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High resistance to rice yellow mottle virus in two cultivated rice cultivars is correlated with failure of cell to cell movement

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Rice yellow mottle virus (RYMV) accumulation in protoplasts and whole plants was investigated in two highly resistant cultivars, Tog5681 (*Oryza glaberrima*) and Gigante (*Oryza sativa*). Three susceptible cultivars, i.e. one *O. glaberrima* Tog5673 and two *O. sativa* (IR64, *Ac.* 2428), and a partially resistant cultivar (Azucena) were used as control. After inoculation, accumulation of coat protein (CP) and viral RNA were monitored on protoplasts, inoculated leaves, sheaths of inoculated leaves and newly infected leaves by serological and Northern blot analysis. Viral RNA accumulated to a similar extent in protoplasts from all cultivars studied. In contrast, three distinct *in planta* behaviors were noted. In susceptible plants (IR64, Tog5673 and *Ac.* 2428), there was high CP and RNA accumulation at 5 d.p.i. in whole plants, suggesting that cell to cell and vascular movements occurred before 5 d.p.i. in inoculated leaves. The second behavior concerned Azucena, which showed a delay (around 7 d.p.i.) of viral accumulation in inoculated leaves. The third behavior involved the highly resistant cultivars Tog5681 and Gigante. CP and viral RNA were not detected in these cultivars. The comparison of viral accumulation in protoplasts and plants suggested that resistance of the highly resistant cultivars Tog5681 (*O. glaberrima*) and Gigante (*O. sativa*) was not due to the inhibition of virus replication but rather to the failure of cell to cell movement.

Keywords: Rice yellow mottle virus; *Oryza sativa*; *Oryza glaberrima*; virus movement; resistance; Sobemovirus.

INTRODUCTION

Rice yellow mottle sobemovirus (RYMV) is very destructive and therefore threatens rice production in parts of Africa and Madagascar where it is prevalent [*12*]. Under natural conditions, RYMV is transmitted by chrysomelid beetles [*4*] and can also be efficiently transmitted under artificial growing conditions through mechanical inoculation of virions or sap. RYMV symptoms include yellow mottling on leaves, reduced tillering, plant stunting, and flower sterility [*4*]. There can even be high mortality in infected susceptible cultivars [*4*]. The RYMV genome is a positive single-stranded RNA of 4450 nucleotides and contains only four open reading frames (ORFs) [*25*]. ORF 1 encodes the protein PI (17·8 kDa) known to be necessary for virus spread in rice plants [*6*]. ORF 2 proteins are necessary for virus replication, ORF 3 encodes proteins of unknown function and ORF 4 codes for the 26 kDa coat protein (CP). RYMV CP is not required for RNA replication but is necessary for virus spread (cell to cell and vascular movements) in rice plants [*7*]. Viruses need viral genetic material such as nucleic acid, proteins and host material to move in their hosts by cell to cell transport within parenchyma cells and spread rapidly through the vascular system [*8*, *13*, *17*]. Cell to cell movement of the virus from initially infected cells is believed to occur through plasmodesmata, which are plasma membrane-lined channels that traverse adjacent cell walls, thus connecting symplasts of adjacent cells [*20*]. Vascular movement normally occurs in the phloem [*22*, *29*] or xylem [*27*] and leads to systemic infection of the susceptible host.

Different degrees of resistance to RYMV have been detected in the cultivated Asian and African rice species *Oryza sativa* and *Oryza glaberrima*. In *O. sativa* species, partial resistance has been found in upland japonica rice cultivars such as Azucena [*2*, *23*]. Evaluation of RYMV

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Abbreviations used in text: BMV, bromo mosaic virus; CaMV, caulimo mosaic virus; CP, coat protein; ORFs, open reading frames; PVY, potato virus Y; RYMV, rice yellow mottle virus.

