Plant Resistance to Geminiviruses

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Nomenclature

AGO Argonaute protein
CAS CRISPR-associated proteins
CMD Cassava mosaic disease
CMG Cassava mosaic geminiviruses
CRISPR Clustered regularly interspaced short palindromic repeats
DCL Dicer-like
dsRNA Double-stranded ribonucleic acid
GM Genetically modified crops
gRNA Genomic RNA
HR Hypersensitive response
LRR Leucine-rich repeat
mRNA Messenger RNA
NGS Next generation sequencing
nt Nucleotide(s)
QTL Quantitative trait loci
R Dominant resistance
RDR RNA-dependent RNA directed polymerase
RdRp RNA-dependent RNA polymerase
RGAs Resistance gene analogs
RIL Recombinant inbred line
RISC RNA-induced silencing complex
RNAi RNA interference
SNP Single-nucleotide polymorphism
ssRNA Single-stranded ribonucleic acid
VIGS Virus-induced gene silencing

Glossary

Allele Allele is a distinct form of a gene at a particular locus, that shows different effect on the phenotype from the other forms of alleles.
Betasatellite A type of circular single-stranded satellite DNA associated with begomoviruses.
CRISPR-Cas The acronym for Clustered Regularly Interspaced Short Palindromic Repeat – CRISPR-associated protein, an adaptive immune system used by bacteria and archaea against viruses and mobile genetic elements, which has been adapted for genome editing in eukaryotes.
Dominant allele An allele expressing its phenotypic effect even when having a recessive allele as its counterpart in a heterozygous system.
NB-LRR protein Plant disease resistance proteins containing a central nucleotide-binding domain (NB) and C-terminal leucine-rich repeat (LRR) domain. Also known as NBS-LRR or NB-ARC-LRR proteins.
Quantitative trait loci Corresponds to genomic regions that is associated with the phenotypic variation of a quantitative trait.
Recessive allele An allele whose phenotypic effect is not expressed in a heterozygote.
Resistance gene Plant gene conferring resistance to a specific pathogen, that possess a corresponding avirulence gene.
RNA interference A broad antiviral defense system used by plants, animals, and fungi. It recognizes double-stranded RNA as a cue of viral presence and induces degradation or silencing of viral transcripts or genomes.
Transcriptional gene silencing A mechanism of gene silencing involving decreased RNA synthesis because of promotor methylation.
Virus immunity Genetic mechanism by which plants are unable to support replication of a virus, and consequently where the virus cannot be isolated by any mean from the infected plant.
Virus resistance Genetic mechanism by which plants are able to defend themselves against a viral invasion (by one or the other mechanism) that leads to reduced viral replication levels.
Virus tolerance Genetic mechanism by which plants are infected and viral replication/infection occurs at wild type levels but without any visual symptoms.
Virus-induced gene silencing It is a technology that exploits an RNA-mediated antiviral defense mechanism.

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Introduction

Domestication of crops from their wild relatives has resulted in the loss of many resistance genes because these are often genetically linked to unwanted traits (such as low yield, poor flavor, small size). With the commercialization of agriculture and increasing production of crop plants in monoculture, genetic diversity has dwindled, which has made crops susceptible to a variety of pathogens. Therefore, breeding for resistance has consisted in reintroducing these resistance genes in the susceptible domesticated crop by crossing and selection. The wild relatives or ancestors of these crop plants present in their centers of origin or diversity are the best sources of resistance genes (R genes). Pathogen resistance in crop plants can also be generated by either natural or induced mutations. For example, Rice yellow mottle virus (RYMV) resistance in cultivated rice spontaneously appeared under RYMV pressure. Cassava varieties such as TME3 are also probably mutants which were selected under cassava mosaic disease pressure.

Geminiviruses are single-stranded, circular DNA viruses that cause economically important diseases in a wide range of crop plants across the globe. Geminiviruses are characterized by small geminate particles (18×30 nm) containing either one (A component, monopartite) or two (A and B components, bipartite) single-stranded circular DNA molecules of $\sim$2.7 kb and sometimes associated with satellite DNA molecules of $\sim$1.4 kb. Around 460 distinct species and a larger number of strains belong to the family *Geminiviridae*. In this article, we briefly discuss the resistance genes and the sources of resistance against the most important geminiviral diseases in cassava, tomato, bean, maize, and cotton.

An Overview of Natural Resistance to Viruses in Plants

Depending on the number of genes involved in providing the resistance phenotype, the host resistance can be classified into monogenic, digenic, or polygenic. Many of the monogenic and digenic types of resistance are qualitative in nature, with phenotypes showing complete absence of pathogen in the host plant. Whereas the polygenic resistance is manifested by several genes. A quantitative trait locus (QTL) is a region of DNA that is associated with a specific phenotypic trait, that varies in degree and can be attributed to polygenic effects, i.e., the product of two or more genes, and their interaction with the environment. Several such QTLs could be associated with one single trait. Such QTLs are mapped by genetic markers by using recombinant inbred lines (RILs) that are derived by crosses between parents with two contrasting resistance phenotypes. The inheritance pattern of the resistance trait can be dominant or recessive in nature and half of the virus resistance traits known hitherto are recessive in nature.

Plant R genes confer resistance to plant pathogens including plant viruses and each R gene confers resistance to a specific pathogen triggered by a specific avirulence (*avr*) gene or an effector protein. More than 80% of the R genes studied so far are monogenic in nature and one third of the R genes are tagged by molecular genetic markers. R-genes can be either dominant or recessive in nature. Studies done so far have shown that dominant R genes are of the NB-LRR (Nucleotide-Binding-Leucine-Rich Repeat) type and that recessive ones encode for translation initiation factors. Dominant gene resistance is usually associated with HR while recessive genes function at single cell level. R genes are known to show two unique patterns of clustering: in one case R genes with similar inheritance and resistance phenotypes are clustered at one locus, while in another case the R genes for virus resistance are clustered with unrelated R genes. Most of the recessive R genes identified to date are against potyviruses. Although there are no reports of R genes showing resistance to geminiviruses, there are reports of genetic mapping of the resistance loci through molecular markers. There are also reports demonstrating the interaction of geminivirus proteins with the trans-membrane receptor kinases and components of brassinosteroid signaling. It was recently shown that silencing of a *Permease I-like protein* gene transmembrane transporter in tomato using the virus-induced gene silencing (VIGS) vector system, rendered it susceptible to Tomato yellow leaf curl virus. In response to the pathogen recognition by the R gene, changes in ion fluxes are observed, leading to activation of signaling pathways, alteration in transcriptional profiles, production of reactive oxygen species (ROS) and generation of nitric oxide (NO).

