Genetic engineering for virus resistance

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Plant virus diseases cause severe constraints on the productivity of a wide range of economically important crops worldwide. In India the Green Revolution ushered in intensive agricultural practices and reduced varietal diversity, resulting in the emergence of viral diseases at an alarming pace in the cultivated crops. Some such diseases, which are especially relevant to India, along with their yield losses, are listed in Table 1.

Strategies for the management of viral diseases normally include control of vector population using insecticides, use of virus-free propagating material, appropriate cultural practices and use of resistant cultivars. However, each of the above methods has its own drawback.

Rapid advances in the techniques of molecular biology have resulted in the cloning and sequence analysis of the genomic components of a number of plant viruses. A majority of plant viruses have a single-stranded positive-sense RNA as the genome. However, some of the most important viruses in tropical countries like India have single-stranded and double-stranded DNA genomes and RNA genomes of ambisence polarity, i.e. genes oriented in both directions. An excellent book is now available on the organization of plant viral genomes[1]. Genome organization, electron-microscopic structures and symptoms caused by some of the viruses, referred to in this review, are briefly illustrated in Figure 1.

Concomitantly, tremendous advances have taken place in our understanding of plant–virus interaction in the process of pathogenesis and resistance. This, along with associated advances in the genetic transformation of a number of crop plants, have opened up the possibility of an entirely new approach of genetic engineering towards controlling plant virus diseases.

There are mainly two approaches for developing genetically engineered resistance depending on the source of the genes used. The genes can be either from the pathogenic virus itself or from any other source. The former approach is based on the concept of pathogen-derived resistance (PDR)[2,3]. For PDR, a part, or a complete viral gene is introduced into the plant, which, subsequently, interferes with one or more essential steps in the life cycle of the virus. This was first illustrated in tobacco by the group of Roger Beachy, who introduced the coat protein (CP) of tobacco mosaic virus (TMV) into tobacco and observed TMV resistance in the transgenic plants. The concept of PDR has generated lot of interest and today there are several host–virus systems in which it has been fully established. Non-pathogen-derived resistance, on the other hand, is based on utilizing host resistance genes and other genes responsible for adaptive host processes, elicited in response to pathogen attack, to obtain transgenics resistant to the virus. The use of non-PDR type of resistance, even though reported much less in the literature in comparison to PDR-based approaches, holds a better promise to achieve durable resistance. Various aspects of the above topics have been reviewed extensively[5–9].

Transgenics with pathogen-derived resistance

In a number of crops, transgenics resistant to an infective virus have been developed by introducing a sequence of the viral genome in the target crop by genetic transformation. Virus-resistant transgenics have been developed in many crops by introducing either viral CP or replicase gene encoding sequences. Resistance obtained by using CP is conventionally called CPMR. Replicase-mediated resistance has been pursued in a number of

Table 1. Important viral diseases of crops in India

Crop	Disease	Yield loss (%)	Virus	Virus group
Cassava	Mosaic	18–25	Indian cassava mosaic virus	Begomovirus
Cotton	Leaf curl	68–71*	Cotton leaf curl virus	Begomovirus
Groundnut	Bud necrosis	> 80	Groundnut bud necrosis virus	Tospovirus
Mungbean Blackgram Soybean	Yellow mosaic	21–70	Mungbean yellow mosaic virus	Begomovirus
Pigeonpea	Sterility Mosaic	> 80*	Pigeonpea sterility mosaic virus	Tenuivirus
Potato	Mosaic	85	Potato virus Y	Potyvirus
Rice	Rice tungro	10	Rice tungro badna and rice tungro spherical viruses	Badnavirus and waika virus
Sunflower	Necrosis	12–17	Sunflower necrosis virus	Ilarvirus
Tomato	Leaf curl	40–100	Tomato leaf curl virus	Begomovirus

*in epidemic years.

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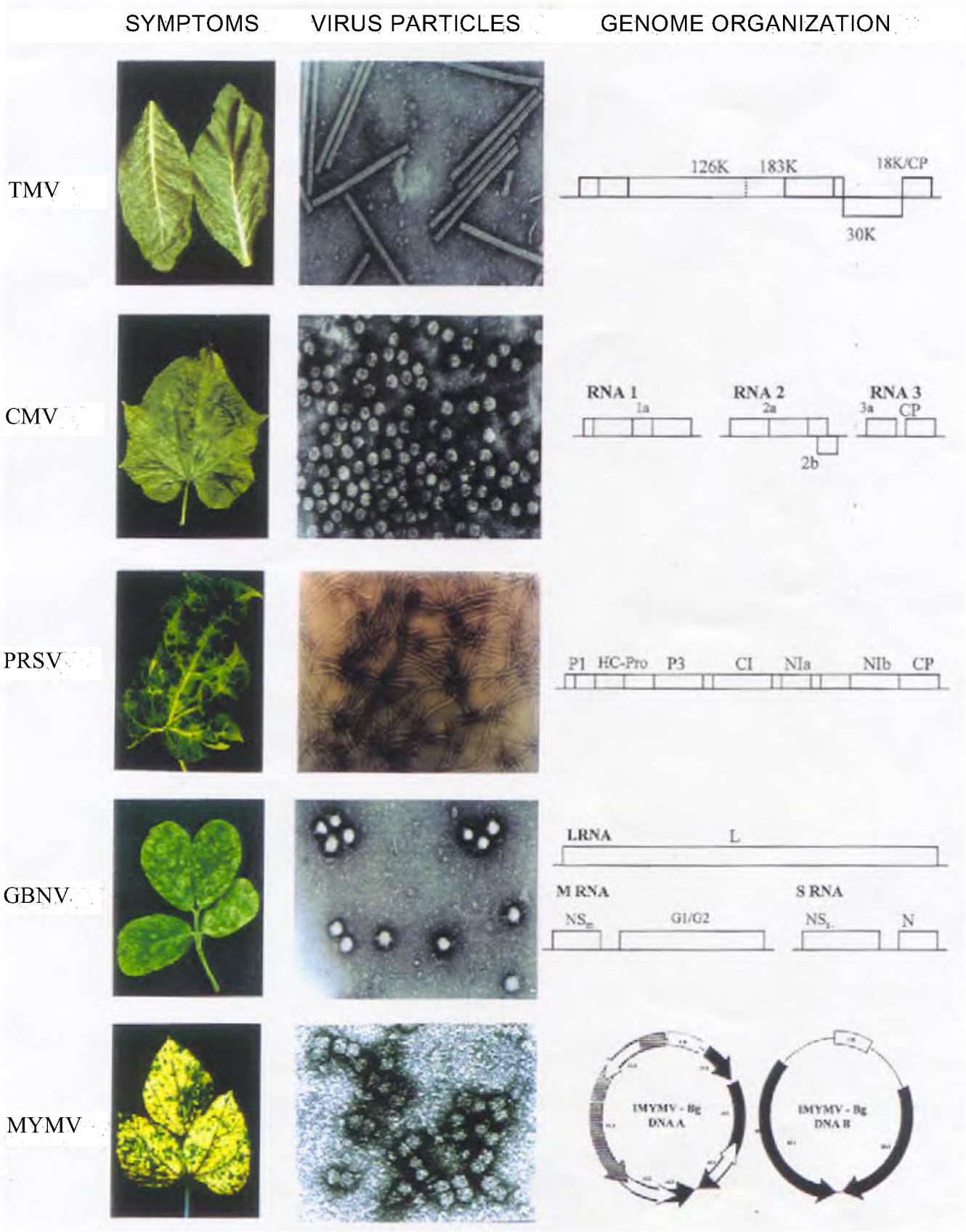

