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Review

Strategies to protect crop plants against viruses: Pathogen-derived resistance blossoms

(coat protein-mediated protection/replicase-mediated protection/satellite RNA-mediated symptom attenuation/defective interfering RNA or DNA protection/sense–antisense-ribozyme RNA-mediated protection)

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ABSTRACT **Since 1986, the ability to confer resistance against an otherwise devastating virus by introducing a single pathogen-derived or virus-targeted sequence into the DNA of a potential host plant has had a marked influence on much of the research effort, focus, and short-term objectives of plant virologists throughout the world. The vast literature on coat protein-mediated protection, for example, attests to our fascination for unraveling fundamental molecular mechanism(s), our (vain) search for a unifying hypothesis, our pragmatic interest in commercially exploitable opportunities for crop protection, and our ingenuity in manipulating transgene constructions to broaden their utility and reduce real or perceived environmental risk issues. Other single dominant, pathogen-derived plant resistance genes have recently been discovered from a wide variety of viruses and are operative in an ever-increasing range of plant species. Additional candidates seem limited only by the effort invested in experimentation and by our ingenuity and imagination. This review attempts to consider, in a critical way, the current state of the art, some exceptions, and some proposed rules. The final impression, from all the case evidence considered, is that normal virus replication requires a subtle blend of host- and virus-coded proteins, present in critical relative concentrations and at specific times and places. Any unregulated superimposition of interfering protein or nucleic acid species can, therefore, result in an apparently virus-resistant plant phenotype.**

SOME RECENT HISTORY

The concept (1–3) and reality (4) of creating virus-resistant crops by genetically engineering them to express part of a viral genome or virus-associated sequence evolved from empirical observations on the use of mild, symptomless or attenuated strains of viruses to protect field crops such as tomato, apple, citrus, or papaya against closely related but severely pathogenic virus strains (3, 5).

Despite extensive and sometimes elegant experimentation, the molecular mechanism(s) of this viral "cross-protection" have remained elusive and controversial. In some cases, the coat protein (CP) of the protectant virus was thought to be primarily responsible, either by preventing particle disassembly or by re-encapsidating the incoming genome of the more severe challenge virus. However, viroids [240- to 380-nt-long, naked circular single-stranded (ss)RNA pathogens] and mutant viruses making assembly-defective or no detectable CP could also cross-protect against their more severe relatives. Such observations prompted models based on inhibitory interactions between sense and antisense RNAs or between the replicational machineries of the two competing pathogens (6). Controversy arose largely because available molecular technology could not resolve which regulatory or coding sequence(s) or polypeptide product(s) from the actively replicating genome of the primary (protectant) pathogen were responsible for interfering with the many replicative processes essential to establish infection by the secondary, related, and more severe virus. In contrast, multiple infections by *unrelated* viruses sharing a common host are very prevalent in nature. Many aspects of this older story have their parallels in current hypotheses and lack of a unified model for pathogen-derived resistance in transgenic plants.

The advent of improved cell and tissue culture techniques, efficient protocols for *Agrobacterium tumefaciens*-mediated transformation and plantlet regeneration in dicotyledonous species (7) [and more recent methods for monocot crops (8–10)], has permitted, among other applications (11, 12), the theory of pathogen-derived virus resistance to be tested in practice.

Collaboration between researchers at Monsanto and Washington University (St. Louis) led to the first report of CP-mediated protection (CPMP) against tobacco mosaic virus (TMV) in tobacco in 1986 (4). Since then, CPMP has been reported for over 20 viruses in at least 10 different taxonomic groups, in a wide variety of dicot plant species, and the list is increasing rapidly. To date, no monocot–virus–CPMP system has been reported, presumably due to technical difficulties with monocot transformation/regeneration, for no fundamental plant physiological or anatomical reasons are predicted to exclude CPMP in these systems. Several groups are attempting CPMP against important virus diseases of rice (rice tungro spherical virus and rice stripe tenuivirus; J. W. Davies and P. Tien, personal communications) and maize. So far, all examples of CPMP have involved viruses with genomes of positive (messenger)-sense ssRNA (for review, see refs. 13–20)—with two exceptions, both predominantly negative-strand RNA viruses having partially ambisense translation strategies, tomato spotted wilt virus [TSWV (21–23)] and rice stripe tenuivirus.* TSWV is a serious pathogen with a very broad host range and is the type species of the new tospovirus genus in the arthropod-borne family *Bunyaviridae* (23). Despite some conflicting claims, available data show little or no evidence for CPMP against plant viruses with double-stranded (ds)DNA or ss-DNA genomes: the caulimo-, badna-, and geminiviruses. Indeed, transgenic *Nicotiana tabacum* plants expressing the CP of abutilon mosaic geminivirus show virus-like symptoms proportional to the level of CP expressed, and no protection against abutilon mosaic geminivirus (H. Jeske, personal communication). No ef-

Abbreviations: CP, coat protein; ss- and ds-, single-stranded and double-stranded, respectively; satRNA, satellite RNA; DI, defective interfering; CPMP, CP-mediated protection; MP, movement protein; mAb, monoclonal antibody; TMV, tobacco mosaic virus; TSWV, tomato spotted wilt virus; PLRV, potato leafroll virus; AlMV, alfalfa mosaic virus; CMV, cucumber mosaic virus; PVX and PVY, potato virus X and Y, respectively; TEV, tobacco etch virus; PEBV, pea early browning virus; BMV, brome mosaic virus; TRSV, tobacco ringspot virus.

