Genome organization of *Tobacco leaf curl Zimbabwe virus*, a new, distinct monopartite begomovirus associated with subgenomic defective DNA molecules

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The complete DNA A of the begomovirus *Tobacco leaf curl Zimbabwe virus* (TbLCZWV) was sequenced: it comprises 2767 nucleotides with six major open reading frames encoding proteins with molecular masses greater than 9 kDa. Full-length TbLCZWV DNA A tandem dimers, cloned in binary vectors (pBin19 and pBI121) and transformed into *Agrobacterium tumefaciens*, were systemically infectious upon agroinoculation of tobacco and tomato. Efforts to identify a DNA B component were unsuccessful. These findings suggest that TbLCZWV is a new member of the monopartite group of begomoviruses. Phylogenetic analysis identified TbLCZWV as a distinct begomovirus with its closest relative being *Chayote mosaic virus*. Abutting primer PCR amplified ca. 1300 bp molecules, and cloning and sequencing of two of these molecules revealed them to be subgenomic defective DNA molecules originating from TbLCZWV DNA A. Variable symptom severity associated with tobacco leaf curl disease and TbLCZWV is discussed.

**Introduction**

Geminiviruses belong to an economically important family of plant viruses that are characterized by having small circular single-stranded DNA (ssDNA) genomes, encapsidated in twinned icosahedral particles (Lazarowitz, 1992). The *Geminiviridae* family consists of four genera that differ with respect to insect vector, host range and genome structure (Fauquet et al., 2000). The genus *Begomovirus* represents the whitefly-transmitted, dicot-infecting geminiviruses that most commonly have bipartite genomes consisting of two ssDNA components, designated DNA A and DNA B. Some begomoviruses, however, have a single genomic component (Kheyr-Pour et al., 1991; Navot et al., 1991; Dry et al., 1993; Noris et al., 1994), which contains all functions necessary for replication and movement in the plant. Defective circular single-stranded DNA molecules about half the size of the genomic DNA have been detected in plants infected with begomoviruses (Stanley & Townsend, 1985; MacDowell et al., 1986; Czosneck et al., 1989; Stanley et al., 1997; Liu et al., 1998; Sharma et al., 1998), and have been shown to delay symptom development and to ameliorate disease symptoms (Stanley et al., 1990, 1997; Frischmuth & Stanley, 1991).

A geminivirus was first reported to be the causative agent of tobacco leaf curl disease in Japan in 1981 (Osaki & Inouye, 1981). In Southern Africa, at least three symptom phenotypes in tobacco have been identified that could be classified as tobacco leaf curl (Paximadis & Rey, 1997). One of these symptom phenotypes has been attributed to a phytoreovirus (Paximadis et al., 1997; Rey et al., 1999) and the other to a begomovirus, *Tobacco leaf curl Zimbabwe virus* (TbLCZWV) (Paximadis & Rey, 1997; Paximadis et al., 1999). Four plants, showing leaf curl symptom severity variation, associated with begomovirus infections in Zimbabwe (Paximadis & Rey, 1997), were phylogenetically compared using coat protein gene (CP) and the common region (CR) nucleotide sequences. Isolates formed a distinct cluster and were ≥ 98% similar in their CP and CR sequences (Paximadis et al., 1999). The present research characterizes TbLCZWV at a molecular level and reports, for the first time, defective DNA molecules associated with TbLCZWV.

Three pairs of overlapping degenerate primers [AV494 and AC1048 (Wyatt & Brown, 1996); PAL1c1960 and PAR1v722 (Paximadis & Rey, 1997); prAV1134 (Idris & Brown, 1988) and PAR1c715 (5’GAT TTC TGC AGT TDA TRT TYT CRT CCA TCC A 3’); D. P. Maxwell, Univ. of Wisconsin, Madison, USA], designed to amplify regions of the DNA A components of most begomoviruses, were employed in PCR to amplify the DNA A from total DNA extracted (Doyle & Doyle, 1987).
from TbLCZWV-infected tobacco. PCR fragments were blunt-end cloned into the pBluescript (KS) vector (Stratagene) and three clones of each were sequenced automatically (ABI Prism 310) to obtain the complete TbLCZWV DNA A. TbLCZWV DNA A was phylogenetically compared to the DNA A sequences of other geminiviruses (GenBank) using the DNAMAN version 4.0 (Lynnon Biosoft, Quebec, Canada) full optimal alignment and neighbour-joining method options with 1000 bootstrap replications. Open reading frames (ORFs) of TbLCZWV DNA A were identified using DNAMAN, and percentage DNA A nucleotide and ORF amino acid sequence identities between virus isolates were calculated using the distances between all pairs of sequences in the multiple alignments.

