

イネおよびツマグロヨコバイ(Nephotettix cincticeps)体内 におけるイネ黄萎病ファイトプラズマの増殖と分布

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
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発行元	日本植物病理學會
巻/号	61巻5号
巻号補足	
掲載ページ	p. 451-455
発行年月	1995年10月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Multiplication and Distribution of Rice Yellow Dwarf Phytoplasma in Infected Tissues of Rice and Green Rice Leafhopper *Nephotettix cincticeps*

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Abstract

Multiplication and distribution of rice yellow dwarf phytoplasma in infected tissues of rice and the vector, green rice leafhopper (*Nephotettix cincticeps*), were demonstrated by dot hybridization using DNA probes for phytoplasma DNA. Phytoplasma DNA was first detected in rice plants at 20 days after the termination of a 3-day inoculation feeding of phytoplasma-exposed insect vectors. The amount of DNA in infected rice tissues started to increase rapidly at 30 days after the feeding, leveled off by 40 days, and remained at the same high level for up to 80 days. In RYD-infected rice tissues, the amount of phytoplasma does not correlate to the severity in chlorosis. In insects, phytoplasma DNA was detected at 10 days after the termination of a 3-day acquisition access feeding on RYD-infected plants, and the amount increased rapidly at 30-40 days after the feeding. Phytoplasma DNA was detected mainly in the abdomen and thorax at 25 days after the feeding, but mainly in the head at 50 days.

(Received March 2, 1995; Accepted July 19, 1995)

Key words: rice yellow dwarf, phytoplasma, mycoplasma-like organism, rice, green rice leafhopper.

INTRODUCTION

The rice yellow dwarf (RYD) phytoplasma agent (mycoplasma-like organism [MLO]), is transmitted by leafhoppers, *Nephotettix cincticeps*, *N. virescens*, and *N. nigropictus*, and occurs mainly in Asian countries^{14,15}. As the organism cannot be cultured *in vitro*, it has been difficult to assay the pathogen and to understand the dynamics of the agent in hosts and in the ecosystem. Assay methods based on DNA hybridization using DNA probes have been applied for the detection and correlation analysis of some phytoplasmas^{3,4,6,7,9,11,17}. Dot hybridization using DNA probes enables a quantitative assay of phytoplasmas in their host plant and insect vector tissues^{1,8,16}. Recently, we developed DNA probes for the RYD phytoplasma, an isolate from Otawara, Tochigi, Japan (RYD-To)^{12,13}. Some of the DNA probes were considered to be derived from the chromosomal DNA, others from the extrachromosomal DNA. The chromosomal DNA probe R 9 and all of the extrachromosomal DNA probes, including R 32, hybridized with all RYD isolates so far collected in Asia, but not with DNA of healthy rice plants. Among all other phytoplasmas tested, excluding the RYD phytoplasma, only the sugarcane white leaf (SCWL) pathogen reacted

to these probes.

In this report, we demonstrate, using the DNA probes, multiplication and distribution of RYD phytoplasma in infected rice plants (*Oryza sativa*, Koshihikari) and the insect vector, green rice leafhopper (*N. cincticeps*).

MATERIALS AND METHODS

Plant and insect materials. RYD-To phytoplasma was maintained in rice plants by successive transfers using *N. cincticeps*. Second instar nymphs of *N. cincticeps* were allowed to feed on RYD-To infected plants for three days. After the acquisition feeding, the insects were maintained on healthy rice plants in a growth chamber (day time: 20,000 lux, 16 hr, 30°C, night: 25°C). Every 10 days after the termination of the acquisition feeding, insects were subjected to dot hybridization to determine the presence of phytoplasma. Also at 25 and 50 days after the feeding, the insects were tested similarly for the distribution of phytoplasma DNA in the insect body.

Rice seedlings at the 3-leaf-stage were inoculated by phytoplasma-exposed insects for 3 days, and grown in a green house (day time: 16 hr, 30°C, night: 25°C). Every 10 days after the termination of the inoculation period, leaves were collected from 20 plants, cut with scissors

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into 2- to 3-mm fragments, frozen at -85°C , and tested for phytoplasma DNA. The DNAs were extracted from randomly collected 1.0 g of small pieces of the leaves. Hybridization tests were repeated four times. At 30 and 70 days after the inoculation, portions of one healthy and three RYD-infected plants were tested for chlorophyll content and then subjected to the hybridization test. Test samples were collected from the top and bottom portions of three youngest leaves, stem, and root.

