Immunodetection of grapevine fanleaf virus satellite RNA-encoded protein in infected *Chenopodium quinoa*

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An antiserum was raised against a fusion protein containing the C-terminal half of the protein (P3) encoded by the satellite RNA of grapevine fanleaf virus (GFLV; F13 isolate) and the N-terminal portion of the CI repressor of phage λ. This antiserum specifically recognized P3 synthesized in the *in vitro* wheatgerm translation system and also in infected *Chenopodium quinoa* plants. In these plants, the amount of virus increased for 10 days, then remained constant for up to 21 days, whereas P3 was detected transiently, reaching its maximum on day 10.

Grapevine fanleaf virus (GFLV) is a nepovirus responsible for one of the most damaging and widespread diseases affecting grapevines. Its natural vector is a nematode, *Xiphinema index* (Hewitt et al., 1958). The bipartite genome of GFLV is composed of two positive-sense ssRNA molecules: RNA 1 of 7342 nucleotides (nt) (Ritzenthaler et al., 1991) and RNA 2 of 3774 nt (Serghini et al., 1990). The virus infects only *Vitis* species in nature but can also be transmitted to herbaceous plants such as species in the family *Chenopodiaceae*. On *Chenopodium quinoa*, GFLV (isolate F13) induces a strong persistent mosaic of the systemically infected leaves leading to deformation and stunting of the plant.

The F13 isolate of GFLV contains a satellite RNA (RNA 3) of 1114 nt, encapsidated in the virions. RNA 3 is dependent for its replication on the presence of the genomic RNAs of the F13 isolate; its replication can also be supported by strain S of arabis mosaic virus (ArMV) (Fuchs, 1989). RNA 3 contains one open reading frame encoding a highly hydrophilic, basic polypeptide of 341 residues and Mr 37275 (Fuchs et al., 1989). In a wheatgerm translation system, RNA 3 directs the synthesis of a protein (P3) with an apparent Mr of 39K (Pinck et al., 1988). However such a polypeptide has never been observed in infected plants. Other nepoviruses, including tomato black ring virus (TBRV) (Fritsch et al., 1978), strawberry latent ringspot virus (Mayo et al., 1982), myrobalan latent ringspot virus (Fritsch et al., 1984), ArMV (Liu et al., 1990) and chicory yellow mottle virus (Rubino et al., 1990), also contain satellite RNAs which encode a satellite protein in the range of 38K to 48K (reviewed in Fritsch & Mayo, 1989). For TBRV, a polypeptide which coelectrophoresed with the *in vitro* translation product of the satellite RNA of TBRV was detected in infected protoplasts but not in plants (Fritsch et al., 1978). This paper describes the preparation and characterization of a P3-specific antiserum and its use for the detection of P3 in infected *C. quinoa*.

