Effect of Inoculum pH on Polyethylene Glycol-mediated Infection of Tobacco Mesophyll Protoplasts by Cucumber Mosaic Virus

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The infectivity of cucumber mosaic virus (CMV) after mechanical inoculation of tobacco leaves is affected by pH values of buffers which suspend the virus as the inoculum; the infectivity was lower at pH 6.0 than at pH 8.0⁸⁾. Because many spherical plant virus particles swell in slightly basic conditions³⁾, this pH-dependent infectivity may be related to some structural change of the virion, uncoating or some other alteration in the early stage of CMV infection processes. In fact, many plant viruses have higher infectivity when the pH of the inoculum is on the basic side. Although CMV shows certain structural transitions when the pH is raised from 5 to 7, the transitions do not result in particle swelling¹⁰⁾. Previously, we pointed out that ribonuclease (RNase) in the sap which exuded from wounded epidermal cells after mechanical inoculation may also be responsible for the pH-dependent infectivity; the RNase binds easier with the virus surface and has higher activity at pH 6.0 than at pH 8.0⁸⁾. Thus, the pH-dependent CMV infection may be affected by several factors. To elucidate the cause of pH-dependent CMV infectivity, we investigated the effect of inoculum pH on the early infection processes in tobacco mesophyll protoplasts that were inoculated using the polyethylene glycol-mediated procedure.

CMV and CMV RNA were purified as previously reported^{8,11)}. Tobacco (*Nicotiana tabacum* L. cv. ky57) plants were grown in a growth chamber at 25°C under $1.0\text{--}1.6 \times 10^4$ lux illumination using a 16 hr photoperiod. Protoplasts were prepared from young tobacco leaves about 5 cm long at the 10-leaf stage by a method modified from that of Otsuki *et al.*⁹⁾. The protoplasts were washed three times by suspending in 0.5 M mannitol and centrifuging ($70 \times g$ for 3 min). For inoculation, the washed protoplasts (5×10^5) were resuspended in 0.1 ml of mannitol solution (0.5 M mannitol dissolved in distilled water) and mixed with 20 μ l of CMV virion (1 mg/ml) or CMV RNA (0.25 mg/ml) suspended in 20 mM potassium phosphate buffer at pH 6.0 or 8.0. After 10 sec, 0.2 ml of 40% polyethylene glycol 1500 (containing 5 mM CaCl_2 , pH 5.5) was added to the mixture⁷⁾. At this stage, the pH of the protoplast suspension was within 5.5-6.0 regardless of the pH of the inoculum added. Then, 2 ml of the mannitol solution was added, and the suspension was incubated on ice for 3 min. After incuba-

tion, protoplasts were washed three times with the mannitol solution and cultured in a medium (0.5 M mannitol solution containing 0.2 mM KH_2PO_4 , 1 mM KNO_3 , 1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 mM CaCl_2 , 1 μ M KI, 0.01 μ M CuSO_4 , pH 6.5) under continuous illumination (*ca.* 3000 lux) at 25°C for 0-48 hr. To analyze the CMV infection process, the protoplasts were collected by centrifugation at $70 \times g$ for 3 min, and the CMV coat protein (CP) was extracted with 50 mM Na_2CO_3 (pH 9.6). The CP concentration was then assayed by ELISA⁵⁾ or by western blotting using ECL detection reagents (Amersham)⁶⁾.

When protoplasts were inoculated with a suspension of CMV at pH 8.0, the amount of CMV CP accumulated in the cultured protoplasts gradually increased up to 48 hr, whereas the amount increased only a little at pH 6.0 (Fig. 1). The percentage of live protoplasts 24 hr after inoculation for inoculum at both pHs was similar (60-70%). When the protoplasts were inoculated with CMV RNA, a similar pH-dependence in CMV multiplication was observed; however, such a decrease in virus multiplication at pH 6.0 was not detected (Fig. 2).

Several possibilities account for the pH-dependent CMV infection of tobacco protoplasts. For example, a slight change in the pH of the protoplast suspension caused by the addition of pH 8.0 inoculum may affect the efficiency of virus infection. In fact, in the case of the polyethyleneimine- or poly-L-ornithine-mediated inoculation of tobacco protoplasts with CMV, the pH level of the buffer was shown to affect the efficiency of virus infection¹²⁾.

To analyze the early infection processes of CMV, the CP in the protoplasts inoculated with the virus suspended at pH 6.0 and at pH 8.0 was measured from 0.5 up to 24 hr after inoculation by western blot analysis. As shown in Fig. 3, regardless of inoculum pH, almost the same amount of CP was detected in both the protoplast samples at 0.5-6 hr after inoculation. The CP then increased markedly in the protoplasts up to 12 hr after inoculation with inoculum at pH 8.0, whereas those protoplasts inoculated at pH 6.0 had a smaller increase. Thus, although nearly the same amount of CMV virion from both inocula adsorbed to the protoplasts, subsequent multiplication varied with the pH of the inoculum.

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Fig. 1. CMV coat protein (CP) accumulation in tobacco protoplasts inoculated with 20 μ g of CMV suspended in 20 mM potassium phosphate buffer at pH 6.0 or 8.0. After inoculation, protoplasts were cultured for 0–48 hr and the quantity of CMV CP was determined by ELISA (absorbance at 405 nm). These results are representative of three experiments.