Dot hybridization. Tissues were homogenized with phytoplasma-extraction buffer (0.6 M mannitol, 20 mM Tris-HCl, pH 7.6, 5 mM magnesium acetate). The homogenate was centrifuged at $15,000 \times g$ for 10 min. DNA was extracted from the pellets by the CTAB method¹¹. DNA extracted from equal amounts of phytoplasma-infected or healthy plant tissues (twofold dilution of DNA extracted from 1.0 mg of tissues) was denatured and blotted onto nylon membranes (Hybond N⁺; Amersham). Cloned DNA fragments R 9 and R 32 of RYD phytoplasma were amplified from the chimeral vector plasmid by a PCR procedure¹³. The amplified DNA molecules were labeled with peroxidase using the Amersham ECL system¹². The blotted membranes were hybridized with labeled DNA probes using the Amersham ECL system.

Quantification of hybridization signal. After the hybridization, the membrane was washed under highly stringent conditions¹³. The absorbance values at 633 nm (A_{633}) of each hybridization spot on the film was measured with an laser densitometer (LKB 2222-020 UltraScan XL).

Measurement of chlorophyll content. Chlorophyll content in portions of leaves was estimated by using a chlorophyll meter (Minolta SPAD). SPAD value (X) was obtained from chlorophyll a/b amount Y (mg/100 cm²) based on the equation, $Y = 0.199 X - 2.869$ ¹⁹.

RESULTS

Symptom development

In plants inoculated at the 3-leaf stage, typical symptoms of RYD consisted of leaf yellowing, plant stunting, and profused tillering. Early symptoms are characterized with leaf chlorosis which appears about 30 days after the inoculation in the green house. Profuse tillering and plant stunting appeared at 60–90 days after the inoculation. The chlorosis was observed first on some young leaves (1st or 2nd youngest leaves) and old leaves (5th youngest or older leaves), and on almost all the leaves at 90 days.

Development and distribution of RYD phytoplasma in infected rice plant

The standard curves showed a parallel relationship between the quantity of probes added and the resulting hybridization signal (A_{633}) until saturation of signals (data not shown). We controlled exposure time for the ECL detection to have hybridization signals unsaturated. In order to compare the absorbance values of signals

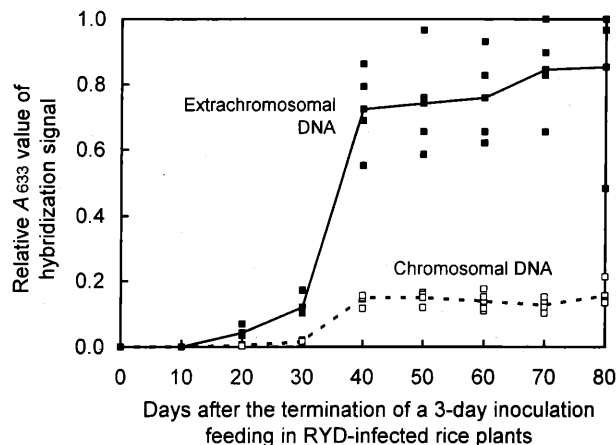
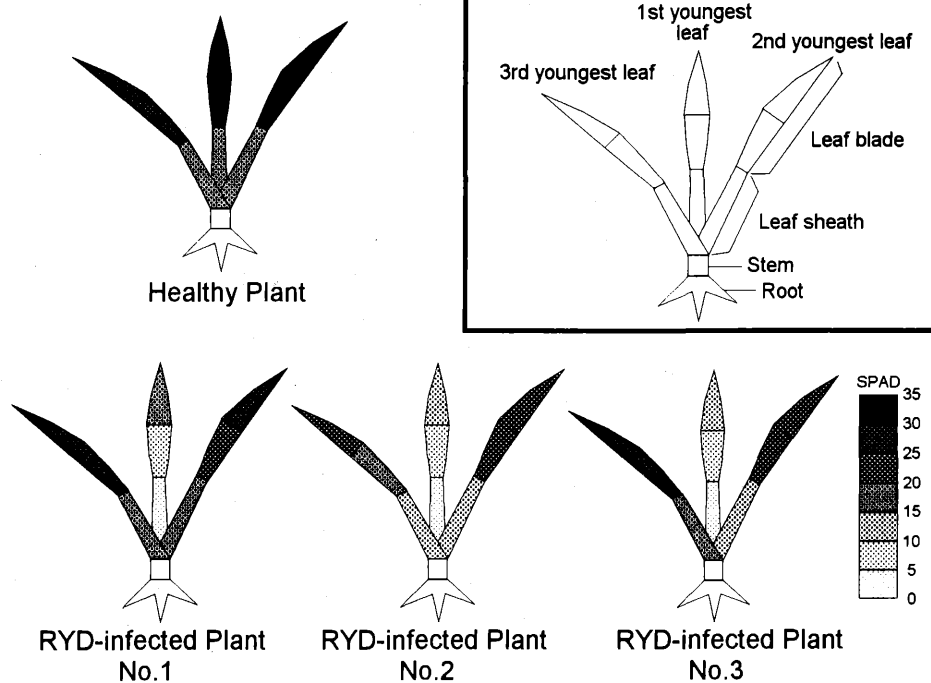


