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# Double-stranded RNA in rice: a novel RNA replicon in plants

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**Abstract** The entire sequence of 13952 nucleotides of a plasmid-like, double-stranded RNA (dsRNA) from rice was assembled from more than 50 independent cDNA clones. The 5′ non-coding region of the coding (sense) strand spans over 166 nucleotides, followed by one long open reading frame (ORF) of 13716 nucleotides that encodes a large putative polyprotein of 4572 amino acid residues, and by a 70-nucleotide 3′ non-coding region. This ORF is apparently the longest reported to date in the plant kingdom. Amino acid sequence comparisons revealed that the large putative polyprotein includes an RNA helicase-like domain and an RNA-dependent RNA polymerase (replicase)-like domain. Comparisons of the amino acid sequences of these two domains and of the entire genetic organization of the rice dsRNA with those found in potyviruses and the CHV1-713 dsRNA of chestnut blight fungus suggest that the rice dsRNA is located evolutionarily between potyviruses and the CHV1-713 dsRNA. This plasmid-like dsRNA in rice seems to constitute a novel RNA replicon in plants.

**Key words** Double-stranded RNA · Plasmid-like RNA · Rice · RNA helicase · RNA-dependent RNA polymerase

## Introduction

Several large (more than 10 kb), linear, double-stranded RNAs (dsRNAs) have been detected in various symptomless plants (Dodds et al. 1984; Brown and Finnegan 1989; Valverde et al. 1990; Wakarchuk and Hamilton 1990; Zabalgogeazcoa and Gildow 1992; Fukuhara et al. 1993; Pfeiffer et al. 1993). These dsRNAs have some intriguing plasmid-like properties, which differ from those of conventional plant RNA viruses. (1) they have no obvious effect on the phenotype of their host plants; (2) their inheritance is vertical (seed-mediated and pollen-mediated); (3) they are present at a constant (low) concentration in their host cells, and (4) they are not associated with distinct virus-like particles. Since there are very few experimental reports (especially sequencing data) that deal with these plasmid-like dsRNAs, the biological functions of these dsRNAs and their evolutionary relationships to known plant RNA viruses are unknown.

We recently found an enigmatic dsRNA in cultivated (*Oryza sativa* L.) and wild (*O. rufipogon*) rice, and reported some plasmid-like properties (including the above-mentioned four properties) of this dsRNA (Fukuhara et al. 1993). Since this dsRNA does not hybridize with host DNA, it appears to be an RNA replicon. The dsRNA detected in cultivated rice (*O. sativa* L., cv. Nipponbare) was homologous, but not identical, to that in wild rice (*O. rufipogon*).

The entire nucleotide sequence of the rice dsRNA has now been determined, and we report here the genetic organization of this dsRNA. To our knowledge, this is the first report of the entire nucleotide sequence of plasmid-like RNA replicon in plants. Furthermore, we compare the deduced amino acid sequence of the ORF encoded by this dsRNA with those of proteins encoded by the genomes of known RNA viruses, and we discuss the possible origin and evolution of this plasmid-like dsRNA.

## Materials and methods

### Isolation of dsRNA

Rice plants (*Oryza sativa* L., cv. Nipponbare) were grown in a greenhouse at 25 °C. Young leaves were pulverized in a mortar after

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freezing in liquid nitrogen, and total nucleic acids were extracted and fractionated on a column of CF-11 cellulose as described by Morris and Dodds (1979). The dsRNA fraction was further purified by treatment with 10 μg/ml of DNase I in 10 mM TRIS-HCl (pH 7.4) and 10 mM $MgCl_2$.

### cDNA cloning of dsRNA

A series of overlapping cDNA clones that covered the entire sequence of the dsRNA was obtained by three different methods. First, random hexanucleotides were used as primers for the synthesis of cDNA, as previously described (Fukuhara et al. 1993). Next, several oligonucleotide primers were synthesized from the sequencing data obtained from the above cDNA clones, and then further cDNA clones were obtained by the method of Gubler and Hoffman (1983). In order to obtain cDNA clones that corresponded to the terminal regions of the dsRNA, we used the 5′ RACE technique (rapid amplification of cDNA ends; Schuster et al. 1992).

