Specific Serological Detection of the Transmissible Virus in Pea Seed Infected by Pea Seed-borne Mosaic Virus

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ABSTRACT


The capsid protein of pea seed-borne mosaic virus (PSbMV), analyzed in mature pea seed, had a molecular mass of 33 kDa in infected embryos but a molecular mass of only 27-29 kDa in seed testas. A proteinase activity, induced in pea seed testas, efficiently cleaved the capsid protein in vitro as did a mild trypsin treatment. The occurrence of this proteinase activity was independent of the infection; its physiological induction in testas occurred at a development stage that corresponds to the active growth of the embryo. The use of an antiserum specific to the deleted part of the protein enabled detection of PSbMV only in embryos. This serological differentiation between the seed-transmitted and seedborne virus may have a direct application in the routine screening of pea seed lots.

RESUME

La protéine capsid de la peau de l'orge-brut de la mosaic-type de la gramine (PSbMV), analysée à partir de grains de pois à maturité, a une masse moléculaire de 33 kDa lorsqu'on l'extrait des embryons infectés et de 27-29 kDa seulement à partir des testas. Une activité protéolytique, induite dans les testas, peut opérer efficacement ce clivage in vitro, comme le fait aussi la trypsin. La présence de cette protéase est indépendante de l'infection; son induction physiologique dans les testas se situe à un stade de développement qui correspond à la croissance active de l'embryon. L'utilisation d'un antiserum spécifique de la partie clivée ne permet de détecter le virus qu'au niveau des embryons. Cette différenciation sérologique du virus transmis par la graine et du virus non-transmissible peut trouver une application pratique intéressante dans une sélection sanitaire des semences de pois basée sur le taux de transmission du PSbMV.

Pea seed-borne mosaic virus (PSbMV) is a potyvirus of pea. The flexuous, rod-shaped virus particle is composed of a 33-kDa capsid protein and a positive-sense, single-stranded RNA molecule 9,924 nucleotides long (7). PSbMV is seed-transmitted at high frequencies, and its economical importance is linked to this biological property (14). Seed transmission is linked to the invasion of the embryo sac before or just after fertilization (23). The virus retains its infectivity in the embryo during seed maturation and storage, but the virus grown in mature testas is not infectious (9). However, the virus can be detected by serological techniques in both mature embryos and testas (13).

The purpose of the present work was to compare the stability of the capsid protein in both seed components, with the hope of serologically differentiating the seed-transmitted virus of infected embryos from the nontransmitted virus of infected testas. As generally observed with the potyviruses, the N- and C-termini of the capsid protein, which are located on the surface of intact particles (2,20), may be cleaved in vivo (6) as well as during storage of purified virus preparations (6,15). Such cleavage induces important differences in serological properties (5), because the N-terminus constitutes the major virus-specific epitopes (2,20).

MATERIALS AND METHODS

Virus. The common strain Sv used in this study was propagated on pea cv. Belinda. Seed collected from plants inoculated before flowering transmitted this strain at a 10-20% rate.

Antiserum. It is a hyperimmune antiserum to PSbMV provided by R. 1. Hamilton (Research Station Agriculture Canada, Vancouver, BC). This serum, prepared with highly purified virus, was obtained by a series of intramuscular injections at 2-wk intervals followed by a booster dose at 10 mo (4).

R1 is an antiserum obtained by intramuscular injection of PSbMV particles purified according to the method of Alconero et al (1) with only one CsCl gradient centrifugation. One milliliter of incomplete Freund’s adjuvant (Sigma Chemical Co., St. Louis) was mixed with 1 ml of virus suspension (500 μg) for the first
intramuscular injection. After a 1-mo delay, intramuscular boosters of freshly prepared virus were administered at 2-wk intervals. R3 corresponds to the bleedings done 10 days after the second booster.

K is an antiserum to the purified capsid protein of PSbMV. The semipurified virus was prepared according to Alconero et al (1) without any CsCl gradient centrifugation and suspended in 0.2 M Tris, pH 8.2. One volume was mixed with 0.5 volume of a Laemmli denaturation solution of 0.2 M Tris, pH 6.8, containing 6% sodium dodecyl sulphate (SDS), 15% mercaptoethanol, 30% glycerol, and 0.03% bromophenol blue (10); heated in boiling water for 3 min; and loaded onto a 12% polyacrylamide preparative gel. Protein bands were visualized by soaking the gel in cold 0.2 M KCl. The portion of the gel that contained the full-length capsid protein was excised, frozen at -20 C, press through a syringe, and stirred in 25 mM Tris and 200 mM glycine, pH 7.4, for 3 h. Two milliliters of incomplete Freund's adjuvant was mixed with 2 ml of capsid protein (200 μg) for the first intramuscular injection. After a 1-mo delay, intramuscular boosters of freshly prepared capsid protein were administered at 2-wk intervals. K1, K2, and K3 correspond to the bleedings done 10 days after the second, third, and fourth intramuscular boosters, respectively.

