1396 Cotton leaf curl disease

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Cotton leaf curl disease is the major biotic constraint to cotton production in Pakistan and India.The disease causes unusual symptoms that include vein swelling, enations and the formation of leaf-like outgrowths. Cotton production during the 1990s was severely curtailed by CLCuD across the sub-continent. This stimulated interest in determining the aetiology and generating resistant varieties. The disease was shown to be caused by begomoviruses in association with a newly discovered single-stranded DNA satellite; such satellites have subsequently been shown to be widespread across the Old World and to be associated with many important diseases of crop plants. In the late 1990s, cotton varieties with natural (host plant) resistant to the disease were developed and were successful in alleviating losses. However, in 2001, symptoms of CLCuD were again evident, indicating the appearance of a resistance breaking strain of the disease. Efforts are now underway to produce transgenic pathogen-derived resistance, a strategy that is showing promise.

Introduction

Cotton is an important crop across the Indian sub-continent. In Pakistan it contributes appprox. 60% of foreign exchange earnings. The disease causes very characteristic symptoms, including leaf curling, darkened veins, vein swelling and enations which

frequently develop into cup-shaped, leaf-like structures on the undersides of leaves (Fig. 1). Early investigations had shown the CLCuD could be transmitted by the whitefly *Bemisia tabaci*, the vector of many begomoviruses (family *Geminiviridae*).

The enations and vein darkening are not symptoms typical of the begomoviruses and this, together with the economic damage it was causing, spurred research into the etiology of the disease in the 1990s. Researchers rapidly identified a number of begomoviruses associated with symptomatic plants (Zhou et al., 1998), but Koch's postulates could not be satisfied; the cloned viruses did not induce typical CLCuD symptoms in plants (Briddon et al., 2000). More detailed investigations identified further novel molecules in CLCuD affected cotton. The most important of these is the symptom modulating satellite known as DNA β, a satellite molecule which is responsible for the typical symptoms of the disease.

History of CLCuD

CLCuD was first reported from Nigeria in 1912, where the disease was a sporadic but minor problem, with a second outbreak in this country occurring in 1924. In North Africa (mainly Egypt and Sudan) *G. barbadense* is the main cotton species cultivated. In these areas CLCuD is endemic, although it is only sporadically a problem. This was not the case during the early part of the 20th Century when the disease caused major crop losses, particularly in Sudan. An epidemic in Sudan in 1927-28 stimulated interest and it was during this time that the disease was shown to be transmitted by the whitefly, *Bemisia tabaci*. Extensive work to understand the disease showed that it was also graft transmissible. Although the causative agent was not identified, a virus-like agent was suspected. The disease in this

case was brought under control by imposing a cotton-free cultivation period and the introduction of virus tolerant *G. barbadense* varieties. The disease continues to occur sporadically throughout the region but does not cause major losses.

CLCuD was noted infrequently across the Indian subcontinent prior to the 1980s. Cotton is the main foreign exchange earner for Pakistan and production suffered heavily from an epidemic of CLCuD which initiated in the vicinity of the city of Multan in the mid 1980s. The disease spread to virtually all cotton growing areas of the country, as well as into western India. The major factor allowing this previously benign pathogen to become epidemic is believed to be the introduction and widespread cultivation of high yielding, but also highly susceptible, exotic cotton varieties such as S-12 and CIM70. The native cotton species, mainly *Gossypium arboreum*, are immune to CLCuD. Although *G. arboreum* is cultivated commercially, this does not produce the high grade cotton lint desired by the processing industry. Although CLCuD remained endemic, losses due to the disease were reduced in both India and Pakistan during the 1990s by the gradual replacement of susceptible varieties with locally developed, tolerant and resistant cotton varieties. By the late 1990s, cotton production was again at record levels, exceeding the output achieved prior to the epidemic. However, in 2001, severe symptoms were noted on resistant cotton varieties signalling a second wave of CLCuD.

Aetiology of CLCuD

The causative agent of CLCuD is a begomovirus complex, consisting of a begomovirus and a satellite molecule belonging to the recently identified group of symptom modulating satellites known as DNA β. In addition, all plants infected with CLCuD were also shown to contain a third single-stranded DNA component known as DNA 1.

