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Direct Stem Blot Immunoassay (DSBIA): A Rapid, Reliable and Economical Detection Technique Suitable for Testing Large Number of Barley Materials for Field Monitoring and Resistance Screening to *Barley mild mosaic virus and Barley yellow mosaic virus*

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Testing a large number of samples from field monitoring and routine indexing is cumbersome and the available virus detection tools were labor intensive and expensive. To circumvent these problems we established tissue blot immunoassay (TBIA) method an alternative detection tool to detect *Barley mild mosaic virus* (BaMMV) and *Barley yellow mosaic virus* (BaYMV) infection in the field and greenhouse inoculated plants for monitoring and routine indexing applications, respectively. Initially, leaf and stem were tested to determine suitable plant tissue for direct blotting on nitrocellulose membrane. The dilutions of antibodies were optimized for more efficient and economical purposes. Results showed that stem tissue was more suitable for direct blotting for it had no background that interferes in the reaction. Therefore, this technique was referred as direct stem blot immunoassay or DSBIA, in this study. Re-used diluted (1:1000) antiserum and conjugate up to 3 times with the addition of half strength amount of concentrated antibodies was more effective in detecting the virus. The virus blotted on the nitrocellulose membrane from stem tissues kept at room temperature for 3 days were still detectable. The efficiency of DSBIA and RT-PCR in detecting BaMMV and BaYMV were relatively comparable. Results further proved that DSBIA is a rapid, reliable and economical detection method suitable for monitoring BaMMV and BaYMV infection in the field and practical method in indexing large scale of barley materials for virus resistance screening.

Keywords **:** *Bymovirus*, *Hordeum vulgare*, nitrocellulose membrane, serology, tissue blot immunoassay

Barley yellow mosaic virus (BYMV) and *Barley mild mosaic virus* (BaMMV) are the two of the most economically important soilborne diseases of winter barley, *Hordeum vulgare* in East Asia and North-western Europe (Huth and Adams, 1990). The causative viruses belong to *Bymovirus* genus and transmitted by root infecting fungus, *Polymyxa graminis* Led. BaMMV and BaYMV caused a serious threat in barley cultivation for they co-infect in nature and symptoms are difficult to distinguish from each other (Adams, 2000; Kashiwazaki et al., 1998). Moreover, during field monitoring, disease incidence assessment become difficult especially at the later growth stages or when temperature rises since at these conditions, symptoms were normally masked resulting to uncertain visual diagnosis (Park et al., 2003). For these reasons, detection techniques such as ELISA or RT-PCR: a protein and nucleic acid based detection tools respectively became very useful. However, these methods were labor intensive, where they require grinding of sample to extract the sap for ELISA or RNA for RT-PCR. In addition, these methods are expensive for they require sophisticated equipment such as ELISA reader and PCR machine, respectively.

Tissue blot immunoassay (TBIA) method is another protein based serological detection technique used to detect wide ranges of viruses and proven to be simple, reliable, economical and sensitive as ELISA (D'Onghia et al., 2001; Hu et al., 1997; Whitefield et al., 2003). Reports showed that TBIA had been employed for BaYMV detection (Kuntze et al., 2000). In their report they have referred it as tissue print immuno-blotting (TPIB) method and they make used of the leaf to detect the presence of the virus. In this study, however specific plant tissue and optimum antibodies (IgG and conjugate) dilutions to efficiently detect the virus were determined. And since, commercial antiserum is very costly, the possibility of re-using the diluted antiserum and conjugate were also tested. Also, the sensitivity of TBIA was compared with RT-PCR. And finally, to apply the established detection technique for monitoring BaMMV and BaYMV infection in the field and indexing barley genotypes for virus resistance screening.