*Hayakawa, T., Zhu, Y., Itoh, K., Kimura, Y., Izawa, T., Shimamoto, K. & Toriyama, S., Third International Congress of the International Society of Plant Molecular Biologists, October 1991, Tucson, AZ, abstr. 1153.

fort has been made at CPMP with cauliflower mosaic virus CP, but work is underway to transform rice with the CP gene of rice tungro bacilliform virus (a dsDNA badnavirus and the second component of the devastating rice tungro disease complex; J. W. Davies, personal communication). Detailed observations and postulated mechanisms for CPMP are discussed further below.

Pathogen-derived molecular interference with challenge virus replication using virus-sense, cis-acting regulatory sequences was demonstrated *in vitro* with the 3′ terminus of turnip yellow mosaic virus RNA (24), and experiments with transformed plants are in progress (A.-L. Haenni and M. Tepfer, personal communication).

Since 1990, some lines of plants transformed with a variety of nonstructural virus-coded proteins {e.g., RNA-dependent RNA polymerase (replicase) holoenzymes or subdomains, cell-to-cell movement proteins (MPs), or other putative RNA-binding proteins [e.g., potato leafroll virus (PLRV) 17-kDa P4]} have shown excellent resistance to challenge with high levels of a homologous or related virus, or viral RNA. However, this phenomenon is not applicable to all viruses examined [e.g., alfalfa mosaic virus (AlMV)] especially when expressing native (and functional) replicase protein(s). The likelihood of obtaining virus-resistant transgenic plants seems to increase when only a replicase subdomain is expressed or if the viral protein (MP, P4, or replicase) is made defective through some cloning accident or by design (refs. 25–34; J. G. Atabekov, D. Baulcombe, W. O. Dawson, J. Donson, C. Hemenway, C. Kearney, W. Rohde, and T. Turpen, personal communications).

Other proven or proposed single, dominant, pathogen-derived resistance "genes" or pathogen-targeted transgenic resistance strategies involve defective interfering (DI) viral sequences, symptom-attenuating satellite RNAs (sat-RNAs), defective viral (auto)protease(s), antiviral ribozymes, antisense RNA or antibodies, or "dormant" antisense phytotoxin (suicide) genes such as ricin, pokeweed antiviral protein, or diphtheria toxin connected to negative-strand virus replication signals (e.g., the CP subgenomic RNA promoter). These are also discussed briefly below.

ON THE NEED FOR NON-HOST-GENE-MEDIATED RESISTANCE STRATEGIES

Individually, most plant species are nonhosts (i.e., resistant) to the majority of the 675 or more plant viruses currently identified (35). In most cases, we do not understand, and so cannot exploit, the molecular biology of this incompatibility. The same is often true even where there is thought to be an active role for the plant (i.e., a dominant host resistance gene) in some accessions of an otherwise susceptible species. Conversely, all crops are susceptible to significant yield and quality losses caused by one or more viruses. This situation has encouraged growers and government agencies alike to adopt or enforce several preventive but imperfect disease-control strategies and phytosanitory regulations: (*i*) planting only certified virus-free perennial stock and eradicating diseased plants; (*ii*) adopting cultural practices to minimize epidemics, including the repeated and extensive use of expensive chemicals to control virus vectors (predominantly insects, less so for soil-inhabiting nematodes or fungi) and to remove possible reservoirs of infection (weeds or feral crop species); or (*iii*) breeding natural resistance against the virus (or its vector) into the crop.

The last strategy, although most desirable and durable in the long term, is at best empirical and can require a prohibitive investment of time and labor, particularly given the plasticity of plant viral genomes and the presence of resistance-breaking virus isolates. We know little to nothing about the genetics of vector–plant feeding preferences and interactions. Insect or nematode antifeedant compounds or unfavorable leaf-surface morphologies are likely to be controlled multigenically. Limited evidence suggests that no resistance exists *per se* to fungal vectors of viruses. Most host-gene-mediated virus resistance is conferred by single dominant genes (36), but none has yet been cloned or sequenced, although efforts in restriction fragment length polymorphism mapping, transposon tagging, and chromosome-walking techniques are closing in on several (e.g., *Tm-1*, *Tm-2*, *Tm-2²* in tomato; *Rx*, *Ry* in potato; N, N′ in tobacco). Studies on *Tm-1* or *Tm-2* and *Tm-2²* resistance-breaking mutants of tomato mosaic virus have mapped point mutations in genes associated with tomato mosaic virus RNA replication or cell-to-cell movement, suggesting these as the inhibitory site of action of the *Tm-1* or *Tm-2* and *Tm-2²* gene products, respectively. Doubtless, many more virus-resistance genes exist in germ-plasm collections, but these are often in wild species that cannot be incorporated into conventional breeding schemes. Thus the ability to identify, isolate, and introduce (at a single step) a target-specific (virus-derived) resistance gene into unlimited elite varieties of a crop, irrespective of their sexual compatibility, without compromising existing desirable agronomic traits, and avoiding the need for extensive back-crossing, has been an extremely attractive commercial goal. Further conventional crossings could then create different multiple resistance gene combinations.

