CHAPTER - V
RESULTS AND DISCUSSION
RESULTS AND DISCUSSION

5.1 Cloned RTBV Indian isolates

Five Indian isolates of RTBV were cloned from tungro infected rice plant materials. The West Bengal tpRTBV200 isolate clone was obtained using the restriction enzyme BamHI. Assam (pRTBV210 and pRTBV211) isolate clones using EcoRI. Andhra Pradesh (pRTBV200) using AatI and Tamil Nadu (pRTBV200) using Hind III enzymes respectively. West Bengal isolate (pRTBV200) was found to contain full-length genome of RTBV of size 8.8kb. The Assam isolate was cloned as two pieces (pRTBV210 and pRTBV211) of size of 6.5kb and 1.5kb respectively. The Andhra Pradesh isolate (pRTBV200) and Tamil Nadu isolate (pRTBV200) was obtained as a small fragment of sizes 1kb and 2kb respectively (Table 4).

5.2 Restriction maps

5.2.1 pRTBV 206

Digestion of pRTBV206 with BamHI restriction enzyme released an insert of 8.8kb and a vector of 2.3kb. Hybridisation of BamHI digested fragment with all different probes showed that it hybridised strongly showing that it was a full-length clone. pRTBV206 when digested with restriction enzyme BglII produced four fragments of sizes 4.7kb, 4.6kb, 1.4kb and 0.2kb respectively. When labelled with probe I, two larger fragments of 4.7kb and 4.6kb respectively hybridised. With probe II, only 1.4kb fragment hybridised. This indicated that this fragment represents the ORF II region and there was a single site of BglII in ORF II region. Hybridisation with probe III, which represented the MP region, only 1.4kb fragment hybridised. The same fragment also hybridised with probe II. This indicated that there was a BglII site within the MP region. Coat protein region of pRTBV206 is represented by probe IV. With this probe, the larger BglII fragment of 1.4kb hybridised. Since the cloning vector, PBSK+ did not have any BglII
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site, this larger fragment of 4.3kb may include the vector pBSK (+) encompassing coat protein region. With probe V, VI and VII, which represented PR/RT RNAseH, ORF IV and intergenic region of pR1BV101, the larger fragment, which can be either 4.7kb or 4.6kb respectively, hybridised. These two fragments were very closely placed. They migrated almost together so it was difficult to differentiate. Hybridisation of single large fragment with these probe indicated that this large fragment had Bgl II restriction site at border of PR/RT RNAseH and towards the end of intergenic region.

Digestion of pR1BV206 plasmid DNA with Bax I restriction enzyme released two fragments of sizes 7.0kb and 3.9kb respectively. The hybridisation pattern analysis with different probes suggested that the smaller fragment of 3.9kb represented the PR/RT RNAseH and ORF IV region. In the vector pBSK (+), Bax I has single site. This 3.9kb fragment may contain a small region of vector. There was a single Bax I site at the end of ORF IV region. The large fragment of Bax I digest covered most of the intergenic region, ORF I, II, MP, part of CP and most part of vector pBSK (+), which was evident from hybridisation pattern analysis. This fragment hybridised when ORF I, II, MP, CP and IR was used as probe.

Digestion with EcoR I restriction enzyme released four fragments of sizes 6.4kb, 3.3kb 0.8kb and 0.4kb respectively. The larger fragment of 6.4kb hybridised with probes IV, V and VI. There is an EcoR I restriction site in vector pBSK (+). This larger fragment may include most of the region of the vector, PR/RT RNAseH and parts of ORF IV region. There are two EcoR I sites one in vector and other some where near end of ORF IV. The 3.3kb fragment of EcoR I showed hybridisation with probe I, II, III and IV respectively. This 3.3kb fragment may thus contain one EcoR I site at border of ORF IV region and intergenic region and other at somewhere in between 'MP' region. The 0.8kb fragment also hybridised with probe III along with 3.3kb fragment. The 0.4kb fragment which may contain part of 'CP' region also showed hybridisation with probe IV along with large fragment of 6.4kb. On the basis of hybridisation pattern analysis, we
can conclude that there were three sites of EcoRI in pRTBV206. One in the middle of ‘MP’ region, one towards end of ‘MP’ region, and one in the ORF IV region. The 0.8kb fragment was faintly visible when hybridised with ORF IV region. This indicated that it covered parts of ‘CP’ region.

Digestion of plasmid DNA pRTBV206 with Hind III restriction enzyme released four fragments of sizes 6.4kb, 2.9kb, 0.9kb and 0.7kb sizes respectively. The largest fragment of 6.4kb when hybridised with all probes separately showed hybridisation with all probes except probe IV, which represented the ‘CP’ region. This indicated that the large fragment covered most of the region of RTBV genome including ‘ORF I’, II, ‘CP’, ‘MP’, PR/RTRNAseH’, ‘ORF IV’ and ‘intergenic region’ respectively. The 2.9kb, 0.9kb and 0.7kb fragment showed hybridisation only with probe IV that represented the ‘CP’ region. This indicated the 2.9kb fragment, which hybridised with probe IV (CP region) contains most part of the vector and part of CP region.