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Materials and Methods

Tissue Assay. Leaf and stem tissues were compared in determining the efficient plant part tissue in detecting the BaMMV and BaYMV infected barley plants. Leaf was excised and was rolled forming a core and subsequently cut with a disinfected scissors and was carefully pressed the cut surface unto Protran nitrocellulose (NC) membrane (Whatman, Schleider and Schuell, Germany). Similarly, excised stem was directly blotted unto NC membrane. It should be noted that hand gloves should be worn in handling the NC membrane to protect from contamination. Entire experiments were repeated twice,

Antibody optimization. In this experiment, the commercial BaMMV and BaYMV-IgG manufactured by Loewe Biochemica (Germany) were used to detect BaMMV and BaYMV infected barley plants in Korea. The efficient dilution prescribed by the kit for ELISA is 1:200. In this study, 1:1000 dilution was used in the entire experiments. To maximize the used of first antibody (IgG), re-used diluted IgG up to 3 times with or without addition of half strength amount of concentrated IgG were tested. Similarly, reused second antibody, alkaline phosphatase-conjugated goat-anti rabbit (KPL, Kirkeganard & Perry laboratories, USA) diluted 1:1000 was tested with or without addition of half strength of concentrated conjugate. Reused diluted antibodies (Abs) were kept in 4°C until use.

TBIA method. TBIA procedure was done according to the reported methods (Hsu et al., 1991; Lin et al., 1990; Makkouk et al., 1994) with some modification (Fig. 1). TBIA consisted of 6 steps and in each step washing of the membrane for 3 times at 5 min each with phosphate buffer saline with Tween 20 (PBS-T) containing 150 mM NaCl, 15 mM KH_2PO_4, 20 mM Na_2HPO_4, 3 mM Na_2N_3 and 0.05% Tween 20, pH 7.4. Washing was done in order to get rid of excess antigen or antibody that failed to adhere in the membrane and also to remove other artifacts that may cause nonspecific reactions. First step was blotting the cut stem tissue unto NC membrane. This step allows the adsorption of antigen unto NC membrane. The second step was blocking the adsorbed antigen in the membrane by soaking the NC membrane with blocking solution containing 1 μg/ml polyvinyl alcohol (PVA, Sigma, USA) in PBS-T for 1 min. Next step was the binding of first antibody diluted 1000 times in conjugate buffer in PBS-T buffer containing 2% Polyvinyl pyrrolidone (PVP-40, Sigma, USA) and 0.2% Egg albumin (Sigma, USA) incubated for 2 hours with constant shaking using adjustable tilt shaker (National Labnet Co., USA). Then, followed by the second antibody diluted 1:1000 in conjugate buffer incubated for 1hr in

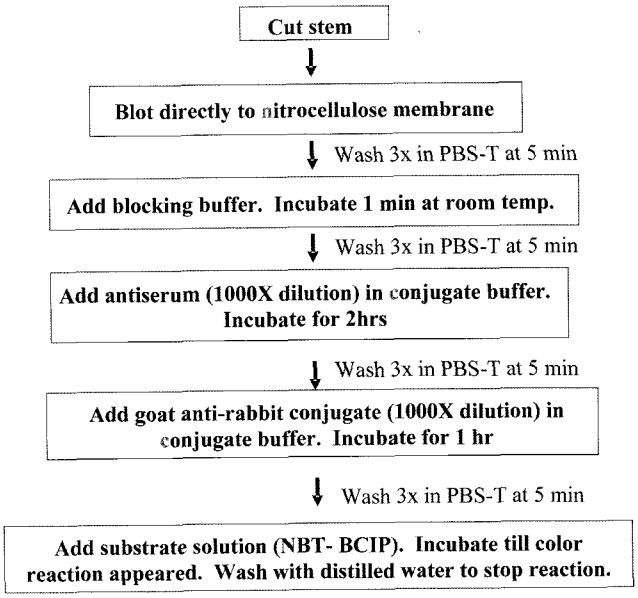

Fig. 1. Step by step procedures of direct stem blot immunoassay method (DSBIA).

constant shaking. Final, step was the detection using the ready mix NBT/BCIP phosphatase substrate (KPL, USA) solution, incubated until coloration appeared. NBT/BCIP mixture is light-sensitive so while waiting for color reaction, container should be covered with aluminum foil. Violet to purple color indicates positive reaction and light green to colorless indicates negative reaction. Initially, coloration appeared in 5 min and reaction was finally stopped after 15 min by washing the membrane with distilled water. Then membrane was laid dried on paper towel. The entire procedures were done at room temperature.