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resistance in *O. glaberrima* revealed five cultivars with high levels of resistance to RYMV [28, 31]. One *O. sativa* rice cultivar (Gigante) was also recently identified by the West Africa Rice Development Association (WARDA) and characterized as highly resistant to RYMV [23]. An analysis of the genetic basis of high resistance to RYMV in Tog5681 and Gigante highlighted the monogenic and recessive nature of this feature. The present study was aimed at investigating the mechanism of high resistance to RYMV in Tog5681 and Gigante cultivars at the cellular and whole plant level. Isolated protoplasts of resistant and susceptible rice cultivars were inoculated with RYMV particles to determine whether the resistance was expressed by inhibition of virus replication within single cells. By comparison, quantification of CP and RNA accumulation in inoculated and new infected leaves of different rice cultivars are also reported. These results suggest that RYMV resistance is correlated with the failure of cell to cell movement.

MATERIALS AND METHODS

Cultivars

Two cultivars (Tog5681 and Tog5673) of the African cultivated rice species *O. glaberrima* were provided by WARDA. Tog5681 is highly resistant to RYMV, as described recently [23]. Four *O. sativa* cultivars were also analysed. They displayed a rice diversity, with a range of reactions from highly susceptible to highly resistant to RYMV: IR64, a susceptible, high-yielding, irrigated *indica* cultivar; Gigante, a lowland *indica* cultivar resistant to RYMV [23]; Azucena, an upland japonica cultivar with partial resistance [2, 19]; and *Ac.* 2428, a temperate *japonica* cultivar which serves as a reference cultivar at the International Laboratory for Tropical Agriculture Biotechnology (ILTAB) for *in vitro* tissue culture.

Virus isolate and plant inoculation

A virus isolate from Côte d'Ivoire (West Africa) was used. This isolate was propagated on the susceptible IR8 cultivar at ILTAB. Four to 6 weeks post-inoculation, virus extraction was performed as described [11]. The inoculum was prepared by dilution of virus particles in phosphate buffer (0·1 M, pH 5) to obtain a 100 μg ml^{-1} solution of virus particles. This purified inoculum was used for all experiments.

Evaluation of RYMV resistance

Tests were carried out in a growth chamber under 28 °C day and 26 °C night temperature conditions, with 12 h of light. Three plants of each cultivar were used and one leaf of each plant was mechanically inoculated as described above. At different post-inoculation times, inoculated leaves, inoculated leaf sheaths and systemically infected leaves from three infected plants were mixed separately and analysed to measure the coat protein (CP) and viral RNA contents. ELISA tests for CP were conducted at 7, 14, and 22 and at 7, 14, 22, 37 and 50 d.p.i. for the limb portion and the sheath portion of inoculated leaves, respectively. The same test was carried out at 14, 22, 37 and 50 d.p.i. in the last systemically infected leaf. The viral RNA assessment was performed at 5, 7 and 10 d.p.i. on inoculated leaves. For the sheath portion of inoculated and systemically infected leaves, the viral RNA content was measured from the samples obtained for CP detection. This experiment was repeated twice.

Symptom scoring

To evaluate the response of the rice cultivars to infection, symptom expression was monitored at 7, 14, 22, 37 and 50 d.p.i. on inoculated and new infected leaves. The following scoring system was adopted: (−) symptomless leaves; (+) light and dispersed mottling; (++) clear mottling over the entire leaf surface; (+++) yellow colouration and beginning of necrosis at the top of the leaves; (++++) orange colouration and severe necrosis. To assess the general impact of inoculation on plant development, tiller emission was also noted and compared to that of uninfected plants (buffer inoculated plants) at the same dates.

ELISA protocols

Virus content was measured in leaves and leaf sheaths using the direct double antibody sandwich enzyme linked immunoabsorbent assay (DAS-ELISA) method [10], with a polyclonal antiserum prepared against the Madagascar strains according to a protocol described previously [23].

The virus concentration was estimated by reference to a standard RYMV concentration curve and expressed as μg g^{-1} of leaf fresh weight. In all of these experiments, uninfected leaves of each cultivar were used as negative control.

