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Strategies for Viral Disease Resistance in Crop Plants

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ABSTRACT

Most crop plant species are susceptible to a number of different viruses, some of which may cause severe systemic infection resulting in significant crop losses. Hence a major preoccupation of both breeders and growers alike has been the development of strategies (pathogen-derived resistance) that protect against infection. Traditional approaches for managing plant virus diseases include avoiding virus-infected material, chemical control of arthropod vectors and, when available, use of virus-resistance in cultivated crops. However, all of these are labour intensive and chemical control of insect vectors is becoming more expensive with potential undesirable side effects, including environmental hazards and the generation of insecticide resistance in vector populations and those of other insect pests. The observation of cross protection, wherein the inoculation of mild virus strains on plants provided protection from more severe strains, suggested that alternative approaches were possible. Transgenic technology opened up environmentally friendly options to engineer plants for resistance to viruses. This includes both protein and RNA-based approaches. One of the earliest approaches through transgenic technology to combat the viruses was the coat protein-mediated resistance. In the recent years, many RNA-based approaches that involve silencing of the viral proteins are in vogue. This involves both the artificial miRNA and siRNA based approaches. This overview is an update on the different strategies used to improve crops against viral diseases. In addition, we would also focus on novel strategies that utilize the multigene concept for virus control which forms the highlight of this review.

Keywords: multigene concept, pathogen-derived resistance, plant virus, transgenics, virus resistance
Abbreviations: CMV, *cucumber mosaic virus*; **CPMR**, coat protein mediated resistance; **MPs**, movement proteins; **PDR**, pathogen-derived resistance; **PTGS**, post transcriptional gene silencing; **Rep-MR**, replicase mediated resistance; **RISC**, RNA-induced silencing complex; **TMV**, *tobacco mosaic virus*

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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 (Anonymous 1999; Kang *et al*. 2005). 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. One of the main reasons for the victory of the pathogens over the plants is the congenial conditions with respect to climate and vectors that transmit viruses (Bos 2000; Hull 2002). Another possible reason could be the importance given to agricultural practices to maximise yield rather than control pests and pathogens. Virus-induced diseases are responsible for major crop losses worldwide.

Most crop plant species are susceptible to a number of different viruses, some of which may cause severe systemic infection resulting in significant crop losses. The focus has been to help the plant fight back the virus. In this direction, there could be two perspectives, one involving strategies that limit crop exposure to potential pathogens and that control pathogen concentration in the environment and the other involving the development of crop improvement programmes for viral disease resistance. The latter strategy of crop improvement involves either breeding or a transgenic approach to develop viral disease resistance. The advent of transgenic technology has allowed the development of crop plants resistant to both biotic and abiotic stress including viral disease resistance. Several strategies are being used by researchers worldwide to develop transgenic plants resistant to viral diseases (Goldbach *et al*. 2003; Lin *et al*. 2007; Morroni *et al*. 2008; Prins *et al*. 2008; Kung *et al*. 2009; Reddy *et al*. 2009; Yun *et al*. 2009). The focus of the review

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Mini-Review

is on the different strategies for engineering virus resistance in plants using transgenic technology.

