Native electrophoresis and Western blot analysis (NEWeB): a method for characterization of different forms of potyvirus particles and similar nucleoprotein complexes in extracts of infected plant tissues

I. N. Manoussopoulos,1 E. Maiss3 and M. Tsagris1, 2

1 Institute of Molecular Biology and Biotechnology, PO Box 1527, Heraklion, 711 10 Crete, Greece
2 Department of Biology, University of Crete, Greece
3 Universität Hannover, Institut für Pflanzenkrankheiten und Pflanzenschutz, Herrenhauser Strasse 2, D-30419 Hannover, Germany

Electrophoretic mobility (EMb) for the study of biological properties, such as electrophorotype formation and interaction of virus particles with other host or virus proteins. We have developed the present methodology [native electrophoresis and Western blot (NEWeB)] for studying the electrophoretic behaviour of two strains of Plum pox virus (PPV) (Potyviridae) – one of Greek origin (PPV-Lar) (Tsagris et al., 1993) and a non-aphid-transmissible strain, PPV-NAT (Maiss et al., 1989) – and the in vivo interaction of virus particles with helper component proteinase (HC-Pro).

Potyviruses have long flexuous filamentous particles (Hollings & Brunt, 1981), and code for at least eight mature proteins, most of which have multifunctional properties (Riechmann et al., 1992). One of these proteins, HC-Pro, is necessary for aphid transmission (Pirone & Blanc, 1996), has been shown to interact with virus particles in vitro (Blanc et al., 1997) and is involved in cell-to-cell and long-distance transmission of the virus in the plant (Rojas et al., 1997; Kasschau et al., 1997)

We studied PPV strain separation in double infections from the same host, interaction of virus particles with HC-Pro and the possible occurrence of electrophorotypes in different organs of the same plant.

PPV-Lar was purified according to the method of Lisa et al. (1981) from Nicotiana clevelandii leaves; two different stocks were used, both prepared by the same method. Inoculations were done by applying 50 µl of PPV-Lar (0·1 mg/ml virus) onto two opposite leaves of 3-week-old Nicotiana benthamiana plants. In experiments with PPV-NAT, crude extract from infected N. benthamiana plants was used as inoculum. For native electrophoresis plant extracts were prepared as follows. Plant material (inoculated or systemically infected leaves, petioles, stems, roots) was homogenized (1 g in 5 ml) in 0·6 M sodium phosphate pH 7·5, or 1 x TBE pH 8·3 (89 mM Tris base, 89 mM boric acid, 2·5 mM EDTA) (extraction buffer). Sap was centrifuged for 5 min at 5000 r.p.m. at room temperature. The supernatant was collected and sucrose,
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Fig. 1. (a) NEWeB on agarose–acrylamide gel in 1 × TBE pH 8.3 of extracts from leaves (L) and stems (S) of N. benthamiana plants infected with PPV-Lar and PPV-NAT. Purified virus particles of PPV-Lar (V) were also used as control. (b) NEWeB analysis of leaf extracts from single and doubly inoculated plants. Electrophoresis was done in mixed agarose–acrylamide gels in 1 × TBE pH 8.3. Lane 1, purified particles of PPV-Lar; lane 2, extract from non-infected plant; lanes 3 and 4, extracts from plants infected with PPV-Lar and PPV-NAT respectively; lane 5, mixed sap of (3) and (4). Lanes 6–8, extracts of leaves with symptoms, from three plants inoculated simultaneously with both strains.

Diluted in extraction buffer, was added to a final concentration of 5%. In some experiments, extract from leaves of two different plants infected with either PPV-NAT or PPV-Lar was mixed 1:1 before it was treated with sucrose and loaded onto the gel. Agarose gels (16 cm length × 11.5 cm width × 0.5 cm height) varied between 0–8 and 1–5% agarose in phosphate extraction buffer. Mixed agarose–acrylamide gels of the same dimensions had a final concentration of 6% agarose, 0.76% acrylamide and 0.04% bis-acrylamide in 1 × TBE extraction buffer. Electrophoresis was performed at 1.5 V/cm for 5–8 h in extraction buffer. Gels were electroblotted (Protein trans blot cells; Bio-Rad) onto nitrocellulose membranes (Schleicher and Schuell) in electrophoresis buffer overnight at 14–20 V, 300 mA in a cold room. After transferring, membranes were treated appropriately with dry-skimmed milk for blocking, coat protein (CP)-specific primary antibody (1:10000) or HC-Pro-specific antibody (1:5000) and secondary antibodies (anti-rabbit alkaline phosphatase-conjugated; Promega) were used for development of the blot. For the study of the HC-Pro–virus complexes, and analysis of particles by immunosorbent electron microscopy (ISEM), two identical gels were always prepared, loaded and run as already described. Antibodies were prepared from recombinant proteins from the PPV-NAT strain (Riedel et al., 1998). NBT/BCIP substrate solution (Promega) was used for colour development. ISEM was performed as described by Roberts (1986). For studying stability of virus particle–HC-Pro interactions urea and sucrose were added to extract from leaves of N. benthamiana plants (see above) so that samples were obtained with a final concentration of 5% sucrose and 5, 2.5 and 1.25 M urea in extraction buffer. Purified particles of PPV-Lar (0.08 mg/ml virus) were mixed at a final concentration of 5 M urea and 5% sucrose in extraction buffer. Samples were left at room temperature for 15 min before they were analysed by NEWeB.

