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Infection of barley protoplasts with bamboo mosaic virus RNA

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Abstract. Protoplasts prepared from barley (*Hordeum vulgare* L. cv. Larker) were inoculated with bamboo mosaic virus (BaMV) RNA. The infection efficiency of protoplasts was assessed by immunofluorescent microscopy, and by assaying extracts for BaMV antigen by immunodot staining and for BaMV RNA by slot blot hybridization. Fluorescent antibody staining demonstrated that 60-80% of viable protoplasts were infected. By slot blot analysis, viral RNA could be detected at a sensitivity level of 13 - 26 protoplasts at 24 h after infection.

Key words: Bamboo mosaic virus; Potexvirus; Protoplast; RNA infection.

Bamboo mosaic virus (BaMV, Hull *et al.*, 1991) has been tentatively grouped as a member of the potexvirus group (Lin *et al.*, 1977; Koenig and Lesemann, 1978). It contains a single-stranded, positive-sense RNA genome about 6.4 kb in length (Lin *et al.*, 1992). In Taiwan, bamboo mosaic virus commonly occurs in rhizomes of the pachymorph type bamboos, such as *Bambusa oldhamii* Munro, *B. beecheyana* Munro var. *beecheyana, B. edulis* Keng and *Dendrocalamus latiflorus* Munro, (Lin *et al.*, 1979). The host range of BaMV is relatively narrow. Besides bamboo species, it infects the local lesion hosts *Chenopodium amaranticolor, C. quinoa* and *Gomphrena globosa* (Lin *et al.*, 1977; Lin *et al.*, 1979). Barley (*Hordeum vulgare* L. cv. Larker) and Bambusoideae are the only known systemic hosts for BaMV (Lin *et al.*, 1979; Lin and Chen, 1991).

The cytopathological effects of BaMV infection have been studied by immunoelectron microscopy (Lin and Chen, 1991), however, little is know about BaMV replication in infected cells. Since infection of protoplasts with viral RNA is a synchronous event, assays of replication and gene expression of viral RNA should be facilitated. Successful infection of protoplasts with potexvirus RNA has been reported in tobacco and potato by potato virus X (PVX) (Shalla and Petersen, 1973; Otsuki *et al.*, 1974; Adams *et al.*, 1985; Prakash and Foxe, 1985) and pea by white clover mosaic virus (Brown and Wood, 1987). In this study, we use barley protoplasts as host system for supporting replication of BaMV RNA.

The BaMV isolate from infected green bamboo (*B. oldhamii*) was propagated in barley (*H. vulgaris* L. cv. Larker) (Lin and Chen, 1991). Leaves were harvested 10 days post-inoculation and the virus was purified as previously described (Lin and Chen, 1991). RNA was isolated from purified virions disrupted in a solution containing 2% SDS, 10 mM NaH_2PO_4, 20 mM Na_2HPO_4 and 1 mM EDTA at 60°C for 5 min. This was followed by phenol-chloroform extraction and ethanol precipitation. The RNA was dissolved in sterile distilled water, quantified by UV absorption and stored at -70°C until use. To check the integrity, the RNA sample was subjected to electrophoresis in a 1% agarose gel in Tris-borate buffer and visualized by staining with ethidium bromide (Maniatis *et al.*, 1982).

Barley protoplasts were isolated from 7-day-old seedlings according to the method of Loesch-Fries and Hall (1980), modified by Kroner *et al.* (1989). Four grams of leaves were sliced transversely with new razor blades into small pieces of 1-2 mm wide. The slided pieces were gently dispersed into the enzyme solution containing Cellulysin, Macerozyme R-10 and mannitol (Loesch-Fries and Hall, 1980). After a 3 h incubation in the dark at 30°C, the solution was filtered and spun at 50x g for 2.5 min. The protoplast pellets were gently resuspended in 1 ml of 10% mannitol, and 8 ml more mannitol was added later. The mixture was then layered over 2 ml of 20% sucrose and spun at 50x g for 10 min. Protoplasts were collected from the interface and transferred to 20 ml of 10% mannitol solution. The yield was approximately 10^6 protoplasts per gm tissue. Protoplasts were then divided into aliquots of 5×10^5 cells and were inoculated separately. Inoculation with BaMV RNA was performed by the polyethylene glycol procedure described by Kroner *et al.* (1989). Inoculated protoplasts were incubated in a growth chamber at 24°C under 24-h daily illumination at light intensity of 50 $uEm^{-2}sec^{-1}$.

