Variation in the genome of rice tungro bacilliform virus: molecular characterization of six isolates

Zaifeng Fan,† Ganesh Dahal,‡ Indranil Dasgupta,§ Joanne Hay¶ and Roger Hull*

John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK

The DNA genomes of isolates of rice tungro bacilliform virus from Bangladesh, India, Indonesia, Malaysia and Thailand were cloned and compared with that of the type isolate from the Philippines. Restriction endonuclease maps revealed differences between the isolates and cross-hybridization showed that they fell into two groups, those from the Indian subcontinent and those from south-east Asian countries. The genomes of isolates from the Indian subcontinent contained a deletion of 64 bp when compared with those from south-east Asia. The implications of this variation are discussed.

Introduction

Rice tungro disease is caused by two viruses, rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) (Hibino et al., 1978; Saito et al., 1976). Under natural conditions, RTSV is transmitted independently by Nephotettix virescens (Distant) and other leafhopper species (Hibino, 1983a, b) but does not produce symptoms. RTBV causes stunting and yellow-to-orange leaf discoloration in infected plants, but depends on RTSV for leafhopper transmission (Hibino et al., 1978); together, RTBV plus RTSV produce typical ‘tungro’ symptoms such as stunted growth, increased tillering and yellow-to-orange discoloration (Hibino et al., 1978).

Rice tungro disease has been reported in more than 10 countries in south and south-east Asia (Hibino, 1987), causing annual losses of about US$1.5x 10⁹ (Herdt, 1991). Much effort has been made to manage the disease by the development and use of conventional ‘tungro resistant’ rice cultivars (Khush & Virmani, 1985; Sama et al., 1991). This has been largely unsuccessful due to the breakdown of ‘resistance’, which was primarily against the vector and not the viruses (Dahal et al., 1990a). The variable reactions of some rice cultivars to tungro in most endemic regions has caused further problems for disease management strategies involving such ‘resistant’ cultivars. Based on apparent symptomatological variability in selected rice cultivars using local populations of leafhoppers, tungro ‘strains’ have been reported from the Philippines and India. In the Philippines three symptomatological ‘strains’, S, M and T, which induced inter-veinal chlorosis, mottle and narrow leaf, respectively were recognized (Rivera & Ou, 1967). Similarly, five tungro ‘strains’, RTV1, RTV2A, RTV2B, RTV3 and RTV4, were reported in India, based on variability in symptomatology and disease severity in selected cultivars (Anjaneyulu & John, 1972; Mishra et al., 1976). Using similar criteria, the existence of tungro ‘strains’ was suspected in Thailand, Malaysia and Indonesia (Hibino, 1987). However, the designation of most of these strains was made before it was recognized that tungro was a complex of two viruses. Studies which indicate that rice cultivars react differentially to RTBV and/or RTSV, and express variable symptoms depending on single or double infection by these viruses (Hibino et al., 1987, 1990; Dahal et al., 1990b) also confuse the recognition of strains. Recently, two variants (strains) of RTSV have been recognized in the Philippines using their reaction in rice cultivar TKM6 (Cabauatan et al., 1994) and four strains of RTBV have been recognized from their symptoms in cultivar FK135 (Cabauatan & Koganezawa, 1994; Cabauatan et al., 1995). Three of the RTBV strains could be differentiated by restriction fragment polymorphism of a PCR fragment (Dolores-Talens et al., 1994). However, because of quarantine restrictions it has not been possible to make a controlled ‘side-by-side’ comparison of virus isolates in any of the south-east Asian countries to delineate how different isolates are related.
Molecular analysis of the RTBV genome indicates that it is probably a member of the badnavirus group (Lockhart, 1990). It has a circular dsDNA genome interrupted by two discontinuities which map to specific sites, one on each strand (Jones et al., 1991; Bao & Hull, 1992; Fig. 1a). The complete sequence of two isolates and the partial sequence of one isolate of RTBV from the International Rice Research Institute (Philippines) have been published. The full sequence reported by Hay et al. (1991) was of an infectious clone (Dasgupta et al., 1991) which was isolated in 1987, while the other full sequence (Qu et al., 1991) was of an isolate made in 1988. Kano et
al. (1992) reported on the sequence of the region encoding the virus coat protein of an isolate collected in 1977 and the full sequence is in the EMBL database (accession number RTBWG). The sequences show that the genome of RTBV is 8.0 kbp and has four ORFs (Fig. 1a). They differ in about 100 nucleotides which do not affect the genome organization.

