Responses of *Oryza sativa japonica* sub-species to infection with *Rice yellow mottle virus*

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The distribution of *Rice yellow mottle virus* (RYMV) was compared in the partially resistant upland rice *Oryza sativa japonica* cv. Azucena and in the susceptible *O. sativa indica* cv. IR64, which are the two parents of a doubled haploid population studied for several traits, including genetic determinants of resistance to RYMV infection. Symptom expression, distribution and accumulation of viral coat protein and nucleic acid were studied in inoculated leaves, systemically infected leaves and apices, and in leaf host tissues. Initially, the resistance was apparent as delayed virus detection and multiplication which led to a lower virus content in inoculated leaves and in systemically infected organs. Later, the resistance was less apparent as virus titers became progressively similar in the two cultivars. Then, tolerance was observed, as symptom expression was less pronounced in cv. Azucena than in IR64, despite similar virus contents. In host tissues, partial resistance was associated with the delayed detection of virus in the bundle sheaths (mestomes) of Azucena, and, later, the mestome invasion paralleled the decline in resistance. The mature leaves of both cultivars always escaped infection. Partial virus resistance resulted from the expression of quantitative trait locus on chromosome 12, whereas tolerance is a characteristic of *O. japonica*, including the expression of QTL1. An impaired cell to cell movement through the mestome, possibly reinforced by slow vascular movement, are proposed to explain the partial resistance.

Keywords: *Rice yellow mottle virus*; quantitative trait locus; bundle sheath; partial resistance; tolerance; *Oryza sativa*; *Oryza glaberrima*.

**INTRODUCTION**

*Rice yellow mottle virus* (RYMV) of the genus *Sobemovirus* is the most damaging pathogen of rice (*Oryza sativa*) [5] in Africa, where it is widespread [31]. First described in Kenya [6], RYMV is now prevalent in all rice growing regions of Africa where it consistently induces serious yield losses [7]. RYMV is transmitted by beetle vectors of the family Chrysomelidae and possibly also abiotically [6, 7]. Infected plants are characterized by leaf mottling and yellowing, together with stunting, partial emergence of panicles and sterility. Early infection of susceptible cultivars often leads to plant death.

*O. sativa* comprises of two groups of cultivars, *indica* and *japonica*, which are analogous to sub-species [29]; the latter includes both upland and irrigated cultivars. *Indica* cultivars are very susceptible to RYMV infection, with the notable exception of cv. Gigante which shows a high resistance that is also found in a few cultivars of the indigenous African rice species *O. glaberrima* [39]. Upland *japonica* cultivars are partially resistant [2, 3, 41].

Coevolution between *O. sativa japonica* cultivars and RYMV cannot account for the natural resistance observed as these rice cultivars originate from Asia, whereas RYMV occurs only in Africa. The use of natural host plant resistance is an appropriate way to control RYMV [14, 41] and breeders have tried to introduce the resistance of upland *japonica* cultivars into productive *indica* cultivars that are adapted to irrigated conditions in Africa [39]. In an alternative approach, transformed lines encoding the RNA-dependent polymerase of RYMV were also found to be resistant to RYMV [36].

The genetic basis of the host responses to RYMV has been studied in a doubled haploid population derived from the partially resistant upland *O. japonica* cv. Azucena crossed with the susceptible *O. indica* cv. IR64 [19]. This is also a reference population for genetic mapping of other characters [16, 43, 44]. A quantitative trait locus (QTL) that is associated with a limited impact of RYMV infection on plant growth and yield is localized on chromosome 1 (QTL1) [3, 4]. Another QTL associated with low RYMV virus titers at early stages of infection and limited symptom expression is located on...
chromosome 12 (QTL12), close to the indica/japonica zone of differentiation, in epistasis with a QTL on chromosome 7 [37], but without links to morphological traits [4, 15]. By contrast to partial resistance, the high resistance found in cv. Gigante and in a few O. glaberrima lines is determined by a monogenic recessive genetic trait [30]. Preliminary studies indicated that partial resistance in upland O. japonica cultivars such as Azucena and IRAT177 and others [3] differed phenotypically and genetically from high resistance in cv. Gigante. In the present study, we characterized the differential responses to RYMV of the parents of the double haploid population used for genetic mapping of the partial resistance, i.e. cv. Azucena and cv. IR64. Differences in responses to RYMV infection between several rice sub-species, including cv. Azucena and cv. IR64, were investigated on whole plants by recording symptom expression and virus titer. The accumulation of both RNA and coat protein of RYMV and localization in leaf host tissues were studied in apices and leaves of cv. Azucena and cv. IR64 at early and late stages of infection. Responses to RYMV infection in cv. Azucena were found to combine (1) partial resistance expressed only at early stages of infection and characterized by delayed and lower virus accumulation in leaves and delayed virus invasion in bundle sheath tissues, and (2) tolerance that was apparent at later stages of infection and characterized by low symptom expression despite high virus accumulation.

