J Gen Plant Pathol (2005) 71:238–242
DOI 10.1007/s10327-005-0189-0

VIRAL AND VIROID DISEASES

Short communication

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Immunolocalization of *Pepper mild mottle virus* in *Capsicum annuum* seeds

Received: July 21, 2004 / Accepted: September 10, 2004

Abstract To clarify the mechanism of seed transmission of *Pepper mild mottle virus* (PMMoV), the virus was immunolocalized in *Capsicum annuum* seeds using fluorescence microscopy. Two distinct patterns were observed: In the first, PMMoV was present in the epidermis and parenchyma but not in the endosperm or embryo; in the second, the virus was restricted to the surface of the epidermis and parenchyma. These findings shed light on the fundamental mechanisms of seed transmission of tobamoviruses and may aid in the design of new methods to prevent the spread of seedborne virus diseases.

Key words *Capsicum annuum* · Immunolocalization · *Pepper mild mottle virus* · Seed transmission · Trisodium phosphate · Tobamoviruses

The first report of a disease caused by seed transmission of a virus, *Bean common mosaic virus*, was published early in the last century (Stewart and Reddick 1917). Approximately 10% of all plant viruses are currently recognized as being seed-transmitted (Fauquet and Mayo 1999; Mink 1993). These viruses can spread between distant locations via contaminated seeds and cause serious economic losses. Accurate diagnoses of viral infections in commercial seed lots and the effective elimination of contaminated seeds are necessary to produce certified seed stocks and minimize the risk of spreading the pathogens.

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There are two distinct types of seed infection by viruses. Seedborne viruses do not infect germinated seedlings produced by the seeds that carry them. In contrast, seed-transmitted viruses infect seedlings produced by the host seeds (Neergaard 1977). Viruses in the latter category generally invade the seeds during embryonic development, resulting in high rates of natural infection among the germinated seedlings. For example, *Pea seed-borne mosaic virus* infects pea embryos during early development (Wang and Maule 1994). The seed transmission of members of the genus *Tobamovirus* is an exception. These pathogens, which are mainly carried on tomato, pepper, and cucumber seed coats, do not infect the developing embryos; however, they can infect germinating seedlings as a result of mechanical contact with contaminated seeds (Broadbent 1965; Crowley 1957; Demski 1981; Nagai et al. 1974; Taylor et al. 1961).

Pepper mild mottle virus (PMMoV) is one of the major viral pathogens of cultivated peppers; this tobamovirus causes mosaic symptoms and malformations on the leaves and fruit of green peppers, reducing the yield of commercial products worldwide (Alonso et al. 1989; Nagai 1981). PMMoV consists of a rod-shaped particle with a length of about 300 nm and a width of 18 nm (Wetter and Conti 1988) in which a positive-sense linear single-stranded RNA (6.3 kb) is encapsidated (Alonso et al. 1991; Kirita et al. 1997). PMMoV is transmitted among plants through mechanical contact with contaminated sources, soil, and seeds (Lewandowski 1999). The infection of pepper plants by one strain of *Tobacco mosaic virus* (TMV), probably PMMoV, was first reported in 1952 when seedlings from seeds harvested from infected plants became contaminated without any artificial inoculation with the virus, demonstrating seed transmission of tobamoviruses (McKinney 1952). Subsequent studies have revealed tobamoviruses, including PMMoV, on and in the seed coat (Crowley 1957; Demski 1981; Sakamoto and Matsuo 1972) but rarely in the embryo-endosperm (Demski 1981), during seed transmission. Transmission on or in the seed coat without infection of the embryo is comparatively rare among viruses (Lewandowski 1999) and might be related to the high stability of the virus