STRATEGIES FOR RESISTANCE TO PLANT VIRUSES

Plant virus life cycle

Plant viruses differ considerably in the morphology of the virus particle and in the form of genetic material used to encode the viral genes. These various genomes include single- or double-stranded DNA, double-stranded RNA, or ssRNA in a message (p1us)-sense or minus-sense format. The general strategies underlying the expression of these genomes are diverse, but ultimately mRNAs are transcribed for translation of structural and nonstructural proteins that are required to fulfil the viral life cycle. Despite differences in their replication strategies, all plant viruses have broadly similar steps in their life cycles: they must enter a host plant cell, generally by penetrating the cell wall, following abrasive mechanical damage, or via fungi, insects, mites, or nematodes that penetrate the plant cell wall during infection or feeding. The virus particle is then thought to swell or partially disassemble, which exposes the viral DNA or RNA to the cellular milieu (Verduin 1992). If the virus possesses mRNA as genetic material, translation will begin to produce the virus-specific proteins required for replication. DNA viruses generally enter the nucleus and utilize host enzymes to produce mRNAs suitable for translation. A critica1 event in infection by most plus-sense RNA viruses is the production of replicase protein(s) that, together with the cellular machinery, produce progeny by replicating the parenta1 genome. Most RNA viruses are thought to spread from cell to cell via the plasmodesmata assisted by a movement protein or, for optimum long-distance movement, in conjunction with a functionally active coat protein (Citovsky and Zambryski 1991). Thus, each stage of the infection cycle has the potential of being perturbed, i.e., at uncoating, translation, replication, and/or movement. The objective in generation of transgenic plants resistant to plant viruses would be to express a portion of the viral genome, either with or without expression of an encoded protein that will interfere with some particular aspect of the multiplication cycle.

Pathogen-derived resistance

The first virus-resistant transgenic plants were generated using a transgene derived from a viral pathogen. The idea that a pathogen's genome might itself be the source of resistance genes was formally proposed by Sanford and Johnson (1985). Thus, engineering resistance to viral diseases using the pathogen-derived genes without affecting essential host function is called as pathogen-derived resistance. Pathogen-derived resistance (PDR) can be successful either by the expression of the viral proteins or just accumulation of viral nucleic acid sequences. Anticipating rapid progress in transgenic plant technology, they proposed that the inappropriate overexpression of wild-type or mutant viral genes in a host plant would disrupt the life-cycle of an incoming virus and so confer resistance-by-default on the host. The phenomenon of viral cross protection, in which plants infected with one strain of a virus are found to be immune to super infection by another strain of the same virus formed the basis for PDR. This natural phenomenon has been exploited for many years by horticulturists in the form of deliberate infection of plants with a mild strain of virus in order to confer protection against more severe strains (Fulton 1986). With the advent of transgenic technology, it became possible to investigate which viral components were capable of conferring a cross-protection-like effect.

Several successful strategies based on pathogen derived resistance to suppress specific events required for infection include expression of coat proteins, replicases, use of antisense RNAs that are the complement to the plus- or minus-sense template of the virus, or use of satRNAs that can presumably overcome the viral RNA replicase (Beachy *et al.* 1990; Gadani *et al.* 1990; Dawson and Hilf 1992; Register and Nelson 1992; Bendahmane and Beachy 1999; Lucioli *et al.* 2008; Gottula and Fuchs 2009; Collinge *et al.* 2010).

Coat protein-mediated resistance

Beachy *et al.* (1986) reported that transgenic plants which accumulate the coat protein of *Tobacco mosaic virus* (TMV) are protected from infection by TMV, and by closely related tobamo viruses. The phenomenon is referred to as coat-protein-mediated resistance (CP-MR), and bears certain similarities to cross protection, a phenomenon described by plant pathologists early in this century. Beachy *et al.* (1986) demonstrated that CP-MR against TMV in tobacco showed that in the transgenics expressing CP, there was interference with disassembly of TMV particles when artificially challenged with the virus. These findings opened new avenues for plant protection in important agricultural crops. In most instances, CP-MR extends only to the virus or to related strains with substantially similar coat protein, but there are a few instances where the expression of the viral coat protein of one virus can provide at least some limited protection of transgenic plants against a heteroloous virus (Beachy *et al.* 1990; Gadani *et al.* 1990; Pang *et al.* 1992).

Several mechanisms have been proposed to account for the observed protection, (1) the protecting virus occupies or depletes host metabolites and/or structures needed by the challenge virus to establish an infection, (2) RNA from the protecting strain hybridizes with nascent RNA of the challenge virus, (3) the coat protein (CP) of the protecting virus inhibits uncoating of the challenge virus genome, (4) the presence of the protecting virus blocks systemic movement of the challenge virus, and (5) replication of the protecting virus activates a host defense mechanism that targets the challenge virus RNA for degradation (Sherwood and Fulton 1982; Palukaitis and Zaitlin 1984; Dodds *et al.* 1985; Ponz and Bruening 1986; Sherwood 1987; Angell and Baulcombe 1997; Kamo *et al*. 2010). Numerous crops have been transformed to show high levels of resistance in comparison to untransformed plants (**Table 1**). Though the actual mechanism has not yet been completely elucidated, this mechanism of viral disease resistance has been used to generate viral disease-resistance plants.