### DNA sequencing and homology searches

DNA sequencing was performed on both strands by the dideoxynucleotide chain-termination method using a 7-deaza Sequenase Ver. 2.0 DNA sequencing kit (United States Biochemicals, Cleveland, Ohio, USA). Analyses of nucleotide and amino acid sequences and homology searches were performed with SDC-GENETYX genetic information-processing programs (Software Development, Tokyo, Japan).

## Results

### Entire nucleotide sequence of the rice dsRNA

The entire sequence of 13952 nucleotides of the rice dsRNA was determined from a series of independent overlapping cDNA clones (Fig. 1). We sequenced more than 50 independent cDNA and RT-PCR (Reverse Transcriptase-PCR) clones to determine the complete nucleotide sequence of the rice dsRNA, and then many base differences among independent cDNA clones were found within the entire sequence of the dsRNA. For example, six base substitutions were detected among four independent cDNA clones in a region of 500 nucleotides between positions 3100 and 3600 from the 5′-end of the coding strand (data not shown). Five out of six substitutions lead to changes of amino acids in an open reading frame (ORF). Furthermore, comparison of the nucleotide sequences of four independent cDNA clones (F76, F403, F413 and F415) revealed an insertion of a single nucleotide in clone F76. Clone F76 contained four adenine residues at nucleotide positions 1231–1233 from the 5′-end of the coding strand, whereas each of the other three clones (F403, F413 and F415) contained three adenine residues at the same site (data not shown). This insertion leads to a frameshift in the ORF and thereby causes a premature termination of the polyprotein. Such phenomena (substitutions and insertion) were also found in cDNA clones that corresponded to the terminal regions of the dsRNA.

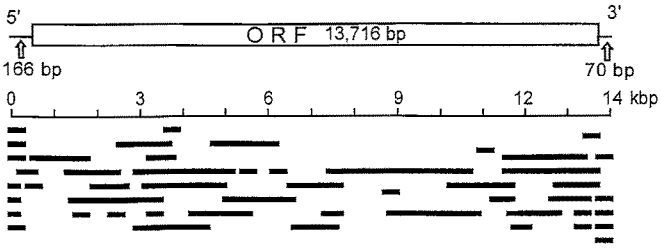


**Fig. 1** Map of cDNA and RT-PCR clones of the dsRNA in rice (*Oryza sativa* L., cv. Nipponbare). Each cDNA and RT-PCR clone is represented by a *filled horizontal line*. The genetic organization of the coding (sense) strand of the dsRNA is indicated at the top of the Figure, and includes the 166-bp non-coding leader sequence, the ORF of 13716 bp and the 70-bp 3′ non-coding sequence. The nucleotide sequence data will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases under the accession number D32136

The observed rate of base substitution and nucleotide insertion seems high, even if one takes into account possible errors during the synthesis of cDNA by reverse transcriptase, and this suggests that the population of dsRNA in a rice plant may be heterogeneous. Similar phenomena have been reported in the case of the W dsRNA of *Saccharomyces cerevisiae* (Rodriguez-Cousino et al. 1991) and the dsRNA of *Leishmania* RNA virus 1 (Stuart et al. 1992).

In a previous report (Fukuhara et al. 1993), we described the identification of two ORFs in the rice dsRNA from sequencing data that included the sequence of clone F76. However, the sequencing data for three other cDNA clones (F403, F413 and F415) lead us to conclude that the majority of dsRNA molecules comprise a single, unusually long ORF of 13716 nucleotides. The AUG codon at nucleotides 167 – 169 in this ORF lies in a favorable context (AAGAAUGGA) compared with the consensus sequence for the initiation of translation in plants (AACAAUGGC) proposed by Lutcke et al. (1987), and is likely to be the initiator AUG for this large polyprotein. This long ORF accounts for 98% of the entire dsRNA and the predicted translation product contains 4572 amino acids. Other reading frames are present on both strands but contain numerous termination codons and, therefore, are not likely to be translated into proteins. This is, to our knowledge, the longest ORF reported to date in plants. The non-coding region preceding the ORF is 166 nucleotides long. The termination codon at nucleotide positions 13883–13885 from the 5′-end of the coding strand is followed by a non-coding region of 70 bp and no poly(A) tail was found in this dsRNA.

