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DETECTION OF LEAF CURL GEMINIVIRUS IN TOMATO AND WHITEFLIES USING NON-RADIOACTIVE DNA PROBE

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Of 24 leaf curl infected samples of tomato, squash and pepper collected from different tomato growing areas of Luzon, 19 showed positive hybrids with the non-radioactive probe developed from the gene clone of tomato leaf curl geminivirus (Philippine isolate). Samples from healthy tomato showed negative reaction with the probe. Squash with leaf curl symptom also reacted positively, suggesting the presence of the geminivirus in squash. Tomato leaf curl geminivirus (TLCGV) was also detected from whiteflies (*Bemisia tabaci*) that were allowed to feed on leaf curl infected tomato and produced positive hybrids with the non-radioactive probe.

The use of non-radioactive probe in nucleic acid hybridization technique can be adopted for routine diagnosis of leaf curl geminivirus in tomato as well as in other crops.

Key words: Tomato leaf curl, non-radioactive DNA probe, geminivirus

INTRODUCTION

Tomato leaf curl is a serious viral disease prevalent in the tropics and subtropical regions of the world including Thailand, Indonesia, Taiwan, the Middle East and Eastern Mediterranean countries (Czosnek *et al.* 1988). The virus caused epidemics in vegetable crops and generally associated with local or regional whitefly (*Bemisia tabaci*) infestations characterized by severe yellow mosaic, leaf curling and stunting symptoms.

In the past, exact diagnosis of viruses involved in this disease was not possible. Traditionally, serological methods have been the primary means of virus detection but the approaches are of little success with whitefly transmitted viruses because they are extremely difficult to purify (Green and Kalloo, 1994). The few polyclonal antisera produced showed high cross reactivity with heterologous antigens (Stein *et al*, 1983). Disease diagnosis has been merely dependent on visual symptoms and whitefly transmission. None of these features are, however virus, specific and will not differentiate isolates among whitefly transmitted geminivirus. In the advent of new technologies, development of nucleic acid probes to accurately detect whitefly transmitted viruses become possible.

Tomato leaf curl virus was isolated and characterized from tomato leaf curl samples obtained from the CES, UPLB Central Experiment Station (Dolores, 1995; Dolores and Bajet, 1995). In 1997, a DNA fragment was cloned from the DNA-A of the Philippine isolate of tomato leaf curl geminivirus and constructed a non-radioactive DNA probe for geminivirus detection (Shih *et al*, 1997). Kon *et al* (2002) also determined the nucleotide sequence identities of an infectious clone from the Philippine isolate of tomato leal curl whitefly transmitted geminivirus, a new monopartite begomovirus. Both studies reported tomato leaf curl geminivirus-Philippine isolate as a distinct whitefly transmitted geminivirus based on the nucleotide sequence identities in comparison with other whitefly transmitted geminivirus.

This study was conducted to detect the presence of tomato leaf curl geminivirus in tomato and whiteflies and be able to learn how to diagnose plant virus using non-radioactive probe in nucleic acid hybridization.

MATERIALS AND METHODS

Virus Source.

Plant samples were obtained from the different tomato growing areas of Liliw and Los Baños, Laguna; Batangas and Baguio City. Samples were collected from youngest leaves of symptomatic and asymptomatic plants.

Tomato leaf curl virus from infected tomato plant was tissue implanted onto healthy tomato seedlings and kept inside insect proof screencage. Ten healthy adult whiteflies reared on seedgrown ampalaya seedlings were transferred to each infected plant and kept inside insect proof screencage until use. Whiteflies were collected and use for virus detection.

Non-radioactive DNA Probe

The non-radioactive DNA probe was developed at AVRDC, Taiwan (Shih *et al*, 1997) from a DNA-A fragment that was cloned in bluescript plasmid vector. Double stranded DNA was excised from the vector at EcoRI site and labeled by oligonucleotide random primed incorporation of DIG labeled dUTP according to Anonymous (1989). This labeled probe was used for geminivirus detection.

