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# Improved method for assaying maize plant resistance to maize rough dwarf disease by artificial inoculation and real-time RT-PCR

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**Abstract**

The study of maize rough dwarf disease (MRDD) and breeding for resistance requires inoculation of maize plants by means of planthoppers. The plant age, insect density and inoculation duration are main factors in the success of maize rough dwarf disease inoculation. These parameters were tested using a susceptible maize inbred line Ye478. Using one or two-leaf plants, 15 planthoppers per plant and a five day inoculation duration, the line Ye478 was the most susceptible with 100% diseased plants; F112132 was moderately susceptible with 60% diseased plants and 90110 and F022411 were resistant without any disease. The results were consistent with those from six years of field studies. Using enzyme-linked immunosorbent assay (ELISA) and real-time quantitative RT-PCR, *rice black-streaked dwarf virus* was detected in severely diseased plants. The plants were rated from 0 to 3 according to their symptoms at the time of flowering. Plants scoring 0, 1 and 2 could not be distinguished by ELISA, only by real-time quantitative RT-PCR. All of the plants with a score of 3 were positive by ELISA and real-time quantitative RT-PCR. The significant differences in the average viral contents in plants with different symptom ratings could be distinguished by using real-time RT-PCR.

## Introduction

Maize rough dwarf disease (MRDD) is a viral disease that is widely distributed in the world and causes great losses in grain yield. MRDD was first reported in Italy and the pathogen was named the *maize rough dwarf virus* (MRDV) (Slykhuis, 1976). In China, MRDD was first reported in the 1950s in Ganshu and Xinjiang provinces and there was an epidemic outbreak in the 1990s in the north, northwest, and middle parts of China. The pathogen was thought to be MRDV, until in 2001 the *rice black-streaked dwarf virus* (RBSDV) was identified as the causal agent of MRDD in China (Bai et al., 2001; Fang et al., 2001; Zhang et al., 2001b).

RBSDV belongs to the *Fijivirus* genus in the family *Reoviridae* and can invade several cereal crops including rice, maize and wheat. The genome of RBSDV consists of 10 dsRNA segments and the complete genome has been sequenced (Zhang et al., 2001a; Wang et al., 2003). RBSDV and MRDV are quite similar in host range, serology, morphology, and the structure and sequence of genome (Luisoni et al., 1973; Azuhata et al., 1993; Marzachi et al., 1995; McMahon et al., 1999).

RBSDV is transmitted in a persistent manner by the insect vector *Laodelphax striatellus* and cannot be transmitted by mechanical inoculation or seeds. In addition, RBSDV is not transmitted congenitally to the progeny of the planthoppers. The virus is retained when the vector moults, and can

multiply in the insect. RBSDV can be found only in the phloem cells of infected plants, and the development of these cells is abnormal, leading to the formation of enations on the veins (Chen et al., 2004).

Currently MRDD is controlled with chemicals, altering the sowing date and improving field management, but these are not ideal measures. Disease resistance could potentially reduce crop losses with minimal efforts by growers, in an environmentally safe, cost-effective manner. It is important to identify the sources of disease resistance and improve this by breeding programmes. Suitable and reproducible techniques for assessing plant susceptibility would be favourable for the study of MRDD and maize breeding. Field assessment has been broadly applied to screening for plant resistance to MRDD. Because of great natural variations in the amounts of insect vectors, the natural source of the virus and the climate in the field, especially temperature, confirmation of results based on field assessment usually takes several years. Inoculation is a relatively stable method for identifying the resistance of plants, but there are few reports using the inoculation method to measure resistance to MRDD.

Rapid and accurate identification of pathogens is critical to predicting and controlling diseases, and breeding for plant resistance. Viral quantification is also an essential tool for the study of resistance mechanisms of plants. Enzyme-linked immunosorbent assay (ELISA), Western blotting (Sun et al., 2004) and dot-blot hybridization (Wang et al., 2001) have been used to identify RBSDV, but the application of these methods is limited by lack of sensitivity and reproducibility. Reverse transcription-polymerase chain reaction (RT-PCR) is more sensitive and can discriminate between RBSDV and MRDV (Wu et al., 2000), but cannot quantify the virus accurately. Real-time quantitative PCR is a method that provides accurate and reproducible quantification of gene copies, does not require post-PCR sample handling and results in much faster and higher throughput assays (Heid et al., 1996). Recently, real-time quantitative RT-PCR was successfully applied to detecting plant pathogens (Eun et al., 2000; Roberts et al., 2000; Cullen et al., 2001; Korimbocus et al., 2002; Balaji et al., 2003; Delanoy et al., 2003; Nicolaisen, 2003; Schena et al., 2004; Pico et al., 2005), but there have been no reports on identifying RBSDV with real-time quantitative RT-PCR.

In this study, we improved a method for screening maize genotypes for resistance to RBSDV. Three important factors affecting the incidence of infection were investigated in a series of trials: maize plant age, the density of insects and inoculation duration. RBSDV was identified by ELISA at the protein level and real-time quantitative RT-PCR using SYBR Green 1 at the RNA level to determine the virus content in individual maize plants.