Three fragments of sizes 5.5kb, 3.5kb and 1.9kb respectively were produced when pRTBV206 was digested with Kpn I restriction enzyme. Hybridisation with different probes showed that the larger 5.5kb fragment showed hybridisation with probe I, II, V, VI, and VII respectively. This indicated that the larger fragment covers PR/RTRNAseH, ORF IV, intergenic, ORF II and ORF I region respectively. This larger fragment has two Kpn I restriction sites at both ends i.e. towards the beginning of PR/RTRNAseH and towards the end of ORF II region. The smallest fragment of 1.9kb hybridised with both ORF II and MP region. This indicates that there is a Kpn I site present in ‘MP’ region. The second Kpn I site must be present near and of CP region. The vector also contains a Kpn I restriction site. The second largest fragment of 3.5kb size showed hybridisation with probe IV that represents ‘CP’ region.

The RTBV DNA of pRTBV206 when digested with restriction enzyme Nco I released two fragments of sizes 8.0kb and 2.9kb respectively. Southern blot analysis of different Nco I digest shows that the 8.0kb fragment showed hybridisation with probe IV, V and VI, which represent the regions of CP, PR/RT
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RNAaseH and ORF IV indicate that there may be two XhoI sites present in pRTB206 genome. One of these two, one may be present near CP region and other may be present near ORF IV region. The rest of probe I, II, III and VII showed hybridisation with 20kb fragment. This hybridisation pattern indicates that 20kb fragment extends from intergenic region to movement protein region.

Digestion of plasmid DNA of pRTB206 with Pvu I restriction enzyme released three fragments of sizes 45kb, 46kb and 1.8kb respectively. Hybridisation with different probes showed that smallest 1.8kb fragment hybridised only with probe III and IV which represents the MP and CP region of RTB virus genome. This suggests that there exists a Pvu I restriction site in MP region and other lies with vector. The larger fragment of 45kb or 46kb showed hybridisation with probe I, II, V, VI and VII respectively. In vector pBR322, there exists single site of Pvu I. The larger fragment were not definable. It could contain large fragment of vector along with parts of PR RT RNAaseH region or may contain region covering part of PR RT RNAaseH region, full ORF I, the whole of intergenic ORF I and ORF II region. The hybridisation pattern using various probes indicated that two Pvu I restriction site occurs in viral genome. One Pvu I site occurs in ORF II region and other exist in PR RT RNAaseH region of the viral genome.

Digestion of RTBV DNA pRTB206, with Xho I restriction enzyme released five fragments of sizes 45kb, 35kb, 15kb, 10kb and 9.5kb respectively. Probe I, II hybridised with 10kb fragment and probe V, VI, VII hybridised with the larger 45kb fragment. Probe III hybridised with 15kb fragment and probe IV showed hybridisation with 35kb fragment. Study of hybridisation pattern using different probes suggests that the largest 45kb fragment represent PR RT RNAaseH, ORF IV and IR region. The 35kb represent the CP region. It covers most of the region of vector and part of CP region. There is one Xho I site in vector and one near CP region. The 10kb fragment represents the MP region. And 15kb fragment is representative of ORF I and ORF II region. There exists 14
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recognition sites of \( \lambda \)II restriction enzyme in the viral genome and single site in vector pBSK (+).

Two fragments were generated after digestion with \( Xho \) I restriction enzyme. This suggest that there occur single sites of \( Xho \) I in viral DNA. The vector pBSK (+), contain this enzyme. Hybridisation with different probes suggests that the larger fragment of 6.5kb represent PR/RT RNAseH region, which includes the vector along with it. The smaller fragment of 4.5kb represents ORF I, II, CP, MP, ORF IV and IR region. This \( Xho \) I site occurs somewhere near border of PR/RT RNAseH and ORF IV region.

Based on the results discussed above, the restriction map of the genome of West Bengal (pRTBV206) was constructed, which is depicted in fig.61.

5.2.2 pRTBV210

Digestion with \( Cfo \) I restriction enzyme released two fragments of sizes 6.5kb, 2.9kb respectively. Differential hybridisation pattern showed that the larger fragment of 6.5kb size hybridised with all five probes. Thus it can be concluded that in pRTBV210, the insert is 6.5kb in size, which is released upon digestion with \( Cfo \) I restriction enzyme. Digestion with \( Bgl \) II restriction enzyme releases four fragments of sizes 4.5kb, 1.9kb, 1.7kb and 1.3kb respectively. The largest fragment of 4.5kb showed hybridisation with probe representing ORF IV, ORF II and movement protein region. As the vector pBSK (+) does not contain this enzyme, the larger fragment contains movement protein region, the whole of vector and parts of ORF IV region. The second largest fragment of 1.9kb size represents ‘PR/RT RNAseH region’ and smaller fragments of sizes 1.7kb and 1.3kb represents ‘CP’ and ‘ORF IV’ region. Based on the differential hybridisation pattern, it was concluded that in 6.5kb fragment of pRTBV210, four sites of \( Bgl \) II restriction enzymes are present.