Gene silencing is another important mechanism which plants have evolved to defend against viruses. In plants, post-transcriptional gene silencing (PTGS) acts as a natural anti-viral defense system and is considered as part of the plant's innate immune system. RNA silencing involves suppression of gene expression by sequence-specific interaction at the transcriptional or post-transcriptional level in diverse eukaryotes. Double stranded RNA (dsRNA) is the trigger for gene silencing, which involves a chain of events to produce small interfering RNA (siRNA) through a diverse range of enzymes and its complexes, including the RISC-complex (RNA induced silencing complex) as well as DCL (Dicer-Like) and RDR (RNA dependent RNA polymerase) proteins. In virus-infected plants, RNA silencing is initiated by the double-stranded (ds) RNA that can be a viral replication intermediate or by 'aberrant' RNAs, or single-stranded RNA (ssRNA) that is converted to dsRNA by host-encoded RNA-dependent RNA polymerase (RdRP). The dsRNAs can also be formed because of bi-directional transcription of these viruses with transcripts occurring from opposite polarity overlapping at their 3′-ends, which partly explains how these geminiviruses induce PTGS in infected plants. Alternatively, the early and abundant transcripts of the AC1 gene of these geminiviruses can serve as the template for the host RdRP to produce dsRNAs. Yet another possibility is the fold-back structures of geminivirus transcripts which can serve as a template for DICERs to cleave at specific locations and produce siRNAs. In plants, some virus-host interactions naturally lead to host recovery that are similar to RNA-mediated virus resistance. This symptom recovery phenomenon is also reported for some of the geminiviruses (Fig. 1). This recovery phenomenon is associated with the production of virus-derived siRNAs, that later become abundant in the newly developed symptom-less recovered leaves. This increase in virus-derived siRNA accumulation is accompanied by a reduction in the levels of both viral DNA and mRNA accumulation.

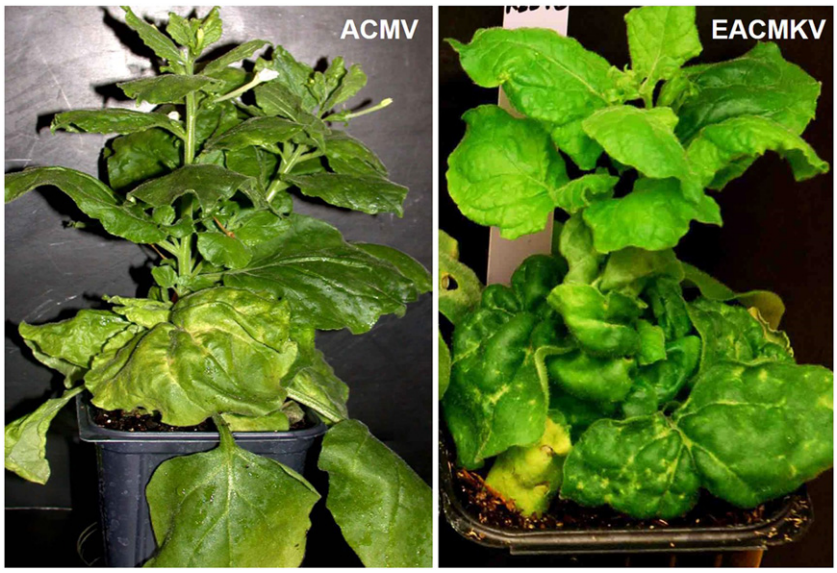

Fig. 1 Symptom recovery in *Nicotiana benthamiana* plants infected by two distinct cassava mosaic geminiviruses (ACMV and EACMKV), as a result of gene silencing.

Since RNA silencing and R gene signaling are both components of the plants innate immunity, it will be interesting to know if there is any cross-talk between these two pathways through one of the systemic signaling systems such as the salicylic acid pathway. It has been shown that the RdRPs *NbRdRp1m*, *NtRdRp1* and *RDR1* are all inducible by both salicylic acid and certain viruses, although recent studies failed to find any overlap between the DCL endoribonucleases involved in silencing and the SA-induced resistance to positive-sense RNA viruses. More recently it has been shown that the virus resistance induced by NB-LRR proteins involved Argonaute4-dependent translational control. It has also been shown that the viral silencing suppressor HC-Pro enhances R-gene mediated resistance to viruses and this resistance is weak at higher temperatures. Geminiviruses are known to be associated with DNA satellites which modulate the viral symptoms and the betasatellites in particular are known to enhance the symptoms through their PTGS suppressor proteins (ßC1).

In this article we discuss in greater detail and on a case by case basis the principal sources of resistance for the major geminiviral diseases of the world (**Table 1**).

Resistance to Cassava Mosaic Geminiviruses

It was recognized from the earliest period of research on cassava mosaic geminiviruses (CMGs) (Family *Geminiviridae*; Genus *Begomovirus*) that resistance would be the most effective method for controlling the cassava mosaic disease (CMD) caused by these viruses. The team responsible for the first biological characterization of CMGs in the former Tanganyika in the 1930s realised that wild relatives of cassava (*Manihot esculenta* Crantz) would provide an important potential source of resistance genes. Resistant progeny from *Manihot esculenta* × *Manihot glaziovii* (Müll-Arg.) crosses were backcrossed with cultivated cassava to produce the first CMD-resistant cassava varieties. Material from this initiative was subsequently used in the 1970s to extend the development of CMD resistance within the cassava breeding program of the International Institute of Tropical Agriculture (IITA). A recurrent selection approach was taken with the primary source of CMD resistance being the cassava clone 58308. It had been suggested since the 1950s that the resistance source derived from the *M. glaziovii* cross was polygenic, but this was also shown to be recessive in the early 1970s. During this period, evaluations of resistant varieties were undertaken in West, Central and East Africa, as well as in south India. Resistance was shown to be effective in all of these regions, providing a strong demonstration of the broad activity of the resistance source as well as its likely durability. We now know that there is significant virus diversity across these regions (ACMV, EACMV-like viruses, ICMV and SLCMV), which confirms the validity of the earlier conclusions about the broad-based activity of the *M. glaziovii*-derived resistance. This polygenic or quantitative recessive resistance source is now referred to as CMD1.

In the 1980s and 1990s in Africa there were extensive trial-based epidemiology studies of CMGs, several of which compared the responses of varieties with contrasting levels of resistance. Different components of resistance were identified, including: resistance to infection, resistance to virus multiplication, resistance to virus movement (leading to incomplete systemicity), and resistance of normal plant function to the effects of virus infection. These were assessed through measurements of virus titre, incidence of infected plants, severity of symptoms of infection, distribution of symptoms through the plant, and yield of healthy versus infected plants. Strong correlations were demonstrated between each of these variables. Resistant varieties were therefore infected less, had

Table 1 List of geminivirus resistant genes/loci and their characteristics for the major geminivirus diseases