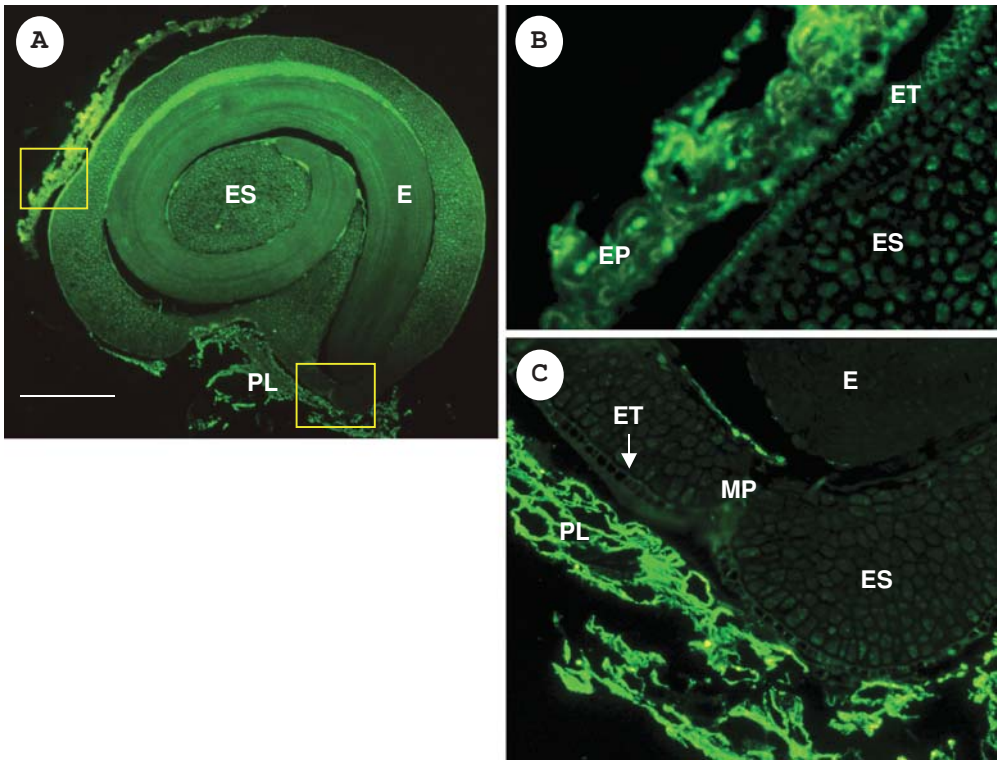

Fig. 1. Longitudinal sections of the disk-shaped face of a *Pepper mild mottle virus* (PMMoV)-positive pepper seed. **A** Immunofluorescence micrograph of the seed. **B** Higher magnification of the seed coat (in upper left *box* in **A**). **C** Higher magnification of section through the micropyle (in lower right *box* in **A**). *E*, embryo; *EP*, epidermis; *ES*, endosperm; *ET*, endothelium; *MP*, micropyle; *PL*, placenta. *Bar* 1 mm

particles. Accordingly, seedling infection by tobamoviruses occurs primarily through mechanical contact with contaminated sources, which is generally a result of handling by farmers.

Effective treatments are required to prevent the spread of tobamoviruses via seeds. Exposure to a 10% solution of trisodium phosphate for 20 min has been put forward as a method to eliminate TMV from the surface of contaminated tomato seeds, although this technique does not destroy TMV particles in the endosperm (Broadbent 1965; Taylor et al. 1961). Furthermore, this method is unable to eradicate tobamoviruses from the seed coats of infected pepper seeds (Crowley 1957; Nagai 1981). To elucidate the mechanism of PMMoV transmission and to evaluate the effectiveness of trisodium phosphate, we used immunocytochemistry to investigate PMMoV infection sites anatomically in green pepper (*Capsicum annuum*) seeds.