Digestion with \( BstXI \) restriction enzyme released two fragments of sizes 6.2kb and 3.2kb respectively. The larger fragment of 6.2kb showed hybridisation with probe representing ‘PR/RT RNAseH’, ‘ORF IV’, and ‘ORF II’ regions
Figure 61. Linearized version of the restriction map of cloned West Bengal isolate (pRTBV206). Legend same as figure 3.
whereas the smaller fragment of 3.2kb showed hybridisation with probe representing ‘MP’ and ‘CP’ region respectively. The vector pBSK(+) contains this restriction enzyme. So it can be said that BvX 1 occurs singly in pRTBV210 genome near PR:RTRNAselI region. The large fragment of 6.2kb extends from between ‘CP’ and ‘PR:RTRNAselI’ region till ORF II including full length ORF IV and most region of vector. The smaller fragment of 3.2kb lies in ‘MP’ and ‘CP’ region. The single restriction site of BvX 1 in pRTBV210 viral genome lies between ‘CP’ and ‘PR:RTRNAselI’ region.

Digestion with ‘EcoR I’ restriction enzyme released three fragments of sizes 4.6kb, 3.5kb, and 1.3kb respectively. The hybridisation pattern showed that the largest fragment of 4.6kb shows strong hybridisation when labelled with probes representing ‘PR:RTRNAselI’ region, ‘CP’ region, and slight hybridisation with probe representing ‘MP’ region. The fragment covers part of vector, full CP and PR:RTRNAselI region. There is an EcoR I restriction site between ‘PR:RTRNAselI’ and ‘ORF IV’ region along with an EcoR I site in vector. The 3.5kb fragment hybridised with probe representing ‘ORF IV’ region. This fragment covered ‘ORF IV’ region along with most part of vector. The smallest 1.3kb represented ‘MP’ and ‘ORF II’ region. Study of EcoR I digestion pattern revealed that two EcoR I restriction site occurred within pRTBV210 genome. One somewhere near beginning of ORF IV or in between ‘PR:RTRNAselI’ and ‘ORF IV’ region and the second EcoR I site occurred towards end of ‘MP’ region.

EcoR V digestion released two fragments of sizes 7.5kb and 1.9kb respectively. The large 7.5kb fragment showed hybridisation with probe representing ‘PR:RTRNAselI’ and ‘ORF IV’ region and ‘ORF II’ region whereas the smallest fragment of size 1.9kb showed hybridisation with probe representing ‘MP’, ‘CP’, and ‘ORF II’ region. From this data, it can be analysed that there occurs single EcoR V site in viral genome and vector pBSK (+). The larger fragment extends from ‘PR:RTRNAselI’ region towards ‘ORF IV’ region and covers parts of ‘ORF II’ region and most parts of vector. The 1.9kb fragment
extends from part of ‘ORF II’ region till ‘CP’ region covering the ‘MP’ region.
This single site of EcoR V lies in between ‘CP’ and ‘PR/RTRNAseH’ region.

Digestion with restriction enzyme Hind III released three fragments of sizes 4.6kb, 2.9kb, and 1.9kb respectively. The largest fragment of 4.6kb showed hybridisation with probe representing ‘ORF IV’ region. The second largest fragment of size 2.9kb showed hybridisation with probe representing ‘CP’, ‘MP’, and ‘ORF II’ region whereas the smallest fragment of size 1.9kb showed hybridisation with probe representing ‘PR/RTRNAseH’ region. The differential hybridisation pattern revealed that the 4.6kb fragment covered ‘ORF IV’ region along with large portion of vector. There occurred two sites of this enzyme in viral genome of pRTBV210, which was at both the extreme ends of PR/RTRNAseH region.

Kpn I restriction digestion released two fragments of sizes 6.5kb and 2.9kb respectively. Hybridisation using different probes indicated that the large fragment of 6.5kb represented ‘MP’, ‘CP’, ‘PR/RTRNAseH’, ‘ORF IV’, and ‘ORF II’ region. It extended from ‘ORF II’ region and covered region of ‘CP’, ‘PR/RTRNAseH’, ‘ORF IV’ and parts of vector. The second fragment of 2.9kb largely represented the vector with very small part of ‘ORF II’ region. The single Kpn I restriction site occurred in ‘ORF II’ region.