Both PPV strains caused distinguishable symptoms in N. benthamiana (PPV-Lar being milder and PPV-NAT causing necrotic patches and small leaves) and in mixed infections symptoms appeared initially milder but later appeared similar to those caused by PPV-NAT. Virus particles were negatively charged when electrophoresed either in 0.6 M phosphate buffer pH 7–5, or in 1 × TBE pH 8.3 (data not shown). Virus particles from plants infected with each of the two strains migrated at different positions in mixed agarose–acrylamide gels, and the migration behaviour was characteristic for each strain (Fig. 1a). PPV-Lar migrated more slowly than PPV-NAT and this seems to be an intrinsic property of the two strains.

When sap of doubly infected plants was subjected to native electrophoresis, the particles of the two strains could be clearly separated (Fig. 1b). Interestingly, in plants inoculated simultaneously with both strains, PPV-NAT particles were present in a higher concentration than those of PPV-Lar. PPV-NAT was detected in all three plants we had doubly infected, whereas PPV-Lar was hardly detected in two of the three plants (Fig. 1b, lanes 6, 7 and 8). In sap from extracts of singly infected plants mixed before electrophoresis, both strains were separated accordingly and detected with the same intensity. The detection of higher concentrations of PPV-NAT particles...
in doubly infected plants was in accordance with the observed symptoms, which were similar to those caused by this strain in *N. benthamiana*. Gel extracts from the position of PPV-Lar showed typical potyvirus particles in EM (data not shown).

We examined profiles of virus particle from different tissues, at different times post-infection (p.i.). The symptom-bearing systemically infected leaves (Fig. 2a, lane L; 2c, lane NL) always showed the highest accumulation of virus particles. It is interesting that in leaf samples virus particles appeared as two electrophorotypes, often migrating as a double band. When the petiole of the same leaf and stem of the same plant were studied, the virus was found to migrate as a single band (Fig. 2a, b, lanes P and S). This indicates that in these samples, at least two different forms of virus-like ribonucleoprotein particles were present. However, in the third, independent experiment, a slight double band was also apparent with virus material from stems (Fig. 2c, lane S). Interestingly, the amount of virus particles in the inoculated leaf was low, in contrast to systemically infected leaves (Fig. 2c, lane L). The appearance of the double bands was dependent on the sample, not on the type of gel material used (data not shown).

We also investigated the presence of HC-Pro in virus-like particles by using a specific polyclonal antibody raised against recombinant HC-Pro protein (originating from the PPV-NAT strain). We analysed extracts of plants infected with PPV-Lar and PPV-NAT. Virus particles were detectable in leaves and stems of plants infected by each virus strain (Fig. 3, CP). When the same extracts were examined with the HC-Pro antibody, a signal at the position of virus particles was visible in extracts of plants infected with PPV-Lar.

However, the HC-Pro signal was not always associated with virus particles in different tissues of the same plant. The HC-Pro signal was weaker on PPV-NAT particles (Fig. 3, HC-Pro, NAT), although the HC-Pro antibody was raised against the (homologous) PPV-NAT strain. The weak binding of HC-Pro to PPV-NAT particles might be a reason for the non-aphid-transmissibility of the PPV-NAT strain. Using SDS–PAGE and a conventional Western blot, we were also able to detect HC-Pro in purified virus preparations, a fact which indicates that HC-Pro–virus complexes can survive the purification procedure through a CsCl gradient (data not shown). We examined the stability of virus–HC-Pro particles after incubation of the extract with different concentrations of urea. Complexes of HC-Pro and virus were detectable at a concentration up to 2.5 M, while all complexes, including virus particles, were destroyed at 5 M urea (data not shown). The above results indicate that the observed virus–HC-Pro association is a stable one, resisting the denaturant activity of urea.

Electrophoresis of plant viruses under native conditions can be a helpful and simple tool for characterization of their particles. We have demonstrated that PPV strains can be separated by electrophoresis directly from plant extracts, and that they can be effectively transferred by electrobolting onto nitrocellulose membranes for further analysis. The method combines the analytical capacity of electrophoresis with the flexibility of using different antibodies for the characterization of the particles. It gives the advantage of dealing with intact virus or similar ribonucleoprotein particles directly from infected tissue and is simpler to perform compared to expensive and time-consuming techniques such as EM and centrifugation.

Using this method we were able to show that virus particles were present in all parts tested (systemically infected leaves, petioles, stems and roots) and that the inoculated leaves contained a lower amount of particles. This is in accordance with current models where replication (and consequently particle formation) in inoculated leaves is limited. We also
observed two electrophorotypes in some extracts. Formation of different electrophorotypes of another virus (Hibiscus chlorotic ringspot virus) in different hosts has been studied and the predominance of some of them in different hosts has been attributed to host selection (Hurtt, 1987). However, purification of virus from each host was necessary. Our study shows that NEWeB is a suitable method for electrophorotype characterization and suggests that there should be an analogous mechanism for the formation of different electrophorotypes of PPV-Lar in different organs of the same plant. Although adaptation of different electrophorotypes in different organs could not be ruled out, an explanation of the different patterns observed between organs could be given by the occurrence of virus forms suitable for long-distance transmission. Thus, the type of particle, which is present predominantly in the petiole and stems, might be the virus particle (or ribonucleoprotein) that is present or assembled in phloem tissues and actually transported over long distance.

Using NEWeB we were able to detect HC-Pro in association with virus particles and we observed that these complexes are stable under low urea concentrations. It is also interesting, however, that a small amount of PPV-Lar virus particles was found in association with HC-Pro in purified preparations and further investigation is needed to determine whether such particles differ from virus particles losing HC-Pro during purification.

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References


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