ACCUMULATION OF HELPER COMPONENT/PROTEINASE AND COAT PROTEIN OF TURNIP MOSAIC VIRUS IN INTACT PLANTS

K. OHSHIMA

Laboratory of Plant Virology, Faculty of Agriculture, Saga University, 1-banchi, Honjo-machi, Saga 840-8502, Japan

Received November 10, 1998; accepted February 1, 1999

Summary. – The helper component/proteinase (HC/Pro) protein of turnip mosaic virus (TuMV) was fused with glutathione S-transferase (GST) and expressed as a fusion protein in *Escherichia coli*. The quality of antiserum raised against the GST-HC/Pro fusion protein was compared to that of antiserum raised against coat protein (CP) by image analyser. The result showed that these antisera were of similar quality. Then the both antisera were used to follow the time course of accumulation of HC/Pro protein and CP in intact TuMV-infected leaves. CP appeared first at day 3 post inoculation (p.i.) and gradually accumulated in uninoculated upper leaves, whereas HC/Pro protein appeared first at day 4 p.i., accumulated up to day 7 p.i. and then gradually decreased. Potyvirus proteins are encoded by a single translation unit spaning most of the genome and are presumably synthesized in equimolar ratios. Therefore, the reduced accumulation of HC/Pro protein in relation to CP at one month p.i. in infected plants is presumed to be the result of its degradation.

Key words: turnip mosaic virus; helper component; proteinase; fusion protein; intact plant

Introduction

Potyviruses form a diverse group of plant viruses that cause diseases worldwide (Shukla *et al.*, 1994). The monopartite genome of potyviruses consists of a single-stranded, positive-sense 10 kb RNA molecule containing a single open reading frame whose translation product is processed by cis- and trans-activated viral proteinases to yield mature viral proteins (Carrington and Dougherty, 1987; Carrington *et al.*, 1988; Hellman *et al.*, 1988; Carrington *et al.*, 1989a; García *et al.*, 1989; Laliberté *et al.*, 1992; Riechmann *et al.*, 1992; Kim *et al.*, 1996, 1998). The proteinases are P1, HC/Pro and NIa proteins of potyviruses. The proteolytic activity has been shown to reside within the C-terminal half of the NIa protein (Dougherty and Parks, 1989) whereas the N-terminal domain represents the VPg which becomes covalently linked to the 5'-end of the genomic RNA (Murphy *et al.*, 1990). The NIa proteinase cleaves between NIb protein and CP, and between several other proteins of potyviruses (Carrington and Dougherty, 1987; Carrington *et al.*, 1988; Dougherty and Carrington, 1988; Hellmann *et al.*, 1988; Dougherty and Parks, 1989; García *et al.*, 1989; Carrington *et al.*, 1993). On the other hand, P1 and HC/Pro proteins autocatalytically cleave their respective C-termini (Carrington *et al.*, 1989a; Carrington *et al.*, 1989b; Carrington *et al.*, 1990; Mavankal and Rhoads, 1991; Verchot *et al.*, 1991). Of other viral proteins with known function it is the P1 protein which binds not only ssRNA but also ssDNA (Brantley and Hunt, 1993; Soumounou and Laliberté, 1994). The cytoplasmic inclusion (CI) protein displays RNA-dependent ATPase and RNA unwinding activities characteristic of RNA helicase (Laín *et al.*, 1991; Ohshima *et al.*, 1993).

Abbreviations: CI = cytoplasmic inclusion; CP = coat protein; EDTA = ethylenediamine tetraacetate; GST = glutathione S-transferase; HC/Pro = helper component/proteinase; IPTG = isopropyl-thio-β-D galactoside; NIa = nuclear inclusion a; NIb = nuclear inclusion b; Me = 2-mercaptoethanol; nt = nucleotide; PCR = polymerase chain reaction; PBS = phosphate-buffered saline; p.i. = post inoculation; PPB = potassium phosphate buffer; RT-PCR = reverse transcritpion-PCR; SDS = sodium dodecyl sulfate; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of SDS; TuMV = turnip mosaic virus; VPg = genome-linked protein

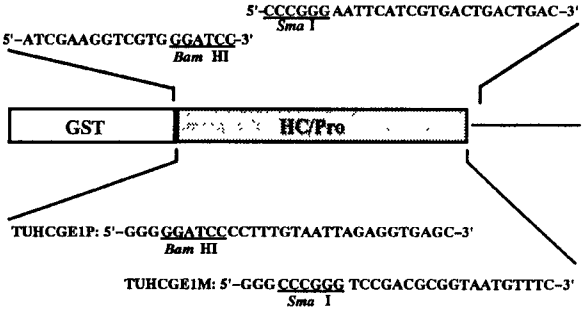

Fig. 1
Structure of the GST-HC/Pro fusion protein gene present in pGETUHC recombinant vector

Nucleotide sequences at the junctions of HC-Pro gene are shown in the upper part and oligonucleotide primers TUHCGE1P (sense) and TUHCGE1M (antisense) with restriction sites (underlined) used for the assembly of the fusion protein gene are shown in the lower part.