TBIA for field monitoring. A total of four sites of barley fields were monitored at Jeonnam Province, South Korea, for BaMMV and BaYMV incidence at varying growth stages. Three plant stems per field were sampled and collected samples were blotted during the trip and in the laboratory, 3 days after returning from the trip. BaMMV and BaYMV-infection were assessed based on visual observation and presence of viruses was determined by TBIA as described above. BaMMV and BaYMV inoculated susceptible check Baegdong plants were also tested as positive controls.

Detection comparisons. TBIA and RT-PCR were compared in detecting field infected and greenhouse inoculated barley plants. RT-PCR primers were based on sequence of the coat protein (CP) region of BaMMV and BaYMV designed by Lee et al. (1996) and Lee (1998) respectively to further confirm CP amplification. Greenhouse inocu-

lation with BaYMV was done following the modified method previously described by Jonson et al. (2007). Inoculated plants were assessed visually and individual leaf and stems were tested by RT-PCR and TBIA after 2 months from inoculation. For RT-PCR, RNA was extracted in the leaf by following kit's protocol (Qiagen, USA) and for TBIA, stem was used for blotting.

TBIA for routine indexing. A total of 54 malting barley lines were inoculated with BaMMV using the improved mechanical inoculation as previously described by Jonson et al. (2006). Naehanssalbori cultivar was used as susceptible check. Ten seedlings were inoculated per line and 1 month after inoculation, individual stem was cut 2-3 times and each cut was directly blotted unto NC membrane.

A petri dish was utilized with a diameter of 10.5 cm and a height of 1 cm. An NC membrane format was designed with a size of 7.5×6.5 cm NC membrane and lines drawn with pencil making a total of 42 lots (1×1 cm). The size of the membrane perfectly fit inside the petri dish and a 10 ml volume of solution was just enough to cover the membrane (Fig. 2).

Results

TBIA method and optimization. In this study we have established the method of TBIA as shown in Fig. 1. Also, we have demonstrated the use of stem as an efficient plant tissue to detect the presence of BaMMV and BaYMV and in this study we referred it as direct stem immunoassay method or DSBIA. The leaf tissue showed a strong background or the green pigment was not eliminated during washing, causing interference in the reaction (Fig. 3). The re-used diluted Abs could still detect the presence of the virus however signals were not as strong as to the original

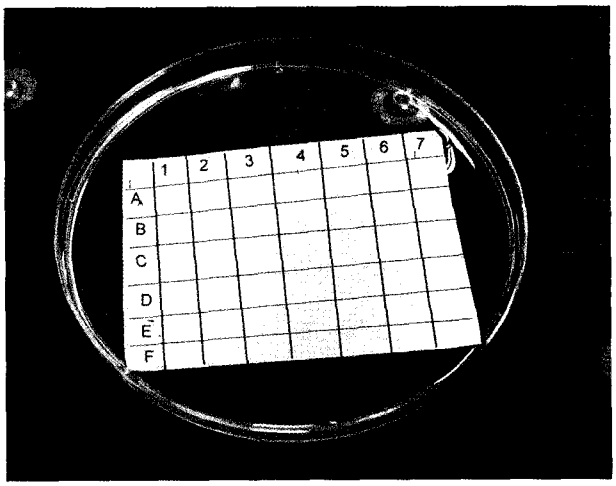

Fig. 2. Nitrocellulose membrane format showing a total of 42 lots and a petri dish as container.