Target virus (es)	*Target host*	*Gene/Locus*	*Nature/Location of resistance gene/locus*	*Source of resistance*	*Type of resistance*
CMGs	Cassava	*CMD1*		*Manihot glaziovii*	Polygenic recessive
		CMD2		West African Cassava landraces	Single dominant gene
		CMD3		Cassava genotype TMS97/2205	Polygenic or quantitative
TYLCVs	Tomato	*Ty-1*	*RdRp*	*Solanum chilense*	Partial dominant
		Ty-2	*NB-LRR*	*S. habrochaites*	Dominant
		Ty-3	*RdRp*	*S. chilense*	Partial dominant
		Ty-4	–	*S. chilense*	Partial dominant
		ty-5	*Pelota*	*S. peruvianum*?	Recessive
		Ty-6	–	*S. chilense*	Partial dominant
		–	–	*S. habrochaites*	Oligogenic (Complete resistance)
ToLCV		*tgr-1*	*Cell-to-cell movement*	*S. chilense*	
ToCMoV		*tcm-1*		*S. lycopersicum*	
ToLCNDV	Cucurbits	CmoCh08G001490	BZIP	*Cucurbita moschata*	Quantitative
PYMV	Potato	–	–	*S. pimpinellifolium*	Bigenic
BCTV	Common bean	*Bct-1*		Red Mexican common bean varieties	Single dominant gene
		–		*Arabidopsis thaliana* (3 ecotypes, Ms-0, Pr-0, and Cen-0)	Monogenic
CaLCuV and TYLCV		*gip-1*		*Arabidopsis thaliana* (Pla-1 ecotype)	
BGYMV	Common Bean	*bgm-1*		*Phaseolus vulgaris* (Red S)	Recessive
		bgm-2			
		bgm-3		*Phaseolus coccineus* (scarlet runner Bean)	
		Bgp-2			Dominant
MYMIV	Black gram	*CYR-1*	Non-TIR-NBS-LRR	*Vigna mungo*	Dominant
	Soybean	Glyma18g02850	LRR-RP (on chromosome 18)	*Glycine max*	Dominant
MSV	Maize	*msv1*	Chromosome-1	*Zea mays* (CML206 × CML312)	Partial dominant effect
		Q1 - PHM5502_31	SNP on: Chromosome-3	*Zea mays* (TZIL07A01005 × TZIL07A01322)	Dominant
		Q2 - PZA02616_1	Chromosome-3		Additive
		Q3 - PZA02872_1	Chromosome-7		Dominant
		Q4 - PHM1766_1	Chromosome-9		Additive & dominant

Abbreviations: CMGs, cassava mosaic geminiviruses; TYLCVs, tomato yellow leaf curl viruses; ToLCNDV, tomato leaf curl New Delhi virus; ToLCV, tomato leaf curl virus; ToCMoV, tomato chlorotic mottle virus; PYMV, potato yellow mosaic virus; BCTV, beet curly top virus; CaLCuV, cabbage leaf curl virus; BGYMV, bean golden yellow mosaic virus; MYMIV, mungbean yellow mosaic India virus; MSV, maize streak virus; NBS-LRR, nucleotide-binding site Leucine-rich repeat; LRR-RP, leucine-rich repeat receptor-like protein kinase.

less severe symptoms which were spatially limited in the plant, had lower virus titers and yielded more, demonstrating that each of these were expressions of the same resistance.

Following the increasing accessibility of PCR and sequencing analyses from the late 1990s onwards, it became possible to examine the molecular characteristics of CMD-resistant cassava varieties. A key development came in 2002 when a novel source of resistance to CMGs was identified from West African cassava landraces. Molecular genetic mapping was used to identify a single dominant gene referred to as CMD2, and this development made it possible for breeding programs to speed up their resistance breeding work through marker-assisted selection (MAS). Several markers were identified which could pick out clones carrying CMD2 with a high degree of reliability. Moreover, similar approaches also led to the identification of a third resistance source – CMD3 – in the genotype TMS97/2205. This clone also carried CMD2, and it was observed that the combination of CMD2 and CMD3 gave the plants very high levels of resistance to CMD. An additional spin-off from the development of MAS was that the technique could be applied in regions where CMGs did not even occur, but where there was an interest in having strategic stocks of CMD-resistant germplasm. This would allow these regions to respond rapidly to any future invasive spread of CMGs. This activity was pioneered by the International Center for Tropical Agriculture (CIAT) in Colombia, to prepare for the potential future introduction of CMGs to Latin America.

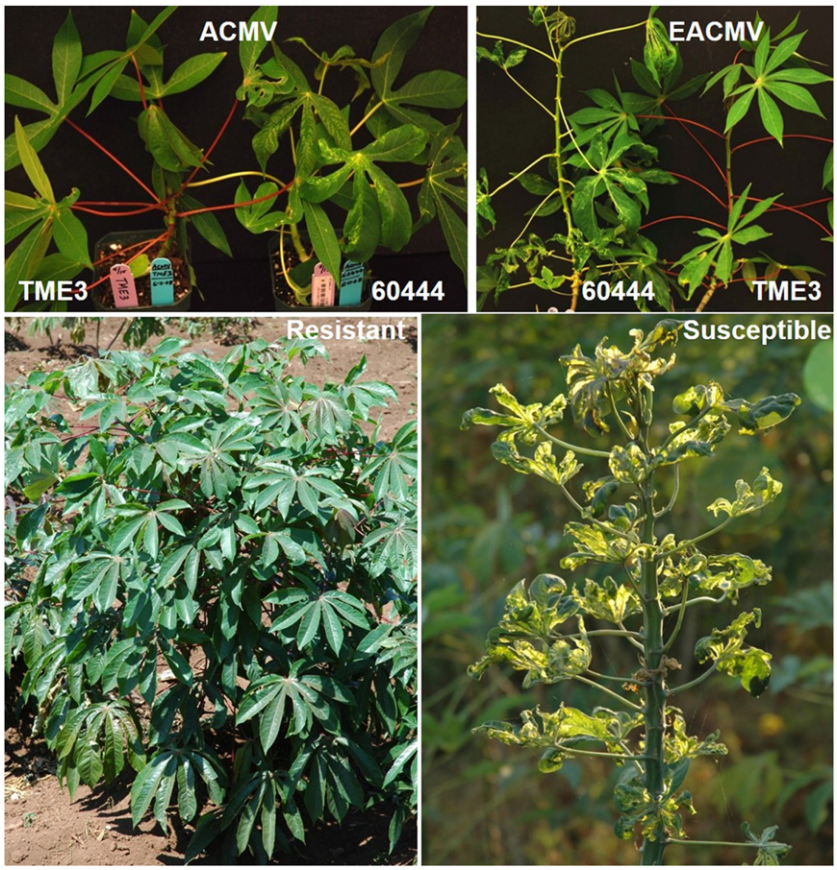

Fig. 2 (Top) CMD resistant cassava variety TME3 showing recovery from ACMV (2 weeks post inoculation) and EACMV (4 weeks post inoculation) infections in glasshouse experiments. Reproduced from Patil, B.L., Fauquet, C.M., 2015. Studies on differential behavior of cassava mosaic geminivirus DNA components, symptom recovery patterns, and their siRNA profiles. Virus Genes. 50 (3), 474–486. doi:10.1007/s11262-015-1184-y. (Bottom) CMD resistant and susceptible varieties of cassava cultivated in fields of Burundi.

In addition to extensive field evaluations for CMD resistance, targeted approaches such as microparticle bombardment using infectious clones have been used to screen for resistance to CMGs. In comparisons of ACMV and EACMV resistance, putative resistant accessions with CMD1, CMD2, or CMD3 were shown to have high levels of resistance to ACMV yet were severely affected by EACMV. The only exception to this was TMS97/2205 which was known to combine CMD1, CMD2, and CMD3. As a consequence of these and related findings, the primary objective of most cassava geminivirus resistance breeding programs is to combine these different resistance sources into varieties that also have favorable yield, organoleptic, and processing qualities.

