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Review Paper

Citrus tristeza virus a Worldwide threat to Citriculture: Advances made in its Research and Future thrust

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Abstract

Citrus tristeza virus (CTV) is one of the biggest oppressions to the citrus industry globally and a major contributor of citrus decline. It is a phloem associated virus of various citrus species, containing positive-sense single stranded monopartite RNA genome with encapsidation by two different capsid proteins and transmitted by several aphid species. It has various pathotypes which exhibits different symptoms on citrus along with symptomless mild strains. Research on CTV has focused on detection and diagnosis of the virus mainly by utilizing serological and molecular methods, molecular characterization through sequencing and their phylogenetic analyses. Utilization of resistance mechanism of host in disease management like cross protection, pathogen derived resistance, RNA interference, etc has been exploited in the recent past and also further advancement of the technologies are going on. A large number of isolates of CTV exist in nature and many new more isolates are developing due to recombination of the isolates which are creating more affects on host infection and virus vector transmission dynamics. Bioprospecting and allele mining of wild citrus germplasms can give valuable insights into some novel genes of resistance against CTV which can be exploited and used for protecting the host. With the development of sequencing technology possible alterations could be carried out in the genome using CRISPR technology or genome editing which has the potential of modification with ease and efficiency of the endogenous genes in host plants and make it possible for them to defend against pathogen attack.

Keywords: CTV, detection, characterization, pathogen derived resistance, RNAi.

Introduction

Among the fruit crops citrus, one of the most widely cultivated and important economically, is grown in around 142 countries in the world. Citrus cultivation is facing decline caused by both biotic and abiotic factors from centuries, of which *Citrus tristeza virus* (CTV) is one of the biggest oppressions to the citrus industry globally and also a major contributor to citrus decline throughout the world. With the first outbreaks of CTV in South America in the 1940s, it has killed more than 100 million trees worldwide[1] and at recent times, there are more than 400 million citrus trees grafted on sour orange rootstocks at the risk to CTV decline[2]. Even though in citrus there are more than 30 diseases of virus and virus-like pathogens known in the world, but among these CTV is arguably the most destructive and most widely distributed[3]. In Spanish and Portuguese, Tristeza which indicates sadness, in origin was used to describe the quick and widely spread death and decline that occurred to millions of trees in Argentina and Brazil on sour orange rootstocks since the 1930's[4].

Biology of CTV

Genomic and morphological characteristics: Of all the known plant viruses CTV, a member of the *Closterovirus* genus in the *Closteroviridae* family, has one of the largest and most complex genome[5], next to genome of animal *Coronaviruses*, the largest known genome worldwide[6]. Bipolar long flexuous particles of CTV observed with an electron microscope in the phloem tissues of infected citrus plants[7] had filaments of 2000 x 11 nm size, helicoidal in shape, with two different capsid proteins of dissimilar sizes that coat the opposite ends of the virions[8-9]. About 95 per cent of the genome of CTV is encapsidated by the 25 kDa CP, considered as major CP, and the rest portion of the CTV genome is encapsidated by a minor CP of 27 kDa[7]. CTV has a genome size of about 19.3 kb, consisting of long positive-sense single-stranded monopartite RNA[10], with two untranslated regions at the 5′ and 3′ termini of 107 and 273 nucleotides, respectively, along with 12 open reading frames (ORFs), encoding for more than 17 proteins. CTV gene expression is controlled three different strategies *i.e.* proteolytic processing, ribosomal frameshifting and generation of a set of 3′-coterminal subgenomic RNAs control, in a combination[11].

Defective RNAs: In CTV, along with the RNA genome it is reported to contain defective RNAs (D-RNAs) in some isolates[12]. CTV D-RNAs bear a genome ranging from 2.0 to 5.0 kb, which varies in its size, relative availability and sequence and are composed of CTV genomic RNA with variable portions

from the 3' and 5' termini having large internal deletions[13]. They have the ability of encapsidation in CTV particles and transmission by aphids[14]. D-RNAs have been reported in Israeli CTV isolates[12]; Spanish CTV isolates[15].

