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RESEARCH ARTICLE

A Model for Seed Transmission of a Plant Virus: Genetic and Structural Analyses of Pea Embryo Invasion by Pea Seed-Borne Mosaic Virus

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Pea seed-borne mosaic virus (PSbMV), a seed-transmitted virus in pea and other legumes, invades pea embryos early in development. This process is controlled by maternal genes and, in a cultivar that shows no seed transmission, is prevented through the action of multiple host genes segregating as quantitative trait loci. These genes control the ability of PSbMV to spread into and/or multiply in the nonvascular testa tissues, thereby preventing the virus from crossing the boundary between the maternal and progeny tissues. Immunocytochemical and in situ hybridization studies suggested that the virus uses the embryonic suspensor as the route for the direct invasion of the embryo. The programmed degeneration of the suspensor during embryo development may provide a transient window for embryo invasion by the virus and could explain the inverse relationship between the age of the mother plant for virus infection and the extent of virus seed transmission.

INTRODUCTION

Approximately 20% of plant viruses are transmitted from generation to generation in the seed (Matthews, 1991; Mink, 1993), and yet very little is known about the mechanism(s) involved. The process of virus seed transmission is environmentally influenced and is a consequence of a specific interaction between the virus and the combined physiology of two generations of the host plant (Carroll, 1981). The host genetic basis for this interaction has been studied in only one case, that of barley stripe mosaic virus (BSMV) in barley; in this host, a single recessive gene was implicated in the regulation of seed transmission (Carroll et al., 1979). In no case has it been determined whether it is the genetic complement of the maternal or progeny tissues that determines the efficiency of seed transmission.

Seed transmission is achieved either by direct invasion of the embryo via the ovule or by indirect invasion of the embryo, mediated by infected gametes. For some viruses in certain hosts (e.g., BSMV in barley), both processes operate simultaneously, although the relative contribution of the two processes will vary depending upon a large number of factors (Mandahar, 1981). For direct embryo invasion, there is currently no explanation for how the virus is able to cross the boundary between the parental and progeny generations in the ovule, and no routes have been identified that lead to the establishment of the virus in the tissues of the developing embryo. Furthermore, genetic and cell biological studies have never been combined to obtain an overall understanding of the principles involved in seed transmission.

Pea seed-borne mosaic virus (PSbMV) is one of many potyviruses that are seed transmitted in legumes and that are of major economic importance (Khetarpal and Maury, 1987). Typical for this group of viruses, PSbMV has a (+) sense RNA genome of 9.9 kb (Johansen et al., 1991) that is encapsidated within a flexuous filamentous particle. Pea cultivars assessed for seed transmission of PSbMV have been shown to vary from high efficiency to zero, although all were susceptible to infection in the vegetative tissues (Stevenson and Hagedorn, 1973; Wang et al., 1993). A recent study (Wang and Maule, 1992) of two cultivars with extremes of seed transmission efficiency showed that neither the male nor female gametes provide the route to embryo invasion, but rather that PSbMV directly invades pea embryos early in development and multiplies within the embryonic tissues. It was suggested that embryo invasion, and hence seed transmission, might occur only during a "window" in the development of the embryo and that such a "window" might be subject to environmental influence.

We provide evidence here that PSbMV invasion of the embryo occurred via the suspensor, which is a transient structure in embryo development that is believed to serve as a conduit for nutritional support of the growing embryo. We propose that degeneration of the suspensor "closes the window" for virus transmission and that resistance to seed transmission is

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effected by maternal host genes, which prevent the virus from reaching this structure before this time.

RESULTS

Seed Transmission of PSbMV Is Determined by the Maternal Genotype

In a previous study (Wang and Maule, 1992), we have shown that PSbMV is not transmitted via pollen in the pea cultivars Vedette and Progreta. To address directly whether it was the genetic complement of the maternal tissues or the genetic complement of the progeny tissues that determined the efficiency of seed transmission, we attempted to introduce either susceptibility or resistance to seed transmission into the heterologous maternal background. This was achieved by reciprocal cross-pollination experiments between cultivar Vedette (40 to 80% seed transmission) and cultivar Progreta (0% seed transmission) when the recipient parent had already been infected with PSbMV. Table 1 details two experiments in which paired pods at single nodes were compared and shows that seed transmission efficiency was similar whether the flowers had been self- or cross-pollinated (Table 1). Hence, the genotype of the progeny embryo did not influence the degree of seed transmission appreciably.

Multiple Host Genes Are Involved in Determining Resistance to PSbMV Seed Transmission

To further investigate the genetic complexity of host determinants controlling PSbMV seed transmission, reciprocal crosses between uninfected plants of cultivars Vedette and Progreta were made. Uninfected F_1 and F_2 plants were subsequently inoculated with PSbMV after the emergence of two leaves and compared for their seed transmission behavior. The data from these crosses are presented in Figure 1. To minimize the effects of environmental influence on the seed transmission character, parental lines (F_0) and the F_1 and F_2 populations were germinated, inoculated with PSbMV, and maintained together in a glasshouse at 18 to 22°C until maturity. Seed transmission of PSbMV was scored as the percentage of transmission in individual mother plants and the mean efficiency in the population as a whole.

The parental lines showed seed transmission efficiencies of 0% (cv Progreta) and 60% (cv Vedette). Both reciprocal F_1 populations showed a reduced efficiency of seed transmission (4 and 7%) when compared to cultivar Vedette, although this was higher than in cultivar Progreta (Figure 1), indicating that resistance to seed transmission was incompletely dominant. Segregation of the percentage of seed transmission in

