Rice tungro bacilliform virus DNA independently infects rice after Agrobacterium-mediated transfer

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In nature, rice tungro disease is caused by an RNA and a DNA virus complex, but we have obtained an independently infectious clone of rice tungro bacilliform virus (RTBV) DNA. Infectivity could be demonstrated only when a more than unit-length copy was cloned in the Agrobacterium binary vector Bin19 and agroinoculated into rice plants. Rice plants thus agroinfected with cloned RTBV DNA showed typical symptoms of tungro disease, presence of viral DNA and bacilliform particles, and could be used as a source of virus to infect healthy plants by the green leafhopper (Nephotettix virescens). The importance of this infectious clone in understanding the molecular biology of RTBV and the rice tungro disease is discussed.

Introduction

Rice tungro is an important viral disease of rice in tropical Asia, causing severe yellowing and stunting in rice. It is a composite disease caused by two viruses, rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV) (Hibino et al., 1978). RTSV and RTBV can apparently occur separately, as assessed by serological reactions. Rice plants naturally infected with RTBV alone show generally severe symptoms as compared to almost no symptoms in the case of RTSV infection but, when the viruses are present together, they cause the even more severe form of the disease (Hibino et al., 1978). However there is no conclusive evidence that the viruses replicate completely independently. In the case of apparent single infection, there is the possibility that the other virus had been present or still is at a level below the limit of serological detection. The viruses are transmitted in a semipersistent manner, from plant to plant, by the green leafhopper, Nephotettix virescens (Hibino et al., 1979). RTSV can be transmitted efficiently and independently of RTBV by N. virescens, but RTBV depends on the presence of RTSV, or more likely a factor from RTSV-infected plants, for its transmission (Cabauatan & Hibino, 1985). Neither RTSV nor RTBV nor the complex is mechanically transmissible to rice.

Recently we showed (Jones et al., 1991) that RTBV contains dsDNA and shows several properties which suggest that, like caulimoviruses, it replicates by reverse transcription. Several potentially full-length clones of RTBV DNA have been constructed and we wished to know whether these included a clone which would be infectious. An infectious clone would facilitate the molecular analysis of the tungro disease, by making it possible to reintroduce mutated and wild-type viral genes into the plant, independent of the insect vector, and thus to test the phenotypic effect of mutations in the viral DNA.

Cloned DNA of caulimoviruses is infectious when mechanically inoculated on host plants, after enzymic excision from the cloning vector (Howell et al., 1980; Lebeurier et al., 1980). Because of the reported similarities between RTBV and caulimovirus DNAs (Jones et al., 1991), mechanical inoculation of cloned RTBV DNA, after suitable restriction enzyme digestions, was tested to see whether it was infectious. Another method of testing infectivity, bombarding rice seedlings with microprojectiles (Klein et al., 1987) coated with RTBV DNA (biolistics) was also tried.

Agroinoculation is the term given to the process of introducing viral or viroidal genomes into plants by the mediation of the soil bacteria, Agrobacterium tumefaciens or A. rhizogenes resulting in an 'agroinfection' (Grimsley et al., 1986; Boulton et al., 1989). It has been used to produce infections in monocotyledons with cloned viral DNAs of maize streak virus (MSV), digitaria streak...
virus and wheat dwarf virus, viruses which are solely insect-transmitted (Grimmley et al., 1987; Donson et al., 1988; Woolston et al., 1988). Here, we report that infectivity of the cloned RTBV DNA was demonstrated by agroinfection only.

**Methods**

**Source of tungro viruses and cloning of RTBV DNA.** Rice (*Oryza sativa*) cv. Taichung Native 1 (TN1) infected with both RTBV and RTSV, and the rice green leafhopper *N. virescens* were obtained from the International Rice Research Institute, The Philippines (IRRI) and maintained under containment glasshouse and insectary conditions. RTBV DNA was isolated from virus particles by Pronase treatment and phenol-chloroform extraction according to the method of Jones et al. (1991), and cloned in pUC18 at the SalI site to obtain pJIIIS2.