## Materials and methods

### *Virus and plant materials*

The maize plants with RBSDV used in inoculations were naturally infected field-grown plants with typical symptoms of MRDD in the Jinan area, Shandong Province, in China. Plants of a wheat cultivar that was susceptible to RBSDV and MRDV infection were used to maintain the virus through the winter.

Ten maize inbred lines with different resistance to RBSDV including Mo17, Ye515, 8112, Luyuan92, P138, 90110, F022411, Ye478, F112132 and 178 were used in field assessments and the inoculation trial. Among them, resistant inbred lines 90110 and F022411, susceptible inbred line Ye478, and F112132 whose resistance was between Ye478 and 90110 based on observations in field assessments were used in serial inoculation and subsequent ELISA and quantitative PCR analysis. The maize lines except F022411 and F112132 were provided by Profs. Chenghe Zhang (Agricultural Academy of Hebei Province in China) and Denghai Li (Agricultural Academy of Laizhou in China), respectively. F022411 and F112132 are the recombinant inbred lines of crossing Ye478 $\times$ 90110.

### Laodelphax striatellus *maintenance and plant inoculation*

Planthoppers were captured in the fields in Jinan of Shandong Province and fed on the plants of susceptible wheat cv. Lumai 23 in plastic cages covered with 80-mesh mothproof net in a culture

room. The culture room was programmed to provide a photoperiod of 14:10 (L:D) h with a temperature of 26:20 °C. The planthoppers had propagated more than 10 life cycles before they were used in the transmission experiments.

Maize is not a suitable host of planthoppers; when planthoppers are fed on maize plants they are only alive for 5–7 days. So the second instar planthoppers were fed on maize MRDD plants obtained in the Jinan area in China for three days to acquire the virus, and afterwards they were kept on young wheat seedlings for 7–10 days during which time the planthoppers developed into imagoes with wings. Uninfected maize seedlings grown in plastic cups with vermiculite were then fed to the planthoppers in a mothproof net (1 m × 1 m × 1 m) in a greenhouse for RBSDV inoculation. The conditions were 14 h of daylight with a temperature of 28:20 °C. Following the inoculation the infected maize plants were sprayed with 5% imidacloprid (1-(6-chlorine-3 pyridine methyl)-*N*-nitroalkane-2-amino) and transplanted into flowerpots that were protected with mothproof netting. The plants were grown using routine management.

In this study, three factors affecting the incidence of infection were studied with susceptible line Ye478, including plant age, insect density and inoculation duration. There were 15 plants for each level of each factor and every experiment was repeated three times. The effects of maize plant age on the success rate of RBSDV inoculation were examined by inoculating Ye478 seedlings at different stages, including the emergence of seedlings, and two-leaf, three-leaf, five-leaf and seven-leaf stages. The inoculation was carried out with 10 planthoppers per plant for 5 days. In the trial of insect density on inoculation, Ye478 two-leaf seedlings were inoculated for 5 days with one, five, 10 and 15 planthoppers per plant, respectively. The effects of inoculation duration were examined by inoculating Ye478 seedlings at the two-leaf stage for 3 h, 12 h, 24 h, 72 h (3 days), 120 h (5 days) and 168 h (7 days), respectively with 10 planthoppers per plant. An appropriate combination of insect density, inoculation duration and seedling stage was selected, based on inoculation results of Ye478, for the inoculation of other inbred lines. In each experiment, at least 25 plants from each line were tested. The plants were arranged in a randomized block design in a mothproof net. Each experiment was repeated three times. The non-inoculated plants of each line were negative controls.

The inoculated plants were categorized based on their symptoms at the time of flowering, and rated from 0 to 3, as follows: 0: healthy plant, 1: 3/4–2/3 of healthy plant height; slightly shortened internodes; crimpled leaves; a small amount of enations found on one or two leaves; a stunting tassel and small ears, and a seedy ear, 2: 2/3–1/2of healthy plant height; shortened internodes; crimpled leaves; enations found on the upper leaves; deficient tassel and ear, and few seeds, 3: 1/2 of healthy plant height or shorter; obviously shortened internodes; dark green leaves; many crimpled leaves; enations found on many leaves, many rough leaves; tassel and ear badly developed, no seeds. The disease incidence was obtained according to following equation:

$$\text{incidence} = [(x_1 + x_2 + x_3)/(x_0 + x_1 + x_2 + x_3)] \times 100\%.$$

$x_n$ represent the number of plants at different rating.

*Assessing the ratio of viruliferous planthoppers*

Samples consisting of different numbers of imago planthoppers were tested by ELISA. The planthoppers that were not fed on diseased maize plants were negative controls and the extract buffer was the blank control. The planthoppers were ground in a 1.5 ml tube with 300 $\mu$l citrate buffer (0.7 mM citric acid, 9 mM ammonium citrate, Tribasic, pH 6.1), and centrifuged for 10 min at 10,000 rpm. Aliquots (100 $\mu$l) were transferred to microtitre plates and kept overnight at 4 °C. The indirect ELISA method was used. Anti-serum against the capsid protein of RBSDV was diluted 1:500 in PBS-Tween solution (NaCl, 1.37 mM; KCl, 2.7 mM; $Na_2HPO_4$, 10 mM; and $KH_2PO_4$, 2 mM; 0.05% Tween 20) containing 3% BSA. The secondary antibody was goat anti-rabbit IgG-AP (Huamei, Beijing, China) and was diluted 1:1000 in PBS-Tween solution. The blocking solution was 200 $\mu$l sterile dd-water containing 2% (w $v^{-1}$) polyvinyl pyrrolidone (PVP-40,000) and 2% (w $v^{-1}$) defatted milk powder. PBS-Tween solution was used as washing solution. *Para*-nitrophenylphosphate (Sangon, Shanghai, China) dissolved at 1 mg $ml^{-1}$ in 10% (v $v^{-1}$) diethanolamine buffer (pH 9.8) containing 0.22 mM $MgCl_2 \cdot 6H_2O$ was used as substrate solution. The optical density of

