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Genetically engineered rice resistant to rice stripe virus, an insect-transmitted virus

(transgenic rice/coat protein/virus resistance)

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ABSTRACT **The coat protein (CP) gene of rice stripe virus was introduced into two japonica varieties of rice by electroporation of protoplasts. The resultant transgenic plants expressed the CP at high levels (up to 0.5% of total soluble protein) and exhibited a significant level of resistance to virus infection. Plants derived from selfed progeny of the primary transformants also expressed the CP and showed viral resistance, indicating stable transmission of the CP gene and the trait of resistance to the next generation. Moreover, the virally encoded stripe disease-specific protein was not detected in transgenic plants expressing CP 8 weeks after inoculation, indicating protection before viral multiplication. These studies demonstrated that CP-mediated resistance to virus infection can be extended to cereals and to the viruses transmitted by an insect vector (planthopper).**

One of the goals in plant biotechnology is production of genetically engineered cereals such as rice, maize, and wheat with improved resistance to diseases and harmful insects (1–3). Because damages to crops are often related directly or indirectly to virus infection, protection against viruses should considerably reduce the yield loss in many crops. Since the report that transgenic tobacco plants expressing tobacco mosaic virus (TMV) coat protein (CP) showed resistance to TMV infection (4), this strategy has been widely used for protection against a number of viruses (5). This strategy, however, has not been applied to cereals, the most important group of plants worldwide, largely because reproducible transformation has not been available until recently.

Rice stripe virus (RSV), which causes severe damage to rice in Japan, Korea, China, Taiwan, and the Commonwealth of Independent States (CIS), is a type member of the tenuivirus group and is transmitted by the small brown planthopper, *Laodelphax striatellus*, in a persistent manner (6, 7). RSV has four species of double-stranded RNA and four species of single-stranded RNA, which are designated RNAs 1–4 in order of decreasing molecular mass. The single-stranded RNAs were predicted to be counterparts of the double-stranded RNAs (8). The CP of RSV was deduced to be encoded on the RNA-3 genome (9), and this was confirmed by RNA-3 sequence analysis (10, 11). In addition to the CP, the rice plants infected by RSV show a large amount of accumulation of an ≈20-kDa protein, called stripe disease-specific protein (SP) (7). The SP was shown to be encoded on the RNA-4 genome of RSV (9).

Recently, we established an efficient method for producing fertile transgenic japonica rice by using the bacterial hygromycin B-resistance gene as a selection marker (12). Here we describe introduction of the CP gene of RSV into two japonica varieties of rice. Transgenic rice plants thus obtained efficiently expressed the RSV CP and exhibited a significant level of resistance to RSV infection. Furthermore, the resistance trait was observed to be stably transmitted to the next generation of transgenic plants.

MATERIALS AND METHODS

Plasmid Construction. To construct a vector efficiently expressing the CP gene in rice plants, the first intron of the castor bean catalase gene was introduced into the expression vector (13). An *Eco*RV/*Bam*HI fragment of pIG221 (14), which contains the upstream region from the initiation codon ATG of the β-glucuronidase (GUS) gene, was replaced by an *Eco*RV/*Bam*HI fragment of pCKR138 (15) to yield pIG221ΔATG. The plasmid pIG221ΔATG has no initiation ATG codon, because the sequence immediately upstream of the *Bam*HI site on pIG221ΔATG (cccggGGATCC) is different from that of pIG221 (aacatGGATCC). A cDNA of the RSV CP gene was cloned and inserted into the *Sma* I site of pUC18 (16) to yield pURSCP (10). The plasmid pLAN150, the CP expression vector, was then constructed by replacing a *Sal* I/*Sac* I fragment of pIG221ΔATG containing the GUS coding region by a *Sal* I/*Sac* I fragment of pURSCP containing 1138 nucleotides from the 5′ end of the viral complementary RNA (Fig. 1*A*).

Rice Transformation. Preparation of rice protoplasts, transformation by electroporation, selection of transformed calli, and regeneration of transgenic plants have all been described (12, 18). The plasmid pLAN150 was digested at the *Hin*dIII site, which is located immediately upstream of the cauliflower mosaic virus (CaMV) 35S promoter, resulting in a linear vector. The linearized plasmid was cotransformed into rice protoplasts by electroporation with the hygromycin-resistance plasmid. Transformants were selected in a medium containing hygromycin B (30 μg/ml), and plants were regenerated from hygromycin-resistant calli (12).