Natural resistance to viruses is manifest at several levels (37): *true immunity*, where no virus replication takes place at all; *subliminal infection*, where virus replication is restricted to the initially infected cell(s); the *hypersensitive response*, where virus is restricted to cells around each primary site of infection, usually with necrosis. In *tolerant* plants, a mild or symptomless systemic virus infection occurs, although virus replication may be unaffected.

The phenotype of virus-derived resistance in transformed plants can vary, case-by-case, from a simple delay in normal symptom development or partial inhibition of virus replication, to complete immunity to challenge virus or viral RNA inoculation. Even the former can be useful if it allows plants (seed or fruits) to outgrow the infection, avoid the damaging disease, and/or diminish their potential to act as sources of inoculum. On hypersensitive hosts, transgenic resistance can be quantified directly by reduced numbers of local lesions (= bioassay for productive sites of infection). Even a reduction in systemic virus titer should help control field epidemics by reducing the efficiency of vector transmission, as with PLRV CPMP and aphids (38, 39). In this case, PLRV CPMP seems to mimic events in some naturally resistant breeding lines of potato by reducing virus titer and restricting virions to sieve tubes and companion cells of the internal (adaxial) phloem (40).

ON CP-MEDIATED PROTECTION AND PROPOSED MECHANISMS

At the outset it is accurate to say that no consensus model exists for the mechanism of CPMP. The vast literature since 1986 reveals many details unique to each virus–plant–CP system and even some patterns common to several viruses, but recent cases add more exceptions than rules, and prudence dictates a closer examination of the precise nature of the viral gene construct used, transgene position effects, copy number and transcriptional activity, the extent and site of uninhibited virus replication and spread within the host plant, and secondary effects of transgenesis on host cell metabolism and general stress/disease resistance responses. We may then come closer to understanding how CPMP works.

Early CPMP experiments involved transgenic expression of virus-sense CP gene transcripts, frequently with the adjacent 3′-terminal sequence of the viral genome (for review, see refs. 13–20). Nonfunctional (−AUG) CP mRNAs or antisense CP mRNAs were inactive or only weakly active (41–43) if they also contained the antisense 3′-untranslated

genomic sequence (43). A direct correlation appeared between the amount of intact, functional CP expressed *in planta* and the efficacy of CPMP. Higher concentrations of virus inoculum or (partially) unencapsidated viral RNA overcame CPMP. However, these simple rules derived from the first model systems [TMV, tobacco rattle virus, tobacco streak virus, or AlMV in tobacco (4, 44–46)] were quickly broken by cucumber mosaic virus (CMV) and potato virus X (PVX), where antisense CP mRNA provided protection (47, 48), and for PVX and potato virus S [a carlavirus (49)], where resistance to naked RNA inocula occurred.

We can now add to these "classic" cases of CPMP several recent examples, particularly among members of the aphid-transmitted potato virus Y (PVY; potyvirus) and luteovirus (PLRV) groups, and the thrip-transmitted TSWV, where intentionally truncated, antisense or nonexpressing (−AUG) CP genes have been transformed into plants and have provided measurable protection or even complete immunity against the appropriate parent virus (50–58). Recent field tests with the untranslatable tobacco etch virus (TEV) CP RNA lines (50, 51) have shown 100% resistance to high disease pressure (W. G. Dougherty, personal communication). In all these cases (50–58), it may be that some direct form of RNA–RNA interference between the transgene transcript and the challenge virus (especially the low titer PLRV) could account for the resistant phenotype. However, with the truncated TEV CP transgenic plants, it was common for the inoculated leaves to develop symptoms and virus titers similar to control plants, but for the plant to outgrow the infection. This result suggested an interference with virus spread, and some data pointed to the C-terminal domain of the TEV CP as being involved (50). However, an exciting and more generic clue to the mechanism of many of these phenomena has recently been found (W. G. Dougherty, personal communication)—the young, virus-free, and symptomless upper parts of such plants were completely immune to further virus challenge. This "virus-resistant state" may be host-encoded and result from interactions between the transgene and the viral and host genomes. For example, to date, all plant lines transformed to express untranslatable potyviral CP sense or antisense RNAs (TEV, PVY, or zucchini yellow mosaic virus) have contained two or more transgenes (Southern blots). Position effects may permit complementary-sense transcription of one transgene from a host promoter because no attempts were made to provide insulating buffer domains of DNA sequence (59). The resulting transgene-derived dsRNA could then induce an antiviral state in the host (52) or cosuppression (60) of host gene expression and viral replication. Whatever the mechanism, the use of dysfunctional virus-derived CP sequences in transgenic crop monocultures is likely to raise fewer regulatory/risk concerns (see below).

Table 1. Lack of reciprocity in CP-mediated protection

Transgenic plant/ protoplasts expressing	Virus inoculum used	Phenotype observed
U1 CP	U1 virus	Protected
U1 CP	U2 virus	Protected
U1 CP	U1 RNA in U2 CP	Protected
U2 CP	U2 virus	Protected
U2 CP	U1 virus	Infected
AlMV wt CP	AlMV wt	Protected
AlMV wt CP	AlMV Ser-2 → Gly*	Protected
AlMV Ser-2 → Gly	AlMV wt (United States or Strasbourg)	Infected
AlMV Ser-2 → Gly	AlMV Ser-2 → Gly*	Protected
U1 CP	SHMV virus	Infected
U1 CP	SHMV RNA in U1 CP	Infected

SHMV, sunnhemp mosaic tobamovirus.
*AlMV Ser-2 → Gly virions were made by mutating the CP gene in a full-length cDNA of AlMV RNA3 and coinfecting plants with wild-type (wt) AlMV RNAs 1 and 2 (+CP).

