Multiple viral determinants affect seed transmission of pea seedborne mosaic virus in *Pisum sativum*

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Two pea seedborne mosaic potyvirus (PSbMV) isolates, P-1 DPD1 (P-1), which is highly seed-transmitted, and P-4 NY (P-4), which is rarely seed-transmitted, and chimeras between P-1 and P-4 were analysed to map the viral genetic determinants of seed transmission. Infectivity of chimeric viruses was evaluated by inoculating *Pisum sativum* with RNA transcribed *in vitro* from recombinant full-length cDNA clones. The chimeric viruses that were used demonstrated that a genomic segment encoding the 49 kDa protease and putative RNA polymerase was responsible for symptom induction. Attempts to determine transmission of the chimeric viruses in *P. sativum* cultivars known to transmit P-1 at high frequencies showed that seed transmission is a quantitative character influenced by multiple viral determinants. Seed transmission frequency did not correlate with accumulation of virus in vegetative tissue. The 5' 2-5 kb of the 10 kb PSbMV genome had a major influence on the seed transmission frequency and was analysed further. This showed that, while the helper-component protease was a major determinant of seed transmission, the potyviral P1-protease exerted no measurable influence.

Introduction

Transmission through seed has been described for 108 plant viruses in one or more of their hosts. For all of these, except tobacco mosaic virus, successful seed transmission depends on the virus entering and surviving in the embryo (Mink, 1993). Seed transmission is precluded when the virus is unable to infect the gametes prior to fertilization, unable to enter the embryo during development, or when the virus is inactivated in the embryo during seed maturation and storage (Maule & Wang, 1996). In virus-host combinations with potential for seed transmission, the frequency of seed transmission depends on both host and virus genotype and may range from 0% to almost 100% (Mink, 1993).

The mechanisms of resistance to seed transmission are not resolved and inheritance of resistance has been investigated in only a few cases. In * Hordeum vulgare* cultivar Modjo, resistance was reported to be conditioned by a single recessive gene (Carroll et al., 1979), whereas resistance to seed transmission of pea seedborne mosaic potyvirus (PSbMV) in *Pisum sativum* is inherited as a quantitative character controlled by the action of multiple maternal genes (Wang & Maule, 1994). In a single host cultivar, different virus isolates are seed-transmitted to different degrees. These differences may reflect differences in virus replication and movement which determine the frequency at which the virus successfully enters the gametes or the embryo (Carroll, 1981).

Pseudorecombination studies with isolated viral RNAs from strains differing in seed transmissibility showed that the seed transmission phenotype was linked to RNA 1 of raspberry ringspot and tomato black ringspot nepoviruses (Hanada & Harrison, 1977) and to RNA 1 of cucumber mosaic cucumovirus (Hampton & Francki, 1992). Analyses of barley stripe mosaic hordeivirus (BSMV) chimeras facilitated mapping of the major determinants of seed transmission in barley (*H. vulgare*) to the 5' untranslated leader of RNA2, a 369 bp repeat in the ya gene, and the yb gene (Edwards, 1995).

The seed transmission frequency of PSbMV is influenced by the genotype of the virus isolate (Kohnen et al., 1995). The complete nucleotide sequences of two isolates of PSbMV which differ widely in seed transmission frequency in several *P. sativum* cultivars have been determined (Johansen et al., 1991, 1996). However, a comparison of the primary sequences...
revealed too many differences to conclude which part of the genome determined seed transmission (Johansen et al., 1996). Therefore a study was undertaken to analyse the 10 kb RNA genome determined seed transmission (Johansen et al., 1996). A HindIII site was introduced into each isolate at nt 1276 of P-I, and the P-4 sequence was modified in three positions, introducing a HindIII site at nt 1234, a BamHI site at nt 2259 (recently corrected), and a PsiI site at nt 5812. All nucleotide substitutions were translationally silent.