A fusion protein containing the C-terminal part of P3 (residues 202 to 341) and the N-terminal portion of the CI repressor of phage λ was produced in *Escherichia coli* strain W3110lacPl8, using the expression vector pEA305 kindly provided by Dr U. Niesbach-Klösgen (Amann et al., 1983; Niesbach-Klösgen et al., 1990). This vector overproduces the CI repressor under the control of the tac promoter (De Boer et al., 1983) upon induction by IPTG. Plasmid pEA305 has a single *Hind*III cloning site within the CI gene portion (John et al., 1985) which allowed insertion of the cDNA of the 3'-terminal part (nt 615 to nt 1114) of RNA 3 including the P3-coding sequence (Fuchs et al., 1989). The recombinant plasmid (pOM305) was used to transform *E. coli* strain W3110lacPl8 as described by Maniatis et al. (1982). Clones containing the inserted cDNA fragment were characterized by appropriate restriction enzyme digestion of plasmid preparations. Those containing the plasmid with the insert in the correct orientation were selected by testing for the expression of a fusion protein (P3CI) of the expected size (Mr, 32K; i.e. 17K for the CI region and 15K for the P3 C-terminal region) in transformed bacteria.
A clone containing the recombinant plasmid was amplified at 37 °C in M9 salts medium supplemented with 0.2% casamino acids and 50 μg/ml ampicillin (Maniatis et al., 1982). When cell density reached an optical density at 600 nm of 0.4, 1 mM IPTG was added to induce synthesis of high levels of the fusion protein. The overproduction of the fusion protein leads to formation of inclusion bodies (Marston, 1986, Mirtaki & King, 1989) which were purified as described in Niesbach-Klösgen et al. (1990) with the following modifications. Bacteria were harvested 4 h after IPTG induction and centrifuged. The pellet was washed in cold 50 mM-Tris·HCl pH 8.0 and resuspended in 50 mM-Tris·HCl pH 8.0 containing 20% sucrose. The cells were lysed by two French press cycles at 77 MPa and centrifuged for 30 min at 8000 r.p.m. in a Beckman JA20 rotor. The pellet (Pel-1) containing the inclusion bodies and cell debris was resuspended in 20% sucrose, 3 mM-EDTA, pH 8.0, and centrifuged through a 22 ml sucrose cushion (40% sucrose in 3 mM-EDTA, 50 mM-NaCl, 50 mM-Tris·HCl pH 8.0) for 20 min at 10000 r.p.m. The pellet (Pel-2), mainly composed of inclusion bodies, was resuspended in electrophoresis sample buffer (ESB; 4.5% SDS, 9 M-urea, 7.5% 2-mercaptoethanol in 75 mM-Tris·HCl pH 6.8) and the proteins were fractionated by 12% preparative SDS-PAGE (Laemmli, 1970). After visualization with ice-cold 0.25 M-KCl, the band corresponding to the fusion protein was excised and the protein was electroeluted and precipitated with acetone (Niesbach-Klösgen et al., 1990). After a 20 min centrifugation at 10000 r.p.m., the pellet (Pel-3) containing the purified fusion protein P3CI was resuspended in 0.1% SDS in PBS (0.15 mM-NaCl, 10 mM-phosphate buffer pH 7.2) and solubilized by a 5 min incubation at 80 °C.

The results obtained at each step of the purification are presented in Fig. 1(a). As a control, total protein extracts obtained from a 200 μl aliquot of induced and non-induced transformed bacteria were centrifuged, treated for 5 min at 100 °C in ESB, and fractionated by 12% SDS-PAGE. Upon IPTG induction, a prominent protein band of apparent Mr, 28K was observed (Fig. 1a, lane 2) which is not present in non-induced cells (Fig. 1a, lane 1). The apparent Mr of this protein is consistent with the value of 32K expected for P3CI. After each
purification step, the amount of this protein increased when compared to that of the bacterial proteins (Fig. 1a, lanes 3 to 5); the final step yielded a highly purified P3CI preparation (Pel-3, lane 5). In this fraction, a second band with an apparent \( M_r \) of 52K, a possible dimer of P3CI, is also detected. Amounts of 0.6 to 0.8 mg of purified P3CI (estimated on a Coomassie blue-stained polyacrylamide gel using known amounts of BSA) were obtained from 41 of IPTG-induced bacterial culture.

A sample of the purified P3CI solution containing 30 \( \mu \)g of protein was emulsified with Freund's adjuvant, either complete (first injection) or incomplete (boosters), by passage through a 21-gauge syringe needle and injected subcutaneously into rabbits at 3 to 4 week intervals. Sera were prepared from blood collected 10 days after each booster and were kept at -20 °C in 50% glycerol. They were tested by the immunoblotting of total proteins from transformed bacteria, fractionated by 12% SDS–PAGE and transferred to a nitrocellulose membrane (Towbin et al., 1979). Immunoreactions were as described (Berna et al., 1986) except that sheep IgG raised against rabbit IgG and conjugated to alkaline phosphatase was used at a 2000-fold dilution and visualized using the NBT-BCIP kit (Sigma). The results (Fig. 1a, lanes 7 to 10) show immunostaining of one major band (lane 8) comigrating with the Coomassie blue-stained purified protein (lane 5) and almost no background of bacterial proteins. A very low amount of this protein was detectable in non-induced bacteria (lane 7) owing to a low basal production of fusion protein with the possible presence of dimers, is also immunostained in total proteins of IPTG-induced cells; this species had a mobility similar to that of the 52K Coomassie blue-stained band present in the highly purified preparation (lane 5). Immunostaining of P3CI in total protein extracts of induced bacteria was totally abolished (lane 10) when antibodies were preabsorbed with 100 ng/ml of purified P3CI. This demonstrates the specificity of the P3CI immunostaining by the anti-P3CI serum.