Naturally infected pepper seeds were obtained from commercial seed lots derived from susceptible pepper plants grown in fields with PMMoV-contaminated soil, which carries no tobamovirus-resistant genes, such as the *L* genes. As a negative control, healthy seeds were obtained from pepper plants that were cultivated in fields with no history of PMMoV infection. Each seed was dissected into two parts, and one sample was subjected to a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) to test for PMMoV infection. If the seed was positive for virus infection, the second sample was then used in an immunofluorescence study to identify infection sites. These samples were prepared for analysis using a polyclonal rabbit antibody to PMMoV (Tsuda et al. 1998) following staining with Alexa Fluor 488 goat anti-rabbit IgG (H + L) conjugate (Molecular Probes, Eugene, OR, USA). Briefly, each sample was fixed with 4% paraformaldehyde and 1% glutaraldehyde in 10 mM phosphate buffer. The samples were then dehydrated, embedded, and sectioned, as described previously (Ohnishi et al. 2001; Tsuda et al. 1996). Finally, after incubation with the antibodies, the samples were observed with a fluorescence microscope (AH3-RFC, Olympus, Tokyo, Japan; or MZ-FL III, Leica, Heerbrugg, Switzerland).

Several previous reports have detailed the internal anatomy and morphology of pepper seeds (Bosland and Votava 2000; Chen and Lott 1992; Kondo 1933; Moeller 1886), which comprise a seed coat, endosperm, and embryo. The seed coat has two layers, epidermis and parenchyma, which are both derived from the maternal plant (as in other solanaceous plants). The outermost layer of the endosperm is the endothelium (an inner placenta) and is derived from the parenchyma (Fig. 1).

In the present study, PMMoV was localized in the epidermis and parenchyma of infected seeds. This was confirmed by observing longitudinal sections of both the disc-shaped (Fig. 1) and fusiform-shaped (Fig. 2) faces of the seed coat, collapsed epidermal cells (EP in Figs. 1B and 2B), the placenta of the maternal plant (PL in Figs. 1C and 2B). The virus was not detected in either the endosperm or the embryo of the PMMoV-positive seeds. In contrast, in a small number of infected seeds, the virus was present only on the outer surface of the epidermis and placenta (EP in Fig. 3B,C), and none of the internal tissues were infected. An orange autofluorescence in the endothelium was often