DNA Extraction of Plant Samples

Healthy and diseased plant tissues were ground each in one ml of 0.1 M tris buffer, pH 8 and centrifuged to removed plant debris. To each 250 ul of supernatant, an equal volume of denatured solution containing 67 ul of 3M NaOH, 50 ul 0.1M EDTA and 133 ul distilled water was added. DNA solution was boiled for 10 min in a waterbath and immediately chilled on ice. Denatured DNA was diluted 1:10 and used for probing.

Squash Blotting of Whiteflies

The procedure of Navot *et al* (1989) with some modifications was followed. For each whitefly sample, the insects were removed from leaf curl infected tomato by a needle and placed on nitrocellulose membrane. To each sample dot, 10 ul of denaturing solution (0.125 NaOH, 0.125 x SSC) was added and the insects were squash using small glass rod. The membrane was air dried and used for probing.

Nucleic Acid Hybridization

DNA extract of each plant samples was blotted onto nitrocellulose membrane and air-dried DNA of both plants and whiteflies was fixed on membrane by vacuum baked at 80^{0}C. Pre-hybridization was accomplished in sealed plastic bags with 20 ml hybridization solution (5xSSC, 1.2% w/v blocking reagent, 0.1% N-Lauroyl sarcosine Na-salt, 0.02% SDS) per 10 cm^2 of filter at 68^{0}C for at least one hr. After that, the filter was added with 2.5 ml of fresh hybridization solution containing 25 ul of freshly denatured TLCGV – DNA probe per 100 cm^2 filter. Hybridization reaction was maintained at least 6 hr at 68^{0}C. Washing was performed at room temperature with at least 50 ml of 2xSSC, SDS 0.1% (w/v), per cm^2 filter followed by 2 x 15 min at 68^{0}C with 0.1 x SSC; SDS, 0.1% (w/v).

Immunological Detection

The membrane was washed briefly in tris buffer 1 (Tris-HCl, 100 mmol/1; NaCl, 150 mmol, pH 7.5) and incubated in 0.5% blocking reagent in the same buffer for 30 min before washing again with tris buffer 1. The filter was then incubated with 20 ml of 1:5000 diluted antibody conjugate (DIG-AP conjugate, 750 u/ml) in tris buffer 1 at 20^{0}C for 30 min. The unbound conjugate was removed and the filter washed 2 times with 100 ml of tris buffer II (Tris-HCl, 100 mmol/l, NaCl, 100 mmol/l; $MgCl_2$, 50 mmol/l, pH 9.5). The filter was incubated with 10 ml color solution in suitable box kept in the dark. Color solution was freshly prepared by mixing 45 ul NBT solution and 33 ul X-phosphate in 10 ml tris buffer solution. The color reaction was observed and stopped by placing the filter in 50 ml tris buffer III (tris HCl, 10mmol/1:EDTA, 1mmol, pH 8.0) for 5 min and the results was recorded.

RESULTS AND DISCUSSION

Detection of Tomato Leaf curl Geminivirus in DNA Extracts of Plant Samples

Tomato leaf curl geminivirus was detected by the DNA probe of TLCGV-Phil. isolate from 19 out of 22 leaf curl infected tomato samples (Table 1; Fig. 1). Squash leaf curl also gave positive hybrids with the probe. Reaction varied from strong to weak signals as visualized on the nitrocellulose membrane of the NBT-X phosphate detection method (Fig. 2). Samples from healthy tomato showed negative reaction with the probe. All the symptomatic tomato and squash with leaf curl reacted positively with the probe. The apparently healthy plants and the other tomato plants with crinkling and yellow spots and fern leaf symptoms exhibited negative results. These findings indicate the association of the geminivirus with the leaf curl symptoms (Green and Kalloo, 1994). The pepper exhibiting crinkling and leaf distortion also gave negative hybrids with the probe.

