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USE OF TISSUE PRINT-IMMUNOBLOTTING FOR DETECTION OF BARLEY YELLOW DWARF LUTEOVIRUS

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Salama[1], M.I.; I.A. Abdel-Hamid[1]; Safaa G. Kumari[2]; and A.S. Sadik[3]

ABSTRACT

Direct tissue blot immunoblotting assay (TBIA) on nitrocellulose membrane was successfully used for the detection of barley yellow dwarf luteovirus (BYDV) in artificially virus-infected stems of barley plants. In this study, the virus antigens on the blots were detected by means of goat antirabbit (GAR) labeled with alkaline phosphatase (AP), as a clear difference between the virus-infected sample (a purplish-blue color) and non-infected control was appeared. The use of GAR labeled with gold was also successfully used for detecting BYDV as the gold-labeled antibody was directly visible because of its pink color in virus-infected blots. Results also showed that the virus was localized in the xylem of virus-infected tissues, while no reactions were seen with tissues from healthy plants. In this technique, the free binding sites on the membrane were blocked by 2 hr incubation in phosphate buffered saline containing 2% bovine serum albumin. The binding of AP and gold-labeled-antibodies were detected on the washed blots by means of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and the silver Enhancer staining complex, respectively. These results paid an attention to that the viral antigens might be identified by TIBA, although they occur in low concentrations in virus-infected plants.

Key words: Barley, Barley yellow dwarf luteovirus (BYDV), Tissue blot immunoassay (TIBA), Gold immunoassay, Luteoviruses

INTRODUCTION

Barley yellow dwarf viruses (BYDV) are phloem-limited luteoviruses that infect members of *Poaceae* family such as barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.) and oat (*Avena sativas* L.) **(Hoffman and Kolb, 1998).** The BYDV originally was classified into 4 strains (PAV, MAV, RPV and RMV)

1- Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), 12619, Giza, Egypt.
2- Genetic Resources Unit, ICARDA, P.O. Box 5466, Aleppo, Syria.
3- Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, P.O. Box 68 Hadayek Shubra 11241, Cairo, Egypt.

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on the basis of vector specificity, host range, and virulence (**Rochow, 1969**). The virus interferes with physiological processes within the plant, which in turn the symptoms of chlorosis, stunting, and yield loss (**Jensen and D'Arcy, 1995**). Since high levels of resistance have not been found in wheat (**Burnett *et al* 1995**), the early and sensitive detection of BYDV can play an important role in limiting yield loss and reduced grain quality due to disease.

A low concentration of pathogenic-specific antigen in a test sample is a limiting factor in using enzyme-linked immunosorbent assay (ELISA) for disease diagnosis in which the causal agent occurs at low concentration in infected plants. Increasing test sample volumes does not proportionally increase the detection limit in ELISA. Also, the volume of a test sample is limited by the size of well in a microtiter plate. Furthermore, not all-viral antigens in test samples are absorbed in doubly antibody sandwich-ELISA (DAS-ELISA) during a sample incubation period (**Hsu *et al* 1992**).

In addition, the detection of very small quantities of the virus as an antigen (Ag) from the crude plant sap requires an optimized Ag capture system. By nitrocellulose membrane (NCM) filters instead of polystyrene, the binding capacity for antibody (Ab) is enhanced (**Bode *et al* 1984**). It was also reported that NCM filters could be dried after spotting of Ag with full retention of antigenicity after up to 20 weeks in a desiccated state (**Lizarraga and Fernandez-Northcote, 1989**).

Recently, **Lin *et al* (1990)** described a tissue-blotting technique for detection of virus and mycoplasma like-organisms in plants. The method uses direct blotting of a freshly cut tissue surface onto a NCM followed by incubation with path gen-specific primary antibodies and e zyme-labeled immunoglobulin-specific secondary antibodies. A sensitive and rapid direct tissue-blot immunoassay (TBIA) was used by **Hsu *et al* (1992); Katul *et al* (1993); Samson *et al* (1993) and Hsu *et al* (1996)** for identification of some plant viruses affecting orchids, faba bean, potato and tomato, respectively.

The immunogold labeling technique (IGLT) was reported by **van Lent and Verduin (1985)**, who described specific gold-labeling antibodies bound to plant viruses in mixed suspensions. In the following **van Lent and Verduin (1986 and 1987)** detected the viral protein and particles in thin sections of virus-infected plant tissue by immunogold-silver staining and light microscopy. Furthermore, this technique was successfully applied for either plant or animal viruses (**Patterson and Verduin, 1987**).