The NIb protein is thought to be the core replicase because of its homology to RNA-dependent RNA polymerase from other positive-stranded RNA viruses (Domier *et al.*, 1987). CP is the major structural viral protein (Shukla *et al.*, 1994) and is involved in aphid transmission along with HC/Pro protein (Atreya *et al.*, 1990). On the other hand, the P3 protein has as yet no known function but has been detected in infected plants (Rodríguez-Cerezo and Shaw, 1991).

The potyvirus proteins are encoded by a single translation unit that spans most of genome and are presumably synthesized in equimolar ratios (Restrepo *et al.*, 1990). However, accurate accumulation rates of these proteins remain still unknown. Moreover, it is known that potyviral proteins such as CP, cytoplasmic and nuclear inclusion proteins are easy to detect and to purify from virus infected plants, but this is not the case of other proteins including HC-Pro protein. Therefore, we investigated in this study accurate accumulation rates of CP and HC/Pro proteins in TuMV-infected plants and found differences in their pattern of accumulation.

Materials and Methods

Purification of virus and CI protein. TuMV strains 1 and 31 (Sako, 1980) were propagated on *Brassica rapa* L. cv. Hakatasuwari and the virus particles were purified by the method described by Choi *et al.* (1977) with some modifications. The infected leaf tissues were homogenized with 2 volumes (w/v) of 0.5 mol/l potassium phosphate buffer (PPB) pH 7.2 containing 0.01 mol/l ethylenediamine tetraacetate (EDTA), 0.1% thioglycolic acid and 15% carbon tetrachloride. The homogenate was clarified by centrifugation at 3200 x g for 10 mins. Virus particles were pelleted by two cycles of centrifugation at 80,000 x g for 90 mins. The pellet was suspended in 0.01 mol/l PPB pH 7.2, laid on 10 – 40 % sucrose density gradient and centrifuged at 90,000 x g for 90 mins. The fraction containing virus particles was collected from the gradient and centrifuged at 111,000 x g for 90 mins. The pellet was resuspended in 0.01 mol/l PPB pH 7.2. TuMV CI protein was purified by the method of Hiebert *et al.* (1984) with some modifications. The infected leaf tissues were homogenized with two volumes (w/v) of 0.5 mol/l PPB pH 7.5 containing 0.2% sodium sulfite. After the homogenate was squeezed through cheesecloth, two volumes (w/v) of the mixture of chloroform and carbon tetrachloride (1:1) were added to the sap and mixed. The mixture was clarified by centrifugation at 14,000 x g for 20 mins. The pellet was re-suspended in 0.05 mol/l PPB pH 8.2 containing 0.1% 2-mercaptoethanol (Me) and 5% Triton X-100, stirred for 60 mins and then centrifuged at 27,000 x g for 20 mins. The pellet was resuspended in 0.02 mol/l PPB pH 8.2 containing 0.1% Me, laid on 50 – 80% sucrose density gradient and centrifuged at 50,000 x g for 60 mins. The CI protein fraction was collected from the gradient and then centrifuged at 50,000 x g for 90 mins. The pellet was resuspended in 0.01 mol/l Tris-HCl pH 8.0.

TuMV RNA was extracted from purified virus according to Rosner *et al.* (1983).

Recombinant DNA construction. The structure of the gene encoding the GST and HC/Pro fusion proteins used in this study is shown in Fig. 1. To assemble this gene, the region of nt 61-1,378 of TuMV genome (Nakashima *et al.*, 1993) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) (Saiki *et al.*, 1988). The RT-PCR conditions were as described previously (Ohshima *et al.*, 1993). The recombinant vector pGETUHC (pGEX3X containing HC/Pro gene) was introduced into *E. coli* XL1-Blue and then extracted by the boiling method (Holms and Quigley, 1981) to confirm its presence. The nucleotide sequence of the junction between GST and HC/Pro genes was analysed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the GEX3X2P primer (5'-GCATGGCCTTTGCAGGG-3').