Begomoviruses associated with CLCuD

The Multan strain of CLCuD has been shown to be caused by one of at least six distinct begomovirus species (Cotton leaf curl Multan virus [CLCuMV], Cotton leaf curl Kokhran virus [CLCuKV], Papaya leaf curl virus, Tomato leaf curl Karnataka virus, Cotton leaf curl Alabad virus and Cotton leaf curl Rajastan virus). Many CLCuD affected cotton plants contain more than one of these viruses (Mansoor et al., 2003b).

In southern Indian another begomovirus species, Cotton leaf curl Bangalore virus, has been isolated from CLCuD affected *G. barbadense*. It is unlikely that this virus is involved in causing disease in the epidemic areas of northern Indian, although the precise geographic distribution of this virus has not been determined.

In northern Africa CLCuD has recently been shown to be caused by a begomovirus, Cotton leaf curl Gezira virus, which is distinct from those associated with CLCuD on the Indian subcontinent (Idris and Brown, 2002). This is typical of geminiviruses, where viruses in an area in different crops tend to be more closely related to each other than to viruses in the same crop from different areas.

The recently identified "Burewala strain" of CLCuD has yet to be fully characterised. However, the evidence suggests that, at this time, only a single begomovirus (a recombinant virus consisting of sequences derived from Cotton leaf curl Multan virus and Cotton leaf curl Khokhran virus), Cotton leaf curl Burewala virus, is associated with resistance breaking in cotton (manuscript in preparation). Indications are, however, that further viruses are already being drawn into this ongoing epidemic.

DNA1 associated with CLCuD

The DNA 1 components are a recently identified group of satellite-like molecules which are associated with the majority, but not all, monopartite DNA β requiring begomoviruses (Briddon et al., 2004; Mansoor et al., 1999). They are circular single-stranded DNA molecules that are approximately half the size (~1375 nucleotides) of their helper begomoviruses and contain a single gene that encodes a rolling circle replication initiator protein (referred to as a replication associated protein [Rep]; Fig. 2). The DNA 1 components are capable of autonomous replication in the cells of host plants. Hence they are defined as satellite-like; by definition satellites are capable of autonomous replication. They require the helper begomovirus for systemic movement in plants and insect transmission between plants.

Significantly, the DNA 1 components are closely related to satellite-like components associated with nanoviruses. Nanoviruses are a second family of plant-infecting, single-stranded DNA viruses (Gronenborn, 2004). They are multicomponent (6 to 8 components are thought to comprise each virus) and transmitted plant-to-plant by aphids in a circulative manner. One of the nanovirus components (known as component DNA-R) encodes the Rep (referred to as the master Rep) which is capable of (trans-)replicating all *bona fide* virus components (Timchenko et al., 2000). In addition, the majority of nanovirus isolates are also associated with additional Rep-encoding components which are not essential for the virus and can be viewed as parasites. DNA 1 components are closely related to these satellite-like nanovirus components from which they are believed to have evolved after component capture (exchange) following co-infection with a begomovirus. The major difference between DNA 1 components and nanovirus satellite-like components is the presence, in DNA 1, of a region of sequence rich in adenine. The requirement for this is thought to be to raise the size of a nanovirus components (typically 1000-1100 nucleotides) to that of half the size of a begomovirus (approx. 1400 nucleotides). Geminiviruses show a strict size selection for their components either for movement or encapsidation. Some evidence suggeststst that half size components (such as DNA 1) may be preferentially encapsidated in monomeric (rather than geminate) capsids (Frischmuth, Ringel, and Kocher, 2001).

DNA β associated with CLCuD

The DNA β molecules are a recently identified group of symptom modulating, single-stranded DNA satellites that are associated with monopartite begomoviruses and occur only in the Old World. Since their identification in 2000, over 200 full-length DNA β sequences have been deposited with the databases (Briddon and Stanley, 2006). They have a highly conserved structure, being approximately half the size of their helper begomoviruses (~1370 nucleotides), encoding a single gene (known as βC1), having an approx. 200 nucleotide region of sequence rich in adenine and a region of sequence approx. 100 nucleotides in length which is highly conserved between all DNA β molecules (known as the satellite conserved region [SCR]; Fig. 2).