the ELISA samples was measured with a microtitre plate reader (BLX800, BIO-TEK) at 405 nm. Results were given as means of three replicate wells minus the OD of the blank control. The sample was considered positive if the ELISA value was twice that of the control.

*Identifying RBSDV in maize plants with ELISA*

Five representative plants at the time of flowering at a disease level were individually detected by ELISA for the lines of Ye478, 90110, F112132 and F022411. Non-inoculated plants of the same lines were negative controls and the extract buffer was the blank control. Leaf tissue (0.5 g) from the uppermost fully expanded leaf was ground in 4 ml PBS buffer. After centrifugation at 10,000 rpm for 10 min, 100 $\mu$l aliquots were added to microtitre plates and kept overnight at 4 °C. Each sample was tested in triplicate. The ELISA protocol was the same as above.

*Real-time quantitative RT-PCR analysis*

Five representative plants of each of the disease levels obtained for lines Ye478 and F112132 and 10 plants of lines 90110 and F022411 were individually detected by real-time RT-PCR. Non-inoculated plants of the lines were negative controls and sterile dd-water was the blank control. Total RNA was extracted from the uppermost fully-expanded leaf (0.1 g) by TRIZOL (Sangon, Shanghai, China) according to the manufacturer's protocol. Before cDNA synthesis, the RNA was treated with RNase-free DNase (Takara, Dalian, China) according to the manufacturer's protocol. Total RNA concentrations were measured with a UV-spectrophotometer (Eppendof Biophotometer) to normalize the nucleic acid concentrations for subsequent cDNA synthesis and PCR amplifications. Random hexamers were used as primers for the first-strand cDNA synthesis, and about 1000 ng total RNA in 10 $\mu$l volume was used to synthesize cDNA using the RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's protocol, except that samples were denatured at 95 °C for 3 min (Wang et al., 2001). cDNA samples were diluted 10-fold as the templates in subsequent PCRs.

Universal primer P1, RBSDV specific primer P2 based on the RBSDV S7 segment and MRDV specific primer P3 based on the MRDV S6 segment were as designed by Wu et al. (2000). The RBSDV segment by P1 + P2, and the MRDV segment by P1 + P3. Both of the two primer combinations were predicted to amplify 510 bp fragments. In this study, the cDNA samples from the field-infected maize plants, diseased plants from inoculation and planthoppers fed on these infected maize plants were detected using these primers.

The RBSDV specific fragment was ligated to the pGEM-T EASY vector (Promega, USA) to transform *E. coli*, and positive colony P510 was sequenced (BIOASIA, Shanghai, China) on a model 3730 sequencer (Applied Biosystems). Another primer, named RP (5′-GCTCCTACTGAGTTGCCTGTC), was designed based on the 510 bp segment. By the P1 + RP primer combination a predicted PCR product of 342 bp was amplified. The amplification with 0.2 $\mu$M of each primer in a total volume of 10 $\mu$l had the highest efficiency from the optimized primer concentration experiment. A pair of internal control primers based on the sequence of maize actin 1 (Accession No.: J01238) in Genbank was designed to allow normalization of products between experiments and were named as MA1 (5′-ATCACCATTGGGTCAGAAAGG, positions 1153–1173) and MA2 (5′-GTGCTGAGAGAAGCCAAAATAGAG, positions 1440–1463) for sense and anti-sense primers, respectively. The internal control primers were predicted to amplify a 311 bp fragment.

Real-time quantitative RT-PCR reactions were done on chromo 4 (MJ Research, USA) with the SYBR® RT-PCR Kit (Takara, Dalian, China), in a 10 $\mu$l reaction volume, which contained 5 $\mu$l SYBR® Green 1 PCR mix, 0.2 $\mu$l (10 $\mu$M) each of forward and reverse primers, 1 $\mu$l diluted cDNA template and 3.6 $\mu$l sterile dd-water. Amplification conditions were: 95 °C for 5 min followed by 40 cycles at 95 °C for 45 s, 60 °C for 45 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. A melting curve from the temperature interval between 65 and 90 °C was generated, and a few PCR products were further analysed by electrophoresis in 2% agarose gel stained with ethidium bromide.

Standard curves based on threshold cycles (Ct) for the serial dilutions of the recombinant plasmid P510 were constructed. The viral content of a plant was quantified by comparing the Ct value with the

standard curve, which represented copy numbers per 100 ng total RNA. Triplicate samples were amplified for each plant cDNA sample.