The dsRNA has a high AU content (66.1%), and analysis of the codon usage of the ORF encoded by this dsRNA (Fig. 2) indicates that the rice dsRNA has a significant bias towards codons with A or U as the third letter, while many rice genes tend to have a GC-rich

**Fig. 2** Codon usage of the ORF encoded within the rice dsRNA.

| | | | |
|---|---|---|---|
| TTT-Phe102 (2.23%) | TCT-Ser 74 (1.62%) | TAT-Tyr115 (2.52%) | TGT-Cys 68 (1.49%) |
| TTC-Phe 75 (1.64%) | TCC-Ser 33 (0.72%) | TAC-Tyr 58 (1.27%) | TGC-Cys 38 (0.83%) |
| TTA-Leu215 (4.70%) | TCA-Ser 71 (1.55%) | TAA-*** 0 (0.00%) | TGA-*** 0 (0.00%) |
| TTG-Leu107 (2.34%) | TCG-Ser 8 (0.17%) | TAG-*** 0 (0.00%) | TGG-Trp 65 (1.42%) |
| CTT-Leu 29 (0.63%) | CCT-Pro 60 (1.31%) | CAT-His 76 (1.66%) | CGT-Arg 17 (0.37%) |
| CTC-Leu 16 (0.35%) | CCC-Pro 24 (0.52%) | CAC-His 45 (0.98%) | CGC-Arg 6 (0.13%) |
| CTA-Leu 51 (1.12%) | CCA-Pro 71 (1.55%) | CAA-Gln112 (2.45%) | CGA-Arg 15 (0.33%) |
| CTG-Leu 24 (0.52%) | CCG-Pro 14 (0.31%) | CAG-Gln 19 (0.42%) | CGG-Arg 4 (0.09%) |
| ATT-Ile133 (2.91%) | ACT-Thr 99 (2.17%) | AAT-Asn227 (4.97%) | AGT-Ser 88 (1.92%) |
| ATC-Ile 53 (1.16%) | ACC-Thr 42 (0.92%) | AAC-Asn 96 (2.10%) | AGC-Ser 48 (1.05%) |
| ATA-Ile202 (4.42%) | ACA-Thr117 (2.56%) | AAA-Lys276 (6.04%) | AGA-Arg124 (2.71%) |
| ATG-Met136 (2.97%) | ACG-Thr 22 (0.48%) | AAG-Lys 79 (1.73%) | AGG-Arg 27 (0.59%) |
| GTT-Val 87 (1.90%) | GCT-Ala 51 (1.12%) | GAT-Asp215 (4.70%) | GGT-Gly 77 (1.68%) |
| GTC-Val 35 (0.77%) | GCC-Ala 19 (0.42%) | GAC-Asp 93 (2.03%) | GGC-Gly 22 (0.48%) |
| GTA-Val 78 (1.71%) | GCA-Ala 43 (0.94%) | GAA-Glu211 (4.62%) | GGA-Gly 84 (1.84%) |
| GTG-Val 64 (1.40%) | GCG-Ala 12 (0.26%) | GAG-Glu 68 (1.49%) | GGG-Gly 32 (0.70%) |

Total number of codons = 4,572

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Potyviruses:
  PPV  QDILIRGAVGSGKSTGLPF..70aa..YKCIIFDECHVH..168aa..ERIQRLGRVGRNK
  TEV  RDFLVRGAVGSGKSTGLPY..70aa..YDFVIIDECHVN..168aa..ERIQKLGRVGRHK
 TVMV  KDIILMGAVGSGKSTGLPT..70aa..YQFIIFDEFHVL..168aa..ERIQRFGRVGRNK

