

Delay in virus accumulation and low virus transmission from transgenic rice plants expressing *Rice tungro spherical virus* RNA

Vidhu Verma · Shweta Sharma · S. Vimla Devi ·
S. Rajasubramaniam · Indranil Dasgupta

Received: 17 May 2012 / Accepted: 9 July 2012 / Published online: 24 July 2012
© Springer Science+Business Media, LLC 2012

Abstract Rice tungro, a devastating viral disease of rice in South and Southeast Asia, is caused by the joint infection of a DNA virus, *Rice tungro bacilliform virus* (RTBV) and an RNA virus *Rice tungro spherical virus* (RTSV). RTBV and RTSV are transmitted exclusively by the insect vector Green leafhopper (GLH). RTSV is necessary for the transmission of RTBV. To obtain transgenic resistance against RTSV, *indica* rice plants were transformed using DNA constructs designed to express an untranslatable sense or anti-sense RTSV RNA. Progeny of primary transformants showing low copies of the integrated transgenes and accumulating the corresponding transcripts at low levels were challenged with viruliferous GLH. Three out of four transgenic plant lines expressing untranslatable RTSV RNA in the sense orientation and two out of the four lines expressing an RTSV gene in the anti-sense orientation showed delayed buildup of RTSV RNA over time. Transmission of RTBV from the above lines was reduced significantly.

Keywords *Rice tungro spherical virus* · RT-PCR · Resistance · Transmission · GLH

Introduction

Tungro is a devastating disease of rice in South and Southeast Asia caused by a complex of two viruses, *Rice tungro spherical virus* (RTSV) and *Rice tungro bacilliform*

virus (RTBV) [1]. Green leafhopper (GLH) (*Nephotettix virescens*) functions as the natural vector for transmission of the viral complex, of which, RTSV contributes the transfer functions [2]. RTBV is responsible for the typical yellow–orange discoloration of the leaves and stunting of rice plants in tungro disease, as observed after introducing cloned RTBV DNA into rice through *Agrobacterium* [3]. RTSV is a member of genus *Waikavirus* within family *Secoviridae* and has a single-stranded polyadenylated positive-sense RNA genome of about 12 kb [1, 4, 5], the sequence being highly conserved among all the isolates reported from India and Philippines [6]. The genomic RNA of RTSV consists of one large ORF and possibly two short ORFs present at the 3'-end, although the latter has not been confirmed. The ORF I of RTSV encodes for a polyprotein, comprising three coat proteins (CP) the genes for which are arranged adjacent to each other (*cp1-3*) towards the 5'-end of the genome, a protease, a gene encoding the replicase (*rep*) which is present near the 3'-end of the genomic RNA and some functionally uncharacterized proteins (Fig. 1) [4].

Rice tungro disease is estimated to be responsible for economic losses of 340 million US dollars annually [7] in Southeast Asia and a production loss of 2 % at the national level in India, although regional losses could be very significant [8]. Several sources of genetic resistance against RTSV are known in rice [9, 10]. However, they are poorly characterized and show rapid breakdown under field conditions, possibly in response to changes in vector population [11, 12]. Except for a single resistance gene against RTSV (*tsv1*), encoding a putative translation initiation factor 4G (eIF4G) in chromosome 7 of rice cv. Utri Merah [13], none of them have been characterized in detail.

Transgenic resistance, where a heterologous gene conferring an advantageous trait is transformed into the plant, offers a viable alternative to breeding efforts aiming at

V. Verma · S. Sharma · S. V. Devi · S. Rajasubramaniam ·
I. Dasgupta (✉)
Department of Plant Molecular Biology, University of Delhi
South Campus, Benito Juarez Road, New Delhi 110021, India
e-mail: indranil58@yahoo.co.in

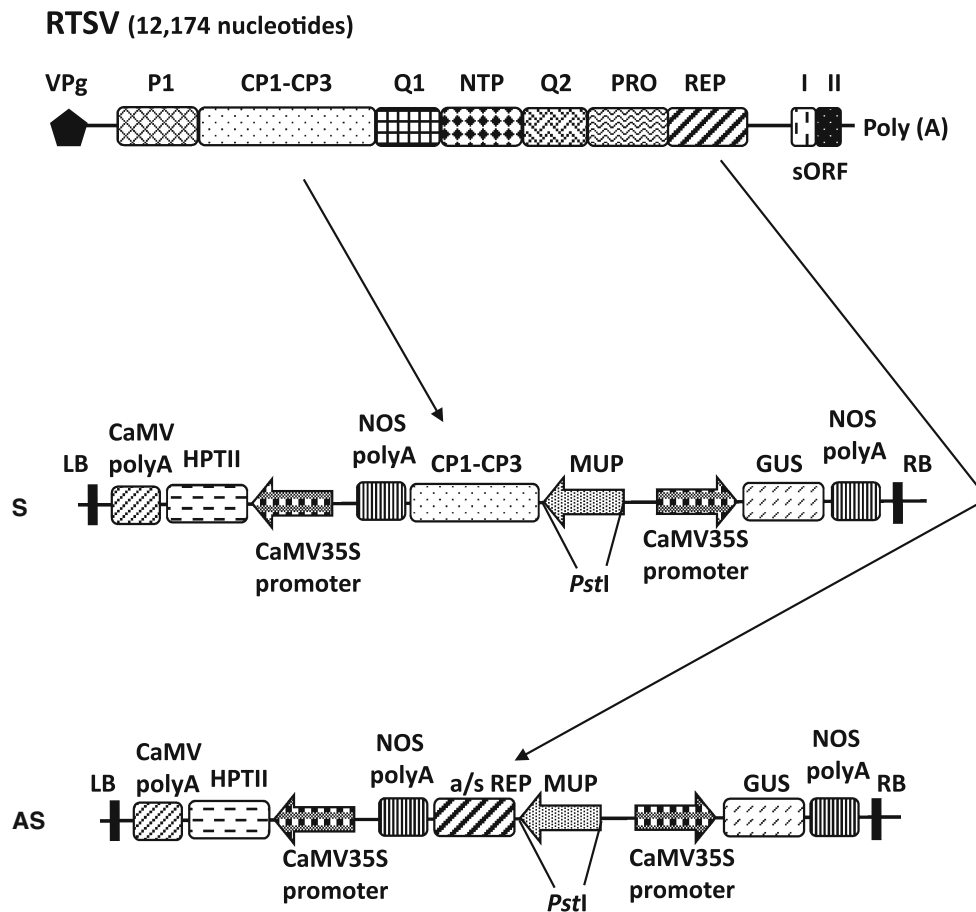


Fig. 1 Schematic representation of the DNA constructs used for the generation of transgenic plants. *Upper portion* shows a schematic diagram of the genomic RNA of RTSV where the polyadenylated RNA is indicated as a *single line* and the coding regions as *shaded boxes*. Transgenic constructs are shown in the *lower portion*. *VPg* viral protein of the genome; *P1* leader protein 1; *CP1-3* coat protein

