Genetic engineering of plants for virus resistance

Brief Review

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Summary. Historically, control of plant virus disease has involved numerous strategies which have often been combined to provide effective durable resistance in the field. In recent years, the dramatic advances obtained in plant molecular virology have enhanced our understanding of viral genome organizations and gene functions. Moreover, genetic engineering of plants for virus resistance has recently provided promising additional strategies for control of virus disease. At present, the most promising of these has been the expression of coat-protein coding sequences in plants transformed with a coat protein gene. Other potential methods include the expression of anti-sense viral transcripts in transgenic plants, the application of artificial anti-sense mediated gene regulation to viral systems, and the expression of viral satellite RNAs, RNAs with endoribonuclease activity, antiviral antibody genes, or human interferon genes in plants.

Introduction

One of the most striking successes in genetic engineering of crop plants has been the introduction of synthetic virus-resistance genes. This work epitomizes two advantages of genetic engineering: (*i*) the ability to transfer single genes directly without linkage to undesired genes, and (*ii*) the ability to construct novel genes that are unlikely to have existed in nature. In this review, we will present several examples of virus resistance introduced by genetic engineering methods. This has been possible with little advancement in our knowledge of natural mechanisms of resistance. We do not minimize the important problem of understanding natural resistance, but simply emphasize the effectiveness of genetic engineering. Genetically engineered, or synthetic, resistance has been achieved largely because of a relatively good understanding of viruses at the

molecular level and the ease with which viral genomes can be manipulated. The latter is a most important point because, as will be discussed, our understanding of how genetically engineered resistance works is limited. This review will present proven approaches for construction of virus-resistance genes as well as a variety of new strategies that show promise but have yet to be tested.

Three major approaches have been developed that employ viral nucleic acid sequences: (*i*) expression of the viral-coat-protein coding sequences in plants to confer resistance [16, 48, 52, 66, 69, 88, 115, 125, 130–132]; (*ii*) expression of anti-sense viral transcripts in transgenic plants, which presumably inhibits virus gene expression by RNA-RNA hybridization [16, 48, 90, 96]; and (*iii*) production of engineered plants that express nucleic acid sequences encoding viral satellite RNAs. These may interfere with efficiency of virus replication and result in host resistance to infection [2, 38, 46, 58, 119].

Other novel approaches directed toward the genetic engineering of plant virus resistance include: (*i*) the use of artificial anti-sense genes transferred to the plant genome [129] or antisense oligodeoxynucleotides ("antimessenger oligos") used as potential chemotherapeutic agents [12, 124]; (*ii*) the introduction and expression of RNAs with endoribonuclease (ribozyme) activity in plants [47, 137]; (*iii*) the use of anti-idiotypic antibodies as receptor-specific anti-viral agents [53, 75, 76] and cloning mouse antiviral antibody genes into plants [50]; (*iv*) the expression in plants of human α- and β-interferon genes and the detection of plant interferon-homologous sequences with antiviral activity [10, 21, 22, 106].

Transformation of plants with coat-protein coding sequences: coat protein-mediated protection

One approach to genetically engineering plants for virus resistance is to mimic the natural phenomenon of "cross-protection", first observed 60 years ago by McKinney [74]. He showed that infection of a host plant with a mild strain of tobacco mosaic virus (TMV) protected the plant against subsequent superinfection by severe strains of the same virus. Cross-protection is used to control some virus diseases of horticultural crops [for reviews see 34, 108]. Although cross-protection is well studied, the mechanism(s) responsible is poorly understood.

The major hypotheses proposed to explain the molecular basis of crossprotection include: (*i*) encapsidation of the challenging viral RNA by free coat protein of the inducing strain [19], or blockage of uncoating [109]; (*ii*) competition between the protecting strain and the challenge virus for a factor present in the host cell, (e.g., the replicase) [39]; and (*iii*) annealing of sense and antisense RNAs of the inducing and challenge virus to prevent replication and/or translation of the severe strain [87].

Hamilton [43] predicted that cross-protection could be induced by introducing cDNAs to various regions of the viral RNA genome into plants. These

would be expressed as stable Mendelian traits. At that time, however, gene transfer methods had not been developed for plants.