Full-length clones for in vitro transcription were assembled in the vector pT7E19(+) (Petty, 1988), which was modified to remove the SphI and HindIII sites from the polylinker. The virus sequences were cloned directly behind the T7 promoter by digesting modified pT7E19(+) with Sall, treating with T4 DNA polymerase and then digesting with XbaI before inserting the 5' terminus of either P-1 or P-4 as a DraI-XbaI fragment. These plasmids were digested with SphI and XbaI, and cloned cDNAs (covering Sphi 965 to BamHII 2300 of P-1 and Sphi 924 to BamHII 2259 of P-4) were inserted as Sphi-XbaI fragments creating plasmids pPa and pPb, respectively. Cloaked cDNAs covering HindIII 8647 to the poly(A) tail of isolate P-1 and HindIII 8585 to the poly(A) tail of isolate P-4 were inserted as BamHI-XbaI fragments 3' to both BamHI 2300 of pPa and BamHI 2259 of pPb resulting in pPc, pPd, pPe and pPf.

pPg (containing cDNA covering BamHI 2300 to HindIII 8467 of P-1) and pPh (containing cDNA covering BamHI 2259 to HindIII 8585 of P-4) were digested with BamHI and PsiI, and the BamHI-PsiI fragment of pPg was inserted into pPh, creating pPf.

Full-length plasmids pP-I, pP-I14 and pP-4111 were assembled by

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**Methods**

**Virus isolates.** PSbMV isolate P-1 DPD1 was recovered from a pea seed sample analysed at the Danish Plant Directorate (Lyngby, Denmark). The PSbMV P-4 NY isolate was recovered from USDA Pisum P. I. accession 471128 and kindly provided by R. Provvidenti (New York State Agriculture Experiment Station, Geneva, NY, USA). For convenience P-1 DPD1 and P-4 NY will be referred to hereafter as isolates P-1 and P-4; however, it should be noted that seed transmissibility is not necessarily linked to the pathotype of PSbMV. Cloning and sequencing of P-1 and P-4 were reported by Johansen et al. (1991, 1996).

**cDNA modifications and construction of full-length clones.**

A cistron map of PSbMV is shown in Fig. 1(a). Locations of natural and engineered restriction sites in the cDNA of isolates P-1 and P-4 are identified by the first nucleotide (nt) of the recognition sequence. Maps and names of isolates P-1 and P-4 intermediary clones are shown in Fig. 1(b).

![Cistron map of PSbMV](image)

**Diagram Description:**

Fig. 1. (a) Cistron map of PSbMV showing the noncoding regions (solid line), the open reading frame encoding the potyvirus polyprotein (open box) and putative proteolytic cleavage sites (vertical lines). Relevant restriction sites in the cDNA are shown above (P-1) and below (P-4) the cistron map. Restriction sites marked with an asterisk were engineered into the sequences. P1-Pro, P1 protease; HC-Pro, helper-component protease; P3, P3 protein; 6k1, 6 kDa protein 1; CI, cylindrical inclusion protein; 6k2, 6 kDa protein 2; 49k-Pro, 49 kDa protease; VPg, genome linked virus protein; RdRp, putative RNA dependent RNA polymerase; CP, coat protein. (b) Maps and names of intermediary clones used in the assembly of the full-length clones pP-I, pP-I14, pP-4111, pP-1144 and pP-4144. The P-1 isolate sequences (light grey) and P-4 isolate sequences (dark grey) contained in the clones are shown as boxes, positioned according to the sequence in the genome they represent. Areas represented by a solid line are not present in the clones. Vector sequences are not shown.
inserting the BamHI-HindIII fragment of pPg into BamHI/HindIII-digested pFc, pFe and pPf, respectively. pF-1144 and pF-4144 were assembled by inserting the BamHI-HindIII fragment of pPf into BamHI/HindIII-digested pFc and pPf. The full-length clones are shown in Fig. 2(a).

Plasmids pP-1(P-4 5'UTR), pP-1(P-4 P1pro) and pP-1(P-4 HCProl) (Fig. 3a) were created by substituting the HindIII 102-BamHI 2259 of pPf with the HindIII 146-BamHI 2300 of pPa; the HindIII 146-HindIII 1276 of pPa with the HindIII 102-HindIII 1234 of pPb; and the HindIII 1276-BamHI 2300 of pPa with the HindIII 1234-BamHI 2259 of pPb, respectively, followed by assembly of the full-length clones as described for pP-1.