RNA extraction and Northern blot analysis

Total RNA was extracted from inoculated leaves and new infected leaves as described previously [9]. Equivalent amounts of total RNA (10 μg for susceptible cultivars and 20 μg for resistant Tog5681 and Gigante) were denatured and loaded on 1·2 % agarose gels containing formaldehyde [15]. RNAs were transferred by blotting to hybond N+ membranes (Amersham Biosciences). Prehybridization and hybridization were carried out with DNA probe corresponding to RYMV ORF4 (nucleotides

TABLE 1. *Disease evaluation by symptom expression on inoculated and new infected leaves and number of tillers per infected plant compared to uninfected plants*

	d.p.i.														
	7			14			22			37			50		
Cultivars	IL	NIL	T	IL	NIL	T	IL	NIL	T	IL	NIL	T	IL	NIL	T
IR64	+	−	1/1	+++	+++	1/2	*	*	*/3	*	*	*/4	*	*	*/4
Tog5673	+	−	1/1	+++	+++	1/2	*	*	*/3	*	*	*/4	*	*	*/4
Ac. 2428	+/−	−	1/1	++	+	1/1	+++	++	2/3	*	+++	2/3	*	+++	2/3
Azucena	−	−	1/1	+	+/−	1/1	++	+	3/4	*	++	3/4	*	+++	3/4
Tog5681	−	−	1/1	−	−	2/2	−	−	4/4	−	−	4/4	−	−	4/4

− symptomless leaves, + leaves with light and dispersed mottling, ++ clear mottles on inoculated and new infected leaves, +++ yellow colouration and beginning of necrosis on the top of leaves, ++++ orange colouration and severe necrosis or * death of the leaf or plant, IL: inoculated leaf, NIL: new infected leaf, T: number of tillers.

3447–4199 [25]). The DNA probe was obtained by PCR amplification using forward and reverse primers purified with Geneclean (Qbiogene, Carlsbad, CA, U.S.A.). DNA was labelled by random primer labelling (Stratagene, La Jolla, CA, U.S.A.) and purified by Nuctrap push columns (Stratagene). The membranes were washed at 65 °C, twice with 2× SSC (standard saline citrate) at 0·1 % SDS for 10 min, twice or thrice with 1× SSC at 0·1 % SDS for 15 min, and once with 1× SSC at 0·1 % SDS for 15 min. Viral RNA was quantitated by exposing the Northern blots to storage phosphor screen plates and counting on a phosphorimager (Biorad, Laboratories Inc.).

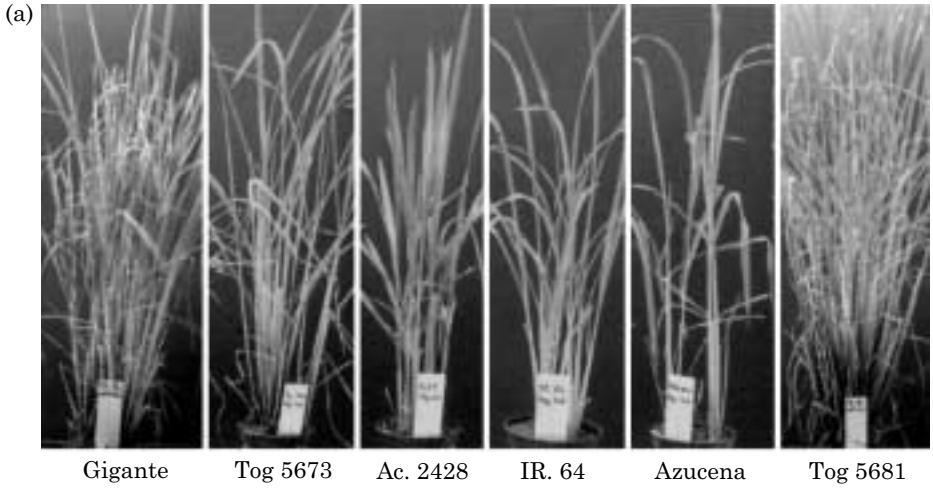
Infection of rice protoplasts using RYMV particles

Protoplasts were isolated from immature embryos of *O. sativa Ac.* 2428, Gigante, *O. glaberrima* Tog5681 and Tog5673 cell suspension cultures as described previously [6, 7]. Protoplasts were washed twice with CPW medium and resuspended in 1 ml R2 medium [26]. One μg of RYMV particles was used to inoculate 1 ml of protoplast suspension at $0{\cdot}5 \times 10^6$ density using the Gibco-BRL Cell-Porator system I [6]. One h after incubation in the dark at 28 °C protoplasts were washed with R2 medium and transferred to Petri dishes (35 × 10 mm) in 1 ml R2 medium and incubated in the dark for 24 h. Total RNA was extracted 1 and 24 h post-incubation from pelleted protoplasts by phenol extraction [14] and 10 μg was loaded on agarose denaturing gel for Northern blot analysis as described above. Integrity and amount of RNA was checked visually under u.v. using ethidium bromide staining. The protoplast experiment was repeated thrice. As all the tested varieties were found equally susceptible to RYMV and showed high replication ability in protoplasts regardless of the resistance, tolerance or susceptibility at plant level, a control of the protoplast experiment with another virus was not found necessary.

