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TRANSGENIC APPROACHES TO DEVELOP RESISTANCE AGAINST GEMINIVIRUSES INFECTING CROP PLANTS

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ABSTRACT

Geminiviruses infect large number of economically important crop plants and cause a devastating yield loss. These are a group of ss-DNA viruses with unique twined icosahedral virus particles. This is the largest genus of plant viruses. Yield losses depend on the growing stage at which infection occurs and ranges from 50-95%. Reduction in exploitable genetic changes for virus resistance in crop germplasm, emergence of disease resistance breaking virus strains, increase in virus vector populations, environmental conditions and emergence of new biotypes in the recent years have made it mandatory to explore alternative means for incorporating virus resistance/tolerance in crop plants. Much progress has been achieved to protect plants against RNA and DNA virus infections using breeding and biotechnological tools. Developing resistant crops against these DNA viruses is one of the major challenges faced by plant virologists and biotechnologists. Current progress in understanding the molecular mechanisms underlying the roles of resistance genes has promoted the development of new anti-virus strategies. This review emphasis on various biotechnological approaches for incorporating virus resistance in to crop plants that include pathogen and non pathogen derived resistance (PDR).It should, however, be possible to achieve robust transgenic geminivirus resistance either by using mixtures of genes targeting multiple virus processes via multiple mechanisms, or by using ''tolerance'' genes that alleviate symptoms but do not selectively favour resistance-breaking virus mutants. Despite moderate successes in the engineering of geminivirus resistance using many of these strategies, no comparative data are available either on the relative merits of different approaches, or on how well the various resistant transgenic plants that have been produced will fare in the field.

KEY WORDS: *Geminiviruses, PDR, Resistance breaking virus strains, biotypes.*

INTRODUCTION

The viruses are intracellular obligate pathogens that do not have the molecular machinery to replicate without a host .The economically important viruses belong to gemini, tospo, poty, cucumo, tobamo, ilar and potex groups. Estimated US$60 billion loss caused by plant viral pathogens in crop yields worldwide per year (Wei et al.,2010; Rao et al.,2008; Sastry and Zitter,2014). Many pathogenic viruses are vectored by insects that include whiteflies, aphids, thrips and leafhoppers. Resistance often observed in wild relatives could not be exploited due to numerous problems associated with incompatibility, sterility, poor seed set and linkage drag associated with yield and other traits. Under these limitations, broad spectrum resistance by combining

conventional and transgenic methods is a sound approach towards alleviating losses due to viral diseases. In this review economically important geminiviruses are described and the efforts currently being made to develop transgenic resistance against them are focused.

1.Diseases Caused by Geminiviruses

The extent of yield loss caused by some geminiviruses has been estimated by Dasguptaet al.,(2003) to be as high as 100%.In India, diseases caused by begomoviruses are the major constraints in the production of tomato, cotton, okra, and chilli pepper (Borah and Dasgupta, 2012).Geminiviruses possess a small genome comprised of one or two circular ss-DNA molecules, each of which is 2.5–3.0 kb. Based on genome structure, sequence, host range, and insect vectors, geminiviruses have been further classified into different genera Begomovirus, Curtovirus, Mastrevirus, Topocuvirus. Monopartitebegmoviruses have only one circular genome, which carries genes C1, C2, C3, V1, V2, which encode for replicase, Trap or replication activator protein and replication enhancer protein/ReNCP, coat protein, pre coat/movement protein, respectively. Bipartite begomoviruses have a single-stranded DNA genome arranged in two circular components. Component A encodes the CP gene (AV1) and the genes involved in the replication process (AC1, AC2, and AC3); component B encodes two movement protein encoding genes (BV1 and BC1). Satellite molecules are associated with both mono and bipartite viruses and are largely responsible for induction of symptom severity. Begomoviruses are transmitted by insect vector whitefly Bemisiatabaciand cause severe diseases in tomato, pepper, cassava, beans, cotton, okra and cucurbits.

