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Synthesis of an Infectious Full-Length cDNA Clone of Rice Yellow Mottle Virus and Mutagenesis of the Coat Protein

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A full-length cDNA clone of rice yellow mottle sobemovirus (RYMV) was synthesized and placed adjacent to a bacteriophage T7 RNA polymerase promoter sequence. Capped-RNA transcripts produced *in vitro* were infectious when mechanically inoculated onto rice plants (*Oryza sativa* L.). Individual full-length clones varied in their degree of infectivity but all were less infectious than native viral RNA. A representative clone, designated RYMV-FL5, caused a disease phenotype identical to that produced by viral RNA except that symptoms were somewhat slower to appear than those induced by viral RNA. The infectivity of RYMV-FL5 was verified by ELISA, Western blot analysis, Northern blot hybridization, RT-PCR, and Southern blot hybridization. Frameshift and deletion mutations introduced into the coat protein cistron demonstrated that the coat protein was dispensable for RNA replication in rice protoplasts. However, the coat protein was required for full infectivity in rice plants, presumably by playing a role in phloem-mediated long-distance movement and possibly in cell-to-cell movement.

INTRODUCTION

Rice yellow mottle virus (RYMV) is a member of the sobemovirus group of plant viruses of which southern bean mosaic virus (SBMV) is the type member (Francki *et al.*, 1991). RYMV is a small isometric virion (25 nm) containing a single-stranded positive-sense monopartite genomic RNA of 4450 nucleotides (Ngon a Yassi *et al.*, 1994). The RNA contains a 5′-linked protein (VPg) and lacks a 3′-poly(A) sequence (Hull, 1988; Ngon a Yassi *et al.*, 1994). RYMV was reported for the first time in Kenya in 1970 (Bakker, 1974) and has since been identified in many western and eastern African countries (Hull, 1988). RYMV is primarily a pathogen of rice; its host range is restricted to the *Gramineae,* principally *Oryzae* ssp. and *Eragrostideae* ssp (Bakker, 1974). Symptoms of viral infection consist of yellow or discolored dots that appear at the base of the youngest systemically infected leaves. These dots expand parallel to the veins; infected leaves discolor and become yellow or orange, leading to reduced tillering, stunting of the plants, and sterility of the flowers (Bakker, 1974; Attere and Fatokun, 1983). RYMV is naturally transmitted by chrysomelid beetles, but mechanical transmission is also effective. Virion stability and high levels of replication, ranging from 1 to 6 g/kg of viral particles in infected rice plants (C. Fauquet, unpublished observation), are characteristic of RYMV.

Recently, the sequence of the RYMV genome (4450 nucleotides, GenBank Accession No. L20893) was reported (Ngon a Yassi *et al.*, 1994). The genome appears to encode at least four distinct proteins with predicted molecular weights of 110.7, 26, 17.8, and 13.5 kDa. Amino acid sequence comparisons with proteins of known function and those encoded by SBMV (Wu *et al.*, 1987) suggest that the 110.7-kDa protein is the putative replicase and the 26-kDa protein is the coat protein. The functions of the 17.8- and 13.5-kDa proteins are not known at this time. *In vitro* translation reactions primed with virion RNA were shown to produce protein products with molecular weights of 110, 70, 29, 20, 19, 18, and 13 kDa (M. Ngon a Yassi, unpublished data).

In order to better study RYMV replication, gene expression, and the function of its proteins, we developed a full-length cloned cDNA of RYMV from which infectious transcripts can be produced. In addition, using site-directed mutagenesis we demonstrate that the viral coat protein is indispensable for full infectivity of the virus. This is the first such report for a sobemovirus.

MATERIALS AND METHODS

Virus isolate and viral RNA preparation

The rice yellow mottle virus isolate used was obtained from a rice field (*Oryza sativa* L.) in the Ivory Coast. The virus was propagated in *O. sativa* L. variety IR8 and purified according to the technique previously described by Fauquet and Thouvenel (1977). Viral RNA was isolated using "RNaid plus" (BIO 101, Inc.) according to the manufacturer's protocol.

