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Expression of rice yellow mottle virus coat protein enhances virus infection in transgenic plants

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Summary. The disease caused by rice yellow mottle virus (RYMV) is a major, economically important constraint to rice production in Africa. RYMV is mechanically transmitted by a variety of agents, including insect vectors. The production of resistant rice varieties would be an important advance in the control of the disease and increase rice production in Africa. We produced transgenic plants of the *Oryza sativa* japonica variety, TP309, to express a RYMV coat protein gene (CP) and mutants of the CP under the control of a ubiquitin promoter. Transgenic plants expressing genes that encode wild-type CP (wt.CP), deleted CP (ΔNLS.CP), mRNA of the CP, or antisense CP sequences of the CP gene were characterised. Eighty per cent (80%) of independent transgenic lines analysed contained CP gene sequences. Transgenic plants were challenged with RYMV and produced two types of reactions. Most of the plants expressing antisense sequences of the CP and untranslatable CP mRNA exhibited a delay in virus accumulation of up to a week, and the level of virus accumulation was reduced compared with non-transgenic TP309 plants. Transgenic plants expressing RYMV wild-type CP (wt.CP) and deleted CP (ΔNLS.CP) accumulated the highest levels of virus particles. These results suggest that antisense CP and untranslatable CP mRNA induced moderate resistance, whereas transgenic CP enhanced virus infection.

Introduction

The disease caused by various isolates of *Rice yellow mottle virus* (RYMV; genus *Sobemovirus*) is one of the greatest threats to rice production in Africa in both lowland and upland rice systems. The disease can cause yield losses of up to 100% depending upon the variety, the growing stage of the plant at the time of

infection, and the virulence of the virus isolate [2, 25, 30]. RYMV is transmitted mechanically, including by chrysomelid beetles [3, 16, 32]. RYMV epidemiology, distribution, and other characteristics have been reviewed elsewhere [20].

The coat proteins (CP) of plant viruses are involved in many aspects of virus-related biology, including encapsidation, virus replication, dissemination, and cell-to-cell and/or systemic movement [9]. In some cases, CP gene sequences can suppress RNA silencing (post-transcriptional gene silencing; PTGS) [29, 35]. The reaction of plants to virus infection is affected by a wide variety of defense mechanisms [14, 26, 35]. For RYMV, two types of resistance mechanisms have been described: a polygenic partial resistance found in the variety Azucena, and a highly effective recessive monogenic resistance gene which has been identified in a few accession lines, such as Gigante and Tog5681 [1, 17]. However, introgression of these traits into cultivated rice has not yet been achieved. Furthermore, both of these types of resistance can be overcome by repeated inoculations or by especially virulent isolates [15, 34].

In some cases, the natural resistance of plants against viruses can be replaced by transgenic resistance, which includes pathogen-derived resistance and the transfer of other natural resistance genes. Pathogen-derived resistance includes CP-mediated resistance (CP-MR) and RNA-mediated resistance. This strategy has been extensively used for protection against many viruses [5, 6, 9, 28] and has been reported as conferring resistance to rice stripe virus in transgenic rice plants [19]. RNA-mediated resistance observed in plants that produce untranslatable CP mRNA derived from a *Tobacco etch virus* isolate is related to PTGS [4, 22]. Transgenic rice plants that expressed a large fragment of ORF2a and 2b coding for an RNA-dependent RNA polymerase (RdRp) of RYMV, were reported to be partially or highly resistant to infection [27]. Nevertheless, these transgenic plants were found to be less resistant to RYMV than rice varieties that contain natural resistance genes [34].

In the present studies, approaches based on the concepts of CP-MR, sense RNA, and antisense RNA-mediated resistance have been used simultaneously with the aim of obtaining resistant plants. CP gene sequences were placed under the control of the constitutive ubiquitin promoter to produce transgenic TP309 rice plants which express genes encoding CP (wt.CP), deleted CP (ΔNLS.CP), antisense CP, and untranslatable mRNA. Two different types of reactions to RYMV inoculation were observed: first, plant lines producing untranslatable mRNA and antisense mRNA of the CP gene sequence showed a delay before infection, and the virus replicated at low levels compared with non-transgenic plants; second, lines producing wt.CP showed virus replication at higher rates than non-transgenic plants, suggesting that the transgenic CP acted as an enhancer of RYMV infection.