The antiserum was tested against P3 synthesized in vitro, using as mRNA a synthetic transcript corresponding to a full-length RNA 3 cDNA insert transcribed from the plasmid 18p5 (Fuchs, 1989; Fuchs et al., 1989) with T3 RNA polymerase. Uncapped transcripts synthesized from 0.4 \( \mu \)g of EcoRI-linearized plasmid DNA were treated with RNase-free DNase I (Stratagene) and translated in the wheatgerm system in the presence of \(^{35}\)S)methionine (Amersham) according to Godfrey-Colburn et al. (1985), except that wheatgerm tRNA and RNasin were omitted and that the final concentrations of magnesium acetate and potassium acetate were 2.4 mM and 40 mM, respectively. In some experiments the translation medium was subsequently treated with 10 \( \mu \)g/ml RNase in 2 mM-EDTA pH 7.2 for 30 min at 37 °C (Berna et al., 1985) and centrifuged at 12000 g for 30 min to eliminate wheatgerm proteins remaining in the supernatant. One volume of double concentrated ESB was added to all samples before analysis by 10% SDS–PAGE followed by immunoblotting.

The blot was autoradiographed and treated with the anti-P3CI serum. The autoradiogram of the transcript-containing translation medium showed a protein band with an apparent \( M_r \) of 39K (Fig. 1b, lane 3) corresponding to the expected translation product and a minor band (P'3) with a slightly lower electrophoretic mobility. This band is probably due to a post-translational modification of P3 (Fuchs, 1989). Upon RNase–EDTA treatment, the translation products were recovered in the pellet fraction (b, lane 1) and undetectable in the corresponding supernatant fraction (b, lane 2). Immunostaining with the anti-P3CI serum (Fig. 1b, lanes 4 to 6) showed immunostained bands which could be superimposed with the radiolabelled translation products of the transcript present either in the complete translation medium or in the pellet obtained after RNase–EDTA treatment (b, lanes 6 and 4 respectively). The antiserum also bound non-specifically to some wheatgerm proteins. However upon RNase–EDTA treatment, most of these proteins were eliminated from the pellet containing P3 (b, lane 4) and were present in the supernatant fraction (b, lane 5). This is clearly visible on the Coomassie blue-stained gel (b, lanes 7, 8 and 9). Additional experiments showed the absence of P3-related products when RNA 3 transcripts were omitted from the translation medium (b, lanes 11 and 12). In addition, after preincubation of the anti-P3CI serum with 50 ng/ml purified P3CI, immunostaining of P3 was abolished (b, lanes 13 and 14). These results prove that the anti-P3CI serum specifically recognized the in vitro translated P3.

The anti-P3CI serum was then used to detect P3 in GFLV F13-infected C. quinoa plants. Inoculations were carried out on three or four mature leaves of C. quinoa with sap from leaves of plants ground in 50 mM-phosphate buffer pH 7.0. The plants were kept at 24 °C (day temperature) and 22.5 °C (night temperature) with illumination of 4000 lux for 16 h each day. Leaves and stems of healthy and infected plants (12 days post-inoculation) were ground in liquid nitrogen and mixed with ESB (1 ml/g), heated at 80 °C for 5 min and centrifuged at 12000 g for 3 min. Aliquots of the supernatants (called crude extracts), were analysed by immunoblotting as above. Controls were run in parallel: these were P3CI and a fusion protein containing the same CI repressor portion but fused to a heterologous viral protein, i.e. the P75 readthrough protein of beet
Thirty μl (equivalent to 15 mg fresh tissue) of crude extract from healthy (lanes H) or virus-infected C. quinoa leaves and stems harvested 13 days post-inoculation (lanes I) were fractionated by 10% SDS-PAGE (4 h at 13 V/cm) and transferred to a nitrocellulose membrane (1 h at 400 mA). Additional controls were run in parallel: (i) a pellet fraction (see legend Fig. 1b) obtained after RNase-EDTA treatment of translation products (lane T) (autoradiography of the nitrocellulose membrane revealed the position of P3 and P'3) and (ii) purified P3CI (2 ng, lanes 1) and P75CI (5 ng, lanes 2). Immunodetections were carried out with the anti-P3CI serum at a 2000-fold dilution without preabsorption (a), after preabsorption with 150 ng/ml purified P3CI (b) and after preabsorption with 125 ng/ml of purified P75CI (c). Marker proteins were as in Fig. 1(b).