Fig. 1. Multiplication of RYD phytoplasma in inoculated plants as revealed by the hybridization using DNA probe R 9 or extrachromosomal DNA probe R 32 against DNAs extracted from plants which was spotted on a nylon membrane. A_{633} values of hybridization spots were measured by a densitometer on four DNA samples extracted from pools of RYD-infected tissues.

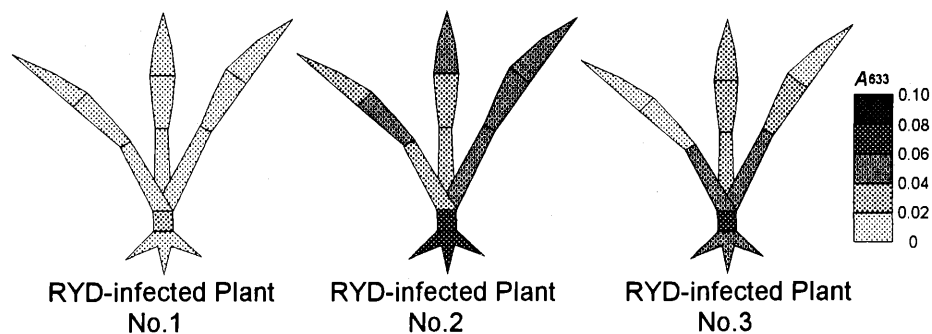
produced with the chromosomal DNA probe R 9 and extrachromosomal DNA probe R 32, readings of A_{633} were done at the same time, and the A_{633} values for R 9 and R 32 were divided with the highest reading in each test, and indicated as "Relative A_{633} values" as shown in Fig. 1. The Fig. 1 shows the increase of relative A_{633} values after inoculation in the inoculated plant using R 9 or R 32 probe against DNA extracted from 1.0 mg of plant tissue. In infected plants, phytoplasma DNA was first detected at 20 days after inoculation. Generally, chlorosis symptoms appeared in rice plants at 30 days after inoculation. Therefore, the hybridization assay using DNA fragments of RYD phytoplasma as probes enabled to detect phytoplasmas at least 10 days earlier than the use of symptomatology. In infected plants, relative A_{633} values increased rapidly at 30–40 days after inoculation, leveled off by 40 days, and remained at the same high level for up to 80 days. The detection efficiency of RYD phytoplasma in infected plants was five to ten times more sensitive for R 9 than for R 32.

For the chlorophyll content and the distribution of RYD phytoplasma in infected plants, portions of the three youngest leaves, stems, and roots of three plants with similar symptoms were assayed by SPAD, and then tested for hybridization using R 9 or R 32 at 40 and 70 days after inoculation. At 40 days after the inoculation, the youngest leaves showed severe chlorosis, the second youngest leaves mild chlorosis and the third youngest leaves did not show any symptoms (Fig. 2). Phytoplasma was detected in all the parts of infected plants. There was no definite correlation between the amount of phytoplasma and the level of chlorosis in infected rice tissues. At 70 days after the inoculation, all the leaves tested showed severe chlorosis, and phytoplasma was detected in all parts of tested plants (data not shown).

