Mechanisms and applications of pathogen-derived resistance in transgenic plants

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Genes that confer viral pathogen-derived resistance (PDR) include those for coat proteins, replicases, movement proteins, defective interfering RNAs and DNAs, and nontranslated RNAs. In addition to developing disease-resistant plant varieties for agriculture, PDR has increased the understanding of viral pathogenesis and disease. Furthermore, significant advances in elucidating the fundamental principles underlying resistance will lead to second and third generation genes that confer increased levels of sustainable resistance.

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Abbreviations

AlMV	alfalfa mosaic virus
CaMV	cauliflower mosaic virus
CMV	cucumber mosaic virus
CP	coat protein
CPMR	coat protein mediated resistance
DI	defective interfering
GFP	green fluorescent protein
MP	movement protein
PDR	pathogen-derived resistance
PVY	potato mosaic virus
Rep-MR	replicase-mediated resistance
TEV	tobacco etch virus
TMV	tobacco mosaic virus
TYLCV	tomato yellow leaf curl virus

Introduction

Known plant viruses number more than 1200, and, although those that cause significant losses in crop yield may number less than 250, the challenges that face plant breeders around the world are substantial. Control of viral disease requires an understanding of the virus, its replication, the vectors that spread the virus, and the deployment of useful genes for resistance in high-yielding varieties. Unfortunately, in all too many cases, particularly in climates in which vectors and hosts are present all year round, the battles are won by the pathogens. In many cases, disease problems are aggravated by agricultural practices that maximize production and yield rather than control pests and pathogens.

The first use of PDR to limit virus infection and disease was reported 11 years ago in the form of coat protein mediated resistance (CPMR) [1]; since that time, there have been growing numbers of examples of resistance as well as the development of a variety of strategies to achieve resistance. The first commercial sale of virus-resistant transgenic crops in the US took place in 1995, with virus-resistant squash by Asgrow Co (Kalamazoo, MI, USA); more examples are expected to reach the market place in the near future. The cellular and molecular mechanisms that are at play in the various types of PDR remain somewhat obscure, although they are not totally hidden. The future challenge for scientists in this field is to develop strategies that broaden the breadth and increase the degree of resistance. This will be achieved through fundamental studies that elucidate the molecular mechanisms of resistance and apply the knowledge thus derived to develop second and third generation resistance genes with increased efficacy.

In this review, I will highlight progress in the various pathogen-derived resistance strategies, indicate the limitations of each strategy, and suggest approaches that are needed if PDR is to provide sustainable resistance to virus infection.

Characteristics of plant viruses

Plant viruses belong to a large number of taxonomic groups with genomes of single- or double-stranded DNA or RNA, in messenger (+) sense, antisense or ambisense polarity. Plant viruses may contain 1–12 genetic segments, with genomes that contain as few as ~2700 or as many as ~20 000 nucleotides. Most are simple in structure and do not contain membranes or glycoproteins, although there are exceptions. A limited number of plant viruses are seed-born, infecting the embryo or the seed coat, thus passing virus to succeeding generations; others are mechanically transmitted during the handling of plant material by workers. More frequently, viruses are spread by insect, fungal, or nematode vectors that distribute the pathogen throughout a locale or across long distances, depending on the factors that control the dissemination of the vector. Most severe epidemics result from dissemination and infection by one or more viruses (or virus strains) that infect a susceptible crop variety and spread unchecked through a locale or region. Interdiction to prevent disease relies on both interfering with the transmission of the disease (e.g. applying insecticides or fungicides to control the vector, reducing access of the vector to reservoirs of virus in alternative hosts) and planting crop varieties that are resistant to virus infection or to the vector.

The past 20 years of research have led to a reasonable degree of understanding of the biochemistry and genetics of replication of many viruses; unfortunately, the large

numbers of different types of viruses preclude obtaining detailed information for each. Nevertheless, there is growing understanding of the function of viral replicases and related enzymes, the role of movement (transport) proteins that enable local and systemic infection, activities of proteinases that process viral polyproteins, assembly of virions by coat proteins (CPs), factors that aid virus acquisition and transmission by vectors.