One of the objectives of studying the molecular organization of RTBV is to gain information in order to develop strategies for non-conventional approaches to resistance (see Hull & Davies, 1992; Hull, 1994) by, for example, making plants transgenic in virus-derived sequences which interfere with virus functions. It is considered most important to target regions of the RTBV genome which are conserved across different isolates and strains of the virus. We made a comparative biological study of isolates of tungro collected from a number of south-east Asian countries and have shown some variation between isolates obtained from India, Malaysia and the Philippines (Dahal et al., 1992). Here we report on the cloning of the DNA of RTBV isolates from five south and south-east Asian countries and the comparison of their genomes with each other and that of the type Philippine isolate, which has been cloned and sequenced previously (Jones et al., 1991; Hay et al., 1991), by restriction endonuclease mapping and cross-hybridization.

Methods

Tungro isolates, virus and DNA isolation. The origin, maintenance and comparative biological characteristics of the Philippine, Indian and Malaysian tungro isolates are described in Dahal et al. (1992); for simplicity these isolates were designated PhL1 (Philippines, Luzon 1), IdD1 (India, Delhi 1) and MaP1 (Malaysia, Peninsular 1) (Table 1). The other isolates used in this study were field isolates from Maros Research Station, South Sulawesi, Indonesia (InS1), the Rice Research Institute, Dhaka, Bangladesh (BaD1), West Bengal, India (IdWB1) and a laboratory isolate from the Department of Agriculture, Bangken, Thailand (ThB1) (Table 1).

The crude preparation of unseparated viruses and the extraction of RTBV DNA were carried out according to the methods described by Jones et al. (1991).

Cloning and manipulation of RTBV DNAs. To identify single restriction sites, the DNAs of each isolate were digested with a range of enzymes (AccI, BamHI, EcoRI, PstI, SalI and XhoI); those which gave a single 8 kbp band on gel electrophoresis were selected for cloning the full genome into pUC18 or 19. All DNA manipulations such as cloning and restriction endonuclease mapping were as described by Sambrook et al. (1989). The cloned DNAs were compared with the infectious clone of the Philippine isolate (pRTBV 102; Hay et al., 1991) (previously designated pJIIS2; Jones et al., 1991).

DNA discontinuities were mapped as described by Jones et al. (1991), the probes being hybridized with homologous clones labelled with 32P by random priming (Feinberg & Vogelstein, 1983) as described by Sambrook et al. (1989).

Cross-hybridization. The overall sequence similarity between the DNAs of the Philippine and other isolates was assessed by cross-hybridization using Southern blotting and dot blotting. For Southern blotting the full-length clones of each isolate were digested with a selection of restriction endonucleases and electrophoresed side by side in 0.8% agarose gels at 20-30 V overnight. Blotting and probing with cloned DNAs of each isolate were as described above.

In the dot blot analysis, the genomes of the respective target DNAs were linearized with EcoRI. The concentration of the digested products was determined by spectrophotometry, electrophoresis and by gel scanning using the UVP Gel Analysis program (Ultra Violet Products). Before blotting, all samples were denatured with 1 M-NaOH and neutralized with 3 M-sodium acetate pH 4.8 as described by Maule et al. (1983). Aliquots of 100 ng of each of the samples were dotted in duplicate onto Hybond-N nylon membrane marked into 2 x 2 cm squares. The membranes were then air-dried and UV cross-linked in a Stratalinker (Stratagene). Blots were probed with labelled virus inserts from which the vector DNA had been removed by gel electrophoresis, washed with 0.1 x SSC at 65 °C and subjected to autoradiography.

DNA sequencing. dsDNA sequencing was carried out by dyeoxy-nucleotide chain termination (Sanger et al., 1977) primed with the universal sequencing 17 nucleotide primer (USB) from the pUC vector or with a specific oligonucleotide primer to the tRNA binding site (Lockhart & Olszewski, 1993), and using [35S]dATP and a Sequenase kit (USB) as recommended by the manufacturer, or by a simplified method (Hsiao, 1991). The nucleic acid sequence data were assembled and analysed using programs from the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

PCR conditions. Amplification by PCR was performed using the primers described in Results. After initial denaturation at 94 °C for 3 min, samples were subjected to 50 cycles of denaturation at 94 °C for 30 s, reannealing at 50 °C for 30 s and extension at 72 °C for 30 s. Aliquots from the reactions were electrophoresed in 1.5% agarose gels and the bands detected by staining with ethidium bromide.