Traditional, functional CP-dependent CPMP has been shown to involve interference with the early events of virus disassembly (61–63); however, data also indicate inhibitory effects on later events in the virus replication cycle (62, 64), especially in cases where CPMP occurs against viral RNA inoculum (48, 49). Opportunities for multiple levels of interference will depend upon the precise nature of virus–plant interactions, including cell or tissue specificity, both where the CP is expressed and where the virus replicates, and how natural infections move cell-to-cell or long distance (17). Using tissue-specific promoters for TMV CP expression it has been shown that the level of CP in tobacco epidermal cells is most significant for protection against mechanically inoculated virus (65, 66).

Notable for its failure, tobaccos transformed with the 60-kDa CP precursor of cowpea mosaic comovirus were not protected (even locally) against cowpea mosaic comovirus inoculation, the precursor was not cleaved, and no virus-like capsids were seen (67).

In general, CPMP operates well only against closely related virus strains (e.g., ref. 68). Using a range of Tobamoviruses, protection was detectable when their CPs were ≥60% homologous in amino acid sequence (69). A low but significant degree of CPMP against unrelated viruses has been claimed for transgenic tobacco plants expressing zucchini yellow mosaic virus, TMV, AlMV, soybean mosaic potyvirus, or CMV CP (58, 70–72). It may be that a mechanism independent of CP, such as discussed above for the TEV (−AUG) constructs (50–52), is operative in these hitherto exceptional cases.

As data accumulate, subtle, and as yet inexplicable, complexities in the traditional CPMP systems become apparent. For example, there is a lack of reciprocity in CPMP between strains or engineered point mutants of TMV or AlMV, as summarized in Table 1 (R. N. Beachy and J. F. Bol, personal communications). Such observations arise from experiments that have attempted to delineate essential CP subsequences which confer CPMP, either by using divergent strains or by creating point mutants or chimeras in the CP transgene or in full-length infectious clones/transcripts comprising the challenge virus. In part, the motivation has been to minimize the assumed risks of transencapsidation by creating assembly-incompetent, but still CPMP-active, constructs. Sometimes, addition of extra N- or C-terminal amino acids has been unavoidable during cloning or expression strategies. Work with TMV, bean yellow mosaic potyvirus, or PVY CP genes has shown that addition of C-terminal amino acids corresponding to sunnhemp mosaic tobamovirus CP, translational fusions of bean yellow mosaic potyvirus CP to 14 *nos*-derived N-terminal residues, or up to 41 amino acids from PVY nuclear inclusion protein NIb, respectively, have little or no effect on CPMP in transgenic plants (73, 74). In contrast, a single Ser → Gly change at position 2 in AlMV CP eliminates CPMP against wild-type AlMV (J. F. Bol, personal communication; Table 1). In helical viruses such as TMV and PVY, the termini are on the outer surface, where their effects on CP assembly/disassembly may be minimal—assuming this to be at least one critical site of action for functional CP in some CPMP systems. While many details and observations accumulate and useful crops are produced, we seem little closer to confirming or agreeing upon a unified mechanism for CPMP.

ON NONSTRUCTIONAL PROTEIN-MEDIATED RESISTANCE

Serendipitously, Golemboski *et al.* (25) discovered that transgenic tobaccos ex-

pressing U1 strain TMV RNA residues 3472–4916, a putative 54-kDa open reading frame corresponding to most of the 126-kDa UAG-codon readthrough domain of the 183-kDa replicase, were highly resistant to inoculation with homologous or closely related strains of TMV or TMV RNA (up to 500 μg/ml or 300 μg/ml, respectively). Resistance was a consequence of a marked yield reduction at all stages of virus replication (26) and, although no 54-kDa polypeptide could be detected in transformed tobaccos despite extensive efforts (25, 26), mutagenesis strongly suggested that the 54-kDa protein and not the transcript was essential for protection (27; for reviews, see refs. 28 and 29). In view of discussions below, it is noteworthy that their T-DNA construct lacked 67 nt of the putative 54-kDa mRNA (I_1-RNA) leader and had five extra C-terminal amino acid residues (polylinker coded).

Independently, but not entirely unconnected, the equivalent region (54-kDa) of the 201-kDa pea early browning tobravirus (PEBV) putative replicase protein was found to confer resistance in some transgenic lines of *N. benthamiana* to PEBV inocula up to 1 mg/ml and against two other close relatives, but not to tobacco rattle virus or pepper ringspot tobravirus (32). Premature termination products of the PEBV 54-kDa protein failed to provide protection, again suggesting a need for most or all of the coded amino acids. How much this reflects the functionality of the polypeptide fragment(s), or simply their stability *in vivo*, to sustain an already vanishingly low, but essential, threshold concentration has yet to be resolved. Despite this evidence, the involvement of the mRNA itself cannot be ruled out completely because even plants of the PEBV-resistant line 54.3 were found to have six PCR-derived point mutations in the 54-kDa "gene" (32).