1-3; *NTP* nucleotide triphosphate binding protein; *Pro* protease; *Rep* RNA-dependent RNA polymerase; *sORF* short ORF; *a/s rep* RTSV replicase gene in anti-sense orientation; *MUP* maize ubiquitin promoter; *nos poly A* nopaline synthase polyA site; *CaMV poly A* CaMV35S polyA site; *HptII* hygromycin phosphotransferase gene. Names of the constructs are indicated at the sides

incorporating resistance to RTSV. RNA-interference (RNAi), is an integral component of many defense responses in plants, involving degradation of specific mRNA [14–19]. RNAi is an important tool for the improvement of several agronomic traits in crop plants [20], including transgenic resistance against viruses [21–30]. The mechanism of transgenic resistance against viruses obtained by RNAi was largely explained using a “threshold model” which proposed that the plant cells have an as yet undefined “threshold level” over-shooting of which activates a cytoplasmic cellular process that specifically target cognate RNA for elimination and results in low steady state levels of the transgene mRNA [31]. However, in several cases, deviation from the threshold model was reported with no correlation observed between the level of transgene expression and RNAi [32, 33]. “Aberrant RNAs”, which originate from incorrect processing of the RNA transcripts and operate through a dsRNA intermediate, inducing the formation of 21–24

nucleotide siRNAs, are proposed to be one such potent trigger of the cytosolic RNA degradation machinery [34]. The aberrant nature such as the formation of truncated, modified, or aberrantly structured transgene mRNA molecules might originate from read-through transcription of the transgenes, multiple-site integrations, abnormal RNA processing, or transcription of a methylated template [35]. The presence of such aberrant RNAs activates the RNA silencing machinery, which, ultimately, results in the degradation of target mRNA in a sequence-specific manner. Therefore, expression of untranslatable or anti-sense viral RNA which could potentially trigger production of aberrant RNA in transgenic plants, is one way of using RNAi against viruses.

In order to obtain RTSV resistance in rice using RNAi, transgenic rice plants were raised using two RTSV genes; one an untranslatable gene in the sense orientation and another in anti-sense orientation, from separate regions of the RTSV genomic RNA. We analyzed the resistance to

RTSV in independent transgenic lines of each of these constructs and observed that a majority showed delayed accumulation of viral RNA and acted as poor source of further transmission of RTBV.

Materials and methods

Growth and maintenance of plants and GLH

Rice seeds of variety pusa basmati-1 (PB-1) were germinated on adsorbent cotton and sown in soil in plastic/earthen pots and watered at frequent intervals. They were grown inside containment glasshouse maintained at a temperature range of 30–32 °C under 14 h/10 h light–dark photoperiod and 80 % relative humidity. GLH collected from the field were reared inside well-aerated cages containing rice plants.

DNA constructs used

For obtaining the untranslatable sense construct a *cp1-3* DNA fragment (~2.2 kb) was amplified by PCR from a cDNA clone representing nucleotide residues 2,431–4,689 of the RTSV-[Ori] RNA [6] by Pwo DNA polymerase (Roche), using primers CP1F (positions 2,431–2,451) and CP3R (positions 4,671–4,689), whose sequences are shown in Table 1. The amplified product was designed to lack a translation initiation codon, making the genes untranslatable. The reaction to amplify CP1-3 was set as follows: 94 °C for 5 min, followed by 10 cycles each of 94 °C (30 s), 58 °C (30 s), and 72 °C (2 min) and 20 cycles of amplification were performed using the same conditions as mentioned for 10 cycles but with an additional 5 s increment in the extension time with a final extension of 10 min at 72 °C. The amplified products were cloned in the *Sma*I site, present between maize *ubiquitin* promoter and *nopaline synthase* terminator, of the binary vector pB4NU [36].

The promoter was cloned in the *Pst*I site and *nopaline synthase* terminator cloned in the *Eco*RI–*Sac*I sites of the binary vector pCAMBIA1301 [37]. The putative clones were screened by colony PCR using CP3F–CP3R (Table 1) and the selected plasmids were confirmed by *Kpn*I–*Sal*I restriction digestion, the resultant construct being called S (sense RNA construct). For anti-sense construct, a 0.5 kb fragment encoding Rep was PCR amplified from a cDNA clone (positions 8,993–9,489) of RTSV-[Ori], by Pwo DNA polymerase (Roche), using primers RepF (positions 8,993–9,009) and RepR (positions 9,475–9,489), whose sequences are shown in Table 1. Thermal cycling involved an initial denaturation at 94 °C for 2 min, followed by 10 cycles of denaturation at 94 °C (15 s), annealing at 62 °C (30 s), and extension at 72 °C (45 s) and 20 cycles of amplification were performed, using the same conditions as mentioned for 10 cycles but with an additional 5 s increment in the extension time, with final extension of 72 °C for 10 min. The amplified product was cloned into *Sma*I site of the binary vector pB4NU under the control of maize *ubiquitin* promoter as described for the S construct. The putative clones were screened by colony PCR using RepF and RepR, as well as by restriction digestion using *Kpn*I and *Bam*HI, whose sites were present on either side of *Sma*I. The completeness and orientation of the construct were determined by nucleotide sequencing and the construct was called AS (anti-sense RNA construct). Representative maps of AS and S are shown in Fig. 1.

