European Journal of Plant Pathology **105:** 389–395, 1999.

Detection of potyviral nuclear inclusion b proteins by monoclonal antibodies raised to synthetic peptides

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Accepted 20 April 1999

Key words: potyvirus, protein overexpression, RNA-dependent RNA polymerase, Western blot

Abstract

Potyviral nuclear inclusion b protein (NIb), the RNA-dependent RNA polymerase, contains three highly conserved regions. Peptides corresponding to these regions were synthesised and used for immunisation. A panel of monoclonal antibodies was obtained. Most of the MAbs reacted with the peptides and a recombinant NIb of PVY in PTA-ELISA. Two of them specifically detected native NIb of potato A, potato V, potato Y, plum pox and turnip mosaic potyviruses in extracts of infected plants in Western blots. Time course experiments revealed that NIb protein can be first detected on the fifth day after infection.

Introduction

The genus *Potyvirus* is the largest genus of plant viruses and belongs to the economically very important family *Potyviridae*. It contains about two hundred definitive and possible members, accounting for more than one-third of all viruses known to infect plant species around the world (Shukla et al., 1994; Ward and Shukla, 1991). Potyviruses are flexuous filamentous particles containing a single stranded positive RNA of about 10 kb with a covalently linked 5′-terminal viral protein (VPg) and a 3′ terminal poly(A) tail (reviewed in Riechmann et al., 1992). The potyvirus genome contains a single open reading frame (ORF) which is translated as a large polyprotein precursor (ranging between 340 and 368 kDa, depending on the individual potyvirus). The precursor is subsequently cleaved by virus-encoded proteolytic enzymes to yield products of different size (Carrington and Dougherty, 1988; Ryan and Flint, 1997). Only two of them, VPg and CP, have been detected in virus particles. During infection some potyviruses induce nuclear inclusion bodies which are co-crystals of two viral-encoded proteins present in equimolar amounts: the smaller nuclear inclusion (NIa) protein is a polyprotein consisting of the VPg and proteinase, the large nuclear inclusion (NIb) protein has amino acid motifs of RNA-dependent RNA polymerase (RdRp) (Koonin, 1991). NIa and NIb are also found in the cytoplasm. Evidence for NIb protein possessing an RdRp activity has been obtained recently (Hong and Hunt, 1996).

Serology of virus particles and coat proteins is a very useful and widely used tool for the identification and classification of plant viruses (Jordan and Hammond, 1991; Shukla et al., 1988). Serology of non-structural proteins including RdRp could serve as an additional criterion for the identification and classification of potyviruses. Despite all efforts, RdRps have been successfully purified only for a limited number of bacterial, animal, and plant viruses (Bates et al., 1995). Consequently, only a few monoclonal antibodies (MAbs) to viral non-structural proteins have been

produced (Slade et al., 1989). Two approaches are often used to overcome this limitation: the use of a recombinant protein or synthetic peptides. Antibodies to synthetic peptides that correspond to linear segments of a protein can react with the homologous sequence in the native folded protein provided it is exposed on the surface and exhibits a structure or conformation similar to the immunising peptide (Cariepy et al., 1986; Craig et al., 1998). Broadly cross-reactive antibody probes were successfully generated using synthetic peptides corresponding to the conserved coat protein regions of potyviruses (Joisson et al., 1992a,b). The advantage of the synthetic peptides approach is that the immune response can be directed to a region of special interest.

The objective was to establish a system to detect potyviral NIb in infected plants with the help of MAbs. To achieve a universal detection system for almost all potyviruses, we chose three highly conserved regions of this protein as targets for MAb production and synthesised the corresponding peptides for immunisation. A panel of MAbs was obtained and tested for use. Further experiments will demonstrate whether the MAb can specifically inhibit the enzymatic activity of this protein, thus raising the possibility to block potyvirus multiplication in transgenic plants by expressing genes coding for corresponding single chain antibody fragments (scFv).

