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1 **Microperforated leaf blotting onto polyvinylidene difluoride and nylon**  
2 **membranes to analyze spatial distribution of endogenous and viral**  
3 **gene expression in plant leaves**

4

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11

1 **Abstract**

2 Leaf blotting to detect proteins and investigate their spatial distribution in leaves has so  
3 far mainly been used to detect viral coat proteins that accumulate abundantly in infected  
4 leaves, but rarely to detect endogenous plant proteins. We improved the method for  
5 detecting endogenous proteins. We found that microperforating leaves with bundled  
6 pins before blotting, then pressing leaves with a rolling pin onto polyvinylidene  
7 difluoride (PVDF) membranes enabled even blotting of sap. This microperforated leaf  
8 blotting (mPLB) was also suitable for use with nylon membranes to detect leaf RNA.  
9 The mPLB revealed that accumulation of two endogenous proteins, calmodulin-like  
10 rgs-CaM and actin, was respectively positively and negatively associated with that of  
11 viral coat protein in tobacco leaves infected with cucumber mosaic virus (CMV). When  
12 a tobacco plant primed with benzothiadiazole was inoculated with CMV, mPLB showed  
13 that the infection was restricted to some areas of the leaf, and that in these areas the  
14 mRNA encoding tobacco pathogenesis-related protein 1, an indicator of salicylic  
15 acid-mediated immune responses, was induced. These results demonstrate the  
16 effectiveness of mPLB for investigating the spatial distribution of endogenous and viral  
17 gene expression in leaves.

18

19 Key words: leaf protein distribution; leaf RNA distribution; leaf microperforation.

20

## 1 **Introduction**

2

3 Leaf blotting, also called press blotting, tissue imprinting or direct tissue  
4 immunoblotting, is a simple protein detection method (Hamada et al. 2003; Jung and  
5 Hahne 1992; Polston et al. 1991; Srinivasan and Tolin 1992; Takahashi et al. 2002).  
6 After a plant leaf has been pressed onto a specific type of membrane, such as filter  
7 paper or nitrocellulose, its proteins adhere to the membrane, and the proteins of interest  
8 can be detected on the membrane using specific antibodies. Leaf blotting can also be  
9 used to detect RNAs expressed in leaves (Takeshita et al. 2001).

10 Leaf blotting is not only simple (i.e., it does not require protein extraction steps), but  
11 also can be used to analyze the spatial distribution of specific proteins in leaves.

12 However, its detection sensitivity is relatively low, which likely explains why it has  
13 mainly been restricted to detecting viral coat proteins, which are strongly expressed in  
14 virus-infected plant cells (Hamada et al. 2003; Polston et al. 1991; Takahashi et al.  
15 2002). There are no examples of the detection of endogenous plant proteins by leaf  
16 blotting except for a single study where this method was used to detect endogenous  
17 Rubisco protein and pathogenesis-related protein 1 (PR1; Jung and Hahne 1992).

18 Several techniques for blotting leaf proteins onto specific types of membranes have  
19 been reported. Srinivasan and Tolin (1992) used direct tissue immunoblotting, whereby  
20 infected leaves or other plant parts were gently pressed onto nylon, cellulose or other  
21 types of membranes. A Carver Laboratory Press (10,000 psi for 2 min) was used to  
22 press leaves onto nitrocellulose membranes (Polston et al. 1991). Takahashi et al.  
23 (2002) sandwiched leaves between filter paper and hit them with a wooden hammer to  
24 blot the proteins onto the paper. In another study, the efficiency of the blotting was  
25 improved by removing the lower epidermis of the leaves using carborundum before

1 pressing the leaves onto a nitrocellulose membrane between two blocks of plexiglass in  
2 a hydraulic press for 5 min at 70 kg cm<sup>-2</sup> (Jung and Hahne 1992).

3 Most previous research has used nitrocellulose membranes or filter paper for leaf  
4 blotting. Polyvinylidene difluoride (PVDF) membranes have rarely been employed.  
5 PVDF membranes have a high hydrophobicity, which may be considered to be  
6 inappropriate for physically attaching the leaf sap to the membrane. This type of  
7 membrane does, however, have several advantages, such as high mechanical strength,  
8 high protein binding capacity via hydrophobic interactions, solvent resistance, and  
9 compatibility with western blotting to detect proteins (Pluskal et al. 1986). In this study,  
10 we investigated methods for blotting leaf sap onto PVDF membranes to facilitate the  
11 detection and mapping of endogenous plant proteins.