Rice transformation and analysis for integration and expression of the transgene

The AS and S constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 by freeze–thaw transformation method [38], followed by transformation of rice variety PB-1 according to a previously reported method [39]. Plants recovered after survival on selection media containing 30 mg L⁻¹ hygromycin were grown on soil and

Table 1 Sequence and origin of primers used in this study

Primers	Position	Sequence (5'–3')
CP1F	2,431–2,451 ^a	<u>GGAATTC</u> AGGCACAAGCTGGTGAGACAG
CP3R	4,671–4,689 ^a	GGGGT <u>ACCCCACTGCCAAATCCTCGAC</u>
REPF	8,993–9,009 ^a	GCTCTAGACTCGAGGCACGTCAGTGGACTAC
REPR	9,475–9,489 ^a	CCATCGATGGTACCAATTCACACTTCGTG
RTBV MP F	999–1,013 ^b	TATGGATCCATGAGTCTTAGACCG
RTBV MP R	1,881–1,898 ^b	GGAGCTCTTCATCAGAATTTATTTTC
CP3F	3,649–3,673 ^a	CTCATGATGTGCTGACCGACAAGA

Underlined nucleotides indicate specific restriction sites incorporated into the primers

^a Genomic position derived from RTSV-orissa isolate (accession number AM234048)

^b Genomic position derived from RTBV-West Bengal isolate (accession number AJ314596)

tested for the presence of the transgene by PCR. Plants testing positive were further analyzed by Southern hybridization using 10- μ g DNA isolated by a standard method [40]. A 2.2 kb fragment corresponding to *cp1-3*, amplified using the primer pair CP1F–CP3R, was used as radioactive probe for southern analysis of S constructs. In case of AS constructs, a 0.5-kb DNA fragment representing part of the *rep* gene fragment amplified using the primer pair RepF–RepR, was used as radioactive probe for southern analysis. To ascertain the transcript levels of the inserted transgene, northern analysis was performed. Progenies (T_1) of selfed primary transformants were tested for the presence of the transgene by PCR, as above, and if positive, used to extract total RNA, using standard methods [41]. The RNA sample was quantified spectrophotometrically at 260 nm using U-2001 spectrophotometer (Hitachi, Japan). Samples were resolved by electrophoresis in formaldehyde–agarose gel. Positively charged nylon membranes (Hybond N⁺, Amersham Pharmacia Biotechnologies) were employed for transfer of the RNA by capillary action using standard protocols [42]. For detecting transgene specific transcript in case of S- and AS-constructs a 2.2-kb RTSV *cp1-3* and 0.5 kb *rep* gene fragments, respectively, were used as radioactive probes. Transcription of the transgene was also studied by RT-PCR of RNA treated with RNase-free DNase using Titan One-tube RT-PCR kit (Roche). Reverse transcription was performed at 55 °C for 30 min followed by 30 cycles of amplification under the following conditions: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s using RepF–RepR (Table 1) for AS and CP3F–CP3R (Table 1) for S-derived lines. As positive control, transgenic plants were assayed for β -glucuronidase (GUS) activity by histochemical staining of leaf tissue [43] or by testing for the accumulation of *gus* transcripts by northern hybridization [42].

Virus resistance assays

Seeds obtained by selfing PCR-positive T_1 plants were germinated in cotton for 10 days, after which PCR was again performed to check for the presence of the transgene and to eliminate the segregants. Five PCR-positive (T_2) plants were used for RTSV resistance assay of the transgenic rice lines. RTBV and RTSV isolates collected from West Bengal, India (RTBV-WB [43] and RTSV-WB [6]), were maintained in rice plants by serial transfer by GLH and was used for the challenge inoculation. Viral resistance assay for RTSV was performed by dot-blot hybridization using RNA for the assay of viral titers. Test plants were inoculated overnight with three GLH per plant. Leaf samples from inoculated and un-inoculated control plants were collected and pooled every 10 days till 40 days post-

inoculation (dpi) and total RNA was extracted from the pool. Approximately 1 μ g of the above RNA was denatured by heating at 65 °C for 10 min followed by spotting on nylon membranes using a dot-blot apparatus (Hoefer). An RTSV cDNA (nucleotide positions 516–2,430), which is neither included in the S construct, nor in the AS construct, was used as the radioactive probe for hybridization to the membrane bearing the blotted RNA samples, using standard protocols [42] to determine the accumulation of RTSV genomic RNA in the tissues.

RTBV transmission from inoculated transgenic and control plants

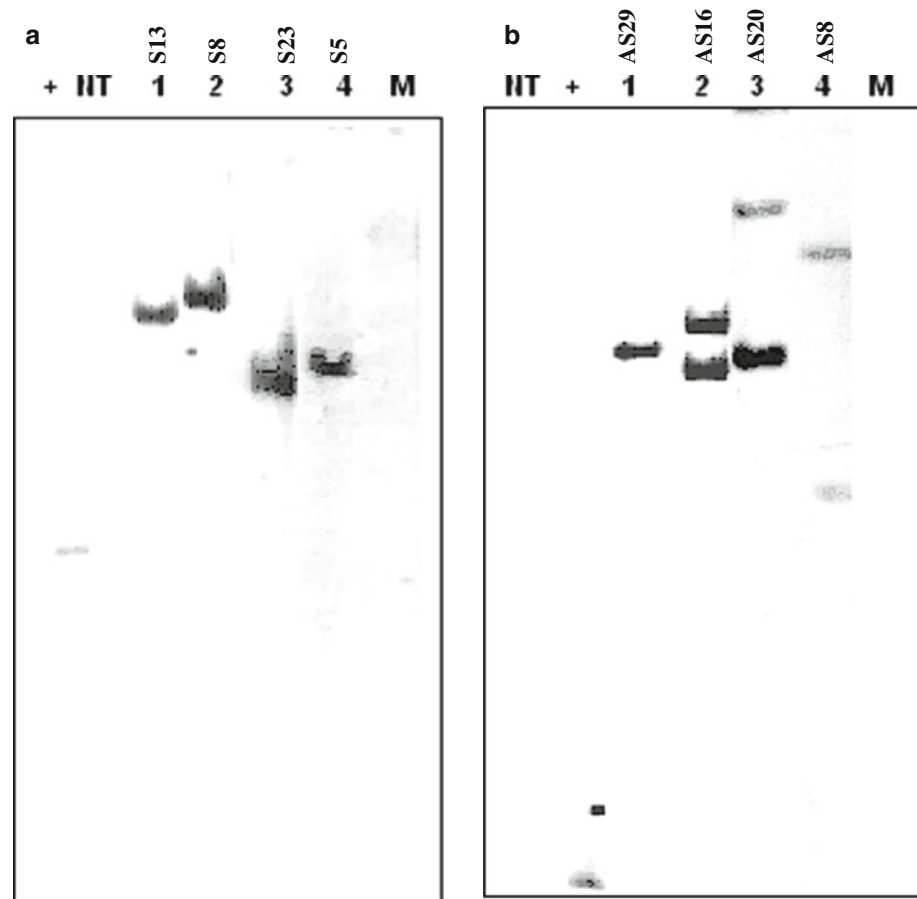
To use plants derived from AS and S as sources of virus inoculum, five PCR-positive T_2 plants were inoculated using viruliferous GLH, along with untransformed controls. The infected T_2 as well as untransformed control plants were used for virus acquisition at 15 dpi and the viruliferous GLH were then used to inoculate five PB-1 plants. All transmission experiments carried out involved both the viruses, i.e., RTBV and RTSV simultaneously. RTBV was assayed at 21 dpi by semi-quantitative PCR using different dilutions of template (undiluted, 1/10, 1/100, and 1/500 dilutions) with the primer pair RTBV MP F–RTBV MP R (Table 1) [45]. The transmission frequency was calculated by percentage of PB-1 plants infected with RTBV by PCR. For each set of transmissions, the percentage of transmission from infected untransformed control plants were taken as 100 and the frequency of five PB1 plants inoculated from a single T_2 line were expressed as its percent fraction.