This work was designed to focus on the use of the TIBA technique as one of the recently described serological techniques to detect the phloem-limited BYDV that used to be found in a low concentration in virus-infected plant tissues.

MATERIAL AND METHODS

Plant materials

Barley plants (BYDV-infected and healthy, as a control), grown in the greenhouse at Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC) were used.

Antisera used

BYDV-specific rabbit polyclonal antibodies (1 mg/ml) and goat anti-rabbit (GAR) antibodies conjugated with colloidal gold, kindly provided by Prof. Dr. K. Makkouk. Virology Laboratory, ICARDA, Aleppo, Syria were used.

Direct tissue blotting immunoassay

In this experiment as reported by **Lin *et al* (1990),** the stem of BYDV-infected barley plants were held in one hand and cut with a sharp (new) razor blade in a steady motion with the other hand to obtain a single plane cut surface. In the following the freshly exposed surface was pressed firmly on the NCM which was either processed immediately or was kept at 4°C until more materials for further blots were available. The NCM was washed three times with phosphate buffered saline-Tween (PBS-T). The free binding sites on the NCM were blocked by a 2-hr incubation in PBS containing 2% bovine serum albumin (BSA) at room temperature (RT). The NCM was then treated with BYDV-specific antibodies at dilution 1/500 in PBS, incubation for 2 hr at RT and then washed as mentioned before. Two membranes were prepared.

GAR-AP detection

In this experiment, GAR conjugated to AP (dilution 1/1000 in conjugate buffer) was added to the membrane followed by incubation at RT in shaking condition for 2 hr. To 10 ml of substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl and 50 mM $MgCl_2$, **Lin *et al* 1990**) 200 µl of the substrate (NBT/BCIP) stock solution were added. Then this freshly diluted substrate solution was added to NCM and incubated (without shaking) for 5-10 min at RT. After color has appeared, the membrane was rinsed quickly with distilled water (d.H_2O) and air-dried.

GAR-G detection

The second antibodies (GAR) conjugated to colloidal gold (dilution 1/500 in conjugate buffer, **Hsu, 1984**) was added followed by incubation at RT for 1 hr. The membrane was washed three times with d.H_2O at 5 min interval. The silver stain Enhancer complex (A and B, 1:1, v:v, freshly mixed before use) was added, then fixed for 2-3 min in a 2.5% sodium thiosulfate and rinsed again.

Light microscopy

The two membranes were subjected to light microscopy (**van Lent and Verduin, 1987**) and photographed to differentiate between the virus-infected and non-infected blots.

RESULTS AND DISCUSSIONS

BYDV, a luteovirus that is obligately transmitted by aphids, is prevalent worldwide and has a wide host range in the *Poaceae* (**D'Arcy, 1995**). In regions of the world where cereal grains are grown, BYDV is the most significant viral pathogen (**Lister and Ranieri, 1995**).

Direct tissue blotting technique

Virus detection can be done on plant material directly blotted on the NCM. Accordingly, plant sections (stems, leaf,

petiole.....etc) can be printed on NCM followed by the appropriate antibodies. The second antibody could be either one of the following, a) GAR conjugated to alkaline phosphatase (GAR-AP), b) protein A conjugated to AP (PA-AP), c) rabbit anti-mouse IgG labeled with AP (RAM-AP), goat AM labeled with AP (GAM-AP) and GAR conjugated to colloidal gold (GAR-G) **(Hsu, 1984; Lin *et al* 1990 and Zagula *et al* 1990)** as shown in **Figure (1).**

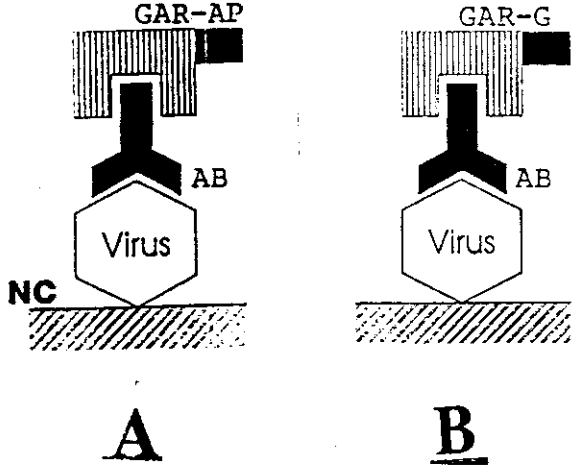

Figure 1. A diagram shows different procedures for virus detection *via* TBIA using GAR-AP (A) **(Lin *et al* 1990)** and GAR-G (B) **(Hsu, 1984)** used in this study.

