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

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Abstract

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

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

Introduction

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

Leaf blotting is not only simple (i.e., it does not require protein extraction steps), but also can be used to analyze the spatial distribution of specific proteins in leaves. However, its detection sensitivity is relatively low, which likely explains why it has mainly been restricted to detecting viral coat proteins, which are strongly expressed in virus-infected plant cells (Hamada et al. 2003; Polston et al. 1991; Takahashi et al. 2002). There are no examples of the detection of endogenous plant proteins by leaf blotting except for a single study where this method was used to detect endogenous Rubisco protein and pathogenesis-related protein 1 (PR1; Jung and Hahne 1992).

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

pressing the leaves onto a nitrocellulose membrane between two blocks of plexiglass in a hydraulic press for 5 min at 70 kg cm^{-2} (Jung and Hahne 1992).

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

Materials and Methods

Preparation of plants and inoculation of viruses and antibodies

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

respectively.

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

Leaf blotting

We first used a conventional leaf blotting method, largely following Takahashi et al. (2002). After sample leaves were sandwiched between three pieces of filter paper (Whatman 3MM; GE Healthcare, Chicago, IL, USA) on each side, and the entire surface was hit with a hammer, the two inner pieces of filter paper, adjacent to the leaf, were dried on cellophane for 30 min, then soaked and gently shaken in a PBS-T buffer (137 mM NaCl, 8.10 mM $Na_2HPO_4 \cdot 12H_2O$, 2.68 mM KCl, 1.47 mM KH_2PO_4, 2% v/v Triton-X) to remove excess leaf materials for 15 min, instead of Tris-based buffer.

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

remove excess leaf materials.

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

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

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

Immunological detection

The pieces of filter paper and the PVDF membranes were shaken in a blocking buffer (PBS-T containing 3% skim milk) for 30 min and then allowed to react with the primary antibodies (2000-fold dilution of antibody solution in blocking buffer) for 1 h at 37 °C. The filter paper and membranes were washed three times in blocking buffer, then reacted with a secondary antibody (anti-rabbit IgG [Thermo Fisher Scientific, Waltham, MA, USA] or anti-mouse IgG [Bio-Rad, Hercules, CA, USA]), conjugated with alkaline phosphatase solution (5000-fold dilution in blocking buffer) for 1 h at 37 °C. They were again washed three times in blocking buffer. After equilibration in AP buffer

(100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), they were packed in sealed bags containing 1 mL of AP buffer and 7.5 μL of CDP-Star solution (Roche, Basel, Switzerland) to develop the chemiluminescent signal, which was detected using an LAS-4000 mini PR Lumino-image analyzer (Fujifilm, Tokyo, Japan). For colorimetric detection, the filter paper and membranes were soaked in AP buffer containing 0.17 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt (BCIP; Roche) and 0.083 mg/mL nitroblue tetrazolium chloride (NBT; Wako, Osaka, Japan) to develop the signal.

RNA detection

The blotted nylon membranes were moistened with distilled water and equilibrated with 5× SSC for 5 min. The membranes were packed in sealed bags containing hybridization buffer [50% formamide, 7% SDS, 1% *N*-lauroylsarcosine sodium salt, 2% Blocking Reagent (Roche), 50 mM Na_2HPO_4 pH7.0, 5× SSC] and stored at 58 °C for 30–60 min (pre-hybridization). The hybridization buffer was then replaced with a buffer containing digoxigenin (DIG)-labeled RNA probes that had been prepared previously (Nakahara et al. 2012), and the bags were incubated at 58 °C overnight. Membranes were then washed twice in 2× SSC and 0.1% SDS for 10 min at room temperature, and then twice more in 0.1× SSC and 0.1% SDS for 20 min at 65 °C. After equilibration with MBS buffer (100 mM maleic acid, 150 mM NaCl) for 5 min at room temperature, they were soaked in blocking solution (MBS containing 1% Blocking Reagent, 0.3% Tween 20) and shaken for 30 min. The membranes were then incubated in a 10,000-fold dilution of anti-DIG-AP conjugate (Roche) in blocking solution for 30 min. They were washed twice in MBS buffer containing 0.3% Tween 20 for 15 min and equilibrated in AP buffer for 5 min, and then packed in sealed bags containing 1 mL of AP buffer and 3 μL

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

Results

Microperforation of leaves for blotting onto PVDF membranes

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

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

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

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

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

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

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

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

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

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

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

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

Discussion

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

detecting endogenous plant proteins.

