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Comparative Analysis of Serological and Molecular Methods for the detection of Rice Grassy Stunt Virus*

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水稻草矮病毒血清学和分子检测方法的比较*

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Abstract: Methods of ELISA, nonradioactive molecular hybridization and RT-PCR were applied in the detection of rice grassy stunt virus (RGSV). The detection sensitivity of indirect ELISA using antiserum against fusion protein GST-NC was 1 mg of infected leaves or 84 ng of purified virus. The method of dot hybridization using NC, a DIG-labelled DNA probe was 50 μg diseased leaves, or 6 ng purified preparations. The detection endpoint of RT-PCR was 10 μg diseased leaves, or 2 ng purified virus preparation. Comparisons of sensitivity and maneuverability were made among these methods.

Key word: Rice grassy stunt virus; serological detection; molecular detection

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摘要：将间接 ELISA、非放射性分子杂交和 RT-PCR 三种方法应用于水稻草矮病毒(RGSV)的检测。结果表明，利用自制的融合蛋白 GST-NC 的抗血清检测 RGSV 的灵敏度为 1 mg 鲜重的病株叶片或 84 ng 提纯病毒，利用地高辛(DIG)标记的 DNA 探针 NC 的点杂交方法检测 RGSV 的灵敏度为 50 μg 病叶或 6 ng 病毒，而 RT-PCR 的检测灵敏度则为 10 μg 病叶或 2 ng 的病毒，对上述三种方法的灵敏度和可操作性也进行了比较。

关键词：水稻草矮病毒；血清学检测；分子检测

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Rice grassy stunt disease, which was identified in the Philippines in 1963[1], is one of the most important virus disease on rice in South and Southeast Asia. During the 1970's and early 1980's the disease caused severe yield losses in the south and southeast Asian countries such as Indonesia, the Philippines and India[2]. In the mainland of China, the disease was discovered in Fujian in 1979[3]. Its pathogen is rice grassy stunt virus (RGSV), a member of the genus *Tenuivirus*[4]. RGSV is transmitted by the brown planthopper *Nilaparvata lugens*, which has the ability of long-distance migration. Though in recent years rice grassy stunt disease can hardly been found in paddy in China, still it is a potential threat to rice production in our country once a large population of viruliferous brown planthoppers moves into South China from prevalence areas. Therefore, virus detection is very important. In this paper the detection methods of indirect ELISA, dot hybridization and RT-PCR were established and the sensitivity and maneuverability of these three methods were compared.

1 Materials and Methods

1.1 Plant and virus source

The RGSV-SX was originally collected at Shaxian, Fujian and maintained on the rice cultivar of Taichung Native 1(TN1) in a green house by periodic transfers to new plants. Leaves from infected TN1 were collected 20-30 days after symptom development.

1.2 Virus purification and RNA extraction

Procedures were performed as described previously[5].

1.3 Establishment of indirect ELISA method

Fusion protein GST-NC was the product of recombinant plasmid pGTNCP harboring the *NC* gene of RGSV in *E. coli*[5]. Preparation of antisera against GST-NC and indirect ELISA were carried out essentially as described by Tian *et al*[6]. To determine the appropriate working concentration, the crude antiserum was diluted to a series of 1:100～1:10,000 (V/V) with dilution of sap extract and IgG-AP (Sigma) being settled to be 1:50 (W/V) and 1:10,000 respectively, and the concentration of substrate to be 1 mg/ml. The diseased sap extract and healthy control were then diluted to a series of 1:10～1:1,000 and boiled for 5 min to determine the detection sensitivity, using an appropriate antiserum concentration set out earlier and the same dilutions of IgG-AP and substrate as before.

1.4 Preparation of DIG-labeled DNA probe

Using plasmid pTNCP harboring the *NC* gene of RGSV as the template and primed by P1 (5'-AGTGTCATATGGGTAAAGTGCAATTTGG-3') and P2(5'-TCACAGGATCCACTACGCTAAAGGC-3')[5], the DIG labeled probe was produced by PCR amplification. The protocol was the same as normal PCR[5] except that DIG-dUTP (Boehringer Mannheim) was mixed with dTTP in a ratio of 1:12. The PCR products were recovered from agarose gels using QIAEX Ⅱ Gel Extraction Kit (QIAGEN) for purification.

1.5 Extraction of total nucleic acids from rice leaves

The method of Nishiguchi *et al*[7] was followed to prepare total nucleic acid extracts from rice leaves.

1.6 Dot-blot hybridization

RNA dot blot hybridization was performed as described in the DIG System User's Guide for Filter Hybridization (Boehringer Mannheim) and the manual for PolyScreen PVDF Transfer Membrane (DuPont). 1 μL of each dilution was applied to the membrane.