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Construction of full-length cDNA clones

RYMV sequence data and nucleotide numbering were derived from Ngon a Yassi *et al.* (1994). Genomic RNA (5 μg) was annealed with an excess of oligonucleotide primer I, 5′dGCATGCAAGCTT**CTCCCCCACCCATCCCGAGAATT** 3′, which contains sequences complementary to the 3′ end of the RNA (in bold) and unique 3′-flanking *Sph*I and *Hin*dIII sites. First-strand synthesis was carried out with Superscript II RNase H⁻ reverse transcriptase (RT; Gibco-BRL) according to the manufacturer's protocol and Gerard *et al.* (1992), except that the RNA–primer mixture was heated to 90° for 10 min and the extension temperatures were 42° for 45 min, 45° for 15 min, and 50° for 5 min. The viral RNA was subsequently degraded by electrophoresis in an alkaline agarose gel (Sambrook *et al.*, 1989) or by RNase H (Gibco-BRL) treatment. Single-stranded cDNA was purified by Glass MAX (Gibco-BRL) and was subsequently annealed with an excess of oligonucleotide primer II, 5′ dATTCTAGACTGCAGTAATACGACTCACTATA**GACAATTGAAGCTAGGAAAGG** 3′. This primer contains 20 nucleotides that correspond to the genomic RNA (in bold), a T7 RNA polymerase promoter sequence (underlined) with an additional downstream G residue to allow for efficient initiation of transcription (Hearne *et al.*, 1990; Xiong and Lommel, 1991; Bujarski and Miller, 1992), and unique *Xba*I and *Pst*I restriction sites. Second-strand synthesis employed Vent polymerase (New England Biolabs) followed by 15 cycles of amplification by polymerase chain reaction (PCR). The full-length double-stranded cDNA was recovered from an agarose gel, purified by GeneClean II (BIO 101, Inc.), digested with *Hin*dIII and *Pst*I, and ligated into pUC19 previously digested by *Hin*dIII and *Pst*I. Recombinant plasmids were transformed into competent *Escherichia coli* strain DH5α cells. The resulting transformants were screened for the presence of the cDNA insert by restriction analysis and nucleotide sequence analysis.

Coat protein gene mutations

Mutations were introduced to an infectious RYMV full-length cDNA (clone FL5) using standard techniques (Sambrook *et al.*, 1989). Enzymes were from Gibco-BRL and Promega. Mutations were confirmed by restriction enzyme digestion, PCR, and nucleotide sequencing.

Construction of FL5-Δ MluI. A frameshift mutation in the RYMV coat protein cistron (nucleotides 3447–4166) was generated by digesting clone FL5 with *Mlu*I (unique site at nucleotide 3989), filling in the 5′ overhang with the Klenow fragment of *E. coli* DNA polymerase I, and blunt-end ligation.

Construction of FL5-Δ 3989–4112. A deletion mutation in the RYMV coat protein cistron was generated by digesting clone FL5 with *Mlu*I (unique site at nucleotide 3989) and *Sna*BI (unique site at nucleotide 4112), filling in the 5′ overhang with Klenow polymerase and blunt-end ligation with a deletion of nucleotides 3989 to 4112.

Polymerase chain reaction

PCR was used to synthesize the second-strand cDNA (using primer II) and to amplify the double-stranded cDNA (using primers I and II). Amplification reactions were carried out in an Ericomp thermocycler for one cycle with step of 94° for 5 min and for three cycles with steps of 94° for 1 min, 42° for 2 min, and 72° for 4 min 30 sec, followed by 12 cycles with steps of 94° for 1 min, 50° for 2 min, and 72° for 4 min 30 sec. The final cycle was followed by an additional 10 min at 72° to complete polymerization.

Reverse transcription and polymerase chain reactions (RT-PCR) were used to reisolate progeny cDNA from total RNA extracted from infected rice plants. First strand cDNA was synthesized using Superscript II RT with primer III which is complementary to the 3′ end of RYMV RNA (5′dCTCCCCCACCCATCCCGAGAATT3′). The coat protein gene was subsequently amplified with primer IV, complementary to nucleotides 3438–3453 of the RYMV RNA sequence (5′dCAAAGATGGCCAGGAA3′), and primer V, which corresponds to nucleotides 4386–4406 (5′dGAATTCGGTTTGGTGAAAGGT3′) according to the following protocol: One cycle with step of 94° for 5 min and 5 cycles with steps of 94° for 1 min, 42° for 1 min, and 72° for 1 min, followed by 30 cycles with steps 94° for 1 min, 60° for 1 min, and 72° for 1 min. These PCR conditions were also used to verify cDNA constructs *FL5-Δ MluI* and *FL5-Δ 3989–4112* with primers III and IV except that annealing temperatures of 50° for 5 cycles and 60° for 20 cycles were used.

In vitro transcription with T7 RNA polymerase

Plasmid DNA, purified with Magic Maxiprep columns (Promega), was digested with *Hin*dIII, which linearizes the plasmid at the 3′ end of the RYMV cDNA. *In vitro* transcription by T7 RNA polymerase (Promega) was carried out in the presence or absence of the cap analogue $m^7G(5')ppp(5')G$ (New England Biolabs) under the reaction conditions described by Nielsen and Shapiro (1986) as modified by Holt and Beachy (1991), except that the concentrations of the reagents in the transcription reactions were reduced by half and 10 μg of DNA template was used for each transcription reaction. For protoplast experimentation, *in vitro* transcripts were digested by RQ1 DNase (Promega) for 2 hr at 37° followed by phenol–chloroform and ethanol precipitation. The absence of DNA template and integrity of RNA were verified by electrophoresis in agarose gels, and the concentration of RNA was estimated by uv absorption and ethidium bromide staining.

