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Short communication

Localization of potato leafroll virus in leaves of secondarily-infected potato plants

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Abstract

Potato leafroll virus (PLRV) antigen was localized by immunogold labelling in semi-thin leaf sections of secondarily-infected potato plants cv. Bintje. Viral antigen was present in all cell types of the phloem tissue, but occurred most abundantly in the companion cells. Detectable amounts of PLRV antigen were found only in the sieve elements in veins with a large number of infected companion cells. Occasionally, parenchyma cells were also found to be infected. PLRV was not exclusively limited to the phloem tissue in the infected potato plants, but was also found in mesophyll cells neighbouring minor phloem vessels. Spread of virus from cell to cell in the mesophyll was not observed. The distribution of PLRV in the potato leaf tissue has implications on its availability for acquisition by aphids.

Potato leafroll virus (PLRV), a member of the luteovirus group [Waterhouse *et al.*, 1988], causes severe losses in potato crops worldwide [Kojima and Lapierre, 1988]. The virus is transmitted in a circulative non-propagative manner by a number of aphid species, of which *Myzus persicae* is the principal and most efficient vector [Sylvester, 1980].

Electron microscopic studies on potato plants infected with PLRV have revealed the presence of the virus-like particles in the phloem tissue, mature sieve elements, and companion cells, and plasmodesmata between these two cell types [Shepardson *et al.*, 1980; Golinowski *et al.*, 1987]. The sieve elements are the major nutritional source for aphids [Pollard, 1973]. As a consequence, aphids acquire virus particles along with phloem sap while feeding on infected plants. Previous work has shown that aphids acquire and transmit PLRV more readily from potato plants with higher titres of the virus than from plants with low titres [Barker and Harrison, 1986; Van den Heuvel *et al.*, 1993]. This correlation was most obvious early in the growing season. However, later in the growing season, PLRV acquisition did not correlate with the titres of viral antigen in the leaves and was markedly reduced [Barker and Harrison, 1986; Harrison, 1981; Van den Heuvel *et al.*, 1993]. A similar phenomenon was observed for PLRV-infected *Physalis floridana*. Leaves with distinct symptoms were poorer sources for PLRV acquisition than those showing faint symptoms [MacKinnon, 1962; Peters and Elderson, 1984; Van den Heuvel *et al.*, 1991]. Since viral antigen was still readily detected in *P. floridana* leaves with distinct symptoms, and in potato plants later in the growing season, apparently the amount of PLRV available for acquisition had decreased [MacKinnon, 1962; Van den Heuvel *et al.*, 1991, 1993]. It is not yet clear what may have caused the reduced availability of the virus in older plants. One of the contributing factors may be the spread of PLRV to plant cells that are not normally feed on by aphids. Barker [1987] reported that

a small number of parenchyma protoplasts obtained from *Nicotiana clevelandii* plants infected with PLRV were stained by fluorescent antibodies to the virus. To investigate whether PLRV also invades non-phloem tissue in potato, localization studies were performed on semi-thin sections of leaves from secondarily-infected potato plants cv. Bintje (plants grown from PLRV-infected tubers), using immunogold-labelling followed by silver enhancement.

The leaf material from glasshouse-grown potato plants, three and five weeks after planting the tubers was used for these studies. Tubers were infected with the Wageningen isolate of PLRV [Van der Wilk *et al.*, 1989]. Three-week-old plants still in the sprout stage were used. At the moment of sampling, sections were made from the nearly symptomless top leaves. Single-aphid transmission experiments with one-day-old *M. persicae* nymphs were performed using these leaves as a virus source; the median acquisition access period (AAP_{50}), i.e. the period of time after which 50% of the nymphs acquired enough virus to become infectious, was less than 48 hours. In these experiments, 30 nymphs were individually tested for their ability to infect a *P. floridana* seedling after acquisition access periods of 48, 72 and 96 h. Bottom leaves taken from five-week-old plants were poor sources for virus acquisition since the AAP_{50} was longer than 96 hours, even though the virus was readily detected in the leaves by ELISA, and interveinal chlorosis and reddening of the leaf margins was clearly visible.