12

### 13 **Materials and Methods**

14

15 Preparation of plants and inoculation of viruses and antibodies

16

17 *Nicotiana tabacum* cv. BY, transgenic tobacco plants expressing HC-Pro of clover  
18 yellow vein virus (Nakahara et al. 2012) and adzuki bean (*Vigna angularis*) plants were  
19 grown in an air-conditioned room at 24 °C. Wild-type tobacco plants at 6 weeks after  
20 germination were mechanically inoculated with cucumber mosaic virus (CMV)-yellow  
21 strain, maintained in our laboratory, in 0.1 M phosphate buffer containing 1% v/v  
22 2-mercaptoethanol. A tobacco plant was primed immediately before inoculation with  
23 CMV by treating the seventh leaf from the bottom of each plant with  
24 acibenzolar-*S*-methyl (benzothiadiazole, BTH), as described by Atsumi et al. (2009).  
25 Tobacco and adzuki bean leaves were analyzed 6 and 13 days post-inoculation,

1 respectively.

2 Anti-HC-Pro mouse monoclonal antibody (Yambao et al. 2003) and anti-rgs-CaM  
3 rabbit polyclonal antibody (Nakahara et al. 2012) were prepared in advance. Anti-CMV  
4 CP rabbit polyclonal antibody, which is available from the Japan Plant Protection  
5 Association (Tokyo, Japan), and anti-actin monoclonal antibody (Sigma-Aldrich, St.  
6 Louis, MO, USA) were used for protein detection.

7

8 Leaf blotting

9

10 We first used a conventional leaf blotting method, largely following Takahashi et al.  
11 (2002). After sample leaves were sandwiched between three pieces of filter paper  
12 (Whatman 3MM; GE Healthcare, Chicago, IL, USA) on each side, and the entire  
13 surface was hit with a hammer, the two inner pieces of filter paper, adjacent to the leaf,  
14 were dried on cellophane for 30 min, then soaked and gently shaken in a PBS-T buffer  
15 (137 mM NaCl, 8.10 mM Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 2.68 mM KCl, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 2% v/v  
16 Triton-X) to remove excess leaf materials for 15 min, instead of Tris-based buffer.

17 For the improved method, termed microperforated leaf blotting (mPLB), that we  
18 developed, sample leaves on cellophane were first perforating thoroughly on both sides  
19 with a rubber-banded bundle of 400 insect pins without heads (0.6 × 40 mm; Shiga,  
20 Tokyo, Japan). PVDF membranes were hydrophilized by soaking them in methanol for  
21 a few seconds and then replacing the methanol with distilled water. The perforated  
22 leaves were then sandwiched between two hydrophilized PVDF membranes, which  
23 were overlaid with two pieces of moistened filter paper on each side. The sandwich was  
24 then covered with cellophane and pressed uniformly using a rolling pin. The PVDF  
25 membranes were then washed for 15 min in a plastic container of PBS-T buffer to

1 remove excess leaf materials.

2 We here note that we did not change or wash bundled pins for every perforation but  
3 obtained contrasting signals on the blot of leaves, in which a target protein appeared to  
4 unevenly accumulate. Thus, we empirically conclude that changing or washing bundled  
5 pins for every perforation is not required to prevent nonspecific signals.

6 We used the same blotting procedures on two other types of pretreated leaves. One  
7 set of leaves was pre-treated with a freeze–thaw procedure in liquid nitrogen, basically  
8 according to Takeshita et al. (2001), without peeling off of the lower epidermis. The  
9 epidermis of another set of leaves was abraded with carborundum (Jung and Hahne  
10 1992).

11 For RNA detection, microperforated leaves were blotted onto nylon membranes  
12 (Hybond-N; GE Healthcare) that had been soaked in 20× SSC buffer (3 M NaCl, 0.3 M  
13 sodium citrate) for 15 min. The blotted RNAs were fixed to the membranes using UV  
14 cross-linker and dried completely.

15

16 Immunological detection

17

18 The pieces of filter paper and the PVDF membranes were shaken in a blocking buffer  
19 (PBS-T containing 3% skim milk) for 30 min and then allowed to react with the primary  
20 antibodies (2000-fold dilution of antibody solution in blocking buffer) for 1 h at 37 °C.

21 The filter paper and membranes were washed three times in blocking buffer, then  
22 reacted with a secondary antibody (anti-rabbit IgG [Thermo Fisher Scientific, Waltham,  
23 MA, USA] or anti-mouse IgG [Bio-Rad, Hercules, CA, USA]), conjugated with  
24 alkaline phosphatase solution (5000-fold dilution in blocking buffer) for 1 h at 37 °C.

25 They were again washed three times in blocking buffer. After equilibration in AP buffer

1 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>), they were packed in sealed  
2 bags containing 1 mL of AP buffer and 7.5 μL of CDP-Star solution (Roche, Basel,  
3 Switzerland) to develop the chemiluminescent signal, which was detected using an  
4 LAS-4000 mini PR Lumino-image analyzer (Fujifilm, Tokyo, Japan). For colorimetric  
5 detection, the filter paper and membranes were soaked in AP buffer containing 0.17  
6 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt (BCIP; Roche) and 0.083  
7 mg/mL nitroblue tetrazolium chloride (NBT; Wako, Osaka, Japan) to develop the signal.