*Data analysis*

Means of one-way analysis of variance were performed (SPSS 10.0) with the data from inoculation, ELISA and real-time quantitative RT-PCR among different inbred lines and different disease levels of each line, to confirm differences between the genotypes and disease groups.

## Results

*Incidence of viruliferous planthoppers*

To determine how many planthoppers were infected with the virus using ELISA, the planthoppers fed on diseased maize plants provided the samples of different numbers of individuals. Three of the 12 samples containing a single planthopper were positive, and two of the three samples with five planthoppers and all three of the samples with 10 planthoppers were positive (Table 1). The results from the samples consisting of a single planthopper suggested that approximately 25% of planthoppers were viruliferous.

*Inoculation of maize plants*

In the flowering period, some of the inoculated maize plants developed the typical disease symptoms of MRDD (Figure 1). The inoculated plants were rated in different disease levels based on their symptoms.

The effects of maize plant age on the success rate of RBSDV inoculation were examined by inoculating susceptible maize line Ye478. The disease incidence was higher at the emergence of seedlings, two-leaf and three-leaf stages with 100, 87.5 and 70% diseased plants, respectively. At the five-leaf stage, the incidence was 25.3%, and there were no diseased plants at the seven-leaf stage. It was concluded that a higher disease incidence could be obtained when younger maize plants were inoculated.

The effects of insect density on the success rate of RBSDV inoculation were assayed by inoculating Ye478 seedlings at the two-leaf stage. The inoculation lasted 5 days. Fifteen plants were inoculated for each insect density, respectively. The disease incidence was higher when the plants were inoculated with 15 and 10 planthoppers per plant with 100% and 96% diseased plants, respectively. When the plants were inoculated with five and three planthoppers per plant, 67.3% and 42% plants were diseased, respectively.

*Table 1.* Detecting *rice black-streaked dwarf virus* in planthoppers by enzyme-linked immunosorbent assay

| No. of planthoppers per sample | Control[a] OD405 nm[b] | Positive[c] | | Negative | |
|---|---|---|---|---|---|
| | | OD405 nm | S/C | OD405 nm | S/C |
| 1 planthopper | 0.164 ± 0.012 | 0.374 ± 0.030 | 2.28 | 0.182 ± 0.018 | 1.11 |
| | | 0.369 ± 0.034 | 2.25 | 0.221 ± 0.017 | 1.35 |
| | | 0.352 ± 0.037 | 2.14 | 0.188 ± 0.009 | 1.15 |
| | | | | 0.206 ± 0.003 | 1.26 |
| | | | | 0.163 ± 0.016 | 0.99 |
| | | | | 0.285 ± 0.027 | 1.73 |
| | | | | 0.161 ± 0.009 | 0.98 |
| | | | | 0.174 ± 0.016 | 1.06 |
| | | | | 0.167 ± 0.023 | 1.01 |
| 5 planthoppers | 0.184 ± 0.032 | 0.372 ± 0.029 | 2.02 | 0.229 ± 0.031 | 1.24 |
| | | 0.489 ± 0.015 | 2.65 | | |
| 10 planthoppers | 0.244 ± 0.037 | 0.519 ± 0.027 | 2.12 | | |
| | | 0.565 ± 0.010 | 2.32 | | |
| | | 0.544 ± 0.040 | 2.23 | | |

S/C: Ratio of sample OD to control OD.

[a]The planthoppers fed on uninfected plants.

[b]Values presented are the means (±SD) for three replicate wells minus the OD of the blank control.

[c]The sample was considered positive if the OD value was twice that of the control, S/C > 2.


*Figure 1*. Maize rough dwarf disease symptoms of an inoculated Ye478 plant. Diseased plant: A plant at two-leaf stage was inoculated for 5 days with 15 planthoppers. CK: A non-inoculated plant.

The effects of inoculation duration on the success rate of RBSDV inoculation were examined by inoculating Ye478 plants for different periods of time. In this experiment, some plants became diseased even after being inoculated for only 3 h (Figure 2), and the longer the inoculation duration, the greater the incidence. Nearly all of the plants were diseased, following inoculation for 5 or 7 days (95.3% and 100%, respectively).

To determine the resistance to RBSDV infection of different genotype maize plants, 10 inbred lines were inspected after inoculation with viruliferous planthoppers under the following conditions: two-leaf plants and 15 planthoppers per plant for 5 days. All of Ye478 plants inoculated exhibited disease symptoms ranging from 1 to 3. All of 90110, P138, 178 and F022411 plants were healthy, and in the F112132 population 60% of the plants had the symptoms of MRDD (Table 2), but there were no plants with a disease level of 3. As a result of inoculation, 90110, P138, 178 and F022411 were MRDD resistant lines, Ye478 was the highly susceptible line and Ye515, F112132, Luyuan92 and 8112 were moderately susceptible to MRDD. The results of field assessments for 3 years were consistent with the results of the inoculations, but