Replicase-mediated resistance

Yet another viral gene that has been used in PDR is the Replicase. 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 (Golemboski *et al*. 1990).

It has been suggested that the production of the replicase protein is apparently required for the effective PDR (Baulcombe 1996; Carr and Zaitlin 1991; Zaitlin *et al.* 1994) and confer resistance to different subgroups of the same virus. 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 (Zaitlin *et al.* 1994).

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 the challenged 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 (Hellwald and Palukaitis 1995); this may reflect inhibition of virus replication leading to a reduction in movement protein. Recently, Azadi *et*

Table 1 Coat protein-mediated resistance to viruses of crop plants (Dasgupta *et al.* 2003; Aragão and Faria 2009; Amudha *et al.* 2011; Klas *et al.* 2011).

Crop	Virus
Maize	Rice stagged stunt virus (RSV)
	Rice tungro spherical virus (RTSV)
Rice	Maize dwarf mosaic virus (MDMV)
	Maize chlorotic mottle virus (MCMV)
Wheat	Wheat sterility mosaic virus (WSMV)
Apricot	Apricot Plum pox virus (PPV)
Cantaloupe	Cucumber mosaic virus (CMV)
	Watermelon mosaic virus-2 (WMV2)
	Zucchini yellow mosaic virus (ZYMV)
Citrus	Citrus tristeza virus (CTV)
Grape	Grapevine chrome mosaic virus (GCMV)
	Grapevine fan leaf virus (GFLV)
	Tomato ringspot virus (ToRSV)
Muskmelon	Zucchini yellow mosaic virus (ZYMV)
Papaya	Papaya ringspot virus (PRY)
Plum	Plum pox virus (PPV)
Squash	Watermelon mosaic virus-2 (WMV2)
	Zucchini yellow mosaic virus (ZYMV)
Pepper	Tomato spotted wilt virus (TSWV)
Tomato	Cucumber mosaic virus (CMV)
	Tomato mosaic virus (ToMV)
	Tomato yellow leaf curl virus (TYLCV)
	Yellow vein mosaic virus (YMV)
Potato	Potato virus X (PVX)
	Potato virus Y (PVY)
	Potato leaf roll virus (PLRV)
	Potato virus A (PVA)
	Potato virus M (PVM)
Lettuce	Lettuce mosaic virus (LMV)
	Tomato spotted wilt virus (TSWV)
Pea	Pea enation mosaic virus (PEMV)
Cucumber	Cucumber mosaic virus (CMV)
Sugarbeet	Bean necrotic yellow mosaic virus (BNYMV)
Common bean	Common bean mosaic virus (CBMV)
Cassava	Cassava common mosaic virus (CMV)
Cotton	Cotton leaf curl virus (CLV)
Groundnut	Groundnut bud necrosis virus (GBNV)
Pigeonpea	Pigeon pea sterility mosaic virus (PSMV)
Sunflower	sunflower necrosis virus (SNV)

al. (2011) reported an increased resistance to CMV in *Lilium* transformed with a defective replicase gene.