There has been limited progress to date in identifying specific genes associated with CMD1, CMD2, and CMD3. However, transcriptome data have revealed differences between CMG-susceptible (T200) and CMG-resistant (TME3; **Fig. 2**) cassava varieties. Moreover, resistance gene analogs (RGAs) were also shown to be expressed differentially during the process of symptom recovery (from CMD infection), suggesting that they may provide a complementary resistance mechanism to RNA silencing. Methylation of viral DNA has been shown in several systems to be an important resistance strategy employed by plants against geminivirus infection and has been demonstrated specifically for ICMV. However, although resistance sources such as CMD2 have been pinpointed down to chromosome and linkage group level through genome-wide sequencing and mapping studies, further research is required before a more comprehensive understanding can be obtained of the genetics of the responses of cassava plants to virus infection.

While studies continue to expand knowledge about natural resistance to CMGs, there are widespread and highly successful programs being implemented to deploy CMD-resistant varieties (**Fig. 2**). These were disseminated throughout East and Central Africa to control the severe CMD pandemic of the 1990s/2000s with great success, and similar programs are now being used to address the more recent CMD pandemic in South-East Asia. Africa-derived CMD resistance has proven to be effective in controlling virus infection caused by the South Asian CMGs – ICMV and SLCMV – so they should also provide effective control for the currently spreading South-East Asian pandemic associated with SLCMV.

Fig. 3 Resistant (green plants) and susceptible tomato plants (dead or yellow plants) from a tomato breeding field of a program for begomovirus resistance in Guatemala, tomato experimental field in Sanarate Guatemala, 2005. Funded by USAID-CDR. Courtesy: Luis Mejia, Douglas P Maxwell, Favi Vidavski, Henryk Czosnek.

Geminivirus Resistance in Tomato

The Case of Resistance to Tomato Yellow Leaf Curl Virus

Tomato crops can be infected by a large number of geminiviruses (family *Geminiviridae*) mainly belonging to the genus *Begomovirus*. Tomato yellow leaf curl disease, one of the most devastating virus disease affecting tomato worldwide, is caused by a complex of begomoviruses originated in the Middle East but expanded initially to the Mediterranean Basin and then to the Far East, the Caribbean, North America, and Australia. Tomato yellow leaf curl virus (TYLCV) was the first monopartite begomovirus characterized and the most successful in spreading the disease worldwide.

Breeding for TYLCV Resistance

Breeding programs aimed at producing tomato varieties resistant to TYLCV started in the late 1960s and have expanded since (Fig. 3). These programs are based on the introgression of resistance found in some accessions of wild tomato species into the domesticated tomato (*Solanum esculentum*). Certain accessions of the wild tomato species *S. chesmanii*, *S. chilense*, *S. habrochaites*, *S. peruvianum*, and *S. pimpinellifolium* are resistant (with mild or no symptoms, but containing virus) to whitefly-mediated inoculation. The discovery of loci, and later on of genes, associated with TYLCV resistance, was facilitated by the development of saturated maps based on DNA polymorphism distinguishing resistance from susceptibility (RFLP, AFLP, SSR, SCAR, etc.).

To date, six loci, coined *Ty-1* to *Ty-6*, have been found to be associated with TYLCV resistance (Table 1). They have been mapped on different tomato chromosomes and five of them are independently inherited. The *Ty-1* and *Ty-3* loci originated from *S. chilense* accessions LA1969 and LA2779, respectively, and they were mapped to the long arm of tomato chromosome 6. Later, *Ty-3* was shown to be allelic to *Ty-1*. *Ty-2* was introgressed from *S. habrochaites* accession B6013 and was located on the long arm of chromosome 11. *Ty-4* was introgressed from *S. chilense* accession LA1932 and mapped to the long arm of chromosome 3. Compared to other *Ty* genes, *Ty-4* is less effective against TYLCV. *ty-5*, the only recessively inherited *Ty* gene known to date, was introgressed from *S. peruvianum* and mapped on chromosome 4. *ty-5* has been reported from different sources including line TY172 (derived from four different accessions of *S. peruvianum* which were crossed with *S. arcanum*) and tomato cultivar Tyking. *Ty-6* originated from *S. chilense* accessions LA1938 and LA2779 and was located on the long arm of chromosome 10. It has been shown that *Ty-6* is effective, in addition to TYLCV, against Tomato mottle virus, a New World begomovirus.

Pyramiding resistance from various wild tomato species in a single tomato line often broadens resistance. Today, most commercial tomato varieties resistant to TYLCV contain the *Ty-1* gene. Upon whitefly-mediated infection in the field and greenhouse, the plants remain symptomless and their yield compares with that of non-infected plants. However, reports in 2008 indicated that *Ty-1*-mediated resistance could be broken under high virus pressure. Worse, it was recorded in 2016 in Jordan and Israel that *Ty-1* resistant varieties suddenly became susceptible to TYLCV. Molecular cloning and sequencing indicated that the symptomatic plants were contaminated with the cotton leaf curl Gezira betasatellite, which in association with TYLCV, overcame the resistance based on *Ty-1*. These finding were later confirmed in the laboratory using cloned virus and betasatellite.

Characterization of Ty Genes

Three of the *Ty* loci have also been associated to genes which have been recently cloned and characterized.

Ty-1/*Ty-3* were identified as coding for an RNA-dependent RNA polymerase (RdRp) belonging to the RdRp type which has an atypical DFDGD motif in the catalytic domain. It has been shown that *Ty-1* confers resistance to TYLCV and other begomoviruses by enhancing transcriptional gene silencing through an increase in cytosine methylation of viral genomes.

Ty-2 encodes a nucleotide-binding domain and leucine-rich repeat-containing (NB-LRR) gene, $TYNBS_1$. Introduction of genomic fragments containing the $TYNBS_1$ gene into susceptible tomato plants resulted in transgenic lines resistant to TYLCV.

ty-5 is a loss-of-function mutant allele of the gene that encodes a messenger RNA surveillance factor Pelota (*Pelo*) located on chromosome 4. Pelota is involved in ribosome recycling phase of protein synthesis. The mutation in *ty-5* is caused by a T-to-G transversion in the coding region.

Ty-6 is located on chromosome 10, that compliments resistance conferred by both *Ty-3* and *ty-5* genes, thus providing resistance to both monopartite and bipartite begomoviruses.

Genetic Engineering for TYLCV Resistance

In some cases, as mentioned above for *Ty-2*, host-derived resistance has been engineered into susceptible tomato plants. Nonetheless, most of the tomato transgenic plants with incorporated begomovirus resistance were produced by expressing viral genes. TYLCV genes or gene segments, whether functional or not, have been used to confer a narrow as well as a broad-range virus-specific resistance (pathogen-derived resistance).

The expression of the TYLCV *CP* gene in an interspecific *S. lycopersicum* x *S. pennellii* hybrid was characterized by the late development of disease symptoms, often followed by their disappearance and recovery. The expression of the TYLCV *Rep* gene in tomato cultivars provided a virus-specific resistance. For example, tomato plants transformed with a truncated TYLCV *Rep* gene exhibited various degrees of resistance to TYLCV infection in the field in Florida. However, these TYLCV-resistant plants were susceptible to the bipartite begomovirus Tomato mottle virus conspicuous in Florida tomato fields. Similarly, tomato plants transformed with a 3′ end-lacking *Rep* gene of Tomato yellow leaf curl Sardinia virus (TYLCSV, a monopartite begomovirus closely related to TYLCV) were resistant to TYLCSV from Italy but were susceptible to a cognate TYLCSV isolate from Spain.