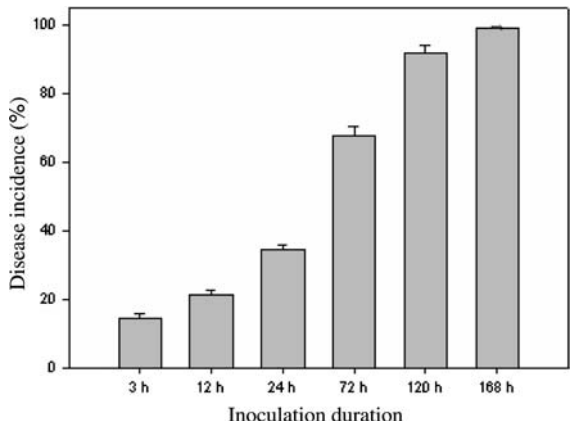


*Figure 2*. Effects of inoculation duration on disease incidence with *rice black-streaked dwarf virus* by planthoppers. Plants of susceptible maize line Ye478 with two leaves were inoculated for different durations with 10 planthoppers per plant. There were 15 plants for each time period. Each value is the mean % (±SE) disease incidence calculated from three experiments.

incidences were lower than those of the inoculations and showed great fluctuations in different years (Table 2).

## *ELISA*

ELISA analysis was performed on plants from each disease level for the lines of Ye478, 90110, F112132 and F022411 in order to compare different diagnostic methods for plants with RBSDV,

*Table 2*. The disease incidence of different inbred lines assessed in the field and by inoculation

| Maize inbred lines | Disease incidence | |
|---|---|---|
| | Field (%)[a] | Inoculation (%)[b] |
| Ye478 | 90 ± 8.3 | 100[c] |
| Mo17 | 85 ± 12.0 | 96 ± 4.0 |
| F112132 | 48 ± 22.7 | 60 ± 4.1 |
| Ye515 | 40 ± 23.0 | 55 ± 5.0 |
| Luyuan92 | 39 ± 23.5 | 53 ± 4.9 |
| 8112 | 35 ± 17.2 | 48 ± 5.3 |
| 90110 | 0 | 0 |
| F022411 | 0 | 0 |
| P138 | 0 | 0 |
| 178 | 0 | 0 |

[a]The disease incidence in the field was the mean of three yearly assessments.
[b]The plants were inoculated at two-leaf stage with 15 planthoppers per plant for 5 days. Each value represents the mean % disease incidence calculated from three replicate experiments.
[c]All the plants were diseased in three replicates.

and the rates of infection of the different lines were calculated. The plants with a score of 3 from line Ye478 were all positive, but there were no positive individuals among the plants with a score of 1 and 2 (Table 3). There was only one positive individual (score 2) among the plants from F112132. No positive samples were found in 90110 and F022411. It could be concluded that differences between healthy plants and diseased plants with a score of 1 or 2 could not be distinguished significantly with ELISA.

*Real-time quantitative RT-PCR analysis*

Total RNA was extracted from maize plants with typical symptoms of MRDD, diseased plants from inoculation and planthoppers fed on diseased maize plants. cDNA synthesis was performed for the identification of viruses. cDNA samples were used as templates to amplify RBSDV specific fragments using P1 + P2 and MRDV specific fragments using P1 + P3. PCR products were obtained only using the P1 + P2 primer combination from the samples of the maize plants from the field and from inoculation and the planthoppers. The sequence of the RBSDV specific fragment from PCR amplification in plasmid P510 was aligned in Genbank with BLASTn, and showed 98% identity to the RBSDV S7 segment (Accession No.: AF397894).

Standard curves based on threshold cycles (Ct) for the serial dilutions (ranging from $4.29 \times 10^7$ copies to $2.14 \times 10^2$ copies) of the RBSDV-containing plasmid P510 were constructed (Figure 3). A standard regression line was obtained by plotting the Ct values versus the logarithm of the starting template copy number of each serial dilution. Amplifications with starting template copies lower than $2.14 \times 10^2$ were inconsistent.

Five representative plants from each of the disease levels obtained for lines Ye478 and F112132 and 10 plants of lines 90110 and F022411 were analysed by quantitative RT-PCR. Means and standard errors were calculated for each disease level of each line. The size of the PCR product was confirmed to be what was predicted with both the melting curve analysis and agarose gel electrophoresis (Figure 4). Non-specific products or primer dimers were not observed in our real-time quantitative RT-PCR.

Among the four maize lines, the average viral content of Ye478 was the highest ($2.12 \times 10^5$

*Table 3*. Detection of *rice black-streaked dwarf virus* in plant samples by enzyme-linked immunosorbent assay

| Maize line | Symptom score | No. of plants | $OD_{405\ nm}$[b] | S/C | ELISA[c] |
|---|---|---|---|---|---|
| Ye478 | Control[a] | 3 | 0.293 | | |
| | 1 | 5 | 0.290–0.347 | 0.99–1.18 | − |
| | 2 | 5 | 0.364–0.478 | 1.24–1.63 | − |
| | 3 | 5 | 0.589–0.780 | 2.01–2.66 | + |
| F112132 | Control | 3 | 0.275 | | |
| | 0 | 5 | 0.243–0.336 | 0.88–1.22 | − |
| | 1 | 5 | 0.288–0.427 | 1.05–1.54 | − |
| | 2 | 1 | 0.584 | 2.12 | + |
| | 2 | 4 | 0.355–0.427 | 1.26–1.55 | − |
| 90110 | Control | 3 | 0.273 | | |
| | 0 | 5 | 0.259–0.302 | 0.95–1.11 | − |
| F022411 | Control | 3 | 0.211 | | |
| | 0 | 5 | 0.208–0.352 | 0.99–1.67 | − |

S/C: Ratio of sample OD to control OD.

