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Comparison of a beet curly top virus isolate originating from the old world with those from the new world

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Abstract

The complete nucleotide sequence of an infectious, insect-transmissible clone of a beet curly top virus isolate originating from Iran (BCTV-I) has been determined. The nucleotide sequence of BCTV-I shows high levels of similarity to the sequences of BCTV strains isolated from North America, and is nearly identical to the CFH strain of BCTV. The symptoms produced by BCTV-I in *Nicotiana benthamiana* and *Beta vulgaris* most closely resemble those of the CFH strain and are distinct from the other isolates. The significance of these findings with respect to the possible geographic origins and evolution of BCTV are discussed.

Introduction

Geminiviruses typically have single-stranded DNA genomes which are encapsidated in characteristic twinned geminate particles. These viruses are transmitted in nature by insects in a circulative manner. The family Geminiviridae is presently divided into three genera (Subgroups I, II and III) based upon such factors as host range, insect vector and genome arrangement (Briddon and Markham, 1995). Members of the genus Subgroup I infect either dicots or monocots, are leafhopper-transmitted and have a typical genome arrangement with two open reading frames on each strand. Geminiviruses of Subgroup III have either monopartite or bipartite genomes, infect only dicotyledonous plant species and are transmitted by the whitefly *Bemisia tabaci* (Genn.). The genus Subgroup II consists of viruses in which recombination has been suggested to have played a part in their evolution (Stanley et al., 1986; Briddon et al., 1996; Klute et al., 1996). Viruses of subgroup II have monopartite genomes and infect only dicots.

The type member of Subgroup II, beet curly top virus (BCTV), is a distinct member of the geminivirus family possessing features associated with both of the other genera. The genes encoded on the complementary-sense DNA strand of the BCTV genome are homologous to those of the DNA A component of the bipartite whitefly-transmitted (Subgroup III) geminiviruses. The coat protein gene, however, shows much closer similarity to those of the other leafhopper-transmitted geminiviruses (Subgroup I), providing strong evidence that for geminiviruses the coat protein is involved in vector specific interactions.

Beet curly top virus has by far the largest host range of any of the characterized geminiviruses, covering some 300 species in 44 plant families [Bennett, 1971). Despite efforts to control the virus by breeding for resistance and by combatting its insect vector, the leafhopper *Circulifer tenellus* (Baker), with insecticides BCTV continues to cause losses in a number of commercially grown crops including sugarbeet and tomatoes (Stenger, 1995; Creamer, Luque-Williams and Howo, 1996; Stenger and McMahon, 1997).

In addition to North America, BCTV has also been reported from countries bordering the Mediterranean and from the Middle East, where the virus causes minor losses in sugarbeet crops (Bennett and Tanrisever, 1957; Monsef and Kheyri, 1992; Abdel-Salam, 1990; Abdel-Salam and Amin, 1990). Initial serological comparisons of the American and Middle Eastern isolates suggested that they were closely related and raised the question of their geographical origin (Oman, 1948; Bennett and Tanrisever, 1957; Bennett, 1971). The following study was initiated to establish the precise relationship of several BCTV isolates from the New World with one from the Old World.

Materials and methods

Origin and maintenance of virus isolate and insect vector

BCTV-infected sugarbeet (*Beta vulgaris*) was collected in Iran in 1986. The virus isolate was maintained by insect transmission in *B. vulgaris* cv. Giant Western. A colony of *C. tenellus*, originating from California (kindly provided by Dr. A.M. Purcell), was maintained on *B. vulgaris* cv. Giant Western in perspex cages at 28 °C with a 16 hour daylength.

The isolate of BCTV obtained from Iran (BCTV-I) is compared to a number of BCTV isolates from the United States of America. These isolates are BCTV-California (BCTV-Cal.; Stanley et al., 1986), BCTV-CFH (Stenger, 1994), BCTV-Logan (S.G. Hormuzdi and D.M. Bisaro, unpublished, as referenced in Hormuzdi and Bisaro 1993), BCTV-Worland (Stenger and Ostrow, 1996).