Resistance was also achieved by mimicking the plant RNA interference (RNAi) antiviral mechanisms relying on pathogen transcriptional (TGS) and post-transcriptional (PTGS) gene silencing. Small interfering RNAs (siRNA) directed against TYLCV genes were designed, based on hairpin RNAi (hpRNAi), and used to produce transgenic tomato plants. It was found that hpRNAi-based resistance was highly sequence-specific. The first set of experiments using PTGS hpRNAi demonstrated that TYLCV and TYLCSV non-coding regions could trigger the accumulation of virus-specific siRNAs, followed by high levels of resistance against these two viruses. TYLCV *Rep* was also the target of hpRNAi. Homozygous transgenic tomatoes expressing hpRNAi TYLCV *Rep* were resistant to TYLCV in greenhouses and fields and carried large quantities of small RNAs. In addition, resistance was associated with changes in the general plant host transcriptome, mainly genes involved in biological regulation, metabolic and cellular processes such as catalytic and binding activities. The hpRNAi strategy has also been used against the IR, CP, V2, and Rep genes of TYLCV from Oman. The transgenic lines obtained exhibited various levels of resistance upon TYLCV agro-inoculation.

The CRISPR-Cas system, which protects prokaryotes from potentially deleterious foreign DNA, is the latest tool in the molecular breeder's panoply aimed at conferring TYLCV resistance. CRISPR-Cas was recently implemented to the making of virus-free crops, demonstrating that this technology can be applied to the molecular breeding of virus-immune tomatoes. Indeed, TYLCV resistance was conferred by editing the tomato genome. Targeting the TYLCV CP and Rep genes with Cas9 single RNA-guided DNA endonuclease resulted in stable resistance to the virus. The Cas9-treated tomato plants carried reduced TYLCV amounts and their progeny was resistant to TYLCV. Therefore, antiviral strategies based on genome editing have the potential to constitute an additional, maybe preferred, tool in the breeding of TYLCV-resistant tomato varieties.

Resistance to *Beet Curly Top Virus*

Beet curly top virus (BCTV, genus *Curtovirus*, family *Geminiviridae*), is a phloem-limited virus that causes curly top disease in several economically important crops such as common bean, pepper, sugar beet, and tomato. This disease is more prevalent and is known to cause heavy economic losses in the western USA, north-central Mexico and countries of the Mediterranean Basin and the Middle East (e.g., Iran and Turkey). Under favorable environmental conditions the disease outbreaks are driven by populations of the beet leafhopper vector (*Circulifer tenellus*).

Sources of curly top disease resistance have been identified in germplasm of common bean, flax, squash and sugar beet. However, the availability of commercially available resistant varieties that can be used in a management program for BCTV depends on the crop plant. Resistant varieties have played a major role in management of curly top in sugar beets and somewhat of a role in common bean, whereas there are no commercially available resistant varieties for pepper or tomato. In the early 1900s in the western USA, curly top disease emerged as a major constraint on sugar beet production, in some cases resulting in sugar refineries having to close. To search for resistance, mass selection from heavily infested fields led to the identification and release of the first curly top resistant sugar beet variety, US1, in 1933. This variety became widely grown in the western USA, despite some shortcomings. The identification and selection of resistant sugar beet varieties has been facilitated by the use of breeding plots, known as curly top nurseries, in which high populations of leafhoppers viruliferous for the most prevalent and virulent strains of BCTV are released. As a result of the high selection pressure in these nurseries, combined with the development of commercial hybrids and molecular breeding technologies, curly top resistance in sugar beet has advanced tremendously, despite this being a complex quantitatively inherited trait. Modern curly top resistant varieties have greater uniformity, higher yield and improved

resistance. However, plants of these resistant sugar beet varieties will develop curly top symptoms and may suffer yield losses when infected at an early stage of development (two to four leaf stage). Therefore, resistant varieties need to be part of an IPM program that addresses the susceptibility of young plants, e.g., seed treatment with systemic insecticides.

Common bean is another crop that can be heavily impacted by curly top in the western USA. Curly top resistance was identified in some common bean varieties (e.g., Red Mexican type). Genetic studies identified a single dominant gene (*Bct-1*) associated with curly top resistance in common bean. Breeding efforts led to the release of the commercial curly top-resistant varieties Great Northern UI 15 and Red Mexican UI 34. Subsequently, snap bean varieties with curly top resistance were developed and released. These varieties have provided acceptable yields under high curly top pressure. Now there is a need to develop curly top-resistant varieties for other classes of common bean.

In terms of tomato and pepper, curly top resistance was not found in commercial cultivars. However, high levels of resistance were found in accessions of some wild species of tomato, including *Solanum chilense*, *S. habrochiates*, S. *lycopersicoides* and *S. peruvianum*. Unfortunately, introgression of this resistance into commercial tomato varieties has been difficult. However, as described in the case of resistance to TYLCV, a series of 6 loci (genes) conferring some degree of resistance to the virus, have been introgressed into tomato breeding lines. These lines have been used to generate commercial varieties with effective resistance against monopartite begomoviruses such as TYLCV. The mechanisms of Ty gene products determined to date, such as the Ty-1/Ty-3 enhancing transcriptional gene silencing (TGS) and cysteine methylation of the viral genome, appear to be common to all geminiviruses rather than genus-specific. Therefore, BCTV agro-inoculation screening was used to assess the response of 15 breeding lines, having different combinations of *Ty* genes (e.g., *Ty-1*, *Ty-2*, *Ty-3*, *ty-5*, and *Ty-6*), kindly provided by the World Vegetable Center. The response was assessed based upon a 0–4 rating scale, with 0 = no symptoms to 4 = severe stunting and leaf curling and vein purpling. In screens with the BCTV-Svr-[US: SVR:Cfh], the susceptible control received a rating of 4.0, whereas 11 of the breeding lines had ratings <2.5, with three lines with rating of 1.5–1.7 (lines with *Ty3* only, *Ty3* and *Ty2* and *Ty5* and *Ty6*). In an equivalent screen with BCTV strain BCTV-LH-71-[US: Cal:10], which was associated with the 2013 curly top outbreak in processing tomatoes in California, only 6 of the *Ty* breeding lines had ratings <2.5, with two lines having ratings of 0.9 and 1.2 (both having the *Ty2* and *Ty3* combination). Together, these results indicate that pyramiding *Ty* genes in tomato can provide resistance to BCTV, consistent with these gene products targeting a general geminivirus property, such as replication or transcription. Importantly, the effectiveness of the resistance was not the same for two strains of BCTV. A similar result was reported in the reverse scenario in common bean, where the *Bct-1* gene that confers qualitative (strong) resistance to BCTV, provided a high level of quantitative resistance to the bipartite begomovirus, Bean dwarf mosaic virus (BDMV). These results suggest the possibility of generating broad spectrum geminivirus resistance.