RESULTS

Plant response to viral infection

Symptom expression and the impact of RYMV disease on plant growth were assessed throughout the experiments (Table 1, Fig. 1). Mottling and yellow colouration appeared clearly on inoculated leaves of the two cultivars IR64 and Tog5673 as early as 7 d.p.i. At 14 d.p.i., a drastic increase in mottling intensity was observed on inoculated and new emerging leaves. Inoculated leaves began rolling up and mottling was also observed on sheaths. Complete leaf necrosis occurred rapidly without further growth. At 22 d.p.i., plants were dead and further evaluation of virus content was no longer possible. *Ac.* 2428 also showed mottling and yellow colouration on inoculated leaves at 7 d.p.i., but with slightly lower intensity than noted on the susceptible cultivars IR64 and Tog5673. Symptoms increased at a slower rate and it took until 22 d.p.i. to affect the whole plant. Inoculated plants produced few tillers. Although inoculation had a very severe impact, plants were able to develop to some extent, and all of them were still alive at 50 d.p.i. Azucena presented mottling and yellow colouration on inoculated leaves only at 14 d.p.i. There was a delayed increase in mottling intensity and no leaf necrosis was observed. Tillering was also reduced. Conversely, no symptoms were detected in inoculated leaves or new emerging leaves of the resistant cultivars Gigante and Tog5681, even at 50 d.p.i. These two cultivars were able to grow and flower normally without any phenotype alteration. Under very drastic virus infection conditions (early inoculation and utilization of a large amount of virus particles), marked differences in plant responses were observed. *Ac.* 2428 was found to be less susceptible than IR64 and Tog5673. Azucena showed the partial resistance pattern usually found in upland japonica rice