Cloning, infectivity and insect transmission

BCTV supercoiled DNA (scDNA) was isolated from infected sugarbeet leaf tissue, as previously described for African cassava mosaic virus (previously known as cassava latent virus; Stanley and Townsend, 1985). Leaf tissue was harvested from sugarbeet plants collected in Iran and maintained in insect-proof glasshouses in the UK. Samples of scDNA were digested with various restriction endonucleases and analysed by agarose gel electrophoresis to determine single cutting sites suitable for cloning the full-length genome. An aliquot of scDNA, linearized by digestion with *Sph*I, was cloned into the bacteriophage vector M13mp18 (Norrander, Kempe and Messing, 1983). A single, potentially full-length, clone (pBCI001; isolated during 1987), was chosen for further analysis.

A partial repeat of pBCI001 was constructed by cloning an approximately 1000bp *Kpn*I-*Sph*I fragment into pUC1318 (Kay, 1987), and subsequently cloning the full-length insert into the unique *Sph*I site of this construct. The partial repeat of the pBCI001 insert was then transferred to the binary vectors pMON521 (Rogers et al., 1987) or pBin19 (Bevan, 1984) as an *Sst*I fragment. The binary vector construct was introduced into *Agrobacterium tumefaciens* strain GV3111 (Rogers et al., 1987) or C58^{nal} (Hepburn et al., 1985) and used to inoculate plants as described previously (Stenger et al., 1991; Briddon et al., 1989). The construction of partial repeats of the full-length clones of BCTV strains CFH, Worland and Logan have been described previously (Stenger et al., 1991; 1994).

The latent period (time between inoculation of seedlings and appearance of symptoms) for BCTV-I, BCTV-Logan and BCTV-CFH were determined following *Agrobacterium*-mediated inoculation of *Nicotiana benthamiana* plants.

Plant-to-plant transmission assays were carried out by caging approximately 20 *C. tenellus* insects on infected *B. vulgaris* plants for 48 h. Insects were then transferred singly to healthy *B. vulgaris*, *Nicotiana tabacum* cv. Samsun and *Lycopersicon esculentum* cv. Kondine Red test seedlings. Insects were given an inoculation access period of approximately three days before they were removed and the plants were sprayed with organophosphorous insecticide. Test plants were maintained in insect-proof glasshouses and monitored daily for symptoms of virus infection.

Determination and analysis of nucleotide sequence

The nucleotide sequence of clone pBCI001 was determined by dideoxynucleotide chain termination sequencing (Sanger, Nicklen and Coulson, 1977) using Sequenase Version II (USB) and (α-^{35}S)dATP (New England Nuclear). Restriction fragments of clone pBCI001 were subcloned into bacteriophage vectors M13mp18 and mp19 for sequence analysis. Regions of the clone lacking suitable restriction sites were sequenced using specific oligonucleotides. Sequencing products were resolved by electrophoresis in denaturing buffer gradient acrylamide gels (Biggin et al., 1983).

Sequence information was stored, assembled and analysed using Version 7 of the program library of the Genetics Computer Group (Devereaux, Haeber-

Table 1. Predicted open reading frames of BCTV-I

ORF	Start	Stop	No. of amino acids	Predicted M_r
V1(CP)	563	1324	254	29.5
V2	343	720	126	15.0
V3	270	530	87	9.8
C1	2836	1778	353	40.2
C2	1983	1465	173	19.5
C3	1754	1346	136	16.0
C4	2836	2437	87	9.8

li and Smithies, 1984). Phylogenetic analyses were conducted on matrices of aligned sequences using the neighbor-joining and bootstrap options of Phylip version 3.5c (J. Felsenstein, Dept. of Genetics, University of Seattle, Washington, USA) running on an IBM compatible personal computer. Sequence alignments were produced using CLUSTAL V (Higgins et al., 1992) running on a VAX mainframe computer.