Recently, a defective version of the multicomponent CMV RNA2 97-kDa putative replicase gene provided homologous protection against CMV Fny (75) and other cucumovirus subgroup I strains (using 500 μg of virus per ml or 50 μg of RNA per ml, inocula) but not those of subgroup II (28). This protein corresponds to the 54-kDa readthrough domains of TMV and PEBV. In contrast, transgenic tobaccos expressing native, functional AlMV RNA1- and/or RNA2-encoded replicase proteins (P1 and P2) were not resistant to virus challenge and could even complement RNA1- and/or RNA2-deficient inocula (76–78). Similarly, transformed protoplasts expressing the equivalent full-length brome mosaic virus (BMV) RNA2 protein had no resistance to BMV replication and would function *in trans* to support RNA2-defective BMV replication (79). Ten independent transgenic lines of tobacco expressing mRNA for the C-terminal domain of the AlMV P2 protein (equivalent to the TMV and PEBV 54-kDa species) were unable to resist AlMV infection (J. F. Bol, personal communication). Transformed tobaccos expressing AlMV P2 with mutations in the conserved GDD motif are now being tested.

Rather unexpectedly, transgenic tobacco lines expressing the 5′-untranslated leader and the complete, or N-terminal half of the 165-kDa open reading frame 1 product of PVX RNA exhibited limited protection (lowered lesion numbers and reduced virus accumulation) against PVX or PVX RNA inocula at 5 μg/ml (30). The C-terminal third and fourth quarters of the 165-kDa polypeptide, containing the conserved NTP-binding and polymerase (GDD) motifs, respectively, conferred no protection (30). Despite high transcript levels (Northern blots), no open reading frame 1 products could be detected at first in any transgenic line (estimated sensitivity = 0.0002% of total soluble protein), as in the TMV 54-kDa story. However, recent work has detected low but equal amounts of 165-kDa protein in all five PVX-susceptible and two PVX-resistant transgenic lines (C. L. Hemenway, personal communication). Three possible modes of action have been proposed, which could apply singly or in combination to all examples of replicase-mediated resistance described above and below: (*i*) the 165-kDa protein expressed is an inactive or low-activity variant from the virus population, being derived from a poorly infectious (0.2%) PVX cDNA clone; (*ii*) the low 165-kDa-protein yield reflects instability/proteolysis *in vivo*, and the degradation products could act as defective replicase subunits, interfering with the assembly/function of what is presumed to be a complex, multicomponent enzyme (80); (*iii*) the transgenic, native replicase mRNA could form RNA–RNA duplexes with complementary-sense PVX RNA during the initial rounds of virus replication (30; C. L. Hemenway, personal communication), or subtle mRNA sequence changes and/or the chimeric nature of the T-DNA transcript could interfere with normal RNA–RNA or protein–RNA interactions during virus replication.

The PVX open reading frame 1 story has been further complicated by Baulcombe and colleagues (31), who showed that changing the GDD motif to GAD, GED, or ADD in transcripts of a highly infectious full-length clone of PVX RNA completely abolished infectivity. When the 84-nt genomic 5′-leader and each of these variant 165-kDa genes (but unfortunately not the wild-type sequence) were expressed in transgenic tobaccos, measurable quantities of PVX 165-kDa protein could be isolated from the 30,000 × *g* pellet fraction (P30), and two out of four lines expressing the ADD mutation were highly resistant to PVX RNA inocula equivalent to 1 μg/ml. Plant lines containing all other gene constructs were susceptible to PVX inoculation. These data suggest a trans-active "dominant negative mutant" strategy for replicase-mediated protection against plant viruses, as shown for bacteriophage Qβ (81, 82).

Further support for this model comes from recent results with transformed tobaccos (both *n* and *N* genotypes) expressing the full-length replicase proteins (126/183 kDa) of U1 strain TMV (33; J. Donson, C. Kearney, T. Turpen, and W. O. Dawson, personal communication). Most plants with cauliflower mosaic virus 35S promoter-driven constructs expressing U1 TMV RNA genome coordinates 1–5086 or 70–5399 (-leader) were not resistant to TMV inoculation; however, five transgenic lines of the latter construct showed complete (four) or partial (one) resistance to mechanical inoculation with TMV U1 at up to 100 μg/ml (20 μg/plant) but, more significantly, were almost equally resistant to TMV U2 and U5, tomato mosaic virus, green-tomato atypical mosaic virus, and ribgrass mosaic virus, some of which are distantly related tobamoviruses (83), and would infect U1 TMV 54-kDa transgenic plants (25). Restriction digests and sequence analysis revealed that all five resistant lines had a 1.4-kbp insert in the TMV sequence at nt 2875 and that the insert was followed by a direct nine-residue repeat of TMV nt 2867–2875. Terminal sequencing identified the insert as the 1.33-kbp *Escherichia coli* IS10 (98% homology). The insert caused premature termination of translation of the TMV replicase gene; thus, a "defective" 126-kDa protein was presumed responsible for the highly virus-resistant phenotype (C. Kearney, personal communication).

Although no data exist, there is no reason to exclude the use of replicase-derived sequences for protection against dsDNA or ssDNA viruses, and some work is underway using bean golden mosaic geminivirus *AL1* gene sequences with site-specific mutations in the NTP-binding domain (84).