Mature seed-sample preparation for enzyme-linked immunosorbent assay (ELISA) and/or Western blot assay. Ten mature seeds of pea cv. Belinda collected from infected plants and 10 healthy seeds were soaked overnight in water. After manual separation of embryos and testas, the pooled embryos and pooled testas were ground and serially diluted in PBST to obtain the 1:1, 1:20, 1:200, and 1:100 (w/v) dilutions. Before loading the ELISA microplate, each of these dilutions was centrifuged (for 10 min at 8,000 g) mixed (1:1 [v/v]) with an infected leaf extract (1:50 [w/v] in PBST) prepared with young leaves collected from peas inoculated 4 wk earlier.

Serological tests. Double antibody sandwich ELISA (ELISA) was conducted as described by Clark and Adams (3). The positive threshold was determined as described previously (13). Protein blots were performed on Bio-Rad Mini-Protein II ready gels (Bio-Rad Laboratories, Richmond, CA) (10% single-percent gels for the experiment reported in Figure 1, 12% for other experiments) and nitrocellulose membranes with the Mini-Protein II electrophoresis and transblot cells. The samples were run along with the Bio-Rad low-range prestained SDS-PAGE (polyacrylamide gel electrophoresis) standards (phosphorylase B, bovine serum albumin [BSA], ovalbumine, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme with respective apparent molecular masses of 106, 80, 49.5, 32.5, 27.5, and 18.5 kDa, respectively; the carbonic anhydrase did not appear clearly) to monitor the duration of electrophoresis; the efficiency of transfer to nitrocellulose membrane, and the integrity of the capsid protein. After saturation with BSA (1%), the membranes were incubated with the (H)IgG-alkaline phosphatase conjugate (1:2,000 [v/v]) or the K conjugate (1:1,000 [v/v]) in 0.05 M Tris, 0.15 M NaCl, and 0.5% Tween 20, pH 7.5; alternatively, they were incubated first with (H)IgG (1 μg/ml) or (K)IgG (2 μg/ml) and then with a 1/6,000 (v/v) dilution of a goat–anti-rabbit conjugate (Biosys, 21 quai du clos des roses F-60200 Compiègne). The alkaline phosphatase was visualized with Fast Red TR salt and Naphthol AS-MX phosphate (Sigma).

RESULTS

Heterogeneity of the capsid protein of PSbMV after storage of a prepared semipurified virus. The capsid protein of strain Sv was characterized by sequencing the 3′-terminal region of viral RNA (11). When compared with the sequence of the pathotype PI (7), the deduced amino acid sequence varied at position 120 with an alanine to arginine substitution. The molecular mass of the intact capsid protein, as deduced from the nucleotide sequence, was 33 kDa.

This 33-kDa protein could be cleaved during storage of leaf extracts or semipurified virus preparations. A range of cleavage products of the capsid protein was observed in Western blots.

(H) conjugate

(K1) conjugate

![Western blot analysis of extracts in phosphate buffered saline plus Tween 20 (1:20 [w/v]) of healthy (h) or infected (i) embryos (E), testas (T), and leaves (L). The lane iLs refers to an infected leaf extract stored 4 days at 4 C. The capsid protein was detected, with either a hyperimmune antiserum to pea seed-borne mosaic virus (PSbMV) (H)IgG-alkaline phosphatase conjugate or an antiserum to the purified capsid protein of PSbMV (K1) conjugate.](image-url)
performed with the H antiserum (Figs. 1 and 2). The fastest migrating band had a molecular mass of 27 kDa. The value calculated for the trypsin-treated core protein, which migrated similarly, was 26.4 kDa, as deduced from the observation that trypsin cleaves the N-terminus of the capsid protein of different potyviruses at the same site: DR/DVDAG (19). These findings support the hypothesis that the N-terminal domain of the capsid protein of PSbMV was cleaved serially. These cleavages of the capsid protein might involve a proteolytic activity from leaves, present in leaf extracts or in partially purified virus preparations.

Interestingly, the K1 polyclonal antiserum did not detect the capsid protein cleavage products as shown in lanes corresponding to the infected leaf extract (Fig. 3); thus, K1 is specific to the intact form of the capsid protein.

