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# Emergence of resistance-breaking isolates of *Rice yellow mottle virus* during serial inoculations

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**Abstract**

Two sources of resistance to *Rice yellow mottle virus* were challenged in host passage experiments. Pronounced changes in pathogenicity occurred over serial passages of virus isolates inoculated to partially or highly resistant cultivars. The changes encompassed the known existing pathogenic variability of field isolates. Ultimately, the high resistance of the *Oryza indica* cv. Gigante was overcome and the partial resistance of the *O. sativa japonica* cv. Azucena broke down. The effect was resistance-specific as different isolates overcame partial and high resistance, and may also be allele-specific as different isolates overcame the resistance of cultivars carrying the same resistance gene. The ability of isolates to break resistance was not linked to a high initial pathogenicity of the isolates and did not result in higher virus content in the infected plants. Implications for resistance breeding and deployment are discussed.

## Introduction

*Rice yellow mottle virus* (RYMV) of the genus *Sobemovirus* causes the most widespread and serious disease of rice in Africa. Identified for the first time in Kenya (Bakker, 1974), it now occurs wherever rice is grown in Africa (Abo et al., 1998). Control of the disease is based primarily on host plant resistance. Two types of natural resistance have been characterised phenotypically and genetically. A partial resistance that was associated with delayed virus accumulation in the host was found in *Oryza sativa japonica* cultivars (Albar et al., 1998). Partial resistance in these cultivars was associated to tolerance which was characterised by low symptom severity despite the high virus content at the late stage of infection (Ioannidou et al., 2000). Partial resistance is polygenic and a major quantitative trait locus (QTL) has been identified on chromosome 12 acting in epistasis with a QTL on chromosome 7 (Ahmadi et al., 2001; Ghesquière et al., 1997; Pressoir et al., 1998). A high resistance characterised by a low virus titre and the absence of symptoms was found in a few cultivars of *O. glaberrima*, including Tog5681 of the Tog series, and in the *O. sativa indica* cv. Gigante (Ndjiondjop et al., 1999). A single recessive gene common to Gigante and Tog5681 is located on chromosome 4 (Ndjiondjop, 1999; Ndjiondjop et al., 1999).

Marker-assisted breeding programmes are currently in progress to introgress the partial and high resistance genes into various cultivars (Ahmadi et al., 2001). The stability and durability of the resistances is a necessary prerequisite of the deployment of such improved cultivars. RYMV is a variable virus, and several strains having different geographical distributions and pathogenic properties have been characterised (N'Guessan et al., 2000; Pinel et al., 2000). Both partial and high resistances are effective after inoculation of various isolates in field and controlled conditions (Albar et al., 1998; Ndjiondjop et al., 1999; N'Guessan et al., 2001). It is known, however, that use of resistant cultivars may lead to the emergence of resistance-breaking virus isolates

(Lecoq and Pitrat, 1984; Masuta et al., 1999; Pelham et al., 1970; Zitter and Murakishi, 1969). This possibility was tested for RYMV and the results are reported here. Resistance-breaking isolates emerged rapidly after serial mechanical inoculations of resistant plants.

## Materials and methods

*Serially inoculated isolates*

Two RYMV isolates (named P and H) of the widely distributed strains S4 and S2 (Pinel et al., 2000), respectively, that induced the most commonly encountered pathogenicity pattern, were chosen for study. Each isolate induced intermediate symptoms on the susceptible *O. sativa indica* cv. IR64, inconspicuous symptoms on the partially resistant *O. sativa japonica* cv. Azucena and no symptoms on the highly resistant *O. sativa indica* cv. Gigante. The viral populations were transferred monthly to virus-free plants of each of the three cultivars for a total of eight passages. These were designated $P_0$–$P_8$ and $H_0$–$H_8$. Furthermore, after each passage, the serially transferred isolates were back inoculated to the other two cultivars. The experiment was duplicated and each replicate was tested independently. For each passage, the replicate consisted of two to four pots of each cultivar and each pot contained 4–6 rice plants.