Figure 1. Symptoms, particle morphology and genome organization of some important viruses discussed in the text. Viral gene products and putative ORFs are indicated.

laboratories and in most of these cases, resistance has been shown to be due to an inherent plant response, known as post-transcriptional gene silencing (PTGS), which is described in more detail later in this article. Because of the essential nature of the viral movement protein for intercellular movement of plant viruses, movement problem sequence has also been used for achieving viral resistance. Other pathogen-derived approaches described in the literature, include the use of satellite RNA and defective-interfering viral genomic components.

Coat protein

The use of viral CP as a transgene for producing virus-resistant plants is one of the most spectacular successes achieved in plant biotechnology. Numerous crops have been transformed to express viral CP and have been reported to show high levels of resistance in comparison to untransformed plants (Table 2 and 3). Powell-Abel *et al.*[4] first reported resistance against TMV in transgenic tobacco expressing the TMV CP gene, as described in the previous section. The resistance was manifested as delayed appearance of symptoms as well as a reduced titre of virus in the infected transgenic plants, as compared to the controls. The resistance against TMV using TMV CP in tobacco was also reported to be effective against other tobamoviruses whose CP was closely related to that of TMV but not effective against viruses which were distantly related to TMV[10]. Transgenic potato, expressing the CP of potato virus X (PVX) also showed resistance against PVX[11]. However, in marked contrast to TMV, this resistance was not broken down when PVX RNA was used as the inoculum, thus indicating several possible mechanisms of CPMR.

The stage of the viral life cycle at which the CPMR is effective has been shown to vary. In TMV, it is at the virus disassembly and in the long-distance transport stage[12]. In the case of alfalfa mosaic virus (AMV), it is only at the disassembly stage, whereas in PVX, it is at multiple stages, including replication, cell-to-cell and systemic movement stages. In tospoviruses, the stage affected is believed to be replication. These mechanistic aspects have been dealt with in greater detail elsewhere[13].

Recently, considerable efforts have been made towards understanding the molecular basis of the CPMR especially in tobamoviruses. These studies may lead to more rational design of CP-derived transgenes. There is now enough evidence to suggest that CPMR results from the propensity of the transgenically expressed CP to form aggregates. For example, if the transgenically expressed CP was mutated such that there was an increase in inter–subunit interactions, the transgenic plant expressed higher levels of virus resistance[14,15]. In the case of resistance to TMV, the transgenically expressed CP sub-units are believed to re-coat the nascent disassembled viral RNA which leads to a decreased pool of the available viral RNA for translation[15,16], resulting in resistance. However, in many other cases of CPMR, the mechanisms are unclear. Hence, further studies need to be conducted to investigate the existence of mechanisms underlying CPMR.

Substantial yield increase observed in field trials of transgenic papaya and squash (Table 3) has established

Table 2. Coat protein-mediated transgenic resistance to viruses in crops

Crops	Viruses*	Field tested**
Cereals		
Maize	MDMV, MCMV	n.r.
Rice	RSV, RTSV	n.r.
Wheat	WSMV	n.r.
Fruits		
Apricot	PPV	n.r.
Cantaloupe	ZYMV, WMV2, CMV	Yes
Citrus	CTV	n.r.
Grape	GCMV, GFLV, ToRSV	n.r.
Muskmelon	ZYMV	Yes
Papaya	PRV	Yes
Plum	PPV	n.r.
Squash	ZYMV,WMV2	Yes
Vegetables		
Pepper	TSWV	n.r.
Tomato	ToMV, YMV, CMV, TYLCV	Yes
Potato	PVX, PVY, PLRV	Yes
Lettuce	LMV, TSWV	n.r.
Pea	PEMV	n.r.
Cucumber	CMV	Yes
Sugarbeet	BNYVV	n.r.
Legumes		
Peanut	TSWV	n.r.
Soybean	BPMV	n.r.
Bean	BPMV	n.r.

*MCMV, Maize chlorotic mottle virus; MDMV, Maize dwarf mosaic virus; RSV, Rice stripe virus; RTSV, Rice tungro spherical virus; WSMV, Wheat streak mosaic virus; CTV, Citrus tristeza virus; GCMV, Grapevine chrome mosaic virus; GFLV, Grapevine fanleaf virus; ToRSV, Tomato ringspot virus; YMV, Yellow mosaic virus; LMV, Lettuce mosaic virus; PEMV, Pea enation mosaic virus; BNYVV, Bean necrotic yellow vein virus; BPMV, Bean pod mottle virus; for rest of abbreviations, consult text

**n.r. indicates not reported.

Source: http://www.bspp.org.uk/mppol/1997/0116fuchs, Sivamani *et al.*[120].

Table 3. Comparative performance of transgenic virus resistant plants

Host	Transgene	Yield increase (%)*
Tomato	TMV CP	40
Tomato	CMV satellite	14
Potato	PVX + PVY CP	38
Squash	CMV + ZYMV + WMV2 CP	97
Squash	ZYMV + WMV2 CP	90
Squash	ZYMV CP	77
Papaya	PRSV CP	90

*Yield increase over susceptible non-transgenic plants.

Source: Gonsalves[121], Mayo[122].

CPMR as the most favoured strategy to engineer resistance against many viruses. The success of CPMR has prompted the production of transgenic plants expressing multiple CP genes from more than one virus. Several important crops have been engineered for virus resistance using CPMR approach and released for commercial cultivation. These include tomato resistant to TMV, tomato mosaic virus (ToMV) and cucumber mosaic virus (CMV); cucumber resistant to CMV, squash resistant to zucchini yellow mosaic virus (ZYMV) and watermelon mosaic virus (WMV2); cantaloupe resistant to ZYMV, WMV2 and CMV; potato resistant to PVX, potato virus Y (PVY) and potato leafroll virus (PLRV); papaya resistant to papaya ringspot virus, PRSV (more details can be found at the website http://www.bspp.org.uk/mppol/1997/0116fuchs). In addition, transgenic tobacco containing the CP gene of three viruses has been shown to develop resistance to all of them[17], namely tomato spotted wilt virus (TSWV), tomato chlorotic spot virus and groundnut ringspot virus.

Since CP plays a major role in vector transmission, CPMR confers additional advantage of resistance to vector inoculation in a majority of cases. For example, potato, which express PVX and PVY CP[18] and tobacco, tomato and cucumber expressing CMV CP were seen to be highly resistant to aphid transmissions. Tomato plants, having TSWV CP transgene were resistant to thrips and plums transformed with PPV CP displayed resistance to *Sharka* virus transmission[18]. Transgenic rice expressing high level of rice stripe virus CP gene expressed resistance to virus inoculation by plant-hopper[19]. However, the mechanism of vector transmission is unclear in many viruses and thus remains a fertile field of research, having potential implications for further effective control of viral diseases.