8

#### 9 RNA detection

10

11 The blotted nylon membranes were moistened with distilled water and equilibrated with  
12 5× SSC for 5 min. The membranes were packed in sealed bags containing hybridization  
13 buffer [50% formamide, 7% SDS, 1% *N*-lauroylsarcosine sodium salt, 2% Blocking  
14 Reagent (Roche), 50 mM Na<sub>2</sub>HPO<sub>4</sub> pH7.0, 5× SSC] and stored at 58 °C for 30–60 min  
15 (pre-hybridization). The hybridization buffer was then replaced with a buffer containing  
16 digoxigenin (DIG)-labeled RNA probes that had been prepared previously (Nakahara et  
17 al. 2012), and the bags were incubated at 58 °C overnight. Membranes were then  
18 washed twice in 2× SSC and 0.1% SDS for 10 min at room temperature, and then twice  
19 more in 0.1× SSC and 0.1% SDS for 20 min at 65 °C. After equilibration with MBS  
20 buffer (100 mM maleic acid, 150 mM NaCl) for 5 min at room temperature, they were  
21 soaked in blocking solution (MBS containing 1% Blocking Reagent, 0.3% Tween 20)  
22 and shaken for 30 min. The membranes were then incubated in a 10,000-fold dilution of  
23 anti-DIG-AP conjugate (Roche) in blocking solution for 30 min. They were washed  
24 twice in MBS buffer containing 0.3% Tween 20 for 15 min and equilibrated in AP  
25 buffer for 5 min, and then packed in sealed bags containing 1 mL of AP buffer and 3 μL

1 of CDP-Star solution (Roche) to develop the chemiluminescent signal. This signal was  
2 detected as for the other membranes.

3

#### 4 **Results**

5

6 Microperforation of leaves for blotting onto PVDF membranes

7

8 To accurately assess improvements to the leaf blotting method, we used a transgenic  
9 tobacco plant expressing the HC-Pro protein of clover yellow vein virus (CIYVV;  
10 Nakahara et al. 2012) and a monoclonal antibody raised against HC-Pro (Yambao et al.  
11 2003). Since that method has been optimized, we should have been able to detect the  
12 HC-Pro protein derived from the transgene in the transgenic plant, using the monoclonal  
13 antibody, but not in the wild-type tobacco plant. As expected, however, we failed to  
14 detect HC-Pro using either the colorimetric or the chemiluminescent detection  
15 procedure when we used the conventional leaf blotting method, by which leaf sap was  
16 blotted onto filter paper with a hammer. In fact, similar colorimetric and  
17 chemiluminescent signals were detected in the leaves of both types of plants (Fig. 1a),  
18 probably because less HC-Pro accumulated in the transgenic tobacco leaves than viral  
19 coat proteins did in the virus-infected plant leaves. This result suggested that the  
20 conventional method must be improved to reduce nonspecific background signals in  
21 wild-type tobacco leaves and enhance specific signals that indicate the expression of  
22 HC-Pro in the transgenic leaves.

23 To enhance the specific signal, we attempted to blot the leaf sap onto a PVDF  
24 membrane, which has a higher protein binding capacity than filter paper does. Leaves of  
25 the transgenic tobacco plant expressing the HC-Pro protein were sandwiched between

1 hydrophilized PVDF membranes. However, when this sandwich was worked over with  
2 a hammer, the leaf sap seeped out unevenly onto the membranes, in contrast to the  
3 process of blotting onto filter paper (Fig. 1a). Despite this, we completed the experiment  
4 to examine whether HC-Pro would be detected among the proteins of the transgenic  
5 plants. Although the colorimetric detection method still resulted in high nonspecific  
6 background signal, a specific signal was detectable using chemiluminescent detection  
7 (Fig. 1a). The PVDF membranes thus showed potential for detecting endogenous  
8 proteins in leaves, but the ineffective blotting of leaf sap was problematic.

9 To overcome uneven blotting of leaf sap to the PVDF membrane, two other types  
10 of pretreated leaves were unsuccessfully tested in trial and error (Fig. 1b): (1) the  
11 freeze–thaw without removing the lower epidermis and (2) epidermal abrasion with  
12 carborundum. Effective, even blotting of leaf sap onto the PVDF membrane was  
13 achieved by first microperforating the leaves with the bundled pins (Fig. 1b) as  
14 summarized in Fig. 2. Pressing the sandwich of leaf, PVDF membranes, and pieces of  
15 filter paper with a rolling pin enabled the leaf sap to spread evenly from the many  
16 microperforations in the leaves and to adhere to the membranes (Figs. 1, 2). The  
17 chemiluminescent signal analysis detected HC-Pro throughout the transgenic tobacco  
18 leaves, as expected for this transgenic variety, but not in the wild-type leaves (Fig. 1a,  
19 lower panels).