Each transmission began by collecting leaf pieces from all portions of infected plants, including both inoculated and systemically infected leaves and both symptomatic and non-symptomatic leaves. Leaf pieces totalling ca. 500 mg per sample were ground using autoclaved mortars and pestles to avoid contamination. The inoculum was obtained by grinding 1 g of leaf extract in 10 ml of inoculation buffer (0.1 M $KH_2PO_4$ and 0.1 M $Na_2HPO_4$, adjusted to pH 7.2). Approximately 1 ml of this slurry was then inoculated manually onto 2-week-old plants. The leaf symptoms were assessed weekly and ranked I1, I2, and I3 for mild, intermediate, and severe symptoms, respectively (Figure 3). Viral titres were determined at each passage by ELISA as described by N'Guessan et al. (2000). Cross-reacting polyclonal antiserum able to detect both isolates with similar efficiency was used (N'Guessan et al., 2000). The experiments were conducted in growth chambers under 13-h illumination at 120 $\mu EM^{-2}\,s^{-1}$ of PAR at 30 °C and 90% relative humidity to facilitate uniform symptom development.

*Control isolates*

To assess the extent of changes in pathogenicity of the isolates used on serial inoculations, control isolates representative of the natural diversity of RYMV were used. Then, symptom development was followed on the partially resistant cv. Azucena and on the highly resistant cv. Gigante inoculated by (i) representative isolates of the various RYMV strains, (ii) isolates freshly collected from the fields that showed contrasting pathogenicity, (iii) isolates of the pathotype BF1 that severely affects susceptible *O. sativa indica* cultivars (Albar et al., 1998), and (iv) the rare pathotype A which induced symptoms on the highly resistant cv. Tog5681 (Konaté et al., 1997).

## Results

*Increase of pathogenicity after serial passages*

Preliminary experiments with representative isolates of the major strains and the other control isolates (except pathotype A) confirmed that cvs Azucena and Gigante were partially and highly resistant, respectively (Table 1). Similarly, both initial isolates $P_0$ and $H_0$ induced intermediate symptoms on the susceptible *O. sativa indica* cv. IR64, mild symptoms on the partially resistant *O. sativa japonica* cv. Azucena and no symptoms on the highly resistant *O. sativa indica* cv. Gigante. Subsequently, the pathogenicity of the viral populations derived from isolates $P_0$ and $H_0$ changed rapidly during the serial inoculations. With isolate P, symptoms became more conspicuous on cv. Azucena after the third passage ($P_3$) with more intense mottling and development of longitudinal streaks on the leaves, and size reduction of the plants. Symptom intensity on the 1–3 scale increased from I1 to I3 between the first and the fifth passage. At the fifth passage ($P_5$), leaf necrosis developed and the plants died (Figure 1). With isolate H, mottling symptoms occurred on the cv. Gigante at the fourth passage ($H_4$) and intensified at the sixth ($H_6$) leading to a marked stunting (Figure 2).

*Specificity of the pathogenicity changes*

The change in pathogenicity of isolates during serial inoculation was cultivar-dependent. Isolate $P_5$ which overcome the partial resistance of Azucena did not

*Table 1.* Symptom expression after inoculation of susceptible, partially, and highly resistant cultivars by control field isolates and by serially inoculated isolates[1]

| Cultivar[2] | Control isolates[3] | | | | | | Serially inoculated isolates[4] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S1 to S5 | I1 | I2 | I3 | BF1 | Path A | Isolate H | | | Isolate P | | |
| | | | | | | | H1 | H4 | H6 | P1 | P3 | P5 |
| *O. sativa indica* IR64 | ++ | ++ | +++ | +++ | † | ++ | ++ | ++ | ++ | ++ | ++ | ++ |
| *O. sativa japonica* Azucena | + | + | ++ | +++ | ++ | + | + | + | + | + | +++ | † |
| *O. sativa indica* Gigante | − | − | − | − | − | ± | − | + | +++ | − | − | − |
| *O. glaberrima* Tog 5681 | − | − | − | − | − | + | − | − | − | nt | nt | nt |

[1]Symptom intensity is ranked '+' (I1), '++' (I2), '+++' (I3) for mild, intermediate, and severe symptoms, respectively, '†' for death of the plant, and '−' for absence of symptoms; 'nt' indicates 'not tested'.
[2]IR64 and Azucena are susceptible and partially resistant cultivars, respectively; Gigante and Tog5681 are highly resistant cultivars.
[3]Isolates S1 to S5 are representative of the five major strains of RYMV, isolates I1, I2, and I3 are field isolates inducing mild, intermediate, and severe symptoms on Azucena, respectively, BF1 is a pathotype inducing severe symptoms on IR64, and pathotype A induces symptoms on Tog5681.
[4]Isolates H1, H4, and H6 correspond to passages 1, 4, and 6 of isolate H, and P1, P3, and P5 correspond to passages 1, 3, and 5 of isolate P, respectively.