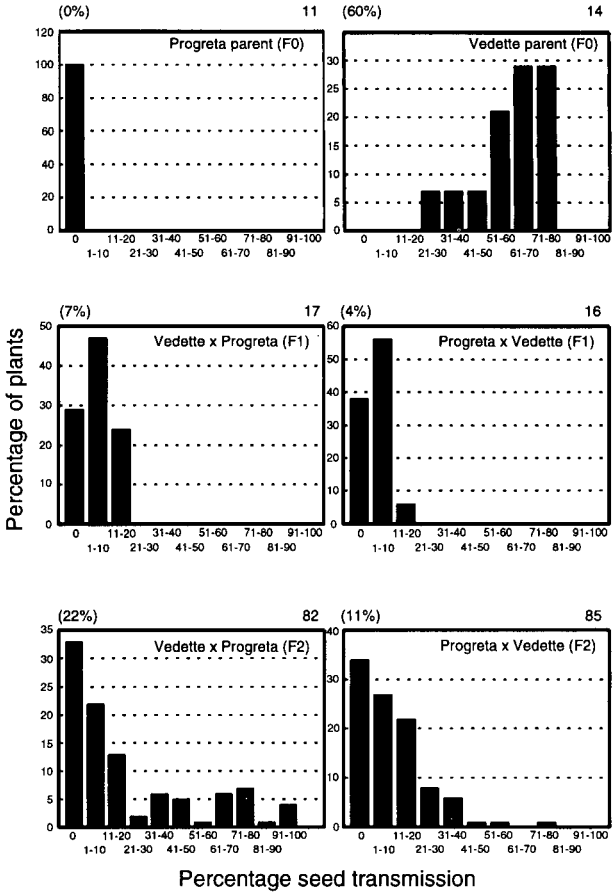

Figure 1. Segregation of the Seed Transmission Character in Reciprocal Crosses.

The distribution of the percentages of seed transmission in individual mother plants in the F_0, F_1, and F_2 populations arising from reciprocal crosses between cultivars Vedette and Progreta is shown. For the F_1 and F_2 crosses, the pollen recipient is listed first. Numbers above each histogram define the number of individual parent plants analyzed (right), and the mean efficiency of seed transmission (left) is given within parentheses. Between 20 and 50 seeds per plant were analyzed.

Table 1. An Assessment of the Role of the Maternal Genotype in Determining PSbMV Seed Transmission

Plant Populations[a]	No. of Seeds Examined[b]	Seed Transmission (%)
$Progreta_I$, Self-Pollination	120	0
$Progreta_{I♀} \times Vedette_{H♂}$	182	0
$Vedette_I$, Self-Pollination	87	42
$Vedette_{I♀} \times Progreta_{H♂}$	74	47

[a] Subscripts: I, Infected plants; H♂, healthy plants as pollen donor; I♀, infected plants as pollen recipient.
[b] Germination rate was 100%.

the F_2 populations was incomplete, which suggested a quantitative character. The pattern of segregation was similar for the reciprocal populations (Figure 1). For the assessment of segregation in the F_2 populations, ~80 plants from each reciprocal cross were used. With this number, the occurrence of individual F_2 mother plants with seed transmission percentages equivalent to the extremes reached by the two parental lines suggested that the trait was controlled by only a few genes, although it was not possible to calculate how many.

Pattern of PSbMV Distribution in the Maternal Tissue Correlates with the Extent of Seed Transmission

The organization of the reproductive tissues and development of the embryo of pea have been described in detail by several authors (Cooper, 1938; Marinos, 1970; Davies and Williams, 1985). Of particular relevance to the work presented here is the organization of the immature seed during the early stages of embryo development. This is illustrated diagrammatically in Figure 2. Briefly, soon after fertilization, which occurs in the micropylar region, the integument tissue of the ovule develops into the testa of the immature seed, and zygotic divisions lead to the formation of a globular terminal cell and a suspensor apparatus. The testa is of maternal origin and maintains vascular continuity with the carpel tissues via the funiculus; the vascular strand terminates one-half to two-thirds around the periphery of the ovule. The suspensor consists of two elongated and expanded basal cells and two globular middle or parabasal cells supporting the developing embryo (in our work with two pea culivars, however, we have only ever observed a single parabasal cell in the suspensor). The basal cells maintain intimate contact with the testa in the micropylar region of the ovule and extend into the embryo sac, providing positional and nutritional support to the developing embryo (Raghavan, 1986). The multinucleate embryo sac contains the fluid endosperm and is lined by the endospermic cytoplasm and a boundary wall derived from the wall of the pre-fertilization megaspore cell (Marinos, 1970). An extracellular sheath (not shown in Figure 2) covering the developing embryo and the suspensor is also deposited by the activity of the embryo sac (Marinos, 1970).

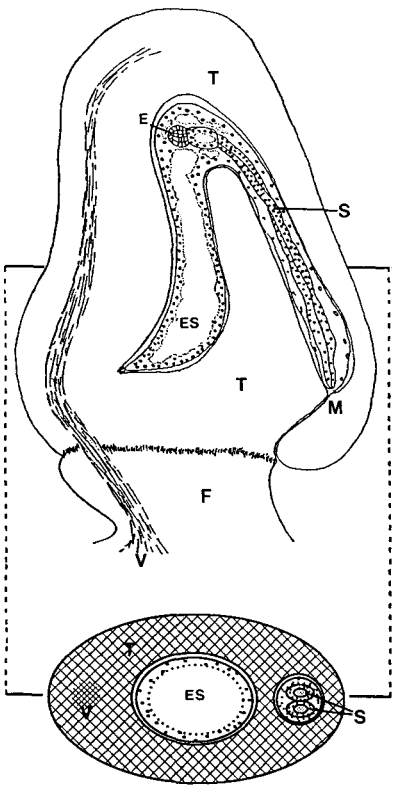

Figure 2. Diagrammatic Representation of an Immature Pea Seed at the Globular Stage of Embryo Development.

The organization of the various tissues of the developing pea seed is shown in longitudinal and cross-sections of the ovule. E, embryo; ES, embryo sac; F, funiculus; M, micropyle; S, suspensor; T, testa; V, vascular strand. Modified from Cooper (1938).

To understand how expression of the maternal genes determines seed transmission, we compared the pattern of PSbMV accumulation in the ovule tissues of cultivars Progreta and Vedette before and after fertilization. Previously, we have shown that PSbMV is not present in mature pollen and, by using electron microscopy, that it cannot be detected in cells of the integument prior to fertilization (Wang and Maule, 1992). Also, we have shown that the vegetative tissues of the two cultivars are equally susceptible to PSbMV, indicating that differences in virus distribution related to seed transmission would probably occur in the reproductive tissues (Wang et al., 1993).