Material and methods

Plant material and virus isolates

Oryza sativa L. Taipei 309 (TP309), a japonica rice variety, was used throughout the production of embryogenic callus and cell suspension cultures. Selection, regeneration of transgenic plants, and culture conditions have been previously described [10, 21, 23]. The

resistance to RYMV infection of these transgenic lines was compared with IR64, a highly susceptible indica variety, Azucena, a partially resistant japonica variety, and Gigante, a highly resistant indica variety. The CP sequence used in this study was generated from RYMV isolate CI17 (RYMV-CI17, accession # AJ279918, S3 strain) collected in Côte d'Ivoire. We used two isolates for challenge inoculation: RYMV-Ma1 (accession # AJ279920, S1 strain) in growth chamber studies, and RYMV-BF1 from Burkina Faso (accession # AJ279901, a highly virulent S2 strain) in greenhouse studies. The antiserum raised against an isolate from Madagascar (RYMV-Mg1; accession # AJ608211, S4 strain) [24] was used in this experiment.

Plasmid constructs and DNA transfer to plants

The CP gene (ORF4 of RYMV, Fig. 1A) was amplified by PCR from the plasmid pCR II containing a cDNA of RYMV CP (ORF4 of RYMV-CI17 isolate), using the forward primer (F) N1 (5′CGCTCTAGATCTAAGATGGCCAGGAAGGGCAA-3′) and the reverse primer (R) N2

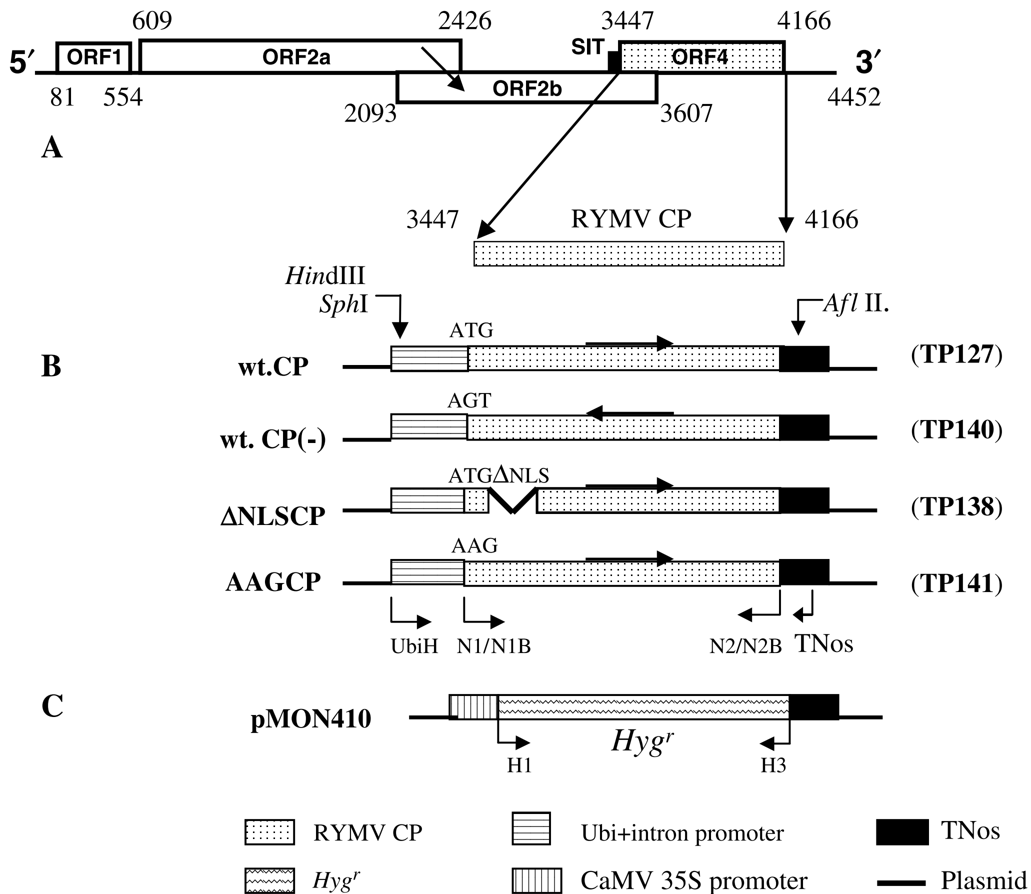

Fig. 1. Schematic diagrams of RYMV genomic organization and CP constructs. **A** Genomic organization of RYMV [18]; **B** Gene constructs used for rice transformation (construct names are given on the left-hand side and the designations of the resultant transgenic plant lines are shown on the right-hand side); **C** pMON410 plasmid carrying hygromycin resistance gene (*Hyg*^{r}) inserted in CaMV 35S promoter and Nopaline synthase polyadenylation site(TNos):. *SIT* transcription initiation site