Sequencing of cloned PCR fragments spanning the complete TbLCZWV DNA A revealed it to be 2767 nucleotides in
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Fig. 2. (a) Agarose gel electrophoresis of products obtained from abutting primer PCR. Lane 1, PstI-cut λ DNA molecular mass markers (selected sizes in bp are indicated). Lanes 2 and 3, PCR of ‘Mild’ (Paximadis & Rey, 1997) TbLCZWV total DNA at 0–5 and 1 µg respectively. Lanes 4 and 5, PCR of ‘Severe’ (Paximadis & Rey, 1997) TbLCZWV total DNA at 0–5 and 1 µg respectively. Lanes 6 and 7, PCR of ‘HG’ (Paximadis & Rey, 1997) TbLCZWV total DNA at 0–5 and 1 µg respectively. Note the ca. 2700 bp (full-length) and ca. 1300 bp fragments amplified (indicated by arrows).

(b) Genome organization of TbLCZWV ‘HG’ defective DNA (GenBank accession number: AF368275). (c) Genome organization of TbLCZWV ‘Mild’ defective DNA (GenBank accession number: AF368274). Arrows in (a) and (b) represent complete ORFs; solid bars indicate incomplete ORFs (percentage of the ORF retained is indicated on the figure); IR, intergenic region.

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size, exhibiting the typical genome organization of begomoviruses originating from the Old World, with six conserved ORFs (Fig. 1 a, b). Phylogenetic analysis of TbLCZWV DNA A placed TbLCZWV in the Old World African and Mediterranean cluster (Fig. 1 c), with the highest DNA A sequence identity (78.4%) being shared with Chayote mosaic virus (ChaMV), a cucurbit-infecting begomovirus from Nigeria, reported in GenBank as having a single genomic component. TbLCZWV also clustered with ChaMV in the DNA A relationship dendrogram (Fig. 1 c), sharing high amino acid sequence identity with this virus in the CP (95.3%) and C3 (77.4%) ORFs. The high CP amino acid sequence identity shared between ChaMV and TbLCZWV might suggest that these are strains of the same virus; however, the unique intergenic region (IR) putative iterons of TbLCZWV (Paximadis et al., 1999) and < 90% DNA A sequence identity shared with ChaMV demonstrate that TbLCZWV is a distinct species within the genus Begomovirus. Begomoviruses sharing < 90% DNA A sequence identity are considered to be distinct species (Rybicki et al., 2000). In both the DNA A and ORF amino acid comparisons, TbLCZWV generally showed high sequence identity with the monopartite Mediterranean tomato yellow leaf curl begomoviruses and with South African cassava mosaic virus (Berrie et al., 2001).

Three strategies were employed to extensively search for a DNA B component in TbLCZWV-infected tobacco material. The first employed PCR and non-overlapping abutting primers [pIRc2671 (5‘ GGG TAC CGA TAT ACC AGG AG 3‘) and pIRv2672 (5‘ AAT ATA TAG TGG GTA CCG AAT GG 3‘)], designed in the IR of TbLCZWV DNA A, a region that includes the TATA box and two putative iterons identified upstream and downstream of the TATA box (Paximadis et al., 1999). Full-length PCR fragments (Fig. 2 a) were blunt-end cloned into the pMOSBlue cloning vector (Amersham Pharmacia) and PCR was used to screen clones for their full-length nature (abutting primers pIRc2671 and pIRv2672) and their DNA A or B status using DNA A-specific primers (AV494 and AC1048). Fragments of ca.1300 bp, also amplified in the abutting primer PCR (Fig. 2 a), were blunt-end cloned into pBluescript (KS) and sequenced (ABI Prism 310). The second strategy employed PCR to attempt to amplify a DNA B-specific fragment using three degenerate DNA B-specific primers [prBV1855 (Idris & Brown, 1998); pBL1v2040 (Rojas et al., 1993); pBR1c800 (5‘ ACG ACT GCA GTT VAC MGT CCC TTT GAA ACG 3‘); D. P. Maxwell], and two CR-specific primers [PCRc1 (Rojas et al., 1993); PCRc4 (5‘ GGC CAT AGA GCT TTG AGG ATC CCG ATT CAT TTC 3‘); D. P. Maxwell], in all possible combinations. In addition, abutting