It has been proposed that seed transmission could be mediated by viral RNA rather than virus particles (Carroll, 1981). To allow for the possibility that detection of either virus coat protein or viral RNA alone may not give a complete picture of the seed transmission process, we used the techniques of immunohistochemistry (with a monoclonal antibody raised against PSbMV particles) and in situ hybridization (with an RNA probe specific for the [+] sense of the viral RNA genome). We applied these techniques to serial sections of infected ovules harvested at different times during the early phase of embryo development. It was always found that similar patterns of PSbMV distribution were obtained in consecutive sections treated, respectively, by the two techniques.

Sections of the ovary tissue from unfertilized flowers of cultivars Progreta and Vedette shown in Figures 3A and 3B revealed that PSbMV was abundant in and around the vascular tissues running the length of the upper and lower edges of the carpel. Invasion of the unfertilized ovule was seen occasionally as patches of infected tissue at the edges of the ovules of both cultivars (Figure 3B, arrow). In agreement with our previous study (Wang and Maule, 1992), virus was never detected in the egg cell, the synergid, or the antipodal cells. Fertilization appeared to trigger the ingress of the virus into the ovule along the vascular strand and into the surrounding

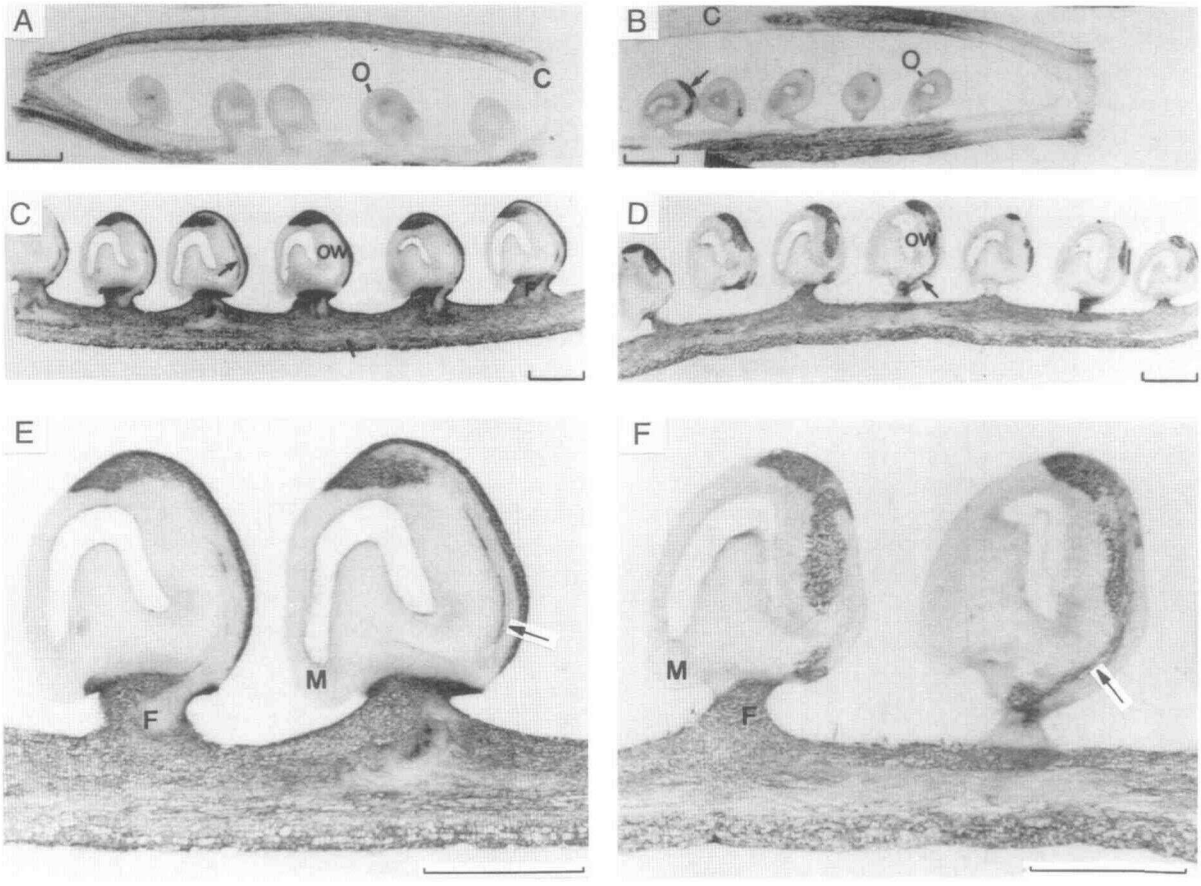

Figure 3. Distribution of PSbMV RNA in the Ovary Tissue before and after Fertilization.

PSbMV was localized in sections of ovaries of pea by in situ hybridization using a PSbMV RNA-specific antisense probe.
(A) Longitudinal section through an unfertilized ovary of cultivar Progreta showing that viral RNA is mostly restricted to the carpel tissues. Bar = 500 μm.
(B) Longitudinal section through an unfertilized ovary of cultivar Vedette showing a similar viral RNA distribution to that seen in cultivar Progreta in **(A).** The arrow shows an extreme case of RNA accumulation in the integument tissues. Bar = 500 μm.
(C) Longitudinal section through part of a fertilized ovary of cultivar Progreta showing the ingress of the virus into the ovule along the vascular tissue (arrow). Bar = 600 μm.
(D) Longitudinal section through part of a fertilized ovary of cultivar Vedette showing a similar distribution of viral RNA to that seen in cultivar Progreta in **(C).** The arrow shows PSbMV using the vascular tissues as the route for ovule invasion. Bar = 600 μm.
(E) Magnified view of two ovules in **(C).** Bar = 600 μm.
(F) Magnified view of two ovules in **(D).** Bar = 600 μm.
C, carpel; O, ovule; OW, ovule wall; F, funiculus; M, micropylar region.

tissues, but at this stage of development, the patterns of virus accumulation in cultivars Progreta and Vedette were similar (Figures 3C to 3F). We have shown previously that the testa tissues of all ovules of both cultivars became infected (Wang and Maule, 1992). However, at later stages in embryo development (e.g., early heart stage), the virus moved from the vascular-associated tissue in cultivar Vedette and progressively invaded the neighboring tissue. This process, illustrated in Figure 4, continued until the tissues around the micropyle became infected (Figures 4A and 4B), whereas the virus concentration in the earlier invaded tissue appeared to diminish. This is in contrast to the situation in cultivar Progreta. Here, after the early replication of the virus in and around the vascular strand, there was very little additional invasion of the surrounding tissue (Figures 4C and 4D), although diminution of existing virus also was observed during the course of embryo development.