For semi-thin sectioning, small pieces of tissue between primary and secondary veins of the PLRV-infected leaves were fixed with 3% (w/v) glutaraldehyde, 2% (w/v) paraformaldehyde and 1.5 m*M* $CaCl_2$ in a phosphate-citrate buffer, pH 7.2 (0.1 *M* Na_2HPO_4 and 2.7 m*M* citric acid) for 1 h under a vacuum of approximately 60 mbar at room temperature, and held at 4 °C for 16 h. Dehydration and low temperature embedding in LR-Gold was done essentially as described by Van Lent *et al.* [1990]. Semi-thin sections (0.5–1 μm) were cut with a glass knife on a LKB Ultratome, transferred onto a droplet of 40% acetone on dimethyldichlorosilane-treated glass slides and dried on a hot plate at 40 °C for about 10 min. Mounted semi-thin sections of PLRV-infected leaf material and appropriate controls were preincubated with PBS-BSA (0.02 *M* sodium phosphate buffer, pH 7.4, containing 0.14 *M* NaCl, 2 m*M* KCl and 1% (w/v) bovine serum albumin). After 1 h, PLRV-specific monoclonal (Mabs) and polyclonal antibodies at a concentration of 0.2 μg per mL PBS-BSA were added and incubated for 2 h. Sections were then washed with PBS and exposed to rabbit anti-mouse antibodies coupled to 10 nm gold particles for 1.5 h. The gold-conjugate was diluted in PBS to an optical density of 0.1 at 520 nm. Next, the sections were washed with PBS and the immobilized immune-complexes fixed in 1% (v/v) glutaraldehyde for 15 min. Glutaraldehyde was removed by extensive rinsing with distilled water. Subsequently, gold particles were silver-enhanced by incubating the treated sections with a 1:1 mixture of initiator and enhancer (Intense BL silver staining kit; Janssen Pharmaceutica B.V., Tilburg, Netherlands) for 8–10 min followed by washing with distilled water and staining with 0.1% (w/v) toluidine blue. All incubation steps were performed at room temperature. Stained sections were examined with epi-illumination in a Leitz Orthoplan microscope equipped with water-immersion objective lenses and a polarization filter block (epipolarization microscopy).

Since glutaraldehyde/paraformaldehyde (GA/PF) fixation of infected tissue can negatively affect the antigenic properties of the viral antigen [Garret *et al.*, 1991], PLRV-specific Mabs were first tested for their ability to detect virus treated with GA/PF in a triple antibody sandwich (TAS) ELISA. In this assay, PLRV was trapped on the solid phase by polyclonal antibodies and incubated overnight at 4 °C with the same concentrations of GA and PF as used for fixation of leaf material. All nine conformation-dependent PLRV-specific Mabs from the WAU-panel [Van den Heuvel *et al.*, 1990], and SCR5 from the Scottish Crop Research Institute [Massalski and Harrison, 1987] were screened. It was noted that the GA/PF treatment greatly reduced the reactivity of the virus with these antibodies as compared to the untreated virus. Only WAU-A6, -A7, -A12, and SCR5 retained their capacity to tag the virus (results not shown). Since none of the WAU-Mabs gave a positive signal in the immunogold labelling experiments, SCR5 was used throughout this study. Western blot analysis showed that SCR5 is directed to a linear epitope of the major coat protein of PLRV. Testing the affinity of SCR5 for intact and degraded PLRV in ELISA revealed that it detected either type equally. Polyclonal antisera to PLRV were unsuitable to be used in the labelling procedures due to a high level of aspecific labelling of the tissue.