Detection using non-radioactive probe developed from a gene tomato leaf curl geminivirus may be considered a practical and reliable diagnostic tool for whitefly transmitted geminivirus which occur in low concentration in plants and extremely difficult to purify (Green and Kalloo, 1994). The technique of DNA extraction was simple and may be used for processing large number of samples (Chiemsombat *et al*, 1990). The utilization of this method in routine diagnosis of virus is highly recommendable.

Detection of Tomato Leaf curl Geminivirus from Whiteflies

Tomato leaf curl geminivirus was also detected by the non-radioactive DNA probe from 8 out of 10 whiteflies previously fed to leaf curl infected tomato. The TLCGV infected tomato (with leaf curl tissue implant) also gave positive hybrids with the probe. Our results corroborated the findings of Chiemsombat *et al* (1990) who reported the presence of tomato yellow leaf curl virus from whiteflies. Using non-radioactive probe, TYLCV was detected in different stages of whiteflies from eggs, larvae and adult whiteflies (Navot and Czosnek, 1989).

This result would be very helpful in studies that would determine virus vector relationship of viruses specifically the whitefly transmitted geminivirus and may be considered an interesting area of research in future virus work.

CONCLUSION

The findings provided evidence that tomato leaf curl geminivirus (TLCGV) is present in tomato and squash with leaf curl symptom. Similarly, the geminivirus was detected from whitefly (*Bemisia tabaci*) that had been fed previously to TLCGV infected tomato. The non-radioactive probe is a very practical and simple method to use in nucleic acid hybridization to detect whitefly transmitted geminivirus and may be applied for routine diagnosis of viruses in tomato and other vegetable crops like squash.

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Table 1. List of collected leaf curl infected plants and their reaction with the Philippine tomato leaf curl geminivirus DNA probe

Sample/ Description	Symptom	Place Collected	Reaction Signal
1. *L. esculentum*	Leaf curl: stunting	IPB Demo, UPLB	++
2. *L. esculentum*	Leaf curl: yellow edge leaf	IPB Demo, UPLB	++
3. *L. esculentum*	Leaf curl: mos; st	IPB, UPLB	++
4. *L. esculentum*	Crinckled; brittle leaf, mottle	Liliw, Laguna	-
5. *L. esculentum*	Severe leaf curl, mosaic	Liliw, Laguna	+++
6. *L. esculentum*	Slight leaf curl, mos	Liliw, Laguna	+++
7. *L. esculentum*	Leaf curl; yellow banding	Liliw, Laguna	+++
8. *L. esculentum*	Yellow netted mosaic	CES, UPLB	+
9. *L. esculentum*	Downward, leaf curl, green mos	CES, UPLB	+
10. *L. esculentum*	Upward,: leaf curl slight	CES, UPLB	++
11. *L. esculentum*	Vein yellowing, leaf curl	Liliw, Laguna	++
12. *L. esculentum*	Leaf curl, yellow mos	Liliw, Laguna	++
13. *L. esculentum*	Leaf curl, yellow mos	Liliw, Laguna	+
14. *L. esculentum*	Severe leaf curl; tiny leaf	IPB, UPLB	++
15. *L. esculentum*	Sever yellow mos; severe leaf curl	IPB, UPLB	+++
16. *C. anuum*	Leaf distortion; mos	Batangas	-
17. *L. esculentum*	Fern leaf, mos; leaf distortion	Baguio City	-
18. *L. esculentum*	Severe leaf curl; resetting	IPB, UPLB	+++
19. *L. esculentum*	Downward leaf curl leaf distortion	IPB, UPLB	++
20. *L. esculentum*	Yellow mos; leaf curl	Lipa, Batangas	++
21. *L. esculentum*	Apparently healthy	Liliw, Laguna	-
22. *L. esculentum*	Crinckle yellow spots	IPB, UPLB	-
23. *L. esculentum*	Vein yellow mos; leaf curl	IPB, UPLB	+
24. *C. maxima*	Leaf curl; tiny leaf' vein enation	IPB, UPLB	+

Legend: mos – mosaic, st - stunting
L. esculentum – Lycopersicon esculentum, C. anuum – Capsicum anuum, C. maxima – Capsicum maxima