To test the possibility that the limited distribution of virus in cultivar Progreta was associated with the inability of the virus to cross the boundary between the maternal and progeny

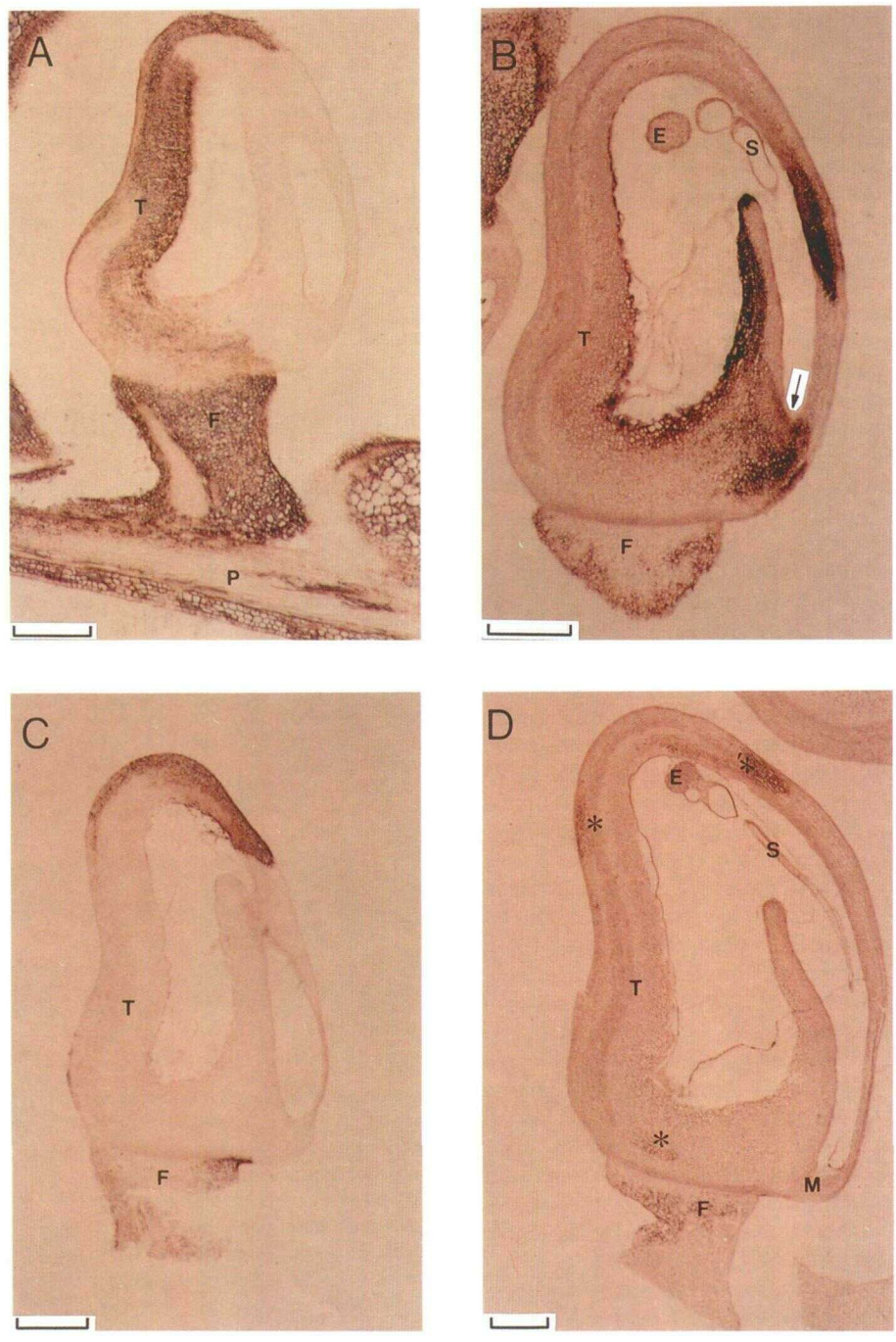

Figure 4. Distribution of PSbMV in Tissues of Immature Seeds.

PSbMV was detected in sections of immature seeds of pea by immunocytochemistry using a monoclonal antibody to PSbMV.
(A) Section of an immature seed of cultivar Vedette. The distribution of PSbMV in tissues away from the primarily invaded vascular tissues is shown. Seed weight is 40 mg. Bar = 500 μm.
(B) Section of an older seed of cultivar Vedette. The section shows that the virus has further invaded the testa tissues to reach the point of contact between the testa and the suspensor in the micropylar region (arrow). Accumulation of the virus in the earlier invaded region of the testa shown in **(A)** has decreased. Seed weight is 50 mg. Bar = 500 μm.
(C) Section of an immature seed of cultivar Progreta. PSbMV is not as widely distributed as it is in cultivar Vedette (cf. distribution in **[A]**) at the same stage. Seed weight is 40 mg. Bar = 500 μm.
(D) Section of an older seed of cultivar Progreta. The overall reduced accumulation of PSbMV and its limited distribution in patches (asterisks) within the testa tissue are shown. Unlike for cultivar Vedette, the virus does not reach the micropylar region. Seed weight is 60 mg. Bar = 500 μm.
E, embryo; F, funiculus; M, micropylar region; S, suspensor; T, testa.

tissues and resistance to seed transmission, immature seeds from individual plants from the segregating F_2 population were analyzed for their "Vedette-like" or their "Progreta-like" distribution of virus in the testa. Embedded seeds were serially sectioned, probed using immunocytochemical staining, and analyzed for their virus distribution patterns. In parallel, F_3 seeds from the same plants were harvested and used to correlate the analysis with the presence or absence of seed transmission. Of 10 plants found to have the restricted distribution of PSbMV, only two showed seed transmission. In contrast, of 29 plants with the Vedette-like distribution, 25 showed seed transmission. These results provided further evidence for a correlation between a restriction of virus invasion of the testa tissue and resistance to seed transmission.