Results

Integration, copy number, and expression levels of transgene

Fertile hygromycin-resistant rice plants were obtained following *Agrobacterium*-mediated transformation and four lines each, from AS and S constructs, produced PCR-amplified products representing transgenes. Southern analysis of the total DNA extracted from four S (S5, S8, S13, and S23) and AS (AS8, AS16, AS20, and AS29) plants, after digestion with *Pst*I, indicated integration of the respective transgenes at low copy numbers in the genome (Fig. 2). Since *Pst*I cuts twice in the T-DNA region without disrupting the transgene, the number of fragments hybridizing with labeled probe indicates the number of transgene copies integrated into the genome of the transgenic plants. Southern analysis of S-derived plants indicated single copy integration of the transgene at independent sites in all the

Fig. 2 Southern blot analysis of genomic DNA from S and AS transformed rice plants, probed with *cp* and *rep* genes. **a** Lanes 1–4 T₁ plants with S construct (S13, S8, S23, and S5) digested with *Pst*I; NT non-transgenic control; “+” PCR-amplified transgene; M marker. A 2.2 kb fragment corresponding to *cp1-3*, amplified using the primer pair CP1F/CP3R, was used as radioactive probes for Southern analysis. **b** Lanes 1–4 T₁ plants with AS construct (AS29, AS16, AS20, and AS8) digested with *Pst*I; NT non-transgenic control; “+” PCR-amplified transgene; M marker. A 0.5 kb DNA fragment, PCR amplified using the primer pair RepF/RepR, representing part of the *rep* gene fragment was used as a probe for Southern analysis



four lines (Fig. 2a). A similar analysis of the transgenic plants raised using AS construct indicated that line AS29 contained single copy integration while the other three (AS8, AS16, and AS20) contained independent double copy integration of the transgene (Fig. 2b). No hybridization signal was obtained in untransformed control plants.

Northern analyses of mRNA from one PCR-positive progeny of each of the four S-derived T₁ transgenic plants were conducted to determine transcript accumulation in the leaf tissues. Transcripts homologous to the transgene could not be detected in any plant (Fig. 3a, first panel). However, RT-PCR performed on the same samples resulted in products of the expected size, in three (S5.1, S13.1, and S23.1) out of the four lines (Fig. 3a, second panel), while no amplification was obtained from the line S8.1 and untransformed control plants. Interestingly, when probed with a DNA fragment encoding *gus* (contained within the T-DNA of pB4NU), the above three lines showed accumulation of the corresponding transcript by northern analysis (Fig. 3a, third panel). The uniformity in RNA loading, as shown by ethidium bromide staining, is illustrated in Fig. 3a, fourth panel. Similar results were obtained with the PCR-positive T₁ progenies of AS transgenic lines, in which transcripts homologous to the transgene could not be detected in any plant (Fig. 3b, first panel). However, RT-PCR performed on the same samples

resulted in products of the expected size in two (AS8.1 and AS20.1) out of the four lines (Fig. 3b, second panel) while no amplification was obtained from RNA extracted from the other two lines AS29.1 and AS16.1, and untransformed control plants. Histochemical staining was used to check the expression of the *gus* gene, which was part of the T-DNA. Line AS8.1, AS20.1, and AS 29.1 showed GUS activity but not AS16.1 (data not shown).

Pattern of accumulation of RTSV RNA in transgenic plants challenged with RTSV

Five PCR-positive T₂ plants derived from each of the eight PCR-positive T₁ lines, representing four lines each from AS and S were challenged with viruliferous GLH and the accumulation of RTSV RNA was monitored by RNA dot-blot analysis till 40 dpi. Each set of dot-blot was performed separately and were repeated once. A representative dot-blot is shown in Fig. 4. The viral RNA levels increased rapidly and reached a peak in ~20 dpi and did not decrease significantly till 40 dpi in the untransformed control plants, although there were differences in the rates of accumulation between the plants. There was a significant delay in accumulation of RTSV transcripts in the progenies of two AS lines (AS8.1 and AS29.1) and in the progenies

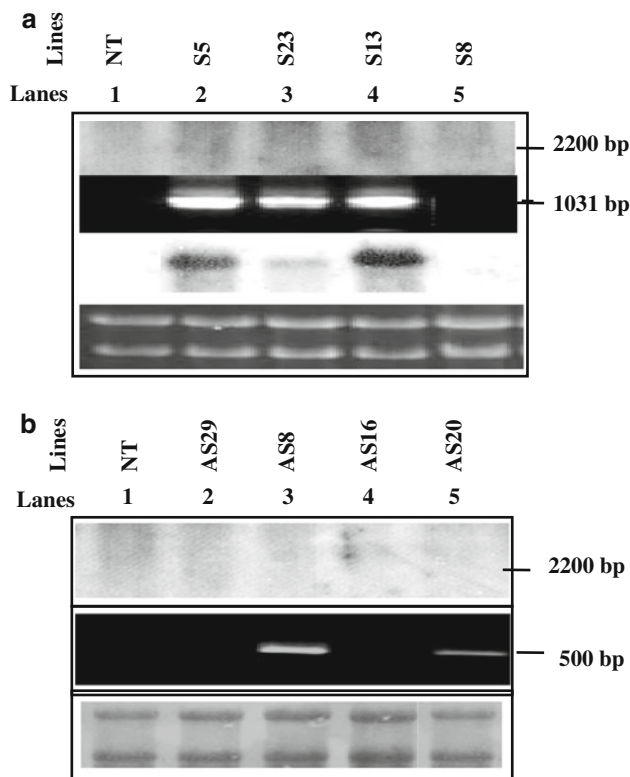


Fig. 3 Expression analysis of transgenes and marker gene *gus* in S and AS transgenic plants. **a** *Top panel* total RNA from S plants (20 µg each) hybridized with *cp1-3* probe; *second panel* RT-PCR products of total RNA of S plant lines, specific to RTSV CP3; total RNA from S plant lines (20 µg each) hybridized with *gus* probe; *fourth panel* ethidium bromide stained gel containing total RNA from S plants. The transgenic lines are indicated on *top* (NT indicates control untransformed plant) and the sizes of the amplified fragments (bp) are indicated at the side, **b** *top panel* total RNA from AS plants lines (20 µg), hybridized with *rep* probe; *second panel* RT-PCR products of total RNA of AS plant lines, specific to *rep*; *third panel* ethidium bromide staining of total RNA isolated from AS plants. The transgenic lines are indicated at the *top* (NT indicates control untransformed plant) and the sizes of the amplified fragments (bp) are indicated at the side