For imuno-assays, protoplasts were collected at 48 h post-inoculation. The procedures for immunodotting and for immunofluorescent microscopic staining (IMS) were the same as previously described (Lin *et al.*, 1989), except that a 1:2000 dilution of rabbit anti-BaMV capsid protein serum (Lin and Chen, 1991) was used as primary antibody, and fluorescein isothiocyanate (FITC) -conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) was the secondary antibody for IMS.

For the preparation of the cDNA probe, cDNA was transcribed from BaMV RNA by AMV reverse transcriptase (Promega, Madison, WI) in the presence of random hexamer as primer (New England BioLabs, Inc., Beverly, MA) and [α-^{32}P] dCTP (50 μCi; 3,000 Ci/mmol; Amersham) (Maniatis *et al.*, 1982). The reaction mixture contained 2 μg RNA and a typical yield was 1 - 2 $\times$ 10^8 cpm of radioactive cDNA.

For slot blot analysis, total nucleic acids were extracted from transfected protoplasts at 24 h post -inoculation. To each sample, 200 μl of extraction buffer (0.33 M glycine, pH 9.5, 0.33 M NaCl, 33 mM EDTA, 3.3% sodium dodecyl sulfate, 8.33 mg bentonite/ml) was added and the sample was then twice extracted with phenol-chloroform (1:1) and then precipitated with isopropanol. Total nucleic acids were collected by centrifugation and suspended in 30 μl of distilled water. Serial dilutions were made in distilled water and 1 μl of the diluted sample was dotted onto a H - Bond hybridization membrane (Amersham Corp., Arlington Heights, Ill.) in the PR 600 Slot Blot (Hoefer Scientific Instruments, CA.). After baking for 2 h at 80°C, membranes were prehybridized for at least 1 h at 65°C in a prehybridization solution (Kroner *et al.*, 1989). Hybridization was usually done in the same buffer containing 5-6 $\times$ 10^7 cpm of P^{32}-labelled cDNA at 65°C overnight. Nonspecific hybridization was removed by washing the membranes in a stringent condition as described by Kroner *et al.* (1989). Hybridized RNA was visualized after the dried membrane was exposed to X -Omat AR film (Eastman Kodak Co., N. Y.) at -70°C by using intensifying screens.

In a preliminary study, the amount of BaMV RNA used as inoculum was varied from 0.13 - 5.0 μg/5 $\times$ 10^5 cells and the infection efficiency was judged by IMS assay. Infected protoplasts stained with anti-BaMV capsid protein serum followed by FITC-conjugated antibodies showed bright green fluorescence over the whole cytoplasm (Fig. 1A) as compared to lack of fluorescence in mock-inoculated protoplasts (Fig. 1B). There was also no fluorescence in the inoculated protoplasts stained with rabbit pre-immune serum (data not shown). Inoculation with BaMV RNA inoculum of less than 0.20 μg/5 $\times$ 10^5 cells usually gave little or no positive staining of the inoculated protoplasts. However, when BaMV RNA was increased to 2-5 μg, 60-80 % of the viable protoplasts became positive with immunofluorescent stain. In these experiments, isolated protoplasts displayed over 80% of viability as judged by fluorescent diacetate staining (data not shown). The percentage of infected protoplasts varied slightly in different experiments but an infection rate of greater than 60% was normally obtained. For the standard inoculation need, 2 μg of BaMV RNA was used.

Immunodot assay showed that diluted anti-BaMV capsid protein serum easily detected BaMV antigen in the crude extracts of BaMV RNA- (Fig. 2, lane A2) or BaMV virion- (lane A3) inoculated protoplasts. These extracts gave purple dots on the blots when immunologically stained, whereas crude extracts from mock-inoculated protoplasts showed brown color when processed similarly (Fig. 2, lane A1). No BaMV antigen was detected in the supernatant of culture medium after inoculated protoplasts were harvested

(Fig. 2, lane A5). All the crude extracts of protoplasts treated with preimmune serum showed only background color development (Fig. 2, lane B1-5).

Slot blot analysis showed that the cDNA probe hybridized the nucleic acid extracts of inoculated protoplasts in the dilution range from 5 - 1,280 × (Fig. 3, lane A1-A9) whereas the hybridization signals were absent in the mock-inoculated protoplasts (lane B). In three separate experiments, BaMV RNA could be detected in 640-fold diluted nucleic acid samples, an equivalent of 26 protoplasts. Occasionally, the detection was positive in samples of 13 protoplasts.