Several studies have suggested that the coat protein (CP) plays a major role in cross-protection [19, 20, 109, 134, 143]. To test this, Powell-Abel et al. [88] introduced the CP gene of TMV into tobacco plants by constructing a chimeric gene containing a cDNA that corresponded to the CP coding sequence of the common U1 strain of TMV, flanked by the 35S RNA promoter from cauliflower mosaic virus (CaMV) and the polyadenylation signal from the *Agrobacterium* nopaline synthase gene. After introduction of the construct into *Nicotiana tabacum* cv. Xanthi by *Agrobacterium* transformation, tobacco cells were regenerated into plants. Accumulation of TMV CP (up to 0.1% of total soluble cell protein) was associated with high resistance to virus infection and a corresponding delay in symptom development in progeny of self-fertilized transgenic plants. The protection was overcome by inoculation of the transgenic seedlings with naked viral RNA and was less effective when a high concentration of virus was used. Therefore, the expression of the TMV CP gene mimicked classical cross-protection. The observation of CP-mediated protection against alfalfa mosaic virus (AlMV) in tobacco and tomato was subsequently reported [69, 125, 131].

As was shown for TMV [81], a dramatic decrease in the number of chlorotic and necrotic lesions was observed in transgenic plants expressing AlMV CP when inoculated with AlMV. These results are consistent with the hypothesis that expression of the CP coding sequence blocks early events of viral infection. As with TMV, inoculation of plants with AlMV RNA partially overcomes protection, which suggests that this resistance operates by interfering with stages of infection not required for infection by naked viral RNA.

Indeed, recent experiments demonstrated that CP is responsible for genetically engineered cross-protection. Transgenic plants that express a chimeric gene encoding the TMV CP sequence but do not produce CP are not protected against TMV [89]. In contrast, introduction of purified TMV CP into protoplasts that do not express the CP gene can induce transient protection when introduced shortly before or at the same time as the virus [95]. Similarly, tobacco plants transformed with a frame-shift mutated CP gene of AlMV [132] accumulated viral transcripts, but the coat protein was not produced in detectable amounts and plants showed no resistance to infection with AlMV virions, in contrast to transgenic plants expressing wild-type AlMV CP. Furthermore, Van Dun et al. showed that CPs of both AlMV [131] and tobacco streak virus (TSV) [132] (an ilarvirus with a genome organization very similar to that of AlMV) that accumulated in transgenic plants are biologically active and result in infection by AlMV upon inoculation of plants with a mixture of AlMV RNAs 1, 2, and 3. CP-engineered protection against TSV also was obtained in tobacco plants transgenic for the CP-gene of TSV [132].

Loesch-Fries et al. [69] obtained tobacco plants expressing AlMV CP in which viral infection was restricted to the inoculated leaves upon inoculation

with either of two strains of A1MV (425 and McKinney). Some of the transgenic plants showed systemic infection, although with a delay in the appearance of symptoms.

Coat-protein expression also has conferred resistance to another important plant viral pathogen, cucumber mosaic virus (CMV), the type member of the cucumovirus group [16]. The subgenomic RNA 4, which encodes the coat protein, was cloned in both sense and antisense orientations and introduced into tobacco plants via *Agrobacterium* transformation. Transgenic plants expressing CP showed protection in both inoculated and systemic leaves with a reduction in virus accumulation only in the inoculated leaves. Interestingly, the degree of cross-protection was independent of the inoculum concentration, which is not consistent with previous reports of other transgenic plants expressing viral CP or with classical cross-protection studies. When the antisense CP gene was employed, protection was less efficient, in agreement with the results shown for PVX [48].

CP-mediated protection also has been extended to the tobravirus group [130]. Transgenic tobacco plants that expressed the CP gene of tobacco rattle virus (TRV), strain TCM, were resistant to infection with TRV-TCM, whereas a severe disease syndrome developed when plants were infected with TRV strain PLB. A possible explanation for this phenomenon may be that the low sequence homology between the CPs of the two strains (39%) is insufficient to give protection. Significant resistance was obtained, however, against pea early browning virus (PEBV), another tobravirus, in plants expressing CP of TRV-TCM. Plants expressing nonstructural genes of TRV were not resistant to the infection by TRV [1]. Therefore, protection only occurred in plants expressing the TRV CP structural gene.