PCR Analysis. Integration of the CP gene in calli was first examined by PCR (19). Two 17-mers of synthetic nucleotides were used as primers; one is located in the CaMV 35S promoter (CTCAGAAGACCAAAGGG) and the other is located in the cDNA of RSV RNA-3 (TCTTCCAGGGAGATATG) (▼ in Fig. 1*A*). Genomic DNAs from hygromycin-resistant calli were extracted (20) and the PCR was performed at 55°C for 30 cycles. The amplified DNAs were subsequently visualized on 1% agarose gels using ethidium bromide.

Southern Blot Analysis. Southern blot analysis was performed according to Southern (21) with slight modifications.

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Abbreviations: CP, coat protein; RSV, rice stripe virus; SP, stripe disease-specific protein; TMV, tobacco mosaic virus; GUS, β-glucuronidase; CaMV, cauliflower mosaic virus.

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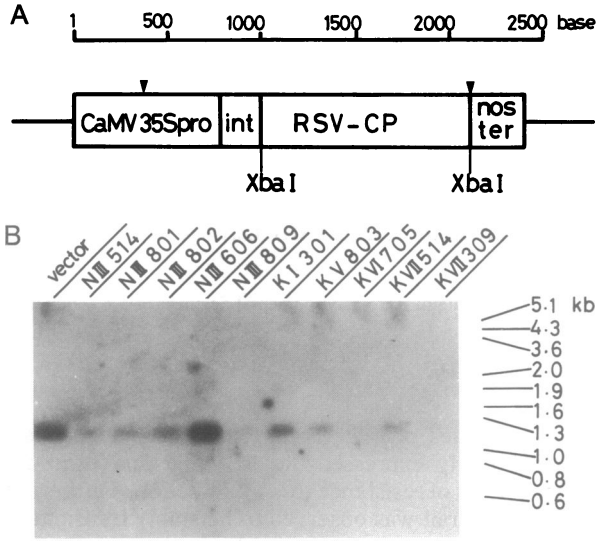

FIG. 1. Diagram of pLAN150 and Southern blot analysis of transgenic plants. (*A*) The RSV CP expression vector pLAN150 consists of the CaMV 35S promoter (35Spro) and a nopaline synthase polyadenylylation signal (nos ter) from pBI221 (17), the first intron of castor bean catalase (int) (14), and a cDNA of RSV RNA-3 (1138 nucleotides) (RSV-CP) (10). The cDNA encoding CP contains 92 bases of untranslated leader sequence. ▼, Primer positions for PCR. *Xba* I sites used for Southern blot analysis are indicated. (*B*) Southern blot analysis of primary transformants. Leftmost lane was loaded with *Xba* I-digested pLAN150 at the equivalent of 2 copies per haploid rice genome (40 ng). Names of transformants are indicated above each lane and K and N are the varieties Kinuhikari and Nipponbare, respectively. Molecular markers are shown on the right.

DNAs were prepared from the leaves of transgenic plants according to Richards (20). Three micrograms of DNA was digested with *Xba* I and separated on 1% agarose gels. After denaturation, the DNAs on the agarose gels were blotted onto a nylon membrane (Hybond-N; Amersham) under alkaline conditions, using a vacuum blotting system (Vacugene; LKB-Pharmacia). An *Xba* I fragment containing the CP gene [1.1 kilobases (kb)] was labeled with [^{32}P]dCTP (111 TBq/mol) by a multiprime labeling kit (Amersham) and was used as a probe for hybridization.

Northern Blot Analysis. RNAs were prepared from leaves of transgenic plants according to Chomczynski and Sacchi (22). The RNAs were treated with 50% formamide and 2.2 M formaldehyde (23) in Mops buffer (20 mM 4-morpholinepropanesulfonic acid/5 mM sodium acetate/1 mM EDTA, pH 7.0) and separated on 1% agarose gels containing 20 mM Mops buffer and 2.2 M formaldehyde. Then the RNAs were transferred onto a Hybond-N membrane by using a vacuum blotting system. After drying at room temperature, the membrane was irradiated by a UV lamp (365 nm) for 2–5 min. A *Bam*HI/*Sal* I fragment of pIG221 containing the intron region and an *Xba* I fragment of pLAN150 were labeled with [^{32}P]dCTP as described above and used as probes for hybridization.