Capsid protein stability in embryos and testas of mature seeds. Embryo and testa extracts from infected pea seed were analyzed by Western blots in comparison with extract of infected leaves. The capsid protein subunit in embryos had a molecular mass of 33 kDa. In testas, the antigen consisted essentially of 29- to 27-kDa degradation products (Figs. 1 and 3).

Unlike the H antiserum, the K1 antiserum did not detect the capsid protein in testas, using either the alkaline phosphatase conjugate (Fig. 1) or, in a different experiment, the immunoglobulins visualized by an indirect method (Fig. 3). It recognized only the intact capsid protein present in infected embryos.

The failure of the K1 antiserum to detect the truncated protein in testas of mature seeds also was found in DAS-ELISA with several antisera resulting from early bleedings (K1, K2, K3, and K4). Whatever immunogen was used for preparing the antiserum, the capsid protein (K1, K2, or K3) or the intact virus particle (K4), these antisera detected the virus in infected embryos but did not detect it in infected testas (Table 1).

Thus, in mature seeds, the capsid protein was present only in its degraded form in testas, and the K1 antiserum, either in Western blots or ELISA, detected only the virus present in embryos (i.e., the seed-transmitted virus). It was interesting, therefore, to investigate at what earlier stage of seed development the K1 antiserum detected the virus in embryos only.

Cleavage of the capsid protein in testas according to seed-development stage. To determine at what stage of seed development the capsid protein is degraded, immature seeds were collected from mechanically inoculated pea plants. Because the weight of a green seed constitutes a reliable criterion of its physiological evolution, these immature seeds were distributed into five classes of weight, t1-t5. PSbMV was analyzed in the corresponding testas by comparing first the reaction of the H and K1 antisera in DAS-ELISA.

The results with the H antiserum indicated a constant level of virus in each of the classes of testa. The K1 antiserum similarly detected the virus in the first three classes, however, a drastic reduction and a complete loss of the ability to detect the virus occurred in the fourth and fifth classes (Fig. 4).

However, in Western blots, the intact form of the capsid protein was observed in each class of testas, as reported for t5 in Figure 5 (lane 2). This result was not consistent with the ELISA results. To test whether degradation was due to incubation, new extracts of testas were obtained corresponding to the t5 class. Two aliquots of the t5 testa extract were mixed with the Laemmli denaturing solution, one immediately after obtaining the extract, the second after an overnight incubation at 6°C to reproduce the conditions of the ELISA procedure: The capsid protein was indeed degraded during the overnight incubation (Fig. 5).

These results showed that although the cleavages of the capsid protein did not occur to a great degree in vivo in t5 immature testas a proteolytic activity capable of degrading the capsid protein to 27 kDa was induced in testas at the t4 and t5 stages.

Protease activity in healthy t5 testa extracts. To determine if the proteolytic activity observed in extracts of immature infected t5 testas was induced by PSbMV infection, similar extracts were prepared from healthy testas and mixed with an infected leaf extract from peas inoculated 4 wk earlier. Two stages of immature testas, t2 and t5, were compared in this experiment. The proteolytic activity from t5 testa extract appeared to be significantly efficient on the virus capsid protein to a dilution of 2.10^-2, whereas the t2 extracts were not able to degrade the capsid protein at any

Fig. 2. Length heterogeneity of the capsid protein analyzed by Western blot with the hyperimmune antiserum to pea seed-borne mosaic virus (PSbMV) (H) conjugate. Lane 1: semipurified virus preparation after storage for 1 wk at 6°C. Lane 2: 10 μg of freshly purified PSbMV treated with 1 μg of trypsin for 1 h at 37°C.

Fig. 3. Western blot analysis of extracts in phosphate buffered saline plus Tween 20 (1:20 [v/v]) of infected embryos (iE), testas (iT), and leaves (iL), with hyperimmune antiserum to pea seed-borne mosaic virus (PSbMV) (H) IgG-alkaline phosphatase conjugate or an antiserum to the purified capsid protein of PSbMV (K1) conjugate and a goat-anti-rabbit immunoglobulin-alkaline phosphatase conjugate.
dilution (Fig. 6). This experiment showed that the factors able
to cleave the capsid protein in the t5 testas exist in healthy seeds.