In vitro transcription and inoculation of RNA. Full-length PSbMV cDNAs were cloned and amplified in Escherichia coli strain SURE (Stratagene). Caesium chloride gradient-purified plasmids were linearized with XbaI, and 2–3 μg of linearized plasmid was used for in vitro synthesis of capped transcripts (inMMESSAGE MACHINE, Ambion). Approximately 2 μg of transcript was inoculated mechanically onto two carborundum-dusted leaves of each P. sativum plant. Two weeks after inoculation, infectivity of the in vitro-synthesized transcripts was assayed by double-antibody sandwich (DAS)-ELISA (Converse & Martin, 1990) using an antisera specific for PSbMV coat protein (Hampton & Mink, 1989). Virus derived from plasmid pP-1 is referred to as vP-1, and chimeric viruses are referred to as vP-1114, vP-4111, etc.

Accumulation of viruses in young, fully expanded, systemically infected leaves was compared by measuring (by DAS-ELISA) the accumulation of coat protein in plants infected with native isolates P-1 or P-4, or transcript-derived viruses, respectively. For each virus, eight plants were tested and confidence intervals (95%) were calculated, assuming a standard normal distribution of A405 values.

Determination of seed transmission frequency. The pea cultivar '549' was used for the first three seed transmission experiments under greenhouse conditions described by Kohnen et al. (1995). 'Vedette' was used for the fourth and fifth experiment because this cultivar matures early and transmits PSbMV P-1 through seed at very high frequencies (Wang et al., 1993). Inoculum was raised in leaves of P. sativum '549' or 'Vedette' infected with in vitro-synthesized transcripts. Infection was confirmed by DAS-ELISA 2 weeks after inoculation. Leaves of infected plants were homogenized in buffer (50 mm-sodium phosphate pH 7.0) and used to inoculate 2–3-week-old P. sativum '549' or 'Vedette'.

### Fig. 2. (a) Cistron maps of isolates P-1 (light grey), P-4 (dark grey), transcript derived vP-1 and chimeras vP-1114, vP-4111, vP-1144 and vP-4144. (b) Symptoms and virus accumulation in infected P. sativum '549', seed transmission frequency and percentage germination of seeds from infected plants. The restriction sites B (BamHI), P (PstI) and H (HindIII) were used to exchange the 5' UTR, P/pro and HCpro N in the assembly of the recombinant full length clones. Severe, P-1-like symptoms; mild, P-4-like symptoms. Virus acc., virus accumulation in the youngest fully expanded leaves of P. sativum 3 weeks after inoculation determined by ELISA (A405 values, mean of eight plants). Inf./Tot., total number of infected progeny seedlings detected/total number of viable progeny seedlings assayed (pooled from three separate experiments). % ST, percentage seed transmission detected (average of three separate experiments). Means followed by the same letter are not statistically different at the 95% level.

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Virus acc.</th>
<th>Inf./Tot.</th>
<th>% ST</th>
<th>% Germination</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-1</td>
<td>Severe</td>
<td>0.66 c</td>
<td>116/464</td>
<td>25 b, c</td>
</tr>
<tr>
<td>P-4</td>
<td>Mild</td>
<td>0.37 d</td>
<td>1/505</td>
<td>0.2 d, 91 c, d</td>
</tr>
<tr>
<td>vP-1</td>
<td>Severe</td>
<td>0.71 b,c</td>
<td>136/366</td>
<td>37 a, 96 a,b</td>
</tr>
<tr>
<td>vP-1114</td>
<td>Severe</td>
<td>0.34 d</td>
<td>93/521</td>
<td>18 b, 88 d</td>
</tr>
<tr>
<td>vP-4111</td>
<td>Severe</td>
<td>0.93 a</td>
<td>28/614</td>
<td>4.6 c, 98 a</td>
</tr>
<tr>
<td>vP-1144</td>
<td>Mild</td>
<td>0.81 a,b</td>
<td>44/626</td>
<td>7.0 c, 95 a,b</td>
</tr>
<tr>
<td>vP-4144</td>
<td>Mild</td>
<td>0.34 d</td>
<td>12/729</td>
<td>1.6 d, 95 a,b</td>
</tr>
</tbody>
</table>