A better understanding of the mechanism by which CP-mediated protection operates has been obtained by recent experiments suggesting that endogenous CP is more likely to prevent capsid disassembly or interfere with events of late virus replication rather than with repackaging the uncoated viral RNA. Tobacco plants and protoplasts transgenic for CP have been reported to be resistant to infection with TMV but not to inoculation with TMV RNA or TMV that has been incubated briefly at pH 8.0 to destabilize virus particles [94]. Furthermore, tobacco plants transgenic for both TMV CP and the TMV origin-of-assembly (OAS) sequence retained resistance to infection by TMV [85].

CP-mediated protection has been successfully applied to commercial cultivars of potato, a crop affected by a large number of serious viral pathogens [48, 52, 66, 128]. Major potato cultivars have no resistance to many of these viruses. The most important viruses are potato virus Y (PVY), potato leafroll virus (PLRV) and potato virus X (PVX). Hemenway et al. [48] inserted a cDNA to the CP coding sequence of PVX, the type member of the potexvirus group, into an expression vector in both sense and antisense orientations between a CaMV 35S promoter and the pea rbc SE gene termination signal. Transgenic tobacco plants expressing CP in the sense orientation were protected from PVX

infection. In contrast to the previous examples of CP-mediated viral resistance in transgenic plants, protection was not overcome by inoculation of plants expressing high levels of PVX CP with PVX RNA. These data suggest that protection of plants by PVX-encoded CP, or its correspondent transcript, may function in a manner different from that described in previous examples. The protection against naked RNA may be explained by the location of the PVX OAS. In PVX, the OAS is located near the 5' end of the RNA. Thus, even small amounts of CP binding could inhibit initial translational events in cells infected by naked RNA or intact virus. In contrast, the OAS in TMV is located near the 3' terminus, and translation of the first protein expressed in TMV infection would not be inhibited by a CP OAS near the 3' end of the genome.

Hoekema and colleagues [52] also genetically engineered the susceptible potato cultivars Escort and Bintje to express the CP gene of PVX. One or two copies of the PVX CP cistron were successfully integrated per tetraploid genome of potato plants. Plants transgenic for CP showed a delay in disease symptom development, along with a reduction of virus accumulation, when inoculated with challenge virus.

Lawson et al. [66] introduced both PVX and PVY CP genes into potato plants. PVY, the type member of the potyvirus group, is a member of the largest and most significant group of plant viruses. Its genome has been cloned and partially sequenced [98, 122, 133]. Transgenic plants that expressed the double construct were protected from infection by both PVX and PVY; however, the resistance to either PVX or PVY was greater in transgenic plants expressing the homologous CP gene. More recently, additional approaches have been used to introduce resistance to PVY. Transgenic plants have been obtained that express either a fragment carrying AUG start codons upstream from the CP gene or the nuclear inclusion NIa (protease) gene in conjunction with the CP gene to produce N-terminally modified PVY CP. Tests for resistance to PVY are in progress (W. Rhode, pers. comm.).

Molecular cloning of cDNA to potato leafroll virus (PLRV), a luteovirus that causes significant yield loss worldwide in potato, has been reported recently [72, 91, 114]. A fragment carrying the PLRV CP gene has been cloned and sequenced [116], and 13 independent transformant potato lines have been obtained that express the CP gene in a stable manner. The resistance test is in progress (W. Rhode, pers. comm.).

The genome of another potyvirus, soybean mosaic virus (SMV), has been partially cloned and studied at the molecular level [23, 24, 35, 42, 70]. SMV CP-mediated resistance to tobacco etch virus (TEV) and PVY, two potyviruses with relatively low CP amino-acid-sequence homology to SMV CP (58% and 61% for TEV and PVY, respectively), has been obtained recently in tobacco [115], which is not a host of SMV. This is the first demonstration that a viral CP expressed in a non-host plant can give protection against infection by heterologous viruses.