Expression and purification of GST-HC/Pro fusion protein. LB medium (100 ml) with 50 μg/ml ampicillin and 12.5 μg/ml tetracycline was inoculated with 2 ml of an overnight suspension culture of *E. coli* transformed with pGETUHC vector. The culture was incubated at 37°C until the A_{600} of 0.5 was reached. The expression of the GST-HC/Pro fusion protein was then induced by addition of 1 mmol/l isopropylthio-β-D galactoside (IPTG) and left to proceed for 5 hrs. The purification of GST-HC/Pro fusion protein from cellular proteins was performed by detergent treatment, sonication (Smith and Corcoran, 1990) and lysozyme treatment (Carrington *et al.*, 1990). The bacterial pellet was resuspended in 0.01 mol/l phosphate-buffered saline (PBS) containing 2% Triton X-100 pH 7.4. The bacterial suspension was then sonicated for 10 mins. The insoluble protein was collected and lysed at room temperature in a lysis buffer (20 mmol/l Tris-HCl pH 8.2, 8% sucrose, 100 mmol/l KCl, 5 mmol/l EDTA, 0.1% Nonidet P-40 and 2.5 mg/ml lysozyme). The viscosity of the lysate was reduced by addition of 10 mmol/l $MgCl_2$ and 10 μg/ml DNase I. The insoluble fraction containing the GST-HC/Pro fusion protein was collected by centrifugation. The pellet was resuspended and washed 5 times with the lysis buffer without lysozyme. The insoluble protein was disrupted by the addition of an equal volume of a dissociation buffer (10 mmol/l Tris-HCl pH 8.0, 1 mmol/l EDTA, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% Me and 0.001%

Bromophenol Blue) and heated for 3 mins in boiling water before electrophoresis. The supernatant was electrophorsed in 10% polyacrylamide gels containing SDS as described by Laemmli (1970). The gels were then soaked in 0.25 mol/l KCl and the band with the GST-HC/Pro protein was cut out by a razor blade, homogenized with PBS in a Teflon homogenizer , centrifuged and the supernatant (purified GST-HC/Pro fusion protein) was used for immunization.

Production of antiserum. The purified GST-HC/Pro fusion protein was emulsified in complete Freund's adjuvant and injected into a New Zealand white rabbit in 5 subsequent doses. Antisera against CP and CI protein were produced using the purified virus and CI protein, respectively.

Purification of HC/Pro protein from TuMV-infected plants. HC/Pro protein was partially purified from TuMV strain 1-infected turnip plants by ammonium sulfate precipitation, Sephadex G-75 chromatography (Sako and Ogata, 1981) and affinity chromatography. Purified anti-HC/Pro IgG was coupled to CNBr-activated Sepharose 4B (Pharmacia) in a coupling buffer (0.1 mol/l $NaHCO_3$ pH 8.3 and 0.5 mol/l NaCl). After blocking with 0.2 mol/l glycine, the gel was washed with the coupling buffer and an acetate buffer pH 4.0 four times using centrifugation. Partially purified HC/Pro protein was added to anti-HC/Pro IgG-coupled Sepharose 4B and incubated for 4 hrs at room temperature. The gel with the bound HC/Pro protein was washed 7 times with TPC (Tris-HCl pH 8.0 and 0.3 mol/l KCl). Then the gel was packed in a 1 ml syringe and HC/Pro protein was eluted with APC (0.2 mol/l NH_4OH and 0.3 mol/l KCl, pH 10.8.

Protein extraction and analysis. Small turnip plants (two-true leaf stage) were dusted with carborundum and inoculated by abrading the 1st true leaves with purified virus (10 or 50 μg/ml). Total SDS-soluble proteins were extracted from leaf tissue at up to day 10 p.i. A leaf was ground in 40 volumes (w/v) of the dissociation buffer and the homogenate was heated for 3 mins at 100°C. The insoluble proteins were removed by centrifugation and the protein extracts were analysed for the presence of CP and HC/Pro protein by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) (Laemmli, 1970). The gels were stained with Coomassie Briliant Blue or using Silver Stain Kit (Wako). Or they were subjected to Western blot analysis using a Bio-Rad Mini Trans-blot apparatus (Towbin *et al.*, 1979). The proteins were blotted onto a PVDF membrane and incubated with anti-CP serum (at 1:1,000 dilution) or anti-HC/Pro serum (at 1:1,000 dilution). Signals on the membrane were visualized using Konika Immunostain HRP Kit. The signals were quantified by measuring their contour (OD x mm^2) values with Image Master (Pharmacia).

Aphid transmission test. Aphid transmission and artificial membrane feeding tests were performed as described by Sako (1980) with some modifications. After starving, the aphids (*Mysus persicae*) were given a 5-min-acquisition access period and then were placed on each test turnip plant for 2 hrs. For artificial membrane feeding test, 10 mg of purified HC/Pro protein and 100 mg of purified virus were mixed and used. 0.02 mol/l PPB pH 7.4 containing 0.15 mol/l NaCl and 0.01 mol/l NH_4OH buffer containing 0.15 mol/l KCl (pH 10.8) were used instead of TPC and APC, respectively, for aphid transmission tests.