1.7 RT-PCR

Primed by P611 (5'-CTGAGCTCTCTTGCAATGGTGAG-3') and P622[8](5'-GCGCATATGTCTAAATC-TCATTCTGACG-3'), RT-PCR analysis of purified viral RNA and total nucleic acid preparation were carried out using M-MuLV reverse transcriptase and Taq DNA polymerase (MBI). PCR parameters were: denaturation at 94 ℃ for 5 min followed by 3 cycles of 94 ℃ for 1 min, 50 ℃ for 2 min and 72 ℃ for 2 min, followed by 30 cycles in which the annealing temperature was raised to 52 ℃, and extension finished by incubation at 72 ℃ for 10 min.

2 Results

2.1 Detection of RGSV by serological method

Antiserum of GST-NC was produced by injecting the protein recovered from SDS-PAGE into rabbit. The antiserum had a titer of 1∶2,560 against the purified GST-NC protein in indirect ELISA.

(1)Feasible working concentration of antiserum

Serial dilutions of antiserum between 1∶100 and 1∶10,000 were prepared for the serological test. Results showed that the dilutions between 1∶200 and 1∶500 served well in the detection (Fig 1a).

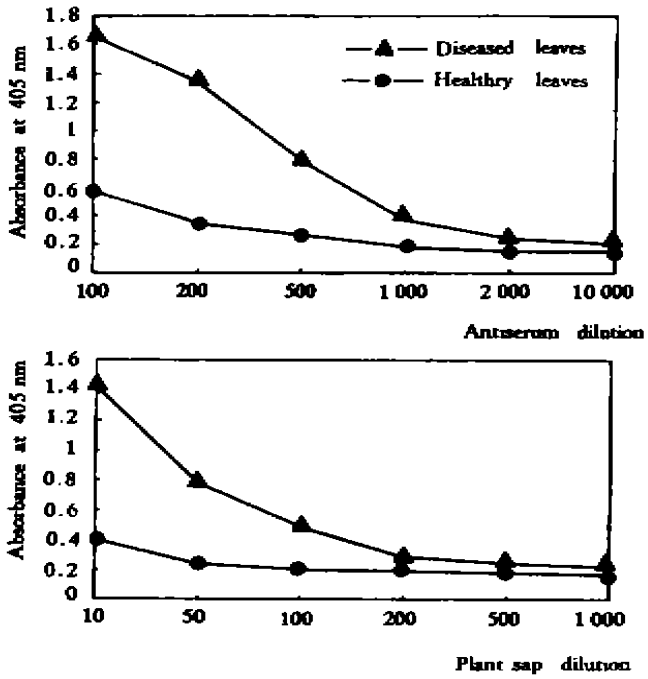

Fig. 1 Indirect ELISA for the detection of RGSV

(a) working concentration of antiserum; (b) detecting sensitivity

(2)Detecting sensitivity

Indirect ELISA was performed on serial dilutions of diseased and healthy saps between 1∶10

and 1∶1,000, using antiserum diluted to 1∶400. It turned out that the detection limit was 1∶100, corresponding to 1 mg, or an equivalent of 10 mg/mL of fresh leaves (Fig 1b). When purified virus preparation with a concentration of 2.7 mg/mL was used, the dilution endpoint was 1∶3,200, corresponding to a minimum amount of virus detected of 84 ng or 840 ng/mL.

2.2 Detection of RGSV by dot-blot hybridization

The concentration of probe NC used in the detection was 8 ng/mL, which was optimized by mock hybridization. The detection limit was determined to be 50 μg of leaf tissue, an equivalent of dilution endpoint of 1∶100 with the original dilution corresponding to a tissue concentration of 5 mg/μL (Fig. 2).