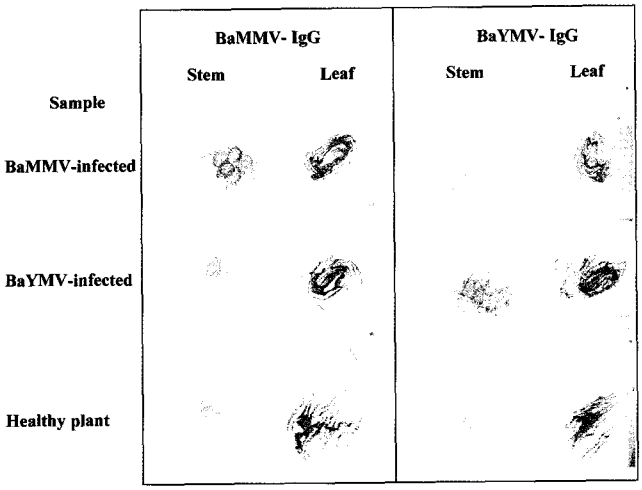

Fig. 3. Detection of BaMMV and BaYMV infection using leaf and stem tissues by TBIA.

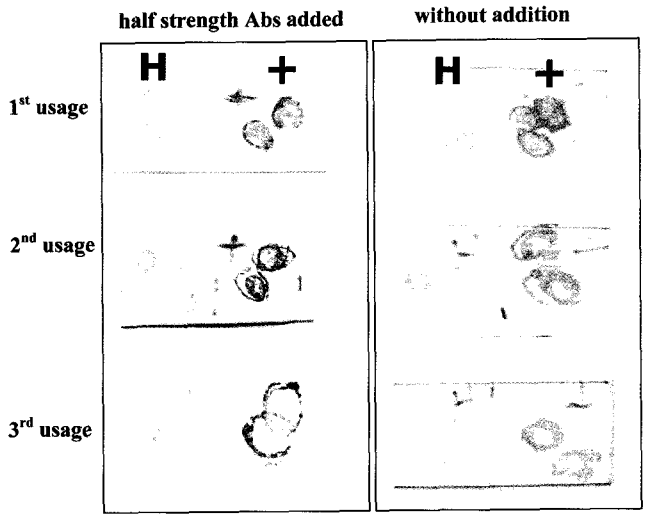

Fig. 4. BaYMV detection signals of re-used antibodies (Abs) between with and without addition of half strength of Abs in 2nd and 3rd usage. H, Healthy control; +, Positive control.

solution. However, when half strength of concentrated Abs added each time it was being re-used; signals from infected samples were as intense as the first usage. While no signal detected from healthy control in both treatments (Fig. 4).

Detection comparisons. The field collected samples also tested for the presence of viruses by RT-PCR showed similar results with TBIA (Table 1). Similarly, detection of inoculated plants by TBIA and RT-PCR were relatively comparable. For those plants showed clear symptoms (sample nos. 1, 4, 6, 7, 8, and 10) also showed strong signal by RT-PCR as well as intense color reaction in DSBIA. However, from 4 samples (nos. 2, 3, 5, and 9) that did not showed symptoms, only single sample (no. 3) showed positive detection , though very weak signal both in DSBIA

Table 1. BaMMV and BaYMV infection detected by DSBIA from samples collected in Jeonnam province, South Korea

Site	Growth Stage	Field No.	Symptoms (visual assessment)	Virus detected by DSBIA[a]	
				BaYMV	BaMMV
Gangjin1	Late tillering-heading	1	No clear symptoms	0/3[b]	0/3
	Late tillering-heading	2	No clear symptoms	2/3	0/3
	Late tillering-heading	3	No clear symptoms	3/3	0/3
	Late tillering-heading	4	No clear symptoms	3/3	0/3
	Late tillering-heading	5	No clear symptoms	0/3	0/3
Gangjin2	Late tillering-heading	6	No clear symptoms	3/3	0/3
Naju1	Late tillering-heading	7	No clear symptoms	2/3	1/3
Naju2	Early tillering	8	Severe yellowing (90%)	6/6	6/6
Greenhouse	Tillering		BaMMV-inoculated	0/6	6/6
Greenhouse	Tillering		BaYMV-inoculated	2/2	0/2

[a]Confirmed by RT-PCR.
[b]Number in fraction indicated as total number sample infected over total number sample tested.