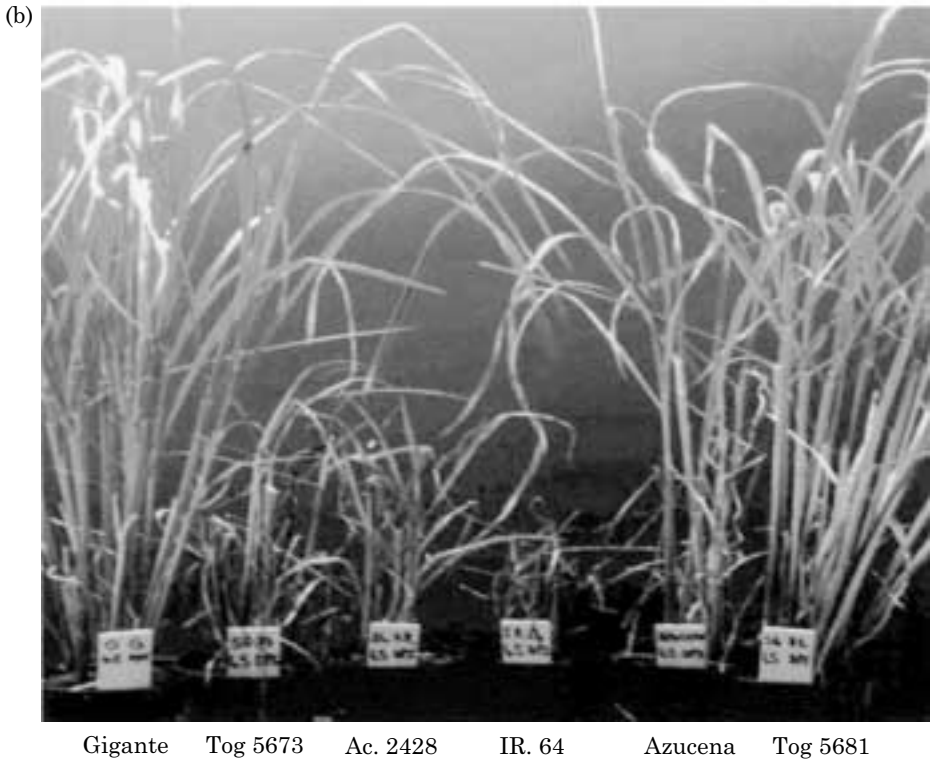


FIG. 1. Impact of RYMV disease on plant growth. (a) Uninfected plants of cultivars Gigante, Tog5673, *Ac.* 2428, IR64 and Tog5681; (b) infected plants at 25 d.p.i. Leaves were mechanically inoculated with a purified RYMV isolate from Côte d'Ivoire or mock-inoculated with buffer at 10 days post-germination.

cultivars, whereas the results confirmed the high resistance of Tog5681 and Gigante to RYMV.

Viral RNA accumulation in rice protoplasts

To investigate whether the high resistance of Tog5681 and Gigante was not due to a RYMV replication failure, rice protoplasts were used to study RYMV accumulation at early stages of infection. Total RNA was extracted from protoplasts of susceptible and resistant cultivars at 1 and 24 h post-infection and the accumulation of newly synthesized viral RNA was determined by Northern blot analyses using a probe corresponding to RYMV ORF4. A very small amount of genomic RNA (4·45 kb) was detected at 1 h.p.i. (Fig. 2). In contrast, at 24 h.p.i., substantial accumulation compared to 1 h was observed in protoplasts from all the cultivars tested, including highly resistant Tog5681 and Gigante. This genomic RNA corresponded to RNA replication and accumulation in infected cells.

TABLE 2. *RNA accumulation in inoculated leaves, leaf sheaths and leaf limbs from different rice cultivars*

	Inoculated leaves			Inoculated leaf sheaths					New infected leaves			
	d.p.i.											
Cultivars	5	7	10	7	14	22	37	50	14	22	37	50
IR64	48	48	27	57	55	*	*	*	96	*	*	*
Tog5673	43	44	63	57	58	*	*	*	83	*	*	*
Ac. 2428	12	12	7	6	10	56	10	11	27	32	20	16
Azucena	0	0	0	6	37	45	56	100	23	14	33	40
Tog5681	0	0	0	0	0	0	0	0	0	0	0	0
Gigante	0	0	0	0	0	0	0	0	0	0	0	0

*: dead plant. Total RNA was extracted at different d.p.i., transferred to membrane, and hybridized with a probe complementary to ORF4. Band intensity was evaluated with a Phospho imaging analyser.

At the protoplast level, viral RNA accumulated to the same extent in the susceptible, partially and highly resistant cultivars.

Viral RNA accumulation in rice plants

In inoculated leaves, viral RNA accumulation was detected at 5 d.p.i. in the susceptible cultivars (IR64, Tog5673 and *Ac.* 2428) (Table 2). At this date, a low level of RNA was observed in Azucena, and this level remained low up to 10 d.p.i. At 7 d.p.i., a high level of RNA was observed for the three susceptible cultivars (IR64, Tog5673 and *Ac.* 2428). After this date, the RNA intensity decreased in the leaf sheaths of *Ac.* 2428. For Tog5673 and IR64, plants died at 14 d.p.i.

Conversely, in the partially resistant cultivar (Azucena), viral RNA accumulation was noted even up to 50 d.p.i. In these experimental conditions, viral RNA was not detected in either of the resistant cultivars (Tog5681 and Gigante).

Viral-encoded protein accumulation of RYMV in inoculated leaves, sheaths and new infected leaves

The time course of infection was monitored by the amount of CP accumulation in different parts of infected plants. Three distinct responses were recorded in the tested cultivars (Fig. 3)..

The first group included susceptible cultivars IR64, Tog5673, and *Ac.* 2428. In these cultivars, the CP content was already high (0·8–1 μg g^{-1}) in the inoculated leaves, sheaths and systemically infected leaves at 7 d.p.i. At 14 d.p.i., cultivars IR64 and Tog5673 had a high virus concentration (1·3–1·5 μg g^{-1}) and plants died. By contrast, the cultivar *Ac.* 2428 remained alive throughout the study, with a higher level of virus compared to the other cultivars (1·4 μg g^{-1} at 22 d.p.i.). However, in this cultivar, the CP content decreased from 22 d.p.i. in sheaths and systemically infected leaves (from 1·4 to 0·9 μg g^{-1}).