CTV pathotypes: A variety of symptoms are exhibited by CTV on different hosts and has three major syndromes *viz.*, Tristeza or Quick decline (QD), Stem pitting (SP) and Seedling yellows (SY) associated with infections in citrus[2,16]. Decline syndrome called Tristeza, results in the quick death and decline of citrus species of commercial importance such as *Citrus sinensis* (sweet orange), *C. reticulate* (mandarins), *C. paradise* (grapefruits), *Fortunella* sp. (kumquats) and *C. aurantifolia* (limes) when these are grafted on rootstocks of *C. aurantium* (sour orange) or *C. limon* (lemon)[11]. During the development of QD, cells close to the bud union between the scion and the rootstock *i.e.* the sieve tubes and companion cells collapse and necroses, producing an excessive amount of non-functional phloem[17] and resulting in an overgrowth of the scion at the bud union, loss of root mass, which exhibited drought sensitivity, dwarfing, leaf chlorosis, poor growth, dieback, reduced fruit size, wilting and finally collapse and death of the infected tree.

The second syndrome resulting due to interaction between CTV and citrus trees is stem pitting (SP). When highly virulent strains of CTV infect commercial sweet orange, lime and grapefruit trees, regardless of the rootstock used, SP is exhibited and can affect both rootstock and grafted varieties[11]. SP usually does not cause tree death but causes high economic losses due to development of deep pits in the wood under depressed areas of bark which chronically limits profitable growth of the various commercial citrus varieties, significant plant vigor reduction, severe dwarfing and low yield of unmarketable fruits[18]. The syndrome observed in young plants due to CTV infection in citrus is SY. It occurs generally in high percentage under greenhouse conditions and also in top–grafted plants in field condition. It results in dwarfing, leaf chlorosis and sometimes complete growth cessation in seedlings of sour orange, grapefruit or lemon[19].

Apart from these, symptomless expression is exhibited by certain CTV strains in most of the varieties of citrus, and also those propagated on sour orange rootstocks, even if the virus multiplies to reach high titers, are called the mild strains[20].

Pest biology: CTV, a phloem-associated virus that replicates in the cytoplasm of companion or phloem parenchyma cells is graft-transmissible and transmitted most widely by aphids in a semi-persistent manner[21]. Propagating materials serve as the long distance transmission of the virus and subsequent secondary spread occurs through the agency of aphid vectors[18,4]. It is not known to be seed- transmitted[22] or pollen-transmitted[11] in any of its hosts.

In nature, the virus is transmitted by several aphid species[11,23] with 30 minutes of acquisition feeding period on virus infected plant, and also of 30 minutes of inoculation feeding period for transmission of the the virus to healthy hosts. The aphid vectors can transmit the virus without any latent period but, because virus multiplication or circulation does not occur in the aphid, the aphid remains viruliferous for only about 24 hours, and infectivity is completely lost within 48 hours of virus acquisition[24]. The most efficient vector of CTV is *Toxoptera citricida* (Kirkaldy)[25,11,23] with *Aphis gossypii*[26], *Aphis spiraecola* and *Toxoptera auranti*[27-28] as other relatively less efficient reported CTV aphid vectors. Both the adults and the nymphs of aphids were found capable of virus transmission[29] while transmission efficiency can vary between virus isolates.
In nature, CTV host range is restricted to plant species of the genera *Citrus*, *Poncirus,* a citrus relative which is widely used as a rootstock and *Fortunella* (subfamily *Aurantioidae*, family *Rutaceae*[11]). The only known natural non-Rutaceae CTV host is *Passiflora* [30]. Depending on the host species, the cultivar and the particular isolate, a variety of symptoms are exhibited by CTV in the host plants.