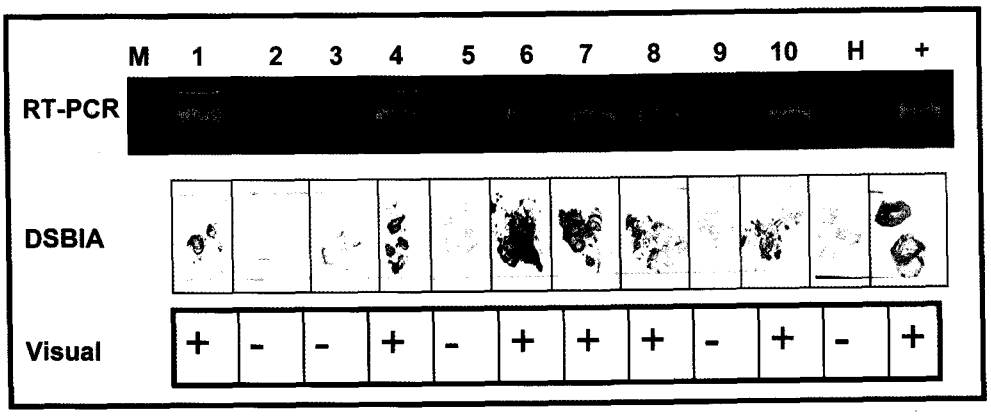

Fig. 5. Comparisons of detection on BaYMV inoculated barley plants by RT-PCR, DSBIA and visual observations. RT-PCR: Lanes 1-10, inoculated plant samples; M, Molecular DNA marker; H, healthy or uninoculated plant; +, BaYMV infected plant. DSBIA: purple colored prints indicate infection; green to colorless prints indicate no infection. Visual: +, with symptoms; –, without symptoms.

and RT-PCR (Fig. 5).

TBIA applications. From the 4 sites monitored, a total of 8 fields were sampled. Based on visual assessment, 7 fields had no clear symptoms and only one field that showed about 90% leaf yellowing symptoms. However, based on DSBIA test, out of 7 fields with unclear symptoms, only two fields were found negative however the rest were infected with one or two viruses. And the field with 90% leaf yellowing was positive to both BaMMV and BaYMV. All positive control plants infected with BaMMV alone and BaYMV alone showed clear positive detection (Table 1). Results further indicate that DSBIA method was efficient in detecting BaMMV and BaYMV in the field. Detection of virus from samples that were blotted while on trip and processed after 3 days was comparable to freshly blotted-membrane (data not shown). This indicates that virus adsorbed in the membrane was stable which is very suitable for remote testing. On the other hand, a total of 54 malting barley lines with a total of 379 plants mechanically inoculated with BaMMV showed clear detection based on DSBIA (membrane not shown). A total of 14 lines did not showed any infection and 3 malting barley lines were identified with no visible symptoms but with high infection rate based on DSBIA (Table 2).

Discussion

TBIA studies showed that tissue-specificity varied among crops for reliable detection. Examples of some crops that identified specific tissues in detecting viruses by TBIA are as follows: leaf mid-rib in sugarcane for *Sugarcane yellow leaf virus* (Comstock and Miller, 2004); tuber in *Ranunculus asiaticus* for *Tomato spotted wilt virus* (TSWV) (Whitefield et al., 2003); ovary in citrus for *Citrus psorosis virus* (D'Onghia et al., 2001); and leaf in barley for BaYMV

Table 2. Malting barley lines mechanically inoculated with BaMMV grown at 10/12°C and tested by DSBIA method, 1month after inoculation.