[a]Non-inoculated healthy plants of each line were used as control.

[b]OD Values present mean values of three replicates minus the value of blank control. CV (coefficient of variation) of every OD value ≤ 12.5%. The highest OD value of three control plants of each line was presented. The range of OD values for each symptom score of each line was shown.

[c]+, Positive ELISA result whose OD value was twice that of the control; S/C > 2; −, negative result.

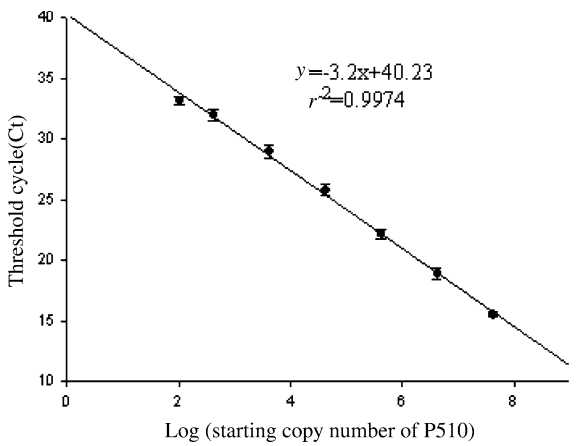


*Figure 3*. Standard regression line obtained by plotting Ct values versus the logarithm of the starting copy number of plasmid. The plasmid P510 was diluted to $4.29 \times 10^7$, $4.29 \times 10^6$, $4.29 \times 10^5$, $4.29 \times 10^4$, $4.29 \times 10^3$, $4.29 \times 10^2$, $2.14 \times 10^2$ copies, and the dilutions were used as templates for amplifying a 342 bp fragment. The Ct value of each dilution was the mean (± SE) of three reactions.

copies per 100 ng total RNA), and that of F112132 was the second highest ($1.26 \times 10^3$ copies per 100 ng total RNA) (Figure 5). RBSDV was detected in all of the plants with symptoms of disease. Six 90110 plants and five F022411 plants were shown to be infected with RBSDV, but the plants did not exhibit the symptoms of MRDD. Out of five F112132 plants without MRDD symptoms there were three plants infected with RBSDV. The viral content was higher as the disease level rose. In line Ye478, the differences between the average viral content of plants scoring