Detection of CTV

For the detection CTV, a number of methods have been developed which includes traditional methods like symptomatology and transmission study, electron microscopy along with serological and molecular methods. But traditional methods are not very reliable and accurate[31] because symptoms vary based on virus strain, citrus species/ cultivar, time of infection and environment and transmission studies are very time consuming, labour intensive and requires a large population of vectors and high chances of human error. Detection through electron microscope is sensitive but it could be used for testing a few samples and use is confined to a few sophisticated laboratories. Therefore, the most efficient and reliable methods of CTV diagnosis are reported to be serological and molecular methods[32-33].

Serology: In detection of CTV, a remarkable advancement has taken place with the development and recent improvements in the serological methods. Serological methods have become the most reliable tools for many global research, extension and quarantine purposes. A great leap has occurred in CTV-specific polyclonal and monoclonal antibodies development in different research laboratories worldwide and is utilized most extensively in different research works and studies[34]. Monoclonal antibody that detects almost all the severe strains were produced for the first time from hybrid cells obtained by fusing a non-secreting myeloma cell line with spleen cells of BALB/c mice which were immunized with T-308 isolate of CTV [35]. ELISA (Enzyme-Linked Immunosorbent Assays), IP-PCR (Immuno Precipitation- Polymerase Chain Reaction), Immunoblotting methods, DIBA (Dot Immuno Binding Assay), TIBA (Tissue Blot Immune Assay), Western blotting, serologically specific electron microscopy, Double Immunodiffusion and Lateral Flow test are the serological techniques available for serological diagnosis of CTV[36].

The DAS-ELISA (Double-antibody sandwich- ELISA), a variant of ELISA[37] and DTBIA (Direct Tissue Blot Immunoassay), are the most widely used virus screening techniques utilized for the rapid detection of CTV[38-40] and both the methods yielded similar rates of CTV infection in Florida and Spain[38]. DIBA was adapted for the detection of CTV and similar results were obtained as of DAS-ELISA and DAS-indirect ELISA[41].

In Florida, ELISA was utilized for the detection of CTV[42] and in Spain direct tissue printing technique was used to test more than 600,000 plants for CTV[43]. For the detection of CTV isolates in Mediterranean region from citrus species of different origin at flowering time[44] and from Turkey in *Citrus unshiu* (Satsuma Owari mandarin trees*)* [45], DTBIA and ELISA were utilized. In India, CTV free citrus mother stocks were identified using Direct antigen coated-ELISA to develop CTV- free planting materials to protect the orchards of Darjeeling hills[46]. Based on ELISA studies, it was found that the accumulation of CTV in all the plant parts is not even, the tender bark of 6 months to 1 year old, petiole and mid rib of young leaves, and apical bud showed highest amount of CTV[47] and also CTV infection in old orchards of 10 years and above was always higher than newer ones of less than 10 years[48]. The DAS-ELISA results showed a CTV incidence of 46.32 per cent in Sikkim Himalayan region[49], 63.50 per cent disease in Assam, India[50] and 52.2% in NE region of India [48] and 87% in Western Crete by immunoprinting-ELISA method[51]. Also strain differentiation of CTV were carried out using monoclonal and polyclonal antibodies[20,52]. A complete kit for the detection of CTV was developed and evaluated utilizing antibodies specific for the recombinant coat protein (rCP) gene *p25* in healthy and CTV-infected tissue[53].

Molecular methods: In CTV detection, the reverse transcription polymerase chain reaction method (RT-PCR) has been the most reliable and accurate method[54]. RT-PCR could detect virus from the semi-hardwood tissues of citrus where virus occurs at low concentration.

RT-PCR, variant of polymerase chain reaction (PCR) where coat protein gene sequence of the CTV Florida isolate T36 was utilized in primer designing for amplification of cDNA, were subsequently used for coat protein gene amplification using RT-PCR of severe California isolate SY568 of CTV[55]. Using RT-PCR, CTV was detected in Satsuma mandarin (*Citrus unshiu)* in Korea[56] and in mandarin from Darjeeling hills in India[46]. For differentiating isolates of CTV inducing decline or not, RT-PCR can be used and was used in CTV infected field trees in Florida[57].