MATERIALS AND METHODS

Plant material

Cultivars IR64 and Apura are of the O. sativa indica sub-species and are used in irrigated cropping systems. IR64 is a highly productive, widely cultivated and rapidly-growing cultivar. Apura is a traditional cultivar having exceptionally long grains. O. indica upland cv. Azucena and cv. IRAT177 are slow-growing cultivars with longer crop cycles and low tillering. They have larger and longer leaves and are taller than O. indica cultivars. Cultivars Honduras and Aichi Asahi belong to the O. sativa japonica sub-species used in irrigated cropping systems. The highly resistant O. sativa indica cv. Gigante [30] was also included in the study.

Plants were grown in a glasshouse under controlled conditions (28-32°C, 15 h of light per 24 h). An isolate of RYMV from Burkina Faso (RYMV-BF1; which induces severe symptoms [2] was used for most inoculations. Such an aggressive isolate was selected to maximize differences in response to infection between resistant and susceptible cultivars. Additional experiments were conducted with two isolates “S” and “M” from Côte d’Ivoire which induced severe and mild symptoms, respectively. Mechanical inoculations were made 2 weeks after planting on the third expanded leaf (leaf III), following the leaf numbering system of Matsuo and Hoshikawa [26], [Fig. 1(a)]. The inoculum was obtained by grinding 1 g of leaf extract in 10 ml of inoculation buffer (0.1 M KH2PO4 and 0.1 M Na2HPO4, adjusted to pH 7.2). Inoculation was performed by rubbing the leaf manually with inoculum mixed with carborundum. Soon after inoculation, the treated leaf was rinsed with water.

Immuno-printing tests

The immuno-printing test was derived from the direct tissue blotting technique [24]. After detaching the expanded leaf, the underside was fully covered with an
adhesive film so that the leaf would not shrink. The upperside of the leaf face was placed in direct contact with the nitrocellulose membrane, wrapped with a cellophane film, maintained at −70°C for 10 min and then pressed hydraulically at 24 bar cm⁻² for 2 min at room temperature. After separation from the rice leaf, the nitrocellulose membrane was dried for 15 min at 50°C.

RYMV was detected using a polyclonal antiserum raised against the capsid protein of an isolate which originated from Madagascar (RYMV-Mg antiserum). Background reactions were reduced by saturation of non-specific binding sites, incubating the membrane for 2 h at 37°C or overnight at 4°C in 5% w/v skimmed dry milk in phosphate-buffered saline with 0.05% (v/v) Tween 20 (PBS-T). The membrane was rinsed with PBS-T before a 2 h incubation at room temperature under agitation with the RYMV antiserum diluted 1:500 in 0.05% PBS-T with 5 U ml⁻¹ heparin (w/v). After three washings with PBS-T, the anti-rabbit secondary antibody conjugated to alkaline phosphatase (Sigma) diluted 1:4000 was added for 2 h under agitation at room temperature. Following three additional washings with PBS-T and two with PBS, the reaction was revealed with nitroblue tetrazolium chloride (NBT, 30 mg ml⁻¹) /bromo-chloro-indolyl phosphate (BCIP, 15 mg ml⁻¹). The enzymatic reaction was stopped by two washes with distilled water. The specific purple color of the immunoenzymatic reaction was assessed visually, photographs were taken and membranes were kept in a dry place.