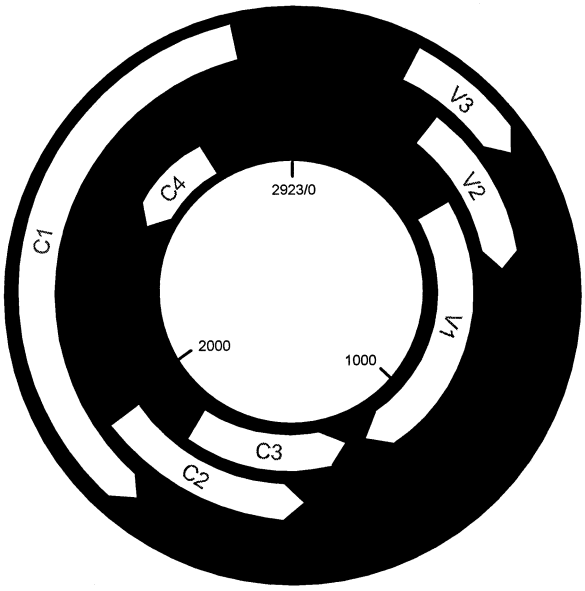

Figure 1. Genome map of BCTV-Iran. Arrows denote the positions and orientation of ORFs with the potential to encode proteins above M_r 9000.

Results

Sequence analysis

The complete nucleotide sequence of both strands of clone pBCI001 were determined. The cloned viral DNA consists of 2923 nucleotides. The sequence is available in the DDJB, EMBL and GenBank nucleotide sequence databases under Accession No. X97203. Nucleotide numbering proceeds from the 3′ A in the conserved sequence TAATATTAC, which forms the nick site of the Rep protein within the origin for virion-strand DNA replication (Laufs et al., 1995).

Both the virion and complementary sense sequences were screened for the presence of open reading frames (ORFs). Seven ORFs with a capacity to encode proteins of predicted molecular mass greater than 9kDa were detected (Table 1) and these are numbered according to the convention of Davies and Stanley (1989). Under this system the coat protein is designated V1 and upstream virion-sense genes are numbered in reverse order. The position and orientation of ORFs on the genome of BCTV-I are shown in Figure 1, starting from the first in-frame methionine codon. No homologue of the V4 (also known as R4) ORF, unique to the BCTV-CFH clone (Stenger,1994), was detected in BCTV-I.

Infectivity, symptoms and insect transmission

Table 2 shows a comparison of the infectivity and mean latent periods of BCTV-I in comparison to BCTV-CFH and -Logan. Clones of all three viruses are infectious in *N. benthamiana* and *B. vulgaris*. However, the latent period between inoculation and appearance of symptoms for BCTV-I in *N. benthamiana* was typical of BCTV-CFH (9 days) rather than BCTV-Logan (18 days).

Symptoms of BCTV-I in both *N. benthamiana* and *B. vulgaris* were typical of BCTV, but overall showed more similarity to BCTV-CFH than symptoms induced by clones of the other BCTV isolates. In *N. benthamiana*, initial symptoms of BCTV-I infection were typically vein clearing, followed by leaf distortion and upward curling of the leaves. Plants were severely stunted. For BCTV-Logan and -Worland, symptoms developed more slowly and vein clearing was much less pronounced. BCTV-Logan induced upward curling of the leaves and leaf distortion, but also induced more generalized yellowing of the leaves in older infections. In contrast, BCTV-Worland tended to induce more downward curling of the leaves. BCTV-I, -CFH and -Logan induced severe symptoms in *B. vulgaris* with initial vein clearing followed by vein swelling, enations, thickening of the leaves and stunted plant

Table 2. Infectivity and latency of BCTV strains following *Agrobacterium*-mediated inoculation

Strain	Infectivity (plants infected/plants inoculated)				Latency[1]	
	N. benthamiana		*B. vulgaris*		(mean days ± SD)	
	exp. 1	exp. 2	exp. 1	exp.2	exp. 1	exp. 2
Iran	8/8	8/8	10/10	10/10	$9.0^a \pm 1.5$	$9.3^a \pm 1.8$
CFH	8/8	8/8	10/10	10/10	$10.1^a \pm 2.2$	$9.4^a \pm 2.5$
Logan	7/8	8/8	10/10	10/10	$18.7^b \pm 5.2$	$18.3^b \pm 3.5$
Mock	0/8	0/8	0/10	0/10	-	-

[1]Determined in *N. benthamiana*.
Means with different letters were significantly different in ANOVA ($P<0.0001$) and Newman-Kuels multiple range ($P<0.01$) tests (Zar, 1970).

growth. The symptoms of BCTV-Worland in *B. vulgaris* were as described for the other isolates but much milder with less pronounced vein swelling, enations and leaf thickening leading to less stunted plants.