20

21 Analysis of spatial distribution of endogenous and viral proteins in infected leaves via  
22 mPLB

23

24 The leaf-blotting process produced two pieces of blotted membrane that are lateral  
25 inversions of each other. They can thus be used for the simultaneous detection of two

1 proteins in the same leaf. We used them here for a comparative analysis of the  
2 endogenous plant proteins calmodulin-like protein rgs-CaM and actin with viral coat  
3 protein (CP) in CMV-infected leaves (Fig. 3). We found that rgs-CaM levels were  
4 higher but that those of actin were lower in the parts of the leaves where CMV CP had  
5 accumulated (Fig. 3a). Although the exact reasons for these patterns remain unclear,  
6 they were consistent with previous studies showing interactions between viral infections  
7 and actin (Harries et al. 2009; Harries and Ding 2011; Hofmann et al. 2009;  
8 Prokhnevsky et al. 2005) or tobacco rgs-CaM (Anandalakshmi et al. 2000; Nakahara et  
9 al. 2012).

10 These results indicate that both endogenous proteins and viral coat proteins can be  
11 detected using mPLB with PVDF membranes. We also confirmed that this method is  
12 applicable to adzuki bean (*V. angularis*) leaves (Fig. 3b).

13

14 Analysis of spatial distribution of endogenous and viral RNAs in infected leaves via  
15 mPLB

16

17 We carried out leaf blotting for RNA detection and found that mPLB is also suitable for  
18 blotting leaf sap onto nylon membranes. We then blotted both CMV-inoculated and  
19 noninoculated upper leaves from the inoculated plants onto PVDF and nylon  
20 membranes to detect CMV genomic RNA and CMV CP (Fig. 4a). In this experiment,  
21 CMV was used to inoculate normally growing tobacco plants and those primed by  
22 treatment with BTH before inoculation. BTH is an analogue of salicylic acid and is a  
23 strong inducer of the type of plant immunity known as systemic acquired resistance  
24 (Friedrich et al. 1996). In untreated plants, CMV CP and genomic RNA were detected  
25 across almost the entire area of both inoculated and non-inoculated leaves. However, as

1 expected, in the BTH-primed plants, CMV CP and genomic RNA were detected only in  
2 restricted but largely overlapping parts of both the inoculated and non-inoculated leaves  
3 (Fig. 4).

4 Using the same set of samples, we then tried to detect endogenous mRNA encoding  
5 pathogenesis-related protein 1 (PR1). PR1 is known to be an indicator gene for the  
6 activation of salicylic acid signaling and is induced strongly by BTH (Friedrich et al.  
7 1996). As expected, we detected PR1 mRNA in the leaves that had been treated with  
8 BTH, especially in the areas infected by CMV (Fig. 4b). Although the method will still  
9 benefit from improvements with respect to reducing strong background signals, we  
10 detected no specific signal indicating the expression of PR1 mRNA in the leaves of  
11 healthy or CMV-inoculated plants that had not been treated with BTH.

12

### 13 **Discussion**

14

15 Plant leaves are composed of thin, flat, fragile tissue and thus highly suitable for  
16 blotting to investigate spatial differences in gene expression within a tissue. To date, leaf  
17 blotting has mainly been used to detect viral coat proteins in infected leaves, and only  
18 rarely to detect endogenous plant proteins. However, viruses are obligate intracellular  
19 pathogens, and their success in infection and multiplication depends heavily on their  
20 host organisms; indeed, host factors involved in virus infection are increasingly being  
21 identified (Nagy and Pogany 2012). To understand the molecular mechanisms  
22 underlying viral infection cycles, we thus should investigate how these host factors  
23 interact with infections. If it could be used to analyze the expression of host factors in a  
24 virus-infected leaf, leaf blotting would contribute to our understanding of virus infection.  
25 In this study, we therefore developed an improved leaf blotting method, with the aim of

1 detecting endogenous plant proteins.