The staining technique used in combination with epipolarization microscopy, revealed the presence of PLRV antigen as a bright blue-coloured signal, mainly in the phloem tissue. Based on the intensity of the colour reaction, viral antigen was most abundant in

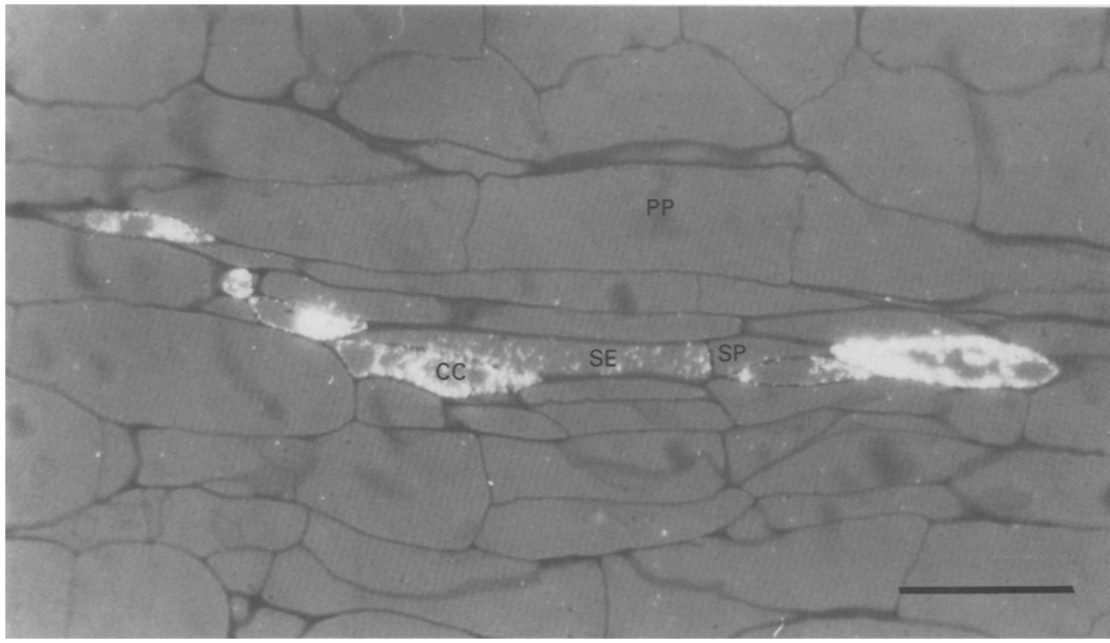

Fig. 1. Leaf tissue from potato plants cv. Bintje secondarily infected with PLRV. Multiple companion cells (CC) are infected and accumulation of viral antigen in a sieve element (SE) is visible. SP, sieve plate; PP, parenchyma. *Bar* represents 50 μm.

the companion cells (Fig. 1). Examination of serial sections from several pieces of a single leaf showed that the virus was quite unevenly distributed within a leaf. The number of companion cells infected varied markedly between the sections. The labelling signal in companion cells of older and younger leaves was comparable. This observation seems to contradict the fact that older leaves were poorer sources for virus acquisition than the younger ones. Unfortunately, it could not be assessed whether the viral antigen in these leaves derived form intact or degraded virus. None of the conformation-dependent Mabs from the WAU-panel could be used in the labelling procedures, and SCR5 recognizes a surface-exposed linear epitope.

Presence of luteoviruses in sieve elements of phloem vessels was previously reported in ultrastructural studies on beet western yellows virus (BWYV) in *Thlaspi arvense*; virus particles in this virus/host combination were most conspicuously found associated with peripheral endoplasmatic reticulum in the developing sieve elements [D'Arcy and De Zoeten, 1979]. In our studies, however, we never observed a clear signal in these areas, irrespective of the age of the potato plants. Apparently, the level of PLRV antigen in functional sieve elements which mediate the systemic spread of the virus in the plant is too low to be detected by the technique used. However, in the bottom leaves of older plants, we infrequently detected PLRV antigen in the lumen of a single sieve element connected to an infected companion cell (Fig. 1). Thickened cell walls at these sites were readily discernable (Fig. 1). It is probable that phloem transport is obstructed in these elements due to excessive callose formation at the sieve plates. Luteovirus accumulation has so far only been reported in the lumina of degenerating sieve elements of *T. arvense* infected with BWYV [D'Arcy and De Zoeten, 1979].