The second group concerns the partially resistant cultivar Azucena which showed a clear delay in virus accumulation. A delay was observed in inoculated leaves (very low level of CP at 7 d.p.i.), then CP amounts increased slowly in inoculated leaves, and sheaths, with the same delay of around 7 days. In systemically infected leaves, the CP content was similar to that in the susceptible cultivars described previously (around 1 μg g^{-1}), and after 14 d.p.i. the virus level remained stable as shown by the CP content in systemically infected leaves.

The third group includes the highly resistant cultivars Tog5681 and Gigante for which the CP was not detected at any time over the course of the study.

DISCUSSION

The high resistance to RYMV was found to be based on a single recessive gene common to the two cultivars Tog5681 and Gigante [23]. This supports the hypothesis that a constitutive resistance mechanism disrupts the infectious process. This study was aimed at assessing the step that could explain the apparent immunity of the two highly resistant cvs Tog5681 and Gigante.

RYMV replication in rice protoplasts

RYMV RNA accumulated to the same levels in protoplasts from the highly resistant cultivars and in protoplasts from the susceptible and partially resistant cultivars at 24 h.p.i. In the experimental conditions, the authors thus assumed that protoplasts from heterotrophic cell suspensions of the resistant cultivars would be able to replicate RYMV to the same extent as the susceptible cultivars. Alternative mechanisms could be proposed to explain the high resistance patterns found in Tog5681 and Gigante

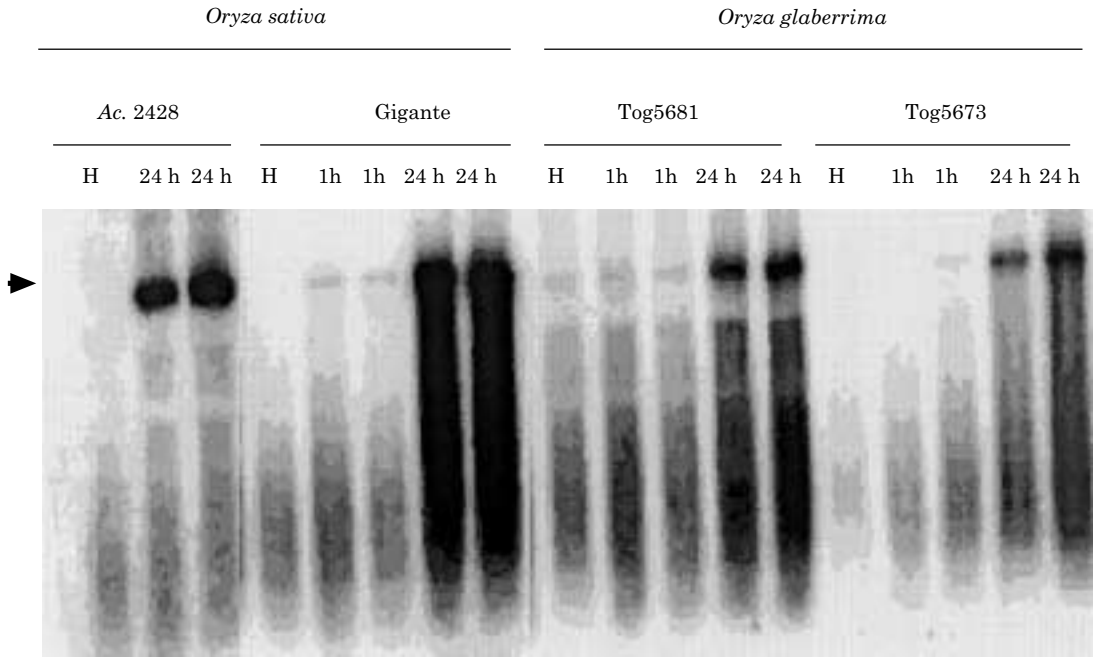


FIG. 2. Viral RNA accumulation in protoplasts produced from cultivars of *O. sativa* (*Ac.* 2428 and Gigante) and *O. glaberrima* (Tog5681 and Tog5673). Northern hybridization analysis with a RYMV CP probe 1 and 24 h post-infection. Protoplasts were infected with virus (1 μg ml^{-1}). Healthy refers to the uninfected protoplast sample (H).