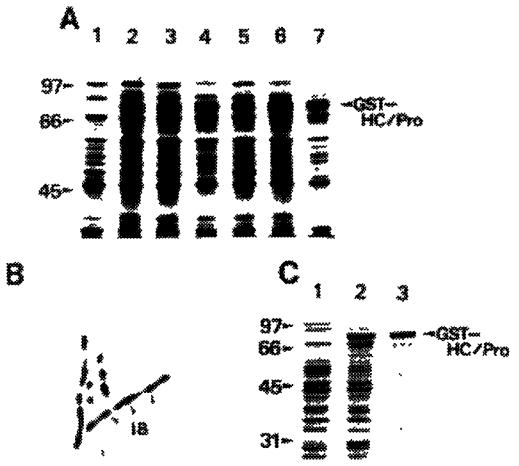

Fig. 2
Expression and purification of GST-HC/Pro fusion protein
A: SDS-PAGE (10% polyacrylamide) of total proteins from *E. coli* harbouring nonrecombinant pGEX3X vector (lane 1) and recombinant pGETUHC vector whose fusion protein gene was induced with IPTG (lanes 2–7). TuMV strains 1 (lanes 2–4) and 31 (lanes 5–7) were donors of the fusion protein genes.
B: A light micrograph of *E. coli* producing insoluble inclusion body protein.
C: SDS-PAGE (12.5% polyacrylamide). Total proteins from *E. coli* harbouring nonrecombinant pGEX3X vector (lane 1) and recombinant pGETUHC vector whose fusion protein gene was induced with IPTG (lane 2), and the fusion protein purified by sonication and lysozyme treatment (lane 3). Positions of the fusion protein shown on the right, and positions and M_r values (K) of size standards shown on the left.

Results

Production and purification of GST-HC/Pro fusion protein

The HC/Pro genes of TuMV strains 1 and 31 were successfully reverse transcribed and amplified by PCR. The amplified fragments were introduced into pGEX3X expression vector which contained Factor Xa proteinase cleavage site. The GST-HC/Pro fusion proteins derived from six recombinant plasmids were successfully produced in *E. coli* (Fig. 2A). It is known that soluble GST fusion proteins are easily purified from bacterial lysates by affinity chromatography using glutathione Sepharose 4B. However, the insoluble GST-HC/Pro protein was found exclusively in inclusion bodies (Fig. 2B) and was not cleaved by Factor Xa proteinase. The insolubility of GST proteins can be addressed by several means. In some cases, these proteins may be solubilized by detergent treatment of cell lysates while in other cases the growth of cells at low temperature has a similar effect (Smith and Corcoran, 1990). We treated the GST-HC/Pro protein with guanidine-HCl or urea, but the

treated protein was still not cleaved by Factor Xa proteinase.When the protein was induced at the low concentration (0.1 or 0.01 mmol/l) of IPTG at 28°C, only a small amount of soluble protein was obtained (data not shown). Therefore, we decided to omit the Factor Xa proteinase cleavage step and to purify the protein in insoluble form. The successful method employed for the fusion protein purification was a combination of sonication, lysozyme treatment and SDS-PAGE (Fig. 2C). The fusion protein was not separated from bacterial proteins when sonication or lysozyme treatment was used alone. The junctions between pGEX3X vector and amplified fragment of HC/Pro DNA was determined by nucleotide sequencing and no framshift was found in the clones (data not shown).

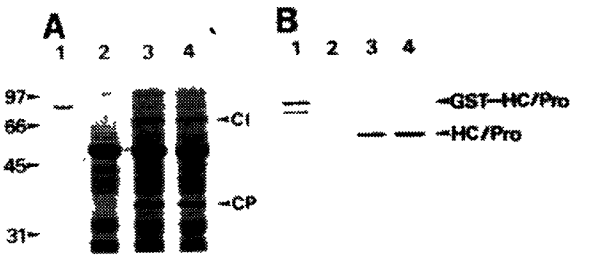

Fig. 3
Detection of CP, HC/Pro and CI proteins in TuMV-infected leaves at one month p.i. by SDS-PAGE (A) and Western blot analysis (B)
Purified GST-HC/Pro fusion protein (lanes 1), total proteins from healthy leaves (lanes 2) and leaves infected with TuMV strain 1 (lanes 3) and strain 31 (lanes 4). Sample volumes: 8 µl (A) and 2 µl (B). Positions and M_r values (K) of size standards shown on the left.