Fig. 2. CMV CP accumulation in tobacco protoplasts inoculated with 5 μ g of CMV RNA suspended in 20 mM potassium phosphate buffer at pH 6.0 or 8.0 as determined by ELISA (absorbance at 405 nm). These results are representative of three experiments.

To investigate earlier infection processes such as uncoating, we analyzed the CP in the protoplasts between 10 and 120 min after inoculation by sucrose density gradient ultracentrifugation. The protoplasts inoculated with CMV suspended at both pHs were washed and left at room temperature for the required period followed by freezing at -80°C . Each sample (1.25×10^5 protoplasts) was homogenized with 200 μ l of 0.5 M citrate buffer (containing 10 mM EDTA, 0.1% 2-mercaptoethanol, pH 6.5) and 200 μ l of chloroform, and then centrifuged at $10,000 \times g$ for 5 min. The supernatant was dialyzed against 50 mM borate buffer (pH 9.0) for 4 hr. After dialysis, the samples were layered on a 10–40% sucrose gradient and centrifuged at $300,000 \times g$ for 1 hr at 4°C . After centrifugation, the gradient was fractionated into 10 fractions from top to bottom⁴⁾ and CMV CP in each fraction was analyzed by western blotting.

Fig. 3. Western blot analysis of CMV CP accumulation in tobacco protoplasts inoculated with 20 μ g of CMV suspended in 20 mM potassium phosphate buffer at pH 6.0 (A) or pH 8.0 (B). Numbers above each lane correspond to the time (hr) after inoculation. M shows 1 ng of CMV CP as a marker.

When purified intact CMV was layered on the sucrose gradient and processed in the same manner as described above, CMV CP was detected mainly in fractions 5–7 (data not shown). As shown in Fig. 4, distribution patterns of the CP extracted from the protoplasts hardly varied. At 10 min after inoculation at either pH, the CP was detected mainly in fractions 5–7. However, at 30–60 min after inoculation, the CP could be detected in fractions 1–2 as well as fractions 5–7. The CP bands appearing in fractions 1–2 contained free CP subunits, possibly from uncoating. The amount of free CP subunits in fractions 1–2 was 42 and 39% of total CP (fractions 1–10) at pH 6.0 and pH 8.0, respectively, 30 min after inoculation (measured by a densitometer, Shimadu CS-9000). At 120 min after inoculation, the bands of free CP subunits disappeared in both the samples. This result suggested that almost the same amount of virus was introduced into the protoplasts either at pH 6.0 or 8.0, and that virus uncoating was not affected by the difference in inoculum pH.

Accordingly, CMV multiplication in tobacco protoplasts inoculated with inoculum at pH 6.0 was lower than that at pH 8.0. The adsorption, entry and uncoating of the virus were not affected by the inoculum pH level. Thus, virus multiplication may be prevented after uncoating of CMV virion suspended at pH 6.0.

Our earlier studies showed that inactivation of CMV was detectable immediately after mixing 0.34 unit/ml of RNase for 2 mg/ml of virus at pH 6.0⁹⁾, and that one RNase molecule was sufficient to inactivate one CMV particle^{1,2)}. In fact, RNase activity in the protoplast suspension (5×10^5 protoplasts/0.1 ml) prepared for inoculation was 0.21 units/ml (measured at pH 7.5 with yeast RNA as the substrate¹³⁾). The RNase activity probably originated from the degraded protoplasts. Thus, the lower CMV infectivity at pH 6.0 might be due to the higher RNase activity in the protoplast-suspension. These observations indicate that the RNase in the protoplast suspension may affect virus multiplication after CMV entry into the protoplasts.

Fig. 4. Distribution of CMV CP extracted from tobacco protoplasts in 10 to 40% sucrose density gradient at 10, 30, 60 and 120 min after inoculation. The gradients were centrifuged at $300,000\times g$ for 1 hr and fractionated into 10 fractions from top to bottom. The CP in each fraction was analyzed by western blot analysis. The protoplasts were inoculated with 20 μ g of CMV suspended in 20 mM potassium phosphate buffer at pH 6.0 (A) or pH 8.0 (B).

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和 文 摘 要

大野 浩・江原淑夫：ポリエチレングリコールを用いたキュウリモザイクウイルスのタバコ葉肉プロトプラストへの感染に与える接種源 pH の影響

pH 6.0 の緩衝液に懸濁したキュウリモザイクウイルス普通系統 (CMV) をポリエチレングリコール法によりタバコ葉肉プロトプラストに接種した場合、pH 8.0 の緩衝液に懸濁したときに比べプロトプラストにおけるウイルス増殖量は少ない。CMV RNA 接種でも同様の結果が得られたが、CMV 粒子接種で見られた pH 6.0 における著しい感染性の低下は認められなかった。接種 10~120 分後のプロトプラストよりウイルス外被タンパク質を抽出し、ショ糖密度勾配遠心分離およびウエスタンブロットにより解析したところ、両 pH でウイルス侵入および脱外被の過程に差は認められなかった。よって pH 6.0 に懸濁したウイルスはプロトプラスト侵入後、脱外被以降の感染過程が阻害されたと考えられる。接種時のプロトプラスト懸濁液中には細胞由来の RNase が存在し、タバコ葉を用いた解析結果⁸⁾ のように CMV のプロトプラストへの感染性に影響を与えている可能性が推定された。

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