2 Three improvements made it possible to detect proteins expressed by transgenes  
3 (Fig.1) and viral and endogenous genes (Figs. 3, 4): (1) use of the chemiluminescence  
4 detection procedure, (2) blotting onto PVDF membranes, (3) microperforation of leaves  
5 with bundled pins before leaf blotting (Fig. 2). Compared with colorimetric detection,  
6 the chemiluminescence procedure generated fewer nonspecific background signals (Fig.  
7 1). However, even with this procedure, we failed to detect the protein expressed by the  
8 transgene (HC-Pro) when the leaves were blotted onto filter paper. The probable reason  
9 is that filter paper insufficiently bind endogenous proteins, which are generally  
10 expressed at lower levels than are viral coat proteins in infected tissues. Using PVDF  
11 membranes instead of filter paper partially resolved this problem (Fig. 1). PVDF  
12 membranes have a higher hydrophobicity, which means they have a greater capacity to  
13 bind proteins (via hydrophobic interactions) than do filter paper or nitrocellulose  
14 membranes (Pluskal et al. 1986), probably resulting in a higher sensitivity for detecting  
15 proteins. However, this high hydrophobicity tends to interfere with the capacity of leaf  
16 sap to adhere to the membrane. This problem was solved by microperforating the leaves  
17 before pressing them onto the membrane; perhaps the leaf sap is distributed more  
18 evenly through the large number of holes, resulting in strong, even blotting onto the  
19 hydrophobic membrane. We also found that mPLB was effective using a nylon  
20 membrane to detect RNAs (Fig. 4). We expect that this procedure will also work well  
21 with other plant tissues such as petals, pericarps, thin stems, bark and roots and with  
22 various types of membranes, including nitrocellulose.

23 Using the mPLB procedure enabled us to detect the endogenous proteins actin and  
24 rgs-CaM, as well as PR1 mRNA, and revealed how their expression related to the CMV  
25 infection in the tobacco leaves. Jung and Hahne (1992) detected endogenous Rubisco

1 and PR1 proteins via leaf blotting. They abraded the lower epidermis by gently rubbing  
2 it with carborundum to increase the transfer of sap onto a nitrocellulose membrane. In  
3 our study, pretreatment by rubbing with carborundum hardly improved the blotting onto  
4 PVDF membranes, and was thus less effective than mPLB, at least with respect to  
5 PVDF membranes (Fig. 1b). Although Takeshita et al. (2001) used leaf blotting to  
6 detect viral genomic RNA in infected bottle gourd plants, to our knowledge this is the  
7 first report demonstrating the detection of mRNA encoding tobacco PR1. Thus, mPLB  
8 may be applicable not only to endogenous plant proteins but also to endogenous  
9 mRNA.

10 Various imaging techniques have been developed to investigate the spatial  
11 distribution of viral and endogenous gene expressions and their intra- and intercellular  
12 interactions in plants (de Ruijter et al. 2003; Salehi Jouzani and Goldenkova 2005).  
13 However, these techniques require the construction of a recombinant viral infectious  
14 clone or expression cassette including a reporter gene such as luciferase, green  
15 fluorescent protein,  $\beta$ -glucuronidase, and Ros1 and subsequent transient or permanent  
16 transfection or transformation of plants that possess these constructs. Ros1 is the  
17 MYB-related transcription factor that activates anthocyanin biosynthesis (Bedoya et al.  
18 2012). Our method obviates the need for these laborious steps, allowing the ready  
19 investigation of the spatial distribution of viral and endogenous gene expressions by  
20 raising antibodies against the target proteins or constructing probes for northern blotting.  
21 Thus, mPLB enables us to analyze leaves of non-model plants that are recalcitrant to  
22 transformation and genetic engineering to express a reporter gene. One possible  
23 drawback of mPLB may be that wound-induced plant genes are expressed, resulting in  
24 some artifactual gene expression because microperforation treatment injures leaf  
25 surface.

1 In summary, pretreatment of leaves with microperforation improves the results of  
2 leaf blotting sufficiently to allow the detection of not only proteins and RNAs of viral  
3 origin, but also those endogenous to plants. This technique is simple and therefore  
4 appropriate to preliminary analysis of viral and endogenous genes suspected to be  
5 involved in the viral infection of a wide variety of plants.

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19

## 20 **Figure legends**

21 **Fig. 1** Comparison of leaf blotting procedures to detect a transgene, the HC-Pro protein,  
22 in transgenic *Nicotiana tabacum* plants expressing HC-Pro (a) and pretreatments before  
23 leaf blotting (b). (a) In upper panels, leaves were sandwiched between filter paper and  
24 blotted using a hammer (conventional leaf blotting); in middle panels, leaves were  
25 sandwiched between polyvinylidene difluoride (PVDF) membranes blotted using a

1 hammer. In lower panels, leaves were microperforated by bundled pins before blotting,  
2 sandwiched in PVDF membranes and pressed with a rolling pin. Blotted membranes  
3 (second columns) were reacted with anti-HC-Pro antibody. After treatment with  
4 secondary antibody conjugated with alkaline phosphatase, the HC-Pro protein was  
5 detected by both colorimetric procedure (third column) and chemiluminescent  
6 procedure (fourth column). (b) Leaves were microperforated, freeze-thawed, or rubbed  
7 with carborundum to abrade the epidermis, then pressing with a rolling pin.