Materials and methods

Viruses

All virus isolates originated from the virus collection of the Institute of Epidemiology and Resistance, Aschersleben, Germany. *Potato virus Y* (PVY; genus *Potyvirus*) isolate CH 605, an N-type strain (PVY-N), was propagated in *Nicotiana tabacum* cv. *Xanthii*, isolate D884 and *potato V potyvirus* were propagated in *N. glutinosa*, *turnip mosaic virus* (TuMV; genus *potyvirus*) was propagated in *Brassica chinensis*, *potato virus A* (PVA; genus *potyvirus*) and *plum pox virus* (PPV; genus *potyvirus*) were propagated in *N. clevelandii.*

Identification of conserved regions

All complete sequences of potyviruses published so far were compared (EMBL Sequence database) and three large conserved regions of NIb protein were identified. The amino acid (aa) sequences are the following: region 1 – ANKTRTFTAAPLDTLLGGKVCVDDENNQFY, region 2–LPENWVYCDADGSQFDSSLTPYLINAVL and region 3 – NLYTEIVYTPILTPDGTIVKKFKGN NSGQPSTVVDNSLMV, located at aa positions 185–214, 240–267, 286–325 (for PVY NIb, EMBL sequence database A08776), respectively. The underlined parts of the three regions contained amino acids which had a predominantly positive antigenic index as determined by computer analysis (Hopp and Woods, 1981), except for most of region 2 (17/26 aa negative index). The underlined peptides were synthesised and purified by HPLC to a purity of ≥95% (Genosys).

Molecular cloning and overexpression of recombinant NIb protein in Escherichia coli

Total RNA was isolated from PVY-N infected *N. tabacum* cv. Xanthii by an RNAzol reagent (Gibco-BRL) according to the manufacturer's recommendations. cDNA and double-stranded DNA of NIb were synthesised and amplified according to standard methods (Sambrook et al., 1989). The primers used were as follows: for the first strand 5′ T_{6984} CGA**TCGCGA**TGTAATGGTGGAGCAAGC (bold and underlined: NruI site added, number: position of the corresponding nucleotide in the genome of PVY-N, EMBL sequence database A 08776), for the second strand 5′C_{8629}TG**CCCGGGATCC**GCATCTTTCTTG (SmaI and BamHI sites added, respectively). The purified DNA was ligated into pCR-script SK(+) and transformed into *E. coli* XL-2 blue (Stratagene). The NIb gene was subcloned in frame into the over-expression plasmid pET30a (Novagen). The recombinant NIb protein was expressed in *E. coli* BL 21 (DE3) pLysS, and purified by immobilised metal affinity chromatography (IMAC) according to manufacturer's instruction (Novagen).

Immunisation and preparation of monoclonal antibodies

Fifty μg of the peptides R1, R2, R3, each mixed with an equal volume of Freund's complete adjuvant (Sigma), was injected into the peritoneal cavity of 8-week-old BALB/c mice. Two mice were used for each peptide. For the second and third injections the same amounts of peptides mixed with equal volume of incomplete Freund's adjuvant (Sigma) were given at

2–3 week intervals. Ten days after the third injection the mice were bled and the antisera titres were evaluated. One mouse from each antigen with the highest antibody titre was boosted with 100–150 μg of the antigen without adjuvant. Three days after the boost, mouse spleen cells were fused with mouse myeloma cell line X-63Ag8.653 (Köhler and Milstein, 1975; Kearney et al., 1979). The fusion product was plated out into 96-well tissue culture plates in the presence of peritoneal macrophages as feeder cells and cultured in Dulbecco's MEM supplemented with HAT and 20% fetal calf serum (both Gibco-BRL).