Plant genetic-engineering techniques are also being used to obtain resistance

in sugar beet to beet necrotic yellow vein virus (BNYVV), the type member of the furovirus group. The 5' terminal CP gene has been cloned and inserted into a plant-expression vector, and transformation of sugarbeet is in progress (J. Brunsted, pers. comm.).

Viral pathogens of vegetable crops are being considered as potential targets of the CP-mediated protection approach. The CP of artichoke mottled crinkle virus (AMCV), a tombusvirus, has been cloned and sequenced [118]. Efforts are in progress to produce transgenic artichoke (E. Benvenuto, pers. comm.).

An interesting alternative strategy to the CP-mediated protection has been described recently for TMV. A full-length cDNA copy of the genomic RNA of a mildly virulent tomato strain of TMV (TMV-L_{11}A) has been introduced into tobacco plants by using a disarmed Ti plasmid vector [135, 136]. The mild isolate used was obtained from the parental, highly virulent, TMV-L and has been used as a classical cross-protecting agent in greenhouse-grown tomatoes in Japan [86]. When challenged with purified TMV-L, transgenic plants containing the TMV-L_{11}A cDNA did not develop symptoms of TMV-L for up to 6 weeks after inoculation, whereas typical mosaic and wrinkling was present on plants expressing the genome of the severe strain L. Moreover, engineered cross-protection was not overcome by inoculation with TMV-L RNA. The protection obtained by this approach was more efficient than CP-mediated protection, presumably because of the high cellular concentration of the mild TMV strain gene products obtained by the expression as well as replication of biologically active viral RNA in the transgenic plants. But the described system may present major disadvantages due to possible yield and quality losses from the mild isolate and the possible occurrence of virulent back-mutants. No such mutations have occurred, however, after years of greenhouse applications.

The previous illustrations have dealt with gene transfer mediated by *Agrobacterium* transformation. In the future, direct gene transfer (DGT) [for a review, see 36] may be possible for induction of virus resistance. One approach to DGT is implementation of treatments to permeabilize cell membranes. These have included electroporation [33, 65, 111], the use of polyethylene glycol [105], or a combination of these treatments. The most important limitation of direct DNA uptake is the requirement for cell wall removal; regeneration from protoplasts remains unreliable and difficult for cereal crops. However, transformed calli [33] and, in some instances, sterile plants, have been regenerated from electroporated maize protoplasts [97]. Recently, regeneration of fertile maize plants [92, 110] and transgenic plants from rice protoplasts has been obtained [123, 142].

A second approach to DGT has been recently developed for general transformation of intact tissue. The process involves use of a particle bombardment accelerator ("particle gun") [102], in which tungsten particles carrying biological molecules (DNA, RNA, etc.) are accelerated to the appropriate velocity and shot into the cell to induce transformation. The most significant advantage of this method is its potential for wide applicability. Tobacco, soybean, and

maize have been successfully transformed by this method [11, 26, 62, 63, 73]. Other transformation methods involve the use of microinjection [15] and viral vectors [4, 32].

Field testing of transgenic plants expressing viral CP

Field testing of genetically engineered plants [17, 29] is necessary to determine if the level of gene activity obtained in the laboratory and greenhouse is maintained under variable environmental conditions that occur in the field. Furthermore, the genetic transformation must not induce detrimental alterations in agronomic traits (e.g., yield, quality, growth). Field tests are currently conducted under strict control of regulatory agencies and are subject to restrictions directed toward preventing adverse environmental effects.

In 1987, the Monsanto Company and Washington University (St. Louis, MO, U.S.A.) obtained permission from the United States Department of Agriculture to test, in the field, tomato plants expressing the CP gene from TMV [82]. Tomato lines expressing TMV CP showed nearly complete protection against TMV in the field. Yields were comparable to control plants that were not infected with the virus. This suggested that the transformation did not affect normal agronomic traits. Interestingly, the plants also were protected against three strains of tomato mosaic virus, a tobamovirus closely related to TMV. Two of the virus strains (2 and 2^2) normally overcome the natural resistance present in many commercial tomato cultivars.