Detection of HC/Pro protein in infected leaves

Total soluble proteins from TuMV-infected and healthy turnip plants were subjected to SDS-PAGE and compared. Only CP and CI protein were clearly detected but no other viral proteins including HC/Pro protein were found (Fig. 3A). This result indicates that CP and HC/Pro protein or CI and HC/Pro proteins did not accumulate in the same amounts in intact plants at one month p.i.

When the anti-GST-HC/Pro serum was used for detecting HC/Pro protein by Western blot analysis, it reacted specifically with HC/Pro protein (52 K) from the infected turnip plants but not with healthy plant proteins.

A prerequisite for comparing the accumulation of HC/Pro protein and CP in the infected plants by Western blot analysis is similar quality of antibodies to HC/Pro protein and CP. Thus, HC/Pro protein and CP were purified from the infected plants and such antisera were used for detecting known amounts of these proteins by Western blot analysis (Fig. 4). Both antibodies reacted with the proteins at 1.25 ng as minimum amount and the contour (OD x mm^2) values of HC/Pro protein and CP signals were similar at each amount. These results indicated that the antisera to HC/Pro protein and CP, respectively, were of similar quality. Unfortunately, the activity of HC/Pro protein purified by affinity chromatography was low when it was used for aphid transmission test through artificial membrane feeding (2 infected plants/48 inoculated plants). The active form of potyvirus HC/Pro protein in plants is known to be a dimer (Thornbury *et al.*, 1985). In this study, the antiserum (antibody) to HC/Pro protein was raised by use of the GST-HC/Pro fusion protein (eluted from SDS-polyacrylamide gel) as antigen. Therefore, the antibody was directed against the

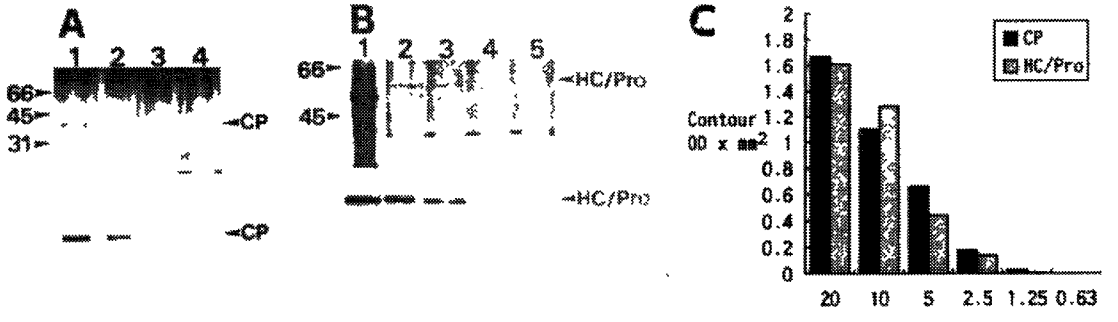

Fig. 4
SDS-PAGE and Western blot analysis of purified preparations of CP and HC/Pro fusion protein
A: SDS-PAGE with silver staining (upper panel) and Western blot analysis (lower panel) of 10 ng (lane 1), 5 ng (lane 2), 2 5 ng (lane 3) and 1.25 ng (lane 4) of CP purificate. Positions and M_r values (K) of size standards shown on the left.
B: SDS-PAGE with silver staining (upper panel) and Western blot analysis (lower panel) of 10 ng (lane 2), 5 ng (lane 3), 2.5 ng (lane 4) and 1 25 ng (lane 5) of HC/Pro protein purificate. Partial purificate of HC/Pro protein (lane 1). Positions and M_r values (K) of size standards shown on the left
C: Contour values (OD x mm^2) of Western blot signals of CP and HC/Pro protein purificates. Abscissa: amounts (ng) of purificates.

epitopes on denatured (primary structure) HC/Pro protein, suggesting that the binding capacity of the antibody to the active form (quarternary structure) of HC/Pro protein was probably low. However, the antiserum to HC/Pro protein reacted well with HC/Pro protein (primary structure) in SDS-PAGE and seemed to be useful for tracing this protein in intact plants.

Accumulation of HC/Pro protein in intact leaves

The time course of accumulation of HC/Pro protein and CP was examined in TuMV-infected turnip leaves. CP was detected first at day 3 p.i. in the uninoculated upper 3rd leaves (Fig. 5A). In contrast, HC/Pro protein was first detected at day 4 p.i. (Fig. 5B).