Molecular characterization of CTV: With the developments in sequencing technology, a revolution occurred in the study of CTV genetics[10] and both complete and partial genome sequencing were carried out. At present, there are twenty full genomic sequences of CTV are available worldwide[13] and these are Floridian isolates, T36 and T30[10,20]; Israeli isolate VT[58]; Californian isolate SY568R[59-60]; Japanese isolate NuagA[61]; Spanish isolates, T385 and T318A[62-63]; Egyptian isolate AY340974 (Qaha); Mexican isolate DQ272579; Indian isolate B165[64]; New Zealand isolates, NZ-M16, NZ-B18, NZRB-TH28, NZRB-TH30, NZRB-M12, NZRB-M17 and NZRB-G9[65-66]; Hawaiian isolates, HA16-5 and HA18-9[67] and Indian isolate Kpg3[68]. But these twenty genomic sequences of CTV based on phylogenetic analysis were placed into seven main genotypes. Genomic sequences, T36-like (T36, Qaha and Mexican); RB group plus HA18-9; VT-like (VT, NUagA, T318A, SY568 and Kpg3); HA16-5; B165 and NZ-B18; and NZ-M16 genomic sequences were the six out of all, inducing severe CTV syndromes. T30-like (T30, T385) consist the seventh group of asymptomatic or mild genotypes[13].

Partial genome sequences were also carried out for the coat protein genes, ORFs like 5' ORF1a to determine the genetic diversity among CTV isolates. Such works were carried out in India[52,69-70], Cyprus[71], Syria[72], Jamaica[73], etc.

One of the factors responsible for genetic variation in CTV isolates was observed to be recombination[74]. Recombination events among the CTV isolates was detected in a recombination analysis performed using RDP3 program involving exchange of sequences between divergent CTV variants[68]. It was observed that the central region in the SY568 genome results from RNA recombination between two CTV genomes, one of which was almost identical to T385[63].

Resistance mechanism against CTV

Cross protection: Cross protection against CTV in Citrus plants utilizing mild strains is a means of extending the economic life of citrus[75]. Grant and Costa[76] were the first to demonstrate CTV disease control by mild strains. The probable mechanisms of cross protection are preventing the cells from entry of the invading virus which are previously infected by one virus[77], competition for host factors and intracellular replication sites between primary and challenging viruses, and also interference of the primary virus in disassembly, translation or replication of the secondary virus[77] and sequence-specific degradation of the challenge virus RNA by the protector virus leading to induction of RNA silencing[78]. In citriculture, one of the most important role in maintaining profitability of citrus cultivation is put forwarded by cross-protection by allowing some citrus species to remain economically viable in a number of countries in the world like Brazil[79-80], South Africa[81-82], Florida[75,83], Australia[84], India[85] etc. Monoclonal Antibody 13 that detects almost all the severe strains causing Quick Decline and Stem Pitting CTV syndromes was utilized to identify non reactive strains to MAC13, which were found to be best candidates for a *Tristeza* disease cross-protection program in Colombia[86].

Pathogen derived resistance: The recent development of transformation techniques in citrus and CTV partial and full

genome characterization provides potentialities in utilization of pathogen derived resistance (PDR) against CTV in citrus. It can be achieved by transformation of plants with coat protein genes or non-structural genes, especially with the genes encoding for proteins associated with movement and replication, like the genes for RNA-dependent RNA polymerase (RdRp).