Saenge Line No.	% rate Inf	Saenge Line No.	% rate Inf	Saenge Line No.	% rate Inf	Saepul Line No.	% rate Inf
3	44.0	18	0.0	34	0.0	3	70.0
4	75.0	19	0.0	39	20.0	4	66.0
5	33.0	20	22.0	41	77.0	5	100.0
6	20.0	21	0.0	43	0.0	6*	55.0
7	75.0	22	40.0	45	0.0	7	90.0
8	55.0	24	16.0	46	37.0	8	70.0
9	22.0	24	12.5	48	14.0	9	40.0
10	87.0	26	30.0	50	0.0	12	75.0
11	25.0	27	40.0	51*	100.0	13	0.0
12	16.0	28	20.0	52	49.0	14	0.0
13	0.0	29	16.0	53*	90.0	12	75.0
14	66.7	30	66.0	Naehan[a]	85.0		
15	25.0	31	11.0				
16	0.0	32	0.0				
17	0.0	33	28.0				

*Barley lines with no visible symptoms but with infection by DSBIA.
[a]Susceptible check cultivar.

(Kuntze et al., 2000).

In this study we have demonstrated that the stem tissue was more efficient than leaf tissue in detecting BaMMV and BaYMV. Therefore, we referred the modified TBIA method as direct stem blot immunoassay or DSBIA in this study. Although, in the report of Kuntze et al. (2000), used leaf tissue for TPIB. In our trials, virus from infected leaf tissue could be also detected but color signal was not as clear with those of stem tissue (Fig. 3). Also, the handling of leaf tissue was a bit cumbersome compared to stem tissue. Since, during blotting unto NC membrane, leaf requires rolling before cutting whereas stem could be cut directly and blotted with ease thus minimizing the assay time.

For those laboratories that are not producing their own antiserum is not a problem anymore, since antisera are now readily available commercially. However, the available antisera are costly especially when testing large number of samples. In this experiment, we have shown that reusing the diluted Abs solution was possible, although signals were weak. However, with addition of half strength of the concentrated Abs the signals were improved comparable to original solution (Fig. 4). In this way it became more economical without affecting the efficiency of the test.

We have applied DSBIA for field monitoring to determine BaMMV and BaYMV incidence in the field and our results showed that our visual diagnosis did not conformed to our DSBIA test (Table 1). Those barley plant samples with unclear symptoms might have been infected but had recovered at the later growth stages (late tillering to heading). Thus, DSBIA test could be used to confirm infection. In addition, due to the ability of NC membrane to retain the virus intact for several days, DSBIA then is also applicable for remote testing. Although, we only tested 3 days in this experiment but most likely virus stability would be more than 3 days since in TSWV tuber samples blotted on NC membrane showed successful detection even after 84 days (Whitefield et al., 2003). In this case, we could recommend this technique to farmers in monitoring their fields for BaMMV and BaYMV infection by simply mailing the blotted membrane to designated laboratories for further testing.

For routine indexing of barley genotypes, DSBIA showed suitability and very economical to use. Since, the commercial 100 µl IgG supposed to test about 96 samples only by ELISA (Kit's instruction). By DSBIA however, using the recommended NC format (42 lots/membrane) it only requires 10 µl IgG/10 ml buffer/membrane, therefore it could test 4 times more of samples or even 8 times more when half strength of concentrated Abs were added in the re-used solutions. This preliminary test using DSBIA for indexing malting barley (Saenge and Saepul) lines showed a promising result (Table 2). Based on DSBIA, we have identified malting barley lines (Saenge line nos. 51 & 53 and Saepul line no. 6) that showed no visible symptoms but were highly infected with BaMMV an indication of tolerant lines. Further tests, however are needed to further conclude its response to BaMMV as well as to BaYMV. In terms of efficiency, we can not say that DSBIA is sensitive as RT-PCR. Although in this study, like RT-PCR, DSBIA could

still detect the virus even at low concentration on plant with no symptoms (Fig. 5). More samples should be tested and the same tissue should be used for direct comparison. Lastly, DSBIA is more advantageous to use when handling large numbers of samples since it get rid of sample grinding for extracting plant sap in ELISA or RNA in RT-PCR. The choice between these methods however depends on the purpose of the study. Certainly, for a study that requires quantification of virus concentration, ELISA test or RT-PCR are recommended. But for only detecting the presence and absence of the target virus, DSBIA is suitable enough for its purpose. However, in case of the presence of strains and for its specific detection, production of antiserum specifically against those strains is recommended.

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