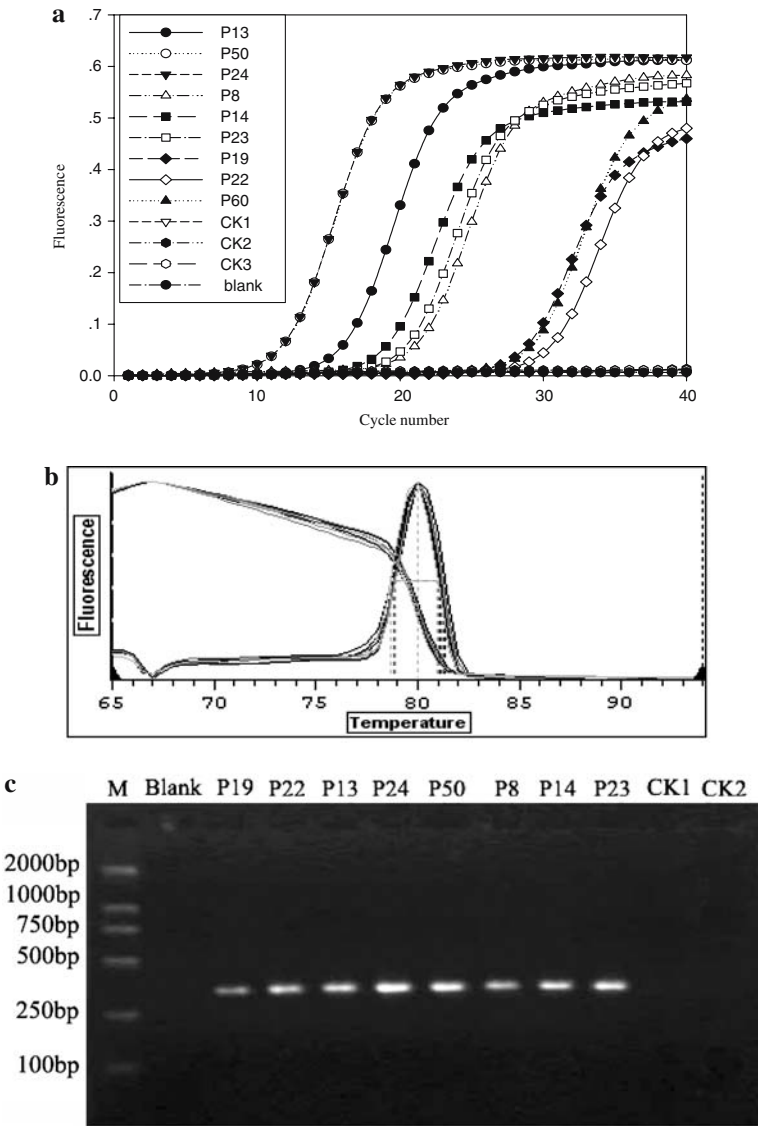


*Figure 4*. Detection of *rice black-streaked dwarf virus* with real-time RT-PCR using SYBR Green 1. Plants from line Ye478 with different disease scores were detected. Score 3: P13, P24, P50; score 2: P8, P14, P23; score 1: P19, P22, P60; uninfected plants: CK1, CK2, CK3; blank: amplification of dd-$H_2O$. (a) Real-time RT-PCR amplification plot based on cycle number versus fluorescence. The amplification curves of P50 and P24 overlap each other. There were no PCR products obtained from CK1, CK2, CK3 and blank control. (b) Melting curve analysis of RBSDV amplicons in Ye478 plants: P13, P24, P50, P8, P14, P23, P19, P22, P60. All of the RBSDV melting curves were grouped at a common melting temperature, indicating that the same RBSDV amplicon was produced in all of their respective polymerase chain reactions. (c) Some of the products were analysed in 2% agarose gels. M: DNA weight marker, DL2000 (Takara, Dalian, China).

3 and those scoring 2 were significantly different ($P = 0.006$), and the average viral content of plants scoring 2 was significantly higher than in thosescoring 1 ($P = 0.008$) (Figure 5). In line F112132, the average viral content of plants that scored 1 was significantly lower than in those scoring 2 ($P = 0.004$), and significantly higher than in plants scoring 0 ($P = 0.002$) (Figure 5). It was concluded that the viral content of the plants measured by real-time quantitative RT-PCR was correlated with the disease level. Furthermore, the moderately susceptible line, F112132, could be distinguished from the other lines. The average viral content of each disease level of Ye478 and F112132 suggested that in susceptible maize inbred lines, the plants with $1 \times 10^3$ viral copies in 100 ng total RNA belonged to susceptible individuals, and the plants with $1 \times 10^6$ viral copies in 100 ng total RNA belonged to highly susceptible individuals.

For individuals, the viral content of some plants was not correlated with their symptom scores, especially between individuals with scores of 0 and 1, or 1 and 2. The viral content of some 90110 plants was as high as susceptible Ye478 plants with scores of 1, but these plants did not show the symptoms of MRDD; in F112132, the viral content of one plant with a score of 1 was much higher than the average. The ELISA-positive plants showed high viral content (the minimum was $7.24 \times 10^5$ copies per 100 ng total RNA) by PCR detection, while in the resistant maize lines the amount of virus was low. Some ELISA-negative plants became positive using PCR detection and the maximum viral content was as high as $2.88 \times 10^6$ copies per 100 ng total RNA.

## Discussion

MRDV, MRCV (Mal de Río Cuarto virus) and RBSDV, which all belong to the *fijivirus* genus cause MRDD in Europe (Dovas et al., 2004), South America (Distéfano et al., 2002) and East Asia (Bai et al., 2001; Fang et al., 2001; Isogai et al., 2001; Zhang et al., 2001b), respectively. RBSDV is also the pathogen of rice black-streaked dwarf disease in East Asia. In this study, maize plants with typical symptoms of MRDD in the field were confirmed to be infected by RBSDV. The virus was then transmitted into maize seedlings via planthoppers and caused MRDD. This result, consistent with that of Bai et al. (2001), Fang et al. (2001) and Zhang et al. (2001b), indicated that RBSDV was the pathogen causing MRDD in the east of China.

The ratio of viruliferous planthoppers was the key parameter for obtaining a reproducible result in artificial inoculation via an insect vector. We estimated the ratio by detecting RBSDV in planthoppers using ELISA. The appropriate insect density was then selected according to that ratio. By studying the susceptible maize line Ye478, we concluded that plant age, insect density and inoculation duration were the three important factors affecting disease incidence of MRDD. A high rate of infection was obtained when young maize plants were inoculated with a high insect density and a 5-day period for inoculation. However, planthopper feeding affected the growth of plants and led to low survival rates after transplantation. We therefore chose a relatively moderate condition with 15 planthoppers per plant infecting two-leaf plants for 5 days. Under these inoculation conditions, 10 maize inbred lines were examined. The results were reproducible and consistent with results from many years of field studies.

In this study, ELISA and real-time RT-PCR were used to detect RBSDV in maize plants. Detected by ELISA, only plants scoring 3 were positive. The slightly diseased plants and healthy plants that were all negative could not be distinguished from each other. However, using real-time RT-PCR, RBSDV could be detected at levels as low as $1 \times 10^3$ copies in 100 ng total RNA from the infected plants. The data we obtained showed that differences in the average viral content among plants with different disease levels were significant. Disease levels on each plant could be determined according to their viral content. Thus, healthy plants could be easily distinguished from infected plants. A small quantity of viral RNA was detected in the infected plants of 90110 and F022411, which means that unknown factors interfere with either the replication of virus or the viral movement between the cells of the plants.