Results

Cloning and restriction mapping of RTBV DNAs

The DNAs of isolates InS1, MaP1 and ThB1 resembled that of PhL1 in having a single SalI site and were cloned.

Table 1. Isolates of RTBV

<table>
<thead>
<tr>
<th>Origin</th>
<th>Region</th>
<th>Isolate</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangladesh</td>
<td>Dhaka</td>
<td>BaD1</td>
<td>pRTBV601</td>
</tr>
<tr>
<td>India</td>
<td>Delhi</td>
<td>IdD1</td>
<td>pRTBV201</td>
</tr>
<tr>
<td>India</td>
<td>West Bengal</td>
<td>IdWB1</td>
<td>pRTBV202</td>
</tr>
<tr>
<td>Indonesia</td>
<td>South Sulawesi</td>
<td>InS1</td>
<td>pRTBV301</td>
</tr>
<tr>
<td>Malaysia</td>
<td>Sadang (Peninsula)</td>
<td>MaP1</td>
<td>pRTBV501</td>
</tr>
<tr>
<td>Philippines</td>
<td>Los Banos (Luzon)</td>
<td>PhL1</td>
<td>pRTBV101</td>
</tr>
<tr>
<td>Thailand</td>
<td>Bangken</td>
<td>ThB1</td>
<td>pRTBV401</td>
</tr>
</tbody>
</table>

Table 2. Mapping of discontinuities

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Restriction site</th>
<th>Sizes of restriction fragments (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaD1</td>
<td>Clal</td>
<td>7.3, 4.5, 3.5, 0.7</td>
</tr>
<tr>
<td>IdD1</td>
<td>BamHI</td>
<td>7.3, 4.7, 2.8, 1.3</td>
</tr>
<tr>
<td>InS1</td>
<td>Clal</td>
<td>7.3, 4.7, 3.7, 0.8</td>
</tr>
<tr>
<td>MaP1</td>
<td>Sphl</td>
<td>7.2, 5.1, 2.9, 0.8</td>
</tr>
<tr>
<td>ThB1</td>
<td>BamHI</td>
<td>7.3, 5.0, 3.0, 0.7</td>
</tr>
<tr>
<td>SalI</td>
<td></td>
<td>7.9, 4.4, 3.6, 0.1</td>
</tr>
</tbody>
</table>
into pUC at the SalI site. The DNAs of BaD1, IdD1, IdWB1, InS1 and ThB1 also resembled that of PhL1 in having a single BamHI site. However, the DNAs of isolates BaD1, IdD1 and IdWB1 differed from that of PhL1 in not being cut by SalI; these were cloned into pUC at the BamHI site. Full-length clones were selected and given the designations shown in Table 1.

The cutting sites of a range of restriction endonucleases in the full-length clones were mapped using single and double digests. The maps shown in Fig. 1(b) detail the sites of 11 enzymes. The map of IdWB1 was the same as that of IdD1. It was apparent that the isolates differed from each other in their restriction endonuclease maps. Among the notable features were MaP1 having two BamHI sites, whereas the other isolates had one, and IdD1 and BaD1 having two and one KpnI site, respectively, and the others having none.

**Mapping the positions of discontinuities**

Electrophoresis of denatured DNA of each isolate revealed just one band of 8 kb, which indicated that they resembled the PhL1 isolate in having one discontinuity in each strand (Jones et al., 1991). To map the positions of...
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these discontinuities, virus DNA was digested with single-cutting enzymes and the denatured products were separated by electrophoresis. The sizes of the single-stranded fragments were measured (Table 2) and the sites located on the map (Fig. 1b).