Fig. 2. (a) Agarose gel electrophoresis of products obtained from abutting primer PCR. Lane 1, PstI-cut λ DNA molecular mass markers (selected sizes in bp are indicated). Lanes 2 and 3, PCR of ‘Mild’ (Paximadis & Rey, 1997) TbLCZWV total DNA at 0–5 and 1 µg respectively. Lanes 4 and 5, PCR of ‘Severe’ (Paximadis & Rey, 1997) TbLCZWV total DNA at 0–5 and 1 µg respectively. Lanes 6 and 7, PCR of ‘HG’ (Paximadis & Rey, 1997) TbLCZWV total DNA at 0–5 and 1 µg respectively. Note the ca. 2700 bp (full-length) and ca. 1300 bp fragments amplified (indicated by arrows).

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Fig. 3. Symptoms seen on tobacco and tomato following agroinoculation with TbcZWV DNA A. (a) *N. benthamiana* (100% infection 2 weeks post-inoculation) exhibiting stunting, severe upward leaf curling, leaf distortion, ventral vein thickenings and bending of petioles. (b) *N. tabacum* cv. Samsun (100% infection 4 weeks post-inoculation), showing extensive puckering of
primers (pIRc2671 and pIRv2672) were used in combination with the DNA B-specific primers. Lastly, restriction enzyme analysis of TbLCZWV DNA A identified enzymes with single (BamHI, SalI) and with no restriction sites (EcoRV, HindIII, KspI, NcoI, NotI, SacI, Smal and XbaI). Supercoiled replicative viral dsDNA (scDNA), presumably a mixture of DNA A and B, purified from total DNA, was digested with these ten restriction enzymes, electrophoresed on agarose gels and Southern blotted onto nylon membranes (Sambrook et al., 1989). Blots were probed with a digoxigenin (DIG)-labelled (Roche Molecular Biochemicals) 437 bp CR-harbouring probe. Additional bands, not corresponding to the expected restriction pattern for DNA A, would be considered to be of possible DNA B origin.

The three strategies employed to search for a DNA B component all failed to indicate a second genomic component associated with TbLCZWV, suggesting TbLCZWV to be a monopartite begomovirus. The search for a DNA B component using IR abutting primers unexpectedly revealed the presence of smaller molecules approximately half the size of the full-length DNA A (Fig. 2a) that proved, upon sequencing, to be subgenomic defective DNA molecules of DNA A origin (Fig. 2b, c). Subgenomic defective DNA molecules, associated with a number of begomoviruses (Stanley & Townsend, 1985; MacDowell et al., 1986; Czosnek et al., 1989; Stanley et al., 1997; Liu et al., 1998; Sharma et al., 1998), seem to be fairly uniform in structure, i.e. in addition to the IR they tend to retain a large portion (5’ end) of the BC1 or C1 ORFs, with a large portion of the BV1 or CP and remaining ORFs deleted. Occasionally they have sequences of unknown origin inserted between the truncated BV1 or C1 and the remaining begomovirus-specific sequence. Similar structures were observed with the defective molecules associated with TbLCZWV in this study (Fig. 2b, c). The interfering role of the TbLCZWV defective DNA molecules has not, however, been established experimentally.