Although successful inoculation of host plant protoplasts with RNA or virions has been reported for a number of potexviruses (Shalla and Petersen, 1973; Otsuki *et al.*, 1974; Adams *et al.*, 1985; Prakash and Foxe, 1985; Brown and Wood, 1987), there have been no reports of infection of protoplasts with BaMV RNA. Our results from IMS, immunodot and slot blot assays provide clear evidence that barley protoplasts are easily infected with BaMV RNA and can efficiently support its replication, e. g. synthesis of RNA and capsid protein to a detectable level 24 or 48 h after inoculation. Compared to previous studies in which a positive detection of PVX infection required 30-70 tobacco or potato protoplasts (Otsuki and Takebe, 1974; Prakash and Foxe, 1985) and of brome mosaic virus RNA infection required 250 barley protoplasts (Rao *et al.*, 1990),

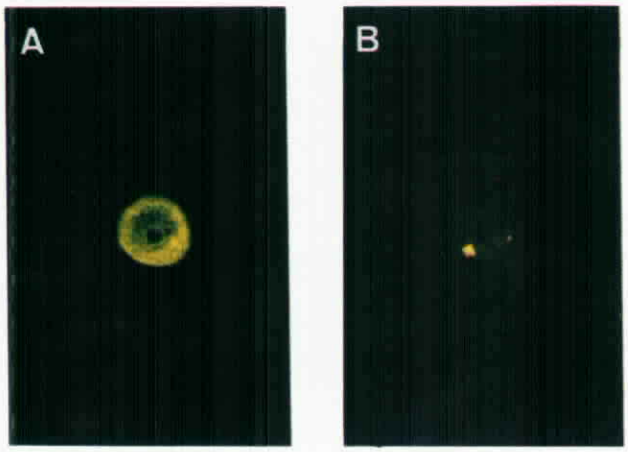

Fig. 1. Fluorescence micrographs of (A) BaMV-inoculated and (B) mock-inoculated barley protoplasts 48 h post-inoculation. Staining was performed with anti-BaMV capsid protein serum followed by fluorescent isothiocyanate-conjugated goat anti-rabbit IgG.

Fig. 2. Dot immunoassays of crude extract from protoplasts 48 h post-inoculation using anti-BaMV serum (A) or preimmune serum (B). Lane 1, mock-inoculated protoplasts. Lane 2, protoplasts inoculated with BaMV RNA. Lane 3, protoplasts inoculated with BaMV virions. Lane 4, purified BaMV virions (0.5 μg). Lane 5, supernatant of protoplast culture inoculated with BaMV RNA.

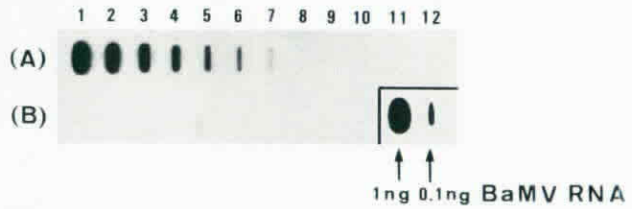

Fig. 3. Slot blot analysis of RNA isolated from BaMV-inoculated (A) and mock-inoculated (B) protoplasts at 24 h post-inoculation. From left to right, RNA samples (1 μl) in two-fold serial dilutions, starting from 1:5, were dotted onto H-bond hybridization membrane in a PR 600 Slot Blot Apparatus. 1 ng and 0.1 ng of BaMV RNA were loaded as control markers. The blot was probed with P^{32}-labelled cDNA of BaMV RNA.

the BaMV-barley protoplast system is far more efficient. Infection with viral RNA, rather than virions, has the advantage that replication events of the individual RNA component or mutagenetically-modified RNA can be studied. Thus, the barley protoplast system should provide a useful tool for studying many aspects of BaMV replication and virus-host interactions (Ahlquist and French, 1988; Allison and Ahlquist, 1989).

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竹嵌紋病毒 RNA 感染大麥原生質體

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本研究以竹嵌紋病毒(Bamboo mosaic virus, BaMV)之 RNA 接種大麥之原生質體。接種後分別利用免疫螢光顯微鏡觀察感染率，墨點免疫染色法偵測原生質體抽出物之 BaMV 抗原及條墨雜交偵測原生質體抽出物之 BaMV RNA。螢光顯微鏡檢結果顯示，在存活之原生質體中感染率達 60-80%。條墨雜交顯示，BaMV RNA 可在大麥原生質體中複製，而且只需 13-26 個原生質體，即可偵測到病毒之複製。