ELISA tests

ELISA tests were performed as described previously [4]. The plant material was stored at −80°C. Leaves were ground in PBS-T 10⁻¹ g ml⁻¹. The extracts were centrifuged at low speed (5 min at 10 000 g), and the DAS-ELISA tests were performed on the supernatants. A 100 μl volume of polyclonal antibodies of the RYMV-Mg antiserum diluted 10⁻² in coating buffer was incubated for 2 h at 37°C in each well. A blocking step with 200 μl of 3% skimmed milk in PBS-T was performed. Then, 100 μl of leaf extract diluted 1:10, 1:100 and 1:1000 and RYMV-Mg antiserum conjugated with alkaline phosphatase diluted 1:1000 were incubated for 2 h at 37°C after three washings of 3 min with PBS-T. A 100 μl volume of para-nitrophenyl phosphate in diethanolamine buffer was added in each well. Absorbance was measured at 405 nm (Dynatech Mr 5000) after increasing incubation periods at room temperature.

Immuno-histolocalization of the viral coat protein

Fragments of systemically infected or healthy leaf tissues (about 2 mm), were fixed for 3 h in 1% (v/v) glutaraldehyde 4% (w/v), paraformaldehyde in 0.1 M cacodylate buffer pH 7.2, and dehydrated using a graded series of ethanol before embedding in LR White (TAAB, U.K., following the manufacturer's recommendations. Samples were semi-thin sections 1.5 µm cut with a diamond knife on a Reichert Ultracut E microtome. Sections were mounted in phosphate-buffered glycerol and examined with a light microscope (Leitz Diaplan). Excitation 430–490 nm, and barrier filters (515 nm), were used for fluorescence observation. For immunolocalization, all solutions were prepared in 20 mM phosphate-buffered saline pH 7.2, with 0.5% w/v bovine serum albumin (BSA) and 0.05% Tween 20 (PBST). After blocking in 5% w/v BSA (20 min at room temperature), sections were incubated for 60 min at 37°C on a drop of the primary polyclonal antibody of the RYMV-Mg antiserum diluted in PBST (1:40). After three washings in PBST, sections were incubated for 60 min at 37°C in fluorescein-labeled goat anti-rabbit antibodies (Biocell, U.K.), diluted 1:20 in PBST. Labeling specificity was assessed through the following control experiments performed on sections from healthy and infected leaves: '1', by incubation with the primary antibody previously adsorbed with the corresponding antigen; and '2', by omitting the primary antibody incubation step. Coat protein labeling was specific in all tissues observed except sometimes in the mesophyll where chloroplast fluorescence hinders the distinction between healthy and virus-infected cells. The inoculated leaves were not tested because the inoculum had been rubbed onto the leaf surfaces.

RT-PCR and Northern blot tests

Genome fragments with the coat protein CP gene of the isolates were transcribed and amplified by RT-PCR after extraction of total RNA from leaves. The protocol was essentially that of Brugidou et al. [9] and nucleotide numbers refer to the RYMV genome reported [32]. Primers A and B amplified 870 bp regions including the coat protein gene from nt 3447 to nt 4166 [32]. Primer A was complementary to the 22 nucleotides from positions 3451 to 3468. Primer B corresponded to the 18 nucleotides containing formaldehyde. RNA was transferred by blotting to Hybond N⁺ membranes. Hybridization was carried out with a randomly labeled DNA probe corresponding to RYMV ORF4 using the Mega prime DNA labeling Kit 'Amersham'. The probe was obtained after RT-PCR amplification of the CP gene as described above. Prehybridization and hybridization were done in 20 ml of prehybridization/or hybridization solution :6 x SSPE, 5 x Denhardt's reagent, 0.5% SDS and 100 μg ml⁻¹ of herring sperm DNA. Hybridization was
carried out overnight at 65°C. After hybridization, membranes were washed twice with 2 × SSC, 0.1% SDS for 15 min at room temperature, once with 1 × SSC + 0.1% SDS for 10 min at 65°C and once with 0.1 × SSC + 0.1% SDS for 10 min at 65°C. The membranes were covered with plastic wrap and exposed to Amersham film for 2 h.

RESULTS

Symptom expression and virus accumulation

Symptom expression, effects on growth and virus accumulation were assessed in experiments with the highly susceptible O. sativa indica cultivars (IR64 and Apura), the susceptible O. sativa japonica irrigated cultivars (Honduras and Aichi Asahi), the upland O. sativa japonica cultivars (Azucena and IRAT177) which have partial resistance associated to QTL128, and the highly resistant O. sativa indica cv. Gigante. Five plants of each cultivar were tested.