Strategies of PDR

One might expect that the development of strategies for PDR to control virus infection and replication would follow from knowledge of the pathogen. In fact, the reverse is true, that is, PDR has increased understanding of virus replication and pathogenesis. Strategies for PDR are divided into those that require the production of proteins and those that require only the accumulation of viral nucleic acid sequences. In general, the former confer resistance to a broader range of virus strains and viruses, whereas the latter provide very high levels of resistance to a specific virus strain. The results of recent studies of protein-mediated and RNA-mediated resistance are summarized below.

Coat protein mediated resistance

CPMR was first reported in the tobacco mosaic virus (TMV)–tobacco model system in 1986 [1], and has since been used to confer resistance to a number of viruses in a variety of different plant species [2,3•]. CPMR can provide either broad or narrow protection; for example, the CP of TMV provided effective levels of resistance to closely related strains of TMV and decreasing levels of resistance to tobamoviruses that share less CP sequence similarity [4]. The CP gene of potato mosaic virus (PVY) strain N605 provided resistance in transgenic potato plants challenged with strain N605 and related strain 0803 [5], but the CP gene of papaya ringspot virus (PRV) strain HA provided resistance in papaya only to strain HA [6]. (It is unclear in the latter case whether resistance was due to the CP or the gene transcript; see discussion of RNA-mediated resistance.) In contrast, the CP of soybean mosaic virus (SMV), which is incapable of infecting tobacco, conferred resistance in tobacco to two unrelated potyviruses, PVY and tobacco etch virus (TEV) [7]. It is unclear why some CPs provide broad or strong degrees of CPMR whereas others provide only narrow or weak resistance. To achieve broad resistance to three different strains of tomato spotted wilt virus, genes encoding the nucleoprotein from each strain were combined in a single construct [8].

CPMR to TMV requires that the CP produced from the transgene is capable of subunit–subunit interactions but not necessarily capable of forming virus particles [9•]. CP apparently interferes with the disassembly of TMV, thereby preventing infection [10]; furthermore, there is a direct correlation between the amount of CP and the level of resistance [11]. Certain mutants of the TMV CP, constructed on the basis of the known structure of the virus [12], can confer much greater levels of resistance than wild-type CP (M Bendahmane, RN Beachy, unpublished data). One of the mutant CPs appears to block disassembly as well as replication and local and systemic spread of challenge virus. In contrast, in a study of CPMR against alfalfa mosaic virus (AlMV), CP mutants that failed to activate AlMV replication were nevertheless conferred CPMR [13•]. The authors suggested that protection was due to the binding of CP to a host factor involved in disassembly; however, it is also possible that disassembly was blocked by CP–virion interactions as proposed for CPMR to TMV (above). Although there are a number of examples of CPMR to potyviruses, there are exceptions. For example, wild-type CP does not protect tobacco plants against TEV unless sequences are deleted from the amino terminus of the protein; however, resistance is lost if the truncated CP is mutated such that self-assembly of CP to form virus-like particles is prevented (A Voloudakis, C Malpica, RN Beachy, unpublished data). It was recently suggested that potyvirus CPs may confer CPMR by interacting in some manner with nuclear inclusion protein b, a replication protein [14••]. This result may indicate that CPs can confer resistance via a variety of mechanisms, and that as yet undetermined structural features of the protein are essential for resistance.

Based upon the successes that have been achieved in improving CPMR against TMV, for which the structure of CP is known, it is clear that knowledge of the three-dimensional structures of other CP molecules and the role of CP in regulating infection and/or replication will significantly aid the design of mutant CPs that have increased efficacy and breadth of protection. The use of transient assay systems will make it possible to test mutant proteins for activity in protection assays and will reduce dependence, at least in part, on transgenic plants to test proteins for CPMR [13•,15••].