The Suspensor Becomes Infected, Providing a Route for Virus Invasion of the Embryo

The process of plant virus seed transmission is neither temporally nor quantitatively synchronized (for example, see the distribution of seed transmission for cultivar Vedette in Figure 1), even within single mother plants. Furthermore, in infected plants especially, the development of embryos within single pods is not synchronous (data not shown). Nevertheless, there is a general negative correlation between the age of the mother plant at the time of virus inoculation and the extent of seed transmission. This is particularly true for older plants, because plants close to flowering when first infected show only low levels or no transmission of the virus into the seed (Carroll, 1981). There are many developmentally linked factors that could regulate seed transmission in this way. However, we have shown previously (Wang and Maule, 1992) that the maximum efficiency of seed transmission is established early in embryo development and that this window of opportunity then remains closed until maturity. One structure that irreversibly breaks the continuity between the maternal and embryonic tissues during development is the suspensor, which is known to degenerate after the major phases of histogenesis are complete (Wardlaw, 1955; Raghavan, 1986; Meinke, 1991; Yeung and Meinke, 1993). To examine this process, embryos with attached suspensors were dissected from ovules of different sizes from uninfected plants of cultivars Vedette and Progreta. Figure 5 shows that the suspensor from cultivar Vedette remained intact until the midcotyledonary stage of embryo development (Figures 5A to 5D) and then degenerated to a vestigial structure (Figure 5E). Suspensors from cultivar Progreta were indistinguishable from those of cultivar Vedette in all respects (data not shown).

Infection of pea embryos with PSbMV early in their development could arise indirectly through infection of the suspensor or by direct infection of embryonic tissues from virus in the embryonic sac fluid. We have shown previously that PSbMV is present in the embryonic sac fluid at the late heart stage of embryo development in cultivar Vedette (Wang and Maule, 1992). To test whether suspensors also became infected, they were isolated from ovules containing embryos at the globular to early heart stage, carefully washed to remove contaminating embryo sac fluid, fixed to slides, and stained with anti-PSbMV monoclonal antibody, using the immunofluorescence staining technique. The accumulation of PSbMV antigen in a suspensor from cultivar Vedette is shown in Figure 6. Of 22 suspensors isolated for cultivar Vedette, 50% were positive for PSbMV antigen (Figure 6A); this percentage is similar to the mean

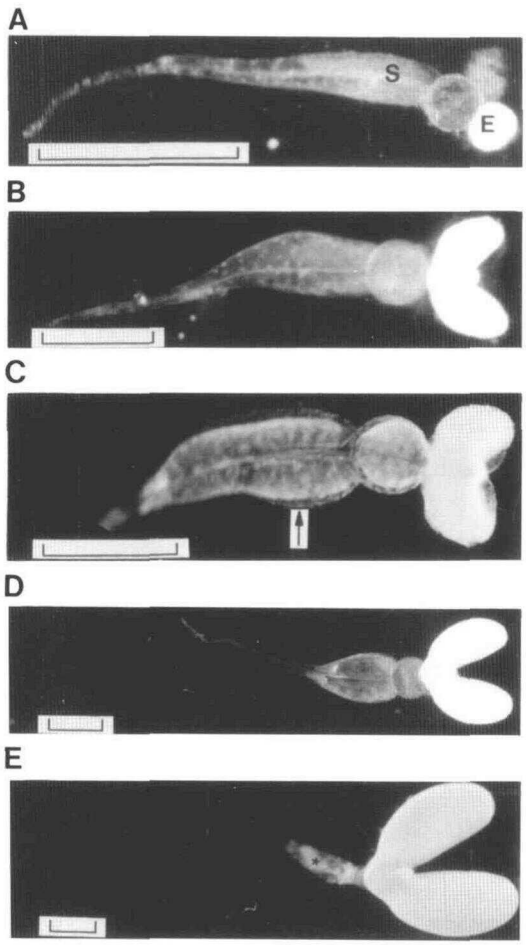

Figure 5. Structure of the Suspensor Apparatus.

Cultivar Vedette embryos with attached suspensor apparatus were dissected from immature seeds at different development stages and viewed with dark-field illumination.

(A) Late-globular stage. Bar = 1 mm.

(B) Heart stage. The multinucleate bicellular nature of the suspensor basal structure of pea is seen clearly. Bar = 1 mm.

(C) Heart stage. The continuous embryonic sheath (arrow) is seen around the embryo and the suspensor. Bar = 1 mm.

(D) Cotyledonary stage. Bar = 1 mm.

(E) Cotyledonary stage. The degeneration of the suspensor occurred during the growth of the cotyledonary stage embryo (cf. **[D]** and **[E]**) leaving a vestigial structure (star) remaining. Bar = 1 mm.

E, embryo; S, suspensor.

efficiency of transmission in this cultivar. No infected suspensors were found among the 20 isolated from infected cultivar Progreta plants.

Two routes for the invasion of embryos by PSbMV are therefore possible, through the suspensor or via the embryo sac fluid. Strong support for the former has come from observations we have made of the accumulation of PSbMV in embryonic tissues. Figure 7 shows the immunohistochemical staining of an immature seed containing a cotyledonary stage embryo from cultivar Vedette. Here, it can be seen that the testa tissues (including those in the region of the micropyle), the endospermic cytoplasm around the embryo sac boundary wall and the embryo sheath, and the suspensor have all become infected. Despite this abundance of virus within the embryo sac, the only tissues of the embryo seen to be infected are those destined to become the radicle (i.e., those at the contact point between the suspensor middle cell and the embryo). Similar analysis of more than 50 infected embryos always showed the source of the embryonic infection to be the radicle end of the developing embryo (data not shown). Embryos of cultivar Progreta did not become infected in any of these experiments. Hence, the position and physiological behavior of the suspensor and its susceptibility to virus infection all suggest that it provides the route taken by PSbMV in the direct invasion of pea embryos.