(5′-GCCTCTAGATCTTCATCGCTGATTCCAATA-3′); the primers include *Xba*I or *Bgl*II sites. Two other forward primers designed to allow several mutations at the 5′ extremity of the CP gene were as follows: N4 (5′-CGCAGATCTAAGATGGCCCCACGTGGGCGTTCG GCG-3′), which includes a *Bgl*II site and allows deletion of a putative nuclear localisation signal (NLS) consensus sequence (aa 3 to 22), and N5 (5′-CGCTCTAGATCTAAGAAGGC CAGGAAGGGCAAG-3′), which produces a mutation of ATG to AAG to prevent translation of the mRNA to protein and includes *Xba*I and *Bgl*II sites. Cloned cDNAs encoding wt.CP or ATG.CP (CP with ATG), ΔNLS.CP (CP with NLS deleted), and mRNA.CP or AAG.CP (CP with ATG mutated to AAG) (Fig. 1B) were thus obtained. The above cDNA fragments were expressed *in vitro* as fusion proteins in *Escherichia coli* (*pTrcHisB* plasmid, Invitrogen). These cDNAs were inserted in the unique *Bam*HI site of pAHC17, a plant transformation vector [11] and placed under the control of the maize ubiquitin (Ubi) promoter and Nos 3′ terminator. The selectable marker used was the pMON410 plasmid containing the hygromycin phosphotransferase gene (*hph* or Hyg^r) placed under the control of the CaMV 35S promoter [31] (Fig. 1C). The DNA mixture of both transformation and selectable marker was introduced by particle bombardment as described by Chen et al. [10]. The following constructs and derived transgenic plants obtained were: wt.CP, with CP inserted in sense orientation, generating TP127 plants; antisense.CP, with CP inserted in antisense orientation, generating TP140 plants; ΔNLS.CP with ΔNLS.CP inserted in sense orientation, generating TP138 plants; and mRNA.CP with AAG.CP inserted in sense orientation and generating TP141 plants (Fig. 1B).

Characterization of transgenic plants

For PCR reactions, DNA was extracted from 25 mg of transgenic rice leaf tissue. The presence of the transgene was confirmed by PCR analysis. Two different pairs of primers were used to amplify the *hph* and the entire ubi-CP-Nos gene cassette in a single reaction. Amplification of the *hph* gene was primed by H1 (F) (5′-CGTCTGTCGAGAAGTTTC-3′) and H3 (R) (5′-TACTTCTACACAGCCATC-3′) (Fig. 1C). The ubi-CP-Nos cassette was amplified in the presence of primers UbiH (5′-ATGCCTGCAGTGCAGCGTGA-3′) and Nos (5′GTAACATAGATGACACCGCG-3′) (Fig. 1B). For RT-PCR, plant total RNA, extracted using TRIzol (Invitrogen™), was reverse transcribed and amplified according to Brugidou et al. [8] except that N2B and N1B primers (underlined sequences of N2 and N1 primers above) were used respectively as first and second primers.

Southern and Western blot analysis

For Southern blot analysis, plant DNA was extracted from 500 mg of transgenic plant leaves using the method described by Dellaporta et al. [13]. Approximately 5 μg of DNA was treated with *Hin*dIII to allow estimation of transgene copy number, and with restriction enzymes *Sph*I and *Afl*II to release fragments containing the entire ubi-CP-Nos cassette (Fig. 1B). Native and digested DNA were subjected to electrophoresis on a 1% agarose gel and transferred to a Hybond-N+ nylon membrane (Amersham). Hybridization reactions were performed using a 560-bp CP cDNA fragment labelled with α-^{32}P-dCTP as a probe. To detect CP accumulation in transgenic plants by Western blot, total proteins extracted from rice leaf tissues were separated on 12.5% SDS-PAGE, transferred to a nylon membrane, and treated with RYMV-Mg1 antiserum. The membrane was subsequently incubated with alkaline phosphatase-labelled goat anti-rabbit IgG and in NBT/BCIP mixture for protein detection.