of three S lines (S5.1, S8.1, and S23.1) as compared to the untransformed controls. The accumulation patterns in progenies of the remaining two AS lines (AS16.1 and AS20.1, data not shown) and one S line (S13.1) were similar to the untransformed controls till 30 dpi, the transgenic lines showing a fall in the viral RNA titers thereafter. The results were reproducible. Table 2 summarizes the results related to transgene copy number, transgene expression and the levels of accumulation of transcripts of inoculated RTSV in various lines tested.

RTBV transmission by GLH from pre-inoculated transgenic plants

PCR-positive progeny (T₂) of each of the six T₁ lines (AS8.1, AS16.1, AS20.1, AS29.1, S8.1, and S23.1) were

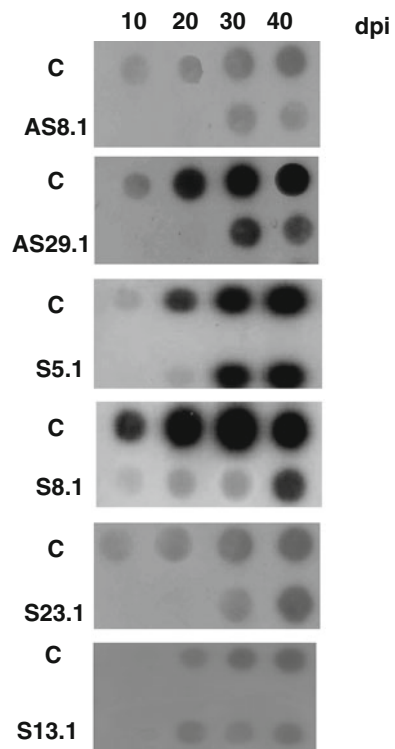


Fig. 4 Accumulation of viral RNA in inoculated control (C) and transgenic rice plants at various time points after virus inoculation. RNA dot-blot was performed using a cDNA region (position 516–2430), which is absent from the transgenic constructs, as the radioactive probe for hybridization to the membrane bearing the blotted RNA samples. Numbers at the *top* indicate days post-inoculation (dpi) at which extraction of the corresponding RNA from inoculated plants was performed and the transgenic lines are indicated at the sides

tested for transmission efficiency of RTBV using GLH and compared with the transmission from untransformed plants. Semi-quantitative PCR analysis of a 900 bp fragment, corresponding to the RTBV DNA, movement protein gene, was employed to assay for the accumulation of transmitted RTBV DNA (Fig. 5a). To obtain a clear indication of the accumulated RTBV DNA levels, the DNA from the inoculated plants were used in three dilutions (1/10, 1/100, and 1/500). While T₂ progenies of AS16.1, AS29.1, and S23.1 inoculated with RTBV and RTSV showed no RTBV transmission at all to PB-1 plants, those of S8.1 showed 62.5 % transmission, considering the transmissions from untransformed controls as 100 %. While progenies of AS8.1 showed 75 % transmission, the progenies of AS20.1 transmitted only at 33.3 % of the controls (Table 2). Consequently, the inoculated PB-1 plants, used to monitor the transmission, showed severe stunting when inoculated using untransformed control plants, whereas, when inoculated using AS and S transgenic lines, the symptoms were either absent or very mild (Fig. 5b).

Table 2 Transgene integration, expression, RTSV resistance and RTBV transmission in transgenic rice plants

Construct	Numbers regenerated following transformation	Transgenic line no. (copy number)	Transgene expression by RT-PCR ^a	GUS expression by northern or histochemical expression	Accumulation patterns of RTSV RNA ^b	Percent transmission of RTBV using the lines as inoculum source ^c
AS	4	AS8 (2)	Y	Y	A	75.0
		AS20 (2)	Y	Y	B	33.3
		AS16 (2)	N	N	B	0
		AS29 (1)	N	N	A	0
S	4	S5 (1)	Y	Y	A	NT
		S8 (1)	N	N	A	62.5
		S13 (1)	Y	Y	B	NT
		S23 (1)	Y	Y	A	0

^a Y indicates transcript detectable by RT-PCR; N indicates transcript not detectable by RT-PCR; NT indicates experiment not performed

^b A indicates delayed accumulation; B indicates fall in viral RNA titer at later stages of infection

^c Percent transmission was calculated as the percent fraction of plants showing RTBV infection by PCR over the plants inoculated using transgenic plants as source. Percent transmissions using untransformed plants as source were taken as 100 %

Discussion

Till date, there have been two reports on the use of viral genes to obtain rice plants resistant to RTSV; one utilizing the expression of CP [46] and the other, *Rep* sequence [47]. The former reported that viral resistance displayed was protein-mediated and the latter, that it was sense RNA-mediated. The investigators reporting CP-mediated resistance did not report any change in the RTBV transmission from the transgenic lines, although the report on the sense RNA-mediated resistance mentioned a decrease in RTBV transmission from the transgenic lines, giving strength to the evidence of helper function of RTSV in the transmission of RTBV. The present report extends the above study to two more constructs; one untranslatable in the sense orientation and another in an anti-sense orientation.

In the two reports [46, 47], viral resistance was assayed by detecting the presence of viral CP by immunological methods at a single time-point, following challenge inoculation. Levels of RTBV in an inoculated plant are reported to fluctuate for several weeks [10, 48], making a single time-based assessment of resistance unreliable. Hence, evaluating resistance by monitoring the temporal pattern of RTBV accumulation in test plants as compared to untransformed controls is considered better as compared to an assay at a single time point. Three investigations on engineering RTBV resistance in transgenic rice plants have used such an assay [26, 48, 49] and the same was used in this study.