8

9 **Fig. 2** Schematic illustration of the microperforated leaf blotting (mPLB) method.

10 PVDF: polyvinylidene difluoride

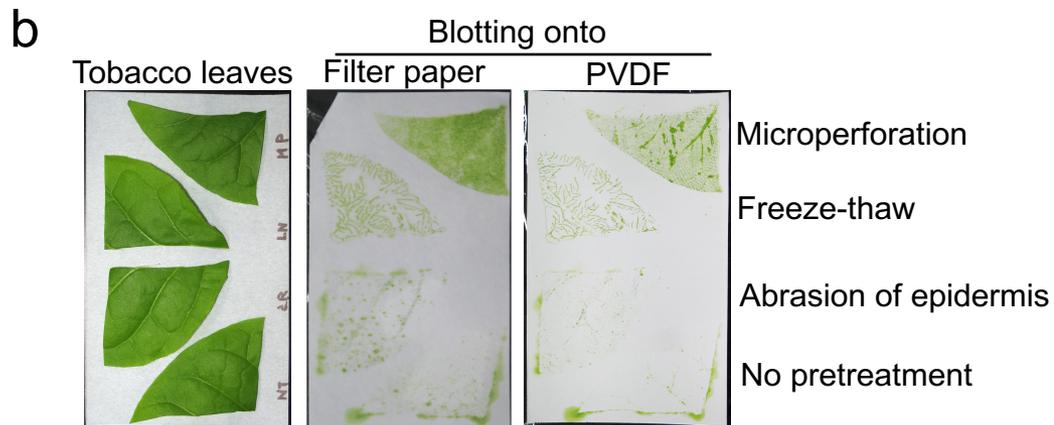
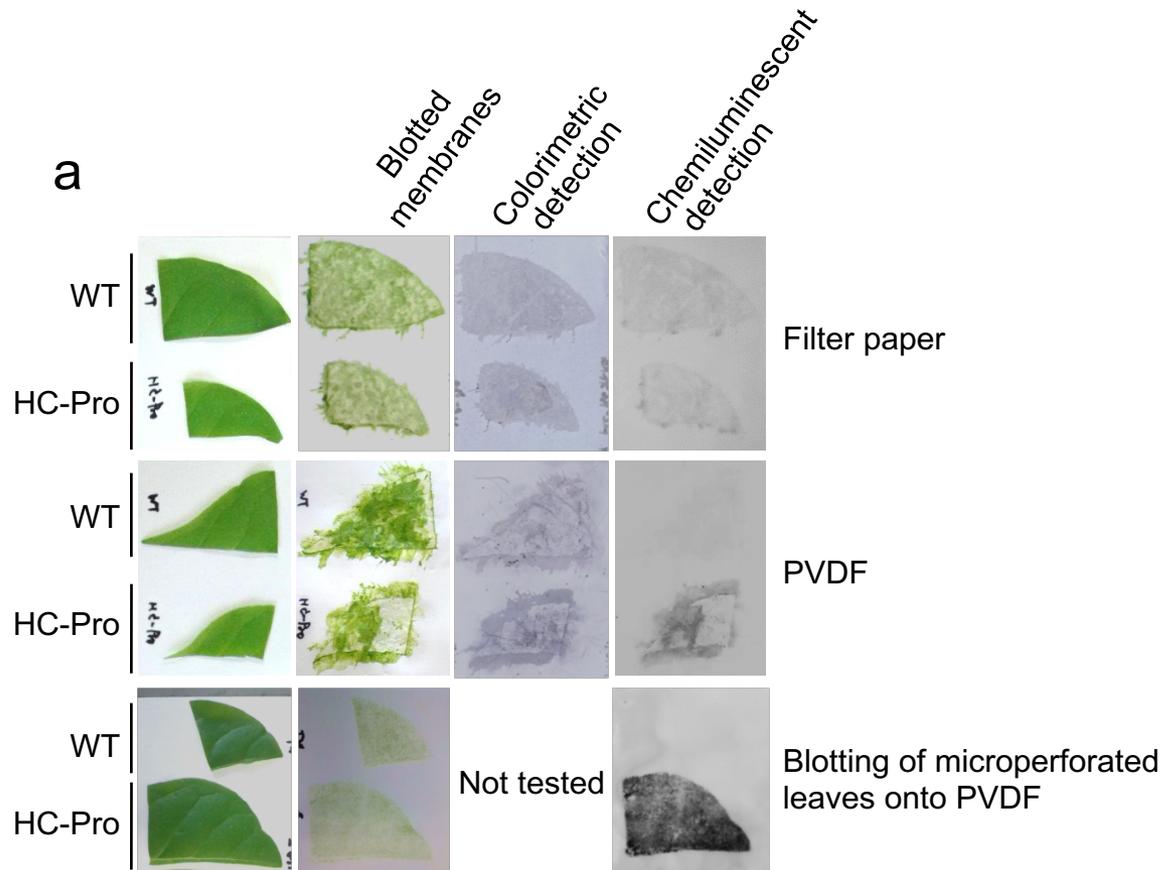
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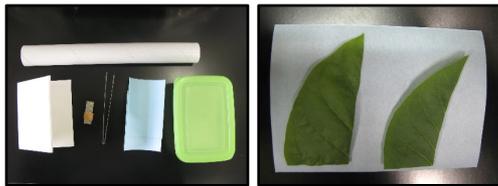
12 **Fig. 3** Analysis of the spatial distribution of endogenous and viral proteins in  
13 noninoculated upper leaves of cucumber mosaic virus (CMV)-infected *Nicotiana*  
14 *tabacum* (a) and adzuki bean (*Vigna angularis*) (b), using the mPLB method. On the  
15 noninoculated upper leaves of tobacco, the recovery phenotype (dark green part) was  
16 partly observed. Six (a) and four (b) replications of the assays yielded similar results  
17 every time. Typical photos are shown.