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

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

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

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

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

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Figure legends

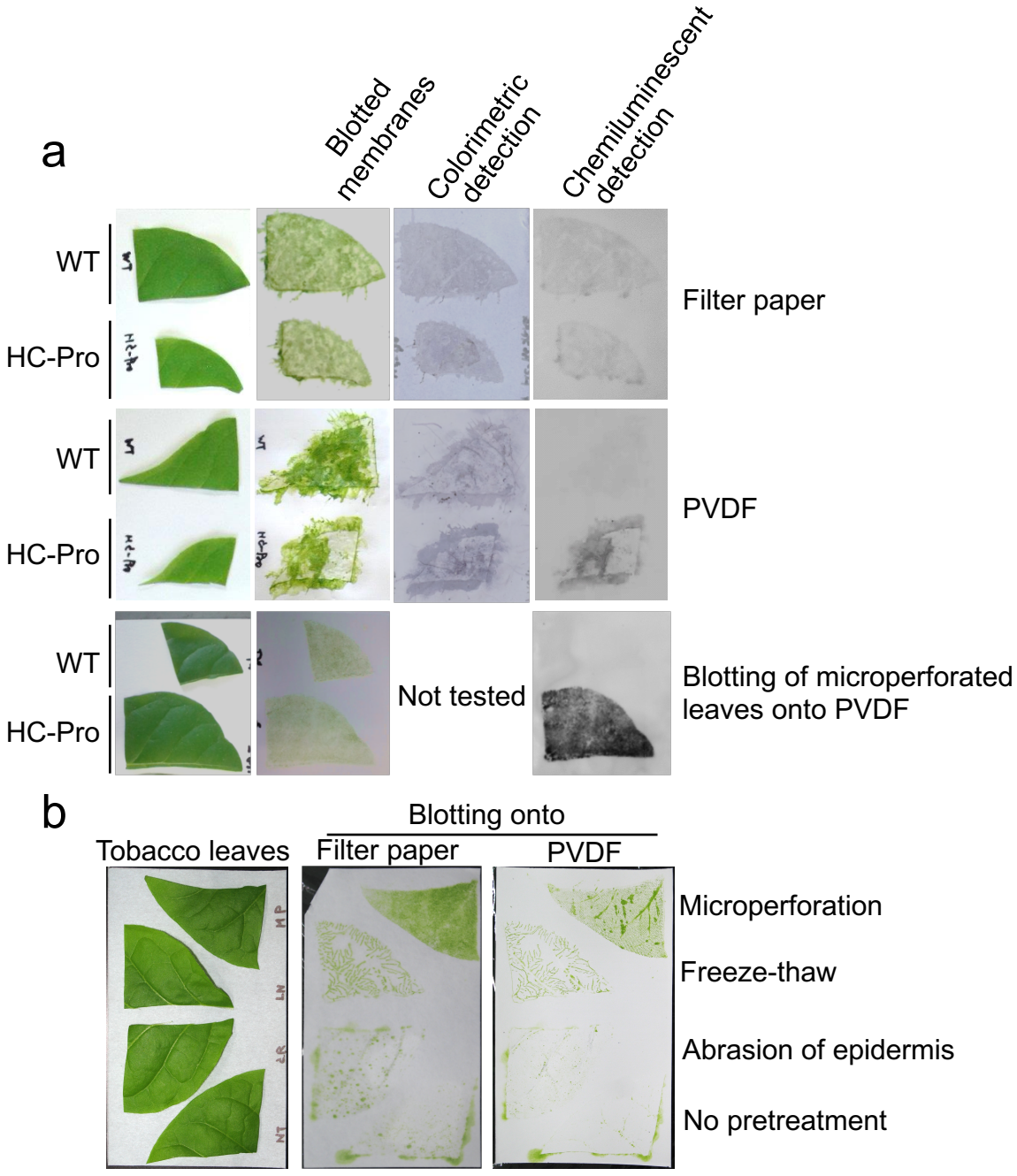
Fig. 1 Comparison of leaf blotting procedures to detect a transgene, the HC-Pro protein, in transgenic *Nicotiana tabacum* plants expressing HC-Pro (a) and pretreatments before leaf blotting (b). (a) In upper panels, leaves were sandwiched between filter paper and blotted using a hammer (conventional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