It is difficult to compare the amounts of proteins on developed Western blots by eyesight. Therefore, the contours of signals on the blots were measured by the Image Master. When the contour values were averaged from three experiments, CP gradually accumulated from day 3 p.i. in TuMV-uninoculated upper leaves. In contrast, HC/Pro protein accumulated until day 7 p.i. and then gradually decreased in the leaves (Fig. 5C). When 10 μg/ml TuMV was used for inoculation, the time course of accumulation of CP and HC/Pro protein was similar to that with 50 μg/ml TuMV except a 2-day delay in the first detection of these proteins in the leaves (data not shown).

Aphid transmission rate from TuMV-infected leaves is shown in Fig. 5C. Aphids started to transmit the virus at day 4 p.i. from the uninoculated upper leaves, and the rate gradually increased. The rate corresponded well to the accumulation of both CP and HC/Pro protein, indicating that both proteins are necessary for aphid transmission (Shukla *et al.*, 1994).

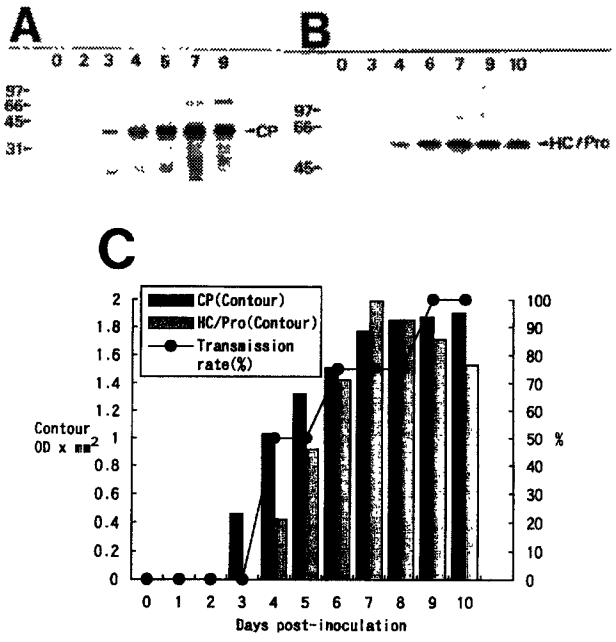

Fig. 5
Time course of accumulation of CP (A) and HC/Pro fusion protein (B) in TuMV strain 1-infected leaves
A, B: Western blot analysis of proteins (10 μl samples) from uninoculated upper (3rd) leaves. Numbers above each lane indicate days p.i. Positions and Mr values of size standards are shown on the left.
C: Contour values (OD x mm²) of Western blot signals of CP and HC/Pro proteins, and aphid transmission rates as determined by placing 10 aphids on each of 6–20 test plants on various days p.i.

Discussion

We describe here the time course of accumulation of HC/Pro protein in turnip plants as determined by an antiserum to TuMV HC/Pro fusion protein expressed in bacteria. CP and CI protein were detected by SDS-PAGE at one month p.i. On the other hand, HC/Pro protein could be detected by Western blot analysis but not by SDS-PAGE. Therefore, we conclude that HC/Pro protein accumulated to a lesser extent than CP in TuMV-infected leaves. Since potyvirus proteins are presumably synthesized in equimolar ratios in infected plants (Restrepo *et al.*, 1990) there is an apparent contradiction between the theory and the results. To investigate the accumulation of CP and HC/Pro protein in the infected leaves more closely, antisera to CP and HC/Pro protein of similar quality were produced and employed for Western blot analysis. The time course of accumulation of CP and HC/Pro protein showed that CP gradually accumulated, whereas HC/Pro protein accumulated initially and then gradually decreased. Moreover, CP was first detected after day 3 p.i., while HC/Pro protein was first detected only after day 4 p.i. Restrepo *et al.* (1990) obtained similar results in experiments with accumulation of tobacco etch virus proteins. They detected HC/Pro protein and CP by immunoblot analysis first on days 4 and 3 p.i., respectively. Our results indicate that HC/Pro protein is more unstable than CP in intact plants and HC/Pro protein degradation probably occurs just after TuMV polyprotein is cleaved to mature HC/Pro protein. However, there was no investigation of time lag of HC/Pro protein (a cleavage site is between HC/Pro and P3 proteins) and NIa proteinase cleavage (NIa proteinase cleaves between NIb protein and CP) so far. Therefore, further detailed analysis using *in vitro* translation system for investigating time lag between these proteins is neccessary. In preliminary experments, a series of inoculated plants were stored in a deep freezer for 10 days and then were employed for Western blot analysis. CP was detected undegraded while HC/Pro protein was not detected at all. Thus in this study, fresh TuMV-infected leaves were homogenized with a dis-

sociation buffer immediately after harvesting and the extracts were stored frozen until use in SDS-PAGE. Results of these experiments also showed that HC/Pro protein is relatively less stable than CP. The concentration of all proteins within plant, bacteria and animal cells is not determined solely by synthesis but also by degradation. Moreover, it is now clear that ubiquitin functions primarily by becoming covalently attached to proteins that are soon degraded in the ubiquitin-dependent proteolytic pathway (Viersta, 1993).