Replicase-mediated resistance

Genes that encode complete or partial replicase proteins can confer near immunity to infection that is generally, but not always, limited to the virus strain from which the gene sequence was obtained. Replicase-mediated resistance (Rep-MR) to TMV was first described in transgenic plants that contain a sequence encoding a 54 kDa fragment of replicase, although the protein fragment was not detected [16]. Although it was suggested that certain examples of Rep-MR are RNA- rather than protein-mediated [17•], other examples require an open reading frame and, apparently, production of protein [18,19]. A truncated mutant of replicase derived from a cucumber mosaic virus (CMV) subgroup I virus conferred high levels of resistance in tobacco plants to all subgroup I CMV strains, but not to subgroup II strains or other viruses [19]. In Rep-MR against PVY [20] and AlMV [21], mutant but not wild-type replicase conferred resistance to infection; a similar approach provided resistance to tomato yellow leaf

curl virus (TYLCV), a geminivirus (single-stranded DNA genome) [22•].

The mechanisms that are involved in Rep-MR are not known, although it was shown that plants exhibiting Rep-MR can strongly repress replication, and, in many cases, are resistant to high levels of challenge inoculum. It is proposed that protein produced by the transgene interferes in some manner with the function of the replicase produced by the virus, perhaps by binding to host factors or virus proteins that regulate replication and virus gene expression. In Rep-MR against CMV, both virus accumulation and systemic infection were inhibited [23]; this may reflect inhibition of virus replication leading to a reduction in movement protein. A hallmark of Rep-MR, like RNA-mediated resistance (described below), is that it is generally quite specific, although in the case of Rep-MR against potato leafroll virus (PLRV) resistance was effective against many different isolates of the virus. In another example, TMV replicase interrupted by an insertion sequence element from Agrobacterium (which occurred fortuitously during vector construction) produced high levels of resistance in tobacco plants to a wide range of tobamoviruses but not other viruses [24]. It is proposed that the mechanism of resistance in these lines is similar to that in those that produce the 54 kDa fragment of replicase [16], although it differs in the breadth of protection.

Movement protein mediated resistance

Movement proteins (MPs) are encoded by plant viruses and enable infections to spread between adjacent cells (local spread) as well as systemically [25•]. Intercellular spread involves plasmodesmata, the channels that traverse plant cell walls and provide symplastic continuity between cells and tissues. Because several MPs were shown to accumulate in plasmodesmata, virus MPs are also used to study protein targeting in plant cells as well as the nature and composition of the plasmodesmata.

It is not known how MPs facilitate the transport of virus particles or viral nucleic acid from sites of synthesis and assembly to and through plasmodesmata. Geminivirus DNA is replicated in nuclei and transport of the single-stranded genome from nucleus to cytoplasm requires one type of viral protein, whereas a second protein transports the DNA to adjacent cells [26•]. In contrast, RNA-containing viruses replicate in the cytoplasm. Many MPs, purified from *Escherichia coli*, bind single-stranded nucleic acids *in vitro* in a sequence nonspecific manner, yet it is likely that high specificity is maintained *in vivo* [25•]. TMV MP fused with the jellyfish green fluorescent protein (GFP) retains its function of spreading TMV from cell to cell and has been used to study MP function in protoplasts as well as in leaf tissues [27••,28••]. Fluorescence microscopy showed that MP accumulates in several different subcellular locations, including microtubules [27••,29••] and plasmodesmata [28••], and that MP is associated with the endoplasmic reticulum (M Heinlein, personal communication); however, the function of MP in each of these sites remains to be determined. MP produced in transgenic plants can enable MP$^-$ mutants of TMV [30,31] to move to adjacent cells, and it was predicted that certain defective mutants of MP (dMP) would restrict infection by TMV and perhaps other viruses [32]. Similar predictions were made based on studies of a geminivirus MP [33]. Indeed, transgenic plants that contain dMP from TMV show resistance to several tobamoviruses as well as to AlMV, cauliflower mosaic virus (CaMV) and other viruses [34,35•]. A mutation that disrupted a putative nucleotide binding site of one of three MPs of white clover mottle virus (WClMV) conferred resistance to several different potexviruses [36]. Although the degree of resistance was not equally high against each virus tested in these studies, it is anticipated that knowledge of MP structure and *in vivo* function(s) will lead to development of other mutant proteins or peptides that act as dominant negative inhibitors to block the local and systemic spread of many different viruses with high efficiency.