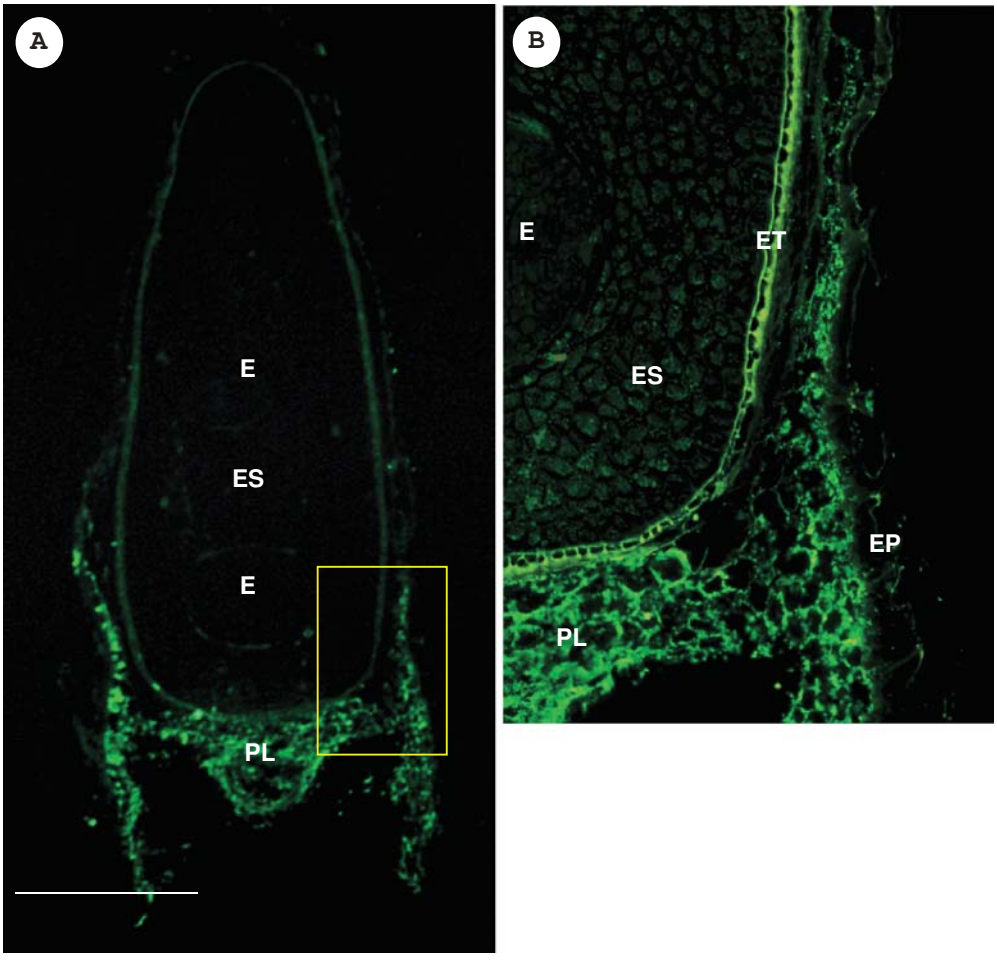

Fig. 2. Longitudinal sections of the fusiform-shaped face of a PMMoV-positive pepper seed. **A** Immunofluorescence micrograph of the seed. **B** Higher magnification of the seed coat (in *box* in **A**). *E*, embryo; *EP*, epidermis; *ES*, endosperm; *ET*, endothelium; *PL*, placenta. *Bar* 1 mm

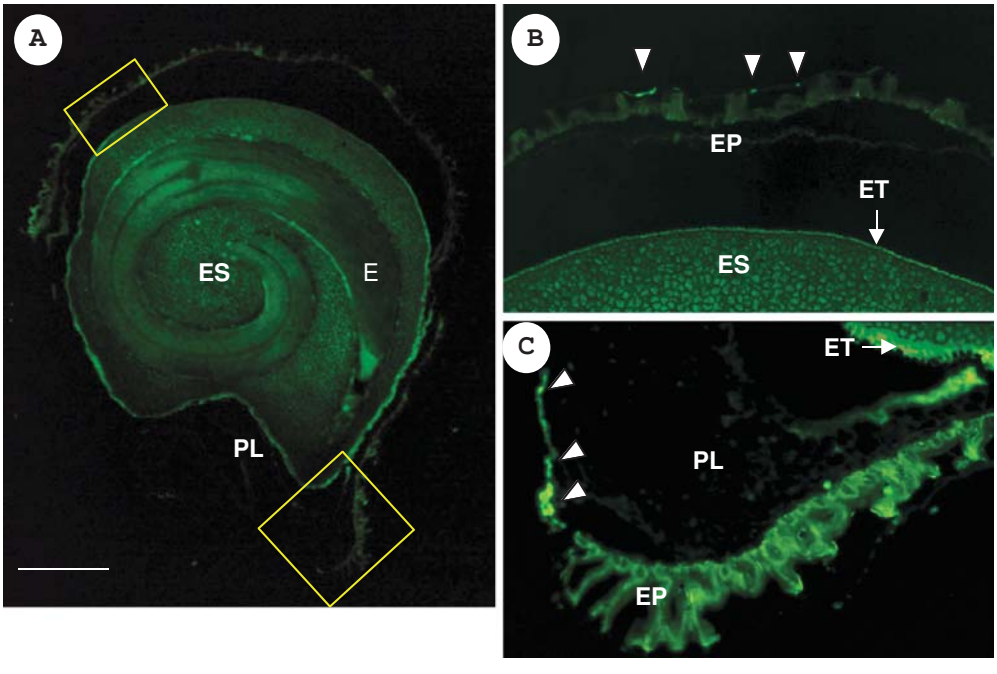

Fig. 3. Longitudinal sections of the disk-shaped face of a PMMoV-positive pepper seed, in which the virus is present only on the outer surface of the epidermis and placenta (*arrowheads*). **A** Immunofluorescence micrograph of the seed. **B** Higher magnification of the seed coat (in upper left *box* in **A**). **C** Higher magnification of the micropyle (in lower right *box* in **A**). *E*, embryo; *EP*, epidermis; *ES*, endosperm; *ET*, endothelium; *PL*, placenta. *Bar* 1 mm