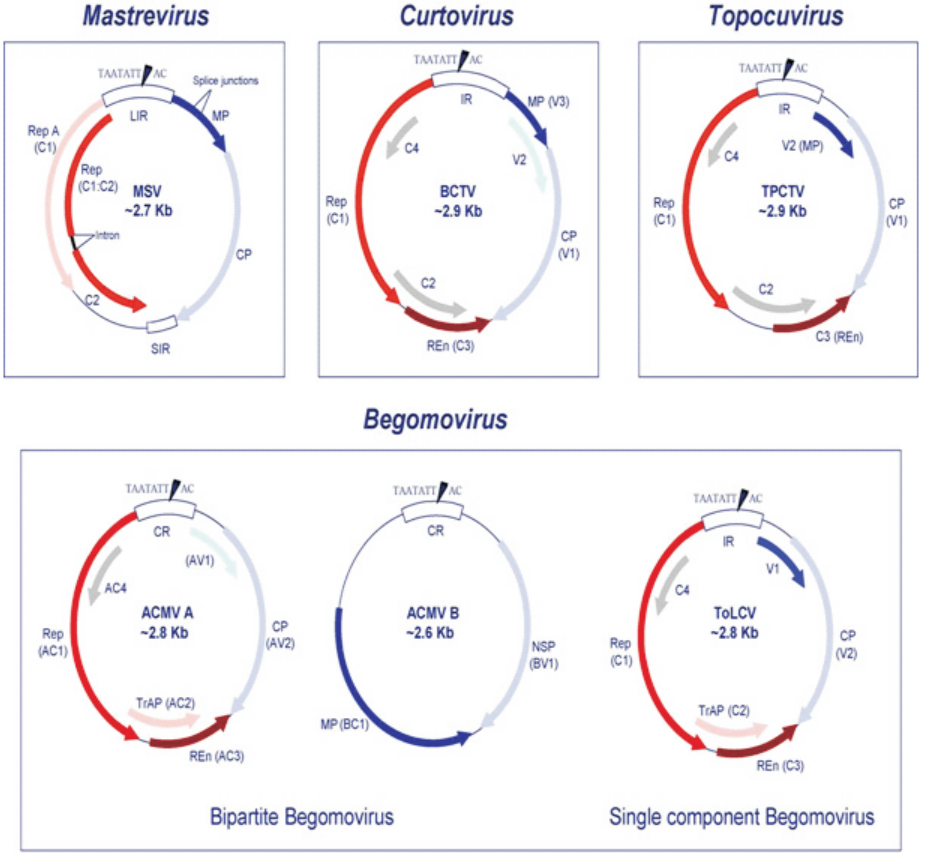

Fig.1. Genome organisation of representative members of the four geminivirus genera. MSV, Maize streak virus; BCTV, Beet curly top virus; TPCTV, Tomato pseudo-curly top virus; (ACMV) African cassava mosaic virus; (ToLCV) Tomato leaf curl virus. Curved arrows indicate open reading frames, diverging in the complementary (C) and virion (V) senses from an intergenic region (IR) in curtoviruses and topocuviruses, a long

intergenic region (LIR) in mastreviruses, and a common region (CR) in bipartite begomoviruses. CP, coat protein; MP, movement protein; TrAP, transcription activator protein; REn, replication enhancer protein; NSP, nuclear shuttle protein. The position of the plus strand rolling circle replication initiation site (TAATATTAC), situated within the loop of a stem-loop structure, is indicated within each genus intergenic region. To initiate RCR, a nick is introduced by the replication associated protein (Rep) at the penultimate A nucleotide of the invariant nonanucleotide sequence. In mastreviruses, bidirectional transcription initiates in the long intergenic region and terminates in the short intergenic region (SIR), which contains signals for polyadenylation. The SIR also functions as the C sense (negative strand) origin of replication (Shepherd et.al.,2009).

2. Strategies for producing genetically engineered geminivirus resistant crops

Resistances can be classified into two groups - pathogen derived and non pathogen derived resistance based on the source from which the transgenes are derived. Sudarshanaet al.(2007) and Reddy et al.(2009) reviewed various options available to develop genetically engineered (GE) virus resistant plants.

2.1. Pathogen Derived Resistance (PDR)

PDR classified into two types based on protein-mediated resistance and RNA silencing mediated resistance.

2.1.1. PDR with protein expression: Coat protein (CP) mediated resistance

The resistance incorporated into plants transformed with the viral CP gene, either full length or truncated with non-translatable regions refers to the CP mediated resistance (CP-MR).First report of CP-MR was reported in tobacco for resistance to *Tobacco mosaic virus* (TMV) (Powell-Abel et al.,1986). Transgenic cotton transformed with antisense coat protein showed considerable resistance against CLCuV (Amudha et al., 2011).CP-MR mechanisms are different for different viruses because some viruses need CP for their movement in addition to MP (movement protein) while others do not.

2.1.2. Replicase mediated resistance (Rep-MR)

Replicase mediated resistance (Rep-MR) was first described in transgenic tobacco containing the sequence encoding for a 54 kDa protein of TMV replicase (Golemboskiet al.,1990). Tobaccco GE with mutated replicase genes from PVY (Audyet al.,1994), *Alfalfa mosaic virus* (AlMV) (Brederode,1995), and tomato GE with TYLCV (Noriset al.,1996) showed resistance to the viruses that were targeted. The protein produced by transgene interfered with the replicase function, perhaps by binding to host factors or virus proteins that regulate replication and virus gene expression (Baulcombe,1996).Successful Rep gene manipulation for engineering resistance was observed against *Tomato yellow leaf curl Sardinia virus*(TYLCSV) in N. benthamiana (Noris et al.,1996) and tomato (Brunetti et al.,1997) and CLCuV(Asad et al.,2003).