The discussion on CPMR would not be complete without reference to the most successful story of resistance to PRSV in papaya. Papaya production in Hawaii, suffered due to high incidence of PRSV in 1950s. Transgenic papaya (var. sunset) with *CP* gene was grown from 1991 to 1993, and remained virus-free for 25 months. Subsequently, it was further crossed with other popular varieties. One such variety, called Rainbow, yielded 112,000 kg/ha marketable fruits in 1995, compared to 5,600 kg/ha from non-transgenic lines. A remarkable increase in the yield clearly established the reliability of CPMR technology (more information at www.plant.uoguelph.ca/safefood/gmo/papayarep.htm).

Replicase (Rep)

Replicase (Rep) protein-mediated resistance against a virus in transgenic plants was first shown in tobacco against TMV in plants containing the 54 kDa putative *Rep* gene[20]. Similar resistances have been developed for several other viruses namely pea early browning virus[21], PVY[22] and CMV[23].

Gene constructs of *Rep* genes that have been used for resistance include full-length, truncated or mutated genes. Many of the above resistance responses have now been shown not to require protein synthesis and to be mediated at the RNA level, which is described in more detail later under 'post-transcriptional gene silencing'. This type of resistance remains confined only to a narrow spectrum of viruses, the spectrum being narrower than that of CPMR. To make the resistance broad-based, it may be necessary to pyramid such genes from several dissimilar virus-sources into the test plant genome. However, the resistance generated by the use of Rep sequences is very tight; a high dosage of input virus can be resisted easily by the transgenic plant.

Movement protein

Movement proteins (MP) are essential for cell-to-cell movement of plant viruses. These proteins have been shown to modify the gating function of plasmodesmata, thereby allowing the virus particles or their nucleoprotein derivatives to spread to adjacent cells. This phenomenon was first used to engineer resistance against TMV in tobacco by producing modified MP which are partially active as a transgene. The conferred resistance is believed to be based on the competition between wild-type virus-encoded MP and the preformed dysfunctional MP to bind to the plasmodesmatal sites[24,25]. The above resistance was moreover seen to be effective against distantly related or unrelated viruses, for example resistance against TMV could be achieved in tobacco using the MP derived from brome mosaic virus, suggesting functional conservation of this protein among several viruses[26].

In contrast to the single MP gene in tobamoviruses, viral movement is mediated by a set of three overlapping genes, known as the triple-gene-block (TGB) in potex-, carla- and hordeiviruses. Expression of the modified central 12 kDa TGB gene of PVX, was shown to confer MP-derived resistance in potato to potexvirus PVX and carlaviruses potato virus M and potato virus S[27]. However, resistance was overcome when inoculated with viruses lacking a TGB, like PVY. This indicated that the resistance depended upon the interaction of the viral-derived and the transgene-derived MPs.

Satellite RNA

Besides using the genomic components of an infectious virus, a strategy exploiting the use of satellite RNA associated with certain viruses received great attention. Some strains of CMV encapsidate satellite RNA (sat RNA) in addition to the tripartite messenger sense, single-stranded RNA genome. CMV sat RNA depends on its helper virus (HV) CMV for replication, movement within the plant,

encapsidation and transmission. The presence of sat-RNA modulates the symptoms induced by the HV and often depresses HV accumulation in different host species. Thus, transgenic tobacco plants expressing multiple or partial copies of CMV sat-RNA showed attenuated symptoms when challenged with CMV[28]. In addition, tobacco plants transformed with anti-sense sat-RNA also showed delayed symptom development with the cognate virus[29].

Sat-RNA was tested as a bio-control agent in field trials in many countries with considerable success[30–32]. Tomato, containing non-necrogenic sat-RNA sequences developed only faint symptoms following CMV infection. The timing of fruit set and fruit yield in transgenic plants was comparable with healthy plants. Thus, high-level of tolerance to CMV conferred by sat-RNA in tomato was demonstrated[32]. This was further improved[33] by combining sat-RNA and CMV CP. The mechanism behind sat-RNA-mediated resistance may be attributed to the reduction in accumulation of the HV and its long distance movement and down-regulation of replication. However, as sat-RNA spreads epidemically, sufficient caution will have to be exercised in adopting this technology.

Defective-interfering viral nucleic acids

In several viruses, truncated genomic components are often detectable in infected tissues, which interfere with the replication of the genomic components. These species of DNA are also called defective interfering (DI) DNA and expression of delayed disease symptoms and recovery, coupled with increased resistance upon repeated inoculation have been observed in plants engineered with DI DNA[34]. For example, incorporation of subgenomic DNA B that interferes with the replication of full length genomic DNA A and B confers resistance to ACMV in *N. benthamiana*[35].

Self-cleaving RNA (ribozymes), seen in viroids and some sat-RNA, were also used with high expectations. There are a few reports like targeting PLRV CP and replicase[36] and 5′ region of TMV RNA[37] and citrus exocortis viroid[38]. In most of the cases, ribozyme sequences were ineffective and the resistant phenotypes observed were duse to antisense RNA.

Transgenics with non-pathogen derived resistance

The following section describes the non-pathogen-derived strategies, i.e. those utilizing genes derived from either the host plant or any other non-pathogenic source. A new phenomenon called post-transcriptional gene silencing (PTGS) has recently been shown to be responsible for the inherent ability of many plants to specifically degrade nucleic acids in a sequence-specific manner, including those of viruses. Thus, this strategy can be very effective in engineering virus resistance. The other non-pathogen derived strategies are the utilization of plant disease resistance genes, the ribosome-inactivating proteins, plant proteinase inhibitors, human interferon-like systems, antiviral antibodies expressed in plants, systemic acquired resistance and secondary metabolite engineering.

Post-transcriptional gene silencing

Post-transcriptional gene silencing (PTGS) is a specific RNA degradation mechanism of any organism that takes care of aberrant, unwanted excess or foreign RNA intracellularly in a homology-dependent manner. It is prevalent in various forms of life, namely plant, fungus and invertebrate animals. This activity could be present constitutively to help normal development or induced in response to cellular defense against pathogens. In this mechanism, the elicitor double-stranded RNA (ds RNA), commonly produced during viral infection, is degraded to 21–25 nucleotides, termed as small interfering RNA (siRNA), with the help of a variety of factors that have already been or are being identified[39]. A complex of cellular factors, namely RNA-dependent RNA polymerase (RdRp)[40], RNA-helicase[41], translation elongation factor[42], RNAse[43], etc. along with the small 21–25 nt RNA (of the elicitor RNA) acting as the guide RNA[44], supposedly degrade RNA molecules bearing homology with the elicitor RNA. This degradation process, initiating from a concerned cell having the elicitor RNA, spreads later within the entire organism in a systemic fashion. This process is generally regarded to have evolved as a plant defense mechanism against invading viruses containing either RNA[45] or DNA[46] genomes.