induce symptoms on the highly resistant cvs Gigante and Tog5681. Nor did it induce increased symptoms on the susceptible IR64 (Table 1). Moreover, pathogenicity of isolate P did not change during five serial passages on cvs Gigante or IR64 (data not shown). Isolate $H_6$ which overcame the high resistance of Gigante did not induce increased symptoms on cvs Azucena or IR64 (Table 1). The pathogenicity of isolate H did not change either throughout five serial passages on cvs Azucena or IR64 (data not shown). Response to infection differed between the two highly resistant cvs Gigante and Tog5681. Isolate $H_6$ did not induce symptoms on Tog5681. Isolates of pathotype A which induced symptoms on Tog5681 rarely induced symptoms on Gigante. Those were inconspicuous and appeared at a late stage of infection (Table 1).

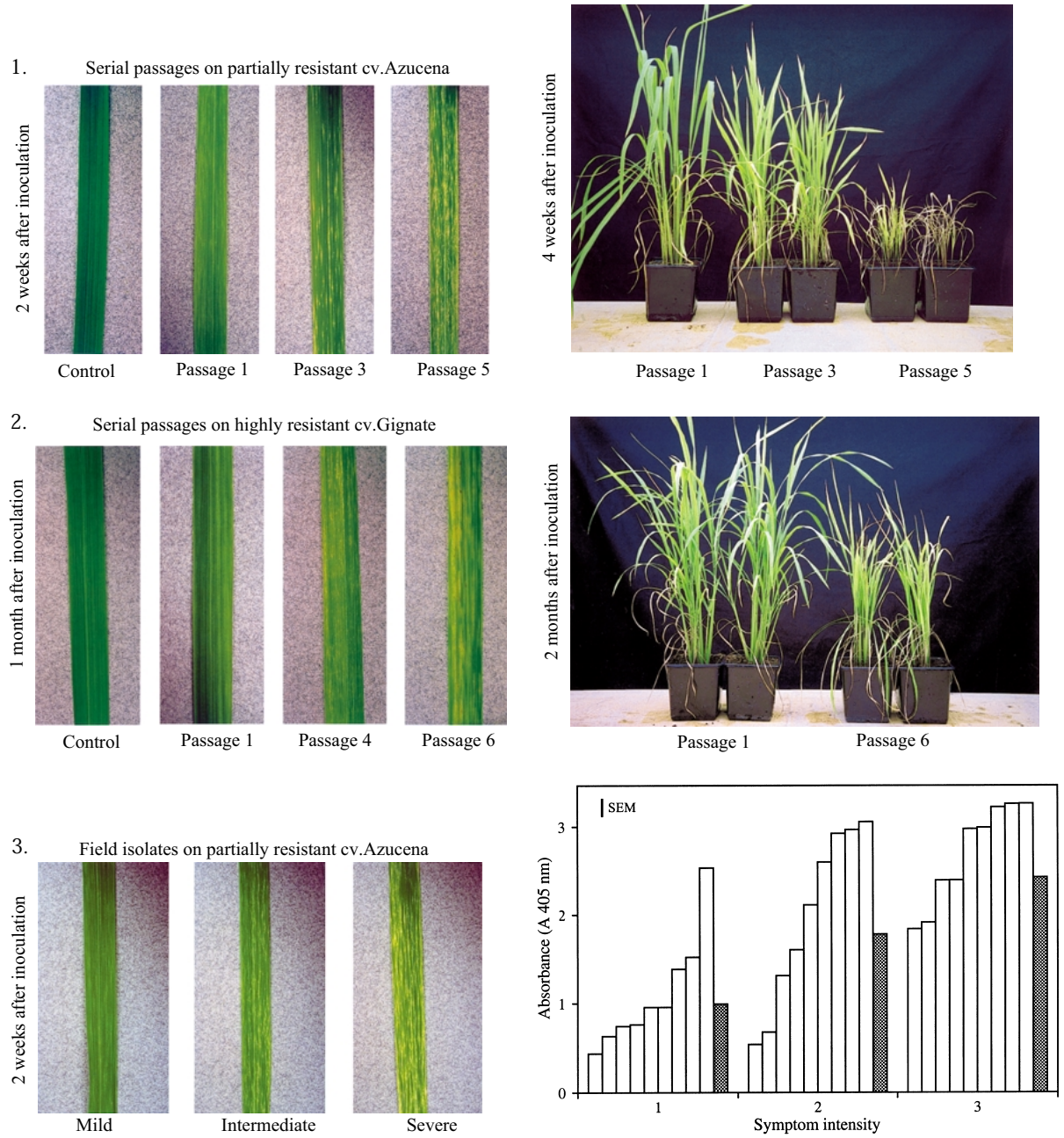
### *Natural diversity of the pathogenicity of field isolates*