TBIA technique is similar to dot blot but the application of samples on the nitrocellulose occurs simply by pressing the freshly cut tissue surface directly on NCM **(van Regenmortel and Dubs, 1993)**. **Hsu *et al*** (1992) detected Cymbidium mosaic potexvirus in crude sap of infected orchid leaves by 4 different techniques, i.e., immunosorbent electron microscopy (ISEM), dot blot immunoassay (DBIA), TIBA and ELISA. They found that TBIA or DBIA was about eight times that of ELISA. They also reported that orchid samples that gave negative or marginal positive results by ELISA were identified as positive for CyMV infection by TIBA.

In other words, TBIA or DBIA were found to be more sensitive than ELISA, as **Graddon** and **Randles (1986)** described a single antibody dot immunoassay for the rapid detection of Subterranean clover mottle sobemovirus. The method was found to be 12 times as sensitive as ELISA in terms of total antigen detectable. **Lange** and **Heide (1986)** reported that dot blot immunoassays might be particularly useful for routine detection of virus in seeds or seed samples, especially for laboratories where an inexpensive and simple test is needed. In **1988, Heide and Lange** have developed dot blot immunobinding using plain paper as a diagnostic method for five potato

viruses using direct extraction from green leaves.

GAR-AP detection

In this study, BYDV-specific antibodies were separately labeled with GAR-AP and GAR-G and used for detection of BYDV antigens, which bound on NCM. The experimental results showed that BYDV antigens could be readily detected in stem of virus-infected barley by tissue blotting on NCM. As a clear difference between the virus-infected sample (a purplish-blue color) and non-infected control was appeared in the case of using the GAR-AP (**Figure 2**). This due to that the GAR-AP was bound with the virus-specific antibodies that reacted with the virus antigens on the NCM. This complex reaction was detected on the washed blots by means of NBT/BCIP substrate as recommended by **Lin *et al* (1990)**.

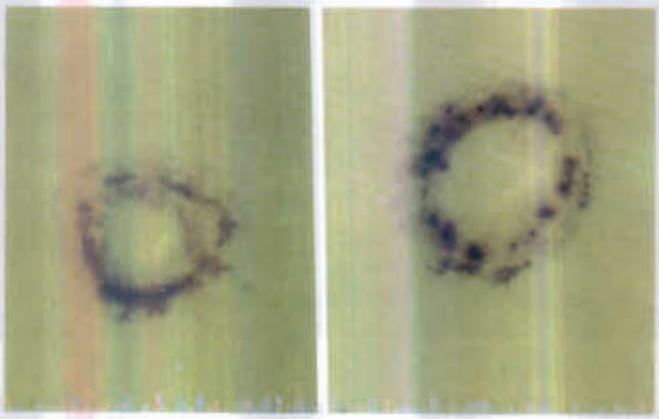

Figure 2. Tissue print-immunoblots of virus-infected (Right) and non-infected (Left) stem of barley plants detected for the presence of virus antigens by means of GAR-AP.

Kaufmann *et al* (1992) reported that the distribution of the coat protein (CP) antigens of beet necrotic yellow vein (BNYVV) and beet soil-borne (BSBV) viruses in tap roots of naturally infected sugarbeets and of BNYVV CP antigen in leaves and petioles of mechanically inoculated sugarbeet seedlings was detected by means of tissue print-immunoblotting.

GAR-G detection

Gold particles are highly electron dense and thus show the position on an EM grid of any particle to which they are attached. Protein A forms a reasonably stable complex with gold particles. This complex can then be used to locate any IgG molecules bound to virus CP on the EM grid (**van Lent and Verduin, 1986**).