As for transgenic resistance conferred by other nonstructural plant viral genes, no resistance was detected in transgenic *N*-gene tobacco plants expressing the overlapping nonstructural (cysteine-rich; Zn-finger?) 13-kDa and 16-kDa protein genes of tobacco rattle virus strain PLB, or the unique 29-kDa gene on RNA2 of tobacco rattle virus strain TCM (85).

Contained within the CP (P3) gene (open reading frame 4) of the luteovirus PLRV, in a different reading frame, is

open reading frame 5 for a 17-kDa polypeptide (P4). During PLRV infection, the 17-kDa protein apparently accumulates to levels seven times higher than CP (34). It may be a precursor for the 5′-viral protein linked to the genomic RNA (VPg). It is also predicted to have an N-terminal domain rich in acidic residues with an amphipathic, α-helix (for dimerization?) and a positively charged C-terminal domain (for binding ssRNA?). It may function as a regulatory protein during virus replication or as a discarded scaffolding protein during virus assembly. Whatever its role, transgenic plants expressing a defective PLRV 17-kDa protein with six additional C-terminal histidine residues (to chelate divalent metal ions, disrupt the C-terminal RNA-binding domain, and allow protein purification on Ni^{2+} columns) were resistant to wild-type PLRV introduced by graft inoculation (34; W. Rohde, personal communication).

A function unique to plant viruses, and consequent upon the architecture of their hosts, is the production of one or three ("triple gene block") polypeptides that "open" the gated intercellular cytoplasmic connections (plasmodesmata) between neighboring cells and allow the infection to spread locally, probably as some viral ribonucleoprotein complex other than a virion (86–90). TMV 30-kDa protein is such a cell-to-cell MP and has been shown to have ssRNA binding activity (91). Plants transformed with a *ts* mutant 30-kDa gene, from the nitrous acid-induced TMV mutant Ni2519, showed a reduction in TMV yield from 15 μg/g of leaf material at 24°C, to 0.2–2.6 μg/g at 33°C, the nonpermissive temperature (92; J. G. Atabekov, personal communication). Compatible interactions between virus-coded MPs and host proteins have been proposed to account for viral host-range; hence, the TMV 30-kDa MP cannot function to mobilize TMV in wheat or barley plants or act *in trans* to complement an MP-defective cereal virus. Conversely, expression of the BMV 32-kDa MP gene in transgenic tobaccos, where it is nonfunctional, can reduce the spread (and hence yield) of a subsequent TMV challenge by ≈40-fold (J. G. Atabekov, personal communication). Presumably the constitutively expressed BMV MP acts as a defective molecular decoy to compete against the native TMV 30-kDa MP for the normal host-protein target, rendering the 30-kDa MP nonfunctional.

MP gene recombinants or chimeras from two geminiviruses, tomato golden mosaic, and African cassava mosaic viruses have recently been shown to act as dominant negative mutants and may serve as useful virus-resistance genes expressed either by plant transformation or even from inducible geminivirus replicons (93).

Cauliflower mosaic virus and most potyviruses are insect (aphid)-transmitted, by becoming attached to the stylets or foregut by a virus-encoded, bifunctional protein that is believed to recognize the viral CP and a surface receptor in the insect. Mutated or heterologously expressed "helper component" or aphid transmission factor has been shown to interfere with normal virus transmission by membrane feeding (94). Thus, it may prove possible to prevent the spread of insect-transmitted (or fungus-, or nematode-transmitted) viruses by engineering crops to express a defective virus transmission protein.

Further permutations on this theme seem endless, limited only by the ingenuity and efforts of researchers and the number of nonstructural viral genes characterized. The production of useful resistance is often a by-product of fundamental virological studies to confirm viral gene function. Indeed, as a simplistic extrapolation from current knowledge on the modular evolution of plant viruses, and the resulting conserved (consensus) functional sequence domains, one could speculate that a completely artificial, chimeric transgene containing an array of several (defective) catalytic motifs (e.g., a consensus GDD box, NTP-binding, helicase, and methyltransferase) on the surface of a "neutral" protein gene (e.g., bovine serum albumin), or in a viral CP gene, might confer resistance to a wide range of viruses.

OTHER STRATEGIES

Satellites and DI Nucleic Acids. The ability of some satRNAs (95) to attenuate the symptoms of their helper virus led to their early and widespread use in spray inoculations of greenhouse and field crops. Concurrent with the development of CPMP, transgenic plants expressing symptom-ameliorating satellites of CMV or tobacco ringspot virus (TRSV) were shown to provide protection from the severe effects of their respective helper virus and to inhibit virus replication (96–99). CMV satRNA also protected against the symptoms of tomato aspermy virus but without causing any reduction in virus replication. This enigma may be explained by recent observations on the ability of attenuating satRNAs to prevent helper virus CP from entering the chloroplasts of infected cells (P. Tien, personal communication). Thus, reduced replication may not be the (sole) mechanism of satRNA protection. Satellite TRSV also interferes with the replication and disease caused by another nepovirus, cherry leafroll virus, even though cherry leafroll virus is not a helper virus for satellite TRSV (100). Transgenic protection against cherry leafroll virus by satellite TRSV was reported in walnut trees in California in 1988; however, the phenomenon proved nonreproducible (E. Bruening, personal communication).