Plant inoculation, symptom scoring, and DAS-ELISA

Plants were grown under controlled conditions (26/24 °C day/night and 16/8h illumination/dark/day and 70/60% day/night humidity) in a biosafety level 2 greenhouse required for transgenic plants (French Commission for Genetic Engineering consent for GMO culture no 4548). Two-week old R2 plants homozygous for the transgene were inoculated with RYMV particles diluted in phosphate buffer (20 mM KH_2PO_4 and Na_2HPO_4, pH 7.2) by finger rubbing the leaves in the presence of carborundum. To challenge the transgenic plants with RYMV, four doses of purified RYMV (0, 50, 100 and 200 ng/plant) were mechanically inoculated onto 15-day old plants resulting from homozygous lines. Scoring of symptom severity was performed using a scale of 1–9 of a standard evaluation system [18]. The level of virus accumulation on different days post inoculation (dpi), was measured in systemically infected leaves by DAS-ELISA as described by N'Guessan et al. [24].

Results

Analysis of transgenic plants

Transgenic R0 TP309 rice lines obtained from each construct are referred to as TP127 (plants expressing wt.CP); TP138 (plants expressing ΔNLS.CP); TP140 (plants expressing antisense.CP); and TP141 (plants expressing mRNA.CP) (see materials and methods). Some of the hygromycin-resistant putative transgenic plants were randomly selected for PCR and Southern analysis. The ubi-CP-Nos gene cassette and the *hph* gene were amplified in a single PCR reaction, and results indicated that all of the tested plants contained the *hph* gene, but only some contained ubi-CP-Nos gene cassettes. Only 60% of Hyg^r R0 plants and 37% of fully transgenic plants were at least partially fertile. Southern blot hybridization confirmed integration of the gene of interest (CP) in 62% of Hyg^r R0 plant genomes with a gene copy number ranging from 1 to 8. Western blot analysis revealed the presence of CP in most of the transgenic plants containing wt.CP and ΔNLS.CP (data not shown). Twenty-six homozygous R1 progenies including one negative segregant (TP127/6-3) and derived from 16 genetically distinct lines were kept to produce seeds.

Challenge of transgenic lines

Preliminary experiments were conducted to establish that non-transgenic TP309 control plants became infected when inoculated with 25 ng RYMV/plant, while 200 ng/plant was the virus saturation limit. For challenge experiments, twenty R2 progeny plants raised from seeds of each of the 26 homozygous R1 plants (16 genetically distinct lines) expressing the RYMV.CP constructs (at the mRNA level) and 6 control varieties were inoculated with RYMV particles (0, 50, 100, or 200 ng/plant). RYMV symptoms appearing on new leaves were scored at 17, 24, and 31 dpi. Leaf samples harvested from five plants growing in the same pot were pooled, and virus accumulation was assessed by DAS-ELISA.

Screening plants by RYMV symptom severity

All of the transgenic rice lines and varieties tested developed symptoms characteristic of RYMV infection except the varieties Gigante and Tog 5681; the variety Azucena exhibited mild symptoms. For the transgenic lines, symptom severity was mild at 50 ng/plant and severe at 200 ng/plant and did not show significant differences between the transgenic plants and control varieties. Differences in symptom severity were more conspicuous at 24 dpi on plants from different lines inoculated with 100 ng of virus (transgenic lines tested, $p < 0.0001$). Therefore, four different types of responses were distinguished according to the symptoms displayed by inoculated plants (Table 1). The first type of response is represented by symptom scale 1 (no symptoms observed): this response is typical of the highly resistant varieties Gigante and Tog 5681. The second type of response was composed of sparse chlorotic spots or streaking on green leaves and is characteristic of scales 2 and 3: plants with this score include the variety Azucena, mRNA.CP transgenic plants and antisense.CP plants. The third type of response consisted of plants exhibiting pale green leaves with mottling (symptom score 5) and included non-transgenic TP309, TP127/6-3 (R1 negative segregant plant), antisense.CP and mRNA.CP plants, wt.CP and ΔNLS.CP plants. The fourth type of response included plants that exhibit yellow to orange leaves (scores 7–9), and is similar to that of the control varieties BG90-2, IR 64, and most of the wt.CP and ΔNLS.CP lines (Table 1).