**Orientation of the maps**

The map of the Philippine isolate is orientated like those of all plant pararetroviruses with the zero position being the discontinuity at the negative-strand DNA primer site (Jones et al., 1991) and the direction of transcription being clockwise (Hay et al., 1991). The map of the cloned DNA of each isolate was orientated by limited sequencing from the cloning vector and around the discontinuity of the negative-strand DNA priming site. The sequences adjacent to the cloning sites were compared to the full sequence of the Philippine isolate (Hay et al., 1991) (Fig. 2a). This showed that the SalI sites of InS1, MaP1 and ThB1 were in similar positions to that of PhL1 at nucleotide 7912. However, the sequences around the BamHI cloning sites of BaD1 and IdD1 aligned with the Philippine isolate sequence at about nucleotide 2930 (Fig. 2b), which is very different from the position of the BamHI site in pPhL1 (nucleotide 7186; Fig. 1b). The section of the genome shown in Fig. 2(b) is in the middle of the ORF for P194. The nucleotide differences between IdD1 and BaD1 and PhL1 do not lead to any amino acid differences. The two BamHI sites in MaP1 mapped to the positions of the single sites in PhL1 and IdD1 (Fig. 1b).

The sequence 5' of the negative-strand priming site for each clone (Fig. 2a) showed that in this region the cloned isolates appear to fall into two groups. PhL1, InS1, MaP1 and ThB1 are very similar to each other, as are IdD1 and BaD1. There are significant differences

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**Fig. 3. Hybridization between genomes of isolates of RTBV. (a) Dot blots of: 1 and 7, BaD1; 2 and 8, IdD1; 3 and 9, InS1; 4 and 10, MaP1; 5 and 11, PhL1; and 6 and 12, ThB1. Probes used were inserts from pRTBV101 (PhL1) (1-6) and pRTBV202 (IdD1) (7-12). (b), (c) Southern blot hybridization between: lanes 1 and 7, BaD1; 2 and 8, IdD1; 3 and 9, InS1; 4 and 10, MaP1; 5 and 11, PhL1; 6 and 12, ThB1. The DNA in lanes 1–6 was cut with EcoRI and HindIII and in 7–12 by EcoRI and XhoI. The blot was probed with (b) insert from pRTBV101 (PhL1) or (c) with pRTBV202 (IdD1).**
between the two groups, the most obvious being a deletion of 64 bp in the genomes of IdD1 and BaD1. This deletion starts at the 5' nucleotide of the SalI site found in the other isolates and explains the lack of cutting by this enzyme in IdD1 and BaD1.

Cross-hybridization

To assess the overall relationships between the genomes of the six isolates, dot blots of 0.1 µg of each of the clones were probed with labelled clone inserts. Probing with the inserts from pRTBV101 and pRTBV601 (Table 1) is shown in Fig. 3(a). It can be seen that the DNA from the PhL1 isolate (pRTBV101) hybridized well with those of the InS1, MaP1 and ThB1 isolates (Fig 3a; spots 3, 4 and 6, respectively) but poorly with those of BaD1 and IdD1 (Fig. 3a; spots 1 and 2). Conversely, the probe from the BaD1 isolate (pRTBV601) recognized the IdD1 isolate (Fig 3a; spot 8) but reacted poorly with the InS1, MaP1, PhL1 and ThB1 isolates (Fig 3a; spots 9–12). Probing dot blots with the other four isolates gave the same results (data not shown).

Southern blots of restriction endonuclease digests of the clone of each isolate were hybridized with probes derived from the other isolates. A representative set of blots is shown in Fig. 3(b) and (c). From these it can be seen that the isolates again fall into two groups. The PhL1 isolate cross-hybridized well with the InS1, MaP1 and ThB1 isolates, but not with the IdD1 or BaD1 isolates. The latter two cross-hybridized with each other.

Southern blotting of restriction fragments showed some heterogeneity in the distribution of relatedness around the genome. For instance, the largest EcoRI–HindIII fragment of InS1 reacted proportionally more with the IdD1 probe than did the other two fragments (Fig. 3c; lane 3).

PCR detection of the deletion

The sequence data in Fig. 2(a) show a deletion in the genomes of IdD1 and BaD1. To determine if this was an artefact of cloning, PCR primers were designed to conserve regions upstream (nucleotides 7747–7765) and downstream (nucleotides 15–1) (Fig. 2a) and the DNA from various sources amplified (Fig. 4). As both the cloned and virus DNAs of PhL1 and IdD1 gave the expected fragments of about 290 and 230 bp, respectively (Fig. 4; lanes 1, 2, 6 and 7) it is unlikely that the deletion was the result of cloning. Isolate IdD1 has been maintained in culture in New Delhi for more than 20 years and thus the deletion may not be representative of field isolates. Virus DNA of IdWB1, a field isolate from West Bengal, was amplified and gave a fragment of the size expected for the deletion (Fig. 4; lane 8). The DNAs of other isolates from the Indian subcontinent showed evidence of the deletion (Fig. 4; lanes 9 and 10) together with ones from Assam in India and Nepal (data not shown). Isolates from south-east Asian countries did not show the deletion (Fig. 4; lanes 3–5). Probing of blots of these PCR products with pRTBV101 and pRTBV202 confirmed the hybridization observations described above (data not shown).