All functions associated with DNA β have been shown to be mediated by the product of βC1. This gene encodes a protein which is the major pathogenicity determinant of the complex. Constitutive expression of CLCuD βC1 in *Nicotiana benthamiana* leads to grossly deformed plants exhibiting virus-like symptoms consisting of swollen veins and occasional enations (Saeed et al., 2004). Expression of the gene from a Potato virus X vector, in either *N. benthamiana* or *N. tabacum*, induces symptoms indistinguishable from CLCuD in these hosts, including vein swelling, vein darkening, enations and the characteristic leaf –like

outgrowths (Qazi et al., 2007). This indicates that βC1, in the absence of both the helper begomovirus and the DNA β backbone, is capable of inducing the full range of symptoms typical of CLCuD. In addition, the product of βC1 has been shown to suppress post-transcriptional gene silencing, bind DNA and possibly have a role in virus movement (Cui et al., 2005).

As well as βC1, DNA β components also contain two other prominent features. The A-rich region is highly conserved in position but not in sequence (other than having an abundance of adenine residues). Its function remains unclear, although it has been shown not to be required for trans-replication or movement by the helper begomovirus. By analogy to DNA 1, it is possible that the A-rich sequence is required to enlarge the component to half begomovirus size. This would suggest that DNA β was captured from another group of single-stranded DNA viruses. However, the only other family of plant-infecting, single stranded DNA viruses, the nanoviruses, have not thus far been shown to have a homolog of DNA β. Possibly the viruses from which DNA β originated have yet to be identified or are extinct. The SCR consists of approx 80 nucleotides of sequence highly conserved between all DNA β molecules. The region of sequence immediately upstream of this consists of blocks of conserved sequence interspersed with sequences that are less conserved. The SCR contains a predicted hairpin structure, containing the loop sequence TAATATTAC. Similar hairpin structures (with the loop sequence TAATATTAC for geminiviruses and TAT/GTATTAC for nanoviruses) form part of the origin (ori) of virion strand DNA replication for geminiviruses and nanoviruses (Hanley-Bowdoin et al., 1999). Additionally, the ori of geminiviruses contains repeated motifs (iterons) that are the sequence specific binding sites of the virus encoded Rep (a rolling circle replication initiator protein). However, DNA β molecules do not contain the cognate iteron sequences of their helper begomoviruses. The nature of and requirement for the SCR thus remains a mystery. Its position on the component, which is analogous to the area containing the iterons on geminiviruses, has led to the suggestion that it may be required for interaction with the helper begomovirus, although this remains to be confirmed.

The "Multan" strain of CLCuD was associated with only a single type of DNA β, despite being assisted by upwards of six distinct begomoviruses (Mansoor et al., 2003b). This finding suggested that distinct begomoviruses were being "captured" by CLCuD DNA β. Despite this, the symptoms induced were always the same, differing mainly by age of plant upon infection and cotton variety; providing further evidence of the overriding nature of DNA β in determining the symptoms of the disease. The newly identified resistance breaking "Burewala" strain of CLCuD is associated with a DNA β closely related to that of the "Multan" strain, containing the same βC1.

Resistance to CLCuD

Conventional host plant resistance

Limited resistance/tolerance is available in *G. hirsutum* germplasm. The cultivars LRA 511 and CP15/2 were the basis for the conventional resistance used to control CLCuD in Pakistan/India during the late 1990s (Rahman et al., 2005). Cultivation of these cultivars was successful in alleviating losses to CLCuD in endemic areas and during this period cotton production in Pakistan showed an upward trend. However, during 2001 leaf curl symptoms were noted on previously resistant cotton varieties. This was the first sign of a change in the prevalent CLCuD complex.

A further source of resistance to the Asian CLCuD complex is available in the form of *Gossypium arboreum*. This species is immune to CLCuD and is grown commercially on a small-scale. However, the cotton fibre it produces is not of a sufficient quality or quantity to make it a viable alternative to G. hirsutum. Although technically demanding, since *G. arboreum* is diploid whereas *G. hirsutum* is tetraploid, introgression of resistance from *G. arboreum* into *G. hirsutum* is underway at this time.

Breakdown of conventional resistance to CLCuD

Recently there has been a change in the prevalent strain of CLCuD across most cotton growing areas of Pakistan. During 2001, in the vicinity of the town of Burewala (Punjab, Pakistan), previously resistant cotton varieties began to exhibit the symptoms typical of CLCuD infection (Mansoor et al., 2003a). In subsequent years the affected area increased and has spread into neighbouring India.