Restriction digestion of Pst I released two fragments of size 5.6kb and 3.8kb respectively. Study of hybridisation pattern revealed that 5.6kb represented ‘ORF II’, ‘PR/RTRNAseH’ and ‘ORF IV’ region of viral genome and smaller 3.8kb represented region of ‘MP’, ‘CP’ and ‘ORF II’. The single Pst I site in viral genome occurred between ‘CP’ and ‘PR/RTRNAseH’ region. The large fragment extended from ‘PR/RTRNAseH’ region and covered ‘ORF IV’ region, parts of ‘ORF II’ and most parts of vector. The smaller region covered part of vector, ‘MP’, and ‘CP’ region.

Digestion with Xba I restriction enzyme released four fragments of sizes 6.6kb, 1.2kb, 1.0kb, and 0.6kb respectively. Hybridisation pattern using different labelled RTBV probes indicated that 6.6kb fragment represented ‘ORF IV’ and
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'PR/RTRNAaseH' region. 1.5kb, 1.0kb and 0.6kb fragment represented 'CP', 'MP' and 'ORF II' region respectively. In pRTBV210 viral genome, there are three sites of Aha I restriction enzymes. One lies in between 'CP' and 'PR/RTRNAaseH'; second site lies in between 'CP' and 'MP' region and third site between 'ORF II' region. The lower 6.6kb fragment covered whole of vector, 'ORF IV' and 'PR/RTRNAaseH' region.

Digestion with Aha I restriction enzyme released two fragments of sizes 3.5kb and 4.0kb respectively. Differential hybridisation pattern using different RTBV labelled revealed that the larger fragment of 3.5kb represents 'CP', 'PR/RTRNAaseH', 'ORF IV' and 'ORF II' regions and smaller 4.0kb fragment represents 'MP' region. It indicated that there occurred single site of Aha I restriction site in viral genome pRTBV210 near the end of 'MP' region. The 4.0kb fragment contained the full vector and 'MP' region.

On the basis of above observation the restriction map of pRTBV210 was constructed, which is shown in fig. 62.

5.2.4 pRTBV211

Digestion of cloned Indian RTBV viral DNA pRTBV211 with Cla I restriction enzyme resulted in production of two fragments of sizes 2.9kb and 1.5kb respectively. Hybridisation pattern using probes representing 'intergenic' region and 'ORF I' region showed that the insert of 1.5kb contained both these regions as this fragment hybridised with both the probes. EcoR I digestion of pRTBV211 produced two fragments of sizes 3.1kb and 1.9kb respectively. The 3.1kb fragment hybridised with both intergenic region and ORF I whereas 1.9kb fragment hybridised slightly with 'ORF I' as well. The 3.1kb fragment covered most of the vector and part of 'ORF I' region.

With Aha I restriction enzyme, two fragments of sizes 3.8kb and 0.6kb were produced. There occurred single site of Aha I restriction enzyme in 'ORF I' region. The large fragment of 3.8kb included vector and intergenic region. Double digestion of pRTBV211 with Cla I and EcoR I restriction enzyme resulted in
Figure 62. Linearised version of the restriction map of cloned Assam isolate (pRTBV210). Legend same as figure 3.
production of three fragments of sizes 2.9kb, 1.8kb and 0.1kb respectively. The 1.3kb fragment showed hybridisation with probes representing both ‘intergenic’ region and ‘ORF I’ region. The 2.9kb fragment represented the vector. The EcoR I site was very close to Cla I site in viral genome. The double digestion of pRTBV211 with Xba I and EcoR I produced three fragments of sizes 3.8kb, 0.4kb and 0.2kb respectively. The hybridisation pattern showed that 3.8kb fragment hybridised with ‘intergenic’ region. This large fragment covered the vector and intergenic region. The 0.4kb fragment contained ‘ORF I’ region. The EcoR I restriction enzyme lies towards one end of the insert, covering ‘ORF I’ region.

Double digestion of viral DNA of pRTBV211 with Xba I and Cla I restriction enzymes released fragments of sizes 2.9kb, 0.75kb and 0.65kb respectively. The 2.9kb fragment represented the vector. The 0.75kb fragment covered the ‘intergenic’ region and part of ‘ORF I’ region. And 0.65kb fragment which had Xba I and Cla I restriction site, represented the ORF I region.

Restriction map study of pRTBV210 isolate revealed that it covered the region from ORF II to ORF VI including regions of ‘MP’, ‘CP’, ‘PR:RTRNAaseH’ and that of pRTBV211 (fig 63) region covers ‘intergenic’ and ‘ORF I’ region. The evidence indicated that, taken together, pRTBV210 and 211 represented the full RTBV genome (fig 64).

5.3 Phylogenetic relationship of different cloned Indian RTBV isolates with Philippines isolate.

The study of Phylogenetic tree indicates that Indian isolates shows a close resemblance with each other. The pRTBV206 Indian isolate showed maximum homology with the Philippine isolate.