Western Blot Analysis. Leaf tissues were ground in Tris buffer (pH 7.0) containing 1% SDS and 1 mM iodoacetic acid as a proteinase inhibitor. The homogenates were boiled for 5 min and clarified by centrifugation. The proteins were separated on 12.5% or 15% polyacrylamide gels containing 1% SDS (24) and transferred onto a nylon membrane (Immobilon; Millipore) by an electroblotting system (Atto). The membrane was incubated for >1 h in Tris buffer [50 mM Tris·HCl (pH 7.5)] containing 150 mM NaCl and 3% bovine serum albumin and treated with an antibody of RSV CP (Japan Plant Protection Association, Ushiku, Japan) or that of RSV SP (25). The CP or the SP bound to the membrane was detected by an alkaline phosphatase-linked goat anti-rabbit antibody (26). Proteins were quantitated according to Bradford (27).

Virus-Resistance Assays. RSV isolate T was used for virus-resistance assays. Maintenance of a colony of the viruliferous planthoppers has been described (8). Viruliferous insects in the colony were shown by ELISA to be ≈80% of adult insects (28). Rice plants were inoculated with 10 nymphs per plant (second or third instar) and were kept in a cage containing 20–40 plants. After incubation at 27°C for 2–3 days under artificial light, the planthoppers were removed or killed with insecticide. Plants were maintained in a growth chamber (10 h at 27°C dark/14 h at 27°C light). The appearance of symptoms on developing leaves was scored after 2–8 weeks (29).

RESULTS

Transformation and Analysis of Transgenic Rice Plants. The plasmid pLAN150 (Fig. 1*A*) was cotransformed with the hygromycin-resistance plasmid into protoplasts derived from two varieties of japonica rice [Kinuhikari (K) and Nipponbare (N)]. Of 238 hygromycin-resistant calli obtained from several experiments, integration of the CP gene was detected in 139 calli by PCR analysis, giving a cotransformation frequency of ≈60% at the DNA level. A total of 187 plants were regenerated from 33 PCR-positive calli (lines).

Southern blot analysis of the primary transformants indicated that one copy of the CP gene was integrated into the genome of most transformants analyzed (Fig. 1*B*). No other fragments of the CP gene were visualized under the conditions used. This observation differs from those reported for transgenic plants generated by direct DNA transfer showing the presence of larger fragments in addition to a fragment of the expected length (30, 31). When the circular plasmid was used for transformation, a variety of integration patterns of the plasmid were obtained (H. Fujimoto, T.I., R. Terada, R. Yu, M. Suzuki, and K.S., unpublished data). Therefore, the simple integration pattern of the RSV CP gene found in this study may be due to linearization of the expression vector before electroporation.

Expression of the CP Gene in Transgenic Rice Plants. We analyzed expression of the CP mRNA in 10 independent transgenic rice plants by Northern blot analysis using the CP coding region as a probe (data not shown). Eight transgenic plants showed mRNA expression of the CP gene; two transformants, NIII606 and NIII809, however, showed no mRNA expression. This observation corresponded to the results of Western blot analysis showing that the CP was not detected in NIII606 and NIII809 (Fig. 2*C*). For further analysis, two probes (the intron region and the CP coding region) were used for Northern blot analysis (Fig. 2 *A* and *B*). When the coding region was used as a probe, a spliced form of mRNA, whose expected size was ≈1.4 kb, was clearly detected (Fig. 2*B*). It also showed that the antisense CP gene (Kα6–11) was expressed as efficiently as the sense CP gene. On the contrary, although the applied RNA in Fig. 2*A* was 5 times that in Fig. 2*B*, no bands were detected with the intron probe (Fig. 2*A*), indicating that the intron was efficiently spliced.