To assess the potential for using *Ty* genes to generate a curly top resistant tomato variety, a breeding line was generated by screening progeny of crosses aimed at pyramiding the *Ty-1*, *Ty-2*, and *Ty-3* genes by agro-inoculation. Plants with a resistance phenotype were then selected and further selection performed by selfing plants and screening progeny by agro-inoculation. The breeding line that was generated, named Line 20#12, carries the *Ty-1*, *Ty-2*, and *Ty-3* genes and has moderate to strong resistance to curly top (**Fig. 4**). Moreover, the resistance in this line involves initial development of mild up-curling and vein purpling in the first true leaves, followed by a recovery phenotype in which all subsequently emerging leaves show no symptoms (although virus can be detected in these leaves). This resistance phenotype is consistent with TGS targeting the BCTV genome for methylation, reducing transcription and replication. Thus, these results suggest that pyramiding *Ty* genes in a commercial tomato background could be a strategy to generate commercial curly top-resistant varieties.

Geminivirus Resistance in Beans

Geminiviruses cause severe diseases in common bean (*Phaseolus vulgaris*), and also in other species of *Phaseolus* such as lima bean (*P. lunatus*). In the Americas, the viral species involved are Bean golden mosaic virus (BGMV), Bean golden yellow mosaic virus (BGYMV) and Macroptilium yellow spot virus (MaYSV), all of which cause similar symptoms of severe yellow ("golden") mosaic. BGMV and MaYSV occur in South America (MaYSV has so far been only reported in Brazil), while BGYMV is present throughout Central America and the Caribbean.

Many institutions in the Americas had breeding programs for resistance to golden mosaic disease in *Phaseolus*. The most successful program was conducted at the International Center for Tropical Agriculture (CIAT) in Cali, Colombia, starting in the mid-1970s. This program targeted BGYMV, although most of the relevant literature refers to BGMV, since the distinction between the two viruses was not completely clear at the time. Indeed, the fact that resistance to BGYMV was not successful against BGMV provided additional evidence for the distinction between the two viruses. A first source of resistance to BGYMV was identified in a black bean landrace named Porrillo Sintetico, originated from El Salvador (Central America). The analysis of segregating populations derived from crosses with this source indicated that resistance was quantitative. Resistance was partial and often broke down under high inoculum pressure. The red-seeded line DOR 364 provided additional quantitative resistance genes, and a number of cultivars containing these two sources, with resistance that was markedly superior to that conferred by either source alone, were released during the 1980s. During that decade, virtually all resistant cultivars that were deployed in Central America and the Caribbean derived from these two sources. These cultivars were largely responsible for the recovery of bean yields in that region. The main limitation with these sources of resistance was that all resistant cultivars produced black seeds, and many of the commercial common bean cultivars grown in disease-prone regions had different seed colors that were changed when crossed with

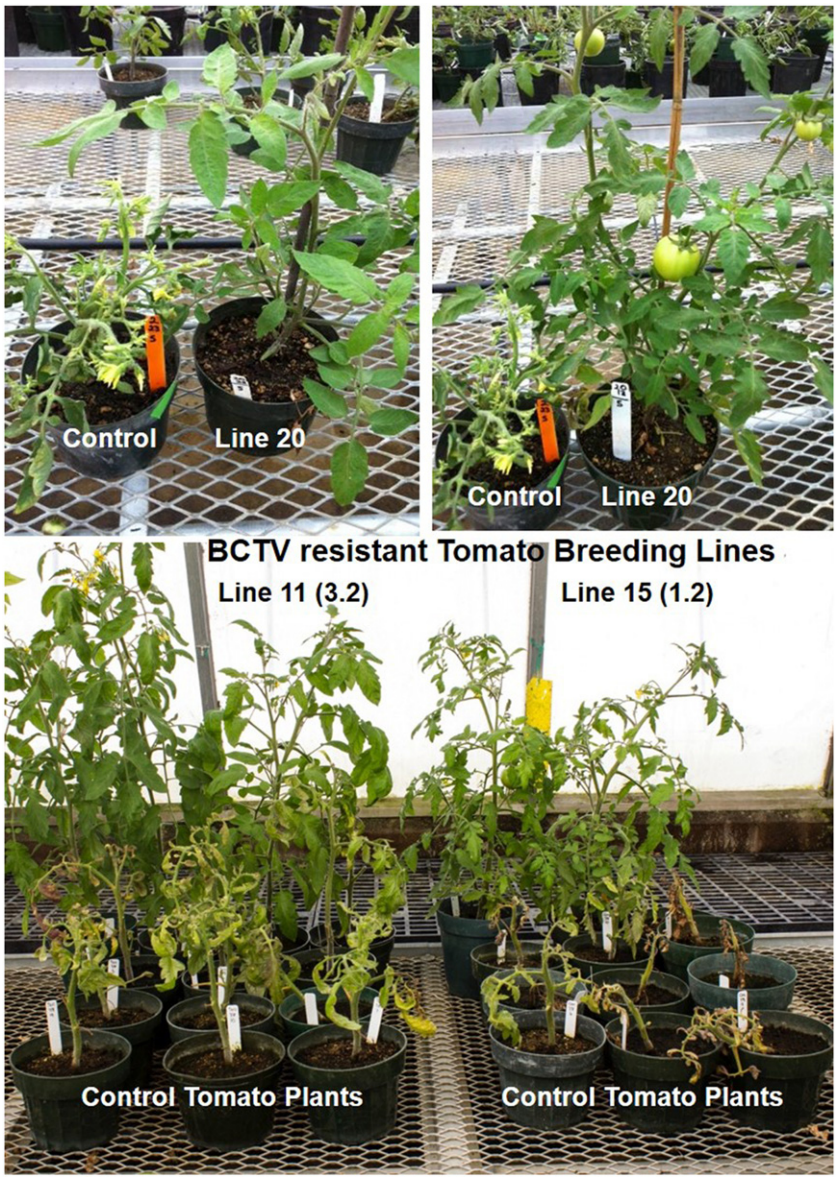

Fig. 4 Advancement and characterization of tomato breeding lines for beet curly top virus resistance, by agro-inoculation of BCTV in glasshouse conditions at the University of California, Davis, CA, USA.

the black-seeded parental sources of resistance. Moreover, resistance was not complete and could still be overcome in years when conditions were highly favorable to the disease.

An improved source of resistance was identified in the early 1990s in Garrapato, a landrace from the Durango group from Mexico. A breeding line derived from Garrapato, named A429, provided the best level of resistance. Inheritance studies indicated that the resistance in A429 was controlled by a single recessive gene, named *bgm-1*. The presence of the *bgm-1* gene strongly reduces the mosaic and yellowing symptoms caused by BGYMV. The *bgm-1* containing DOR lines developed by the breeding program at CIAT remain the best source of durable and stable BGYMV resistance (**Fig. 5**).

A co-dominant sequence-characterized amplified region (SCAR) marker, SR2, which is tightly linked to *bgm-1*, was developed by genetic mapping. The position of the *bgm-1* gene was estimated to be close to that of *bc-1*, which confers resistance to the potyvirus Bean common mosaic virus (BCMV), on chromosome 5. Interestingly, the mapping study indicated that *bgm-1* may be sub-telomeric. Sub-telomeric regions tend to be highly recombinogenic, a characteristic that is advantageous in the context of host-pathogen co-evolution.