Screening plants by RYMV accumulation levels

Accumulation of RYMV-BF1 CP in leaf samples from plants inoculated with 100 ng of virus/plant was determined at 17 dpi using anti-RYMV-Mg1 antibodies in DAS-ELISA. A diagram has been constructed using 18 representative lines

Table 1. Symptom severity at 24 dpi of transgenic plants inoculated with RYMV-BF1

Transgenic lines and varieties	Symptom severity	Phenotype
Gigante, **Tog5681**	1e	HR
Azucena, TP141/9-2, TP141/16-4, TP141/16-7, TP140/20-17, TP140/20-12, TP140/25-2, TP140/39-2	3–3.5d	R
TP309, TP127/6-3, TP127/10-4, TP127/59-6, TP127/64-6, TP140/20-6, TP140/20-19, TP141/11-28, TP141/11-19, TP141/14-9	5–5.5c	MR
TP127/57-10, TP138/2-9, TP138/5-13, TP127/6-9, TP127/57-6, TP138/2-11, TP127/6-2, TP127/37-11, TP127/37-5, TP127/70-19	7–8b	S
IR64, **BG90-2**	9.00a	HS

Twenty plants per transgenic line (5 plants/pot) were inoculated with RYMV (100 ng/plant); average symptoms were scored for each pot at 24 dpi. *HR* highly resistant; *R* resistant; *MR* moderately resistant; *S* susceptible; *HS* highly susceptible. Symptom severity was analyzed according to the Newman-Keuls test. Numbers followed by the same letters are not significantly different at the 5% level. Control varieties appear in bold

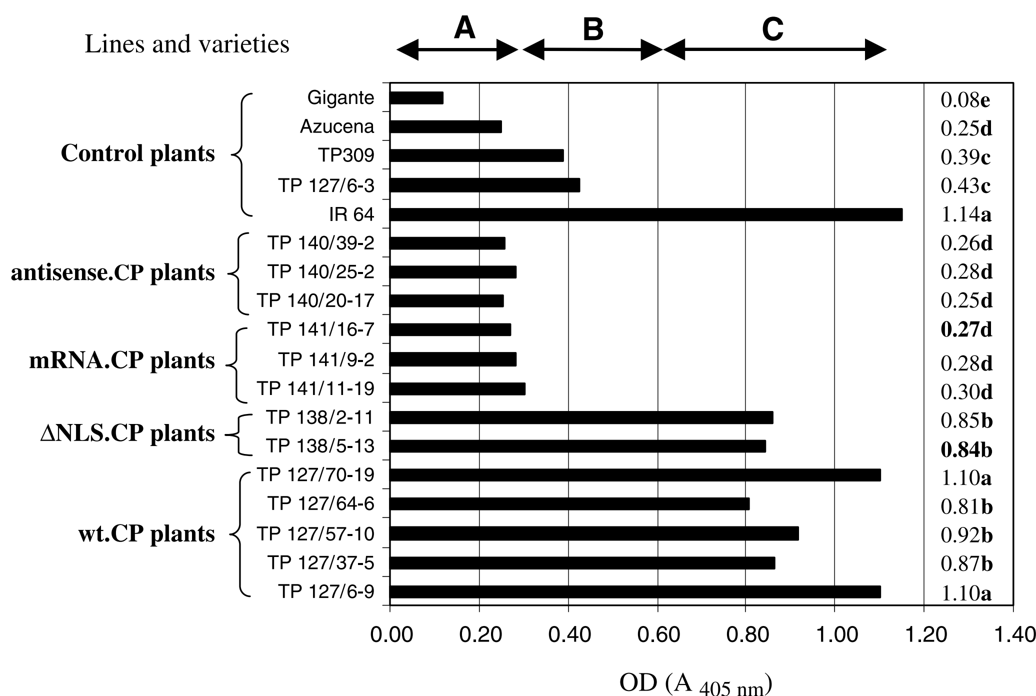

Fig. 2. Diagram illustrating RYMV-BF1coat protein (CP) accumulation in 18 transgenic lines and varieties at 17 dpi. Each bar represents the level of RYMV CP accumulation in transgenic lines or varieties tested. Numbers indicates the value of Newman-Keuls test for CP titer in transgenic plants. Numbers followed by the same letters are not significantly different at 5% level. **A**, **B**, **C**: levels of CP accumulation in inoculated plants; low (**A**), medium (**B**), high (**C**). Twenty plants per transgenic line (5 plant/pot × 4 pots) were inoculated with 100 ng of RYMV virions per plant; plant leaves from each pot were pooled and extracted as one sample. Thus four samples were tested for each line and the mean ELISA value was used for the diagram