Symptom intensity increased rapidly after inoculation in cv. IR64 and cv. Apura leading to intense yellowing, which became necrotic [Fig. 1(b)]. By contrast, in cv. Azucena and cv. IRAT177 leaf symptoms were observed 2–4 days later, were milder and often restricted to the distal part of the leaf. The plants continued to grow and the impact on size and growth was limited [Fig. 1(b)]. Similar moderate symptoms and low impact on growth were observed with the irrigated O. japonica cv. Honduras and cv. Aichi Asahi. Symptoms were never observed in cv. Gigante. Immunoprinting tests also detected RYMV in the leaves of cv. IR64 and cv. Azucena at the same intensity and with the same distribution pattern [Fig. 2(b) and (d)], although symptoms were less pronounced or not seen in cv. Azucena [Fig. 2(a) and (c)]. Immunoprinting tests of infected leaves of cv. Gigante indicated no differences from leaves of healthy plants.

Virus titer was assessed by ELISA 14 and 21 days post-inoculation (d.p.i.) in systemically infected leaves. Cultivar (F = 12, df = 6, P < 0.001) and date (F = 52, df = 1, P > 0.001) effects as well as the cultivar × date interactions (F = 11, df = 6, P < 0.001) were significant in two-way variance analysis. At 14 d.p.i., cv. IR64, cv. Apura, cv. Honduras and cv. Aichi Asahi had similar high virus titers [Fig. 3]. IRAT177 and Azucena both showed a significantly lower virus content, although still significantly higher than in the highly resistant cv. Gigante. At 21 d.p.i., by contrast, the virus titer of cv. IRAT177 and cv. Azucena did not differ significantly from the susceptible cultivars. This decline of the partial resistance in Azucena and IRAT177 was clearly associated with an increase in virus content 14–21 d.p.i., whereas the virus content remained stable between these two dates in cv. IR64, cv. Apura and cv. Honduras (Fig. 3).

Altogether, four patterns of reaction to RYMV infection were observed: (1) susceptibility in the two O. indica cultivars with high virus titer and intense symptoms; (2) tolerance in the irrigated O. japonica cv. Honduras and cv. Aichi Asahi with moderate symptoms and high virus...
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The experiment was performed twice, each with a minimum of two plants. Patterns of detection clearly differed between infected Azucena and IR64 plants (Table 1, 1. In the inoculated leaf III, RNA (by RT-PCR, and CP (by ELISA, were detected concurrently from 5 d.p.i. in both cultivars (Table 1). Nevertheless, a lower virus content at 5 d.p.i. was found in Azucena, as determined by the failure to detect virus RNA by Northern blot (Fig. 4, 2. In the mature leaf II, neither RNA nor CP was detected at any time in either cultivar (Table 1, 3. In systemically infected apices and leaves of Azucena, there was a delay in virus detection. This delay was most pronounced in apices, as RNA was detected by RT-PCR from 9 d.p.i. in Azucena, compared with only 5 d.p.i. for IR64. By using Northern blots (Fig. 4), differences in band intensities indicated that RNA content was higher in IR64 leaves than in Azucena at 11 d.p.i. RNA was never detected in leaf extracts from Gigante by RT-PCR or Northern blot experiments.

Virus detection at later stages of infection

Immunoprinting and ELISA tests were used to study RYMV distribution in plants from 8 to 25 d.p.i. Two plants per treatment were tested and the experiment was repeated. At this later infection stage, virus was detected both in mechanically and systemically infected leaves, except in mature or almost mature leaves that were present at inoculation (discussed below). However, virus

Table 1. Spatio-temporal distribution of RYMV RNA and capsid protein (CP) revealed through RT-PCR and ELISA tests, respectively, in the susceptible cv. IR64 and the partially resistant cv. Azucena at early growth stages

<table>
<thead>
<tr>
<th>Testing date (d.p.i.)^a</th>
<th>1</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>9</th>
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<td>Leaf number II</td>
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</tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CP</td>
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<td>0</td>
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<tr>
<td>Leaf number III^c</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CP</td>
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<td>0</td>
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<td>1</td>
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</tr>
<tr>
<td>CP</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

^a Days post inoculation (d.p.i.,

^b 0/1 indicates differences between repeats, some being negative others positive, a blank that the leaf was not exerted sufficiently to be tested.