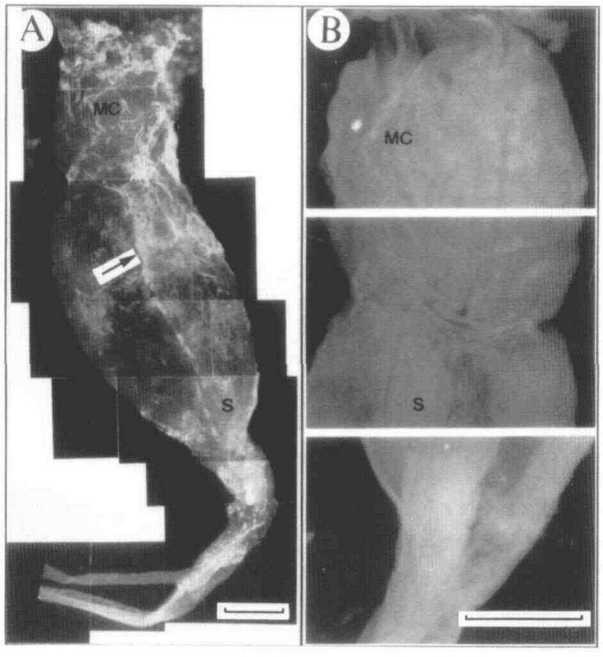

Figure 6. PSbMV Infection of the Suspensor Apparatus.

Immunofluorescent staining of PSbMV in the embryonic suspensor apparatus (embryo at early cotyledonary stage) from an immature seed of cultivar Vedette is shown.

(A) Suspensor from an infected cultivar Vedette plant. The arrow indicates an area of positive fluorescence. Bar = 250 μm.

(B) Suspensor from a healthy cultivar Vedette plant. Only three representative areas of the healthy suspensor are shown. Bar = 250 μm.

MC, middle cell; S, suspensor.

DISCUSSION

The interaction between PSbMV and pea has several features that make it amenable to resolving the mechanisms involved in seed transmission. These include the absence of virus transmission via the gametes in cultivar Vedette, the availability of one cultivar (cv Progreta) with 0% seed transmission, the size of the reproductive tissues for ease of experimentation, and the extent of existing knowledge of pea embryology. We have used these features to propose a model for the invasion of pea embryos by PSbMV. Any such model must take into account several properties of virus seed transmission common to a wide range of host–virus interactions (Carroll, 1981; Mink, 1993). It is generally accepted, although not always rigorously recorded, that the efficiency of seed transmission is environmentally influenced such that, for example, a variation of 20% in the mean percentage of transmission of PSbMV can occur between experiments in cultivar Vedette (D. Wang, unpublished data). As described earlier, the age of the parent plant at the time of infection is important. Variation in the physiology of individual plants presumably accounts for the distribution of seed transmission efficiency within a population (e.g., Figure 1), and the model must take into account the variability in seed transmission between different parts of the same plant, even between different pods at the same node. As with other viruses, the transmission of PSbMV varies widely with the genotype of the host (Wang et al., 1993), so the model must also accommodate the genetic control of seed transmission and the heritability of resistance to seed transmission. It is unlikely, however, that the principles determining direct embryo invasion will be the same as those determining indirect invasion via the gametes.

We propose that after fertilization, PSbMV invades the ovule wall via the main vascular strand. During the later developmental stages, the arrival of the virus in tissues close to the micropyle provides access to the interface between the testa and the suspensor cells. If the suspensor is still functional (i.e., prior to its programmed degeneration), it can act as a channel for transmission of the virus to the embryo proper. Genes in cultivar Progreta that effect resistance to seed transmission do not influence the functioning of the suspensor but, in some way, limit the extent of PSbMV accumulation and/or spread into the testa tissue, at least until suspensor degeneration has occurred. The quantitative influences on seed transmission can take effect through an alteration in the relative timing of virus ingress into the testa tissues and degeneration of the suspensor.

Key to the interpretation of this model was the finding that the seed transmission character was a function of the maternal tissues and particularly the reproductive tissues (because