Screening and cloning of hybridomas

Hybridoma culture supernatants were tested for specific antibodies by plate-trapped antigen ELISA (PTA-ELISA) as described by Mowat (1985). Microtiter plate wells (Costar, high binding) were coated with the corresponding antigen (2 μg/ml peptide in PBS) overnight at 4 °C and blocked with PBS/BSA. After three washes the supernatants from culture wells were added and the plates were incubated for 1.5 h at room temperature. After three washes with PBST, rabbit anti-mouse IgG, conjugated to horseradish peroxidase (DAKO-Immunoglobulins), was added and the plates were incubated for 1 h. After three washes, substrate solution (1 mg/ml o-phenylenediamine, 0.06% hydrogen peroxide in citrate buffer, pH 5.0) was added. After 5–15 min incubation at room temperature, the reaction was stopped by adding 50 μl of 1 N H_2SO_4 and A_{492nm} values were measured. Positive clones were subcloned twice by the limiting dilution method. Monoclonal antibody isotypes were determined by ELISA using subclass-specific goat antibodies and rabbit anti-goat immunoglobulins conjugated to horseradish peroxidase (Sigma).

Antigen specificity assays

The antigen specificity of the MAbs was tested in PTA-ELISA and Western blots. Microtiter plates (Polysorp, Nunc) were coated respectively with the purified recombinant NIb of PVY (2 μg/ml) and PVY (isolate CH605) infected leaf extract diluted in 0.05 M sodium carbonate buffer at pH 9.6 (1:10 dilution, 100 μl per well) overnight at 4 °C and blocked with 1% skimmed dried milk in PBS at 37 °C for 2 h. After three washes the hybridoma culture supernatants containing antigen-specific antibodies were added and the plates were incubated for 2 h at 37 °C. After four washes with PBS/Tween, alkaline phosphatase conjugated rabbit anti-mouse IgG + IgM (Dianova) was added and the plates were incubated for 1 h at 37 °C. After five washes, substrate solution (1 mg/ml pNPP) was added. After 2 h incubation at room temperature, the A_{405nm} value was measured. The procedure for the Western blot analysis of recombinant NIb was the same as described for SDS-PAGE and Western blotting.

Detection of native NIb in potyvirus infected plants

(i) ELISA

The extract of systemically infected leaves of potyvirus infected plants was used 2–4 weeks after infection in PTA-ELISA as described above.

(ii) SDS-PAGE and Western blotting

Leaf tissue (1 g) of the same potyvirus infected plants as tested in PTA-ELISA was ground to a fine powder in liquid nitrogen and total proteins were extracted with 4 ml of extracting buffer (9 M urea, 4.5% SDS, 7.5% β-mercaptoethanol, and 50 mM Tris-HCl, pH 6.8). The extracts were boiled for 10 min and then centrifuged at 10 000 × *g* for 10 min. Equivalent amounts of the supernatant were directly loaded onto a 12% polyacrylamide gel containing 0.1% SDS (Laemmli, 1970). Proteins were transferred from gels onto PVDF membranes (Roth) at 13 V for 2 h in transfer buffer (39 mM glycine; 48 mM Tris-HCl; 0.037% SDS; 20% methanol; pH 8.3) using a semidry electroblotter. After blocking the membranes with 0.2% I-block (Tropix), blots were probed with the MAbs/alkaline phosphatase conjugated anti-mouse antibodies (IgG/IgM) followed by chromogenic detection with NBT/BCIP as substrate (Sambrook et al., 1989).

(iii) Time course studies

In each case four young *N. glutinosa* plants (at the five leaf stage) were inoculated with PVY isolate CH605, and *N. cleverlandii* plants were inoculated with PVA. The infection was confirmed for coat protein by DAS-ELISA (Clark and Adams, 1977). During a serial days post infection systemic leaves were extracted for SDS-PAGE and Western blot.

(iv) Influence of healthy plant extract on binding ability of NIb

Known amounts of affinity purified NIb were diluted with sap from healthy *N. glutinosa* and used for PTA-ELISA and Western blot.