### Fig. 3. (a) Cistron maps of isolates P-1 (light grey), P-4 (dark grey), transcript derived vP-1 and chimeras vP-1114, vP-4111, vP-1144 and vP-4144. (b) Cistron maps of isolates P-1 (light grey), P-4 (dark grey), transcript derived vP-1 and chimeras vP-1114, vP-4111, vP-1144 and vP-4144.
Infected plants were grown to maturity and seeds were harvested as the seeds matured. Seeds from infected plants were sown and seedling infection was determined by DAS-ELISA 3 weeks after emergence. Confidence intervals (95%) were calculated for the probability of the observed seed transmission frequency, assuming a binomial distribution of the data.

Results and Discussion

Infectivity of RNA transcripts from native and recombinant full-length cDNA clones

Inoculation of *P. sativum* with *in vitro*-synthesized transcripts of pP-1, pP-1114, pP-4111, pP-1/444 or pP-4144 (Fig. 2a), and pP-1(P-4 5'UTR), pP-1(P-4 P1pro) or pP-1(P-4 HCpro N) (Fig. 3a) all resulted in infection of 50 to 100% of the inoculated plants. In subsequent mechanical transfers, using infected plant material as inoculum, the infectivity of vP-1 and the chimeric viruses was comparable to native isolates P-1 and P-4. In contrast, transcripts of pP-4 and reciprocal chimeras to those shown in Fig. 2a were not infectious. All these plasmids contained cDNA covering nts 2259-5812 of P-4, suggesting that this cDNA segment was defective. However, substitution of this region with cDNA from a new cDNA synthesis reaction did not result in the generation of biologically active transcripts.

Symptom induction and accumulation of transcript-derived viruses

Symptoms induced by vP-1, vP-1114, vP-4111, vP-1(P-4 5'UTR), vP-1(P-4 P1pro) and vP-1(P-4 HCpro N) were similar to those of native P-1 virus (transient vein clearing, downward rolling of leaflets and shortened internodes). Symptoms of vP-1144 and vP-4144 were mild, causing only a slight growth reduction similar to plants inoculated with native P-4 virus (Fig. 2b).

These observations suggest that the region of the PsbMV genome between *Psl* 5874/5812 and *HindIII* 8647/8585 has a major influence on symptom severity in *P. sativum*. This region contains the potyviral 49 kDa protease and the putative RNA dependent RNA polymerase, both proposed to be involved in replication of the potyvirus genome (Riechmann et al., 1992).

To determine whether the differences in symptom induction correlated with differences in virus accumulation, the relative accumulation of virus in systemically infected leaves was determined 3 weeks after inoculation (Fig. 2b).

vP-4111 accumulated to a higher concentration than P-1, vP-1 and vP-1144, which, in turn, accumulated to higher concentrations than P-4, vP-1114 and vP-4144. These data demonstrate that P-1, P-4 and the chimeras accumulated to different levels in vegetative tissues, but that the accumulation of virus was not correlated with symptom induction. Therefore, the symptoms induced were not merely a result of competitive inhibition of host growth, but rather might have been due to an interference with host growth regulator metabolism and gene regulation (Fraser et al., 1986; Wang & Maule, 1995).

Seed transmission of vP-1114, vP-4114, vP-4144 and vP-4111 in *P. sativum* 'Vedette'

The seed transmission frequencies of vP-1114, vP-4114, vP-4144 and vP-4111 were determined in three experiments. The absolute seed transmission frequency varied between experiments, which was probably a result of seasonal changes affecting greenhouse conditions. However, in all three experiments, vP-1 was transmitted at the highest frequency, followed by P-1, vP-1114, vP-4114, vP-4111 and vP-4144. P-4 was transmitted in only one seed. Results of the combined data are shown in Fig. 2b. The seed transmission frequency of vP-1 was higher than P-1 in all three experiments. The difference, which was significant (P < 0.05), could be a result of the maintenance of P-1 by repeated mechanical transfers without selection for seed transmissibility. In contrast, vP-1 was derived from a cDNA of viral RNA isolated from P-1 shortly after the virus isolate was first recovered from an infected seed sample.