When leaves of primary transformants were examined for CP expression at the protein level, they showed a good accumulation of RSV CP (Fig. 2*C*). The transformed plant expressing an mRNA from the antisense CP gene (Kα6–11) was used as a negative control. CP expression in primary transformants was detected in 19 of 33 lines containing the CP gene. We estimated by densitometric measurement of stained bands that the amount of CP expressed in the rice leaves of the primary transformants was up to 0.5% of the total soluble protein. Comparison with previous reports of CP expression

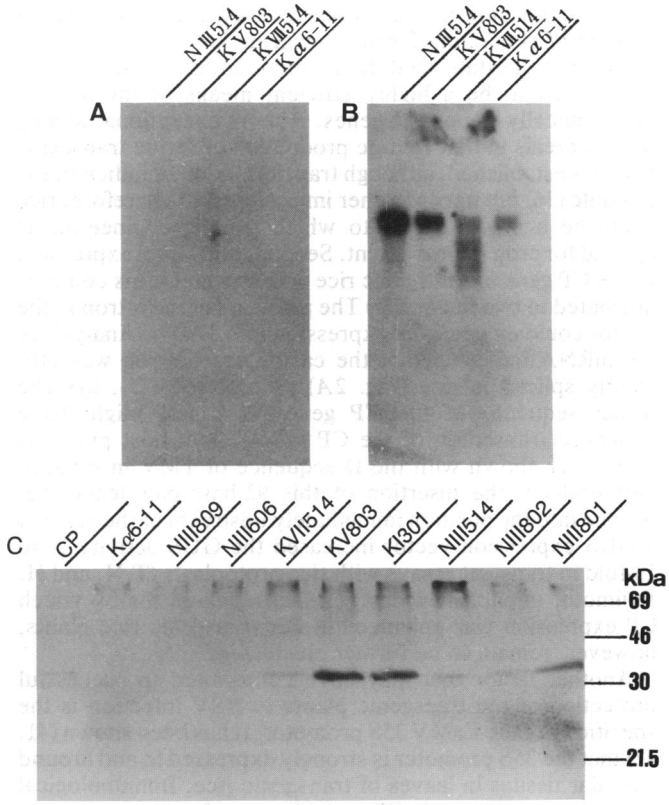

FIG. 2. Expression of the RSV CP gene in transgenic rice. (*A* and *B*) Northern blot analysis of transgenic rice. ^{32}P-labeled fragments of the intron region (*A*) and the CP coding region (*B*) were used as probes. Ten micrograms and 2 μg of extracted total RNAs were applied for *A* and *B*, respectively. Names of transgenic rice are shown above each lane. (*C*) Western blot analysis of transgenic rice. The extracted proteins (15 μg per lane) from the transgenic rice and purified CP (5 ng) were applied. Names of transgenic rice are indicated above each lane. The line Kα6–11 is a transgenic rice expressing the antisense CP gene. Although NIII809 and NIII606 were regenerated from PCR-positive calli, they showed no CP expression.

in dicotyledonous species (5) suggested that the expression level of RSV CP in most transformants was similar to other transgenic plants in which virus protection was observed (33).

Virus-Resistance Assays. Since RSV infects plants only through transmission by planthoppers, the assay for viral resistance was performed by using viruliferous small brown planthoppers as vectors. In our rice culture system, several plants can be regenerated from each callus line. Therefore, these plants are assumed to be clones with respect to the location and the copy number of the CP gene. Three or four plants (clones) from five independent primary transformants were examined for resistance to viral infection. Of the four clones derived from each of the transgenic plants expressing the RSV CP (lines KI301, KVII514, and NIII801) none showed symptoms (Table 1). However, two lines carrying the CP gene, which did not express the CP (lines NIII606 and NIII809), demonstrated clear disease symptoms in all the clones examined. We therefore concluded that virus resistance is conferred by expression of the CP in primary transgenic rice plants.

More than 220 plants derived from selfed progeny of six primary transformants (KV8031, KVII3015, KVII3016, KVII5071, KVII5072, and KVII3092) were examined for inheritance of the CP gene by Western blot analysis. Results indicated that the CP gene was stably transmitted to the next generation in five of six transformants examined. Among

Table 1. Virus-resistance assays of primary transgenic rice plants

Clone	CP expression	No. of plants showing disease symptoms
KI301	+	0 (4)
KVII514	+	0 (4)
NIII801	+	0 (4)
NIII606	−	3 (3)
NIII809	−	3 (3)

Primary transgenic rice plants just after transfer to soil (three- to four-leaf stage) were used for infectivity assays. In control experiments, 25 untransformed rice plants were inoculated under the same conditions and 19 plants (76%) showed disease symptoms in 3 weeks. +, CP expression was detected by Western blot analysis; −, CP expression was not detected. Numbers in parentheses are sample size.

them progeny of three transgenic plants (KVII3016, KVII5071, and KVII5072) showed 3:1 segregation, suggesting the presence of one active CP gene in primary transformants (data not shown). On the other hand, the progeny of KV8031 did not show any detectable level of expression of CP. PCR analysis of these progeny plants demonstrated that the CP gene was not transmitted to the progeny at all.