When the viral RNA is either the elicitor or target of PTGS, the degradation mechanism is known as virus-induced gene silencing (VIGS). VIGS comes into play when plants recover from initial viral infection (viral recovery) or plants resist superinfection of viruses with genomes bearing homology with those of the viruses used as primary inoculum. If tobacco rattle virus (TRV) infects *N. benthamiana*, the plant develops initial symptoms of viral infection at the inoculated region. But the plant shows signs of recovery later and newly emerging leaves are free of TRV. It was shown that viral replicative RNA forms are degraded during the process of recovery, thus indicating the presence of PTGS-related mechanisms[47].

In nepovirus-infected *Nicotiana* sp., there are severe viral symptoms on the inoculated and first systemic leaves. However, the upper leaves that develop after systemic infection are symptom-free and contain a lower concentration of virus than symptomatic leaves. Similarly, *N. clevelandii* inoculated with tomato black ring nepovirus (W-22 strain) initially shows symptoms and later recovers by PTGS mechanism[48]. In addition, if a secondary inoculum of W22 is applied to the recovered

leaves, no additional accumulation of W22 RNA above that resulting from the primary inoculation is seen and the plants remain symptom-free. This kind of resistance is not observed with secondary inoculation of viruses that are unrelated to the genomic sequence of W22. Thus, the resistance of the recovered leaves to subsequent viral challenge depends upon the homology-dependent process. A similar resistance involving PTGS applies not only to RNA viruses but also to DNA viruses[45,49].

Viruses can also induce silencing of host endogenes and transgenes that are similar in sequence to the inoculated virus. The applicability of this principle has been demonstrated by using a fused transgene containing *TSWV–N* gene and the PTGS-inducing turnip mosaic virus (TuMV) *CP* gene[50]. The transgenic *N. benthamiana* showed resistance to both viruses by a PTGS-dependent phenomenon. Silencing can be achieved when the silenced gene is present in either sense or antisense orientation. During silencing, not only the target host gene transcripts but also the viral RNA forms are degraded. Thus it is easily conceivable that the infecting viruses could be inactivated by PTGS mechanisms if the host carries the transgene(s) of the same or similar virus. In fact, such phenomena of recovery/resistance can be explained using PTGS. In a majority of Rep-mediated resistance, mentioned earlier, resistance is now known to occur utilizing the PTGS mechanisms[51], which provide the molecular basis of such phenomena. For the majority of the transgenic plants showing PDR phenotypes using antisense, untranslatable or non-coding regions of the virus, PTGS have been well documented and the level of resistance parallel the level of silencing. Direct correlation between the viral recovery or resistance and PTGS has been demonstrated using the mutant plants that are deficient in one or some of the components required for PTGS[40,41].

Resistance generated against PPV in *N. benthamiana* is a good example of application of this principle for virus control using PTGS. About 10-kb-long RNA genome of PPV is shown in Figure 2. Isolated viral transgene(s) have been chosen from almost every segment of the genome and the transgenic plants are able to resist PPV. Since all the events of recovery or resistance were linked to the loss of viral replicative RNA and the transgenic RNA forms, PTGS must have played its part in conferring the resistance to PPV[51].

Antisense-mediated gene silencing (ASGS) and PTGS with sense transgenics are remarkably similar in mechanistic terms. Both forms of silencing are involved in production of 20–25 nt long degraded RNA (siRNA) and both forms are suppressible by the same viral proteins known to inhibit PTGS[52] (as mentioned later). However PTGS works effectively only when both the sense and antisense RNAs are simultaneously present in the plant cell. Transgene constructs engineered to produce dsRNA as opposed to single stranded sense(s) or antisense (a/s) RNA cause higher incidence of RNA silencing[53,54]. The Pro-gene sequence of PVY was used to demonstrate this effect[55]. Tobacco plants were generated using gene constructs encoding the 'Pro' sequence in the s, a/s or in both

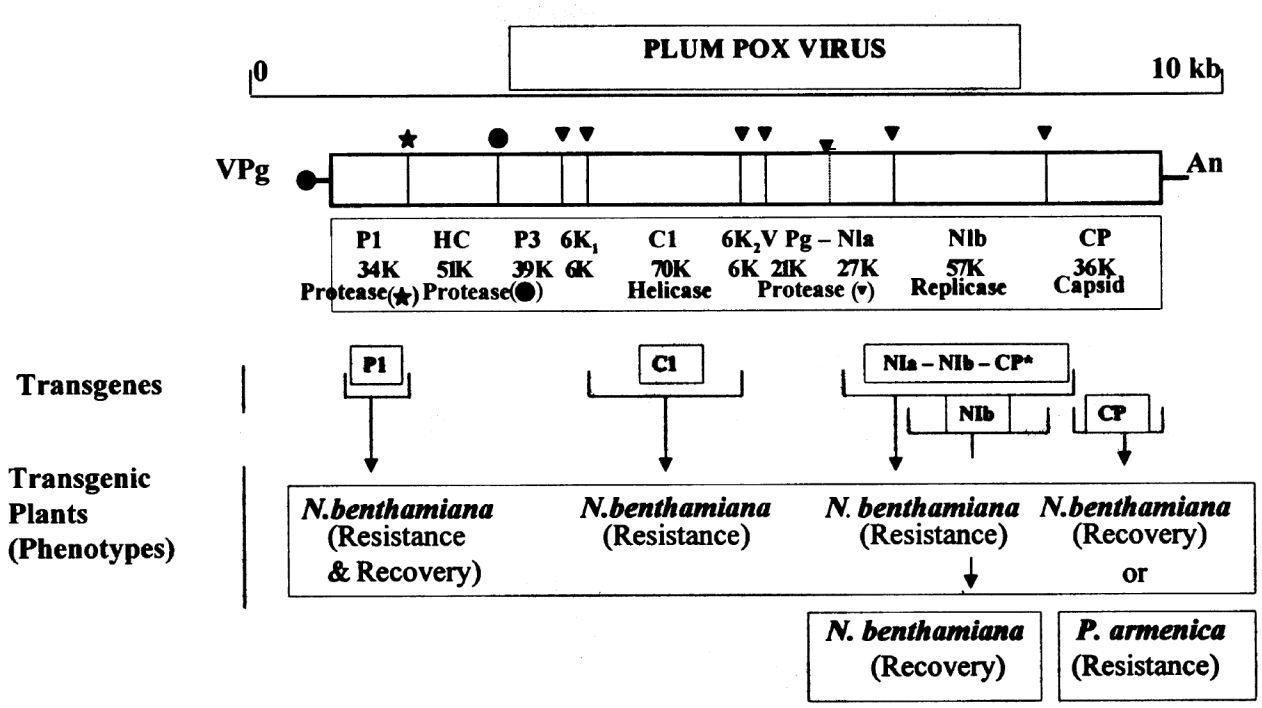

Figure 2. Schematic diagram showing genetic map of PPV with the regions used for the production of transgenic plants for virus resistance. Gene products and the phenotype of the plants are indicated.

the orientations. The plants challenged with PVY were scored for symptoms and tested for PVY replication by ELISA. Results of progeny segregation analysis indicated that, unlike some of the simple s or a/s constructs, the s plus a/s constructs gave stable immunity to PVY, which was inherited in a Mendelian fashion. PVY immunity could also result when the sense and antisense *Pro* gene transcripts of the PVY-susceptible tobacco transformants were brought together by sexual crossing. Such findings confirm that the simultaneous expression of the sense and antisense RNA in the plant was responsible for enhanced PVY immunity.