^c Inoculations were done on leaf III.
The virus was detected throughout the inoculated area. By contrast, it was not detected in the leaf portion which grew after inoculation, except in the basal part of cv. IR64, where RYMV was detected along the central vein [Fig. 2(b)]. In the systemically infected leaf, virus was detected when the leaf was exerted, but in cv. IR64 virus was often restricted to the basal portion (Fig. 2). A high rate of virus accumulation was detected by ELISA as early as 8 d.p.i. in the inoculated leaf III of cv. IR64. A significantly lower virus titer (F = 36, df = 1-12, P < 0.001 in variance analysis) was found initially in the inoculated leaves of cv. Azucena, but from 1 to 21 d.p.i. a similar high virus accumulation rate was noted [Fig. 5(a)]. In the systemically infected leaf of cv. IR64, an early and rapid increase in virus titer was observed between 11 and 14 d.p.i. In leaf of cv. Azucena, high rates of accumulation occurred from 8 to 25 d.p.i. in both

distribution and accumulation patterns differed between cv. Azucena and cv. IR64. RYMV was detected consistently from 8 to 25 d.p.i. in inoculated leaf III of both

| Table 2. Spatio-temporal distribution of RYMV capsid protein revealed through immunoprinting (IP) and ELISA tests in the susceptible cv. IR64 and the partially resistant cv. Azucena |
|---|---|---|---|---|---|---|---|
|  | 8 | 11 | 14 | 17 | 21 | 25 |
| IR64 | Azucena | IR64 | Azucena | IR64 | Azucena | IR64 | Azucena | IR64 | Azucena | IR64 | Azucena |
| Leaf number II | ELISA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | IP | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Leaf number III | ELISA | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | IP | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Leaf number IV | ELISA | 0 | 0/1* | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 |
| | IP | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Leaf number V | ELISA | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | IP | 0 | 0 | 1* | 1* | 1* | 1* | 1* | 1* | 1* |
| Leaf number VI | ELISA | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| | IP | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

ELISA reactions were scored as “1” when above 0.1 twice the average background reaction and “0” otherwise.

* Days post-inoculation d.p.i.

** Leaf III was inoculated.

* indicates that the distribution of the labelling in IP was restricted to the basal portion of the leaf, 0:1 that there were differences between repeats, some being negative, others positive.

**-** indicates that the leaf was dry and not tested, a blank that the leaf was not exerted sufficiently to be tested.
17 d.p.i., leading to virus titers similar to those of IR64 at 25 d.p.i. At 14 d.p.i. and by reference to a dilution curve from a quantified purified virus preparation, the virus content was estimated to be four-fold higher in IR64 than in Azucena in leaf III and double that in leaf IV.

Leaf V in cv. Azucena was exerted 5-7 days later than in IR64. Azucena plants were inoculated 6 days later than IR64 to take into account this difference in rate of leaf production. Accordingly, Azucena and IR64 plants had the same number of exerted leaves when inoculated and were at a comparable physiological stage [21]. A lower virus content was again observed in the mechanically infected leaf III of the cv. Azucena compared to IR64 [Fig. 5(b)]. In the systemically infected leaves of cv. IR64, high virus titers were reached as early as 11 d.p.i., whereas virus accumulation followed the same trend in Azucena but with a delay. At 21 d.p.i., similar virus titers were reached in both cultivars. Irrespective of the cultivar, virus titer was higher in the systemically infected leaves than in the inoculated leaves \( F = 9, df = 1-19, P = 0.004 \). At 21 d.p.i., IR64 plants were necrotic, resulting in lower virus titers than at earlier dates [Fig. 5(b)], whereas the plants of the tolerant cv. Azucena supported a higher virus titer [Fig. 1(b)].

\[ \text{Days post-inoculation (dpi)} \]

![Absorbance (A 405 nm)](Fig. 5) Virus accumulation in mechanically inoculated leaf III squares, and systemically infected leaf \( V \) triangles, in the susceptible \( O. \ indica \) cv. IR64 (black symbols), and the partially resistant \( O. \ japonica \) cv. Azucena; open symbols, inoculated concurrently [a, top], and when Azucena plants were inoculated 6 days later than IR64 [b, bottom]. Arrows indicate that leaves of IR64 became necrotic.