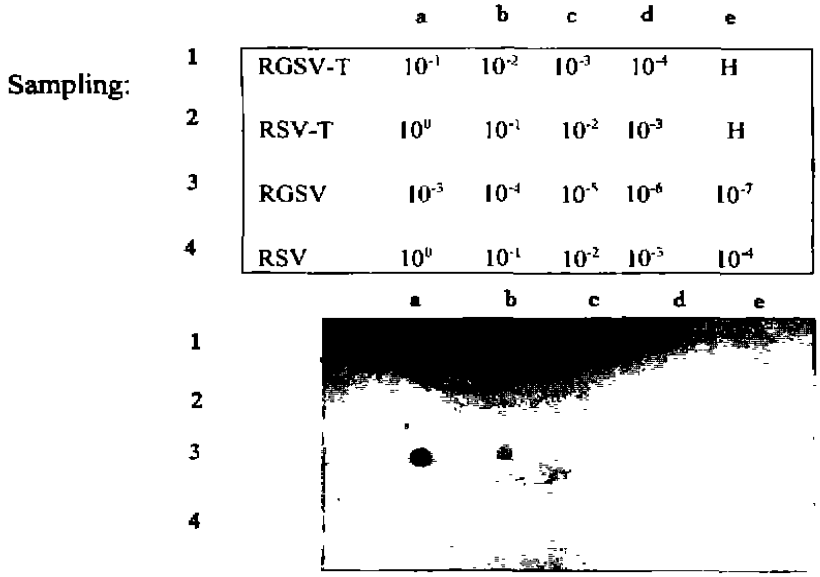

Fig.2 Detection of RGSV in infected leaf extracts by dot blot hybridization

Samples were: RGSV-T& RSV-T: total RNAs extracted from RGSV & RSV infected rice leaves respectively; H: healthy rice plant; RGSV & RSV: RNAs extracted from purified virions.

The probe also hybridized with tenfold-diluted RSV(rice stripe virus)RNA, indicating that the two tenuiviruses shared certain homology in the *NC* gene. However, the homology level was not high since for RGSV-RNA the detection endpoint was 10^{-4}, corresponding to 6 ng of purified virus preparation with an initial RNA concentration corresponding to 60 mg virus/ml.

2.3 Detection of RGSV by RT-PCR

Total nucleic acids extracted from 10 mg of diseased rice leaves were dissolved in 20 μL TE. 2 μL of serial dilutions of the above preparation were used as templates. The PCR products were electrophoresed and a single band of 550 bp was detected in samples up to 10^{-2} dilution, representing 10 μg of infected leaves. When non-infected material was used, no band could be detected

(Fig. 3). When purified viral RNAs were used as templates, the detection limit was 2 ng of virus preparation (data not shown).

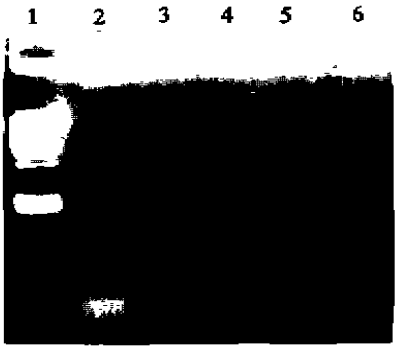

Fig. 3　1% agarose gel electrophoresis for RGSV detection in diseased rice leaves by RT-PCR
Lane 1: λDNA/*Hind* Ⅲ marker; lane 2-5: RT-PCR products from RGSV-infected rice leaves with dilution of 10^{0}, 10^{-1}, 10^{-2} and 10^{-3} respectively; lane 6: uninfected sample (10^{0}).

3 Discussion

Serological methods have been proved to be sensitive and specific in the detection of RGSV[9-11]. Some of the detection techniques have also been compared[11]. Antisera against disease-specific protein (SP), apart from purified RGSV filaments, can be used in the detection as well[12,13]. However, the application of molecular detection has not been reported. Detection by RT-PCR method was established here with two specific primers in the *NS6* gene on RGSV-RNA6. The dot hybridization method was set up following the preparation of DIG-labelled DNA probe. Results showed that both of the two methods could detect RGSV in rice leaves sensitively and efficiently.

A serological method of indirect ELISA was set up after the preparation of antiserum against the protein product of RGSV-*NC* gene in *E. coli*. The three methods were then compared simultaneously using the same material. It turned out that the sensitivity with RT-PCR is the highest, being 5 times (for fresh leaves) or 3 times (for purified virus preparations) higher than that with dot hybridization, and 100 times (for fresh leaves) or 40 times (for purified preparations) higher than that with indirect ELISA.

Molecular hybridization and RT-PCR have been widely used in the detection of virus and viroid because of their high sensitivity, specificity and repeatability. However, when expenses, time and practicability are taken into consideration, they are not as good as ELISA. Serological method can be applied as a conventional method for disease monitoring and for laboratory research since it is easy to perform without special equipments. When sensitivity is in demand, molecular hybridization can be used instead. The major drawback of hybridization with non-radioactive labeling is time-consuming. It normally needs two days to carry out a detection because overnight is

required for the steps of hybridization and color developing. In addition, the step of extraction of *nucleic acid confines the capacity of sample processing. It will be more convenient to deal with* large amounts of samples if the procedures can be reduced or crude extraction can be used instead[14]. The method of RT-PCR is the least affordable and special instrument is needed in addition. The application of this technique might be limited to situations when high sensitivity is required.

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