Analysis of the resistance breaking strain of CLCuD is still in the early stages. Known as the "Burewala strain", this is a typical begomovirus-DNA β complex (although the presence of a DNA 1 component has not been confirmed). Only a single begomovirus has been identified with resistance breaking, which has since been named Cotton leaf curl Burewala virus (CLCuBV). CLCuBV is a recombinant virus consisting of sequences derived from the previously occurring CLCuMV and CLCuKV (L. Amrao, manuscript in preparation). The DNA β is also a recombinant. In this case the molecule consists, for the most part, of sequences derived from the original, "Multan strain", CLCuD DNA β. However, there is a small fragment of the SCR (approx. 80 nucleotides) derived from a DNA β first identified in tomato exhibiting tomato leaf curl disease symptoms (Amin et al., 2006). Although these components have been shown to be infectious to experimental hosts, it has thus far not been possible to infect resistant cotton varieties with clones of CLCuBV and the recombinant DNA β. Thus Koch's postulates have so far not been fulfilled and the precise molecular basis for resistance breaking remains to be identified.

Engineered resistance to CLCuD

Genetically engineered resistance to the CLCuD complex prevalent on the Indian subcontinent is under development. A major challenge to all forms of resistance to CLCuD is the diversity of viruses which cause the disease. It is essential to introduce a broad-spectrum resistance, which is effective against all the viruses present in the field, if the approach is to stand any chance of durability in the field. The strategies under investigation rely almost exclusively on post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS). These are homology dependent, RNA mediated phenomena which stimulate the plants own defences to target the invading virus. Since the one "target" present in all CLCuD-affected plants is CLCuD DNA β, initial studies attempted to induce PTGS/TGS against this molecule, with little success. More promising have been efforts that have targeted the Rep and AV2 genes; by antisense expression as either full-length (AV2) or truncated (Rep) coding sequences (Asad et al., 2003; Mubin et al., 2007; Sanjaya et al., 2005). Both these strategies are presently being assessed in cotton under field conditions. However, it remains to be seen whether the sequences being used provide a sufficiently broad-spectrum resistance to all the CLCuD-associated begomoviruses to be effective and durable under field conditions.

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Fig. 1. Typical symptoms of cotton leaf curl disease in cotton (*G. hirsutum*).

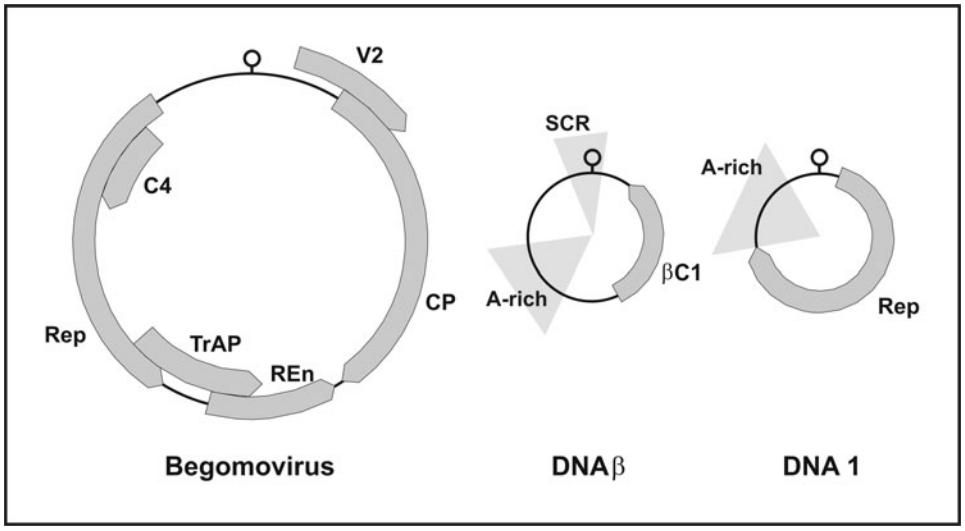

Fig. 2. Components of the cotton leaf curl disease begomovirus complex. Shown are the essential components, the begomovirus and the satellite (DNA β), and the non essential, satellite-like DNA 1. The position and orientation of genes is shown by arrows. The genes encode the replication-associated protein (Rep; a rolling circle replication initiator protein), the transcriptional activator protein (TrAP; involved in activation of late [virion-sense] genes), the replication enhancer protein (REn; involved in up regulating virl DNA replication), the coat protein (CP; involved in movement within and between plants by interaction with plant- and insect-enocded factors, respectively) and the pathogenicity determinant of the satellite known as βC1. The functions of the virus encoded V2 and C4 genes remain unclear. Sequence features (satellite conserved region [SCR] and adenine-rich sequence [A-rich]) are shown as slices.