5.4 Variability

Variability in RTBV DNA has been documented earlier. Cabanatan (1999) found approximately 300 nucleotide changes in six full-length cloned RTBV
Figure 63. Restriction map of Assam (pRTBV211) isolate. Legend same as figure 3.
Figure 6A. Restriction map of Assam isolate (pRTBV210 + pRTBV211). Legend same as figure 3.
molecules from Philippines and Malaysia. Heterogeneity due to nucleotide substitution in virus isolates maintained at IRRI glasshouse was observed by Hull, 1996. Recent work by Nath et al., 2002 has also reported similar levels of variation between two Indian isolates. The RTRV DNA fragment between nucleotide residues 5444-6553 was chosen to study genomic variability on the basis of sequence comparison of two cloned viral DNAs (pRTRV203 and pRTRV204) representing the WB and AP isolates. The only significant difference between the two genomes, in the coding region, was the deletion of 30 nucleotides in the pRTRV204, in the region mentioned above. An MspI digestion of this amplified region produced the expected differences in the banding pattern, as deduced from the sequence information (fig.51, lane 203 and 204). Another cloned WB isolate (pRTRV206) showed a pattern identical to pRTRV203, indicating homogeneity of the cloned DNAs. In addition, a clone representing an AS isolate (pRTRV210) also gave identical pattern with pRTRV204, indicating that pRTRV210 also carried the above deletion (fig.51, lane 210). However, patterns obtained from infected plants, representing the different RTRV isolates, showed the presence of unexpected bands. The bands of approximately 240bp in leaves infected with AS, AP, PB, OR, and TN isolates was not seen in the cloned viral DNAs. Another such band was the 150bp in the WB isolate infected leaves. The expected bands, representing the 30nt deletion were shown clearly by the WB and TN isolates, indicating the presence of deletion in the latter (fig.51, lanes WB and TN). However, the leaf samples representing AS isolate showed a pattern representing both the deleted as well as undeleted viral populations. The banding pattern between the WB isolate, as obtained from infected plants and its cloned counterpart differed only in the 130bp band (fig.51, lane 206, 203 and WB). pRTRV203 and pRTRV206 were cloned from tungro-infected plants obtained from the same field locations but at different times (Joshi and Dasgupta 2001). pRTRV206 was cloned from the same batch of field-infected plants, which were used to obtain amplified fragments for the RFLP analysis. This difference noted above probably represented a molecular species, residing in the infected plant.
which was not represented in either of the clones pRTBV206 and pRTBV203. Similarly, the AS and AP isolates also exhibited molecular species of RTBV, which were not represented in the corresponding cloned forms (fig.51, lanes 204, 210, AS and AP). This supported the earlier data of Villegas et al. (1997) from locations in Philippines, where mixed infections were noted in several plants and reinforces the concept of quasi-species in RTBV field populations. The data clearly indicated the presence of mixed population of the viral genome within the same plant at the field level (fig.51, lane AP and AS). The difference in the intensities of these bands indicated the presence of variable quantities of different population of the viral genome, representing each pattern.

5.5 Promoter study analysis

The RTBV promoter and various transcription factors that interact with it may serve as a model system to study plant tissue-specific gene expression. The RTBV has a simple promoter, which was found to have phloem specific activity in transgenic rice plants (Bharacharyya-Patrasri et al. 1999). The RTBV promoter has been analysed in both transformed rice plant (Yin and Beachy, 1995, Yin et al. 1997 and Kiriti et al. 1999) and transfected rice protoplasts (Chen et al. 1994, 1996). Multiple upstream elements have been identified as being required for phloem specific gene expression in the context of the -164 to +45 promoter in transformed rice plant. In RTBV, the promoter element lies in large intergenic region. Studies carried out by various groups have shown that both upstream and downstream elements are important for gene expression. To test whether a putative promoter region in cloned WB isolate (pRTBV206) is functional, a GUS PCR amplified fragment was cloned using specific primers PD5 and PD7 in promoter-less GUS vector pCambia1381. The specific activity of GUS in recombinant rice calli indicated that pRTBV206 promoter was higher as compared to control where gus genes was used without any promoter. These results indicated that fragment of RTBV promoter present in pRTBV206 showed
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4.5-fold higher GUS activity. This experiment proves that cloned RTBV DNA (pRTBV206) contained a functional promoter.