Many viruses have evolved mechanisms to suppress host PTGS activity. The balance of the pro-PTGS and anti-PTGS activities probably determines the outcome of virus–plant interaction. Table 4 shows the known plant and viral genes inducing or repressing PTGS. PVX does not encode for any strong anti-PTGS activity by itself. Hence PVX-based recombinant viral vectors containing test genes from various viruses have been used for infecting silenced GFP-transgenic plants to screen for PTGS suppressing activity of the viruses[56]. None of the genes shown in Table 4 has been used yet for plant transformation studies to develop or modulate viral resistance. Once the biochemical steps of PTGS are revealed, it may be easy to sort out the appropriate genes and target them to engineer viral resistance.

Plant disease resistance genes

A number of disease resistance genes (R) have been reported against viruses of crop plants (Table 5). They encode products which respond to viral signals (avirulence (*avr*) gene products) culminating in a number of resistance responses in the plant. As shown in Table 5, many of the corresponding viral *avr* genes have also been identified. Some of the *R* genes have been shown to complement the disease susceptibility phenotype in the corresponding cultivars when used as transgenes, furnishing a direct proof of their action. The following section describes the current knowledge about *R* genes against viruses and their mechanisms of action. Excellent reviews on this subject are available in the literature[57–59].

R genes in plants are defined by the classical gene-for-gene hypothesis[60], which states that for every incompatible host pathogen interaction, there exist matching *R* genes in the host and *avr* genes in the pathogen. Resistance reaction against pathogen results generally by direct interaction between the products of *R* and *avr* genes. This interaction, in many cases, results in a resistance reaction, known as hypersensitive reaction (HR), which can be defined as a specific response of a host towards a pathogen. HR results in localized cell death, appearing as necrotic lesions at the site of pathogen entry. HR results in the arrest of pathogen spread, thereby effectively restricting it to the dead cells.

All known *R* genes encode products having two basic functions: to act as sensors for the corresponding *avr* factors/elicitors and to initiate signalling cascades for the expression of defence-related genes. A number of structural features are conserved across several *R* gene products[57]. These include leucine-rich repeat (LRR), nucleotide-binding site (NBS), serine-threonine kinase, leucine zipper, toll-interleukin region (TIR), etc. These structural features are believed to have important roles to play in the execution of the above functions[61]. The following sections describe different types of resistance responses initiated due to *R* genes against viruses and their mechanisms.

One of the *R* genes against a viral pathogen (which has been analysed in great detail) is the *N* gene of tobacco[62] and provides resistance against TMV. The *N* gene product has a prominent TIR (a signalling domain) at the

Table 4. Plant and viral genes inducing or repressing PTGS

Genes	Biochemical function	Source	Possible PTGS-related role
Plant genes inducing PTGS			
Sde1 or *SgS2*	Replication of RNA template	*Arabidopsis*	Synthesis of cRNA, amplification of dsRNA, signalling of methylation, synthesis of systemic signal, viral defence
Ago1	Translation elongation (eIF2C-like)	*Arabidopsis*	Target PTGS to ribosome, signalling of methylation, development
Sgs3	Coiled-coil protein	*Arabidopsis*	Viral defence
Rgs–CaM	Calmodulin-like protein	*Nicotiana tabacum*	Suppression of PTGS, development
Viral genes repressing PTGS			
HC–Pro	Replication/proteinase	PVY TEV	Blocks accumulation of 25-mer RNA
P25	Viral movement	PVX	Blocks generation of systemic signals of PTGS
2b	Viral movement	CMV	Blocks initiation of PTGS at the nuclear step
AC2	Virion-sense transcription enhancer	ACMV	PTGS inhibitor

amino-terminus and a LRR (a recognition domain) at the carboxyl-terminus of the polypeptide. The TIR domain exhibits a strong homology with the *Drosophila* toll receptor protein, which is a well-characterized signalling molecule. The *N* gene product recognizes the TMV replicase as the *avr* factor. Transposon mutagenesis was performed to obtain HR^- lines of the tobacco cultivar Samsun, which were then used to clone the *N* gene adjacent to the sites of transposon insertion[62]. The cloned *N* was shown to be sufficient for the production of a typical HR by complementation analysis. Transgenic tomato plants, expressing the cloned *N*, were also shown to develop resistance against the virus[63]. The *N* gene was thus seen to retain its effectiveness for initiating a HR even in a heterologous system and was the first example of the use of a *R* gene in providing transgenic protection against virus in a useful crop plant.

Turnip crinkle virus (TCV) resistance in *A. thaliana* is mediated by an altogether different mechanism. The *RTM* gene, present in ecotype Columbia-O, brings about a HR-independent resistance against TCV by affecting its long-distance movement and is present as two alleles, *RTM1* and *RTM2*. Both the above allellic forms were cloned by map-based approach and shown to complement the TCV-susceptibility of the *rtm* mutant[64,65]. The RTM protein is believed to interfere directly with an essential component of the long-distance movement of the virus. Thus, model plants like *Arabidopsis* can help us in looking for related *R* genes in crop plants.

Another type of resistance response is seen against PVX in certain varieties of potato carrying the *Rx* gene. This response, termed extreme resistance, is characterized by the rapid arrest of virus accumulation at the sites of infection and by the absence of HR. Gene *Rx* was cloned from potato cultivar Cara by a map-based cloning approach. The functionality of the gene was demonstrated by its ability to prevent the replication of a PVX-derived vector in tobacco *N. benthamiana* using a transient assay[66]. The cloned DNA fragment was used to produce transgenic potato cultivar Maris Bard (*rx* genotype), which developed resistance against mechanically inoculated PVX. Moreover, the above resistance resembled that mediated by *Rx*. Similar results were also demonstrated in transgenic tobacco[66,67].

The other anti-viral *R* genes which have been identified are *Sw-5* (ref. 68) and *Tsw*[69] against TSWV from tomato and pepper respectively, *Ry*[70] against PVY, from *Solanum stoloniferum*, *Va* against tobacco vein mottling virus (TVMV) from *N. tabacum* cultivar Burley[71], *TuRB01* from *Brassica napus* against TuMV[72], *I* against bean common mosaic virus (BCMV) from *Phaseolus vulgaris*[73], *L2* and *L3* against pepper mild mottle virus (PMMV) from *Capsicum* sp.[74,75], *Nx* and *Nb* against PVX from *Solanum tuberosum*[76] and *Tm1*, *Tm2* and *Tm2(2)* against TMV from *Lycopersicon esculentum*[77–79]. There is, however, no report of use of the above resistance genes in engineering resistance against viruses in crop plants.