**Direct DNA inoculation.** The viral DNA insert from pJIIIS2 was excised with SalI and used either directly or after gel purification and religation. DNAs were inoculated at a concentration of 250 µg/ml and, to some samples, denatured calf thymus DNA was added at a final concentration of 1 mg/ml. The leaf surfaces of 3-week-old TN1 plants were dusted with ‘celite’ for abrasion and 20 µl of the DNA sample was applied. Plants were tested for the presence of viral antigens 3 to 4 weeks after the sample application.

**Bombarding rice seedlings with tungsten microprojectiles coated with cloned viral DNA.** TN1 seeds were sterilized with 10% Domestos bleach (Lever Brothers) for 30 min, followed by four washes in sterile water. Seeds were germinated on the surface of solidified nitrogen-free agar medium at 27°C in light for 5 days (Al-Mallah et al., 1987). The seedlings were laid side by side in batches of 20 on filter papers in Petri dishes and placed either 3.0 or 4.5 cm from the stopper plate of the ‘Biolistic Gun’. Samples were prepared as described in the previous section. Sample DNA, prepared exactly as above (in the second section of Methods) (1 µg/ml) but without the calf thymus DNA was mixed with 10 times the volume of 2.5 M-CaCl₂ and four times the volume of 0.1 M-spermidine free base. The sample was then mixed with an equal volume of tungsten particles, incubated at room temperature for 10 rain, vortexed and 2.5 gl was used for each treatment.

**Construction of plasmids for agroinoculation.** It has been reported by Grimsley & Bisaro (1987) that for successful agroinfection, it is necessary to have cloned viral DNA of more than unit-length. The DNA manipulations leading to the construction of the partial (pRTB-1106) and the unit-plus-partial clone, ‘one-and-a-bitter’ (pRTB1162), of RTBV in the binary Agrobacterium-**Escherichia coli** vector Bin19 (Bevan, 1984) are shown in Fig. 1. Bin19 DNA (12 kb) was doubly digested with SalI and SalI and ligated with pJIIIS2 (10.6 kb) doubly digested with EcoRV and SalI (Fig. 1, step 1), and was used to transform *E. coli* JM83 cells. To confirm the presence of RTBV sequences in the transformants, the smallest BglII fragment of pJIIIS2 (fragment a, Fig. 1) was radioactively labelled with [³²P]dCTP by the random priming method (Feinberg & Vogelstein, 1983) and used to screen plasmid DNA from transformed JM83 cells by dot blot hybridization (Sambrook et al., 1989). A clone (pRTB1106) (13 kb) containing the 1-3 kb EcoRV-SalI piece of pJIIIS2 in the multicloning site of Bin19 was obtained. SalI-linearized pRTB1106 was ligated with SalI-linearized pJIIIS2 and used to transform JM83 (step 2, Fig. 1). Plasmid DNAs were isolated from transformed clones and screened using the 1-0 kb EcoRV fragment of pJIIIS2 as the radioactive probe (fragment b, Fig. 1) by dot blotting. Samples showing hybridization with that probe were analysed by restriction digests and those with the insert in an orientation producing a direct repeat of the viral DNA at the ends were selected. Recombinant plasmid pRTB1162 was such a clone, and had the 1-3 kb EcoRV-SalI fragment of pJIIIS2 directly repeated at the ends.

**Conjugal transfer of plasmids from *E. coli* to Agrobacteria.** Transfer of plasmids pRTB1106 and pRTB1162 from *E. coli* to Agrobacterium was by triparental mating (Ditta et al., 1980). Since different Agrobacterium strains were shown to have different efficiencies in transferring viral DNA to monocotyledons (Boulton et al., 1989), six Agrobacterium strains (Table 1), having different opine utilization characters, chromosomal backgrounds and plasmid DNAs, were used for conjugation with *E. coli* carrying pRTB1162 to maximize the chances of DNA transfer to rice plants. One of the strains, LBA4301(pTiC58), was recombination-deficient (Klapwijk et al., 1979), which ensured a better stability of the plasmid construct by preventing homologous recombination. All *E. coli* and *A. tumefaciens* strains were grown on Lennox (L) medium (Lennox, 1955) and *A. rhizogenes* strains were grown on YM medium (Ooms et al., 1985).
Where necessary, kanamycin, rifampicin or nalidixic acid were added to the medium at a final concentration of 50 μg/ml.