despite the apparently normal virus replication capability in protoplasts. Viral compounds such as movement proteins may contribute substantially to the virus infection process, thus explaining why plant protoplasts are frequently able to support the replication of viruses unable to infect intact plants [5]. Such a resistance mechanism was observed in the interaction between *Arabidopsis thaliana* and the turnip crinkle virus, which showed normal virus replication in protoplasts of the resistant ecotype Di-0, but inhibition of movement and absence of symptom expression when the whole plant was infected [30]. This is further supported by the capability of protoplasts prepared from infected plants or virus-inoculated protoplasts to replicate potato virus Y (PVY) in the pepper cultivar Yolo Wonder Y, bearing the recessive resistance gene y^a which confers resistance to the pathotype 0-PVY [3].

Virus spread in the highly resistant rice varieties Tog5681 and Gigante

There are two different modes of viral transport within the plant: (1) cell to cell movement via plasmodesmata; and (2) long distance movement via the sieve tube network of the phloem [8, 17], and between xylem cells through pit membranes [27]. In this paper, the spread of RYMV in rice plants was compared between a partially resistant cultivar (Azucena) and different susceptible cultivars (IR64, Tog5673 and *Ac.* 2428) and two highly resistant rice cultivars (Tog5681 and Gigante). Using very drastic inoculation conditions (100 μg ml^{-1} of virus), the virus was detectable as early as 5 d.p.i. in sheaths and the limb portion of inoculated leaves, and in new infected leaves of all the susceptible cultivars. The results suggested that there is no limitation to virus spread in a susceptible cultivar such as IR64, and leads rapidly to plant death (Fig. 1). In this cultivar, cell to cell and vascular movement occurred before 5 d.p.i. Replication in vascular tissues and vascular movement in new infected leaves were supposed to be achieved at the same moment [27]. Conversely, the partial resistance of Azucena was clearly expressed by the delay in virus spread in inoculated plants. Virus content was not significantly detected before 7 d.p.i., and it was correlated with a lower RNA accumulation up to 14 d.p.i. Nevertheless, the kinetics of virus spread in Azucena showed that the delay was rapidly overcome. The different responses to RYMV infection in rice thus suggested that partial resistance in *O. sativa* could come from different components involving morpho-physiological and genetic traits relative to the genetic differences between *indica* and *japonica* rice cultivars. The identification of morphologically-dependant and morphologically-independant QTLs for RYMV resistance in the (IR64 × Azucena) double haploid populations supports this hypothesis [2].

In contrast, all viral compounds revealed in the two highly resistant cultivars did not differ from the negative control. The absence of a detectable CP level could have been due to a lower number of infected sites in inoculated leaves, i.e. insufficient to allow the detection of these