Many of the *R* genes studied so far are clustered in plant genomes and can induce resistance to diverse pathogens as exemplified by the *Rx* and the *Gpa2* genes, which are tightly linked, specifying resistance against PVX and nematode[80]. Such a scenario can be expected to be more widespread, encompassing more than one viral pathogen. Thus, understanding the molecular interactions between the various *R* genes products and their elicitors would help in a better and more effective design for their use in providing resistance against a wide spectrum of pathogens at the field level.

Strategies to achieve broad-spectrum pathogen resistance utilizing the *R* genes are also being developed and tested. Resistance in tomato to the bacterial pathogen *Pseudomonas syringae* pathovar tomato requires *Pto* and *Prf* genes. *Prf* belongs to the NBS-LRR superfamily of plant disease resistance genes. Overexpression of *Prf* in tomato cultivar lacking the gene leads to enhanced resistance to a number of pathogens, including TMV[81].

Table 5. *R* genes against viruses and corresponding *avr* gene products

Resistance gene	Source plant	Avr product of the virus	Pathogen
HRT	*Arabidopsis thaliana* ecotype Dijon	Coat protein	TCV
I	*Phaseolus vulgaris*	n.d.	BCMV
L2	*Capsicum* sp	Coat protein	PMMV
L3	*Capsicum* sp.	Coat protein	PMMV
N	*N. tabacum* cultivar *Samsun*	Replicase	TMV
RRT	*Arabidopsis thaliana* ecotype Dijon	Coat protein	TCV
RTM	*Arabidopsis thaliana* ecotype *Columbia*-O	n.d.	TCV
Rx, Nx, Nb	*Solanum tuberosum* cultivar *Cara*	Coat protein	PVX
Ry	*Solanum stoloniferum*	NIa protease	PVY
Tm1	*Lycopersicon esculentum*	Replicase	TMV
Tm2	*L. esculentum*	Movement protein	TMV
Tm2(2)	*L. esculentum*	Movement protein	TMV
TuRB01	*Brassica napus*	Cylindrical Inclusion protein	TuMV
Va	*Nicotiana tabacum* cultivar *Burley*	Covalently-linked viral genomic protein	TVMV

n.d. not determined; PMMV, Pepper mild mosaic virus; BCMV, Bean common mosaic virus; TVMV, Tobacco vein mottling virus; for rest of the viruses, consult Table 2 and text.

The most exciting approach towards engineering improved resistance to multiple diseases may be the development of new *R* genes having multiple specificities[82,58]. The *Fen* (resistance to the insecticide Fenthion) and *Pto* genes are located in the same *R* gene cluster in the tomato genome and they are 86% identical in nucleotide sequence. A functional gene was made by domain swapping of the two genes[83], thus raising the possibility of creating a hybrid gene containing multiple specificities[82]. Another novel strategy, termed two-component approach, has been developed lately and holds lot of promise for introducing broad-spectrum resistance. This strategy involves generation of transgenic plants that express a pathogen *avr* gene under the control of a heterologous infection-inducible promoter. If the plant carries the matching *R* gene, it will respond with an HR at the site of infection thus limiting the pathogen. The key to this approach is the identification of suitable promoters that respond or are induced only following infection by broad-range pathogens. Such promoters have been described in the literature[84–86] and the validity of this transgenic approach has also been demonstrated[85,87].

Ribosomal inactivating proteins

Several plants have been found to contain antiviral proteins, commonly termed as ribosome-inactivating proteins (RIPs). RIPs inhibit the translocation step of translation by catalytically removing a specific adenine base from 28S ribosomal RNA. They are synthesized either as pre- or pre-pro-proteins[88] and targeted to vacuoles. Because of their specific intracellular localization, RIPs do not affect the endogenous 28S RNA. It is supposed that RIPs enter cells together with the viruses and exert the damage to the host ribosome or possibly viral RNA[89].

The antiviral activity of several types of RIPs has been well-documented[90]. When purified RIPs are mixed with viruses and applied on plants, virus multiplication and symptom development are dramatically suppressed. A broad range of viruses can be suppressed in this manner. Some RIPs not only inhibit local virus multiplication in RIP-treated leaves but also block viral multiplication systemically. Hence RIPs release a signal that induces systemic resistance to viruses. The development of systemic resistance was reported following studies on induction of a 34 kDa basic protein from the RIP (CA-SRI) treated *Cyamopsis tetragonoloba* plants[91].

The genes for RIPs have been isolated from a number of plant sources. The cDNAs for PAP (Pokeweed), MAP (*Mirabilis jalapa*), Trichoxanthin (*Trichoxanthes kirilowi*), Dianthin (*Dianthus caryophyllus*), Momorcharin (*Momordica charantia*), CA-SRI from *Clerodendrum aculeatum*[92], Ricin (*Ricinus communis*), etc. have been isolated and characterized. These cDNAs have also been used to transform plants and in many cases the transgenic plants have shown broad-range antiviral activities. Transgenic *N. benthamiana* plants expressing PAP have been shown to offer broad-spectrum virus resistance, to both mechanical and aphid transmission[93]. In another experiment, the toxin gene, dianthin was placed downstream of a trans-activatable geminivirus promoter from ACMV[94]. When transgenic *N. benthamiana* plants were inoculated with ACMV, dianthin was synthesized only in the virus-infected tissues where it inhibited virus multiplication.

Protease inhibitors from plants

Many viruses, namely poty-, tymo-, nepo-, como-, and closteroviruses need cysteine protease activity to process their own polyproteins for their replication and propagation. Hence plants expressing cysteine protease inhibitors might resist the growth of viruses as mentioned above. This idea was tested[95] by using cysteine protease inhibitors (oryzacystatin) of rice to successfully engineer resistance against potyviruses in transgenic tobacco plants. Tobacco lines expressing the rice cysteine protease-inhibitor gene were examined for resistance against tobacco etch virus (TEV) and PVY infection. A clear, direct correlation between the level of oryzacystatin message, inhibition of papain (a cysteine protease) and resistance to TEV and PVY in all tested transgenic lines was observed. Expectedly, no protection has been found against the TMV infection because this virus does not require polyprotein-processing for its growth. These results indicate that plant proteinase inhibitors can be used against different potyviruses and potentially also against other viruses, where protein cleavage is an essential part of their life cycle.

Interferon-like systems

Higher vertebrates resist virus infections in part by catalysis of RNA decay using the interferon regulated 2-5A system. The 2–5A system consists of two enzymes, namely a 2–5A synthetase that makes 5′ phosphorylated, 2′-5′-linked oligoadenylates (2–5A) in response to double-stranded DNA, and the 2–5A dependent RNAse L. In plants, homologues of this system are not yet known but the inducers, i.e. interferon-like molecules have been reported. The above human enzymes have been co-expressed in transgenic tobacco plant[96]. The transgenic tobacco produced low-level but functional 2–5A synthetase and activated RNAse L. These transgenic lines were tested positive for their proficiencies to resist at least three different types of viruses: TEV, TMV and AMV.