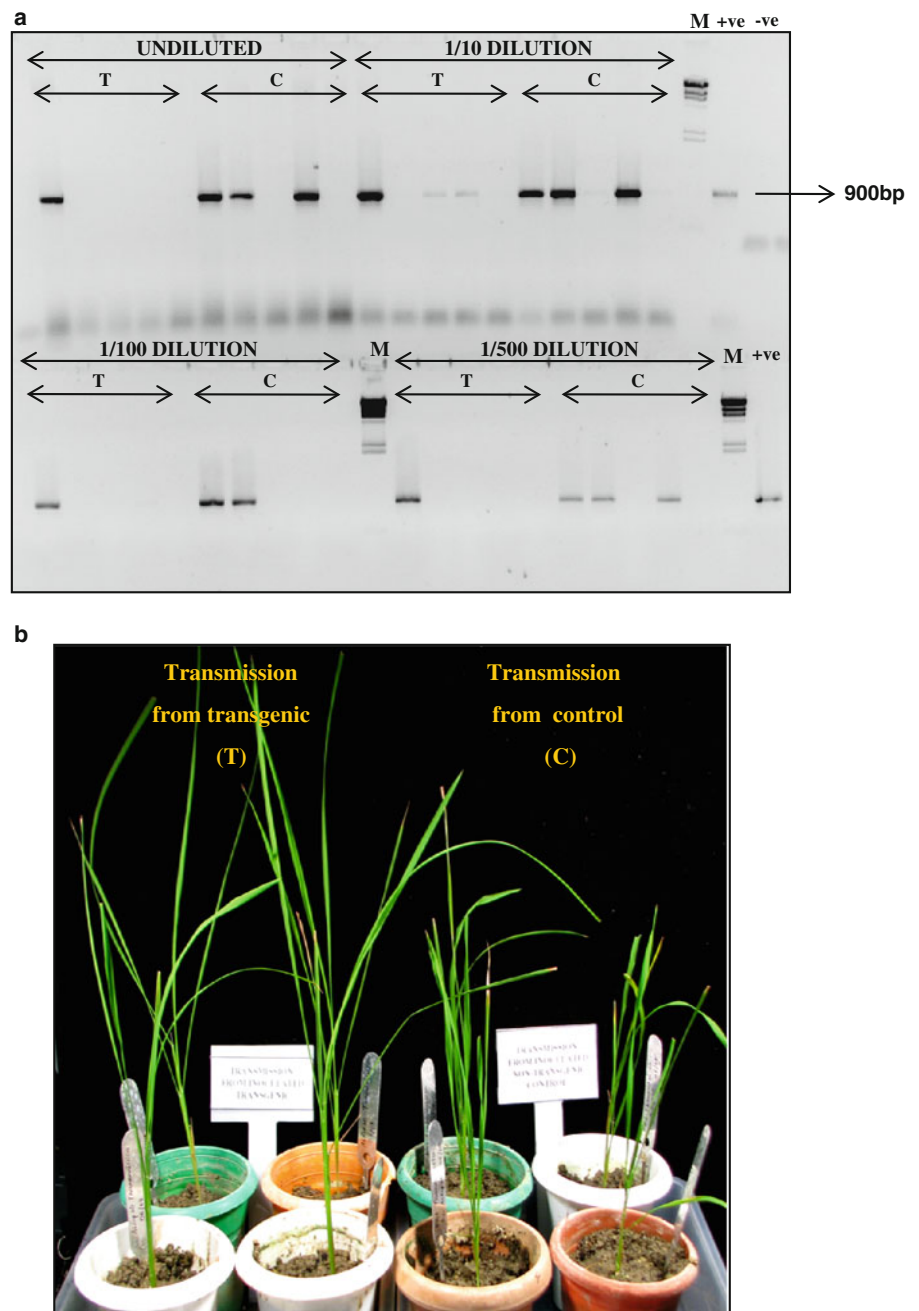
None of the tested transgenic rice lines showed detectable transcript accumulation from the respective transgenes by northern analysis, an observation similar to that reported earlier [47]. This could be attributed to the unstable nature of the transcripts. On the other hand, accumulation of *gus*

transcripts in a high proportion of the plants indicated that the instability of the transcripts may be due to their untranslatable or anti-sense nature. However, RT-PCR-mediated amplification of products of expected size corresponding to the transgene transcript indicated low accumulation of the transcripts, in more than 50 % of the above lines. Surprisingly, the transgenes were under the control of the constitutive maize *ubiquitin* promoter, which is known for its high levels of expression in rice [50].

No clear relationship was found between the transgene copy number, the level of expression of transgene and resistance to RTSV in AS transgenic plants. The fact that independent transgenic lines showed dissimilar patterns of resistance may indicate that different mechanisms maybe responsible for the resistance phenotype. Similar findings with viral replicase-mediated resistance against *potato leaf roll virus* (PLRV), where the expression levels of the transgene were unrelated to the resistance levels, has been reported earlier [51]. Other characteristics, such as viral sequence specificity, configuration, size and genome position of the transgene, etc., have been attributed as important in defining targets for gene silencing [52]. These factors might be playing a part in determining resistance to RTSV.

The levels of signal seen in each set of dot-blot varied, as is clear from the various control blots (Fig. 4), most likely because of the radioactive probes being of different specific activities, representing different sets of experiments. However, it needs to be emphasized that the same probe was used for dot-blot from the transgenic and control samples of each set, making it possible to compare the viral titers in them. Delayed accumulation of inoculated RTSV RNA in two out of four AS lines (AS8 and AS29) and three S lines (S5, S8, and S23), as compared to

Fig. 5 RTBV transmission test for transgenic AS20 and control untransformed plants.
a Amplification of RTBV DNA from rice plants 21 days after transmission from RTSV transgenic line AS20 and non-transgenic control plants infected with RTBV + RTSV. *M* λ /HindIII marker; –ve negative control; +ve positive control; *T* PB1 plants 21 days after transmission from infected RTSV transgenic plants; *C* PB1 plants 21 days after transmission from infected non-transgenic control plants. The size of the amplified fragment (in bp) is indicated at the side,
b symptoms of stunting in rice plants at 15 days after inoculation with leafhoppers, which acquired viruses from infected transgenic line AS20 (*T*) and from infected untransformed control plants (*C*)



untransformed controls, indicated interference in the normal kinetics of viral RNA accumulation. These results are similar to those obtained with *Rice yellow mottle virus* where transgenic plants capable of producing untranslatable mRNA, as well as anti-sense mRNA of the CP showed delayed accumulation and the virus replicated at low levels compared with non-transgenic plants [55]. Two of the remaining AS lines (AS16 and AS20) and one S line (S13) showed a fall in the viral RNA accumulation late in the infection. Both these viral accumulation patterns, i.e., delayed accumulation and fall during late stages of infection resembled those reported for RTBV in transgenic rice

lines containing RTBV-derived double-stranded RNA-expressing constructs [26].