Agroinoculations. These were performed by the method of Boulton et al. (1989). Typically, TN1 plants 4 to 6 weeks old were injected at the base of their stems with 50 μl of a viscous suspension (more than 10^10 c.f.u/ml) of the relevant bacterial strain, using a Hamilton microsyringe. Plants were grown in the glasshouse under containment conditions at 22 °C with supplementary lighting to give a 16 h day for another 4 to 6 weeks before testing them for the presence of viral antigens.

Tests for RTBV infection
(i) Symptoms. Treated plants were observed at regular intervals for signs of yellowing and stunting and were compared with controls.
(ii) Detection of viral antigens. Plants were tested for the presence of antigens of RTBV and RTSV by ELISA as described in Jones et al. (1991) using the method of Barbara & Clark (1982). Specific polyclonal antibodies against RTBV and RTSV (a gift from Dr H. Hibino, IRRI) were used to detect the viral antigens.
(iii) Electron microscopic visualization of the virus particles. Immunosorbent electron microscopy (ISEM) was used to detect virus particles in plants by a modification of the method of Roberts & Harrison (1979). Carbon-coated copper grids (TAAB Laboratories) were floated on 10 μl of anti-RTBV antiserum diluted 10^−2 in 0.06 M-phosphate buffer pH 6.5, on a wax surface at 25 °C for 1 h. Leaf samples to be tested were ground in 0.06 M-phosphate buffer pH 6.5 at a ratio of 1:10 (w:v). Antibody-treated grids were washed and placed over 10 μl of the above leaf extract at 4 °C overnight. The grids were then washed, stained with 2% uranyl acetate and observed using a JEM 1200 EX (JEOL) microscope.

Transmission experiment. This experiment was performed to investigate whether the virus particles in the agroinfected plants could be transmitted by N. virescens to healthy plants. One batch of non-viruliferous insects was allowed to acquire virus from agroinfected plants and another batch from RTSV-infected plants. Half of the insects of the first batch were used directly to inoculate healthy plants and the other half were used for acquiring virus from agroinoculated plants before inoculating healthy plants. Insects were allowed to acquire virus for 48 h and the inoculation access time was 6 days. Typically, two insects were used for each plant.

Detection of discontinuous DNA. Viral DNA from agroinfected and insect-infected plants was isolated by the method of Jones et al. (1991). Denaturation was carried out by immersing samples in a boiling water bath for 2 min, followed by 2 min rapid cooling on ice. Gel electrophoresis and Southern blot analysis, using pJIIS2 as the radioactive probe, were carried out by standard procedures (Sambrook et al., 1989).

Results

Construction of plasmids for agroinoculation
Initially, cloning the tandem dimers of pJIIS2 in Bin19 was tried, but was unsuccessful, probably due to instability of the construct. pPRTRB1162 was found to be stable in both E. coli and Agrobacterium.

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Opine utilization†</th>
<th>Chromosomal background</th>
<th>Plasmid</th>
<th>Plants infected/ inoculated‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. tumefaciens</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C58</td>
<td>nop</td>
<td>C58</td>
<td>pTiC58</td>
<td>3/60</td>
</tr>
<tr>
<td>LBA4301 (pTiC58)§</td>
<td>nop</td>
<td>Ach5</td>
<td>pTiC58</td>
<td>29/60</td>
</tr>
<tr>
<td>LBA4404</td>
<td>oct</td>
<td>Ach5</td>
<td>pAL4404</td>
<td>0/60</td>
</tr>
<tr>
<td>A. rhizogenes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A4</td>
<td>agr</td>
<td>A4</td>
<td>pRiA4</td>
<td>108/200</td>
</tr>
<tr>
<td>LBA9402</td>
<td>agr</td>
<td>1855</td>
<td>pRi1855</td>
<td>31/60</td>
</tr>
<tr>
<td>NCPPB8196</td>
<td>man</td>
<td>8196</td>
<td>pRi8196</td>
<td>0/60</td>
</tr>
</tbody>
</table>

* All the strains carried the plasmid pRTRB1162. Control inoculations were performed with all strains carrying the plasmids pRTB1106, and E. coli JM83 carrying pPRTRB1162. None of the control plants was infected.
† nop, Nopaline; oct, octopine; agr, agropine; man, mannopine.
‡ Infection was detected by testing for the presence of viral antigens. § This was a rec− strain.