Preparation and infection of *Oryza sativa* L. protoplasts

Rice (*O. sativa* L.) (8–10 g) cell suspension cultures at 3–5 days after subculture were plated in 75 ml of filter-

sterilized cell protoplast washing solution (Frearson *et al.,* 1973) containing 3% (w/v) cellulase RS (Yakult Honsha Co., Tokyo, Japan) and 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan). The mixture was kept in the dark at 25–26° for 4–5 hr. The treated cells were filtered through 25-μm nylon mesh, and the filtrate was centrifuged at 750 rpm for 5 min. After three successive centrifugation cycles, protoplasts were counted with a hemocytometer.

The purified protoplasts were suspended in PHBS (10 m*M* HEPES, pH 7.2, 150 m*M* NaCl, 4 m*M* $CaCl_2$) containing 0.4 *M* mannitol to a final density of about 2.5×10^6 cells/ml. One milliliter of freshly prepared protoplasts was inoculated with viral-RNA or *in vitro* transcripts by a Cell-Porator electroporation system I (Gibco-BRL) according to the manufacturer's protocol. Approximately 10 μg RNA was added and the suspension was immediately transferred to a cold 0.4-cm path-length cuvette and subjected to a 16-msec high-voltage pulse provided by discharge of a 1600 μF capacitor set to 300 V.

After electroporation, the protoplasts were incubated for 30 min on ice. The mixture was centrifuged at 75 rpm for 5 min to pellet the protoplasts. The supernatant was carefully removed, and the protoplasts were gently mixed with R_2 medium (Ohira *et al.,* 1973) containing 1 mg/liter 2,4-D, 137 mg/liter maltose, and 1.2% (W/V) sea plaque agarose type VII (Sigma) at 40°. One milliliter medium was mixed with protoplasts for a final protoplast density of 1×10^6/ml. The mixture was plated into a 5-cm diameter disposable Falcon plastic petri dish. When the medium was completely solidified, 100 mg of nurse cells (an OC cell line derived from root explants of *O. sativa* L. c5924, kindly provided by Dr. K. Syono, The University of Tokyo) in 5 ml of the protoplast liquid medium was added to each petri dish. The petri dish was placed in the dark at 25–26° for 48 hr.

Inoculation of plants

Transcription reaction mixtures were diluted 1:2 with 20 m*M* sodium phosphate buffer, pH 7.0, and directly used for inoculation of 2-week-old *O. sativa* plants (variety IR8). The surfaces of the leaves were first lightly dusted with Carborundum (330 grit, Fisher Scientific) and each plant was mechanically inoculated with 20 μl of the diluted reaction mixture corresponding to approximately 5 μg of RNA. Control plants were mock-inoculated with phosphate buffer, 200 ng of RYMV RNA, or 1 ng of purified RYMV.

Usually, 24 plants were inoculated with transcripts, viral RNA, virion, or buffer for each experiment. Plant populations inoculated with the product of different transcription reactions, viral RNA, or virus were physically isolated from each other. Immediately after inoculation, plants were rinsed with water and placed in a growth chamber (Percival) under low light conditions (photoperiod 16 hr light/8 hr dark and 2×20 W Philipps cool white illumination, 20–30 $\mu M/m^2$/sec) and approximately 80% humidity.

After inoculation, virus infection was followed by observing disease symptoms and analysis of plants by indirect ELISA (Lommel *et al.,* 1982), Western immunoblot reactions, Northern blot hybridization reactions, RT-PCR, and Southern blot hybridization; Each experiment was repeated at least three times.

Sequence analysis

Dideoxynucleotide sequence analysis was carried out as described by Sanger *et al.* (1977) using double-stranded plasmid DNA as template and Sequenase according to the manufacturer's recommendation (US Biochemical). Primers included the universal M13 forward and reverse sequencing primers and RYMV-specific primers I through V.

Protein extractions and Western blot analysis

Protein samples were prepared according to Sandstrom *et al.* (1987) and were separated on a 15% polyacrylamide gel containing SDS. Proteins were subsequently transferred onto BA 85 nitrocellulose membranes (Schleicher & Schuell) with a Trans Blot apparatus (Bio-Rad). After blocking with 2% bovine serum albumin in Tris buffered saline–Tween (10 m*M* Tris, pH 8.0, 150 m*M* NaCl, and 0.05% Tween 20), the membranes were incubated for 1 hr at room temperature in a 10^{-3} dilution of rabbit anti-RYMV antiserum which is a polyclonal antibody prepared against purified RYMV. The membranes were washed three times and then incubated with anti-rabbit IgG–alkaline phosphatase conjugate (Promega). Detection of immunoreactive protein bands was performed with P-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad) as recommended by the manufacturer. The routine limit of detection was approximately 50 ng of RYMV coat protein.