A virus-resistance assay was performed with the progeny. Fourteen and 17 progeny plants of KVII5071 and KVII5072, respectively, all of which showed CP expression, were chosen and inoculated with viruliferous small brown planthoppers. As controls, 20 progeny plants of KV8031 expressing no CP and 17 nontransformed plants were used. More than 80% of nontransformed plants showed the disease symptoms 15 days after inoculation. At the same time, >90% of the progeny of KV8031 showed disease symptoms. In contrast, the progeny of KVII5071 and KVII5072 showed a significant level of reduction in the number of plants showing disease symptoms (Figs. 3 and 4). The virus-resistance assay using a similar number of progeny plants was repeated with the line KVII5072 and a significant level of resistance against RSV was observed (data not shown). These data demonstrated that virus resistance was transmitted to the next generation and was conferred by expression of the CP.

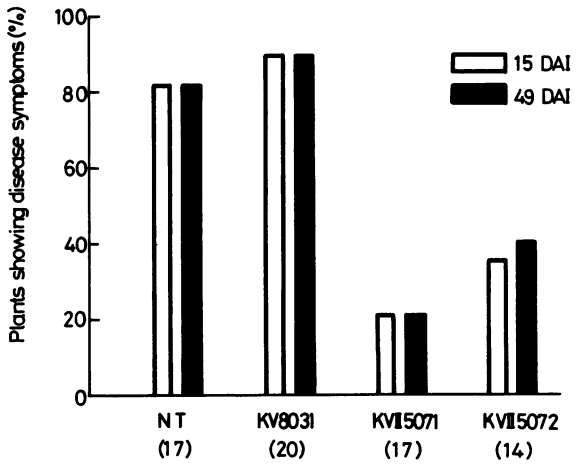

FIG. 3. Virus-resistance assays of selfed progeny of transgenic rice plants. Rice seeds harvested from the transgenic plants were treated at 42°C for 10 days to break dormancy. Seedlings at the four-leaf stage were used for virus-resistance assays. Percentage of plants showing disease symptoms 15 (open bar) and 49 (solid bar) days after inoculation (DAI) is shown. Progeny expressing the CP (KVII5071 and KVII5072) and not expressing the CP (KV8031) were used for the assays. NT, nontransformed plants. Numbers in parentheses are sample size.

FIG. 4. Resistance phenotype of a progeny plant derived from transgenic rice expressing the RSV CP. A resistant progeny showing healthy growth and fertility (*Left*) and a susceptible progeny with severe disease symptoms (*Right*) 4 months after virus inoculation.

Analysis of RSV Multiplication in Transgenic Rice Plants. There is not necessarily a parallel between the appearance of disease symptoms and viral multiplication in host plants (34). Thus, we examined whether the virus multiplied in the transgenic rice plants with an asymptomatic phenotype. The SP of RSV is encoded on RNA-4 (9) and accumulates in leaves when RSV infects the rice plants (7). The detection of SP should reflect viral multiplication. We measured SP accumulation in the 11 progeny plants of a transgenic rice plant (KVII5072; see Fig. 3) 8 weeks after inoculation of RSV by Western blot analysis (Fig. 5). A nontransformed rice plant (lane 12) and a progeny of the transgenic rice, which lacked the CP gene because of segregation (lane 11), showed high-level SP accumulation, whereas 9 of 10 asymptomatic progeny plants expressing the CP showed no SP accumulation (lanes 2–10). In one progeny plant (lane 1), a greatly reduced amount of the SP was detected. These results show that protection was manifested by inhibition of viral multiplication.