observed and was easily distinguishable from virus-specific signals that fluoresced green (ET in Figs. 2B and 3C). Two distinct distribution patterns were therefore observed among the PMMoV-positive pepper seeds, which might reflect different methods of virus transmission. Viral particles from an infected maternal plant could enter the precursor tissues that develop into the epidermis, parenchyma, and placenta during seed development, leading to viral invasion of mature tissues in the seed (as seen in Figs. 1 and 2). This type corresponds to the seed-transmission route. Conversely, mechanical contact from contaminated sources during the harvesting of pepper seeds might result in the attachment of viral particles to the seed coat surface (i.e., the outer surface of the epidermis and placental tissues) (arrowheads in Fig. 3). This type corresponds to seed-borne virus transmission. The endothelium appeared to mark a strict boundary between the endosperm and the parenchyma, with the placenta acting as a defensive border against virus invasion into the internal seed tissues. Of the 10 seeds examined, PMMoV was present in the epidermis, parenchyma, and placenta of nine seeds, whereas the virus localized to the outer surface of one seed.

A small number of previous studies have addressed the localization of TMV in pepper seeds. Crowley (1957) reported that TMV was detected only in the testa or seed coat of infected pungent pepper seeds. In addition, Sakamoto and Matsuo (1972) showed that a TMV-contaminating seed coat spontaneously released from pepper seedlings in bud did not lead to infection during germination. Our results revealed the absence of PMMoV in the embryo, which supports the findings of previous studies.

In a TEM investigation to localize TMV in infected petunia plants, large numbers of virus particles were found in partially degenerated integument cells of immature seeds (Hosokawa and Mori 1983). These aggregates were also detected in the integument cells of mature seeds; however, TMV was not detected in either the endosperm or embryo of infected plants during seed development. Interestingly, PMMoV retains its infectivity toward pepper plants when the seeds are stored for long periods of time, whereas the seed transmission of TMV is not established in the petunia. Hosokawa and Mori (1983) suggested that TMV might be inactivated through catabolism of the integument cells rather than persisting in a dehydrated state in the testa of maturing seeds. In pepper seeds, although the parenchyma derived from the integument degenerates during seed development, an abundant layer of placental cells persists. TMV might be absent in petunia seeds because they lack the infected layer of the placenta, suggesting that the virus might be unable to spread throughout the seeds. PMMoV infection of the placental layer might therefore account for the successful transmission of this pathogen in pepper seeds.

Several chemical treatments have been proposed to eradicate tobamovirus infections in pepper seeds, including 9% hydrochloric acid solution, calcium hypochlorite solution, 4.2% sodium hypochlorite solution, and 10% trisodium phosphate solution; however, none of these methods has proved effective (Demski 1981; Nagai 1981). For example, Nagai (1981) showed that the application of 10% trisodium phosphate solution did not eliminate TMV on the back of the seed coat or in the parenchyma of seeds. In conjunction with these previous findings, our results point to modifications that might improve the use of chemicals in the treatment of contaminated seeds. We have shown that viruses might localize in the deeper tissues of infected pepper seeds (e.g., epidermis, placenta, parenchyma) derived from the maternal plant. Chemicals applied to the seed surface might be unable to reach these pathogens, and PMMoV particles that survive in the placenta and maintain their infectivity toward seedlings remain a risk. Additional reagents or treatment methods that enable antiviral chemicals to penetrate deeper into the placenta are therefore necessary to increase the effectiveness of such treatments.

Acknowledgment The authors thank Eisho Nishino of Chiba University, Japan for valuable information about pepper seed anatomy.

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