Northern and Southern blot analysis

Total RNA was extracted from leaves by previously described methods (Logemann *et al.,* 1987). The same quantity of total RNA (20 μg) was denatured and fractionated on formaldehyde gels as described by Gerard and Miller (1986). Hybridization and washing conditions were the same as those for Southern blots.

Southern blot hybridization analyses were performed to confirm the identity of RT-PCR products. After electrophoresis in agarose gels, DNAs were transferred to Hybond N+ membrane (Amersham). Prehybridization and hybridization were done according to the manufacturer's recommendations using a denatured probe corresponding to the coat protein gene. Probe DNA was obtained by PCR amplification from the parental FL5 plasmid using primers IV and V and was labeled by random primer labeling (Stratagene) and purified by Nuctrap push columns (Stratagene).

RESULTS

Characterization of infectious RYMV cDNA clones

Cloning of RYMV cDNA. Full-length double-stranded cDNAs of RYMV were produced by standard techniques using sequence information as determined by Ngon a Yassi *et al.* (1994). Specific oligonucleotide primers complementary to the 3′ end and corresponding to the 5′ end of the viral RNA were used. Each primer introduced unique flanking restriction sites (5′ *Hin*dIII and 3′ *Sph*I) to allow subsequent cloning and manipulation of genomic cDNAs. The 5′ primer also contained a bacteriophage T7 RNA polymerase promoter with a single nonviral G residue 3′ of the promoter sequence to allow for the efficient production of *in vitro* transcripts. Following first-strand cDNA synthesis, second-strand cDNA was synthesized using a thermostable DNA polymerase and the product was subsequently amplified by PCR. The full-length RYMV cDNA thus produced was cloned into a bacterial plasmid (pUC19) for propagation in *E. coli.*

In vitro transcription of RYMV cDNA. To select infectious full-length clones of RYMV, *in vitro* transcription reactions were performed using mixtures of *Hin*dIII-linearized full-length DNA templates. A pool of five templates (2 μg each) was used per reaction. There were no obvious differences in size or amount of product between transcription reactions (data not shown).

In general, the yield of RNA transcripts was approximately 10 μg/2 μg of DNA template. RNA yield was comparable between two full-length clones designated FL5 and CB12, in both the absence and the presence of the m7GpppG cap analog (data not shown). RNA transcripts generated *in vitro* from the T7 promoter sequence of the full-length RYMV clones were predicted to contain a single nonviral 5′ G residue and not more than one additional 3′ nucleotide.

Sets of transcripts that showed infectivity on rice plants (see below) were subsequently evaluated to identify individual cDNA clones that yielded high levels of infectivity.

Infectivity analysis of in vitro transcripts. Twenty-four 2-week-old rice plants were inoculated for each independent transcription reaction. Each rice plant was inoculated with approximately 5 μg of RNA transcript. This amount of transcript was required to infect a rice plant, whereas 0.2 μg of virion RNA was sufficient to cause infection. The first disease symptoms (chlorotic spots on new leaves) appeared 1 week after inoculation on plants inoculated with RNA purified from virions. However, plants inoculated with capped FL5 RNA transcript exhibited equivalent disease symptoms 15 days after inoculation. Rice plants that were inoculated with either capped CB12 RNA transcript or FL5 RNA transcript that was not capped showed no disease symptoms 4 weeks after inoculation.

Infection was confirmed by ELISA 4 weeks after inoculation. Plants that failed to exhibit symptoms typical of RYMV infection were also negative in ELISA (data not shown). Plants inoculated with either virion RNA or FL5 transcript were infected but accumulated different amounts of virus. Plants infected by virion-derived RNA generally showed areas of yellow or orange coloration, whereas plant infected by FL5 transcripts showed a number of chlorotic spots. This visual observation was confirmed by ELISA and virus purification. From 24 plants infected with virion RNA, the yield of virus was 13-fold greater than from plants infected with FL5 RNA transcript (approximately 1.6 and 0.12 mg virus/g tissue, respectively) at 4 weeks postinoculation. By Western blot analysis (Fig. 1), RYMV coat protein antigen was readily detected (single band of approximately 30 kDa) in inoculated and systemic leaves of plants inoculated with either virion RNA (Fig. 1, lane 4) or FL5-RNA transcript (Fig. 1, lane 5). In contrast, no coat protein was detected in extracts prepared from plants inoculated with noncapped FL5 transcript (Fig. 1, lane 3).