The mechanisms underlying the use of satRNAs (free or transgenic) seem even more empirical and enigmatic than the other approaches described here. Risk of mutation to a more severe satRNA (a single nucleotide change can be enough), their limited occurrence in nature, and possible changes in helper virus relations have detracted from widespread use of the transgenic satRNA protection strategy.

DI RNAs, while common in animal viruses, occur naturally only in members of the Tombusvirus and Carmovirus groups of plant viruses (101–103) and represent complicated rearrangements of genomic sequences. Like satRNAs, they can intensify (103) or ameliorate the symptoms of their helper virus and interfere with its replication (104). Recently, in barley protoplasts, deletion mutants of BMV RNA2 have been shown to act as artificial DI RNAs (105, 106), or "parasitic RNAs." Interference with BMV RNA replication has also been demonstrated with antisense transcripts of the regulatory intercistronic region from BMV RNA3 (107). With the advent of routine monocot transformation/regeneration doubtless these DI constructs will be tested *in planta*. Paradoxically, the first demonstration of a natural DI molecule attenuating virus disease symptoms in a transformed plant involved a defective, subgenomic ssDNA of the B component of African cassava mosaic geminivirus (108, 109), which interfered with the replication of both African cassava mosaic virus DNA components A and B but did not interfere with those of another geminivirus (tomato golden mosaic virus). DI RNAs of the negative-sense L-segment of TSWV RNA have been identified (110, 111) and shown to reduce symptoms. These may also be future candidates for transgenic protection, although creating TSWV-tolerant rather than immune lines of plants (23).

Plantibodies. Since the first demonstration that plant cells could chaperone and assemble functional mouse monoclonal antibodies (mAbs) (112), plant pathologists have been attracted by the possibility of providing protection against fungal, bacterial, or viral diseases by expressing an appropriate IgG, Fab_2 fragment, or single-chain F_v antibody in transgenic plants (for review, see ref. 113). Recent confirmation of the biological activity of an antiphytochrome mouse monoclonal single-chain F_v in transgenic tobacco (114) supports this overall strategy; however, the level of transgene expression is usually low (although >1% of soluble leaf protein was claimed in ref. 112), and problems have been encountered with

PCR-cloning and expression levels of full-length heavy chains in many transformed lines (R. S. Nelson and J. L. Sherwood, personal communication; A. Hiatt, G. Cowan, and T.M.A.W., unpublished work). IgG and Fab_2 molecules against TSWV nucleoprotein or TMV CP have been studied so far. Numerous research groups are studying other antiviral mAb genes. To target the viral CP requires a large molar excess of mAb. *In vitro* experiments with a variety of neo-, crypto-, or meta-tope-specific mAbs to TMV CP have shown that ratios in excess of 50:1 and 18-hr incubations at 4°C are required to cause significant inhibition of subsequent cotranslational disassembly of TMV particles (115). It may prove more effective to use mAbs targeted against nonstructural (catalytic) viral proteins where the antigen concentration would be lower and there would be more time to interfere with virus replication, rather than attempting to inhibit uncoating of every incoming virion at the point and moment of inoculation. There are also compartmentalization issues to consider. IgG (and Fab_2) with leader peptides are exported from plant cells into the apoplastic space (112), whereas single-chain F_v molecules will remain in the cytoplasm to bind the appropriate epitope. Preliminary evidence suggests that plant cells may be unable to chaperone assembly of, and thereby stabilize Fab_2 molecules (A. Hiatt, G. Cowan, and T.M.A.W., unpublished results). "The jury is still out" deciding on the utility of this strategy for plant protection!

Antisense RNA and Ribozymes. As described, data from transgenic plants expressing antisense CP mRNAs from CMV, PVX, or TMV (refs. 47, 48, and 43, respectively) showed only limited protection against inoculum concentrations even lower than those required for CPMP. These antisense constructs also contained part (PVX) or all (CMV and TMV) of the viral 3'-noncoding sequence, which may have accounted for their efficacy by hybridizing to early replication signals on the challenge virus genome. Similarly, transgenic plants expressing antisense RNAs to other regions of the CMV genome were generally not resistant to CMV infection (116), except one line that paradoxically had low transcript levels. Until recently, therefore, transgenic protection using antisense RNA against RNA virus target sequences remained largely unproven. However, an exclusively cytoplasmic RNA-virus replication cycle, high genome-sense RNA copy numbers, and association with proteins at all stages of replication suggest that a simple antisense antiviral strategy is unlikely to be successful.

As discussed above, recent data (50–58) from plants transformed with several potyvirus, luteovirus, or tospovirus CP antisense constructs have rekindled hope for antisense RNA protection against viruses. Use of an antisense intercistronic control sequence from BMV RNA3 (107) to interfere with virus replication in protoplasts has also been mentioned above.

Antisense inhibition of plant nuclear gene expression is well-documented (117), supporting some utility against viruses with a nuclear phase in their replication cycle—for example, geminiviruses (118, 119), caulimoviruses, or badnaviruses.