Occasionally, we found PLRV antigen stained in the cytoplasm of phloem parenchyma cells of either old and young leaves (results not shown). More frequently, however, PLRV antigen was present in mesophyll cells neighbouring infected companion cells (Figs. 2a, b) in older leaves. Serial sectioning revealed that these mesophyll cells were always immediately adjacent to companion cells in minor veins protruding into the mesophyll tissue (Fig. 2b). Since the intensity of staining in both the mesophyll and companion cells was comparable, we concluded that either cell type supports PLRV replication to a similar extend. Evidence for cell to cell spread of PLRV within the mesophyll tissue was not observed. Even though we did not approach the distribution of PLRV in a quantita-

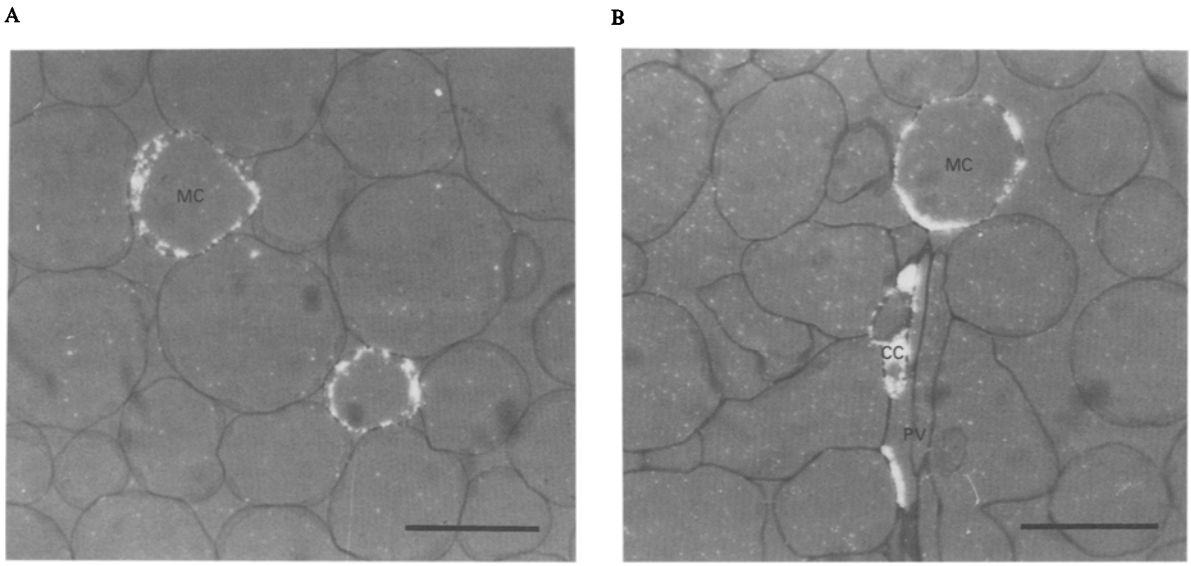

Fig. 2. (A) Mesophyll cells (MC) of a PLRV-infected potato plant cv. Bintje; (B) Protrusion of a minor phloem vessel (PV) into mesophyll tissue. CC, comparison cell. *Bar* represents 50 μm.

tive manner, it could be concluded that the number of infected mesophyll cells was too low to explain the reduced availability of the virus in older potato plants.

The localization studies revealed an uneven distribution of PLRV in the phloem tissue. In some veins or part of veins infected companion cells were observed only occasionally, whereas in others many of these cells displayed clear labelling signals (Fig. 1). Only in the latter situation, frequently seen in old leaves, the lumen of individual sieve elements contained detectable amounts of viral antigen. The uneven distribution of PLRV in the phloem tissue suggests that systemic transport of the virus in heavily virus-infested vessels is considerably impaired. While feeding on PLRV-infected potato, aphids apparently avoid these vessels, and as a result of increasing disease development and cytopathological changes in infected plant tissue, fewer aphids would acquire virus thus reducing the likelihood of virus transmission. Electronically monitoring of aphid penetration behaviour [Tjallingii and Hogen Esch, 1992] combined with localization studies of the virus in the penetrated tissue might provide further information on this matter.