In transgenic plants expressing virus coat protein genes, delay in development of disease or resistance to infection is exhibited upon inoculation with the virus. Forty-two transgenic lines of Mexican lime plant were produced by incorporating CTV *p25* coat protein (CP) gene. In 25 lines, severe T-305 CTV strain *p25* CP gene and in 17 lines, the mild strain T-317 was introduced. 10 to 33% of the transformed plants were CTV resistant and rest showed a significant delay in accumulation of virus and onset of symptom. In non-homologous CTV strains the protection was highest and high p25 CP accumulation was observed in the protected lines suggesting CP-mediated protection mechanism[87]. Similar results of transformation with genes of major CP, translatable and untranslatable versions, were obtained in 'Duncan' grapefruit[88-89].

RNA interference (RNAi): CTV contains three silencing suppressor proteins in citrus in order to overcome the strong antiviral defense exhibited by host, where proteins p23 and p20 act at intra-cellular level and proteins p20 and p25 at inter-cellular level. Therefore, mechanisms to trigger RNA silencing using dsRNA, RNA interference (RNAi), are used for the development of CTV resistant transgenic citrus plants. Untranslatable versions of *p25*, *p20*, *p23* genes carried in intron-hairpin vector and T36 CTV strain 3'-UTR were utilized for the transformation of Mexican lime plants for the purpose of silencing the expression in CTV-infected cells and three transgenic lines were observed to exhibit complete resistance to the viral infection and all the non-transgenic rootstock or the transgenic scion used for propagation were found to be symptomless and virus-free even after inoculation with CTV-T36 grafts[90].

In citrus resistance was observed due to the mechanism of post transcriptional gene silencing (PTGS). In transgenic grapefruit plants which are transformed with different CTV genome constructs resistance against the virus, levels of transgene expression and accumulation of siRNA were tested and no transgene mRNA, accumulation of siRNA and no RNA of CTV were observed in one plant exhibiting resistance from a partially resistant line, indicating post transcriptional gene silencing (PTGS) resistance mechanism to be[89]. Also, enhanced defense by RNAi-mediated gene silencing was utilized to develop transgenic CTV-resistant Mexican lime[91].

Conclusion

Worldwide devastation caused by CTV on citrus fruit crops has generated strong research interest across the globe. Decades of research on this virus has deciphered vital facts about the pathogen, its biology and various detection and management strategies. A large number of isolates of the virus exits in nature due to recombination among the isolates effecting host infection and virus vector transmission dynamics. The methods of controlling CTV infections are expanding. Most of the management strategies are undertaken to reduce sources of infection, to limit the vectors associated and reduce the effect of infection on yield to the least possible degree. Abolishment or refrainment of sources of infection is given prime importance with production of virus free planting material, bud wood certification. Also control or avoidance of vectors is invariably a very important strategy adopted to reduce viral infection which is a subject of intense study. Any strategy to reduce CTV inoculum yields good result but the pathogen is always present in the ecosystem having the capability to cause outbreak when susceptible hosts are available for attack.

Mechanisms to protect viral inoculated plants from developing systemic disease are the most efficient method of its management and efforts in this line of research would always give promising results. Development of resistant cultivars against viruses has been the safest and best alternative to control losses. Resistance from various sources including the pathogen itself has been increasingly used. Bioprospecting and allele mining of wild citrus germplasms can give valuable insights into some novel genes of resistance against CTV which can be exploited through biotechnological approach like marker assisted selection and their introgression in commercial species.

The development of the sequencing technology has made possible to study genome and find out possible alterations that could be carried out in the genome by utilizing Clustered regularly interspaced short palindromic repeats (CRISPR) technology for gene editing or genome editing with engineered nucleases (GEEN). Editing of targeted genome using artificial nucleases has the potentiality of modification with ease and efficiency of the endogenous genes in host plants and making it possible for them to defend against pathogen attack. The interaction of CTV with its aphid vector, a crucial step in the spread of the virus in the orchard, also needs to be carefully looked at. Approaches to disrupt the virus vector transmission relationship may open up new avenues of CTV control in the near future. Concerted effort needs to be done in this line of research to reduce huge stress posed by this pathogen on citrus growers around the world.

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