Movement protein mediated resistance

Movement proteins (MPs) are encoded by plant viruses and enable infections to spread not only between adjacent cells (local spread) but also systemically (Carrington *et al.* 1996). These proteins either interact with secondary plasmodesmata, the intercellular connections between adjacent plant cells, or form tubules to allow intercellular trafficking of virions and/or ribonucleoprotein complexes comprising viral RNA and one or more of virus-encoded proteins. In addition, MPs also bind to RNA and/or DNA. The mutants of MPs were unable to bring about the MP-mediated plasmodesmatal trafficking of virus RNA/DNA. Therefore, in the movement protein-mediated resistance, the transgenic plants would bring about a stall in the trafficking of the viral genetic material. A conspicuous advantage of this strategy is a broad-spectrum resistance to diverse plant viruses that are dependent on the same type of plasmodesmata for the establishment of infection. Expression of a defective TMV 30-kDa MP, in addition to conferring resistance to TMV, was also able to confer resistance to *Tobacco rattle virus*, *Tobacco ringspot virus* (Family Comoviridae), *Alfalfa mosaic virus* (Family Bromoviridae), *Peanut chlorotic streak virus* (Family Caulimoviridae), and CMV (Cooper *et al.* 1995; Duan *et al.* 1997; Hou *et al.* 2000). 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. 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. Also, because plants have evolved plasmodesmata for intercellular communication, interference by MPs may affect plant communication leading to undesirable transgene effects.

RNA-mediated resistance

1. siRNA-mediated resistance

Post transcriptional gene silencing (PTGS) was first observed in transgenic *Petunia* plants as a coordinated and reciprocal inactivation of host genes and transgenes encoding homologous RNA (Napoli *et al.* 1990). However, PTGS or RNA silencing is a recently recognized strategy for developing virus-resistant plants. Double-stranded RNA generated from a replicating virus, a transgene, or an aberrant RNA can act as a key initiator molecule that is subsequently processed by an RNaseIII-like enzyme to produce 25 nt RNAs known as small antisense RNAs (Hamilton and Baulcombe 1999), which were subsequently recognized as small interfering RNAs (siRNAs). The RNA-induced silencing complex (RISC), a key component of which is an endonuclease, is then guided by the siRNAs to specifically cleave homologous RNAs (Hammond *et al.* 2000).

The involvement of PTGS in virus protection was first evident in transgenic plants using potyviral CP cDNA sequence (Lindbo and Dougherty 1992; Van der Vlugt *et al.* 1992). Lindbo *et al.* (1993) first proposed PTGS as an antiviral state in plants. This is best achieved when plants are transformed with constructs that express a self-complementary RNA, containing sequences homologous to the target plant virus. Transgene constructs encoding intron-spliced RNA with hairpin structure provided stable silencing to nearly 100% efficiency against homologous plant viruses (Smith *et al.* 2000; Tyagi *et al.* 2008; Vanderschuren *et al.* 2009; Fahim *et al.* 2010; Yong *et al.* 2010). In addition to transgene expression, transient expression of double-stranded RNA corresponding to viral sequences, either by mechanical inoculation or by *Agrobacterium*-mediated leaf infiltration, can also impart resistance to plant viruses and has been reviewed recently (Tenllado *et al.* 2004).

2. miRNA-mediated resistance

miRNAs, a class of noncoding (untranslated) RNAs of 20–24 nucleotides, are another type of small RNA products processed from dsRNA hairpin precursors by Dicers. They function as negative regulators of gene expression in plants. So far, more than 200 miRNA genes have been identified in animals and plants, which are mainly derived from the regions between protein coding genes (Lagos-Quintana *et al.* 2001; Lee and Ambros 2001; Lagos-Quintana *et al.* 2002; Reinhart *et al.* 2002; Bartel and Bartel 2003). The loci that encode miRNAs, the MIR genes, can occur in clusters in the genome and may even be transcribed polycistronically, processed sequentially into pre-miRNA and miRNA (Lee *et al.* 2002).

The involvement of the microRNA (miRNA) pathway in RNA silencing is a notable feature in plants. In *Arabidopsis*, endogenous developmental signals may trigger the formation of some imperfect dsRNAs, which are subsequently diced by DCL1 and/or other DCLs into double-stranded miRNAs. These miRNAs participate in a variety of regulatory processes: some serve as siRNA molecules in the RNA silencing pathway with perfect or near perfect base complementarity to their mRNA target; some might be recruited into the microRNA ribonucleoprotein complex (miRNP) that further regulates other post-transcriptional gene silencing (PTGS) processes, such as translational inhibition, with imperfect base-pairing interaction with their targets. The interaction between DCL and ARGONAUTE (AGO) proteins may mediate the identification and processing of different dsRNA precursors, which produce different types of small RNAs required for either plant defense or development.