and varieties according to the amount of RYMV that accumulated (13 genetically distinct lines and 5 control varieties). Three types of response are apparent. Firstly, lines which tested negative in ELISA or reacted weakly (OD < 0.30, see A, Fig. 2) did not accumulate RYMV at all, or only at low levels. This group includes the varieties Gigante, Tog5681 and Azucena, and the transgenic lines TP140/25-2, TP140/20-17, TP140/39-2 (lines expressing antisense.CP), TP141/16-7, TP141/9-2, and TP141/11-19 (lines expressing mRNA.CP) (Fig. 2A). Secondly, the lines which gave intermediate ELISA reaction (OD values of 0.30–0.60, Fig. 2B) include TP309 and TP127/6-3 (negative segregant). The third group (OD > 0.60, Fig. 2C) includes RYMV susceptible control variety (IR64), lines expressing wt.CP (TP127/70-19, TP127/64-6, TP127/57-10, TP127/37-5, and TP127/6-9) and lines expressing ΔNLS.CP lines (TP138/2-11, TP138/5-13) (Fig. 2C).

When a comprehensive analysis was conducted, combining symptom development and virus titer according to the nature of the transgene (wt.CP, ΔNLS.CP, mRNA.CP, antisense.CP) and comparing with control varieties, three different

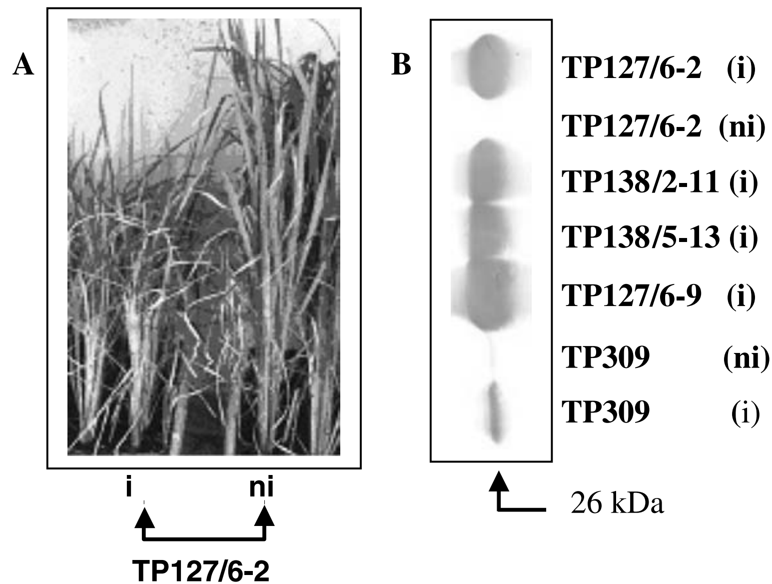

Fig. 3. Susceptibility of transgenic plants expressing the RYMV coat protein (CP) **A**: Transgenic plant challenged with RYMV-Ma1. TP127/6-2 is a wt.CP transgenic line **B**: Western blot analysis of total proteins from transgenic plants infected by RYMV-Ma1 at 17 dpi: (TP127/6-2 and TP127/6-9 are wt.CP plants; TP138/2-11 and TP138/5-13 are ΔNLS.CP plants; TP309 is a non-transgenic control). Arrow indicates the position of the 26-kDa CP of RYMV detected with polyclonal antibodies to RYMV-Mg1. *i* Inoculated; *ni* non-inoculated

groups were identified. In the group labelled as highly resistant (HR), the virus was undetectable by ELISA; this group includes resistant varieties Gigante and Tog 5681. The group of moderately resistant (MR) plant lines is composed of Azucena and transgenic plants that contain the antisense.CP and mRNA.CP constructs and TP309; this group showed low levels of virus replication. The group of highly susceptible (HS) plant lines includes transgenic plant lines that contain wt.CP, ΔNLS.CP, and the highly susceptible variety IR 64.

A subsequent experiment was conducted in a growth chamber using 48 plants per line of both control and inoculated plants. In this study, the plants were inoculated at 100 ng/plant of RYMV-Ma1, a mild RYMV isolate. At 42 dpi, the inoculated plants showed disease symptoms that included stunting and reduced leaf size, characteristic of RYMV infections (Fig. 3A). As in the studies with RYMV-BF1, we observed the three distinct groups of reactions (i.e., HR, MR and HS) in the plants tested (data not shown). Western blot analysis at 17 dpi demonstrated high levels of RYMV CP in transgenic lines (TP127/6-2, TP127/6-9) that contain wt.CP and in plants (TP138/5-13) carrying ΔNLS.CP constructs compared to non-transgenic TP309 plants (Fig. 3B).