Chlorophyll Content



Chromosomal DNA



Extrachromosomal DNA

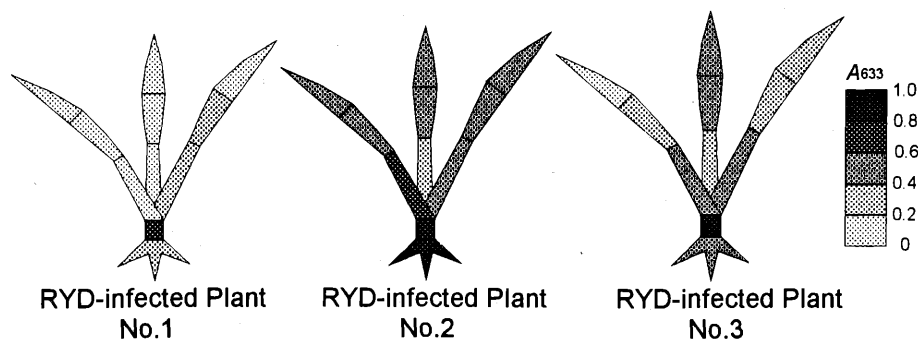


Fig. 2. Chlorophyll and RYD phytoplasma DNA contents in portions of three rice plants at 40 days after inoculation. DNA extracted from 1.0 mg tissues of each portion was spotted on a nylon membrane and hybridized with chromosomal DNA probe R 9 or extrachromosomal DNA probe R 32. Hybridization was quantified by measuring A_{633} values of spots, which were then recalculated in relative values as described in the text.

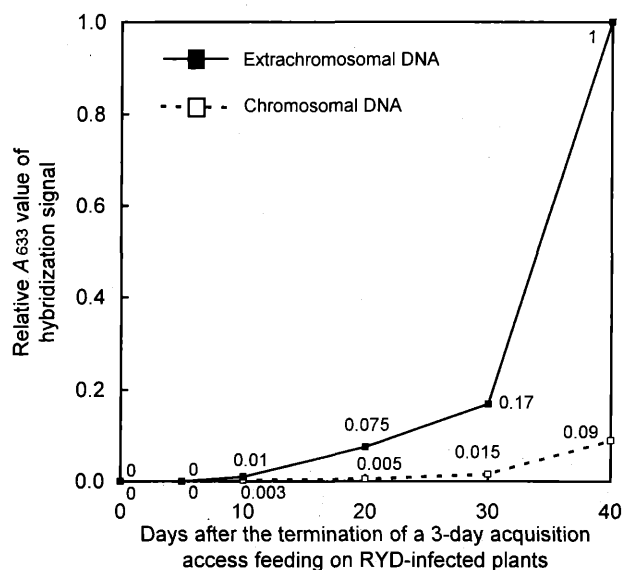


Fig. 3. Multiplication of RYD phytoplasma in green rice leafhoppers *Nephotettix cincticeps* which were exposed for 3 days to RYD phytoplasma, as revealed by the hybridization using chromosomal DNA probe R 9 or extrachromosomal DNA probe R 32 against DNAs which were extracted from 10 insects and spotted on a nylon membrane. The hybridization was quantified by measuring A_{633} values which were then recalculated in relative values as described in the text.

There was also no definite correlation between the amount of phytoplasma and chlorophyll content in each part of infected plants.

Development and distribution of RYD phytoplasmas in insects

By the hybridization test, RYD phytoplasma was first detected at 10 days after the termination of a 3-day acquisition access feeding, and increased rapidly at 30 days after the feeding (Fig. 3). In other tests, the abdomen and thorax gave strong signals in most RYD-exposed insects at 25 days after the feeding, whereas the head did so only at 50 days (Fig. 4).

DISCUSSION

Early symptoms of RYD in rice are characterized by chlorosis in newly emerging leaves. A characteristic symptom of SCWL in sugarcane is severe chlorosis in young leaves. Not all phytoplasmas pathogenic to gramineous plants induce such chlorosis in younger leaves. Rice orange leaf (ROL) phytoplasma, which is genetically distant from RYD phytoplasma¹⁰⁾, induces an orange discoloration in older rice leaves, but not in younger leaves¹⁵⁾. Development of two types of symptoms in phytoplasma-infected plants indicates that phytoplasmas may induce symptoms through a secretion of toxic compounds in the phloem cells, but not simply by blocking phloem flow. In these experiments,