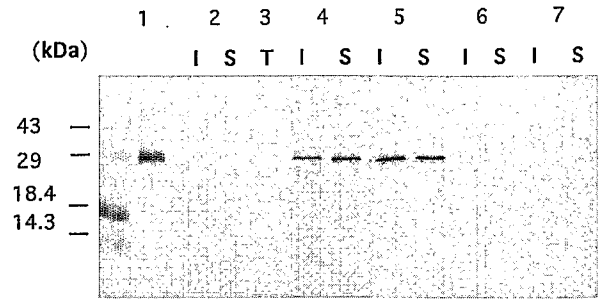

FIG. 1. Detection of RYMV coat protein by Western blot analysis of protein extracts from inoculated leaves (I) and upper uninoculated (systemic) leaves (S) of rice plants (*Oryza. sativa* L. variety IR8) inoculated with either virion RNA or synthetic transcripts. Lane 1 shows the position of coat protein from purified RYMV virions (3 μg) mixed with proteins extracted from mock-inoculated plants. Rice plants were mock-inoculated (2); inoculated with uncapped FL5-RNA transcripts (3), virion RNA (4), capped FL5-RNA transcripts (lane 5), *FL5-Δ MluI* RNA transcripts (6), or *FL5-Δ 3989–4112* RNA transcripts (7). Protein ladder used was prestained protein molecular weight standards from Gibco-BRL. T, total extract from combined inoculated and upper uninoculated leaves.

Virus purified from plants inoculated with transcript showed typical RYMV particles by electron microscopy. These particles were composed of a single protein with the same electrophoretic mobility and antigenic reactivity as RYMV CP and contained RNA of the expected size (data not shown).

Mutation of the coat protein ORF

The FL5 cDNA clone was mutagenized by inserting the nucleotide sequence CGCG between nucleotides 3989 and 3990 to generate clone *FL5-Δ MluI* (Fig. 2). This insertion was predicted to introduce a frameshift in the CP open reading frame, leading to premature termination and a truncated CP with 185 amino acids (compared with the 239 amino acids coat protein of RYMV). A second type of mutation was created by deleting the nucleotides between the *Mlu*I restriction site (nucleotide