Transgenic resistance against viruses obtained via RNAi could be initiated either by the presence of homologous transcripts that accumulate above a certain threshold level or alternatively by the presence of aberrant RNA transcripts [34]. Aberrant RNAs has been proposed to serve as a template for plant encoded RNA-dependent RNA polymerase (RdRp), which produces a pool of complementary RNA (cRNA) molecules. The cRNA molecules are capable of forming duplex structures on homologous mRNAs such as incoming viral RNAs, which in turn could induce a

nucleolytic cleavage that would render RNA molecules accessible for further degradation [33, 54, 60]. As observed in case of co-suppression, RNAi triggered in response of aberrant RNA transcripts has been the underlying principle in some of the examples of successful transgenic resistance obtained against plant viruses [53, 56–59]. In this study, two constructs viz. S and AS, both capable of producing aberrant RNA were tested for providing resistance against RTSV. It was observed that some of the transgenic lines obtained using these two constructs displayed delayed buildup of the viral RNA which possibly is a result of the targeted degradation of viral RNA by RNAi machinery. Therefore, in this study the aberrant RNA mechanism was found successful in controlling the levels of RTSV in transgenic rice plants.

Since RTSV is responsible for the transmission of the tungro virus complex, plants showing RTSV resistance should act as poor sources of transmission of the viral complex. The results of transmission of RTBV from transgenic lines showed that the transmission was reduced in all six lines tested. The plants inoculated using non-transgenic plants as source of RTBV showed PCR-amplified RTBV DNA even after several fold dilutions indicating high RTBV titers. On the other hand, inoculations from transgenic plants resulted in either no amplifications or amplifications only either at undiluted or at low dilutions, not at higher dilutions of the plant DNA. This indicated low or undetectable levels of RTBV, pointing towards the transgenic plants being poor sources of RTBV. This is the first time that a direct relationship was seen between the levels of RTSV accumulation and transmission of RTBV–RTSV complex by GLH.

Tungro disease has been attributed to be a major factor in the steady loss of rice production in India in the recent years [8]. The results of this investigation strongly indicate that deployment of sense- and antisense-based transgenic resistance against RTSV, in addition to those described earlier [47], is a viable option for reducing the tungro incidences in the field. Reports of low nucleotide sequence variability in RTSV isolates across India and Southeast Asia [6] encourage us to believe that the strategy used in this study could be deployed for achieving tungro resistance for entire South and Southeast Asia. It has been reported that even moderate levels of resistance have a potential to greatly reduce the spread of virus disease by insects in field conditions [61]. The delay in infection by RTSV that was observed in our study may be enough to allow the rice crop to escape disease owing to late viral infection. Combining the above with parallel ones targeting the GLH [62] and RTBV [26, 48, 49] promises to significantly reduce the threat of tungro in the near future.

Acknowledgments This study was funded by Department of Biotechnology, Government of India. VV and SS are grateful to the

Council for Scientific and Industrial Research, New Delhi for Research Fellowships.

References

- H. Hibino, M. Roechan, S. Sudarisman, *Phytopathology* **68**, 1412–1416 (1978)
- P.Q. Cabauatan, H. Hibino, *Philipp. Phytopathol.* **21**, 103–109 (1985)
- I. Dasgupta, R. Hull, S. Eastop, C. Poggi-pollini, M. Blakebrough, M.I. Boulton, J.W. Davies, *J. Gen. Virol.* **72**, 1215–1221 (1991)
- P. Shen, M. Kaniewska, C. Smith, R.N. Beachy, *Virology* **193**, 621–630 (1993)
- H. Sanfaçon, J. Wellink, O. Le Gall, A. Karasev, R. van der Vlugt, T. Wetzler, *Arch. Virol.* **154**, 899–907 (2009)
- V. Verma, I. Dasgupta, *Arch. Virol.* **152**, 645–648 (2007)
- D. Nagadhara, S. Ramesh, I.C. Pasalu, Y.K. Rao, N.V. Krishnaiah, N.P. Sarma, D.P. Bown, J.A. Gatehouse, V.D. Reddy, K.V. Rao, *Plant Biotechnol. J.* **1**, 231–240 (2003)
- K. Muralidharan, D. Krishnaveni, N.V.L. Rajarajeswari, A.S.R. Prasad, *Curr. Sci.* **85**, 1143–1147 (2003)
- O. Azzam, T.C. Chancellor, *Plant Dis.* **86**, 88–100 (2002)
- F.C. Sta Cruz, R. Hull, O. Azzam, *Arch. Virol.* **148**, 1465–1483 (2003)
- I. Manwan, S. Sama, S.A. Rizvi, *Indones Agric. Res. Dev. J.* **7**, 43–48 (1985)
- G. Dahal, H. Hibino, R.C. Cabunagan, E.R. Tiongco, Z.M. Flores, V.M. Aguiro, *Phytopathology* **80**, 659–665 (1990)
- J.H. Lee, M. Muhsin, G.A. Atienza, D.Y. Kwak, S.M. Kim, T.B. De Leon, E.R. Angeles, E. Coloquio, H. Kondoh, K. Satoh, R.C. Cabunagan, P.Q. Cabauatan, S. Kikuchi, H. Leung, I.R. Choi, *Mol. Plant Microbe Interact.* **23**, 29–38 (2010)
- D.C. Baulcombe, *Curr. Biol.* **9**, 599–601 (1999)
- V. Vance, H. Vaucheret, *Science* **292**, 2277–2280 (2001)
- H. Yu, P.P. Kumar, *Plant Cell Rep.* **22**, 167–174 (2003)
- P. Susi, M. Hohkuri, T. Wahlroos, N.J. Kilby, *Plant Mol. Biol.* **54**, 157–174 (2004)
- F. Vazquez, *Trends Plant Sci.* **11**, 460–468 (2006)
- S.W. Ding, O. Voinnet, *Cell* **130**, 413–426 (2007)
- S. Mansoor, I. Amin, M. Hussain, Y. Zafar, R. Briddon, *Trends Plant Sci.* **11**, 559–565 (2006)
- M. Pooggin, S.V. Shivaprasad, K. Veluthambi, T. Hohn, *Nat. Biotechnol.* **21**, 131–132 (2003)
- B.L. Lennefors, E.I. Savenkov, J. Bensefelt, E. Wremmer-Weich, P. Van Roggen, S. Tuveson, J.P.T. Valkonen, J. Gielen, *Mol. Breed.* **18**, 313–325 (2006)
- K. Bonfim, J.C. Faria, E.O. Nogueira, E.A. Mendes, F.J. Aragão, *Mol. Plant Microbe Interact.* **20**, 717–726 (2007)
- S.V. Ramesh, A.K. Mishra, S. Praveen, *Oligonucleotides* **17**, 251–257 (2007)
- K.S. Ling, H.Y. Zhu, D. Gonsalves, *Transgenic Res.* **17**, 733–740 (2008)
- H. Tyagi, S. Rajasubramaniam, M.V. Rajam, I. Dasgupta, *Transgenic Res.* **17**, 897–904 (2008)
- H. Ben Tamarzizt, S. Gharsallah Chouchane, R. Lengliz, D.P. Maxwell, M. Marrakchi, H. Fakhfakh, F. Gorsane, *Acta Virol.* **53**, 99–104 (2009)
- Z.Y. Zhang, L. Yang, S.F. Zhou, H.G. Wang, W.C. Li, F.L. Fu, *J. Biotechnol.* **153**, 181–187 (2011)
- N. Nahid, I. Amin, R.W. Briddon, S. Mansoor, *Virol. J.* **8**, 499 (2011)
- T. Shimizu, E. Nakazono-Nagaoka, T. Uehara-Ichiki, T. Sasaya, T. Omura, *Plant Biotechnol. J.* **9**, 503–512 (2011)