In this paper, we have found differences in the pattern of accumulation of HC/Pro protein and CP. To date, the kinetics of viral proteins in plants was investigated only with tobacco mosaic and alfalfa mosaic viruses (Godefroy-Colburn *et al.*, 1986; Lehto *et al.*, 1990; Moser *et al.*, 1988). To examine regulation of the level of potyviral proteins it is necessary first to define the kinetics of their translation and post-translational modification.

Acknowledgements. This work was supported by Grant-in-Aid for Scientific Research No.09660052 from the Ministry of Education, Science and Culture, Japan. We thank the Saga University for enabling use of the Structure-Function Analysis System for Useful Biological Macromolecules.

References

Atreya CD, Raccah B, Pirone TP (1990): A point mutation in the coat protein abolishes aphid transmissibility of a potyvirus. *Virology* **178**, 161–165.

Brantley JD, Hunt AG (1993): The N-terminal protein of the polyprotein encoded by the potyvirus tobacco vein mottling virus is an RNA-binding protein. *J. Gen. Virol.* **74**, 1157–1162.

Carrington JC, Dougherty WG (1987): Small nuclear inclusion protein encoded by a plant potyvirus genome is a proteinase. *J. Virol.* **61**, 2540–2548.

Carrington JC, Cary SM, Dougherty WG (1988): Mutational analysis of tobacco etch virus polyprotein processing: cis and trans proteolytic activities of polyproteins containing the 49-kilodalton proteinase. *J. Virol.* **62**, 2313–2320.

Carrington JC, Freed DD, Sanders TC (1989a): Autocatalytic processing of the potyvirus helper component proteinase in *Escherichia coli* and *in vitro*. *J. Virol.* **63**, 4459–4463.

Carrington JC, Cary SM, Parks TD, Dougherty WG (1989b): A second proteinases encoded by a plant potyvirus genome. *EMBO J.* **8**, 365–370.

Carrington JC, Freed DD, Oh C-S (1990): Expression of potyviral polyprotein in transgenic plants reveals three proteolytic activities required for complete processing. *EMBO J.* **9**, 1347–1353.

Carrington JC, Haldeman R, Dolja VV, Restrepo-Hartwig MA (1993): Internal cleavage and trans-proteolytic activities of the Vpg-proteinase (NIa) of tobacco etch potyvirus *in vivo*. *J. Virol.* **67**, 6995–7000.

Choi JK, Maeda T, Wakimoto S (1977): An improved method for purification of turnip mosaic virus. *Ann. Phytopathol. Soc. Jpn.* **43**, 440–448.

Domier LL, Shaw JG, Rhoads, RE (1987): Potyvirus proteins share amino acid sequence homology with picorna-, como-, and caulimoviral protein. *Virology* **178**, 285–288.

Dougherty WG, Carrington JC (1988): Expression and function of potyviral gene products. *Ann. Rev. Phytopathol.* **26**, 123–143.

Dougherty WG, Parks TD (1989): Molecular genetic and biochemical evidence for the involvement of the heptapeptide cleavage sequence in determining the reaction profile at two tobacco etch virus cleavage site in cell-free assays. *Virology* **172**, 145–155.

García JA, Riechmann JL, Laín BS (1989): Proteolytic activity of the plum pox potyvirus NIa-like protein in *Escherichia coli*. *Virology* **170**, 362–369.

Godefroy-Colburn T, Gagey MJ, Berna A, Stussi-Garaud C (1986): A non-structural protein of alfalfa mosaic virus in the walls of infected tobacco cells. *J. Gen. Virol.* **67**, 2233–2239.

Hellmann GM, Shaw JG, Rhoads RE (1988): *In vitro* analysis of tobacco vein mottling virus NIa cistron: evidence for a virus-encoded protease. *Virology* **163**, 554–562.

Hiebert E, Purcifull DE, Christie RG (1984): Purification and immunological analyses of plant viral inclusion bodies. *Methods Virol.* **8**, 225–280.

Holms DS, Quigley M (1981): A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* **114**, 513–524.

Kim D-H, Hwang DC, Kang BH, Lew J, Han J, Song BD, Choi KY (1996): Effect of internal cleavages and mutations in the C-terminal region of NIa protease of turnip mosiac potyvirus on the catalytic activity. *Virology* **226**, 183–190.