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

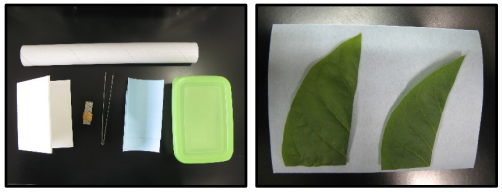
Fig. 2 Schematic illustration of the microperforated leaf blotting (mPLB) method. PVDF: polyvinylidene difluoride

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

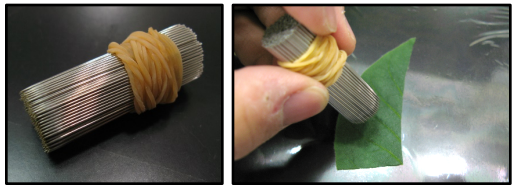
Fig. 4 Detection of viral (a) and endogenous (b) RNA and CMV viral coat protein (CP), via mPLB. The distribution of the endogenous pathogenesis-related protein 1 (PR1) overlaps that of the areas of CMV CP accumulation to a large extent (arrows). One of two assays is shown.


a
Blotted membranes
Colorimetric detection
Chemiluminescent detection
WT
HC-Pro
Filter paper
WT
HC-Pro
PVDF
WT
HC-Pro
Not tested
Blotting of microperforated leaves onto PVDF
b
Blotting onto
Tobacco leaves
Filter paper
PVDF
Microperforation
Freeze-thaw
Abrasion of epidermis
No pretreatment