  CHV  GHVTVAAKTASGKSTFFPA..68aa..DNLVFFDEFHEM..160aa..TNEQRVNRVGRTM

Cons.  ------G--G-GKST----.......------DE----.........--IQR-GR-GR--

  RDR  RSNLTLGPAGYGKSTMISK..43aa..PNTIFVDEATMI..144aa..LVILRADRTGKWD
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**Fig. 3** Amino acid sequence alignment of putative RNA helicase domains encoded by potyviruses, the CHV1-713 dsRNA in chestnut blight fungus and the rice dsRNA. PPV, plum pox virus; TEV, tobacco etch virus; TVMV, tobacco vein mottling virus; CHV, CHV1-713 dsRNA in chestnut blight fungus; RDR, rice dsRNA; Cons., consensus sequence

nucleotide sequence (T. Fujimura, personal communication). The implications of the AU-rich nucleotide sequence in the dsRNA are unknown, but this may result from constraints imposed by the mechanism of dsRNA replication and/or transcription.

## Comparison of the putative polyprotein encoded by the rice dsRNA with those encoded by other RNA replicons

Comparison of the nucleotide sequence of the rice dsRNA and of the amino acid sequence of the ORF it encodes revealed no extensive sequence similarities to any currently reported DNA, RNA or protein sequences in the standard databases (EMBL, GenBank, NBRF and SwissProt). Because the most striking molecular feature of the rice dsRNA is the unusually long ORF it encodes, we performed a detailed search among RNA viruses with similar characteristics. Potyviruses, for instance, are RNA-containing plant viruses whose large (about 10 kb) single-stranded (ss), positive-sense (+) RNA genome encodes a single long ORF (Allison et al. 1986). Similarly, the sequence of the 12.7 kb hypovirulence-associated dsRNA of chestnut blight fungus also revealed a long ORF of about 3000 amino acid residues [CHV1-713 dsRNA (Hillman et al. in press), previously known as HAV (Shapira et al. 1991)]. These long ORFs encode polyproteins containing conserved RNA helicase and RNA-dependent RNA polymerase (replicase) domains (Koonin et al. 1991).

Figure 3 shows the amino acid sequence alignment of the deduced RNA helicase-like domains of three potyviruses, CHV1-713 dsRNA and rice dsRNA. GXXGXGKS and DE have been proposed as a consensus motif around phosphate-binding pockets in many NTP-binding proteins (Gorbalenya and Koonin 1989). This configuration has been found in the putative RNA helicase domains of polyproteins encoded by three potyviruses and CHV1-713 dsRNA, and we also found it in the putative product of the rice dsRNA.

Figure 4 shows a comparison of the putative RNA-dependent RNA polymerase (RDRP) domain of rice dsRNA with those of several positive-strand RNA and dsRNA viruses. Two characteristic regions, (T/S)GXXXTXXXNXXXN and GDD, thought to form the core of the RDRPs of positive-strand RNA

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(+) strand RNA viruses:
  TMV    LDISKYDKSQ..48aa..RKSGDVTTFIGNTVIIA..14aa..GAFCGDDSLLY
  BMV    ADLSKFDKSQ..48aa..RRTGDAFTYFGNTLVTM..14aa..AIFSGDDSLII
  PVX    NDYTAFDQSQ..42aa..RLTGEGPTFDANTECNI..14aa..QVYAGDDSALD
BaYMV    GDGSRFDSSI..50aa..GTQRQPSTVVDNTLVLM..24aa..FVCNGDDNKFA
  TEV    ADGSQFDSSL..51aa..NNSGSPSTVVDNTLMVI..18aa..YYVNGDDLLIA
 TVMV    ADGSQFDSSL..51aa..NNSGQPSTVVDNTLMVV..22aa..FFANGDDLIIA

dsRNA viruses:
  ROT    TDVSQWDSSQ..60aa..VASGEKQTKAANSIANL..20aa..IRVDGDDNYAV
  REO    IDISACDASI..86aa..FPSGSTATSTEHTANNS..32aa..YVCQGDDGLMI
  RDV    ADCSSWDQTF..73aa..MWSGRLDTFFMNSVQNA..23aa..FQVAGDDAIMV
  BCV    LDWSSFDSSV..52aa..IPSGSYYTSIVGSVVNR..17aa..CYTQGDDSLIG
 ScLA    DGASSFCFDY..61aa..LLSGWRLTTFMNTVLNW..16aa..SVHNGDDVMIS
 Phi6    TDVSDHDTFW..58aa..LSSGQGATDLMGTLLMS..40aa..QISKSDDAILG
  CHV    ADATAYDSNC.180aa..GGTGQSATSWDNTATFK..23aa..LYNTSDDTVWW