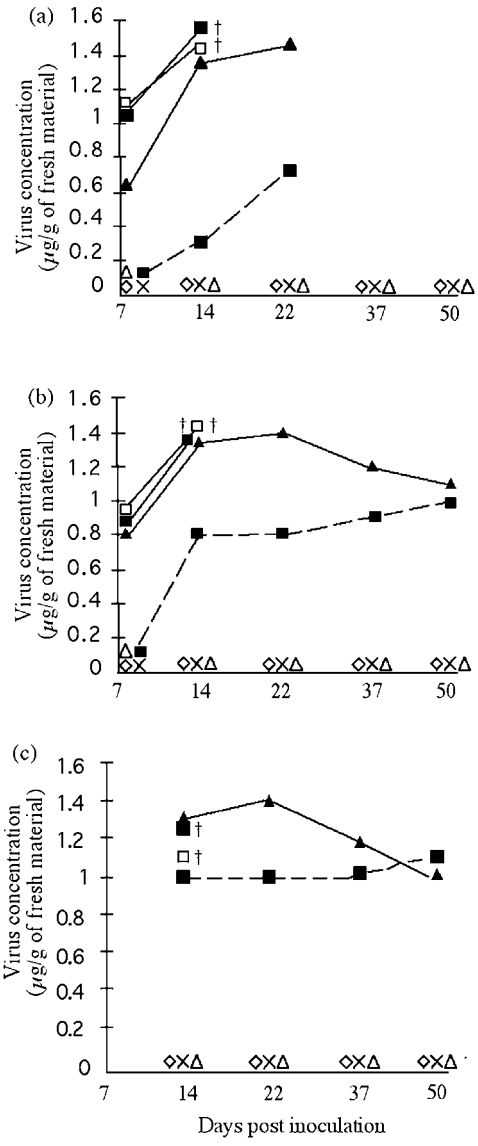


FIG. 3. Virus concentration patterns in (a) inoculated leaves, (b) sheaths of inoculated leaves and (c) systemically infected leaves of susceptible and resistant rice varieties. The leaves were mechanically inoculated with a purified RYMV isolate from Côte d'Ivoire at 10 days post-germination, and the RYMV coat protein (CP) was monitored 7 to 50 d.p.i. Variety: IR 64, ■; Tog5673, □; *Ac*. 2428, ▲; Azucena, ■; Tog 5681, △; Gigante, ◇; Healthy ×. Death of the leaf or plant, †.

proteins. Moreover, it is well known that cell to cell and vascular movements of plant viruses are not passive events and most plant viruses need a protein or a set of proteins to mediate movement [*5, 24*]. At least two proteins are required for RYMV movement in the plant, i.e. CP [*7*] and the movement protein P1 [*6*]. Assuming that a critical amount of virus and/or viral gene products is required for an efficient movement [*18*], altered cell to cell movement could be due to the absence of sufficient virus accumulation in the initially inoculated cells. One or both phenomena would result in a delay of virus spread in the plant. Similar patterns have been reported for the tobacco mosaic virus (TMV) in cowpea, the bromo mosaic virus (BMV) in *Raphanus sativus* and the caulimo mosaic virus (CaMV) in cotton [*18*]. In this model, if it is considered that (1) artificial inoculation by rubbing leaves with carborundum was very efficient for creating multiple inoculation sites, and (2) protoplasts could to some extent reflect what happens in normal cells, then the absence of viral proteins and RNA accumulation in inoculated leaves should have been due to substantial limitation of cell to cell movement in the mesophyll and parenchyma cells. Cytological information confirmed these observations, i.e. only occasional and very weak immunofluorescence was detected in xylem and phloem parenchyma, but no accumulation in mestome accompanying the increase of virus content in systemically infected leaves [*19*].

Most resistance to potyviruses involves monogenic recessive traits. Two cases showed impaired cell to cell movement despite a normal viral replication ability: the resistance to tobacco vein mottling virus (TVMV) and tobacco etch mottling virus (TEV) found in a tobacco mutant and governed by the *va* gene [*16*]. The present results suggested that RYMV resistance in Tog5681 and Gigante was caused by the absence or mutation of a plant factor necessary for the transport of the virus within mesophyll cells, since the inheritance of RYMV resistance in these cultivars is monogenic and recessive [*23*]. This mutation inhibits virus spread in mesophyll cells. The very rare occurrence of high resistance to RYMV in *O. sativa* and *O. glaberrima* also strongly supports the hypothesis that the resistance phenomenon would have evolved from a mutation of the same host factor required for spreading the virus in mesophyll cells. In IR64, this primary factor and probably other factors present along the pathway from the inoculation point to the vascular system promote rapid and efficient movement of the virus towards the vessels. Normal vascular movement of the virus also probably occurs in Gigante and Tog5681 since, when using a sap inoculum, leaves of these two cultivars can be systemically infected at an extremely low level, but sufficient to provide a new infectious inoculum that is efficient against the susceptible line BG90-2 [*23*]. Based on the protoplast experiments, and since vascular movement was efficient, the substantial limitation in cell to cell movement could explain the mechanism involved in the resistance of these two cultivars. As there are close similarities in resistance mechanisms based on short-distance movements during different plant virus interactions, mapping and cloning of the gene involved in the short-distance movement of RYMV could greatly help in identifying corresponding genes in more complex plant genomes and determining the unknown interactions of their products with the movement protein.

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