### Escape to infection in mature leaves

In the mature leaf II, CP was never detected in either IR64 or Azucena cultivars. The CP was not generally detected in the almost mature leaf IV of IR64. Traces of virus were sometimes found in Azucena using ELISA, but immunoprinting tests revealed that the distribution was then restricted to the basal portion of the leaf [Table 2]. The ability of leaves—such as leaf IV—to escape virus invasion was investigated in relation to the age of the plants when inoculated. RYMV titer was then assessed by ELISA in plants inoculated 15, 20, and 25 days post-germination [d.p.g.]. When inoculation was done early at 15 d.p.g., virus was again detected in the inoculated leaf III and in the younger systemically infected leaves V and VI, but not in leaf IV of IR64 [Table 3]. When the inoculation was done later, additional leaves were almost mature at inoculation and remained virus-free. In IR64 plants inoculated at 20 d.p.g., virus was not detected in leaves IV or V [testing date at 20 d.p.i.], or in leaves VI, VII or V at 25 d.p.g. [testing date at 25 d.p.i.]. This increasing tendency of leaves to escape the virus with later inoculation dates was less marked in the slowly growing cv. Azucena as only leaves IV and V escaped virus 20 or 25 d.p.g. [Table 3].

### Cytological detection of RYMV in host tissues

The experiment was duplicated and two plants were used per treatment. Sections were made across the middle of the systemically infected leaf \( V \). Three blocks were treated and at least three semi-thin sections per block were observed. The observations by immuno-fluorescence microscopy revealed RYMV localization at different times in the vascular tissues, the mesophyll and the bundle sheaths of systemically infected leaves \( V \). In rice, as in other monocotyledons, the bundle sheath is divided into two parts: an inner sheath with thickened walls mestome, and an outer sheath with thin walls [13]. Using immunofluorescence microscopy, no morphological differences in leaf structure were apparent between cv. IR64 and cv. Azucena. Eight days after inoculation, no difference in fluorescence was revealed in sections from infected leaves as compared to controls. At 11 d.p.i., RYMV was detected consistently in xylem and phloem parenchyma cells of cv. IR64 and cv. Azucena [Table 4]. From 14 d.p.i., a strong fluorescence was noted in mestome cells of IR64 leaves, in the mestome of irrigated cv. Honduras and cv. Aichi Asahi [data not shown], but not in those of cv. Azucena [Table 4, Fig. 6]. In xylem vessels and phloem sieve tubes, a weak fluorescence was detected erratically, mostly at the late stages, probably reflecting lower virus concentration. No fluorescence was observed in cells of the upper and lower epidermis, the
TABLE 3. Distribution of RTMV capsid protein assessed by ELISA at different leaf stages in the susceptible IR64 and the partially resistant Azucena rice cultivars according to the inoculation date

<table>
<thead>
<tr>
<th>Inoculation date (d.p.g.)</th>
<th>Testing date (d.p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>I</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>IR64</td>
<td>Azucena</td>
</tr>
</tbody>
</table>

Leaf number
- III
- IV
- V
- VI
- VII
- VIII
- IX

**a** Early inoculation “E” was done 15 days post-germination (d.p.g.); intermediate inoculation “I” 20 d.p.g. and later inoculation “L” 25 d.p.g. All inoculations were done on leaf III.

**b** ELISA tests were carried out 15 days post-inoculation (d.p.i.). for inoculation E, I and L (see below), 20 d.p.i. for inoculation I and 25 d.p.i. for inoculation L. Reactions were scored as “1” when above 0.1 (twice the average background reaction, and “0” otherwise.

“---” indicates that the leaf was dry and not tested, a blank that the leaf was not exerted enough to be tested.

TABLE 4. RTMV distribution in different leaf tissues as detected by immunofluorescence of the capsid protein in the susceptible cv. IR64 and the partially resistant cv. Azucena

<table>
<thead>
<tr>
<th>Testing date (d.p.i.)</th>
<th>8</th>
<th>11</th>
<th>14</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR64</td>
<td>Azucena</td>
<td>IR64</td>
<td>Azucena</td>
<td>IR64</td>
</tr>
</tbody>
</table>

**a** The systemically infected leaf V was tested.

**b** Fluorescence intensity was coded as follows: “0” fluorescence not higher than the healthy section of the same tissue; “1” weak fluorescence (a few cells were infected); “2” medium fluorescence (half or almost half the cells were infected); “3” strong fluorescence (all the cells were infected).

**c** Days post-inoculation (d.p.i.),

vascular bundle sheaths, or the sclerenchyma. No fluorescence was observed in sections of cv. Gigante.