Antisense nucleic acid technology against viral infection

Antisense RNA has been shown to play an important role in prokaryotic gene regulation by functioning as a highly specific inhibitor of gene expresson [for reviews, see 41, 57]. Natural antisense RNA was first discovered in *E. coli* [78] and designated "micRNA" (mRNA-interfering complementary RNA) because it was found to inhibit translation by hybridizing to mRNA, probably by blocking the ribosome binding site and the start codon. Because this regulatory RNA is complementary to the target mRNA, it has been named "antisense" RNA. The genes directing its synthesis are called "antisense" genes.

The existence of naturally occurring antisense genes has not been demonstrated in eukaryotic cells, but artifical antisense regulation of gene expression has been obtained in animal systems [reviewed in 41, 57] as well as in plants [for a review, see 128]. Inhibition of gene expression in eukaryotes can occur by one or both of the following mechanisms: (*i*) hybridization may occur in the nucleus and prevent processing and/or transport of the target message or, (*ii*) antisense RNA may hybridize to the sense message in the cytoplasm, causing blockage of translation of specific mRNAs. The construction of an artificial antisense RNA gene can be obtained easily by positioning a DNA fragment coding for the target mRNA in reverse orientation between a strong promoter and a termination signal.

The application of antisense-mediated gene regulation in viral systems represents a new and promising approach toward genetically engineered control of viral infections as well as to anti-viral therapy. In several instances, the development of heritable antisense antiviral genes has induced protection from viral infection by interfering with virus translation and/or replication [13, 51, 117].

There have been several applications of this technology to the control of plant viral disease. A CMV antisense CP was introduced into tobacco plants via *Agrobacterium* transformation. Transgenic plants expressing the antisense transcript showed protection against CMV infection only at low inoculum concentrations, suggesting that antisense CP RNA is much less effective than the CP for preventing viral infection [16]. Low-level antisense-mediated protection also has been obtained against PVX and TMV [48, 90]. The lack of protection at greater inoculum concentrations may be caused by insufficient expression of the antisense transcript, because a clear gene dosage effect has been shown to occur in antisense RNA regulation. In addition, the antisense RNA used in this study was against the CP gene that directs synthesis of significant amounts of CP late in the infection cycle. Use of antisense RNA to stop the initial translation and replication events of the infection cycle may be more effective. The efficiency of the inhibitory reactions also may be increased by repeating copies of the same antisense gene in tandem under single or multiple strong promoters.

The effectiveness of some other antisense constructions for inhibition of CMV genes has been tested [96]. Tobacco plants were transformed with three different antisense genes corresponding to genomic regions of the putative replicase, movement protein and the 3' site of replication initiation. Only one tobacco line expressing a relatively low amount of one of the antisense constructs (corresponding to the putative replicase) showed resistance to CMV infection. Other tobacco lines expressing the same gene supported CMV replication as much as the non-transgenic plants.

The mechanism of action of viral antisense RNA in eukaryotes is not well understood at present, but several hypotheses can be proposed: (*i*) inhibition of CP synthesis by formation of an antisense-sense RNA hybrid, when antisense CP genes are used; (*ii*) prevention of replication by binding of the antisense RNA to the origin of replication; (*iii*) competition with the viral negative strand for viral or host components needed for replication.

A different strategy, called "sense RNA", is being attempted to interfere with the replication in vivo of turnip yellow mosaic virus (TYMV) [79]. The strategy is the use of small "sense" viral RNAs, containing the 3'-terminal region of the TYMV genome, which comprises the recognition site of the replicase, to act essentially as a defective-interfering (D.I.) RNA. Such "sense" RNAs have been shown to act as competitive inhibitors of replication of TYMV genome in vitro and are currently being tested for in vivo activity in *Brassica napus* [127].

Application of the antisense RNA technology to plant-virus disease control may open new and exciting possibilities for "gene therapy" in plants, despite initial poor efficiency. Synthetic oligodeoxyribonucleotides ("oligos") complementary to viral RNAs have been shown to function as antiviral compounds in animals and humans, specifically inhibiting or controlling viral gene expression by interfering with the replication, transcription, and translation machinery [12, 124]. The efficiency of synthetic antimessengers can be increased by chemical modifications designed to: (i) allow delivery to the cell [67], (ii) improve resistance against cellular nuclease attack, or (iii) enhance the affinity for the target RNA [40]. Specific antiviral activity of antisense oligomers has been observed in mammalian cell cultures against influenza virus [141], human immunodeficiency virus [40, 71, 138], Rous sarcoma virus [139], herpes simplex virus type 1 [113], and encephalomyocarditis virus [103].