Direct DNA inoculation and biolistics
Eighty plants from the direct DNA inoculation experiment and 200 from the biolistics experiment were tested for the presence of viral antigens. None of the DNA-inoculated plants was positive; one plant from the biolistics experiment showed the presence of RTBV antigens but died before it could be retested.

Agroinfection
The results of agroinoculation with pPRTRB1162 using three strains of A. tumefaciens and three of A. rhizogenes are shown in Table 1. Of the six Agrobacterium strains used, one A. tumefaciens strain, LBA4301(pTiC58), and two A. rhizogenes strains, A4 and LBA9402, each gave rise to almost 50% infected plants. Control inoculations with pRTB1106 in all six Agrobacterium strains failed to produce a single infected plant, out of the 30 to 40 injected with each strain. JM83, containing pPRTRB1162, did not infect any of the plants inoculated, demonstrating that the process was Agrobacterium-specific. No RTSV antigens could be detected in any of the plants tested. Infected plants showed pronounced stunting but little yellowing, within 4 to 6 weeks after injection (Fig. 2). As in insect-infected plants, symptoms were noticeable within 2 weeks after injection. Agroinfected plants showed the presence of bacilliform virus particles of the same dimensions as that of RTBV, when leaf homogenates were observed under the electron microscope (Fig. 3). No RTSV particles were found.
Table 2. Virus transmission by *N. virescens* using agroinoculated and/or RTSV-infected rice plants for acquisition and healthy plants for inoculation

<table>
<thead>
<tr>
<th>Acquiret</th>
<th>First plant</th>
<th>Second plant</th>
<th>No. of inoculated plants</th>
<th>No. of infected plants*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agroinoculated</td>
<td>RTSV</td>
<td>None</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Agroinoculated</td>
<td>None</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RTSV</td>
<td>None</td>
<td>10</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>RTSV</td>
<td>Agroinoculated</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
</tbody>
</table>

* No plants infected with RTBV alone were detected.
Discussion

The data presented in this paper show that a full-length clone of RTBV DNA was infectious when delivered into rice plants using Agrobacterium. Agroinfection produced typical symptoms of tungro bacilliform virus infection on rice, and virus particles were observed in extracts from the infected tissue. These particles could be transmitted to healthy rice plants by N. virescens only when the insects had been fed on RTSV-infected plants. These data show also that RTBV can replicate, spread and cause symptoms in rice independently of RTSV, which is however required for transmission. In the agroinfected plants, the virus retained its normal transmission characteristics. The observation that virion DNA from agroinfected plants, introduced as covalently closed circular molecules, possessed discontinuities strengthens the belief that it replicates by reverse transcription of an RNA species (Jones et al., 1991), as reported in cauliflower mosaic virus (CaMV) (Hull & Covey, 1983).

However, unlike CaMV, cloned DNA of RTBV was not mechanically transmissible. RTBV is phloem-limited (Saito et al., 1986), whereas CaMV spreads through most tissues of the infected plant. Biolistics is an established method of delivering DNA into plant cells and has been used to obtain expression of the input DNA (Klein et al., 1987). Although, to date, there has been no report of delivery of viral DNA into plant cells by biolistics, it was attempted with cloned RTBV DNA on young seedlings of rice in the hope that the DNA could be introduced directly into the phloem. Absence of infection in those seedlings indicated that the DNA was probably unable to replicate under such conditions.