The range of changes in symptom expression by cv. Azucena during the serial inoculations of isolate P was compared to the existing symptom variability recorded after single inoculation of field isolates. Thirty isolates collected in different parts of West and Central Africa were inoculated. Symptoms in cv. Azucena ranged from the mild I1 to the severe I3 (Figure 3). By contrast to inoculation with $P_5$, these isolates never led to plant death. This indicates that the change of pathogenicity of isolate P during five serial passages in cv. Azucena was wider than the range of pathogenicity occurring naturally in the fields. In cv. Gigante, pronounced leaf mottle and reduction in plant size were induced by inoculation with isolate $H_6$ whereas no field isolates ever caused such symptoms.

### *Pathogenicity and virus content*

Virus content was assessed in cv. Azucena after inoculation with nine representative isolates of each symptom class. There was a wide variability in virus content between individual infected plants showing similar symptoms (Figure 3). On average however, the more severe the symptoms, the higher the virus content ($P \leq 0.01$ after multiple mean difference with Fisher test). The differences in virus titre were significant between isolates inducing mild and intermediate symptoms ($P = 0.02$), and between those inducing intermediate and severe symptoms ($P = 0.05$).

Infection of cv. Azucena with isolate P was associated with a higher virus content than after the inoculation with the H isolate (Figure 4). Infection of cv. Gigante with isolate H was associated with virus content greater than with isolate P, the latter giving absorbances values not significantly greater than the background reactions (Figure 4). However, there was no trend of increase in virus accumulation with the resistance-breaking isolates despite symptom intensification over the five first passages of either cv. Azucena or cv. Gigante (Figure 4). Neither was there an increase later between the 5th and the 8th passage (data not shown).




*Figures 1–3.* (Figure 1) Detached leaves (left) and whole plants (right) of the partially resistant *Oryza sativa japonica* cv. Azucena two and four weeks after inoculation, respectively, with isolate P after one, three, and five serial passages. (Figure 2) Detached leaves (left) and whole plants (right) of the highly resistant *Oryza sativa indica* cv. Gigante one and two months after inoculation, respectively, with isolate H after one, four, and six serial passages. (Figure 3) Leaves of the partially resistant *Oryza sativa japonica* cv. Azucena two weeks after inoculation with field isolates inducing mild, intermediate, and severe symptoms (left). Absorbances ($A_{405\,nm}$) assessed by ELISA test of the corresponding leaf extracts one month after inoculation (right). Open histograms represent absorbances of extracts for individual isolates and the grey histograms indicate the average absorbance for the isolates causing symptoms of similar intensity. The vertical bar indicates the standard error of the mean (SEM).

## Discussion

Host passage effects on virus pathogenicity, transmission and host range are encountered frequently under certain conditions (Garcial-Arenal et al., 2001; Yarwood, 1979). In particular, resistance-breaking isolates can emerge after serial inoculation to virus-resistant plants (Lecoq and Pitrat, 1984; Masuta et al., 1999; Pelham et al., 1970; Zitter and Murakishi, 1969). With RYMV, rapid changes in the pathogenicity of isolates occurred during serial passages. Such changes led to partial or total breakdown of the resistances to RYMV. This occurred whatever the genetics of the resistances, whether it was monogenic or polygenic, recessive or dominant, with or without an associated tolerance. This illustrates the ability of RYMV for host adaptation.

These changes were highly specific as different isolates overcame the partial or high resistance of the cultivars assessed. Moreover, different isolates overcame the high resistance of Gigante and Tog5681, although the same single recessive gene occurs in both cultivars (Ndjiondjop et al., 1999). This suggests that two resistance alleles occur at this locus. This is consistent with recent findings derived from fine mapping of the high resistance gene (L. Albar and A. Ghesquière, unpublished results) and suggests that RYMV interactions with resistant rice cultivars are allele-specific.