The difficulty of effective passage of antisense oligomers from blood into tissues and penetration into cells represents the major disadvantage of using this approach in animals. However, possible insertion of antiviral sequences into the plant genome makes these compounds possible candidates for large-scale use as antiviral agents in plants.

Expression in plants of viral satellite RNAs

Genes encoding virus satellite RNA have conferred tolerance to plant viral infection. A satellite RNA is a small RNA that requires a helper virus to replicate in host plants [30]. With the exception of satellite C of turnip crinkle virus, which seems to be a molecular hybrid between a D.I. particle and a satellite RNA [112], the satellite RNA contains no nucleic acid sequences homologous to that of the helper virus. Satellite RNAs are encapsidated in the coat protein of the helper virus; other satellites, called satellite viruses, differ from satellite RNAs by encoding their own CP gene.

Resistance to CMV has been induced by introduction of a DNA copy of CMV satellite RNA into tobacco by using *Agrobacterium*-mediated transformation [3, 97]. The constructs contained either 1.3 or 2.3 tandem copies of satellite sequences, under the control of the CaMV 35 S promoter. Transgenic tobacco plants contained small amounts of transcribed RNA, which was amplified upon inoculation with CMV. Presence of the satellite RNA decreased CMV replication and largely suppressed symptom development. When the transgenic plants were inoculated with the closely related tomato aspermy virus, the satellite RNA was replicated and symptoms were suppressed. However, virus yield was not reduced. The data suggest that symptom suppression does not necessarily depend on a decrease in virus replication. This study demonstrated that, although mechanisms are unclear, protection by virus satellite-nucleotide sequences can be a viable strategy.

In a similar approach, Gerlach et al. [38] introduced multiple DNA copies of tobacco ringspot virus (ToRSV; nepovirus group) satellite RNA (STobRV)

into tobacco. Both the negative and positive sense strand concatamers undergo self-cleavage at unique sites in vitro. The DNA copies were placed under the transcriptional control of the CaMV 35 S promoter in such an orientation that either the positive or negative sense strand was transcribed. After infection with TobRSV, no alteration in levels of satellite RNA occurred in transgenic plants expressing a permuted monomer of the satellite RNA compared with untransformed plants. However, the level of monomeric satellite RNA increased to relatively high levels in plants that contained multimeric DNA copies of satellite RNA that resulted in positive or minus strand satellite RNAs. This indicates replication of the satellite RNA. Inhibition of disease development in plants producing the positive strand satellite RNAs was more immediate than in plants producing minus strand satellite RNAs. Transgenic plants containing multimeric copies of satellite RNA showed resistance to infection by TobRSV that correlated with amplification of the satellite RNA to high levels during virus infection.

The data are consistent with the observations of Jacquemond et al. [58] that a monomeric copy of the CMV satellite RNA induced tolerance. Moreover, the tolerance occurred whether transgenic plants were inoculated mechanically or by aphid vectors. In all instances, tolerance in transgenic plants containing genes for satellite RNA has been independent of virus strain, inoculum concentration, the use of intact virus or viral RNA, and the level of satellite RNA gene transcription. Although this method of inducing virus resistance has been quite successful, its potential is limited to the few plant viruses possessing a satellite RNA that can limit virus replication. Also, as illustrated with CMV satellite RNA, only a few base changes are required to change a symptom-reducing strain into one that increases disease symptoms [2, 18, 59, 64, 119]. This also may limit its potential for induction of virus-disease resistance.