Kim D-H, Han JS, Lew J, Kim SS, Kang BH, Hwang DC, Jang DS, Kim W, Song BD, Choi KY (1998): Effect of mutations in the C-terminal region of NIa protease on cis-cleavage between NIa and NIb. *Virology* **241**, 94–100.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage. *Nature* **227**, 680–685.

Laín S, Martin MT, Riechmann JL, García JA (1991): Novel catalytic activity associated with positive-strand RNA virus infection: nucleic acid-stimulated ATPase activity of the plum pox potyvirus helicaselike protein. *J. Virol.* **65**, 1–6.

Laliberté JF, Nicolas O, Chatel H, Lazure C, Morosoli R (1992): Release of a 22-kDa protein derived from the amino-terminal domain of the 49-kDa NIa of turnip mosaic potyvirus in *Escherichia coli*. *Virology* **190**, 510–514.

Lehto K, Bubrick P, Dawson WO (1990): Time course of TMV 30K protein accumulation in intact leaves. *Virology* **174**, 290–293.

Mavankal G, Rhoads RE (1991): In vitro cleavage at or near the N-terminus of the helper component protein in the tobacco vein mottling virus polyprotein. *Virology* **185**, 721–731.

Moser O, Gagey MJ, Godefroy-Colburn T, Stussi-Garaud C, Ellwart-Tschürtz M, Nitschko H, Mundry KW (1988): The fate of the transport protein of tobacco mosaic virus in systemic and hypersensitive tobacco hosts. *J. Gen. Virol.* **69**, 1367–1373.

Murphy JF, Rhoads RE, Hunt AG, Shaw JG (1990): The VPg of tobacco etch virus RNA is the 49-kDa proteinase or the N-terminal 24-kDa part of the proteinase. *Virology* **178**, 285–288.

Nakashima H, Sako N, Hori K (1993): Nucleotide sequences of the helper component-proteinase genes of aphid transmissible and non-transmissible isolates of turnip mosaic virus. *Arch. Virol.* **131**, 17–27.

Ohshima K, Inoue AK, Shikata E (1993): Molecular cloning, sequencing, and expression in *Escherichia coli* of the potato virus Y cytoplasmic inclusion gene. *Arch. Virol.* **128**, 15–28.

Restrepo MA, Freed DD, Carrington JC (1990): Nuclear transport of plant potyviral proteins. *The Plant Cell* **2**, 987–998.

Riechmann JL, Laín S, García JA (1992): Highlights and prospects of potyvirus molecular biology. *J. Gen. Virol.* **73**, 1–16.

Rodríguez-Cerezo E, Shaw JG (1991): Two newly detected nonstructural viral proteins in potyvirus-infected cells. *Virology* **185**, 572–579.

Rosner A, Ginzburg I, Bar-Joseph M (1983): Molecular cloning of complementary DNA sequences of citrus tristeza virus RNA. *J. Gen. Virol.* **64**, 1757–1763.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988): Primer-directed enzymatic amplification of cDNA with a thermostable DNA polymerase. *Science* **230**, 1350–1354.

Sako N (1980): Loss of aphid transmissibility of turnip mosaic virus. *Phytopathology* **70**, 647–649.

Sako N, Ogata K (1981): A helper factor essential for aphid transmissibility of turnip mosaic virus. *Ann. Phytopathol. Soc. Jpn.* **47**, 68–70.

Sanger F, Nicklen S, Coulson AR (1977): DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Shukla DD, Ward CW, Brunt AA (1994): *The Potyviridae*. CAB International, Wallingford, Oxon, p. 516.

Smith DB, Corcoran LM (1990): Expression and purification of glutathione-S-transferase fusion protein. In FM Ausubel, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith, K Struhl (Ed.): *Current Protocols in Molecular Biology.* Suppl. 10. John Wiley and Sons, New York, pp. 16.7.1–16.7.8.

Soumounou Y, Laliberté JF (1994): Nucleic acid-binding properties of the P1 protein of turnip mosaic potyvirus produced in *Escherichia coli*. *J. Gen. Virol.* **75**, 2567–2573.

Thornbury DW, Hellmann GM, Rhoads RE, Pirone TP (1985): Purification and characterization of potyvirus helper component. *Virology* **144**, 260–267.

Towbin H, Staehelin T, Gordon J (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.

Verchot J, Koonin EV, Carrington JC (1991): The 35-kDa protein from the N-terminus of the potyviral polyprotein functions as a third virus-encoded proteinase. *Virology* **185**, 527–535.

Viersta RD (1993): Protein degradation in plants. *Ann. Rev. Pl. Physiol.* and *Pl. Mol. Biol.* **44**, 385–410.