As for other virus/host plant models (Saenz et al., 2001), the link between initial pathogenicity, virus content, and the ability to overcome resistance was complex. Changed pathogenicity was not associated with a particular strain as the tested isolates were of different strains. Breakdown of the resistance was not linked to high pathogenicity of the initial natural isolates. Increased pathogenicity on the resistant cultivars over successive passages was not associated with an increased virus accumulation, although a limited virus increase masked by individual variation in cv. Azucena cannot be entirely excluded.

As RYMV populations are genetically variable (Pinel et al., 2000), an explanation for the change of pathogenicity over serial passages is a selection within

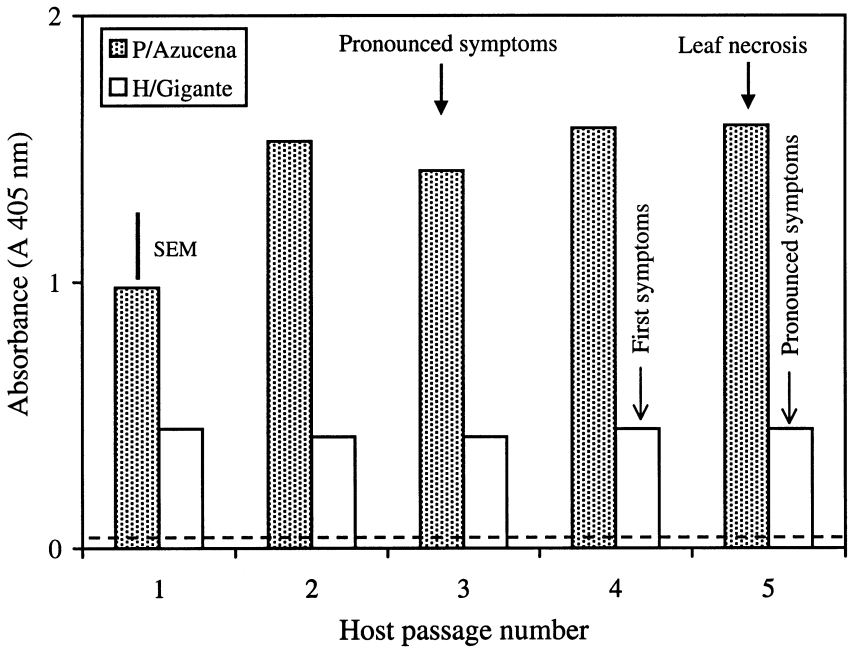



*Figure 4.* Absorbances ($A_{405\,nm}$) assessed by ELISA test of leaf extracts of the partially resistant cultivar *Oryza sativa japonica* cv. Azucena (grey histograms) one month after inoculation and of the highly resistant cultivar *O. sativa indica* cv. Gigante (open histograms) with the P and H isolates, respectively, over five successive passages. Arrows indicate at which passages the changes in symptoms occurred. Vertical bars indicates the standard error of the mean (SEM) in Azucena extracts. SEM in Gigante was 0.01. Extracts of infected cvs Azucena and Gigante were diluted 1 : 1000 and 1 : 20, respectively. Absorbance of cv. Azucena infected with isolate H ranged between 0.1 and 0.7 with an average of 0.3. Absorbance of cv. Gigante infected with isolate P was below 0.04 and was not distinguishable from the background reaction (dotted lines).

a population of RYMV molecules of pre-existing variants that can overcome the resistance. However, our results do not exclude the possibility of the emergence of new variants due to mutations occurring during the serial inoculation process. As partial and high resistance have been attributed, respectively, to an impairment and a blockage of virus movement (Ioannidou et al., 2000; Ndjiondjop et al., 2001), variants with different mobilities may have been selected during the serial passages. This hypothesis is now being tested.

The experiments were done using inoculum dosage that could result in multiple infection greater than that resulting from natural transmission in the field. However, the presence of natural isolates able to break host resistance (Konaté et al., 1997) indicates that the breakdown of RYMV resistance found in our laboratory experiments is relevant to field situations. In particular, the large-scale deployment of resistant cultivars may impose a selection pressure similar to that occurring in our serial passage experiments and lead to partial or total breakdown of resistance. Consequently, the durability of resistance should be assessed not only against naturally occurring isolates, but also by sequential inoculation of commonly occurring isolates. More generally, attention should be paid to the possible impact of large-scale deployment of resistant cultivars on the virus population structure and the associated risk of emergence of resistance-breaking isolates. Additional sources of resistance with different genetic determinants should also be sought and pyramided to increase the stability and durability of the resistance.

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