Cons.    -D-(S/T)--D---.........--(S/T)GQ--T---N---N-.........----GDD----

  RDR    NDLTKQDRQT..46aa..RLTGQATTALGNCITNM..16aa..ALFLGDDMCMG
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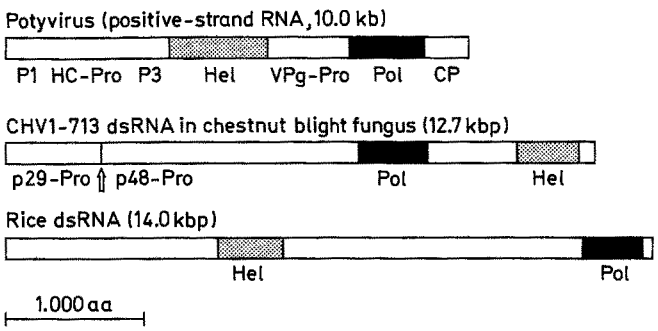


**Fig. 4** Alignment of the amino acid sequence of the putative RNA-dependent RNA polymerase domain encoded within the rice dsRNA and related domains encoded by positive-strand RNA viruses and dsRNA viruses. TMV, tobacco mosaic virus; BMV, brome mosaic virus; PVX, potato virus X; BaYMV, barley yellow mosaic virus; TEV, tobacco etch virus; TVMV, tobacco vein mottling virus; ROT, bovine rotavirus; REO, reovirus serotype 3; RDV, rice dwarf virus; BCV, beet cryptic virus; ScLA, *Saccharmyces cerevisiae* L-A virus; Phi6, bacteriophage phi6; CHV, CHV1-713 dsRNA in chestnut blight fungus; RDR, rice dsRNA; Cons., consensus sequence

**Fig. 5** Schematic comparison of the organization of the (putative) polyproteins of potyvirus, CHV1-713 dsRNA in chestnut blight fungus and rice dsRNA. Related domains are highlighted by identical shading. The boundary between ORFs A and B in the CHV1-713 dsRNA is indicated by an *arrow*. Pol, RNA-dependent RNA polymerase domain; Hel, RNA helicase domain; Pro, protease domain; CP, capsid protein domain

and dsRNA viruses (Bruenn 1991; Koonin et al. 1991), were also identified in the C-terminal region of the polyprotein encoded by the rice dsRNA (Fig. 5).

The locations of the RNA helicase-like domain and the RDRP-like domain within the putative polyprotein of rice dsRNA are similar to those of potyviruses, which encode, in addition, a capsid protein domain located in the C-terminal region of the polyprotein (Fig. 5). However, the C-terminal region of the polyprotein encoded by the rice dsRNA would be insufficient to encode a capsid protein, a result that is consistent with the previously described failure to detect distinct virus-like particles in dsRNA-containing rice plants by the standard methods for preparation of virus particles (Fukuhara et al. 1993). Consequently, rice dsRNA probably does not encode a capsid protein.

## Discussion

In a previous report (Fukuhara et al. 1993), it was shown by Southern hybridization that the rice dsRNA was not transcribed from host DNA and might be an RNA replicon. The existence of RNA helicase-like and RDRP-like domains within the polyprotein encoded by the ORF supports the previous experimental results since these enzymes play key roles in the replication of RNA replicons. Since it is generally accepted that RDRPs replicate their genomes with lower fidelity than DNA polymerases, the heterogeneity of dsRNA molecules within a rice plant is probably caused by the low fidelity of its own RDRP. Furthermore, since the plasmid-like dsRNA does not affect the phenotype of its host plant, base substitutions and nucleotide insertions within the rice dsRNA molecules might tend to accumulate at a higher rate than in other RNA replicons because of the lack of selection pressure.