To explore the tendency of partial resistance to decline in cv. Azucena at 14–21 d.p.i., changes in patterns of virus distribution in host tissues of leaf V of cv. IR64 and cv. Azucena were investigated further. Each cultivar was inoculated separately with “M” and “S” isolates known to induce mild and severe symptoms, respectively. Greater ELISA absorbances were noted 14 and 21 d.p.i. in plants infected by the S isolate than by the
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M isolate \((F = 9, \text{df} = 1-32, P = 0.007, \text{in variance analysis})\). At 14 d.p.i., virus distribution was restricted to the xylem and phloem parenchyma cells of plants infected by the M isolate. Virus was also detected in the mestome of IR64 infected by the S isolate (Table 5).

Higher ELISA absorbances were noted at 21 d.p.i. than at 14 d.p.i. \((F = 29, \text{df} = 1-32, P < 0.001)\). Moreover, there was no difference in virus content or distribution in the leaf tissues of cv. IR64 and cv. Azucena. The virus was detected in the xylem and phloem parenchyma and sometimes at high intensity, but also in phloem sieve tubes and in the bundle sheaths (Table 5).

**DISCUSSION**

The cultivars tested showed a wide range of reactions to RYMV infection. These ranged from susceptibility characterized by intense symptoms associated with high virus titer, to partial and high resistances, or to tolerance with moderate symptoms despite high virus content. Partial resistance with low virus titers was apparent only at early stages of infection. This contrasted with high resistance where RYMV, although present, was not detectable at all plant infection stages [30]. Partial resistance to RYMV also differed from that encountered in many other viral pathosystems in which resistance is expressed late [27]. It was transient and no longer apparent, usually 3 weeks after inoculation, regardless of isolate pathogenicity; high virus titers similar to those found in the susceptible cultivars were then reached.

Delayed virus accumulation in the upland *O. japonica* cv. Azucena at early stages of infection was unlikely to be due to morpho-physiological differences with the *O. indica* IR64 cultivar, the delay being apparent both in the inoculated and in the systemically infected leaves, in leaves of the same age or in leaves at the same phyllochronic stage. Tolerance that was not associated

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**FIG. 6.** Immunofluorescence localization of virus particles in semi-thin sections of LR white-embedded rice tissues using anticoat protein polyclonal antibodies. In sections from infected susceptible of cv. IR64 plants \(a\), at 14 d.p.i., strong green fluorescence was observed in the cytoplasm of vessel-associated parenchyma cells \(\text{small arrows}\), and in phloem parenchyma cells \(\text{arrowheads}\). Mestome \(\text{large arrowheads}\), also displayed fluorescence in the cytoplasm at the edge of cells. Labeling was not significant in vessels \(v\), sieve tubes \(p\), and bundle sheaths \(s\). Following labeling of sections from infected resistant cv. Azucena plants \(b\), at 17 d.p.i., weak fluorescence was observed only over vessel-associated parenchyma cells \(\text{small arrows}\); and on phloem parenchyma cells \(\text{arrowheads}\), but absent from vessels \(v\), mestome cells and bundle sheaths \(s\); (see Table 4). Sections from healthy susceptible plants \(c\) did not show significant fluorescence as compared to infected tissues. \(a, b, c\) bars = 8 µm.
with partial resistance was found in irrigated and upland O. japonica cultivars, whereas partial resistance was found only in upland O. japonica [4]. Partial resistance to RYMV resulted from the expression of QTL\textsubscript{12}, whereas tolerance is a physiological characteristic of O. japonica associated with expression of QTL\textsubscript{11}. These hypotheses on distinct genetic determinism between partial resistance and tolerance were confirmed by preliminary results from quasi-isogenic lines of IR64 introgressed with QTL\textsubscript{11} from CY. Azucena. Partial resistance with slower virus accumulation at early stages of infection has been transferred successively into the quasi-isogenic IR64 lines, but not tolerance: as leaf necrosis and plant death occurred at later stages of growth, as in the cv. IR64 [N. Ahmadi, personal communication]. For agronomic use, cultivars combining partial resistance with tolerance would suffer less from RYMV infection, but would be potent virus reservoirs.