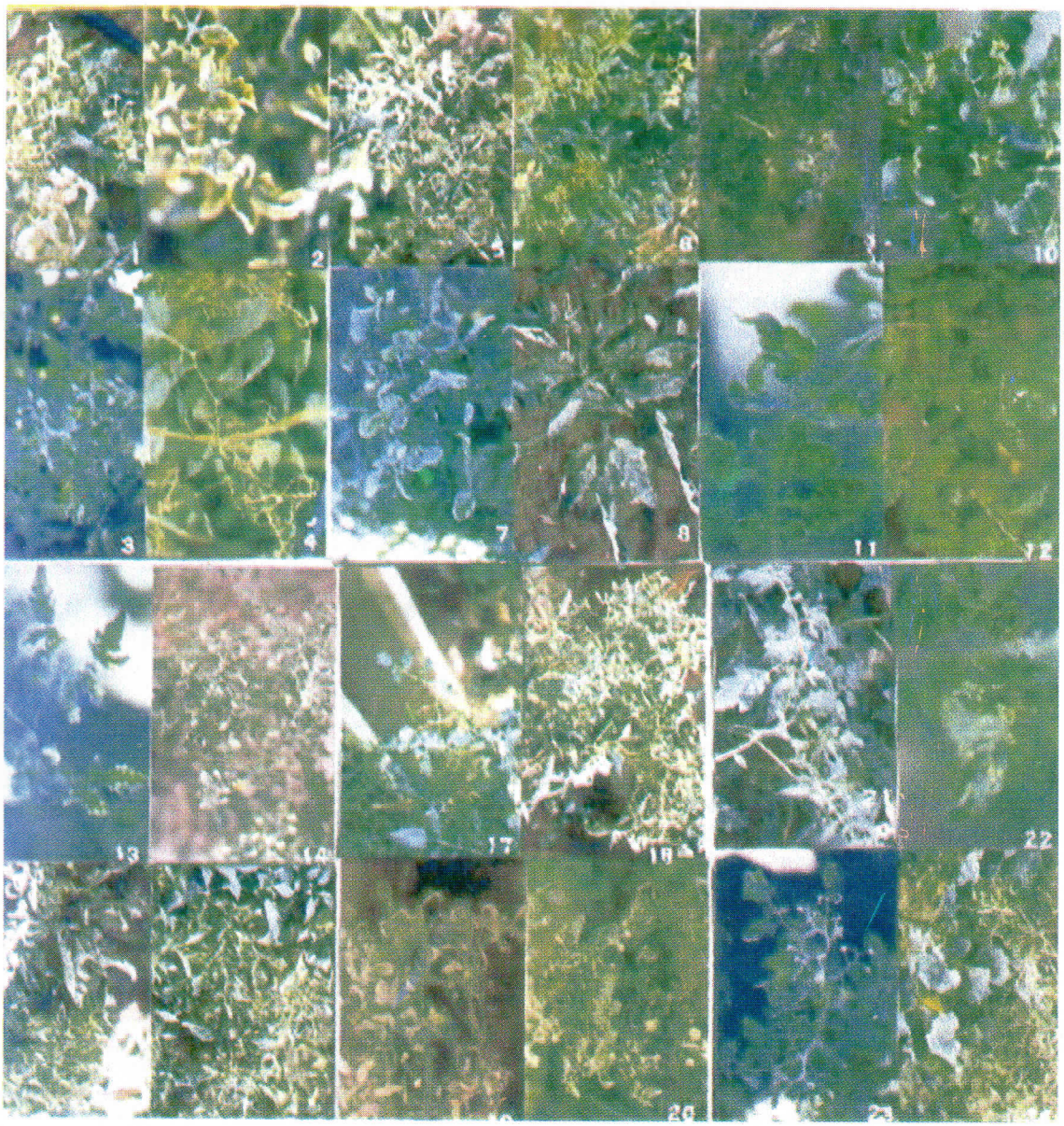

Figure 1. Symptoms of plant samples tested for DNA hybrids using TLCGV probe.