In single insect transmission assays from *B. vulgaris*, infected with BCTV-I by agroinoculation, six out of 20 *B. vulgaris*, two out of four *N. tabacum* (cv. Samsun) and two out of four *Lycopersicon esculentum* (cv. Kondine Red) test plants became infected with BCTV-I by transmission using *C. tenellus*. Symptoms of infection typically appeared within 10 days of inoculation.

Comparison to other BCTV isolates

The levels of conservation of amino acid sequence between the predicted ORFs of BCTV-I and their counterparts in the other Subgroup II geminiviruses are shown in Table 3. In addition, Figure 2 shows pairwise dot-matrix comparisons of the nucleotide sequences of the genomes of BCTV isolates. These comparisons show the genome of BCTV-I to be nearly identical to that of BCTV-CFH. The sequence of the other BCTV isolates differ significantly from those of BCTV-I and -CFH, with the majority of the variation occurring in the intergenic and complementary-sense coding regions. For the virion-sense genes, the amino acid sequence similarity between BCTV-I and the other BCTV isolates was above 96%. For the complementary-sense genes the similarity drops to less than 89% to the non-CFH strains but remains high (over 98%) to CFH. The most variable gene amongst the BCTV isolates is C4, which has been shown to play a major role in the pathogenicity of BCTV-Cal (Stanley and Latham, 1992). The overall nucleotide sequence similarity between BCTV-I and BCTV-CFH, -Worland, -Logan, -Cal, horseradish curly top virus (HRCTV; Klute et al., 1996) was 98.3%, 83%, 82%, 82% and 63% respectively.

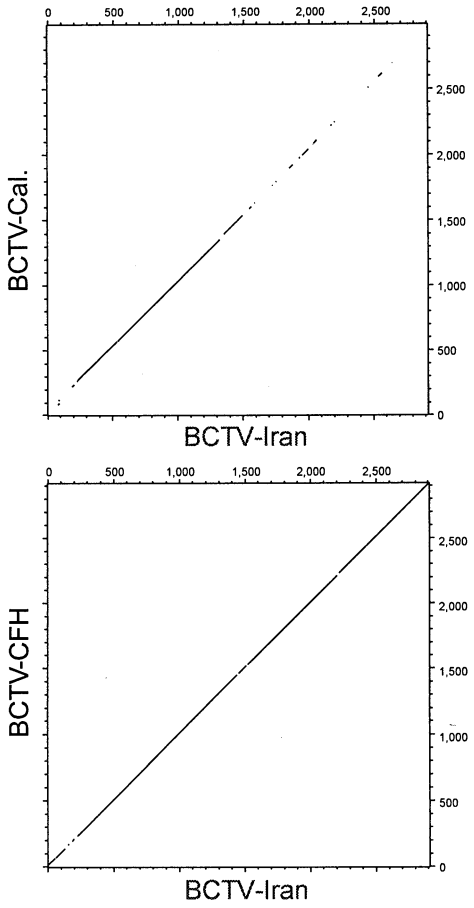

Figure 2. Dot matrix comparisons of the sequence of BCTV-I to those of BCTV-Cal. (A) and BCTV-CFH (B). Comparisons used a window size of 21 and a stringency of 19.

Phylogenetic analyses of the predicted amino acid sequences of the coat protein, Rep protein and C4 pro-

Table 3. Percentage amino acid sequence similarities (identities) between predicted gene products of BCTV-I and other Subgroup II geminiviruses

Virus	Open Reading Frame						
	V1	V2	V3	C1	C2	C3	C4
BCTV-Cal.	98.0	96.1	97.7	81.9	82.7	88.2	54.0
	(97.2)	(93.1)	(97.7)	(70.3)	(70.5)	(80.8)	(46.0)
BCTV-Worland	98.4	98.4	98.9	87.8	77.4	75.7	57.6
	(98.4)	(95.2)	(98.9)	(79.6)	(61.4)	(67.6)	(49.4)
BCTV-Logan	98.4	96.1	97.7	82.7	73.0	89.0	54.0
	(98.0)	(93.1)	(97.1)	(70.5)	(62.4)	(81.6)	(46.0)
BCTV-CFH	99.2	97.6	100.0	99.7	98.8	98.5	98.9
	(99.2)	(96.0)	(100.0)	(98.6)	(98.8)	(97.8)	(98.9)
HRCTV	88.5	76.8	85.1	68.8	39.1	–*	42.2
	(80.6)	(65.6)	(72.4)	(54.1)	(20.3)		(24.1)
TPCTV	45.4	28.2	–*	79.3	44.8	60.3	51.8
	(21.8)	(16.3)		(67.6)	(20.9)	(35.9)	(43.5)