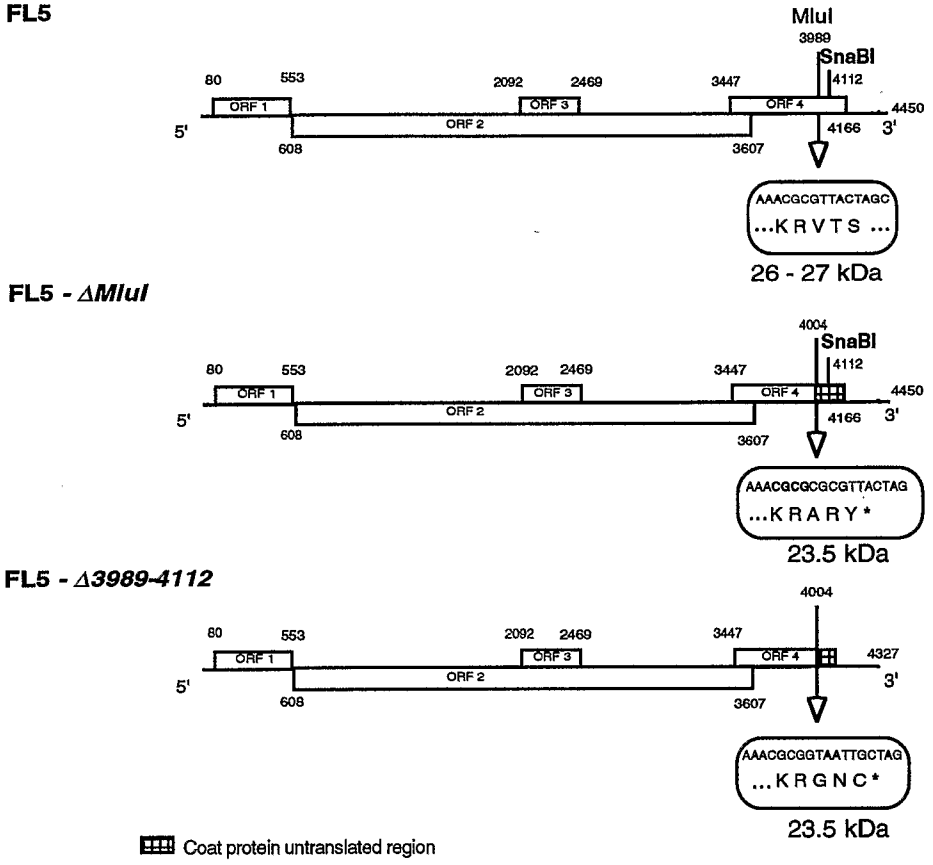

FIG. 2. Schematic diagram of mutations introduced into the FL5 full-length ds cDNA (4450 nt). Nucleotide and amino acid numbering are from Ngon a Yassi *et al.* (1994). ORF1 codes for a protein containing 157 amino acids (aa) with a corresponding molecular weight (MW) of 17.8 kDa; ORF2, 999 aa and MW 110.7 kDa; ORF3, 126 aa and MW 13.7 kDa; and ORF4, 251 aa and MW 27 kDa. Stop codon TAG(*), *FL5-Δ MluI*, coat protein frameshift; *FL5-Δ 3989–4112*, coat protein deletion.

3989) and the *Sna*BI site (nucleotide 4112) to generate clone *FL5-Δ 3989–4112* (Fig. 2). This mutation was predicted to lead to a premature termination codon and truncation of 54 amino acids from the coat protein.

Viral RNA, capped transcripts derived from clones FL5, *FL5-Δ MluI*, and *FL5-Δ 3989–4112* and uncapped transcripts from clone CB12 and FL5 were electroporated into rice protoplasts (Fig. 3). Forty-eight hours after electroporation, RNA was recovered. After reverse transcription and PCR amplification using primers specific for the RYMV coat protein gene region, Southern blotting and subsequent hybridization with a radiolabeled coat protein gene probe verified the specificity of the PCR amplification (Fig. 3). Replication in protoplasts was observed for transcripts from both the frameshift (band of 968 bp, lane 5) and deletion (band of 845 bp, lane 6) clones as well as viral RNA and parental clone FL5 transcripts (band of 968 bp, lanes 1 and 2, respectively). By contrast, the coat protein gene cistron was not detected after 35 cycles of PCR from total RNA of protoplasts electroporated with uncapped CB12 transcripts (Fig. 3, lane 4).

Viral RNA, capped *in vitro* transcripts of FL5, *FL5-Δ MluI*, and *FL5-Δ 3989–4112* and uncapped transcripts of FL5 were also inoculated to rice plants. Four weeks after inoculation, no signs of infection were visible on plants inoculated with capped transcripts from *FL5-Δ MluI* and *FL5-Δ 3989–4112* clones, while rice plants

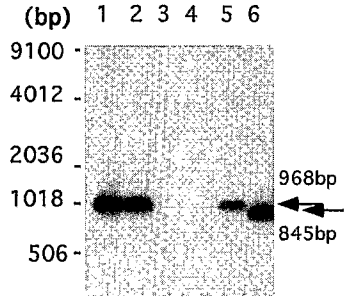

FIG. 3. Rice yellow mottle virus RNA replication in protoplasts. Transcripts were synthesized and used to inoculate *Oryza sativa* L. protoplasts as described under Materials and Methods. Total RNA was extracted at 48 hr post inoculation and analyzed by Southern blotting with a RYMV coat protein probe (968 bp) after reverse transcription and RYMV CP gene amplification by polymerase chain reaction. The position of coat protein gene (968 bp) and coat protein deletion (845 bp) are marked. Protoplasts were electroporated with viral-RNA (lane 1), capped FL5 transcripts (lane 2), no RNA (lane 3), uncapped CB12 transcripts (lane 4), capped *FL5-Δ MluI* (lane 5), or capped *FL5-Δ 3989–4112* (lane 6).

