 ^{c-g}
Honda et al. 2003	2-5Aase + RNaseL	Tobacco	CMV-Y (I)	Virions 100 µg/ml	ND
<i>Hammerhead ribozyme</i>					
Kwon et al. 1997	Conserved sequences of RNA1 and 2 of CMV-Y	Tobacco	CMV-Y (I)	Virions 10 µg/ml	10 to 85
<i>Plantibodies</i>					
Villani et al. 2005	ScFv antibodies ^h	Tomato	CMV-F100 (I), CMV-CAR (II)	Sap	≈50 (CMV-F100)
Aebig et al. 2006	ScFv antibodies ^h	Tobacco	CMV	ND	DS ⁱ

^a PVY = *Potato virus Y*, PVX = *Potato virus X*, TMV = *Tobacco mosaic virus*, PMMV = *Pepper mild mottle virus*, bacteria = *Xanthomonas campestris*, and oomycete = *Phytophthora capsici*.

^b Percentage of number of noninfected plants over total number of inoculated plants. ND = no data and DS = delayed symptoms.

^c Decrease in coat protein amount.

^d Average reduction in the coat protein yield in leaves of transgenic plant compared with nontransformed.

^e Amount of local lesions in *Chenopodium quinoa*.

^f Calculated on marketable yield.

^g Referred to CMV.

^h Single-chain variable fragments.

ⁱ Lost in subsequent generations.

resistance, though levels of hairpin transcript were variable (Kalantidis et al. 2002). More recently, using inverted-repeat CP or RNA 2 sequences of LILY-CMV (subgroup I) separated by an *Arabidopsis* actin2 intron spacer, R₁ transgenic progeny were produced that were 100% resistant (Chen et al. 2004). Comparing this with single constructs (without inverted repeats), it was found that, whereas 20% of the RNA 2 R₁ progeny were resistant, no CP R₁ lines were resistant. All resistant plants produced readily detectable amounts of siRNAs specific to LILY-CMV, the immunity observed being completely overcome by a subgroup II strain (Chen et al. 2004). Because the CMV 2b gene is a potent suppressor of PTGS, the absence of PTGS-mediated resistance in CP—and perhaps also replicase—constructs may be attributed to activity of the 2b gene.

A more recent attempt to confer resistance to CMV using a small RNA pathway focused on transgenic artificial microRNAs (miRNA) that targeted a 21-nucleotide sequence within the 2b gene by using an inverted-repeat construct (Qu et al. 2007). When expressed in *Arabidopsis*, 64% of the plants were resistant to the target strain. This work, along with that of Niu and associates (2006), opens up the possibility of developing even smaller transgenes that target specific pathways of the small RNA regulatory network.

CMV satellite-RNA-mediated resistance.

The discovery of CMV satellite RNAs (satRNAs) in the 1970s came about because of their involvement in various outbreaks of lethal necrosis in tomato in southern Europe (Simon et al. 2004). In spite of this, subsequent work showed that the majority of CMV satRNAs attenuate rather than increase symptoms, and even those that cause lethal necrosis in tomato cause attenuation in tobacco and other species. The ability of satRNAs to attenuate CMV symptoms has been used to protect plants from severe symptoms by preinoculating plants with satRNA prior to infection with CMV (Jacquemond 1982; Montasser et al. 1998; Sayama et al. 1993), a technique that was developed for commercial use in both Japan (Sayama et al. 1993) and China (Stommel et al. 1998) (Table 3). Similarly, plants transgenic for CMV satRNA developed attenuated symptoms in tobacco (Harrison et al. 1987; Jacquemond et al. 1988; Yie et al. 1992), petunia (Kim et al. 1995), pepper (Kim et al. 1997), and tomato (McGarvey et al. 1994; Stommel et al. 1998). However, concerns were voiced quite early about the overall safety of such an approach on account of the necrogenic properties of certain satRNAs. These concerns were reinforced when it became apparent that only very minor sequence differences distinguish necrogenic from benign satRNAs (Jacquemond and Tepfer 1998). In addition, in passage experiments with tobacco it was found that not only could a shift from benign to necrogenic occur spontaneously but also that the latter phenotype could rapidly dominate the satRNA population within the host (Palukaitis and Roossinck 1996). With such a potential risk, albeit laboratory based, the use of transgenic satRNAs has, for all intents and purposes, been abandoned (Jacquemond and Tepfer 1998).