Although the failure to detect a DNA B component strongly suggested that TbLCZWV is a monopartite begomovirus, the ability of the single genomic component to move and cause disease symptoms in its original host (Nicotiana tabacum) had to be shown to unequivocally prove the monopartite nature of this virus. A full-length DNA A genomic clone (pBSZW-A) was prepared by linearizing TbLCZWV scDNA with BamHI, and cloning into the BamHI site of pBluescript (KS). A full-length head-to-tail dimer of the genomic DNA A was constructed in pBluescript (KS) and subcloned into plant transformation vectors pBin19 (Bevan, 1984) and pBI121 (Clontech), yielding clones pBinZW-DA and pBIZW-DA respectively. Agrobacterium tumefaciens C58C1 RifR (pMP90 GmR) (Koncz & Schell, 1986), transformed with pBinZW-DA and pBIZW-DA using the freeze-thaw method of Holsters et al. (1978), was used to agroinoculate Nicotiana benthamiana and two cultivars of N. tabacum, namely Samsun and HG (a Zimbabwean cultivar). Lycopersicon esculentum cv. Moneymaker was also agroinoculated with A. tumefaciens C58C1 RifR (pMP90 GmR) transformed with pBIZW-DA. Exponential-phase recombinant Agrobacterium was pelleted, washed with sterile water and resuspended in 200 μl of Luria broth per ml of original culture. A 30 gauge needle was used to transfer A. tumefaciens cultures to plants by injecting the stems and prickmg the leaves of seedlings at the four to six leaf stage. Plants were monitored for symptoms. All four hosts developed leaf curl symptoms similar to those noted in the field samples (Fig. 3a–f), and both plant transformation vectors used were equally infectious. Southern hybridizations (using a DIG-labelled full-length TbLCZWV DNA A probe) verified the presence of both double-stranded and single-stranded forms of TbLCZWV-specific DNA in the agroinoculated plants, and PCR, using TbLCZWV-specific abutting primers, amplified expected full-length fragments, as well as ca.1300 bp fragments (only in tobacco), thought to be defective DNA molecules.

A perplexing feature of tobacco leaf curl since its earliest reports has been the observed variation in symptom severity (Storey, 1932; McClean, 1940). The begomovirus-infected tobacco plants used in the current study also showed variation in symptom severity ranging from mild to severe (Paximadis & Rey, 1997), and a variation of symptoms was also seen following agroinoculation of the cloned TbLCZWV DNA A, with N. tabacum cv. Samsun exhibiting symptoms that closely resemble the mild form B described by McClean (1940) and N. tabacum cv. HG exhibiting symptoms more typical of the mild form C (McClean, 1940). The severe form of the disease was not seen in N. tabacum, but N. benthamiana did exhibit severe leaf curl symptoms. Although the full-length genomic DNA A used for the agroinoculations was derived from a field tobacco plant exhibiting mild symptoms in N. tabacum, we have previously shown, using CP and CR nucleotide sequence comparisons between the four isolates (Paximadis et al., 1999), that different viral strains or viruses are unlikely to be found in dorsal leaf surfaces. (c) N. tabacum cv. Samsun showing leaf margins of newly developing leaves curling upwards. (d) N. tabacum cv. Samsun ventral leaf surfaces of healthy (H) and infected (I) plants, showing vein thickenings present on smaller veins of the infected plants that are absent in the healthy plants. (e) L. esculentum cv. Moneymaker (92% infection 2 weeks post-inoculation) showing mild yellow mottling of dorsal leaf surface. (f) L. esculentum cv. Moneymaker ventral leaf surfaces of healthy (H) and infected (I) plants, showing vein thickenings present on smaller veins of the infected plants that are absent in the healthy plants. N. tabacum cv. HG (not shown in figure) developed symptoms 8 weeks post-inoculation (only 43% infection) and symptoms were essentially identical to N. tabacum cv. Samsun, only considerably milder.
the four plants. The discovery of defective DNA molecules in leaf curl-affected tobacco plants may possibly explain the variable symptom severity. It was noted that the defective DNAs from two different plants (‘Mild’ and ‘HG’; Paximadis & Rey, 1997) were different in structure (Fig. 2b, c), and since defective DNAs are known to cause symptom amelioration, it could be hypothesized that the defective DNAs may be playing a role in symptom modifications. Both Storey (1932) and McClean (1940) suggested that environmental factors contributed to symptom severity. One cannot therefore exclude the possibility that the environmental stresses experienced by an individual plant, the type of cultivar and defective DNAs present may all play a role in the severity of the symptoms displayed. One also cannot rule out the possibility however that other unidentified factors, like a satellite virus similar to DNA β associated with Ageratum yellow vein virus (Saunders et al., 2000) and Cotton leaf curl virus (Briddon et al., 2001), may be contributing to symptom severity.

In conclusion, TblLCZWV is a distinct, previously uncharacterized new member of the monopartite begomoviruses, associated with subgenomic defective DNA molecules that originate from its single genomic component. A monopartite genome organization has also been reported for Tobacco leaf curl Japan virus (Shimizu & Ikegami, 1999) and Tobacco leaf curl China virus (GenBank); however, these viruses are phylogenetically distantly related to TblLCZWV (Fig. 1c) and furthermore, the infectious nature of their single genomic components has not been reported.

References


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