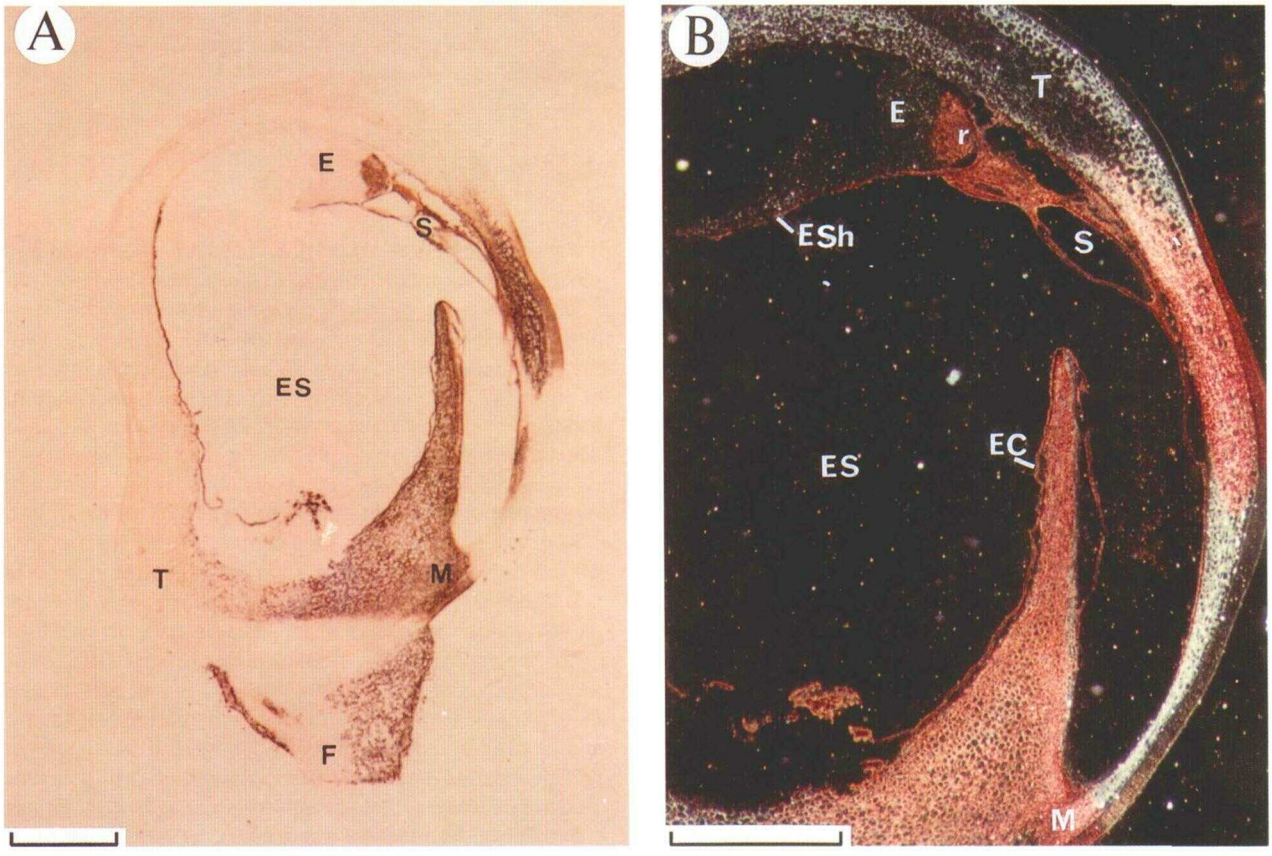

Figure 7. Localization of the Initial PSbMV Infection Site to the Radicle End of the Developing Embryo.

Sections of an immature seed collected from an infected cultivar Vedette plant were stained with a PSbMV-specific monoclonal antibody. The seed contained a cotyledonary stage embryo.

(A) Complete longitudinal section of an immature seed showing PSbMV accumulation in the embryo, the suspensor, and in the testa tissue, including tissue around the micropyle. Seed weight is 100 mg. Bar = 1 mm.

(B) Magnified view of an adjacent section to that shown in **(A)** from the same seed. This section was photographed under dark-field illumination. PSbMV accumulation (red color) is seen in the testa tissue around the micropyle, in the endospermic cytoplasm that lines the embryo sac boundary wall (Marinos, 1970), in the embryonic sheath, and in the suspensor. Despite the presence of virus in the embryonic sheath surrounding the embryo, PSbMV invasion of the embryo was only seen in the tissues destined to become the radicle (i.e., those tissues in contact with the suspensor). Bar = 1 mm.

E, embryo; EC, endospermic cytoplasm; ES, embryo sac; ESh, embryonic sheath; F, funiculus; M, micropylar region; r, radicle; S, suspensor; T, testa.

the vegetative tissues of the two cultivars were equally susceptible). For another potyvirus, soybean mosaic virus, a comparison of seed transmission efficiencies shown by different soybean mosaic virus isolates in soybean also showed reduced accumulation of one isolate in the maternal vegetative and floral tissues as a contributory factor in reduced seed transmission (Iwai and Wakimoto, 1990).

For PSbMV, the determinant of resistance to seed transmission was not revealed as a simple Mendelian character by genetic segregation, although the similar behavior of the reciprocal crosses between cultivars Progreta and Vedette eliminated the complication of non-nuclear factors. However, the significant although incomplete resistance to seed transmission seen in the F_1 population indicated that such resistance genes may nevertheless be accessible. The segregation in the F_2 population showed that PSbMV seed transmission is inherited in a quantitative manner probably involving multiple but relatively few genes. This finding is consistent with the observation that the PSbMV determinant of seed transmission, which was analyzed by the construction of hybrid virus genomes between transmissible and nontransmissible strains, mapped to multiple loci (E. Johansen, personal

communication). The host genetic determinant controlling the seed transmission of BSMV has been defined as a single recessive gene (Carroll et al., 1979). Our findings for PSbMV in pea suggest that seed transmission is controlled differently by the host genome in different host–virus combinations. However, in the BSMV study, crosses were made using infected plants, thereby preventing a separate assessment of the relative contribution of pollen transmission to the overall level of seed transmission. Also, the design and interpretation of the crosses seemed to be based upon the presumption that seed transmission was controlled by the progeny rather than the maternal tissues (Carroll et al., 1979).

The identification of a close correlation between different patterns of PSbMV accumulation in the maternal testa tissue and transmitting and nontransmitting phenotypes suggests that the multiple host genes involved in determining PSbMV seed transmission probably function in the testa tissue and that the products of these genes affect seed transmission by influencing the extent of virus persistence and spread.

The model identifies the functionality of the suspensor as the "window" that determines the extent of seed transmission in the parent plants. This raises an important question regarding how the potyvirus or its genome can cross the boundary between the maternal and progeny generations. It is documented for dicotyledonous plants (e.g., *Capsella* spp.; Schulz and Jensen, 1969) that although there is symplastic continuity between the suspensor, the middle cell, and the embryo, there is no symplastic connection between the suspensor cells and testa tissue cells; the transport of low molecular weight nutrients from the maternal tissue to the developing embryo occurs apoplastically (Raghavan, 1986; Wolswinkel, 1992). From our current knowledge of the mechanisms of virus movement (Citovsky and Zambryski, 1991; Maule, 1991; Deom et al., 1992), it is unlikely that the virus crosses the maternal boundary apoplastically. A symplastic pathway would be possible if the virus induced the formation of plasmodesmata at the maternal tissue–embryo interface. Plant virus–encoded movement proteins modify plasmodesmata structurally (Maule, 1991) and functionally (Wolf et al., 1989), but de novo induction of plasmodesmata by viruses has not been recorded.