Fig. 2. Immunodetection of satellite protein P3 in infected C. quinoa. Thirty μl (equivalent to 15 mg fresh tissue) of crude extract from healthy (lanes H) or virus-infected C. quinoa leaves and stems harvested 13 days post-inoculation (lanes I) were fractionated by 10% SDS-PAGE (4 h at 13 V/cm) and transferred to a nitrocellulose membrane (1 h at 400 mA). Additional controls were run in parallel: (i) a pellet fraction (see legend Fig. 1b) obtained after RNase-EDTA treatment of translation products (lane T) (autoradiography of the nitrocellulose membrane revealed the position of P3 and P'3) and (ii) purified P3CI (2 ng, lanes 1) and P75CI (5 ng, lanes 2). Immunodetections were carried out with the anti-P3CI serum at a 2000-fold dilution without preabsorption (a), after preabsorption with 150 ng/ml purified P3CI (b) and after preabsorption with 125 ng/ml of purified P75CI (c). Marker proteins were as in Fig. 1(b).

necrotic yellow vein virus (kindly provided by K. Richards).

The results are presented in Fig. 2(a to c). The purified P3CI fusion protein and its dimeric form are clearly visible (a, lane 1). The purified P75CI fusion protein (a, lane 2) was strongly immunostained by the anti-P3CI serum and migrated as a 66K protein; several degradation products were also detected. This suggests that the anti-P3CI serum contains antibodies reacting with the CI portion of the P3CI fusion protein.

In infected plant extracts, a 39K double band was immunostained (Fig. 2a, lane I) comigrating with the translation products P3 and P'3 (lane T). These proteins were absent from crude extracts of healthy plants (Fig. 2a, lane H). The antiserum gave a low background with cellular proteins except for species of 94K, 24K, 23K and sometimes 70K, which were strongly immunostained (a, lanes H and I).

Several attempts to preabsorb the antibodies reacting with the cellular proteins were performed by preincubating the antiserum with (i) a supernatant of healthy plant extract, produced by centrifugation at 30000g, (ii) an acetone powder of total proteins of healthy plants (prepared by addition of acetone to C. quinoa pulverized in liquid nitrogen, followed by filtration and washes with PBS under vacuum on Whatman 3MM filter paper and drying) and (iii) an acetone powder obtained as above, but after treatment with acetone of a pellet (centrifugation at 1000 g) extracted from healthy C. quinoa plants (up to 300 mg powder per ml of antiserum at a 40-fold dilution). In all cases, the preincubations did not diminish the immunostaining of the host proteins (not shown) probably because the latter were not present in sufficient amounts to preabsorb all the antibodies capable of reacting with them.

After preabsorption with 150 ng/ml of purified P3CI (Fig. 2b), the anti-P3CI serum no longer reacted with the 94K, 24K or 23K host proteins (lanes I and H) or with P3 (as already observed in Fig. 1), or with P3CI (lane 1); immunostaining of P75CI was almost completely abolished (lane 2). This indicates that the anti-P3CI serum contained some antibodies reacting specifically with the satellite protein as well as other antibodies that react with several host proteins, suggesting the possible presence of common epitopes between these cellular proteins and the fusion protein used as antigen source, i.e. the CI portion or the P3 portion of the protein.

Preabsorption of anti-P3CI serum with 150 ng/ml of P75CI (Fig. 2c) also abolished immunostaining of the 94K, 70K, 24K and 23K host proteins (lanes I and H) and P75CI (lane 2). In this case, however, it did not affect the immunostaining of P3 (lane I) and only slightly that of P3CI (lane 1).