5.6 Infectious clone

The two strategies discussed in the text were tried several times but no clones showing the expected digestion pattern were obtained. The possible reason may be that the insert may be unstable or cells were unviable. In second strategy where no signals were obtained, it may be possible that digestion was partial due to which it did not ligate completely. Further strategies for testing the infectivity of the full-length clone may involve use of some other binary vector with more compatible restriction enzyme sites.
SUMMARY
SUMMARY

The main aim of present investigation was to study the infectivity of cloned RTBV DNAs from field isolates in eastern India. An infectious clone of a virus helps in understanding many of the mechanisms involved in the host-pathogen interactions, which form part of the process of pathogenesis. As a first step towards the above, a study was conducted on the genomic organisation of some isolates of RTBV from eastern India. The genomic DNA of different Indian RTBV isolates were cloned and analysed by restriction mapping and partial sequencing. The restriction map and sequences of cloned Indian RTBV isolates were compared among them and also with Philippine isolate, which has been extensively studied earlier. The variability studies of different cloned Indian RTBV isolates and field isolates were also carried out. The putative promoter region of one of the Indian isolate was cloned and its activity was tested in rice calli. Several methods were tested to obtain infectious clones of RTBV.

The thesis is divided into five chapters. Chapter I deals with the introduction of RTBV. Chapter II focuses on the review of literature. Chapter III deals with the material and methods used in the experiments. In chapter IV and V observation and the results and discussion have been incorporated.

The overall studies carried out in this thesis can be summarised as follow:

- Four different Indian isolates of RTBV were cloned from tungro infected rice plants representing different geographically distinct regions of India. These clones were named as follow - Assam isolate (pRTBV210 and pRTBV211), Andhra Pradesh (pRTBV209), Tamil Nadu isolate (pRTBV208), and West Bengal (pRTBV206) respectively. Out of these four cloned Indian isolates, only West Bengal isolate was found to contain full-length clone of size 8.0kb. Others were obtained as a partial clone. Assam clone (pRTBV210 and pRTBV211) was obtained in two pieces of sizes 6.5kb and 1.5kb respectively. Andhra Pradesh (pRTBV209) and Tamil Nadu (pRTBV208) clones were obtained as small fragment of sizes 1.0kb and 2.0kb respectively. Both the Assam clones...
(pRTBV210 and pRTBV211) were obtained in Cla I restriction enzyme sites. Andhra Pradesh (pRTBV209), Tamil Nadu (pRTBV208) and West Bengal (pRTBV206) clones were obtained in Xba I, Hinf III and BamH I restriction enzyme sites respectively.

- All the above clones obtained were partially sequenced (approximately 10kb) from the ends using automated DNA sequencer. The sequences generated were compared with Philippines isolate. The sequence study showed identity of 75% in Assam isolate, 99% in Andhra Pradesh isolate, 70% in Tamil Nadu isolate and 80.2% in case of West Bengal isolate. The sequence alignment indicated that West Bengal isolate represent full-length clone. Assam isolate was obtained in two pieces. Assam isolate (pRTBV210) represented region of ORF II to ORF IV including the regions of ‘MP’, ‘CP’, ‘PR/TRNAaseH’ and that of pRTBV211 represented ‘intergenic’, and ‘ORF I’ region. These two clones (pRTBV210 and pRTBV211) when taken together, represented the full RTBV genome. Andhra Pradesh clone (pRTBV209) represented the region of part of ‘intergenic’ and full ‘ORF I’ region. Tamil Nadu clone (pRTBV208) represented PR/TRNAaseH region.

- The percentage identity at the nucleotide level and amino acid level was determining by aligning the sequences of different regions (ORF I, ORF II, MP, CP and intergenic) of cloned Indian RTBV isolates with Philippines isolate. It was found that Indian isolates showed approximately 60-90% identity with Philippines isolate. However, exception was seen in case of Andhra Pradesh isolate (pRTBV209), which showed 100% identity with Philippines isolate at both nucleic acid and amino acid level.

- Restriction map gives a physical location of different restriction enzymes sites on DNA molecules. As the clones of Andhra Pradesh (pRTBV209) and Tamil Nadu isolate (pRTBV208) isolates were very small, restriction maps were not
constructed. Only clones of West Bengal (pRTBV206) and Assam (pRTBV210 and pRTBV211) isolates were processed for constructing restriction maps. The restriction maps of different cloned Indian isolates and Philippines isolate were compared with each other. The Indian isolates showed more resemblances among them as compared to Philippines isolate along with few differences. EcoRI was the only restriction enzyme which had same number of recognition sites in all compared isolates including Philippines isolates. SalI restriction enzyme was found to be absent in all cloned Indian isolates.