*HRCTV and TPCTV lack the C3 and V3 ORF, respectively.

tein of selected geminiviruses are shown in Figure 3. The coat proteins of all the BCTV isolates are closely related with very short mutation distances. Although transmitted by the same leafhopper vector as BCTV, the coat protein of HRCTV has diverged from the BCTV isolates but is still most closely related to them. For the tree inferred from the Rep protein sequence, the BCTV isolates -I, -CFH and -Worland are distinct from -Cal and -Logan, with BCTV-I showing the closest relationship to -CFH. The unexpected behaviours of tobacco yellow dwarf virus (TobYDV), HRCTV and tomato pseudo-curly top virus (TPCTV) in these comparisons have been noted previously (Morris et al., 1992; Klute et al., 1996; Briddon et al., 1996). The C4 gene sequences yielded trees in which BCTV-I, CFH and Worland co-segregate and show a closer relationship to the C4 gene of ACMV (for which no function has thus far been ascribed) than they do to BCTV-Cal and -Logan. In this comparison, HRCTV segregates with a Subgroup III geminivirus; this has been noted previously (Klute et al., 1996). For phylogenetic trees derived from other gene sequences, BCTV-I always clustered with and showed the closest relationship to BCTV-CFH (results not shown). The divergence of HRCTV from the other *C. tenellus*-transmitted viruses is highlighted in all three trees.

It is also interesting to note that both BCTV-I and -CFH have the shortest genomes (2923nt and 2927nt respectively) amongst the leafhopper-transmitted curly top viruses. This is followed by the next most related virus, BCTV-Worland, at 2930nt. The less related curly top viruses have considerably longer genomes at 2993nt, 3038nt and 3080nt for BCTV-Cal., BCTV-Logan and HRCTV, respectively.

Discussion

Beet curly top virus has received much attention due to the novel symptoms it produces in plants and its continuing economic impact. First reported in the USA in the late 1800s, four isolates of the virus from the United States have now been characterized at the nucleotide sequence level. In addition, the nucleotide sequence of a related virus species, HRCTV, which shares BCTV's leafhopper vector, has recently been determined (Klute et al., 1996). Phylogenetic comparisons of these sequences suggests that these viruses may represent four distinct species; namely HRCTV, BCTV-Logan/Cal, BCTV-Worland and BCTV-CFH (Stenger and Ostrow, 1996; Klute et al., 1996). Analyses conducted by Stenger and McMahon (1997) have shown that BCTV-CFH and -Worland are the predominant strains in the western USA at this time.

Our analysis of the nucleotide sequence of the cloned genomic component of a BCTV isolate originating from Iran clearly demonstrates that one of the strains of the virus currently present in the United States also occurs in the Middle East. This conclusively proves the contention, as first proposed by Bennett and Tanrisever (1957), that the American and Middle Eastern viruses have a common origin. Howev-