These results prove that the anti-P3CI serum contains at least two types of antibodies, those reacting specifically with P3 and those reacting with CI, and that the N-terminal CI region of phage λ shares common epitopes with the three immunostained host proteins.

Another interesting observation was made in the experiments shown in Fig. 2(b), lanes I and H: the anti-P3CI serum preabsorbed with P3CI gave significant immunolabelling of a 26K host protein in both healthy and infected samples. This staining may derive from binding of P3CI (itself absorbed to antibodies) to the 26K protein. The variation in intensity of this 26K band is explained by differences in the total amount of cellular proteins present in the samples loaded on the gel. As P75CI did not react with this 26K protein (Fig. 2c, lanes I and H) one can reasonably assume that the P3 region of P3CI is responsible for this interaction. This would indicate a strong affinity of P3CI for the 26K cellular protein.

To determine the subcellular location of P3 in infected plants, subcellular fractions [cell walls, a pellet (centrifugation at 1000 g), a second pellet, and supernatant (centrifugation at 30000 g)] of GFLV F13-infected C. quinoa were prepared and analysed by immunoblotting as described in Godefroy-Colburn et al. (1986). P3 was detected mostly in the particulate fractions sedimenting at 1000 g and to lesser extent in those at 30000 g. It was detectable neither in the cytoplasmic fraction (30000 g supernatant) nor in the cell wall fraction or in the...
region of phage 2 present in the fusion protein used as the gous fusion proteins. 

detected on day 7p.M. and increased up to day 10. proteins in stems harvested under the inoculated leaves buds, stems and roots. Kinetics of accumulation of both serum. The CP and P3 were first detected on day 5 post- 

proteins were analysed in crude extracts as described in the text. Eight percent (for CP) and 10% (for P3) polyacrylamide gels were loaded with aliquots of crude extracts equivalent to 5 mg fresh tissue. Electrophoresis was followed by a Western blot analysis for both proteins. Immunoreactions were carried out using an anti-GFLV F13 serum and the anti-P3CI serum at 4000- and 2000-fold dilutions, respectively. (a) Immunodetection of the CP. (b) Immunodetection of P3. Lanes H, healthy extract. Lane numbers 1 to 21 indicate the number of days post-inoculation. Lane CP, 5 ng purified virus.

corresponding healthy fractions (data not shown). However it should be noted that degradation of P3 in the pellets (1000 g and 30000 g) was rapid.

Kinetics of coat protein (CP) and P3 accumulation in different parts of the plant were followed for 21 days after inoculation. Apical buds, stems, leaves and roots were harvested at different times after infection and total proteins were analysed in crude extracts as described above. The CP was visualized with an anti-GFLV F13 serum. The CP and P3 were first detected on day 5 post-infection (p.i.) in inoculated leaves, and 24 to 48 h later in buds, stems and roots. Kinetics of accumulation of both proteins in stems harvested under the inoculated leaves are shown in Fig. 3(a and b). The CP and P3 were detected on day 7 p.i. and increased up to day 10. Thereafter the amount of CP remained constant or decreased slightly until day 21 (a). On the other hand, the amount of P3 decreased rather rapidly and was almost undetectable after day 15 (b). Similar kinetics of accumulation were obtained in leaves, buds and roots.

The results presented in this paper show that the CI region of phage λ present in the fusion protein used as the antigen source gave rise to a significant amount of specific antibodies which unexpectedly reacted with three cellular proteins. Therefore, with fusion proteins, specific controls should always be performed with antiserum preabsorbed with homologous and heterologous fusion proteins.

This anti-P3CI serum allowed us for the first time specifically to detect a satellite RNA-encoded protein in infected plants. This protein appears to be bound to particulate fractions, especially those sedimenting at 1000 g and containing mostly nuclei and chloroplasts. The concomitant accumulation of P3 and CP at the beginning of infection and the transient expression of P3 during the first 10 days of infection suggest a role during the active phase of virus multiplication, on RNA replication and/or symptom expression. Finally, the ability of P3 to bind to a 26K cellular protein may be of importance for understanding the role of P3.

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References


Short communication


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