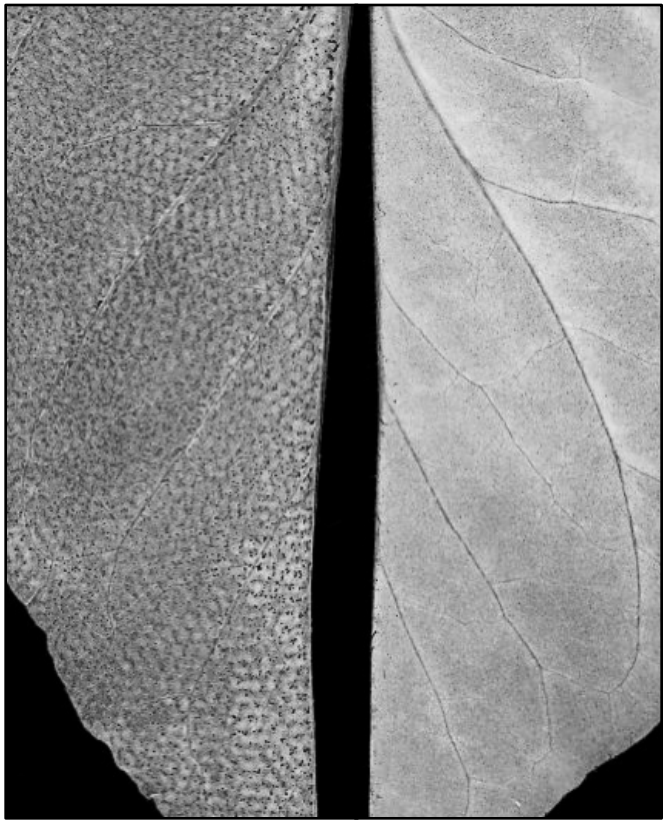
Murakami et al., Figure 2



Materials and detached, trimmed leaves

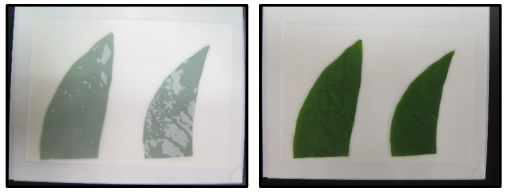


Microperforation of both sides of leaves using bundled pins

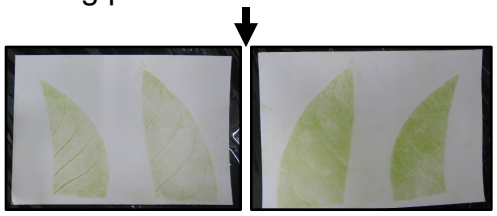


Microperforated leaf Nonperforated leaf

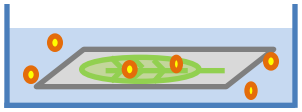
PVDF-leaf sandwich between two layers of filter is pressed with a rolling pin



Leaves sandwiched between PVDF membranes

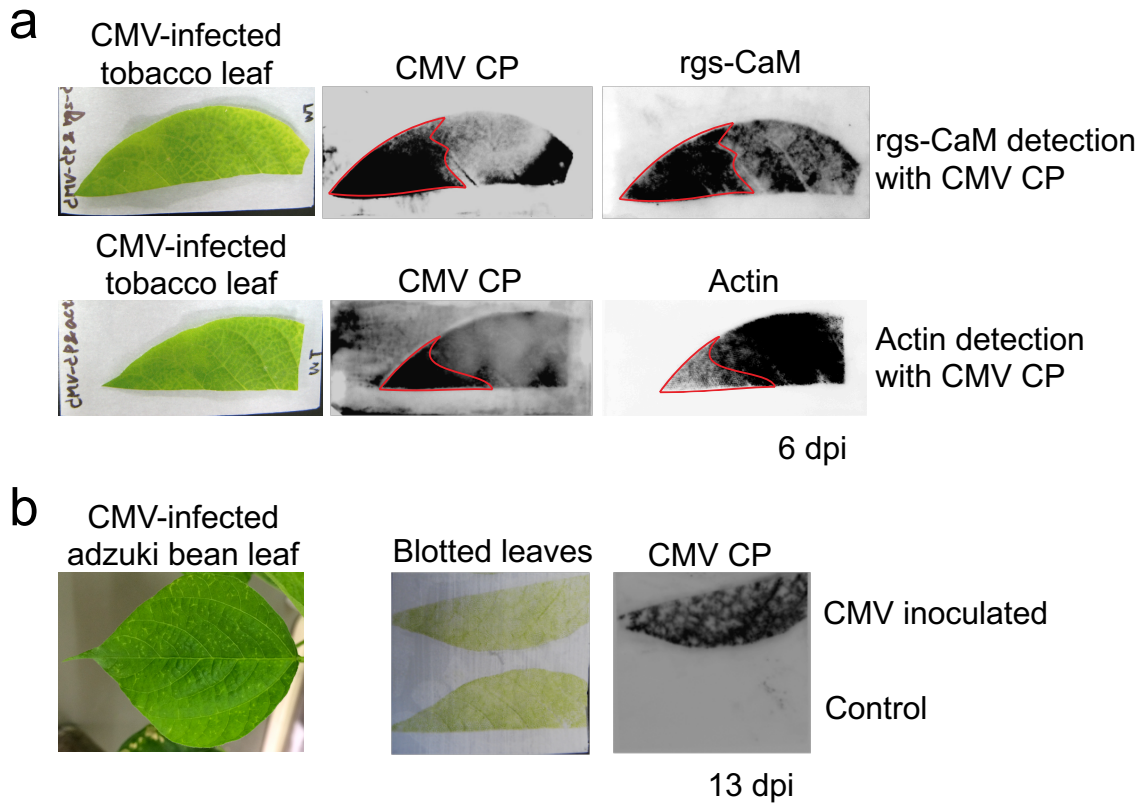


Blotted membranes following removal of the leaves



Membrane reacted with antibody, proteins detected using chemiluminescence

Murakami et al., Figure 3



Murakami et al., Figure 4