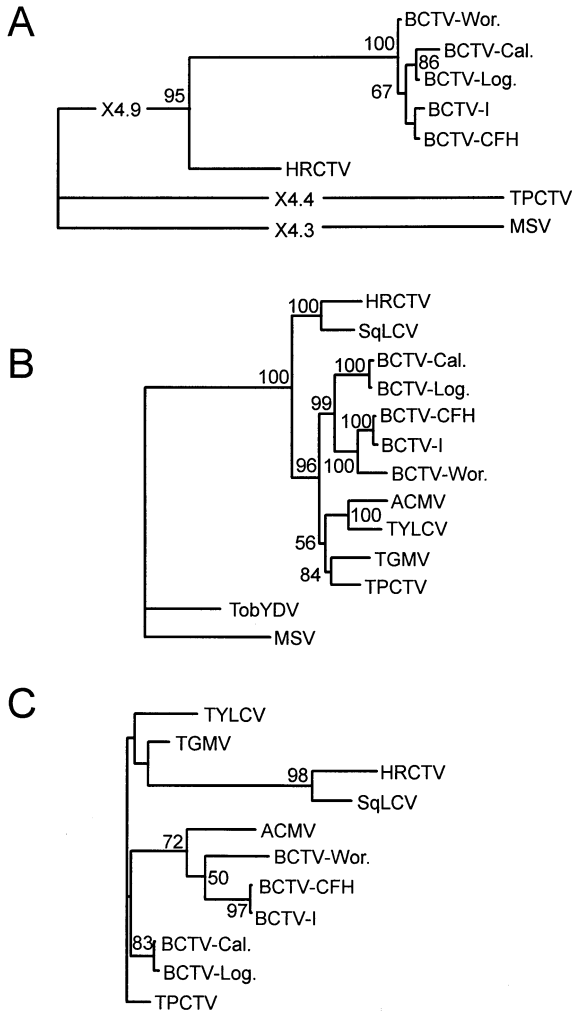

Figure 3. Phylogenetic dendrograms depicting topology and distance relationships between BCTV-Iran, other subgroup II and selected geminiviruses for the predicted amino acid sequences of the coat protein (A), C1 ORF (B) and the C4 ORF (C). Trees were determined by neighbour-joining option of the Phylip package. Vertical distances are arbitrary. Horizontal branches are proportional to calculated mutation distances, unless otherwise indicated. Numbers at nodes indicate percentage bootstrap scores (if $\geq$ 50%).

er, these results do not provide evidence as to their original geographic origin. It is now clear that BCTV has been moved either from America to the Middle East/Mediterranean or vice versa. The evidence in favour of an Old World origin for this virus includes the apparent resistance of some wild beet species to BCTV in the Middle East and Mediterranean (Bennet, 1971). In addition, the genetic diversity of both *Circulifer* spp. and beet suggest a Middle Eastern origin. The interbreeding experiments conducted by Freitag et al. (1955) provided convincing evidence that Old and New World *C. tenellus* populations are of the same species, despite some differences in taxonomic characters (Oman, 1970).

Stanley et al. (1986) suggested that BCTV may have arisen by recombination between a whitefly-transmitted geminivirus (donating the complementary-sense genes involved in replication) and a Subgroup I leafhopper-transmitted geminivirus (donating the coat protein); although it is possible that Subgroup III geminiviruses evolved from a Subgroup II progenitor. Certainly the coat protein of BCTV shows far more similarity to other leafhopper-transmitted geminiviruses, belonging to Subgroup I, than to the coat protein genes of any whitefly-transmitted geminivirus (Stanley et al., 1986; Rybicki, 1994; Padidam et al., 1995), and no Subgroup I geminiviruses occur in the New World. Evidence in favour of a New World origin for BCTV includes the apparent diversity of the virus in the New World. A number of apparently distinct BCTV-like viruses have been identified in South America (Bennett, 1971), although these have yet to be fully characterised or even be confirmed as belonging to the Geminiviridae. However, we should note that this is the first in-depth analysis of an Old World BCTV isolate and the actual diversity of the virus in this region therefore remains to be determined.

The close similarity between BCTV-I and BCTV-CFH provides convincing evidence for the contention that they have a common origin. If the Old World proves to be the origin of BCTV, it is possible that this virus has been introduced to the New World on more than one occasion. The symptoms of BCTV have been described in crops from America since the 1880s (Bennett, 1971). However, the similarity between BCTV-I and BCTV-CFH would appear too high for them to have been geographically separated for this length of time. Possibly BCTV-CFH was introduced much later. Certainly the literature contains evidence that BCTV was imported into the USA for experimental purposes within the last 50 years (Bennett and Tanrisever, 1957).

BCTV is not the only geminivirus for which mankind has been responsible, at least in part, for their geographical dispersal. *Abutilon* and honeysuckle plants, infected with Abutilon mosaic and honeysuckle yellow vein mosaic geminiviruses respectively, have been spread worldwide for their attractive variegated foliage. More recently tomato yellow leaf curl virus has been introduced into the Dominican Republic and has subsequently spread to Jamaica, Cuba and Florida

(McGlashan, Polston and Bois, 1994; Zubiaur et al., 1996; Nakhla et al., 1994).

More detailed analysis of the genetic diversity of BCTV in the Old World should provide us with a baseline for assessing the divergence of the virus in the two geographically distinct regions in which it occurs and the mechanisms which are responsible for it.

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