2.1.3. Movement protein mediated resistance

Movement proteins (MPs) encoded by plant viruses are responsible for the systemic movement and cell to cell spread of the virus. Several MPs have been shown to be localized to plasmodesmata (Lucas,2006). Viral nucleic acids are too large to pass through the plasmodesmata. The knowledge of MP structure and in vivo function would help in designing mutant MPs or peptides that could function as dominant inhibitors to block the local and systemic spread of many different viruses with high efficiency. The MPs interact with host proteins to dilate plasmodesmata to enable viral genome movement from cell to cell (reviewed in Harris and Biao, 2011). Transgenic plants that contain dysfunctional MP from TMV showed resistance to several to begmoviruses as well as Cauliflower mosaic virus (CaMV) and other viruses (Cooper et al.,1995).

2.2. PDR Without Protein Expression

2.2.1. RNA silencing mediated resistance

The first discovered natural function of RNA silencing was antiviral response in plants (Lindboet al.,1993; Ratcliff et al.,1997). The molecular basis of RNA silencing is reviewed in detail by Staigeret al.(2012); Zvereva and

Pooggin (2012); Pumplin and Voinnet, (2013). During infection of viruses, small RNAs formed due to cleavage of double stranded RNA intermediate by DICER like protein accumulate, target and cleave viral messenger RNAs of the invading virus in a sequence specific manner (Hamilton et al.,1999). Degradation of viral mRNA leads to recovery of plants from virus infections (Ratcliff et al.,1997; Baulcombe,1999). Expression or introduction of dsRNA in eukaryotic cells can trigger gene silencing of transgenes, endogenes, and viruses in a sequence specific manner (Montgomery and Fire,1998; Sijen and Kooter,2000; Vaucheret and Fagard,2001).Transgenic plants can be designed to activate RNA silencing against viral genomes. For effective application of RNAi to produce GE virus resistant plants, it is critical to select the appropriate RNAi targets. Different studies have shown that truncated viral protein sequences, sense sequences, non-coding viral sense sequences (like satellite RNAs), antisense and inverse repeat (IR) sequences from viral genomes, when used as transgene,confer varying degree of resistance in plants.

2.2.2. Antisense RNA mediated resistance

PDR using antisense technology has been attempted to induce resistance to both RNA and DNA viruses.Antisense RNA (asRNA) is a single-stranded RNA that is complementary to amessenger RNA (mRNA) strand transcribed within a cell. When asRNA is introduced into a cell, it base pairs with complementary mRNA to form double stranded RNA in a dose dependent manner. The dsRNA will then be targeted by RNA silencing machinery of the cell to induce silencing of the target gene. Effectiveness of an antisense construct in targeting AV2 gene of Tomato leaf curl New Delhi virus was tested by (Mubinet al., 2007) in tobacco. Transgenic lines of wheat expressing the antisense-Nib replicase of Wheat yellow mosaic virus (WYMV) were found to have broad spectrum disease resistance to different isolates of the virus and gave increased yield when compared to the controls (Chen et al.,2013).When DNA fragments from TYLCV,TYLCV-mild, *Tomato yellow leaf curl Sardinia virus* (TYLCSV), *Tomato yellow leaf curl Malaga virus* (TYLCMalV), and *Tomato yellow leaf curl Sardinia virus-Spain* (TYLCV-ES1) in sense and antisense orientations were used in transient assays to check their efficacy, broad resistance was seen against three different isolates of the virus (Abharyet al.,2006). This technology has the potential in slowing down the virus multiplication and in providing resistance and few disadvantages, such as less stability in resistance perhaps due to low accumulation of transgene derived siRNA or suppression of gene silencing by suppressor proteins of invading viruses.

2.2.3. Inverted repeat or hairpin mediated RNA silencing

Inverted repeats (IR) containing transgene having viral target sequences with a spacer intron for making a self complementary hairpin RNA in plants, to trigger the RNA silencing machinery to generate siRNA specific to the viral transgene. When virus infects, siRNA already present in the plant, will provide the primer for viral RNA dependent RNA polymerase to generate dsRNA and siRNA by transitivity of silencing (Dunoyeret al., 2004). Amplification and systemic movement of silencing signal will then target the genome of invading virus resulting in its degradation and elimination. *Bean golden mosaic virus* infection was suppressed in bean plants expressing a hairpin RNA transgene derived from a replicase (AC1) coding sequence of the virus (Arago and Faria, 2009). Sequence homology, length of the transgene and number of copies to the transgene construct in a plant influence the degree of resistance (Pang et al.,1997; Jan et al.,2000; Dalakouraset al.,2011). The geminiviruses can evolve rapidly by mutation, recombination and pseudo recombination, resulting in strain which will overcome RNAi induced resistance in transgenic plants. In addition, mixed viral infections are frequent in nature and virus encoded suppressors of gene silencing interfere with different steps of the RNA silencing pathways to significantly alter the extent of resistance induced.