Ribozymes are small RNA molecules derived from satellite TRSV, or certain viroids and viroid-like satRNAs, which are capable of highly specific catalytic cleavage of RNA (for reviews, see refs. 120 and 121). Although the cleavage is normally intramolecular, the catalytic domain (a hairpin or hammerhead structure, respectively) and flanking antisense arms can be designed (122, 123) to cleave a specific target RNA *in trans* (before or after a GUC triplet, respectively). The hammerhead will also cleave 3' of GUA or GUU. Ribozymes can be visualized as "warheaded" antisense RNAs; however, the length and base composition of the two arms will affect the hybridization on/off rates at a given temperature and, hence, the kinetics and efficiency of RNA cleavage. Although much success has been achieved *in vitro*, progress *in vivo* has been markedly slower, requiring ribozyme/target RNA ratios between 100:1 and 1000:1 to detect activity, and a significant proportion of the down-regulation has been attributed to the antisense arms rather than to RNA cleavage. Most recently in plant protoplasts, neomycin phosphotransferase activity was completely abolished by a transiently expressed ribozyme (124) and, in mouse mammary cells, a ribozyme against α-lactalbumin expressed at a ratio of 1000:1 *via* the T7–vaccinia virus delivery system, reduced α-lactalbumin activity by >80% (125). Stably transformed plants will express much lower levels of ribozyme. Some delay in challenge virus symptoms or in virus replication at the protoplast level has been observed in tobaccos expressing ribozyme(s) targeted to essential TMV gene sequences (ref. 121; R. S. Nelson, personal communication). Experiments have been done in an effort to exploit a high copy-number viral replicon (126) to produce increased cytoplasmic levels of a ribozyme against a second viral RNA (PLRV) or against model mRNAs (e.g., chloramphenicol acetyltransferase or β-glucuronidase; J. Lamb, M. A. Mayo, G. Evans, and T.M.A.W., unpublished results). For plants, this may prove the most effective way to enhance classical "cross-protection"—using a mild virus strain to amplify, as a subgenomic RNA, a ribozyme against another (related or unrelated) severe virus.

Latent Suicide Genes. When a plant cell is transformed to express a low, constitutive level of antisense RNA for a highly phytotoxic protein (e.g., diphtheria toxin A fragment, pokeweed antiviral protein, or ricin) with a minus-sense plant viral subgenomic RNA promoter at its 3'-end, then infection by the cognate virus will, during the production of progeny plus-strands and subgenomic RNAs, transcribe the nonsense RNA into mRNA, allowing expression of the phytotoxin and killing that cell.

By use of the PVX subgenomic RNA promoter and diphtheria toxin mRNA, transgenic tobaccos showed a 20-fold reduction in PVX concentration in upper, systemic leaves, and the PVX-inoculated leaves turned yellow and fell off 6–7 days after inoculation (J. G. Atabekov, personal communication). Comparable constructs for transient gene expression (but containing an antisense ricin or bacterial exotoxin gene and TMV replication signals) were toxic to protoplasts, even without a virus challenge—presumably due to low-level transcription in the opposite direction from the 35S promoter (T. Hohn, personal communication). This general approach may be of questionable utility in the field but can provide a useful and sensitive probe for transcriptional activity.

THE FINAL WORD—ON RISK ISSUES CONCERNED WITH PATHOGEN-DERIVED RESISTANCE

Transgenic plants expressing viral pathogen-derived sequences have been considered sites for hyperevolution of viruses through recombination between a mild or defective viral genome (DNA or RNA) and the transgene or its transcript (127). To date, there is no experimental evidence to confirm that this can occur. On the contrary, evidence against such events exists through one (128–130) or eight (15) viral passages. Nevertheless, transencapsidation or heteroencapsidation of viral RNAs by transgenically expressed CP does occur (128–131). Most recently, the CP of plum pox potyvirus has been shown to confer aphid-transmissibility on a nontransmissible isolate of zucchini yellow mosaic virus (130). Although this may alter the vector relations and spread of a virus in a CP-transgenic monoculture or even the host range of a virus (if a different vector species became involved), the effect would apply only for a single acquisition–transmission cycle. Any long-term, stable effects, genetic or epidemiological, would seem remote—but are now amenable to direct experimentation and more accurate risk assessment using the exten-

sive range of plant species transformed singly or combinatorially (132–134) with viral sequences for enhanced resistance. For example, tobaccos transformed separately with both the AlMV P3 and CP genes have been found to support replication of AlMV RNAs 1 and 2, delivered as cDNAs fused to cauliflower mosaic virus 35S promoters (cf. ref. 78). However, in addition the plants also accumulated AlMV RNA3 and RNA4, which must have arisen by RNA recombination between the two transgene transcripts, perhaps during replication of AlMV RNAs 1 and 2 (J. F. Bol, personal communication).

A statistical analysis of the 393 defined field trials of transgenic plants (25 species) between 1986–1991 (in 21 countries) reveals that 50 involved "virus-resistance" traits (135). Field releases have shown that CPMP, for example, may not behave as predicted from laboratory/growth chamber experiments (132, 133) and, in general, exhibits greater susceptibility to virus challenge, probably through added environmental stresses. Nevertheless, useful virus-resistant lines can be selected (133).

In conclusion, I am confident that we shall continue to be amused and amazed by the ingenuity and unpredictability of future strategies for pathogen-derived resistance to viruses. Let us hope that all this effort, new information, and experimental material also contribute to our knowledge and understanding of conventional virus–plant interactions.

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