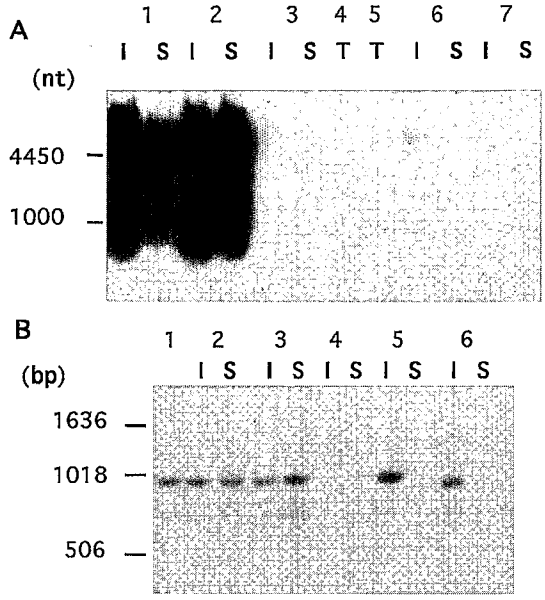

FIG. 4. Rice yellow mottle virus RNA replication and movement in rice plants. Transcripts were synthesized and used to inoculate rice plants as described under Materials and Methods. Total RNA was extracted at 4 weeks post inoculation and analyzed by Northern (A) and Southern blotting after reverse transcription (RT) and RYMV CP gene amplification by polymerase chain reaction (PCR) (B). I, inoculated leaves; S, upper uninoculated (systemic) leaves; and T, inoculated leaves plus upper uninoculated leaves (A) Northern hybridization analysis with a RYMV coat protein probe. Plants were inoculated with viral RNA (1), FL5 RNA transcripts (2), no RNA (3), uncapped FL5-RNA transcripts (lane 4), *FL5-Δ MluI* RNA transcripts (5 and 6), or *FL5-Δ 3989–4112* RNA transcripts (7). Twenty micrograms of total RNA extracted from rice plants was subjected to electrophoresis, blotted onto nylon membrane, and hybridized with a RYMV coat protein probe (968 bp). Exposure time, 24 hr. (B) Southern hybridization analysis with a RYMV coat protein probe (968 bp) following RT and RYMV coat protein gene amplification by PCR. Lane 1 shows the position of the coat protein gene obtained by RT-PCR from purified virion RNA. Plants were inoculated with viral RNA (2), FL5-RNA transcripts (3), no RNA (4), *FL5-Δ MluI* RNA transcripts (5), or *FL5-Δ 3989–4112* RNA transcripts (6). PCR products from plants infected by viral RNA and FL5-RNA transcripts were diluted fivefold.

inoculated on the same date with virion RNA or capped FL5-RNA transcripts were fully infected. To determine the effect of mutations in the coat protein on the movement of the virus, inoculated (old leaves) and systemic, uninoculated leaves (new leaves) were analyzed by Western blot (Fig. 1). While CP was present in both inoculated and systemic leaves in plants infected with RYMV (Fig. 1, lane 4) and FL5 transcripts (Fig. 1, lane 5), no coat protein was detected in either type of leaves on plants infected with transcripts from *FL5-Δ MluI* (Fig. 1, lane 6) or *FL5-Δ 3989–4112* (Fig. 1, lane 7).

Northern blot analysis was used to determine if viral RNA replicated and if long-distance movement had occurred in plants infected with transcripts from *FL5-Δ MluI* or *FL5-Δ 3989–4112* (Fig. 4). As in plants infected by virion RNA or FL5-RNA transcripts, genomic viral RNA (around 4450 nt) was identified in plants inoculated with *FL5-Δ MluI* transcripts (Fig. 4A, lane 5) and the deletion mutant *FL5-Δ 3989–4112* transcripts (data not shown). However, for both mutants, genomic RNA was detected only in inoculated leaves (Fig. 4A, lanes 6 and 7). In addition, the amount of RNA detected was very low in comparison with leaves inoculated with RYMV or FL5-RNA (Fig. 4A, lanes 1 and 2). These results were confirmed by RT-PCR and Southern blot analysis (Fig. 4B). The coat protein gene was amplified from RNAs isolated from inoculated leaves of plants infected with *FL5-Δ MluI* transcripts (band of 968 bp) and *FL5-Δ 3989–4112* transcripts (band of 845 bp) (Fig. 4B, lanes 5 and 6) but not from noninoculated leaves. Two bands are visible in each positive lane, reflecting amplification products of the upstream primer (IV) with each of the downstream primers (III and V).

DISCUSSION

In this paper we describe the construction of full-length cDNA clones of RYMV. These clones, when placed under the control of the bacteriophage T7 RNA promoter, were used to produce transcripts *in vitro* that were infectious on *O. sativa* L. This is the first report of the production of infectious transcripts for a member of the sobemovirus group.

Capped transcripts produced from different individual full-length cloned cDNAs exhibited varying degrees of infectivity and were less infectious than viral RNA. We have studied transcripts from one clone, FL5, in greater detail than those of other clones because the former were moderately infectious. Rice plants inoculated with viral RNA were fully infected within 2 weeks after inoculation while plants inoculated with capped transcripts of FL5 showed a delay in the onset of symptoms, with yellow spots on upper leaves first appearing 3 weeks postinoculation. The infectivity of FL5-RNA transcripts demonstrated that a VPg moiety is not absolutely required for infectivity, as shown for other viruses that possess a VPg (Young *et al.*, 1991; Veidt *et al.*, 1992). In contrast, FL5-RNA transcripts required capping to be infectious in rice. The necessity of a 5′ cap has been previously described for viruses with a 5′ terminal VPg (Vos *et al.*, 1988; Domier *et al.*, 1989; Riechmann *et al.*, 1990) as well as naturally capped viruses (Dawson *et al.*, 1986; Janda *et al.*, 1987; Petty *et al.*, 1989). However, it is not absolutely necessary for all viruses since uncapped poliovirus transcripts are infectious (Van der Werf *et al.*, 1986).