2.2.4. Artificial micro RNA mediated resistance

It is the endogenous gene silencing mechanism to target a gene of interest. The ability of the expressed miRNA to deliver resistance against heterologous viruses was dependent on the levels of complementarity between them .The advantage of using amiRNA for virus resistance when compared to other RNAi methods in that a 21nt sequence can be designed for high target specificity avoiding off target effects. The amiRNA

transgene is unlikely to be silenced by an invading virus or the silencing triggered by amiRNA may be overcome by suppressors of gene silencing. The chance of the transgene recombining with an invading virus to generate new viruses will also be comparatively low. However, a major drawback of amiRNA induced virus resistance is that, if the virus mutates in the sequence of the target amiRNA employed to engineer plants, the resistance can be easily overcome by the targeted virus. Hence, while designing amiRNAs, it is prudent to target multiple regions in a viral genome and particularly those genes that are conserved and most essential for a virus or a group of viruses to replicate in plants. The use of amiRNA to target and destroy pathogenic plant viruses was first described by Niuet al.(2006). They showed that transgenic Arabidopsis expressing amiRNAs targeting the viral mRNA sequences encoding gene silencing suppressors P69 of *Turnip yellow mosaic virus* (TYMV) and HC-Pro of*Turnip mosaic virus* (TuMV) were specifically resistant to TYMV and TuMV.

2.3Expression of Heterologous Foreign Proteins/ Peptides

2.3.1. Peptide mediated resistance

The majority of approaches described so far, either do not confer high levels of field resistance or are limited to providing resistance to similar and closely related viruses. Broad spectrum resistance to several strains of a virus or to distantly related and unrelated viruses especially, in the case of DNA viruses that readily recombine to evolve into newer and virulent strains is desirable. Recombinant/synthetic proteins that bind to and can inactivate a protein of interest in a specific manner (Hoppe-Seyler and Butz, 2000; Hoppe-Seyleret al.,2004) are employed for developing transgenic resistance. Lopez-Ochoa et al., (2006) identified several peptide aptamers that bind to the replicase of *Tomato golden mosaic virus* (TGMV) and *Cabbage leaf curl virus* (CaLCuV) using yeast dihybrid screens and found that these interfered with viral replication in transgenic arabidopsis plants.

2.3.2. Plantibodies: Single domain antibodies

Among the various antibody gene combinations tried so far, the single chain variable fragment (scFv) antibody expressing only the variable regions of one heavy and one light chain are the most favored. These small antibodies are relatively easily expressed in plants. Plantibodies generated against the replicase/RdRp of *Tomato bushy stunt virus* (TBSV) in N. benthamianagave high levels of resistance not only to TBSV and members of the family Tombusviridae, *Red clover necrotic mosaic virus* (RCNMV), *Cucumber necrosis virus* (CNV) and *Turnip crinkle virus* (TCV) (Boonrodet al., 2004). The development of the phage display approach and the generation of synthetic scFv libraries have greatly improved the applicability of this strategy (Ziegler and Torrance, 2002) for inducing GE resistance.

2.3.3. Ribosome inactivating proteins (RIP)

RIPs are predominantly found in plants, bacteria and fungi. They inhibit protein synthesis by damaging ribosomes in a catalytic manner. Proteins that inactivate or damage ribosomes by other mechanisms like RNases or protease activity are not considered as RIPs. The RIPs have been shown to have a role in plant defense. The genes coding for RIPs have been isolated from a number of plants belonging to the families Cucurbitaceae, Euphorbiaceae, Poaceae and from super order Caryophylles (Stirpe, 2004; de Virgilioet al., 2010). Antiviral activity of RIPs is partly attributed to the deadenylation of viral RNA.

CONCLUSION

Viruses are unique as they take control of the host cellular machinery and are difficult to eliminate once they invade. Hence viral diseases cause a serious problem in food security. An effective way to deal with this problem is to identify potential genetic sources of resistance in cultivated as well as wild relatives of crop species. Resistance transfer from wild relatives may bring in additional genetic resources and resistance against viruses. Biotechnological interventions allow the tapping of heterologous sources of resistance proteins to transform crops plants with desired traits. Many options are currently available to produce transgenic virus resistant crops. All these require efficient transformation techniques. For example pepper and okra are difficult to transform.

Multiple virus resistance can be developed through the combinational strategy by using multiple transgene and stacking or pyramiding genes by convention breeding and developing the super resistant plants will be the only means of creating durable resistance to geminiviruses in the world's most important food crops.

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