Anti-viral plantibodies

Another approach to control plant viruses is to express specific anti-viral antibodies in plants, commonly known

as plantibodies. The efficacy of this approach has been demonstrated[97] against Artichoke mottled crinkle virus in transgenic *N. benthamiana*. A panel of monoclonal antibodies was raised against AMCV and the gene for the most reactive of the above panel was cloned and expressed in *N. benthamiana*. The above transgenic plants and their progeny showed lower virus accumulation, reduced incidence of infection and delayed symptom appearance, compared to non-transgenic plants.

A similar approach was utilized to test *N. benthamiana* plants expressing single-chain antibody against the CP of beet necrotic yellow vein virus[98]. A significant delay in symptom development in the above transgenic plants was reported, following mechanical inoculation and inoculation with the natural vector *Polymyxa betae*. Monoclonal antibodies against various gene products of TSWV have been introduced into tomato to generate continued resistance to both TSWV and root knot nematode[99].

Systemic acquired resistance

Following viral infections, plants develop an active resistance which is at first localized only at the site of infection, but spreads systemically in due course. This resistance, called systemic acquired resistance (SAR), is characterized by the coordinate activation of several genes in uninfected, distal parts of the inoculated plants. SAR is characteristically associated with accumulation of salicylic acid (SA), enhanced expression of pathogenesis-related (PR) proteins activation of phenylpropanoid pathway, leading to the synthesis of higher phenolic compounds, increase of active oxygen species and reinforcement of cell wall by the deposition of lignin and suberin. Involvement of SA in TMV resistance has been shown by expressing the bacterial salicylate hydroxylase (*NahG*) gene in tobacco plant, thus decreasing its endogenous salicylic acid, and causing susceptibility to TMV infection[100].

The discovery that SA-binding protein is a catalase, whose activity is blocked by SA led to the proposal that the mode of action of SA is to inhibit the hydrogen peroxide degrading enzyme catalase, resulting in elevation of hydrogen peroxide levels. Transgenic tobacco plants were developed[101] that expressed catalase 1 (*Cat1*) or catalase 2 (*Cat2*) gene in an antisense orientation. Antisense catalase transgenic plants exhibiting severe reduction in catalase activity (approximately 90% or more), developed chlorosis or necrosis on lower leaves. These plants also showed high level of SA and PR accumulation as well as enhanced resistance to TMV.

In another experiment[102], tobacco was transformed with two bacterial genes coding for enzymes that convert chorismate into SA by a two-step process. When the two enzymes were targeted to the chloroplast, the transgenic plants showed 500- to 1000-fold increased accumulation of SA and SA-glucoside, compared to control plants. The level of PR-proteins was enhanced and these plants showed resistance to viral and fungal infection, in a mode similar to SAR in nontransgenic plants.

Secondary metabolite pathways

Metabolic pathways which are important in viral pathogenesis are key targets for intervention against viral infection. One such step is mediated by *S*-adenosyl homocystein hydrolase (SAHH), which is a key enzyme in trans-methylation reactions that take place, using *S*-adenosylmethionine as the methyl donor. It is suggested to play a role in 5′ capping of mRNA during replication. The antisense RNA for tobacco SAHH was expressed in transgenic tobacco plants. Though 50% of the plants showed stunting, they were resistant to infection by various plant viruses. Analysis of the physiological changes in these plants showed that they contained excess level of cytokinin. Since cytokinin is known to induce acquired resistance, increased resistance observed might be attributed to increased level of cytokinin.

Another novel approach of interference with viral pathogenesis is to inhibit tetrapyrrole biosynthesis by expressing antisense RNA of uroporphyrinogen decarboxylase or coporphyrinogen oxidase in *N. tabacum*[103]. The plants were characterized by accumulation of photosensitizing tetrapyrrole intermediates, accumulation of highly fluorescent Coumarin scopolin, PR proteins and reduced levels of infecting viral RNA.

Essential considerations for developing virus-resistant transgenics

Variability

Viral genes show high levels of variability. This may be due to lack of proof reading function of viral replicases and the high recombination rates of viral genomes during the progress of infection. Symptomatic variants or strains of viruses, as well as geographically distinct isolates, not showing such variations in symptoms, have been nevertheless, documented to contain significant variability in their genes. Under field conditions, most of the viruses are believed to exist as collection of variants, or 'quasi-species', as documented in cassava-infecting geminiviruses in Uganda[104] and rice tungro bacilliform virus, a double-stranded DNA virus in southeast Asia[105,106].

As with naturally occurring virus resistance genes, when considering virus resistance under field conditions, strain specificity and breadth of protection are important questions. There is often a general correlation between the extent of protection and the relatedness between the challenge virus and virus from which the transgene was derived. It is clear from the case of transgenic papaya that the level of resistance is dependent upon the homo-

logy between the prevalent viral isolate and the transgene[107]. It is imperative that in any viral transgene strategy, sequence of the aggressive prevalent strain of the virus in that region is used. Sufficient information on the degree of diversity amongst the biologically indistinguishable viral strains needs to be collected before designing the transgene. It is especially true of whitefly transmitted geminiviruses, where the evolution of the virus is rapid[108]. A wide variety of virus genotypes may be present, either maintained in different cultivated hosts or on endogenous weed species. Depending upon change in the vector behaviour, e.g. feeding on to a new host more frequently than it was doing earlier and vector population build-up, viruses of different populations may start infecting new hosts leading to further changes in their genotype.

The success of any transgenic strategy is dependent upon the level of resistance to multiple inoculation of the same or related strains, by vector transmission. In recent years, efforts have been made to identify the variants and to assess the genetic relatedness between them. However, frequency distribution of these variants in a given virus population needs to be assessed to develop a transgenic strategy targeting any virus causing an economically important disease. The population structure of the virus is determined by evolutionary factors affecting its life cycle, the major factor being selection pressure on the gene products that interact with host and the vector. Variability may result due to host component as new host genotypes are introduced, or by vector component as they adapt to new host system or by the virus itself by mutation, complementation or recombination. A periodical assessment of population structure is mandatory if virus-derived transgenic resistance strategy is adopted for the control of the disease. It is especially true of India, where strain variability is observed and which would result in breakdown of resistance[109].

Biological risks

The concept of using pathogen-derived genes to induce transgenic resistance has no doubt raised a number of ecological concerns[110]. Risk perceptions boil down to two major items, (i) recombination between viral-derived transgene and non target virus[111], (ii) transmission/vector host range changes brought about by heteroencapsidation, i.e. encapsidation of the genome of non-target virus with the transgenically expressed CP. Field trials conducted so far with transgenics[17] have not indicated that expression of viral transgenes leads to the emergence of new super strain or change in transmission behaviour of common viral pathogens. However, sufficient care should be taken to avoid any risks due to heteroencapsidation while designing the constructs.