18

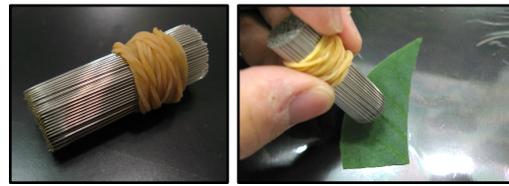
19 **Fig. 4** Detection of viral (a) and endogenous (b) RNA and CMV viral coat protein (CP),  
20 via mPLB. The distribution of the endogenous pathogenesis-related protein 1 (PR1)  
21 overlaps that of the areas of CMV CP accumulation to a large extent (arrows). One of  
22 two assays is shown.

23

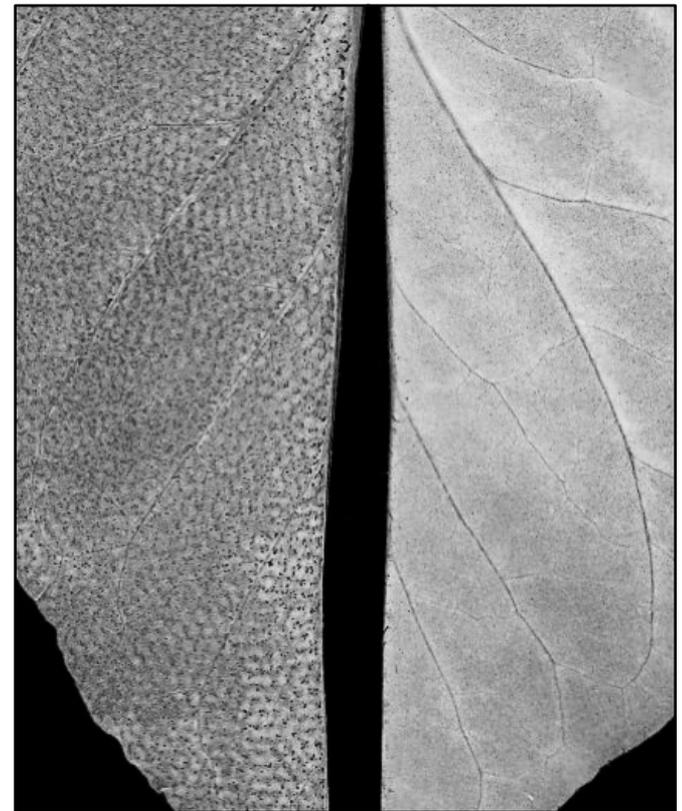




Materials and detached, trimmed leaves



Microperforation of both sides of leaves using bundled pins



Microperforated leaf      Nonperforated leaf



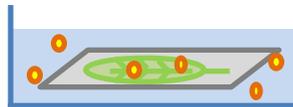
PVDF-leaf sandwich between two layers of filter is pressed with a rolling pin