Discussion

The results presented in this study clearly demonstrate that RTBV isolates fall into two groups, those from the Indian subcontinent and those from south-east Asian countries. While there is close similarity between isolates within each group, as evident from cross-hybridization, there is much less similarity between each group. The stringency of washing after hybridization would detect between 1% and 5% mismatches. Hybridization on Southern blots of restriction endonuclease fragments gave some evidence of variation in relatedness in different parts of the genome. However, for a more detailed analysis of this, full sequence data are needed.

One important difference between the two groups of isolates is the presence of the deletion in those from the Indian subcontinent. This has proved useful in diagnosis of isolates. Natural deletions are found in members of the other pararetrovirus group, caulimoviruses. Cauliflower mosaic virus (CaMV) isolate CM4–184 has a deletion of 421 bp (Howarth et al., 1981), which is thought to be the result of recombination. Splicing events leading to deletions in reverse transcripts have been detected in CaMV (Hirochika et al., 1985; Vaden & Melcher, 1990) and in figwort mosaic virus (Scholthof et al., 1991). The splicing deletions are not encapsidated and that in CaMV CM4–184 would not function naturally as most of the aphid transmission factor is deleted. The deleted genomes of the Indian subcontinent
isolates of RTBV are encapsidated and are obviously functional as they are found in field isolates. Splicing has also been shown to be essential for the expression of CaMV (Kiss-Lázsló et al., 1995) and of RTBV (Fütt er et al., 1994) but again the products are not encapsidated. There is no evidence in the RTBV sequence for consensus splice donor (AG'GTAAGT) or acceptor (YAG'G) sequences (Padgett et al., 1986; Fig. 2a), nor is there sequence homology which could lead to a recombinational event. Thus, it is not possible to suggest a mechanism by which the deletion in the Indian subcontinent isolates arose.

The differences between the isolates from the Indian subcontinent and those from south-east Asia are such that they can be considered to be two strains. It remains to be seen whether the differences are such that they should be considered to be two distinct viruses.

The distinction between the Indian subcontinent and the south-east Asian strains raises the question as to why they are not intermixed. The nearest that the two strains have been found is Assam in India (Indian strain) and central Thailand, a distance of about 1500 km. These two regions are not separated by a rice-free area, as there is considerable rice production in Myanmar. Furthermore, there appeared to be no significant differences in the susceptibility of rice cultivars to the two strains (Dahal et al., 1992). One possible explanation could be related to the semi-persistent transmission of RTBV (in association with RTSV) by the leafhopper, N. virescens. It has been suggested that N. virescens is a poor flier (Cook & Perfect, 1989) and only a small percentage of a population disperses more than a few kilometres, the dispersal being dependent on the prevailing winds. Leafhopper populations are related to the availability of host plants, which in turn is affected by rainfall. In tropical Asian countries, where one main rice crop is grown, there is a distinct annual peak associated with south-west monsoon rains. Although north-eastern India (including Assam) and central Thailand have the same overall climatic characteristics, both being in the Asian monsoon zone, there are some differences in the detailed wind-flows which put them in distinct climatic zones (see Nieuwolt, 1981). The south-west monsoon winds blow parallel to the east coast of India and up the Bay of Bengal to Bangladesh and north-eastern India, including Assam. Further east these winds interact with the south-east trade winds and so the monsoon winds in central Thailand originate from the south-east and do not interact with those flowing up the Bay of Bengal. Thus, there appears to be a climatic interface between north-eastern India and Thailand.

The finding of two distinct strains of RTBV is of significance in designing approaches to and testing for non-conventional protection against the virus. It will be necessary to obtain the full sequence of the Indian strain of RTBV to identify targets conserved between the two strains. From the hybridization data it seems unlikely that there is significant variation within each strain. Promising transgenic lines should be tested against both strains.

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References


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