Discussion

We considered pathogen-mediated resistance, with specific focus on CP-mediated resistance, as a possible means of inducing resistance to RYMV, based on positive

results of similar studies with other RNA viruses [5, 9, 28]. In this study we produced transgenic rice plants of the japonica variety, TP309, using various constructs of the RYMV CP gene (i.e., wt.CP, ΔNLS.CP, mRNA.CP, and antisense RNA) to increase the probability of obtaining resistant plants. Co-integration efficiencies of the target and *hph* gene of about 62% were obtained in this experiment, which is consistent with earlier reports using biolistic methods for rice transformation [21].

The transgenic plants that contained these constructs were challenged by inoculation with RYMV virions and revealed two different types of reactions to RYMV inoculation. Plants expressing antisense CP and untranslatable CP mRNA exhibited a delay in symptom development of up to a week and accumulated the virus at a low level compared with non-transgenic TP309 plants, suggesting that antisense CP and untranslatable CP mRNA induced moderate resistance. In contrast, transgenic plants expressing genes encoding RYMV wild-type CP (wt.CP) and deleted CP (ΔNLS.CP) accumulated RYMV CP at the highest levels, suggesting that transgenic wt.CP with or without the putative NLS acts as an enhancer of infection. However, the low level of resistance observed in these studies is lower than the natural partial resistance exhibited in the variety Azucena.

Screening experiments were conducted at two different sites (La Jolla, CA, USA, and Montpellier, France) and used different virus isolates: the moderately virulent isolate, RYMV-Ma1, was used in growth chamber studies and a virulent isolate, RYMV-BF1, was used in greenhouse experiments. The overall results of the two experiments were consistent, leading to the conclusion that the virus accumulation/resistance was not correlated to the aggressiveness of RYMV isolate. Furthermore, our results demonstrated that NLS included in RYMV CP was not required for this virus accumulation enhancement.

Based on the observation that expression of the CP enhanced virus accumulation in transgenic rice plants, we formulated three hypotheses which are mutually exclusive: (i) RYMV CP suppresses plant silencing of RYMV infection in transgenic plants, and thereby enhances plant susceptibility; (ii) RYMV CP enhances virus replication and cell-to-cell spread of infection; and (iii) RYMV CP may enhance infection by recruiting specific host factors that are required for replication and, when present, increase susceptibility to infection. Suppression of PTGS has been demonstrated with the RYMV P1 gene, which encodes a movement protein [7, 35]. There is no direct evidence that RYMV CP can silence suppression of virus infection.

In contrast, the moderate level of resistance in plant lines that accumulated antisense CP sequences may be related to the phenomenon of PTGS: an RNA band that may include siRNA was suspected (data not shown) only in moderately resistant antisense CP plants. The strategy of RNA-mediated resistance has been developed for RYMV at the Sainsbury Laboratory for widely grown, RYMV-susceptible varieties (Bouake189, ITA212, and BG90-2) using an ORF2a-ORF2b construct encoding a large part of the RYMV RdRp. The authors considered that the mechanism of homology-dependent resistance was the key factor of this resistance [27]. Nevertheless, additional experiments demonstrated that resistance in these transgenic plants was of moderate degree [33]. Like the transgenic lines

that expressed the RYMV RdRp messenger, the plant lines that accumulated antisense CP sequences showed a temporary, moderate degree of resistance against RYMV. Taken together, these results suggest that the mechanism of gene silencing is efficient at the beginning of infection and is subsequently overcome, probably by expression of viral silencing suppressors such as the RYMV P1 protein.

The results of these studies clearly indicate that the transgenic translated CP gene acts as an enhancer of virus accumulation, whereas antisense CP induces moderate resistance. Furthermore, the degree of resistance reported from the pathogen-derived resistance strategies tested here and in previous reports [27] is less effective than high levels of resistance in the varieties Gigante and Tob5681 or the partial resistance found in 'Azucena'. The transgenic lines containing RYMV CP sequences produced in this study cannot be used as resistant material in a breeding programme. However, these lines may be useful for biotechnology studies, including those investigating suppression or activation of PTGS.

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