- Viruses are variable in nature. The lack of proof reading mechanisms in retroviruses during their genome replication have resulted in generation of population of quasi-species. Studies of variabilities in viruses are very important from epidemiological as well as from evolutionary point of view. Analysis of variation makes possible the identification of conserved as well as variable regions in the genome, which are essential for virus diagnosis. Studies carried out by Fan et al., 1996 have indicated that Indian RTBV isolates contain a deletion of 64bp in non-coding region when compared with those from Southeast Asia. Recent analysis (Nath et al., 2002) of two full-length cloned genome of RTBV from India has revealed a 30 nucleotide deletion within the coding region in one of the them which might have a correlation to the strainal variation noted earlier. A variability study using techniques of PCR-RFLP was carried out in cloned RTBV DNA and naturally infected field rice samples from different field locations in India. Between two Indian isolates [West Bengal (pRTBV203) and Andhra Pradesh (pRTBV304)], the pRTBV304 isolate, contained a deletion of 30bp in coding region. Within the above 30bp deletion region, there exists one recognition site for AgeI restriction enzyme. Thus the differences in AgeI restriction digestion patterns of the amplified RTBV DNA was used as a tool to detect genetic variability. The result indicated the presence of mixed population of viral genome in field samples studied. Thus reinforcing the concept of quasi-species in RTBV field population.
SUMMARY

- Plant viral promoters have played an important role in determining the functions of various foreign gene expressed in transgenic plants. RTBV promoter has the potential to be used to drive transgene expression in rice for agronomic improvement. The first report of RTBV promoter analysis was reported by Bhattacharyya-Pakrashi et al., 1993. They studied the promoter activity in transgenic rice and tobacco and showed that RTBV promoter was phloem specific and its activity was comparable to CaMV35S promoter. To initiate studies on the gene expression of Indian RTBV, expression of the putative promoter region of one of the Indian isolate (pRTBV206) was investigated. The putative promoter region of pRTBV206 isolate was cloned and its activity was tested in rice calli. The results indicated that cloned RTBV DNA of pRTBV206 isolate contained a functional promoter. The promoter activity was found to be three times higher (9.765 nmoI/mg protein/h) as compared to control i.e. with vector alone which showed activity of 2.47 nmoI/mg protein/h.

- When any genomic material is able to replicate inside host cell and able to cause disease, it is said to be infectious. An infectious clone helps to study the function of various genes. Any gene can be mutated and reintroduced as a part of above clone so that its phenotypic effects can be analysed and compared with wild type. RTBV DNA cannot be mechanically inoculated in host plant. However, agromoculation has been shown to be able to successfully initiate RTBV infection in rice (Dasgupta et al., 1991). This involves cloning of RTBV DNA as more-than unit length in binary vector so that more-than full length transcript is formed. For this promoter is required to be duplicated. In RTBV, the promoter element lies in large intergenic region. Two different strategies were followed in an attempt to construct infectious clone from West Bengal isolate (pRTBV206), but none of the strategies were successful.
CONCLUSION
CONCLUSION

In this study, four Indian isolates of RTBV were cloned from tungro infected rice plant material collected from four different geographically distinct regions of India. Out of the four cloned Indian isolates, West Bengal isolate (pRTBV206) was found to contain full-length genome of RTBV. The Assam isolate was cloned as two pieces (pRTBV210 and pRTBV211). The Andhra Pradesh isolate (pRTBV209) and Tamil Nadu isolate (pRTBV208) were obtained as small fragment of sizes 1kb and 2kb respectively. The restriction maps of Assam and West Bengal isolates were constructed. Comparative study of restriction map showed some homology with Philippine isolate. These cloned RTBV DNA were found to be more closely related among each other as compared to Philippine isolate.

Studies carried out by Nath et al., 2002 has revealed a 30nt deletion with the coding region of one of the two full-length cloned genomes of RTBV from India, which might have a correlation to a strainal variation noted earlier. This study of variation using PCR-RFLP technique was carried out in cloned RTBV DNA mentioned above and RTBV from naturally infected rice leaf samples collected from diverse field locations in India. The result illustrated the presence of complex population of RTBV molecules in field samples studied. This method can be used in the future as tool for surveying the population structure of tungro epidemics in Indian sub-continent and it can be modified and used anywhere in the world, wherever monitoring a virus population is to be done.

The function of promoter of pRTBV206 was investigated by transient expression assay in callus and was found to be comparable to GUS expression vector pCAMBIA1381. This promoter in future can be used to drive expression of some important genes and their function can be studied.

An attempt was made to construct infectious clone from West Bengal isolate pRTBV206, but no success was obtained.
APPENDIX I
APPENDIX I

Composition of reagents
30% Acrylamide solution
* 29.2% Acrylamide
  0.8% N,N-Methylene-bis Acrylamide

Bradford's Dye
* 0.01% w/v Coomassie brilliant blue G-250
  10.0% Orthophosphoric acid
Coomassie brilliant blue G250 was dissolved in 0.05 volume 95% ethanol.