Studies on more general factors affecting virus seed transmission appear to lend support to a role for the suspensor in determining seed transmission efficiency. The suspensor apparatus is ubiquitously present in all the angiosperm species so far studied and has been demonstrated to be ephemeral during angiosperm embryogenesis (Wardlaw, 1955; Raghavan, 1986; Meinke, 1991; Yeung and Meinke, 1993). The dependence of seed transmission on inoculation of the mother plant at a young age is correlated with the loss of function of the suspensor at a fixed developmental age. The importance of the environment in determining the rate of growth and development provides a link between environmental effects and the suspensor, seed transmission efficiency, and the quantitative nature of the genes controlling seed transmission observed in this study.

METHODS

Virus Isolate and Plant Cultivars

Two pea (*Pisum sativum*) cultivars, Progreta and Vedette, were used during the study. Plants were inoculated with a seed-transmissible isolate of pea seed-borne mosaic virus (PSbMV), PSbMV-28, when they had two fully expanded leaves, as described in Wang and Maule (1992). Healthy and inoculated plants were grown in a glasshouse at 18 to 22°C with a light period of ~14 hr.

Genetic Analysis

To determine the relative contribution of maternal and progeny tissues to seed transmission, reciprocal cross-pollination between cultivars Progreta and Vedette was performed using PSbMV-infected plants as pollen recipients and healthy plants as pollen donors. Using 15 infected plants of each cultivar, single flowers at each node were emasculated and cross-pollinated from the healthy donor. Second flowers at the same nodes were allowed to self-pollinate and were used as controls. After maturation, F_1 hybrid seeds were collected, germinated, and scored for the amount of seed transmission by symptom expression and ELISA (Wang et al., 1993).

To investigate the host genetic complexity controlling PSbMV seed transmission, reciprocal crosses were prepared between healthy plants of cultivars Progreta and Vedette. F_1 and F_2 generation seeds (20 to 50 per plant) were harvested and grown together with the parental lines in the same glasshouse under identical conditions. F_0, F_1, and F_2 seedlings were inoculated with PSbMV and the progeny seed collected as families from individual mother plants. Penetration of the seed transmission character into the F_1 and F_2 generations was assessed by indexing F_2 and F_3 progeny seedlings, respectively, for virus infection as described above.

In Situ Hybridization

In situ hybridization was performed on tissues from intact ovaries dissected from unfertilized flowers and immature seeds collected after fertilization. Nonradioactive in situ hybridization was performed essentially as described by Coen et al. (1990), except that the wax embedding medium was replaced by a low-temperature polyester wax to maintain protein antigenicity in the tissue block (Labacq and Ritter, 1979; Roholl et al., 1991). The temperature for infiltration of polyester wax was 42°C. Sections (15 μm) were prepared using a cooled (10 to 15°C) microtome. Control tissues from uninfected plants were always run in parallel; these samples always gave a negligible signal.

The digoxigenin-labeled antisense RNA probe was prepared from a cDNA clone corresponding to nucleotides 5928 to 9899 of the PSbMV-28 genome (Wang and Maule, 1992) after transcription with T7 RNA polymerase in the presence of digoxygenin-11-UTP (Boehringer Mannheim). The concentration of the probe used for in situ hybridization was 300 ng/mL. After hybridization and washing, the sections were treated with alkaline phosphatase–linked antibody to digoxigenin (Boehringer Mannheim), washed, and the color was developed as described previously (Coen et al., 1990) to identify locations of hybridization. Dried sections were preserved under a plastic mount (DePex; Serva, Heidelberg). Photographs were taken using a Carl Zeiss Stemi-SV11 microscope.

Immunohistochemical Staining

For immunohistochemical staining, a monoclonal antibody (John Innes monoclonal; JIM87) specific for the coat protein of PSbMV-28 was prepared. LOU/c rats were immunized with purified virus particles (Wang et al., 1992), and the spleen lymphocytes were fused with the IR983F myeloma cell line (Bazin, 1982). JIM87 was selected based on its specific reaction to the coat protein of PSbMV-28. Antibody isotyping using a commercial kit (Serotec; Oxford, U.K.) showed that JIM87 belonged to the rat IgG2b class.

Sections for immunohistochemical staining were dewaxed and rehydrated as was done for in situ hybridization. They were then briefly immersed in TBS buffer (150 mM NaCl, 100 mM Tris-HCl, pH 7.5) and incubated in TBSTB buffer (TBS containing 0.3% [v/v] Triton X-100 and 1% [w/v] BSA). After 1 hr, the slides were washed in TBS and treated for 1 hr with the antibody solution (1/10 dilution of cell culture supernatant of JIM87 in TBSB [TBS containing 1% BSA]). After several washes in TBS, sections were treated for 1 hr with goat anti-rat IgG-alkaline phosphatase conjugate (Sigma) at a concentration of 0.1 μg/mL in TBSB. Specific signal was developed in a substrate solution (17.5 μg/mL 5-bromo-4-chloro-3-indolylphosphate and 33.5 μg/mL nitro blue tetrazolium in 100 mM NaCl, 500 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5). All the immunostaining steps were performed at room temperature. The slides were mounted and photographed as for in situ hybridization. Uninfected control tissues always gave a negligible reaction with JIM87.

Immunofluorescence Staining

For immunofluorescence staining of PSbMV particles in the suspensor apparatus of cultivar Vedette plants, globular- and heart-stage pea embryos with attached suspensors were dissected from immature seeds, washed in distilled water, and fixed in 4% formaldehyde in PBS buffer (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4) for 1 hr. They were then dried onto poly-L-lysine–coated slides on a 42°C hot plate and the embryos carefully removed, leaving the suspensors still attached on the slides. The samples were immunostained as given above, except that the second conjugate was fluorescein-labeled goat anti-rat IgG conjugate (Sigma) at a 1:100 dilution in TBSB buffer. At the end of the incubation, the slides were washed and mounted in Citifluor (Agar Scientific Ltd., Stanstedt, U.K.). The result was recorded using a Zeiss Axiophot microscope and Kodak Ektapress 1600 ASA film. All the immunostaining steps were conducted at room temperature.

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