An RDRP activity has been known in healthy plants for about 10 years (Fraenkel-Conrat 1983), and the catalytic properties of this enzyme from tomato leaves were recently reported (Schiebel et al. 1993a, 1993b). However, the physiological role of this enzyme is still unknown. Brown and Finnegan (1989) proposed that these enzymes were associated with, and possibly encoded by, dsRNA plasmids. We detected the plasmid-like dsRNA in apparently healthy rice plants and found the RDRP-like domain within the ORF encoded in it; therefore, our results tend to support the proposal of Brown and Finnegan.

There are many reports of dsRNA genetic elements in phytopathogenic fungi: these dsRNAs (especially large dsRNAs) have some plasmid-like properties

(Nuss and Koltin 1990; Ghabrial 1994). By comparing the amino acid sequence encoded by the CHV1-713 dsRNA of the chestnut blight fungus (*Cryphonectria parasitica*) with those encoded by potyviruses, Koonin et al. (1991) suggested that the CHV1-713 dsRNA and potyvirus-like, positive-stranded RNA plant viruses sheared a common ancestry. However, no sequence data for plant plasmid-like dsRNA were available at that time. We are now able to compare the amino acid sequences of the RNA helicase-like and RDRP-like domains (Figs. 3 and 4) and the entire genetic organization (Fig. 5) of the plasmid-like dsRNA in rice with those of a potyvirus and CHV1-713 dsRNA. The respective locations of the RNA helicase-like domain and the RDRP-like domain in the rice dsRNA-encoded polyprotein are identical to those in the potyvirus-encoded polyprotein but the reverse of those in the CHV1-713 dsRNA-encoded polyprotein (Fig. 5). Whether this is related to the fact that the hosts of both the rice dsRNA and potyviruses are higher plants is not known. From the above-mentioned properties of the various genetic elements, the plasmid-like dsRNA in rice seems to be located evolutionarily between the fungal dsRNA genetic elements (e.g., the CHV1-713 dsRNA) and the potyviruses.

Many kinds of plant virus have an RNA genome, but very few plant viruses have a single genomic RNA component larger than 10 kb (Matthews 1991). Citrus tristeza virus (CTV), a member of the closterovirus group, has the largest (+) ssRNA genome (about 20 kb) among plant viruses studied to date (Pappu et al. 1994), and CTV-specific genome-length dsRNA was detected in CTV-infected citrus tissues (Dodds et al. 1987), but no sequence data are available with which, to evaluate the evolutionary relationship between rice dsRNA and CTV.

All plant dsRNA-containing viruses characterized to date have several segmented genomic dsRNAs, which are smaller than 5 kb. For example, the genome of rice dwarf virus (RDV) consists of 12 segmented dsRNAs, which vary in size from 1000 to 4500 nucleotides (Nuss and Dall 1990). The rice dsRNA as described here is 13952 nucleotides long and codes for a 13716 nucleotide ORF, which is the longest among those of plant genes reported to date. Consequently, the rice dsRNA appears to be unique among RNA replicons and may be regarded as a novel RNA replicon in plants.

Large dsRNAs have been detected in various higher plants, for example, *Phaseolus vulgaris* (Mackenzie et al. 1988; Wakarchuk and Hamilton 1985), *Vicia faba* (Pfeiffer et al. 1993), barley (Zabalgogeazcoa and Gildow 1992) and pepper (Valverde et al. 1990). These dsRNAs share common plasmid-like features: (1) plants harboring them are symptomless; (2) the dsRNAs are vertically inherited, (3) they are present at constant concentration and (4) no distinct virus-like particles are found in infected hosts. In the case of rice dsRNA, the single, unusually long ORF is the most striking feature. Once sequence information for other large dsRNAs is published perhaps a single long ORF can be added to the list of common features of these plasmid-like dsRNAs and we may find that these dsRNAs can be regarded as a novel group of plant RNA replicons.

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