DISCUSSION

The capsid protein of PSbMV does not have the same structure
in both components of mature pea seed. It is intact in embryos
(33 kDa) and partially cleaved in seed testas (29–27 kDa), as
might result from a mild trypsin treatment. This cleavage in testas
results from a proteolytic activity that is inconstant but develops
with time. Thus, the protease involved has some similarities
with a protease induced in Ipomea nil, which partially cleaved
the capsid protein of sweet potato feathery mottle potyvirus (19).
In that system, the induction of a protease was associated with

TABLE 1. Detection of pea seed-borne mosaic virus in testas and embryos
with antisera (H, K1, K2, K3, and R3) able or unable to detect the cleaved forms of the capsid protein*  

<table>
<thead>
<tr>
<th>Sample</th>
<th>H</th>
<th>K1</th>
<th>K2</th>
<th>K3</th>
<th>R3</th>
</tr>
</thead>
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<tr>
<td>Testas</td>
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<td></td>
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<tr>
<td>Testas (h)</td>
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<td></td>
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<td>Embryos</td>
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<tr>
<td>Embryos (h)</td>
<td>0.015</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Underlined values indicate positive reactions.

n = 10 for each sample. h = healthy.

Double antibody sandwich enzyme-linked immunosorbent assay value

Fig. 4. Comparative detection of pea seed-borne mosaic virus (PSbMV)
capsid protein, with hyperimmune antiserum to PSbMV (H) (□) and
an antiserum to the purified capsid protein of PSbMV (K1) (■), in infected
immature testas at different stages of seed development.

Fig. 5. Western blot analysis of the capsid protein of pea seed-borne
mosaic virus (PSbMV) in t5 (seed weight class 5, 268 mg) testa extracts.
Lane 1: healthy extract; lane 2: infected extract immediately denatured
in the Laemmli solution; lane 3: infected extract denatured after overnight
incubation at 6 C. The capsid protein bands were visualized with the
hyperimmune antiserum to PSbMV (H) conjugate.

Fig. 6. Detection of proteolytic activity in healthy t5 (seed weight class
5, 268 mg) (■) and t2 (seed weight class 2, 42 mg) (□) testas. Four
dilutions of testa extracts in phosphate buffered saline plus Tween 20
were mixed (v/v) with an infected leaf extract and incubated overnight
at 6 C in a microplate. The enzyme-linked immunosorbent assay was
performed with the antiserum to the purified capsid protein of pea seed-
borne mosaic virus (K1).
some involvement in the remobilization of seed-testa proteins for supplying nitrogen to the growing embryo (16, 18).

The conversion of the capsid protein to a 27-kDa form occurs in vivo between the mature stage and the immature testa stage (i.e., during seed maturation). Indeed, at the t5 stage of immature green seeds, the predominant form was still a 33-kDa form. However, at that stage, the ELISA test indicated that the amount of proteinase synthesized was high enough for cleaving all the molecules of capsid protein present in infected testas during overnight incubation of the antigen at 6 C. Such a cleavage did not occur at t2 stage in the conditions of the ELISA test.

Therefore, the DAS-ELISA test performed with both antisera—an antiserum (H) specific to both 33- and 27-kDa forms and an antiserum (K) that detects only the 33-kDa capsid protein—appears to be a sensitive assay for detecting this proteinase at early stages of development. In further experiments, we observed that the detection of an extremely low proteolytic activity at the t2 stage needs a much longer incubation period (1 wk at 6 C).

The antiserum (K) specific to the 33-kDa capsid protein resulted from an early bleeding. Presumably, the antibodies are, as demonstrated in similar cases, directed to the degraded N-terminus, which constitutes the major immunogenic part of the capsid protein of potyviruses (21).

From a practical point of view, it has been shown that the cleavage of a part of the capsid protein can cause a failure of potyvirus detection in the context of the production of virus-free propagation material (22). Concerning potyvirus detection in pea seed, this phenomenon should also be taken into account, and the antiserum selected according to the objectives of the programs. In the context of detection experiments in which seed testas are used as representative of the mother plant, a hyperimmune antiserum should be selected. This was the case while screening pea germ plasm for resistance to PSHMV, where most of the susceptible lines were eliminated on the basis of an ELISA test on a few testas from seed obtained directly from the germ plasm collection (8). In the context of quality control programs, the simultaneous presence of a complete capsid protein in the embryo and of a deleted form in the testas enables a serological differentiation to be made between the seed-transmitted and seedborne virus, using for seedborne a definition adopted for most pathogens carried in, on, or with the seed but not necessarily transmitted (17). The specific detection, by appropriate antisera, of the virus in embryos avoids the prior decortication of thousands of seeds for screening infected seed lots on the basis of their seed transmission (12).

LITERATURE CITED