The delay of symptoms of infection caused by *in vitro* transcripts from clone FL5 compared with virion-derived RNA might be explained by the extra 5′ nonviral G residue that was added to increase levels of *in vitro* transcription. Indeed, as demonstrated for other viruses lacking a native 5′ G residue, addition of a single G at the 5′ end of the transcript may be responsible for part of the low infectivity (Bujarski and Miller, 1992). When rice was inoculated with progeny virus derived from plants inoculated with FL5 transcripts, the time of appearance

of symptoms was not significantly different from plants infected by wild-type RNA (data not shown). This could be due to a process described for other viruses in which extra nonviral 5′ nucleotides were lost during virus replication, thus restoring full infectivity (Ahlquist *et al.,* 1984; Heaton *et al.,* 1989; Bujarski and Miller, 1992; Boyer and Haenni, 1994). This is consistent with data indicating that viral replication can repair and remove nonviral sequences (Xiong and Lommel, 1991). Nevertheless, we observed the rate of symptom spread in the systemic leaves of plants infected with FL5 progeny virions to be different than that in plants infected with wild-type virus. The reason for the low infectivity of FL5 transcript may be related to a number of factors related to the *in vitro* transcription reaction or to errors related to PCR amplification. Resequencing of the coat protein cistron from FL5 virions revealed that the FL5 coat protein gene differed from the CP sequence derived previously (Ngon a Yassi *et al.,* 1994) at two amino acids; Leu 34 was changed to proline, and Gly 156 was changed to valine (C. Brugidou, unpublished results). This may suggest that these are PCR-generated mutations leading to varying degrees of infectivity in different clones. Alternatively the full-length clone could have been derived from an RNA molecule different from those from which the viral RNA sequence was derived.

We used the FL5 full-length cDNA clone to generate two different coat protein mutants, *FL5-Δ MluI* and *FL5-Δ 3989–4112* (frameshift and deletion, respectively). Rice plants inoculated with transcripts derived from these clones did not show typical RYMV symptoms and both mutations in the coat protein gene were deleterious to virus accumulation. The RYMV CP was not detected and viral RNA accumulated poorly and only in inoculated leaves. To determine if RYMV CP is required for replication and/or movement, we studied the replication of transcripts in rice protoplasts and in whole plants. In rice protoplasts, transcripts of *FL5-Δ MluI* and *FL5-Δ 3989–4112* replicated as well as did viral RNA or FL5-RNA. In rice plants, RNA replication for both mutants was detected in inoculated leaves 4 weeks after inoculation (data not shown). However, these RNAs were poorly detected by Northern blots. We do not yet know if the low level of detection resulted from instability of the RNA or from RNA extraction techniques. Nevertheless, these results suggest that the carboxy-terminal region of the coat protein is not essential for viral replication. This result agrees with those published for the small RNA virus such as turnip crinkle virus (Hacker *et al.,* 1992).

In contrast to its dispensibility for replication, CP is essential for systemic virus movement. Rice inoculated with *FL5-Δ MluI* or *FL5-Δ 3989–4112* did not develop symptoms or accumulate virus particles 4 weeks after inoculation. The truncated coat protein (predicted to be 23.5 kDa) could not be detected by Western blot analysis in inoculated leaves or systemic leaves, suggesting that the immunoreactive epitopes were affected by the mutations or that the truncated protein is unstable. In the case of SBMV, Fuentes and Hamilton (1993) demonstrated in *Phaseolus vulgaris* L. that assembly of SBMV-C virions is required for long-distance movement. We have not yet determined if the CP mutants are able to move from cell-to-cell in infected leaves. It is known that many viruses encode movement proteins, which are responsible for cell-to-cell spread of virus via plasmodesmata (Hull, 1989). Fuentes and Hamilton (1993) suggested that short-distance and long-distance movement of SBMV-C are distinct and separate processes. In the sobemovirus group (RYMV and SBMV) a protein encoded by the first open reading frame could be the protein required for local movement. However, the differences in the amount of viral RNA accumulation in leaves inoculated with transcripts of the CP mutants and leaves inoculated with viral RNA or FL5-RNA (Fig. 4) suggests that RYMV CP is required along with a movement protein(s) for efficient cell-to-cell movement. Encapsidation is also likely to be essential for phloem-mediated long-distance movement. We are currently attempting to identify the roles of both movement and coat proteins in the RYMV life cycle.

The availability of infectious RNA transcripts opens the way to study replication and gene expression of RYMV. Sobemoviruses encode several proteins whose functions are not yet understood, particularly these corresponding to ORF1, ORF2, and ORF3 (Ngon a Yassi *et al.,* 1994). It is now possible to introduce mutations in specific ORFs and determine their effects on symptom development, RNA replication, cell-to-cell spread, and long-distance movement.

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