31. H.A. Smith, S.L. Swaney, W.G. Dougherty, *Plant cell* **6**, 1441–1453 (1994)
32. R. Van Blokland, N. Van der Geest, J.N.M. Mol, J.M. Kooter, *Plant J.* **6**, 861–877 (1994)
33. M. Stam, R. de Bruin, S. Kenter, R.A. van der Hoorn, R. van Blokland, J.N.M. Mol, J.M. Kooter, *Plant J.* **12**, 63–82 (1997)
34. D.C. Baulcombe, *Plant Mol. Biol.* **32**, 79–88 (1996)
35. J.J. English, E. Mueller, D.C. Baulcombe, *Plant Cell* **8**, 179–188 (1996)
36. S. Raghuvanshi, Ph.D. Thesis, University of Delhi (2001)
37. C.S. Roberts, S. Rajagopal, L.A. Smith, T.A. Nguyen, W. Yang, S. Nugroho, K.S. Ravi, M.-L. Cao, K. Vijhayachandra, V. Patell, R.L. Harcourt, L. Dransfield, N. Desamero, I. Slamet, P. Keese, A. Kilian, R.A. Jefferson, pCAMBIA vector release manual version 3.05 (1998)
38. H. Chen, R.S. Nelson, J.L. Sherwood, *Biotechniques* **16**, 664–668 (1994)
39. M.B. Wang, P.M. Waterhouse, *Plant Mol. Biol.* **43**, 67–82 (2000)
40. S. Dellaporta, J. Wood, J.B. Hicks, *Plant Mol. Biol.* **1**, 19–21 (1983)
41. P. Chomczynski, N. Sacchi, *Anal. Biochem.* **162**, 156–159 (1987)
42. J. Sambrook, D.W. Russel, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, New York, 2001)
43. R.A. Jefferson, T.A. Kavanagh, M.W. Bevan, *EMBO J.* **6**, 3901–3907 (1987)
44. N. Nath, S. Mathur, I. Dasgupta, *Arch. Virol.* **147**, 1173–1187 (2002)
45. S. Sharma, I. Dasgupta, *J. Virol. Methods* **181**, 86–92 (2012)
46. E. Sivamani, H. Huet, P. Shen, C.A. Ong, A.D. Kochko, C. Fauquet, R.N. Beachy, *Mol. Breed.* **5**, 177–185 (1999)
47. H. Huet, S. Mahendra, J. Wang, E. Sivamani, C.A. Ong, L. Chen, A.D. Kochko, R.N. Beachy, C. Fauquet, *Phytopathology* **89**, 1022–1027 (1999)
48. U. Ganesan, S.S. Suri, S. Rajasubramaniam, M.V. Rajam, I. Dasgupta, *Virus Genes* **39**, 113–119 (2009)
49. S. Dai, X. Wei, A.A. Alfonso, L. Pei, U.G. Duque, Z. Zhang, G.M. Babb, R.N. Beachy, *Proc. Natl. Acad. Sci. USA* **105**, 21012–21016 (2008)
50. M.J. Cornejo, D. Luth, K.M. Blankenship, O.D. Anderson, A.E. Blechl, *Plant Mol. Biol.* **23**, 567–581 (1993)
51. N. Ehrenfeld, E. Romano, C. Serrano, P. Arce-Johnson, *Biol. Res.* **37**, 71–82 (2004)
52. G. Hutvagner, L. Mlynarova, J.P. Nap, *RNA* **6**, 1445–1454 (2000)
53. I.L. Ingelbrecht, J.E. Irvine, T.E. Mirkov, *Plant Physiol.* **119**, 1187–1198 (1999)
54. W.G. Dougherty, T.D. Parks, *Curr. Opin. Cell Biol.* **7**, 399–405 (1995)
55. N.K. Kouassi, L. Chen, C. Sifé, M. Bangratz-Reyser, R.N. Beachy, C.M. Fauquet, C. Brugidou, *Arch. Virol.* **151**, 2111–2122 (2006)
56. G.P. Lommossoff, *Ann. Rev. Plant Pathol.* **33**, 323–343 (1995)
57. D.C. Baulcombe, *Plant Cell* **8**, 1833–1844 (1996)
58. Y.M. Pinto, R.A. Kok, D.C. Baulcombe, *Nat. Biotechnol.* **17**, 702–707 (1999)
59. A.L. Jones, I.E. Johansen, S.J. Bean, I. Bach, A.J. Maule, *J. Gen. Virol.* **79**, 3129–3137 (1998)
60. T. Sijen, J. Wellink, J.B. Hiriart, A. VanKammen, *Plant Cell* **8**, 2277–2294 (1996)
61. P.E. Thomas, W.K. Kaniewski, E.C. Lawson, *Plant Dis.* **81**, 1447–1453 (1997)
62. P. Saha, I. Dasgupta, S. Das, *Plant Mol. Biol.* **62**, 735–752 (2006)