Nucleic acid mediated resistance

A variety of PDR strategies involve the expression of genes encoding nucleic acids that lack the capacity to encode proteins. One of the earliest approaches was the expression of antisense RNA sequences to reduce the replication of RNA viruses: in some cases, virus infection was affected little if at all [37,38], whereas in others, infection was more strongly inhibited [39••,40]. Although it is possible that RNA-mediated suppression (discussed below) was responsible for some of the (–) sense mediated resistance reported to date, other mechanisms may also be responsible, including the interruption of template selection by the replicase, or the formation and subsequent degradation of double-stranded RNA. Antisense RNA-mediated resistance is expected to be relatively narrow, providing protection to the virus from which sequences are derived but not to strains that have regions of significant variation from sequences of the transgene.

Antisense RNA strategies were also somewhat effective in controlling geminivirus infections [41], in which replication and transcription take place in the nucleus. The fact that some transgenic lines exhibit significant degrees of resistance indicates that the transgene can provide sufficient levels of antisense RNA to reduce the rates of virus replication and/or gene expression.

Other strategies involve sequences that represent defective interfering (DI) RNAs and DNAs [17•]; such molecules are produced during replication of certain viruses by deleting selected viral sequences while retaining sequence elements that ensure replication by the virus. DIs redirect replication from the genome in favor of the DI molecule and can dramatically reduce the amount of infectious virus and disease symptoms. Satellite RNAs are similar in some ways to DI molecules, but

generally retain little sequence similarity with the virus from which they are derived and on which they depend for replication. Transgenes encoding DI RNAs or DNAs and satellite RNAs have been tested for their capacity to reduce replication and disease [42,43] with some degree of success. In successful cases, both virus replication and disease symptoms are depressed, a result that indicates that it may be possible to control infection under field conditions while preserving productivity of the crop. In other cases, although disease symptoms are suppressed, virus replication is not affected, a situation that would do little to contain the further spread of the virus.

The most widely studied example of nucleic acid mediated resistance is referred to as RNA suppression, a phenomenon that describes the targeted post-transcriptional destruction of RNA sequences [17•,44]. Although a relationship between RNA suppression and the more general phenomenon of transgene silencing [45] has been strongly implicated [46], the similarities and differences between these phenomena remain to be explored [47].

RNA suppression mechanisms generally employ genes that encode (+) sense RNAs but do not lead to the accumulation of protein. Recent studies have demonstrated the following: a high correlation between RNA-mediated resistance and multiple copies of gene inserts and/or complex arrangements of DNA fragments at one or more genetic loci [48••]; little or no accumulation of transcript from the transgene, but moderate to high levels of gene transcription [49••]; and the methylation of transgene sequence in promoter regions, coding sequences, or both [44]. The favored model for PDR by gene silencing mechanisms is that the cell detects abnormally elevated levels of RNA sequences, or, more likely, aberrant RNA structures or modified nucleotides, and activates a destruction mechanism that involves nuclease digestion of the gene transcript [17•,44,47]. By an unknown mechanism, perhaps related to base pairing between the aberrant RNA and viral (+) or (–) sense RNA, viral RNA as well as transgene RNA is destroyed. Although levels of RNA-mediated resistance can be extremely high, its limitation may be that resistance is effective only against viruses with genomes that contain sequences that are similar or identical to the transgene. In addition, as resistance relies on transgenes whose structure is not yet defined, and that may include methylation or other modifications, there is concern that resistance may not be readily controlled.

Conclusions

A variety of PDR strategies have been used to develop virus resistance in crop plants, many of which have been tested under field conditions for eventual commercial release. CPMR has been the most widely applied PDR strategy, although other strategies are also under development. To improve the degree and breadth of resistance as well as its durability will require greater knowledge of the cellular and structural bases of resistance. Once the structural features of the molecules that confer resistance are known, it will be possible to construct sequences that confer increased efficacy and breadth of resistance. If principles of protein design are applied to construct dominant negative mutants of virus proteins such as MPs, it is likely that genes that confer durable PDR to control many different viruses will represent the next generation of PDR genes.

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