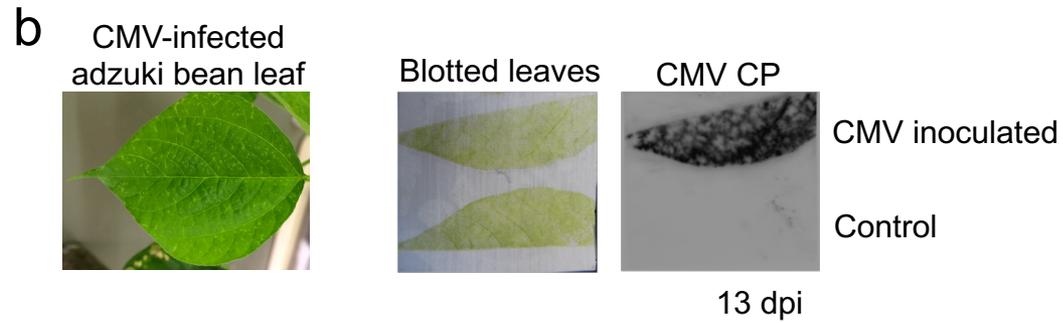
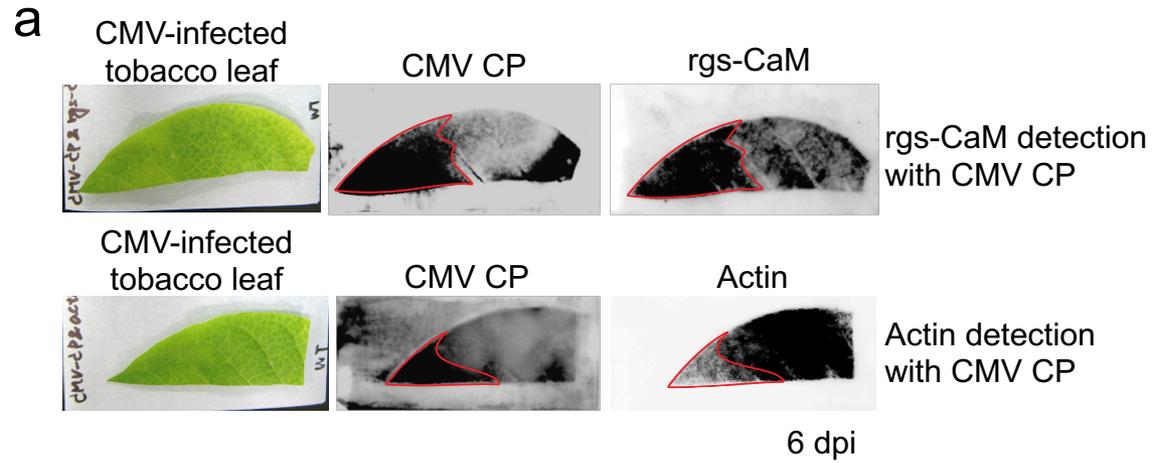
Leaves sandwiched between PVDF membranes



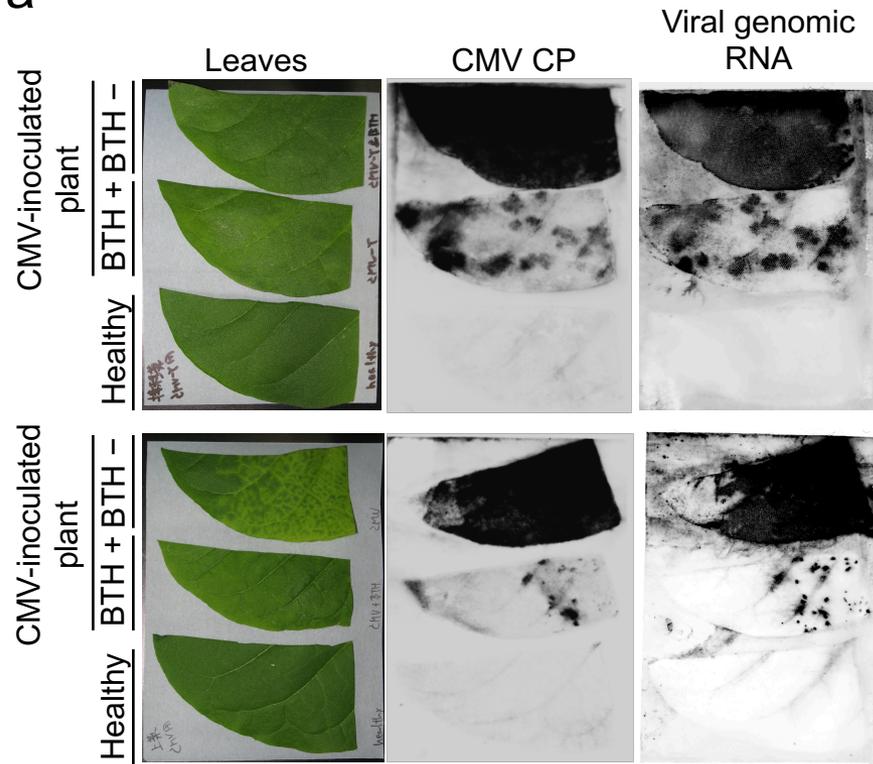
Blotted membranes following removal of the leaves



Membrane reacted with antibody, proteins detected using chemiluminescence



**a**



**b**