The success at obtaining resistant varieties to BGYMV was not, unfortunately, replicated in South America for BGMV (a different virus with a low degree of sequence identity with BGYMV). Breeding for resistance to BGMV initiated in the 1970s at the Campinas Agronomical Institute (IAC in the Portuguese acronym) in Campinas, Brazil, and also at center for bean research at Embrapa, in Goiânia. Although a number of varieties with delayed incidence of the disease were obtained, inheritance was complex, yields were

Fig. 5 The "Mosaico Dorado Amarillo de Frijol" (Bean golden yellow mosaic virus) nursery established at San Andrés Experimental Station of the Centro Nacional de Tecnología Agropecuaria y Forestal "Enriques Álvarez Córdova" in El Salvador (Central America), as part of the breeding program conducted by CIAT. The nursery contains a large number of landraces and breeding lines of black and red beans with different levels of resistance to Bean golden yellow mosaic virus (BGYMV). Courtesy MA Grajales.

unremarkable, and their agronomical traits were not generally favored by growers. The most promising varieties were released during the 1990s by the breeding program at the Paraná Agronomical Institute (IAPAR) in Londrina. However, the quantitative, partial resistance provided by these lines would break down under the high BGMV incidence levels that often occurred in southern Brazil. The lack of success at obtaining cultivars with good natural resistance to BGMV led to the development of a genetically modified common bean, based on RNAi technology, which provided excellent resistance.

Resistance to Mungbean Yellow Mosaic Viruses

In black gram (*Vigna mungo*) Mungbean yellow mosaic India virus (MYMIV) resistance is governed by a single recessive gene having NB-ARC domain with normal functional group. A resistant gene (CYR1) for MYMIV has been characterized, similarly its truncated allele (cyr1) was also found in susceptible black gram. Moreover, a SNP in LRR-like protein kinase gene responsible for a G/C transversion was identified in soybean. The yellow mosaic disease (YMD) was found to be monogenic. The comparative transcriptome analysis showed an upregulation in NAC transcription factor, Argonaute, NB-LRR, Ankyrin and WRKY33 genes. Full length cDNA of black gram MAPK (Mitogen-activated protein kinases) designated as *VmMAPK1* was found increased during MYMIV infection, which helps in autophosphorylation and restricting the multiplication of MYMIV by mediating salicylic acid signaling pathway. MAPK16 and MAPKK3 genes are down regulated during MYMIV infection. Moreover, the G2/mitotic specific cyclin-1-like and Cyclin-B1 (CYCB1) were found up-regulated. MYMIV interacts with proliferative cell nuclear antigen, recombination-dependent replication (RDR51, RDR54), minichromosome maintenance protein subunit 2 (MCM2) and replication protein A, respectively. Micro RNAs (miRNAs) also confer resistance against MYMIV such as gma-miR5785 found in soybean, that helps in cleavage of BC1 gene of MYMIV. In Mungbean RAPD markers OPP 07_{895} and OPP 07_{900} are associated with Mungbean yellow mosaic virus (MYMV) resistance, while OPP 07_{730} was found in French bean.

A resistance gene analog (RGA) marker has been associated with MYMV resistance in mungbean and black gram. Moreover, the MYMIV-resistant markers YR4 and CYR1 were found in urdbean and mungbean. In black gram the SSR marker (CEDG180) was found closely linked with YMD resistance. The QTLs (qTMIV1 to qYMIV5), qMYMIV2 and qMYMIV were identified for MYMIV resistance in *Vigna radiata*. The SSR marker CEDG044 was found highly linked with MYMIV resistance. The MYMIV resistance gene was mapped on chr 6 (LG C2), which is present 3.5-cM on genomic region between two SSR markers GMAC7L and Satt322. Similarly, in black gram the ISSR8111357 marker was developed and confirmed for MYMV resistance. **Fig. 6** illustrates the screening of soybean germplasm for resistance to Mungbean yellow mosaic virus.

Resistance to Cotton Leaf Curl Virus in Cotton

Livelihoods of many rely on cotton in many countries across the world. Cotton leaf curl viruses (CLCuVs), members of the genus *Begomovirus*, in the family *Geminiviridae*, and their natural vector whitefly (*Bemisia tabaci*) pose serious threat to cotton production. In the Indian subcontinent, whitefly-mediated cotton leaf curl disease (CLCuD) stands a devastating factor that limits overall production of cotton. Multiple strategies based on pathogen-derived resistance have been tried but huge diversity of geminiviruses has resulted in limited success under field conditions. *Gossypium hirsutum*, tetraploid cotton cultivars produce high quality lint and fiber but are susceptible to CLCuD. On the contrary, one of the diploid progenitors of cotton, *G. arboreum* (A genome) is resistant to CLCuD, making it a valued source for novel genes to improve CLCuD resistance. Whole transcriptome analysis of *G. arboreum* under graft-mediated CLCuD infection revealed the genetic basis and underlying mechanism of resistance. The genes related to oxidative stress, transcription factors, R-gene family, phytohormone signaling, membrane transporters and channel proteins were found differentially expressed and may be involved in resistance to CLCuD. Correlation

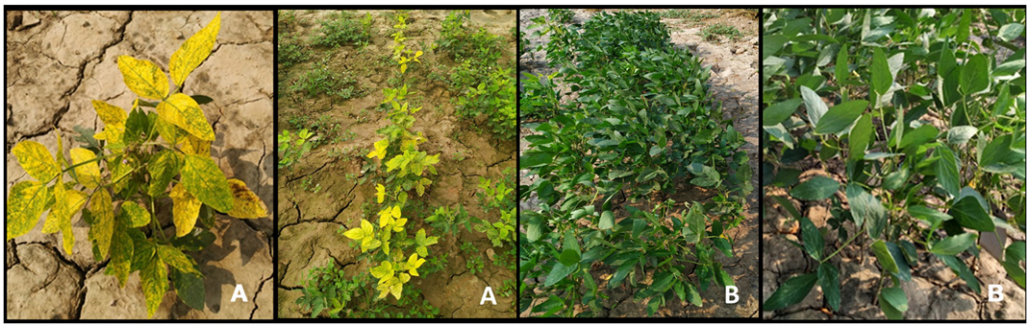

Fig. 6 *Mungbean yellow mosaic disease resistant germplasm.* The panels (A) show soybean (*Glycine max*) line V-47 showing susceptibility to Mungbean yellow mosaic virus. The panels (B) show soybean line SG-16, which is resistant to Mungbean yellow mosaic virus.

Fig. 7 *Cotton germplasm showing resistance to CLCuD.* Mac7 plants grown in field condition showing resistance to Cotton leaf curl disease (CLCuD), while all the susceptible varieties grown around are showing typical symptoms of CLCuD. The black panel represents plant of CLCuD resistant Mac7 accession and susceptible varieties grown in glasshouse conditions. On the left side of panel is the closer look of asymptomatic leaf of Mac7 (Upper) and diseased or symptomatic leaf of susceptible (lower) cotton varieties.

of these identified genes with available QTLs (associated with CLCuD resistance) can help in picking the desirable genes further to impart CLCuD resistance in cotton.

Several efforts have been made for the identification of resistance sources among large collections of available cotton germplasm (Fig. 7). Around 5000 cotton USDA germplasm accessions from their different breeding programs were screened against a high pressure of CLCuD. This massive screening revealed a disease resistant accession Mac7.