The strong linkages shown by CP with insect transmission of viruses, have made possible heteroencapsidation, an important factor to be considered while designing CP-based transgenes. Coat protein genes have been designed[112] from PPV, such that a 'DAG' motif in the CP, believed to play an important role in vector transmission, was deleted to prevent any further insect transmission of heteroencapsidated virions. The use of these constructs in producing transgenic plants has shown that heteroencapsidation of ZYMV was significantly reduced without compromising virus resistance of the plants. Similar results have also been reported recently in transgenic *N. benthamiana* expressing mutated PPV CP, which were not only resistant to PPV, but were also suppressed in heteroencapsidation, when infected with chilli vein mottle virus and PVY[113].

Comparison of anti-viral strategies

The success of transgenic approach varies for any specific host/virus combination. A range of phenotypes is observed amongst the virus-resistant transgenic plants. While CPMR confers broad-spectrum, less complete resistance, Rep-mediated resistance produces immunity against the virus, but to a limited spectrum of strains. Similarly, in RNA-mediated resistance, antisense RNA targeting mRNA of DNA viruses has more potential than against positive-stranded RNA virus. Any antisense RNA/ribozyme strategy should bear in mind the association/dissociation parameters of the molecules. Pyramiding of different transgenes or combination of transgenes with natural resistance targeting different events in viral life cycle will increase the confidence level in the management of viral diseases and will ensure stability of resistance at the field level. Durability, broad-spectrum character of the transgene-derived resistance coupled with enhanced crop yield of the transgenics *viv-à-vis* healthy, untransfomed plants, etc. are some of the essential parameters, which any important strategy must incorporate.

Economically important plant viruses in India and future outlook

In India, the post-green revolution era saw an upsurge in agricultural operations all over the country. Practices like introduction of new genotypes, indiscriminate use of insecticides, change in cultivation practices, etc. tilted the balance in favour of vector-transmitted diseases in several crops. For example, cultivation of soybean as an industrial crop in large areas, continued cropping of *moong* in summer months, without leaving any time lapse, introduction of susceptible germplasm of Nigerian cowpea, etc. led to the perpetuation of the vector whiteflies and to the availability of the viral inoculum throughout the year. The above reasons have been speculated to give rise to epidemics of yellow mosaic diseases of legumes[109]. The scenario changes every year. In 1980s, the diseases

caused by potyviruses and whitefly transmitted geminiviruses were the prominent ones resulting in considerable yield loss (Table 1). In the last five years, Ilarviruses causing severe necrosis and destruction of crop in sunflower and grain legumes[114] and tospoviruses producing severe bud necrosis in groundnut, tomato, melons and grain legumes have emerged as serious pathogens[115]. The host range of these viruses is spreading and in future, many more crops may get infected. We have listed ten most important viral diseases observed in crops extensively grown in India (Table 1). Wide range in yield loss data given indicates the changes from year to year in the incidence and severity of the disease. The disparity is also due to diversity within particular virus and crop genotypes. Beside the viruses listed, viral diseases of horticultural crops like banana bunchy top disease in banana, tristeza virus disease in citrus, papaya ring spot viral diseases in papaya have also assumed serious and unmanageable proportions.

For most viral diseases, resistant lines have been developed by conventional breeding and along with judicious insecticide sprays to control the vector population, help in management of the disease. Some of the examples include cultivar Sree Vishakam in cassava against ICMV, LR5166 in cotton against CLCuV, K-134 in groundnut against bud necrosis virus, Kufri Chandramukhi in potato against PLRV and PVY and Vikramarya in rice against the tungro virus disease. However, when the source of resistance is not available, a biotechnological approach becomes necessary. For the whitefly-transmitted geminiviruses like ToLCV, CLCuV, ICMV and yellow mosaic virus in legumes, results obtained in many laboratories with transgenics containing replication initiation protein are encouraging and this approach could be adopted. CPMR for Ilarviruses, both CPMR and PTGS for potyviruses, have shown promising results, which could be adapted for viruses of India. The *NS* and *NM* genes, similarly, have been used for tospoviruses. Characterization of *R* genes associated with the well-established resistant lines, if achieved, will lead to a long-lasting solution.

Efforts initiated in various research organizations in India towards the development of virus-resistant transgenics have been summarized in the following section.

Following the availability of molecular information on viruses, initiatives have been taken in some leading institutions in India towards the development of transgenic virus resistance in important crops. At the Indian Institute of Science, Bangalore, success has already been reported in controlling physalis mottle virus using pathogen-derived resistance in tobacco[116,117] and tomato[118]. A similar approach has been recently shown to result in resistance to PVY in tobacco in a collaborative research programme between the Central Potato Research Institute, Shimla and the Bhabha Atomic Research Centre, Mumbai[119]. Tobacco and tomato transformation using TLCV CP and replicase genes is being attempted at the National Botanical Research Institute, Lucknow. Similar approaches are also being used to generate resistance against viruses of important crops like cotton, rice, tomato and mungbean at the Indian Institute of Science, Bangalore, University of Delhi South Campus, New Delhi, the Indian Agricultural Research Institute, New Delhi, Madurai Kamaraj University, Madurai and Maharshi Dayanand University, Rohtak. Incorporation of *PVY CP* gene into tobacco and potato has been achieved by the Indian Agricultural Research Institute, New Delhi.

In conclusion, it can be said that genetic engineering of crop plants for virus resistance is undoubtedly a key biotechnological tool which can be used to minimize the losses to crop production incurred due to viral diseases in our country. Most of the important viruses have already been identified and the cloning and molecular characterization of their genomic components is at advanced stages. However, to successfully develop and test a series of virus-resistant transgenic crops, the following bottlenecks need to be removed: (i) Absence of transformation and regeneration systems for all the major crops of the country, (ii) Insufficient variability studies of important viruses, (iii) Lack of basic research on the functional genomics of pathogenesis.

Of all the major crop plants in our country, transformation systems are available for only a few cereals, vegetables, fibre crops and oilseed varieties. A major push needs to be given for transformation of pulses and legumes, which incur some of the heaviest losses due to viruses. The dominant and virulent strains of each important virus in the country need to be identified for obtaining genes for resistance engineering. Studies should also focus on the degree of variability and the recombination of viral genomes. This will help in the design of suitable constructs that will ensure durable resistance across the country. Emerging techniques of functional genomics need to be harnessed to understand the molecular interactions between the viral pathogen and the resistant and susceptible plants leading to resistance or pathogenesis. This is bound to result in novel insights at disease control. Insect-proof glasshouses and insectaries require to be modernized with facilities to provide ambient conditions for plant growth in our country. This needs to be looked into by funding agencies.

It is also clear that the effort for producing viral-resistant transgenic crop plants needs to be multidisciplinary, with a close cooperation among virologists, molecular biologists, tissue-culture specialists, agronomists and the government. Their combined effort is sure to deliver to the Indian farmers, a range of virus-resistant crops in the near future, which will help mitigate the losses in crop yields due to viruses in India.

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ACKNOWLEDGEMENTS. We thank Dr K. Dhandapani and Dr R. Selvakumar of NCIPM, New Delhi, Mr Kaushik Ghosh and Mr Punjab Singh Malik of ICGEB and Ms Saloni Mathur of UDSC for help in the preparation of figures and materials related to this article.