It has also been shown that expression of artificial miRNA targeting viral sequences can efficiently inhibit viral gene expression conferring resistance to the virus in transgenic plants (Parizoto *et al*. 2004; Alvarez *et al*. 2006; Schwab *et al*. 2006; Qu *et al*. 2007; Duan *et al*. 2008; Naqvi *et al*. 2008; Ossowski *et al*. 2008; Pant *et al*. 2008; Molnar *et al*. 2009; Ruiz-Ferrer *et al*. 2009; Zhang *et al*. 2011).

STRATEGIES FOR BROAD SPECTRUM VIRAL DISEASE RESISTANCE

Multigene concept for viral disease resistance

Gene pyramiding is emphasized to obtain many complex biochemical pathways in plants for crop improvement and durable resistance. Approaches can involve conventional sexual crossing, re-transformation, co-transformation and the use of linked transgenes. The level of expression of a transgene is variable and is influenced by various factors, such as the site of integration. Transgene stability also varies among transformants and some plants show a variety of instability even in subsequent generations. To obtain cultivars with durable and broad-spectrum resistance, the pyramiding of major genes (multigene strategy) implying a different mode of action with insecticidal and disease resistance genes against target organisms may be a powerful strategy (Chen *et al*. 1998; Halpin *et al*. 2001; Etienne *et al*. 2006; Wakasa *et al*. 2006).

There had been significant advances made in the field of multigene insertion into plants using conventional as well as novel techniques. Various strategies have been employed in multigene manipulation, including iterative, co-transformation, multigene-linking, polycistron, and polyprotein strategies (Halpin 2005). In the multigene-linking strategy, multiple transgenes are introduced simultaneously into plants by linking multiple transgene expression cassettes onto a single T-DNA or transformation vector (Slater *et al*. 1999; Goderis *et al*. 2002; Lin *et al*. 2003). In polycistron techniques, multiple transgenes, separated by internal ribosome entry sites (IRESs) from various viruses, are fused into a single transcriptional unit in which translation is initiated by IRESs that can directly recruit ribosomes to internal positions within mRNAs (Urwin *et al*. 2000; Jaag *et al*. 2003). Multiple transgenes can be also fused into a single polyprotein via short linker sequences that are substrates for a proteinase either from host cells (Urwin *et al*. 1998; Francois *et al*. 2002) or from within the polyprotein itself (Dasgupta *et al*. 1998; Ceriani *et al*. 1998). The MultiSite Gateway, which was designed for concerted assembly and cloning of multiple DNA segments using the Gateway recombination (Cheo *et al*. 2004; Sasaki *et al*. 2004), provides a candidate method for multigene stacking in the recent years (Chen *et al*. 2006).

Broad spectrum resistance towards virus can be strategized by targeting the virus itself and also the vector in parallel. This can be achieved by a multigene construct with genes like coat protein/movement protein/replicase towards the virus and genes that make the plants tolerant to the vectors of these virus. All these techniques provide valuable addition to the existing understanding about gene stacking or gene pyramiding. Together with the increasing knowledge about the metabolic pathways and identification of genes involved, it is possible to produce tolerant crops that could thrive in adverse environmental conditions.

FUTURE PERSPECTIVE AND CONCERNS

Although the various strategies outlined can bring significant economic benefits, several concerns have been raised (Fuchs and Gonsalves 2007). They include generation of new pathogens due to recombination between transgene and non-target viruses; *trans*-encapsidation resulting in transmission of unrelated viruses to host plants; synergism between products of the transgene and viral proteins; gene flow between pollen from the transgenics to weedy relatives and production of new allergers and proteins (Tepfer 2002; Latham and Wilson 2008). These concerns provide an opportunity for the acceptance and utility of newer strategies for virus resistance. In this direction, RNA-mediated approaches such as the siRNA, and amiRNA strategies for viral resistance could be of utility. However, focus should be to develop novel mechanisms for viral disease resistance with better biosafety regulations under field conditions.

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