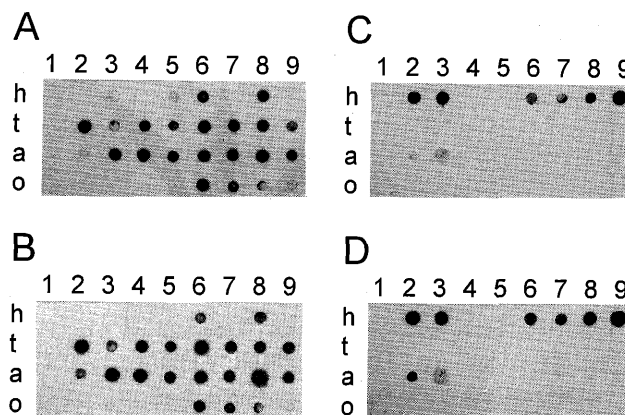


Fig. 4. Detection of RYD phytoplasma in parts of individual *N. cincticeps* at 25 days (A and B) and 50 days (C and D) after a 3-day acquisition access feeding as revealed by the hybridization test using chromosomal DNA probe R 9 (A and C), and extrachromosomal DNA probe R 32 (B and D) against DNA extracted from head (h), thorax (t), abdomen (a), and other parts (o) including legs and wings, which were spotted on a nylon membrane. No. 1; healthy leafhoppers, No. 2-9; leafhoppers exposed to RYD phytoplasma.

there was no correlation between the amount of RYD phytoplasma and the severity of chlorosis in RYD-infected tissues. Similar results were obtained in sugarcane with SCWL phytoplasma¹¹⁾. Apparently, phytoplasma concentration in newly emerging leaves with severe chlorosis was not always high. Our results suggest that possible symptom-inducing metabolites synthesized by RYD or SCWL phytoplasmas may be translocated through the phloem elements from other parts of plants to the meristems. On the other hand, it has been reported that the highest concentration of two strains of aster yellows (AY) phytoplasma were consistently detected in the expanding, symptomatic shoots of infected periwinkle plants⁹⁾. A similar observation was reported also in strawberry plants infected with clover phyllody phytoplasma, in which the phytoplasma titer was highest in areas with symptoms²⁾. Douglas⁵⁾ reported that there was a close correlation between pathogen titers and symptom severity in peach and chokecherry infected with eastern X-phytoplasma. In these cases where phytoplasmas other than RYD or SCWL are involved, the highest concentrations of phytoplasmas occurred in regions of infected plants where symptoms were most severe, suggesting that these phytoplasmas may be colonized locally and synthesize metabolites to induce localized symptoms. Compared to these phytoplasma-diseases, the mechanisms of symptom expression may be different in RYD and SCWL.

Phytoplasma DNA was detected mainly in the abdomen and thorax of insects at an early stage of infection, whereas it was detected mainly in the head later. A similar phytoplasma distribution was observed in leafhoppers *Macrostelus fascifrons*, a vector of AY phyto-

plasma, in which the phytoplasma was first recovered from the alimentary canal and later from the salivary glands¹⁸. The concentration of the AY phytoplasma increased rapidly in the alimentary canal, but then declined sharply, whereas the concentration increased gradually in the salivary glands. Colonization of RYD phytoplasma in specific portions of *N. cincticeps* may be related to its transmissibility of the pathogen. This requires further studies.

The authors thank Dr. T. Shiomi, National Agriculture Research Center, Tsukuba, Japan, Mr. S. Iwanami, National Institute of Agrobiological Resources, Tsukuba, Japan, Mr. S. Kato, formerly at National Agriculture Research Center, Tsukuba, Japan, and Dr. N. Murata, JICA, Islamabad, Pakistan for their useful suggestions.

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和 文 摘 要

中島一雄・林 隆治：イネおよびツマグロヨコバイ (*Nephotettix cincticeps*) 体内におけるイネ黄萎病ファイトプラズマの増殖と分布

イネおよびツマグロヨコバイ (*Nephotettix cincticeps*) 体内におけるイネ黄萎病ファイトプラズマ DNA の増殖と分布を、DNA プローブを用いたドットハイブリダイゼーションにより調べた。イネ体内においては、ファイトプラズマ感染虫を用いて3日間吸汁接種した後、20日目にはファイトプラズマのDNAが検出された。ファイトプラズマ DNA 量は30日後から急速に増加し、40日後から80日後まで同程度のレベルであった。組織内ファイトプラズマ量と黄化症状の激しさの間の相関関係は認められなかった。ツマグロヨコバイ体内においては、3日間獲得吸汁させてから10日後にはファイトプラズマ DNA が検出され、30~40日後に、その量は急増した。虫体内では、ファイトプラズマ感染25日後には主に腹部および胸部から、50日後には主に頭部からファイトプラズマのDNAが検出された。