Ribozymes

RNA molecules have been found to act as enzymes in catalyzing specific RNA cleavage in a variety of living systems [reviewed in 7, 8, 9]. These RNA enzymes are termed ribozymes [140]. The smallest known ribozyme structures are those involved in cleavage of some plant-virus satellite RNAs. Multimeric and circular forms of these RNAs are generated during replication [55]. They can self-cleave at a specific site into linear monomers in a protein-free reaction that requires only a divalent metal cation and a pH of 7 to 10 [56, 93]. Each of these RNAs has a similar structure called a "hammerhead" (Fig. 1), containing conserved bases flanking the self-cleavage site [28]. These hammerhead structures are found in the satellite RNAs of TobRSV, the sobemoviruses (virusoids [28]), and barley yellow dwarf virus (BYDV) [77]. They are also found in avocado sunblotch viroid [56] and transcripts of repetitive DNA in newt [25].

Uhlenbeck [126] showed that the hammerhead structure can be separated into enzyme and substrate components that function in a bimolecular reaction.
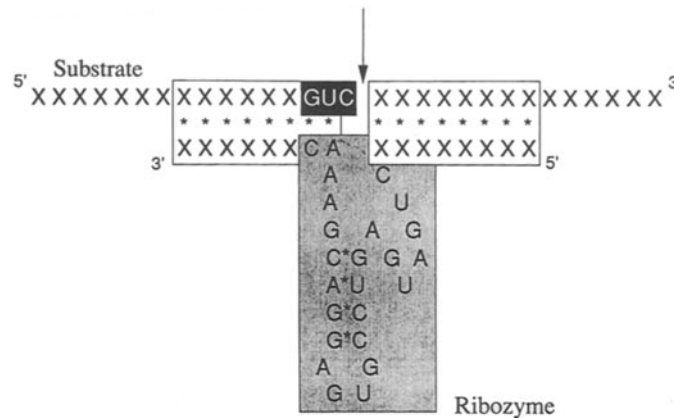
Fig. 1. Model for design of ribozymes (from Haseloff and Gerlach [47]; reprinted by permission from Nature vol. 334. Copyright© 1988 Macmillan Magazines Ltd. Substrate RNA can have any sequence (×) flanking GUC at the cleavage site, as long as base pairing forms with the ribozyme as shown. Arrow indicates cleavage site. Conserved bases in ribozymes are boxed. In naturally occurring hammerheads, the substrate and ribozyme portions are connected by a loop, resulting in an intramolecular cleavage

Haseloff and Gerlach [47] then showed that the only sequence conserved in the substrate RNA is a GUC adjacent to the cleavage site. All the other conserved sequences are in the enzyme portion of the cleavage structure. They exploited this to construct ribozymes that contained the conserved primary sequence in the enzyme fragment, flanked by sequences that could base-pair with the desired (nonsatellite) RNA sequence (Fig. 1). They constructed three different ribozymes that specifically cleaved chloramphenicol acetyl transferase (CAT) mRNA in vitro at three predicted different sites that have only the GUC sequence in common. The ribozymes behaved as true enzymes (i.e., they remained unchanged and performed several rounds of RNA cleavage).

Work is under way to optimize the cleavage reaction and understand the limiting parameters. Gene-specific ribozymes vary widely in cleavage efficiency, due to unpredictable secondary structural and perhaps other unknown parameters [27]. Ribozymes can be designed to cleave at sites other than GUC [14]. In fact, the minus sense strands of lucerne transient streak virus satellite cleaves at GUA [28], and the plus strand of BYDV satellite cleaves at AUA [77]. Gerlach et al. [37] have found that increasing the length of the "arms" that base pair with the substrate to a hundred or more nucleotides increased cleavage efficiency. They created "catalytic antisense" RNA consisting of several ribozyme moieties incorporated in a long antisense RNA and showed that it effectively cleaved CAT mRNA in vitro and in vivo.

Hammerhead-derived ribozymes have been shown to work in vivo in vertebrate cells [6, 14]. Of most importance to this review, ribozymes seem to be effective inhibitors of human immunodeficiency virus in human cells [104].

A ribozyme with a completely different structure also may prove to be

effective as a gene-specific nuclease. The "hairpin" structure of the self-cleaving minus strain of STobRV [5], which bears no structural similarity to hammerheads, cleaves at the 5′ side of the G of a GUC sequence [44]. It has been modified to work as a sequence-specific ribozyme that can function more efficiently than hammerheads under physiological conditions in vitro [45]. These results, combined with the recent demonstration that the *Tetrahymena* ribozyme can be modified to cleave a variety of substrates [80], including DNA [49, 99], suggest that a battery of structurally unrelated ribozymes may soon be available for use as antiviral agents.