Denhardt's solution (100X)
* 2.0 % w/v Ficoll
  2.0 % w/v BSA
  2.0 % w/v PVP

Denaturation solution
1.5 M NaCl
0.5 M NaOH

DNA loading dye (10X)
* 0.25 % Bromophenol blue
  0.25 % Xylene cyanol
  50 % Glycerol

GUS extraction buffer
**50 mM Sodium phosphate buffer pH 7.0
  10 mM EDTA pH 7.0
  0.1% Triton X-100
  10 mM β-ME

GUS assay buffer
* 0.1 mM MUG in GUS extraction buffer

Luria Bertani medium (LB)
1 % w/v Bacto-tryptone
0.5 % w/v Yeast extract
APPENDIX I

1 % w/v NaCl
(The pH of the medium was adjusted at 7.0 with 1 N NaOH solution).

Neutralisation solution
1.5 M NaCl
0.5 M Tris-HCl pH 7.2
0.001 M EDTA

Prelhybridization solution:* (For Southern and colony hybridisation)
5 X SSC
5 X Denhardt solution
0.5 % SDS
100 µg/ml HS DNA

Solution I
50 mM Glucose
25 mM Tris - HCl pH 8.0
10 mM EDTA

Solution II
1 % SDS
0.2 N NaOH

Solution III
3 M CH₃COOK pH 5.2

SSC (20X)
3 M NaCl
0.3 M Sodium Citrate

TBE (10X)
0.9 M Tris-borate
0.02 M EDTA pH 8.0
APPENDIX-I

TE Buffer
10 mM Tris-Cl pH 8.0
1 mM EDTA pH 8.0

10X TAPS Buffer
100mM TAPS (N-Tris hydroxymethyl methyl 3 azinopropane sulfonic acid) pH 8.0, 20mM MgCl₂
500mM KCl
0.1% BSA

Solution I
2XSSC
0.1% SDS

Solution II
1XSSC
0.1% SDS

Solution III
0.5X SSC
0.1% SDS
### CHEMICAL COMPOSITION OF CULTURE MEDIA USED.

<table>
<thead>
<tr>
<th>Compound</th>
<th>[N₁ Medium]</th>
<th>[R₂ Medium]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chu et al.,</td>
<td>Ohira et al.,</td>
</tr>
<tr>
<td></td>
<td>1975</td>
<td>1973</td>
</tr>
<tr>
<td></td>
<td>mg/l</td>
<td>mg/l</td>
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<tr>
<td><strong>Major Salts</strong></td>
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<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>166</td>
<td>147</td>
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<tr>
<td>KH₂PO₄</td>
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<tr>
<td>KNO₃</td>
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<td>4040</td>
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<tr>
<td>MgSO₄·7H₂O</td>
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<tr>
<td>NaH₂PO₄·2H₂O</td>
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<tr>
<td>(NH₄)₂SO₄</td>
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<td><strong>Minor Salts</strong></td>
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<td>CuSO₄·5H₂O</td>
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<td>KI</td>
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<td>MnSO₄·4H₂O</td>
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<tr>
<td>Na₂MoO₄·2H₂O</td>
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<tr>
<td>ZnSO₄·7H₂O</td>
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<td><strong>Organic Compounds</strong></td>
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<td>Thiamine Hydrochloride</td>
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<tr>
<td>Pyridoxine Hydrochloride</td>
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<tr>
<td>Nicotine acid</td>
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<tr>
<td><strong>Iron Source</strong></td>
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<tr>
<td>FeSO₄·7H₂O</td>
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<td>Na₂EDTA</td>
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<td><strong>Carbohydrates</strong></td>
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<tr>
<td>Sucrose</td>
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<tr>
<td>Casein hydrolysate</td>
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<td>2,4-D</td>
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<tr>
<td>Proline</td>
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<tr>
<td><strong>pH</strong></td>
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<td>6.0</td>
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</tbody>
</table>

* Sterilisation of the solution was done by autoclaving at 15 lb/inch² for 15 minutes

** Solution, which cannot be autoclaved.

*** Solutions were autoclaved before adding β-ME and Triton X-100.
Source of chemicals and enzymes

General analytical grade salts and reagents were obtained mostly from Glaxo India Ltd., Mumbai, India, Sisco Research Laboratories, Pvt., Ltd., India or E-Mere (India) Ltd. Bacterial culture are procured from Hi-Media Laboratories Pvt. Ltd., India, Hispan lab, S.A., Madrid, Spain. Molecular biology grade reagents were purchased from Roche Molecular Biochemical, Germany, Sigma Chemical Co., St. Louis, USA, United States Bio chemicals, USA or Amersham Pharmacia Biotech, UK. Restriction enzymes, T4 DNA polymerase, Klenow DNA polymerase and kits were purchased from Roche Molecular Bio molecules Germany, Promega USA or New England Bio labs, Hertfordshire, UK. Radioactive Chemicals ($^{32}$P) were supplied by Board of Radiation and isotopes Technology (BRIT) India. Nylon based membranes were procured from Amersham Pharmacia Biotech, UK.
APPENDIX II
APPENDIX II

ABSTRACTS:


APPENDIX II

PUBLICATIONS


REFERENCES


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