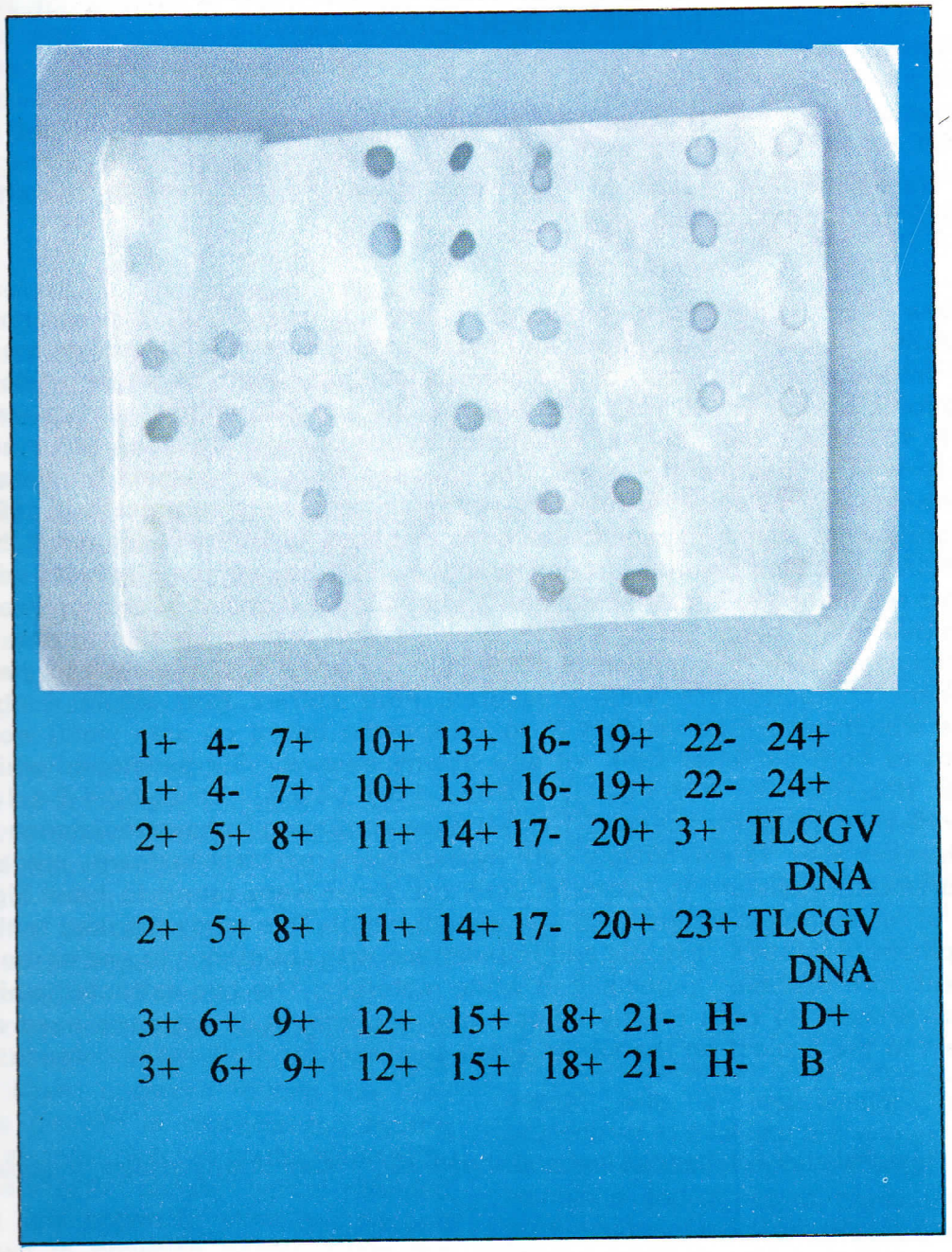

Figure 2. Results pf DNA hybridization between leaf curl infected samples and tomato leaf curl geminivirus probe as visualized on a nitrocellulose membrane.