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References

Barker H (1987) Invasion of non-phloem tissue in *Nicotiana clevelandii* by potato leafroll luteovirus is enhanced in plants also infected with potato Y potyvirus. J Gen Virol 68: 1223–1227

Barker H and Harrison BD (1986) Restricted distribution of potato leafroll virus antigen in resistant potato genotypes and its effect on transmission of the virus by aphids. Ann Appl Biol 109: 595–604

D'Arcy CJ and De Zoeten GA (1979) Beet western yellows virus in phloem tissue of *Thlaspi arvense*. Phytopathology 69: 1194–1198

Garret A, Kerlan C and Thomas D (1991) Visualization of a luteovirus in the vector aphid's body by two gold immunolabelling techniques: a comparative study. J Virol Methods 35: 93–107

Golinowski W, Tomenius K and Oxelfelt P (1987) Ultrastructural studies on potato phloem cells infected with potato leafroll virus – comparison of two potato varieties. Acta Agric Scand 37: 3–19

Harrison BD (1981) Two sensitive serological methods for detecting plant viruses in vectors and their suitability for epidemiological studies. Proceedings 1981 British Crop Protection Conference – Pests and Diseases, pp. 751–757

Kojima M and Lapierre H (1988) Potato leafroll virus (PLRV). In: Smith IM, Dunez J, Phillips DH, Lelliot RA and Archer SA (eds) European Handbook of Plant Diseases, pp. 23–24. Blackwell Scientific Publications, Oxford

MacKinnon JP (1962) Comparative studies between the aphid transmission of potato leafroll and turnip latent viruses. Can J Bot 40: 525–531

Massalski PR and Harrison BD (1987) Properties of monoclonal antibodies to potato leafroll luteovirus and their use to distinguish virus isolates differing in aphid transmissibility. J Gen Virol 68: 1813–1821

Peters D and Elderson J (1984) Some observations on the mechanism of the persistent virus transmission – a study with potato leafroll virus. Acta Bot Neerl 33: 238–239

Pollard DG (1973) Plant penetration by feeding aphids (Hemiptera, Aphidoidea): a review. Bull Entomol Res 62: 631–714

Shepardson S, Esau K and McCrum R (1980) Ultrastructure of potato leaf phloem infected with potato leafroll virus. Virology 105: 379–392

Sylvester ES (1980) Circulative and propagative virus transmission by aphids. Annu Rev Entomol 25: 257–286

Tjallingii WF and Hogen Esch Th (1993) Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. Physiol Entomol 18: 317–328

Van den Heuvel JFJM, Boerma TM and Peters D (1991) Transmission of potato leafroll virus from plants and artificial diets by *Myzus persicae*. Phytopathology 81: 150–154

Van den Heuvel JFJM, De Blank CM, Goldbach RW and Peters D (1990) A characterization of epitopes on potato leafroll virus coat protein. Arch Virol 115: 185–197

Van den Heuvel JFJM, Dirven JAAM, Van Os GJ and Peters D (1993) Acquisition of potato leafroll virus by *Myzus persicae* from secondarily-infected potato plants of different genotypes. Potato Res 36: 89–96

Van der Wilk F, Huisman MJ, Cornelissen BJC, Huttinga H and Goldbach R (1989) Nucleotide sequence and organization of potato leafroll virus genomic RNA. FEBS Lett 245: 51–56

Van Lent JWM, Groenen JTM, Klinge-Roode EC, Rohrmann GF, Zuidema D and Vlak JM (1990) Localization of the 34 kDa polyhedron envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. Arch Virol 111: 103–114

Waterhouse PM, Gildow FE and Johnstone GR (1988) Luteovirus group. CMI/AAB Descriptions of Plant Viruses. No. 339