Preliminary sequence data on the full-length RTBV clone pJIIS2 indicated the presence of a TATA box between the SalI and BamHI sites and a tRNA^Met-binding site, close to the SalI site (unpublished observations) (for map positions, see Fig. 1). These features and their distribution are similar to those of CaMV, where the tRNA^Met binds to the primary transcript and acts as the primer for the synthesis of the positive-sense DNA strand during replication (Hull & Covey, 1983; Guilley et al., 1983; Pfeiffer & Hohn, 1983). For agroinfection with CaMV, it is necessary to have a 'one-and-a-bitmer' clone, comprising an uninterrupted sequence giving rise to the full-length 35S transcript with terminal repeats (Grimsley & Bisaro, 1987). It was thought that a similar situation might exist in RTBV and the same approach was sought while making the 'one-and-a-bitmer' construct (pRTRBl162) for agroinfection. To obtain a construct of RTBV which would give rise to a transcript of more than unit-length with terminal repeats, the SalI-EcoRV fragment was chosen as the fragment which would be repeated in the final construct. Because of the terminal repeats, the above transcript could act as the template for viral DNA synthesis.

Raineri et al. (1990) have shown that embryos of two rice cultivars, Nipponbare and Fujisaka 5, can be transformed using the wide host range supervirulent Agrobacterium strain A281(pTiBo542) and the limited host range strain A856 resulting in the integration and expression of foreign DNA. Our present study indicates that it is possible to transfer foreign DNA to cultivar TN1 using at least three Agrobacterium strains, LBA4301(pTiC58), A4 and LBA9402. We have not yet analysed agroinfected plants for viral DNA integration.

Table 1 shows that the efficiency of agroinfection with
RTBV varied significantly with the strain of Agrobacterium used. A. tumefaciens strain LBA4301(pTiC58), utilizing nopaline, and A. rhizogenes strains A4 and LBA9402, utilizing agropine, were found to mediate agroinfection, whereas agroinoculation using octopine-type strain LBA4404 did not result in infection. Since the chromosomal backgrounds of LBA4404 and LBA4301 are the same, the Ti plasmid appeared to determine the ability to transfer DNA, which was in agreement with the observations on agroinfection with MSV (Boulton et al., 1989). The difference in the efficiency of agroinfection with strain C58 and LBA4301 suggests that the chromosomal background of the bacterium may also be important; this finding requires further study. The strain specificity of Agrobacterium and the inability to produce infection using E. coli containing pTR162 indicate that, whether or not the viral DNA integrates, there is a close interaction between the Agrobacterium and rice.

Agroinfection with RTBV gives a sensitive means of studying this interaction.

Since rice tungro viruses are not mechanically transmissible, it had not been possible to demonstrate conclusively that these viruses are the sole causative agents of the disease. Membrane-feeding of purified viruses to leafhoppers is difficult and not reproducible (Hibino & Cabauatan, 1987; Galvez, 1968). Hibino & Cabauatan (1987) showed that viruliferous leafhoppers, when fed on anti-RTBV or anti-RTSV immunoglobulin, were unable to transmit the respective viruses to plants and concluded that the infectivity neutralization of the viruses, as observed by them, showed that RTBV and RTSV were the virus agents which caused the rice tungro disease in combination. The data presented in this paper represent the first direct evidence that RTBV alone is the causative agent for the severe form of tungro disease.

Agroinfection of rice with RTBV makes the routine production of RTBV-infected plants a much simpler exercise compared to the insect transmission process. Agrobacterium can be cryopreserved indefinitely and used whenever required. Rice plants agroinfected with RTBV can be used as a source of virus for the production of antisera specifically against that virus. This is a major improvement over the procedure of neutralizing RTSV in the insect by feeding them anti-RTSV immunoglobulin through membranes, or that of separating the two viruses during purification (Cabauatan & Hibino, 1988; Omura et al., 1983). Agroinfection can be used to test the tungro-resistant character of rice cultivars, without using the insect vector and thus avoiding the problem of insect resistance (Dahal et al., 1990).

Agroinfection opens up the possibility of mutation analysis of the RTBV genome which would not be feasible with insect transmission. This is likely to become an important method of gene analysis of RTBV and the tungro disease; the knowledge of its molecular biology is still at a very rudimentary stage.

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Infectious clone of RTBV


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