The seed transmission frequencies of the chimeric viruses were intermediate between P-1/vP-1 and P-4. Of the chimeras, vP-4144 was transmitted at the lowest frequency (slightly, but not significantly, higher than P-4). The increasing contribution of sequences from P-1 found in vP-4114, vP-1114 and vP-1114 correlated with increasing seed transmission, demonstrating that seed transmission of PsbMV is influenced by determinants contained in each of the exchanged genome fragments. The 5' region (nt 1-2259) of P-4 appeared to have the strongest influence on the seed transmission frequency, reducing the seed transmission frequency of vP-4111 to 4.6%, compared with 37% for vP-1.

The seed transmission frequency was not correlated to the virus concentration in infected vegetative tissues (Fig. 2b). This is in agreement with results obtained by Wang et al. (1993) who found no obvious relationship between virus content and the efficiency of seed transmission of PsbMV in different pea cultivars. Also, Ligat & Randles (1993) observed that repeated transfers through seed in the cultivar ‘Dundale’ resulted in a gradual reduction of PsbMV accumulation in vegetative tissue to a level where it was not detectable by ELISA, while the seed transmission frequency exceeded 90%.

The percentage germination of seeds from infected plants ranged from 88% (pP-1114) to 98% (pP-4111). However, there was no apparent correlation of seed viability with symptom severity, virus accumulation or seed transmission frequency.

Seed transmission of vP-1(P-4 5'UTR), vP-1(P-4 P1pro) and vP-1(P-4 HCpro N) in *P. sativum* 'Vedette'

As described above, the region of isolate P-4 from nt 1-2259, which covers the 5' untranslated leader (UTR), the P1 protease (P1pro) and the N-terminal two-thirds of the helper-component protease (HCpro N) appeared to have a
major effect on seed transmission. Three recombinant, full-length clones were therefore created in which the 5' UTR, the PIpro and HCproN of P-1 were exchanged with the corresponding region of P-4. Seed transmission of the resulting chimeric viruses was assayed in ‘Vedette’, and the combined data from experiments four and five are shown in Fig. 3(b). The seed transmission frequencies of vP-1(P-4 5'UTR) and vP-1(P-4 HCproN) were reduced to 50% and 20% of vP-1, respectively, while vP-1(P-4 PIpro) was seed transmitted at the same frequency as vP-1.

The 5' UTRs of P-1 and P-4 are 143 and 99 nt in length, respectively, and there are 32 amino acid differences in the HCproN region between the two viruses. The 5' UTR is likely to affect both translation and replication of the potyvirus genome (Riechmann et al., 1992). The region of HCpro contained in HCproN affects aphid transmission, replication (Atreya et al., 1992) and long-distance movement (Cronin et al., 1995).

Wang & Maule (1994) demonstrated that the suspensor can act as a channel for transmission of PSbMV to the embryo proper. A prerequisite for embryo infection is, therefore, that virus reaches the micropylar region of the testa before disintegration of the suspensor. This suggests that the HCproN region may affect replication and/or long-distance movement in reproductive tissues and thus influence the number of embryos which become infected and hence, the final seed transmission frequency.

The observation that seed transmission is affected by multiple determinants in the viral genome agrees well with the results of Wang & Maule (1994) who demonstrated that resistance to seed transmission in P. sativum involves several nuclear genes. This suggests that several virus–host factor interactions determine the spread and accumulation of PSbMV in the maternal testa tissue. Whereas PSbMV can enter the embryo after fertilization (Wang & Maule, 1992), seed transmission of BSMV depends mainly on the ability of the virus to reach the reproductive tissues prior to fertilization (Edwards, 1995). Despite this difference, replication and movement also play central roles in seed transmission of BSMV, the major determinants being the RNA1 5' UTR, a 369 repeat in the yA gene, and the yB gene. Therefore, seed transmission of both PSbMV and BSMV appears to be determined by the ability to replicate and move in the reproductive tissues and to reach the suspensor or megagametophyte, respectively, before a certain critical point in development.

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