Responses to RYMV infection in cv. Azucena and cv. IR64 varied in: 1. inoculated leaves; 2. mature and almost mature leaves; and 3. systemically infected apices and young growing leaves. This could be interpreted in the light of virus movement mechanisms, including RYMV: 'a', vascular movement of virions is closely associated with the movement of assimilates from "source" to "sink" leaves [23]; 'b', in inoculated leaves, RYMV moved as an RNA complex with the capsid and movement proteins, whereas RYMV moved as virions in the vascular tissues [8, 9]; 'c', leaf tissue infection patterns assessed by immuno-fluorescence microscopy confirmed previous ultrastructural observations that xylem and phloem parenchyma cells are the main sites of RYMV accumulation. Coat protein was detected erratically by immuno-fluorescence microscopy in the xylem vessels, probably reflecting the low virus content at these stages of infection, which is consistent with earlier reports from electron microscopy studies [34] where the virions present in the xylem vessels were at a much lower concentration than in adjacent xylem parenchyma cells; 'd', vascular and mesophyll tissues differed functionally [28, 38], and the bundle sheath-phloem interface was found to be an efficient barrier to virus movement in analyses of viruses having impaired systemic movement [18, 30, 40, 42].

1. RYMV was detected early in the inoculated leaves of both cultivars, which seems to be a consequence of applying inoculum to the leaf surface. The slower virus accumulation in inoculated leaves of the partially resistant cv. Azucena could reflect a delayed cell-to-cell movement,
possibly when passing through the vascular bundle sheaths. Consequently, the vascular tissues of inoculated leaves would be invaded later. Once invaded, however, the vascular tissues of the cv. Azucena supported rapid virus multiplication which explains why RYMV titers similar to those of cv. IR64 were reached. This is consistent with the finding that RYMV replicates as efficiently in cv. Azucena as in IR64 protoplasts [M.-N. Ndjindjop and C. Brugidou, unpublished results]. RYMV would then be transported to apices and other leaves as virus particles in the vascular system [33, 39]. The correlation between amounts of RNA and CP that were detected is consistent with the hypothesis that virus assembly is required for vascular movement [9].

2. Mature leaf II, and almost mature leaves 'leaf IV', escaped virus infection. They would be 'sources' for assimilates at inoculation and thus would not be invaded systemically, as observed in other plant-virus models [22, 27]. In rice, the number of leaves before flowering is predetermined [21], which explains why the proportion of leaves which escape infection increases at later inoculation dates. The basipetal sequence of leaves changing from photosynthetic sinks to sources would also account for the observed virus restriction at the basal part of the leaf, as noted in maize infected by maize streak virus [25, 35]. The lower number of leaves which escaped RYMV invasion in cv. Azucena reflected differences in leaf production sequences as O. sativa japonica cultivars grow slowly and produce leaves less rapidly than O. indica cultivars including IR64 [27]. The tendency of maturing leaves to escape infection illustrates the close relationship between rice physiology-morphology and virus movement in the plant. This phloem-mediated vascular movement of the virus is atypical as RYMV has been reported in both xylem and phloem cells. This possibly reflects that in rice plants the differentiation of vessels is complex [29], can occur only when the leaf is fully expanded [29, 34], and a more detailed study of the differentiation behavior is needed to elucidate the respective role of the phloem and the xylem in virus movement at early stages of infection.

3. In apices and young growing leaves which are strong "sinks" for assimilates [10, 17, 28], RYMV was detected at early as 5 d.p.i., even before much virus had accumulated in inoculated leaves. This time lag is consistent with that observed with Tobacco mosaic virus in tobacco [11, 12], which was interpreted as the time necessary to enter the vascular system or, alternatively, the time needed for the virus to reach a sufficient titer to initiate systemic infection [22]. Accordingly, delayed detection of RYMV in apices and in the systemically infected leaves of the slow-growing resistant cv. Azucena would reflect the initial delay of virus accession into the vascular system of the mechanically inoculated leaf; a delay possibly reinforced by slower vascular movement of RYMV and impaired movement of RYMV in the bundle sheaths which separate vascular tissues from the mesophyll. This delayed invasion of bundle sheaths, including the mesome, is reminiscent of the possibly delayed movement of the virus in the inoculated leaves from mesophyll through the bundle sheath toward the vascular tissues. After this delayed phase, high virus concentration was reached in both cultivars, probably resulting from similar high rates of multiplication in systemically infected leaves. This was reflected by immuno-cytolocalization of the virus in the central and minor veins where virus occurs. Moreover, the delayed invasion of bundle sheaths in systemically infected leaves suggests that additional factors in the mesome sheath may limit movement of the virus between the vascular tissues and the mesophyll in the partially resistant cv. Azucena.

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