Anti-idiotypic antibodies as receptor-specific antiviral agents

In 1974, Jerne proposed the Immune Network Theory to describe the regulation of the immune response in an antigenically stimulated animal. The theory suggested that an antigen can be regulated by a series of anti-idiotypic reactions that can either enhance or suppress the immune response to a particular antigen [60]. Antigen binding sites (paratopes) on antibody molecules are located in the idiotypic region of the molecule. Antibodies directed against the idiotypic region of other antibody molecules are called anti-idiotypic antibodies (anti-ids). If the anti-id recognizes the paratope and inhibits its recognition for an antigenic site (epitope) on the antigen, the anti-id may possess a structure similar to the epitope of the antigen. In this situation, both the epitope of the antigen and the anti-id can bind to an antibody molecule at the same site (Fig. 2). Such anti-ids are called internal image anti-ids. The idiotope represents (mimics) the three-dimensional configuration of the antigen.

Speculation concerning the exploitation of anti-ids for vaccine development [83, 100] (reviewed by Thanavala [120]) has resulted in development of several examples. The most successful of these systems involve *Trypanosoma* surface glycoprotein [101], hepatitis B surface antigen [61, 121], and the reovirus hemagglutinin [107].

Although vaccine development probably is not applicable to plant viruses, there have been three reports of the production of anti-ids [31, 53, 75, 76]. In a futuristic application of anti-ids to induction of resistance to plant viruses, Mernaugh et al. [75] suggested that the gene encoding the variable light chain
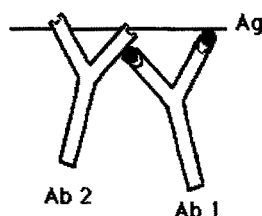

Fig. 2. Diagrammatic representation of interactions that may occur in an antigenically stimulated animal. — *Ag* antigen, ● epitope, *Ab 1* antigen-specific antibody, *Ab 2* antigen-mimicking antibody

of an anti-id representing an epitope of the coat protein of one strain of a plant virus could be introduced into and expressed in plants to provide cross-protection. This would be intended to act as CP-mediated protection in transgenic plants expressing the CP of a virus strain. Expression of active antibodies in transgenic tobacco plant has been demonstrated recently [50].

Expression of human interferon genes in plants

Human α- and β-interferon (α-and β-IFN) activity in plants has been investigated by several groups [54, 68, 84, 106], although its inhibitory effect on plant single strand positive-sense RNA virus infection remains controversial. Recently, transgenic tobacco plants expressing the α-INF gene have been obtained via *Agrobacterium* transformation [10]. However, high-level expression of α-IFN gene in turnip plants did not inhibit replication of turnip yellow mosaic virus [21]. Monoclonal antibodies to human β-INF have been used to purify two plant proteins by immunoaffinity chromatography. These proteins significantly inhibited TMV multiplication, but no sequence homology was found to any known protein, including interferon [22].

Conclusions

Historically, plant virus disease has been controlled by naturally occurring resistance or other kinds of evasive procedures. These measures have included resistance to and control of virus vectors such as insects, nematodes, and fungi; heat therapy; meristem culture; quarantine; eradication; maintenance of virus-free planting stock; sanitation; cross protection; and, depending upon the crop, various cultural practices. Opportunities now exist for development of additional novel control procedures. These developments will depend upon adaptation of technology developed by molecular biology to control virus disease. As illustrated by this review, numerous opportunities exist. At present, CP-mediated protection has been most widely examined and seems to be an effective control measure for disease caused by viruses in several different plant virus groups. It is apparent, however, that numerous other opportunities exist for effective development of additional strategies. The genetic stability of these novel forms of resistance will largely determine their effectiveness in the environment. Use of combined approaches may provide an effective way to enhance the durability of resistance in the field. The challenge to effectively deploy enhanced virus disease resistance will continue for numerous decades in the future!

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