Results in **Figure (3)** showed that immunogold labeling was particularly valuable in locating viral antigens in blots of infected cells As the gold-labeled antibody was directly visible because of its pink color in virus-infected blots The technique has been adopted for localization of viral antigens by light microscopy. **Hsu (1984)** used gold-labeled goat antirabbit IgG to detect rabbit antibodies bound to tobacco mosaic tobamovirus (TMV) on nitrocellulose paper By means of light microscopy using virus-specific immunogold-silver staining. **Giunchedi and Poggi Pollini (1988)** detected BNYVV only in the xylem vessels of sugarbeet tap roots. However, using ELISA technique the virus was often not detectable as its concentration was very low **(Sayama *et al* 1991)**

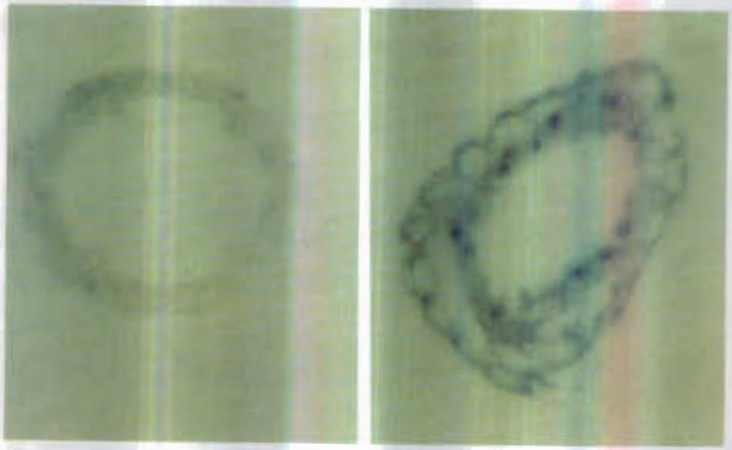

Figure 3 Tissue print-immunoblots of virus-infected (Right) and non-infected (Left) stem of barley plants detected for the presence of virus antigens by means of GAR-AP.

Finally, this study also demonstrated that a direct tissue-blot technique for virus detection not only retains specificity and sensitivity but also provides simplicity and accurancy for detection of BYDV in barley.

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مجلة حوليات العلوم الزراعية ، كلية الزراعة ، جامعة عين شمس ، القاهرة ، م٤٨ ، ع(١) ،٤٧-٥٥ ، ٢٠٠٣

استخدام بصمة النسيج-النقل المناعي للكشف عن فيروس التقزم الأصفر في الشعير

[٣]

محمد إبراهيم سلامة[١]- إسماعيل علي عبد الحميد[١]- صفاء غسان قمري[٢]- عاطف شكري صادق[٣]

١- معهد بحوث الهندسة الوراثية الزراعية - مركز البحوث الزراعية - ص.ب. ١٢٦١٩ - جيزة - مصر

٢- وحدة المصادر الوراثية -أيكا ردا- ص.ب. ٥٤٦٦ - حلب - سوريا

٣- قسم الميكروبيولوجيا الزراعية - كلية الزراعة - جامعة عين شمس - ص.ب. ٦٨ حدائق شبرا ١١٢٤١ - القاهرة - مصر

في هذه الدراسة تم استخدام تكنيك استخدام بصمة النسيج-النقل المناعي بنجاح للكشف عن فيروس التقزم الأصفر في الشعير وذلك في سيقان نباتات الشعير المصابة بفيروس الـ BYDV المنماة تحت ظروف الصوب. وقد تم الكشف عن انتيجينات الفيروس المرتبطة علي غشاء النيتروسليلوز وذلك باستخدام الأجسام المضادة المنتجة في الماعز والمتخصصة لتلك المنتجة في الأرانب (GAR) والمعلمة بأنزيم الأكالين فوسفاتيز (AP) حيث ظهر فرق واضح بين العينات النباتية المصابة بالفيروس (والتي أظهرت لون أزرق-بنفسجي) وبين العينات الغير مصابة. وبالإضافة إلى ذلك فقد استخدم أيضا الـ GAR ولكنها معلمة بالذهب للكشف عن الفيروس حيث ظهر لون قرمزي في العينات النباتية المصابة بالفيروس حيث يتواجد الفيروس والذي وجد متمركزا في أنسجة الخشب لتلك العينات بينما لم يظهر ذلك في العينات السليمة. ومن مميزات الطريقة أن الأماكن الخالية في الغشاء يتم إغلاقها بواسطة البيومين سيرم البقر بتركيز ٢% لمدة ساعتين. وقد تم الكشف عن كل من الـ GAR المعلم بالـ AP أو الذهب والمرتبط بما في الغشاء بواسطة الـ NBT/BCIP ومخلوط الصبغ الفضي المحفز علي التوالي. وفي النهاية فان النتائج تثير الأنتباة إلى إمكانية تعريف فيروسات النبات بواسطة هذا التكنيك علي الرغم من تواجد الفيروسات بتركيزات منخفضة في النباتات.

تحكيم: أ.د محمد أحمد أبو النصر أ.د سيد سلامه