DISCUSSION

In this study, we demonstrated that CP-mediated resistance to virus infection is applicable to rice, a major cereal. Two

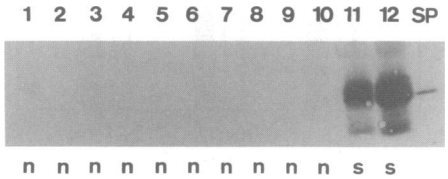

FIG. 5. Analysis of viral multiplication in transgenic rice plants. Viral multiplication 8 weeks after inoculation was analyzed by measuring SP with the antibody against the RSV SP. Ten micrograms of extracted proteins and 5 ng of purified SP were applied. Lanes: 1–10, progeny plants from a transgenic rice plant (KVII5072) expressing the CP; 11, progeny plant not expressing the CP; 12, nontransformed rice plant. Phenotype of disease symptoms for each plant is indicated below each lane. n, No disease symptoms; s, showing disease symptoms.

factors contribute to the present results. First, an efficient method of generating fertile transgenic japonica rice plants has been well established. In addition, cotransformation (12, 35) proved to be a highly efficient means of introducing agronomically important genes. Rice is exceptional among major cereals in that routine production of fertile transgenic plants is established, although transformation of indica rice is possible (36) but needs further improvement. Therefore, rice could be a model cereal to which genetic engineering is applied for crop improvement. Second, high-level expression of the CP gene in transgenic rice was attained. This could be attributed to two factors. (*i*) The presence of the intron in the vector could enhance CP expression (13, 37–39). Analysis of CP mRNA indicated that the castor bean intron was efficiently spliced in rice (Fig. 2*A*) as reported (13). (*ii*) The leader sequence of the CP gene (92 bases) might have enhanced translation of the CP mRNA in a host plant, as previously shown with the Ω sequence of TMV in tobacco (40). Indeed, the insertion of this 92-base-pair leader sequence into the position immediately upstream of the ATG of a GUS expression vector increased the GUS activity 2- to 10-fold in transient assays with rice protoplasts (T.H. and H. Fujimoto, unpublished data). Exact mechanisms by which CP expression was enhanced in our transgenic rice plants, however, remain to be further elucidated.

Another factor that may have contributed to successful protection of the transgenic plants to RSV infection is the specificity of the CaMV 35S promoter. It has been shown (41, 42) that the 35S promoter is strongly expressed in and around vascular tissues in leaves of transgenic rice. Immunological localization of the RSV CP in the leaves of transgenic plants indicated that the RSV CP, which is transcribed from the CaMV 35S promoter, was also expressed around vascular bundles (data not shown). Because RSV is assumed to infect and spread through phloem tissues, localization of CP expression at vascular bundles appears to be preferable for generating viral resistance.

Inheritance of the CP gene in the next generation was examined for progeny of six transgenic rice plants. Although three transformants exhibited Mendelian inheritance, two showed a skewed segregation pattern, in which the number of CP-positive progeny was much less than expected. Furthermore, no progeny carrying the CP gene were recovered in one line—KV8031. One interpretation of these results is linkage of the CP gene and semidominant or dominant lethal mutations. Further analysis of transmission of the CP genes to later generations is necessary to understand the cause of these aberrant segregation patterns.

The results of virus-resistance assays with the progeny of the transgenic plants demonstrated that there was a significant level of protection against RSV infection. Nevertheless, in spite of the fact that all the progeny seedlings used for the assay were CP positive, some plants were sensitive to virus infection (Fig. 4). Concentrations of the inoculated RSV, physiological conditions, and expression level of the CP at the specific stage of leaf development when RSV was infected may affect virus resistance.

With regard to a possible mechanism(s) for CP-mediated resistance, several lines of evidence indicate that resistance is in part the result of interference with early events of the infection process, especially at the uncoating step of the virus particles (43, 44). In the case of TMV (45), alfalfa mosaic virus (46), and tobacco streak virus (47), resistance can be overcome by inoculation with viral RNA. However, inoculation with potato virus X RNA did not overcome CP-mediated resistance (48). These complicated results suggest that interference with virus infection operates at different steps depending on the virus.

Most transgenic plants expressing the CP did not show any accumulation of the SP even 8 weeks after inoculation (Fig.

5), indicating no multiplication of the virus. It appears that the protection is due not to simple tolerance but to resistance against virus infection and it is suggested that interference of RSV infection operates at a step before viral multiplication.

The bioassay for virus resistance using viruliferous vectors seems to mimic natural conditions, although exact concentrations of the inoculated virus cannot be estimated. Several reports (32, 34, 49) have described a successful virus-resistance assay by using viruliferous aphids; we used viruliferous small brown planthoppers for the assay. The present results demonstrate that CP-mediated resistance is applicable to cereal crops and to viruses transmitted by planthoppers.

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