Transgenes based on nonviral sources of resistance.

Besides the major strategies described above, which are based on pathogen-derived resistance, some effort also has been devoted to resistance derived from nonviral sources. For CMV, these include ribosome inactivating proteins, ribonucleases, ribozymes, pathogenesis-related proteins, and plant expressing antibodies directed against viral proteins (Table 3). All of these strategies were covered in sufficient detail in a recent review (Prins et al. 2008) and, thus, will not be described here, particularly because there are no CMV-specific features of these types of resistance. Although some of them possibly could lead to broad-range resistance to different viruses, most also have seri-

ous flaws, such as toxicity (e.g., ribosomal inactivating proteins) or induction of undesirable morphological defects (e.g., ribonucleases). The major exception to this is the use of antibodies directed against viral proteins, which can confer strong resistance to the target virus, although the breadth and durability of antibody-mediated resistance will need to be fully investigated.

Concluding remarks.

As we have seen from this survey, virus-derived transgenes continue to be the favored means for conferring virus resistance. However, from the very beginning, transgenic plants expressing viral sequences have been used for two purposes; not only do they often confer virus resistance but they also are a powerful tool for understanding plant-virus interactions. However, in spite of the number of publications that have appeared, we still have a limited understanding of the mechanism or mechanisms of resistance involved, except when resistance is PTGS mediated. Considering the numerous viruses that have been targeted by CP transgenes, it is striking that only for TMV has protein-mediated resistance been well characterized. In the case of plants expressing a CMV CP gene, there is an apparent lack of evidence for PTGS-mediated resistance and some suggestion that interference with virus movement is primarily involved. Unfortunately, this leaves us today in a situation where creating VRTP remains highly empirical. This problem can be overcome only through a clearer understanding of at least two things: how modifications in CMV-host interactions in VRTP lead to resistance and why a given transgene confers different degrees of resistance in different plant species.

From a biotechnological point of view, it is highly desirable to create CMV resistance with the following characteristics: efficacy against all strains of the target virus and durability in the field, while incorporating all the features that will minimize biosafety concerns. From the point of view of efficacy, in many cases, complete resistance to the diversity of strains in both subgroups of CMV may require two transgenes, one targeting each subgroup, or otherwise a single gene that will induce PTGS to members of both subgroups. Concerning the durability of resistance, our present knowledge is insufficient, because the only CMV-resistant VRTP that has been grown at a scale greater than small field trials is the CZW-3 squash line. Although there have been no reports of resistance breakdown in CZW-3, it is grown only on a modest scale in the United States; therefore, we cannot conclude that the resistance has been challenged in the field by the full range of CMV strains to which it might be exposed, particularly on other continents. PTGS-mediated resistance has many favorable features; therefore, it would be extremely important to determine the durability of this type of resistance, because one could suspect that fairly minor sequence variations could lead to resistance-breaking strains. In order to minimize this potential problem, it is particularly important to target regions of the viral genome in which the minimum sequence variability is observed. In the comparison between PTGS- and CP-mediated resistance, one of the favorable features of the former is the absence of detectable transgene mRNA, because this eliminates messenger and viral RNA recombination as a source of potentially novel recombinants. However, it has been shown recently that the populations of recombinant CMV RNA3 molecules observed in transgenic plants expressing a CMV CP gene are not different from those in nontransgenic controls, suggesting that recombination should not be an issue for plants expressing a CMV CP gene (Turturo et al. 2008). Therefore, at this time, there are no compelling reasons to favor either type of resistance although, in the future, the issue of field durability of the different types of resistance may give a clear advantage to one resistance strategy over the other.

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