Inbred lines Ye478, F112132, 90110 and F022411 show different resistances to MRDD. One explanation is that multiple genes control the plant resistance to RBSDV and the different lines may have different numbers of resistance genes. Using the improved inoculation procedure, all plants of susceptible line Ye478 became infected,

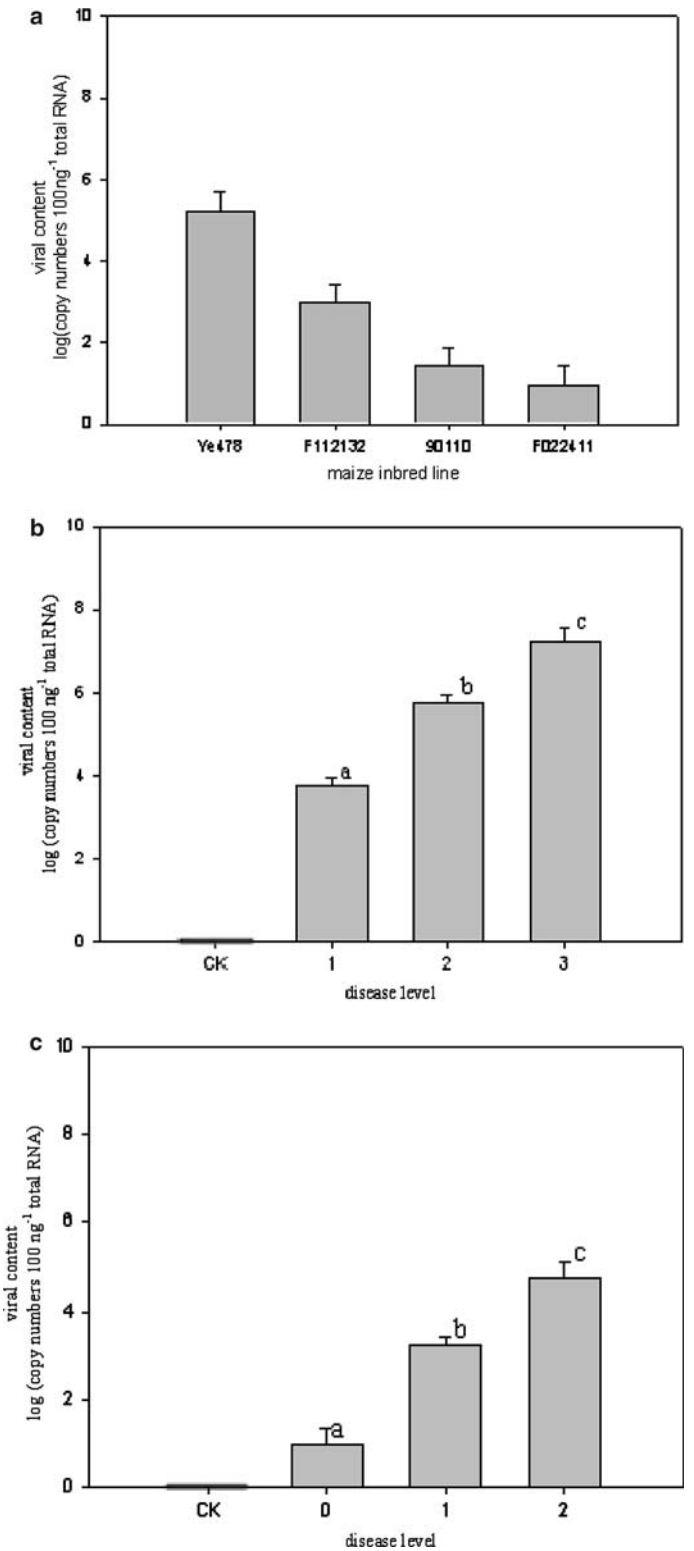
a
viral content
log(copy numbers 100ng-1 total RNA)
Ye478
F112132
9D110
FD22411
maize inbred line
b
viral content
log (copy numbers 100 ng-1 total RNA)
CK
1
2
3
disease level
c
viral content
log (copy numbers 100 ng-1 total RNA)
CK
0
1
2
disease level

*Figure 5*. Content of *rice black-streaked dwarf virus* in inoculated maize plants. Maize plants were analysed by real-time quantitative RT-PCR using SYBR Green 1. The viral content of a plant was quantified by comparing the Ct value with the standard curve. Each column represents the mean (±SE) of the analysed plants. Non-inoculated plants of each genotype were negative controls. Viral RNA was not detected in the controls. Different low case letters in panels b and c denote means (±SE) that differ significantly ($P < 0.05$). (a) Viral content in different inoculated maize lines. (b) Viral content in Ye478 plants with different disease levels. (c) Viral content in F112132 plants with different disease levels.

while the plants of the resistant lines were either healthy (or the virus replicated to a level below the limits of detection) or asymptomatic, and the plants with moderate susceptibility were also identified. The results of the inoculation were also consistent with results from field assessments. Therefore, this inoculation method could be used to screen for RBSDV resistance genotypes among maize inbred lines.

Combined with artificial inoculation, plants in a segregate population of MRDD resistance could be accurately identified as diseased, or not, by using real-time RT-PCR. This will make a contribution to the study of localization of MRDD resistance genes and will be useful in breeding for resistance. In addition, the primers P1 and RP were fully identical with the RBSDV isolates from Japan (S63917) and China (AF397894, AJ297428, AJ297427, AY147039) at the corresponding positions; it is therefore likely that RT-PCR will be appropriate for the detection of RBSDV in wheat and other grasses in early spring to estimate the natural levels of RBSDV and so predict MRDD.

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