The understanding of mechanisms underlying the interaction of virus, whitefly and host cotton plant can help in devising robust strategies for controlling the virus and vector (Fig. 8). Therefore, transcriptome analysis of Mac7 under whitefly-mediated CLCuV infestation has shed light on the molecular insights of CLCuD and whitefly resistance in cotton. This study has pinpointed the involvement of heat shock proteins and geminivirus-interacting genes in Mac7 resistance to CLCuD. Virus-induced gene silencing (VIGS) based silencing of HSC80, E3 ligase and serine threonine kinase (STK) genes exhibited increased susceptibility to whitefly and CLCuD in Mac7 cotton. These identified genes in resistant cotton accession could be used as useful sources of resistance to CLCuD and can help in improving the whitefly and virus resistance in cotton germplasm.

Understanding and exploitation of host plant resistance against whitefly can also help in control of geminiviruses. In this context, a study in cotton infested with whitefly showed differential gene expression of WRKY40, GhMPK3 and copper transport protein. Furthermore, VIGS of GhMPK3, validated the suppression of Jasmonic acid (JA) and Ethylene (ET) signaling related genes leading to the whitefly susceptibility. Small RNA-seq and genome-wide miRNA analysis of whitefly resistant and susceptible cultivars of *G. hirsutum* has been done recently, that revealed the involvement of miRNAs related to leucine-rich repeat (LRR) protein, MYB transcription factors and auxin response factor (ARF). VIGS of ARF8 from whitefly resistant cotton targeting miRNA390 enhanced the auxin and JA accumulation that increased the whitefly tolerance. Susceptible cotton variety of *G. hirsutum* when infected with CLCuD viruliferous whiteflies indicated the differential gene expression of genes including transcription factors (NAC, bHLH, MYB), heat shock proteins, methyl-transferases and metabolism related genes like cytochrome p450. Thus the genes identified and highlighted in these studies can be used in developing and enhancing cotton resistance to whitefly.

Now in the era of new plant breeding technologies, CLCuD/whitefly resistant cotton cultivars harboring ample genetic diversity can be achieved using CRISPR/Cas9 system. CRISPR/Cas9 based gene targeting is now becoming a method of choice with high reliability and efficiency for cotton genome editing. It would help in targeting CLCuD related susceptibility genes in cotton to

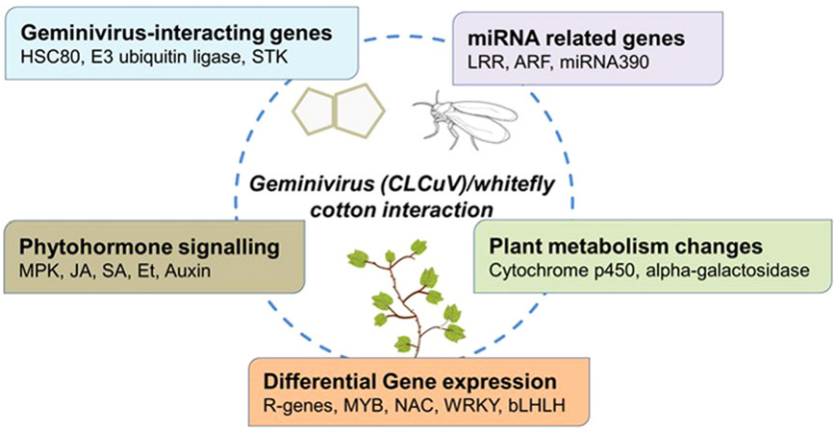

Fig. 8 *Geminivirus (CLCuV)/whitefly – cotton interactions and mechanisms of host plant resistance.* Cotton leaf curl geminivirus disease complex entry into cotton through whitefly mediates different layers of mechanisms in cotton to deal with pest and virus including interaction of geminivirus proteins with plant genes, activation of differential gene expression, miRNA machinery, metabolic changes and phytohormone signaling pathways.

Fig. 9 *Maize streak virus* (MSV) induced stunting in a susceptible maize variety Gusau pool 16 (bottom) and resistant variety TZL Composite 4 CL (top). *Source*: Taiwo *et al.*, 2006. Plant Disease 90, 199–202.

counter the whitefly and fast evolving viral genomes. Moreover, development of improved resistant crop varieties by bridging the conventional and new genetic approaches along with pest/disease awareness to the farmers, capacity building of researchers would be helpful in controlling the geminiviruses and related diseases effectively.

Resistance to *Maize Streak Virus*

Maize streak virus (MSV, genus *Mastrevirus*, family *Geminiviridae*) causes maize streak disease (MSD), one of the most important viral diseases of maize in sub-Saharan Africa. MSV largely remains uncontrolled in most of the African continent and during epidemic years it can lead to widespread yield losses and famine. **Fig. 9** depicts field performance of a MSV resistant maize variety "TZL composite 4L" along with a MSV susceptible maize variety "Gusau Pool 16" in Nigeria. Resistance to MSV is associated with up to five different QTLs located on chromosome 1, 3, 7, and 9, that are either dominant or additive in nature (**Table 1**). None of these QTLs in isolation can prevent MSV infections, however combinations of them can resist the MSV infections. Although maize

genotypes that tolerate MSV infection without compromising much with yield losses have been developed, there has been only limited success in the field. The MSV resistance seems to be specific to certain geographic locations, selection made of MSV resistance at one location may fail to tolerate MSV infections in another location. Thus the breeding efforts and screening for MSV resistance have to be specific to different geographical areas or the agro-climatic zones of Africa. One of the major concern is that the improved MSV tolerant maize genotypes don't have the desirable agronomic traits, such as good yields. Thus developing maize genotypes that possess both MSV tolerance and high yields remains a big challenge to the maize breeders. Further involvement of multiple MSV tolerant QTLs makes it difficult to introgress each of them in one single maize genotype and development of such a variety may take several years of breeding. Despite the humungous efforts made in development of MSV resistant maize varieties, most farmers prefer to use high-yielding maize varieties.

Conclusion

The current knowledge on the genes that confer resistance to geminiviruses is scarce when compared to the information available for plant infecting RNA viruses. Although the geminivirus resistant locus/loci are mapped in several cultivated plant species, the actual genes associated with the resistance are yet to be cloned and characterized. The few resistant genes that have been identified, the underlying resistant mechanism is yet to be understood. In this article we have summarized the sources and genes for geminivirus resistance in the most important crop plants that are heavily infested by geminiviruses. The geminivirus resistant genes identified so far are diverse in their characteristics, they are monogenic and polygenic, dominant and recessive, and specific as well as broad spectrum in nature. Introgression of these resistant genes in cultivated crop varieties has not been very successful since some of these genes are associated with poor agronomic traits. Loci or genes conferring resistance to the whitefly vectors are not yet identified and identification of such genes may boost the breeding for begomovirus resistance. Revolution in genome editing technologies and employment of CRISPR-Cas based technologies for precise editing of host genes are promising for manipulating the recessive resistant genes that makes them immune to geminivirus infections. Transgenics by employing CRISPR-Cas technology that destroys the geminiviral DNA genome have been developed, however their durability remains uncertain with reference to the fast mutating geminiviruses which may evade the targeting by sgRNAs that are key for Cas9 based cleavage. However, these are considered as transgenics and their social acceptance is questionable. Thus emphasis on identification and characterization of novel geminiviral resistant genes offers more promise for development of improved geminivirus resistant crop varieties.

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