Table 1. Characteristics and reactivity of monoclonal antibodies specific for potyviral NIb

Cell culture supernatant	Specific to region	PTA-ELISA									
		Synthetic peptides (2 μg/ml)			Leaf extracts of infected plants						
		R1	R2	R3	NIb	PVY	Healthy	PVA	PPV	PVV	TuMV
1G5	R1	1.16	0.11	0.09	0.03	0.03	0.07	nt	nt	nt	nt
2C3	R1	1.66	0.12	0.10	0.03	0.26	0.30	nt	nt	nt	nt
2G10	R1	1.33	0.10	0.08	0.33	1.92	1.32	1.89	1.88	1.88	1.79
3C6	R1	1.13	0.12	0.11	0.32	0.45	0.08	0.47	0.38	0.33	0.27
3G4	R1	1.24	nt	nt	0.66	0.25	0.07	0.26	0.23	0.25	0.23
1B7	R2	0.16	1.69	0.19	0.68	0.25	0.08	0.34	0.22	0.24	0.26
1C8	R2	0.14	1.94	0.15	0.55	0.37	0.15	0.45	0.37	0.37	0.33
1G12	R2	0.12	1.80	0.11	0.78	0.30	0.07	0.39	0.30	0.33	0.31
1B9	R2	0.22	1.41	0.21	0.02	0.20	0.14	nt	nt	nt	nt
3B4	R3	0.16	0.11	0.32	0.02	0.04	0.05	nt	nt	nt	nt
3E11	R3	0.11	0.10	0.31	0.01	0.04	0.05	nt	nt	nt	nt

nt = not tested; isotype of all MAbs was IgM.

Results and discussion

Production and characterisation of monoclonal antibodies against different regions of NIb

After several fusions, six hybrid cell cultures for region 1, five for region 2, and two for region 3, were selected for cloning and further propagation. Characteristics of these hybrid lines established after three cloning steps are summarised in Table 1. All MAbs belonged to isotype IgM. Probably, it is a feature of small molecules which mainly stimulate B cells to produce the first class of antibody and not effectively induce the synthesis of later classes of antibodies. In each case the MAb gave the highest OD values with their corresponding peptide and no signals were obtained with the two other peptides as well as buffer control.

Expression and analysis of recombinant NIb protein in E. coli

Sequence analysis of two PVY NIb clones revealed that at their extreme 3′-ends 360 nt were missing. Since the clones contained the sequences necessary to express all three regions of interest, no new cloning experiments were conducted. The truncated recombinant NIb protein was over-expressed in *E. coli* and purified by IMAC to near homogeneity. Optimal expression of the NIb was achieved with 1 mM IPTG for induction when the cells were cultured at 30 °C and harvested after 3 h. The recombinant NIb was specifically detected by Western blot by MAb 1B7, 1G12, 3C6, and 3G4. An example for the MAb 3G4 (R1) and 1G12 (R2) is shown in Figure 1. The sensitivity of these MAbs in chromogenic detection with NBT/BCIP as substrate was sufficient to visualise about 100 ng of purified recombinant NIb, limit of detection was 50 ng. Interestingly, the signal of detection of the recombinant NIb is dramatically reduced on Western blots in the presence of healthy plant extract (Figure 2). The same holds true for ELISA detection of NIb (results not shown). Probably, the plant proteins affect the binding of NIb to PVDF membranes or ELISA plates.

Detection of the native NIb proteins in potyvirus infected plants

The ability of the MAbs to detect viral NIb in PTA-ELISA was tested. The results are shown in Table 1. Two MAbs obtained to region 3 gave only a weak reaction with peptide R3 and could not detect the recombinant NIb. These as well as MAb 1G5, 1B9 and 2C3 were not reactive with the native NIb from PVY infected plants and were omitted from further experiments. The best results in detecting recombinant NIb and native NIb in plants infected with different potyviruses were obtained with MAb 3C6, 3G4, 1C8, 1B7 and 1G12 in PTA-ELISA. MAb 1G12 and 3G4 were able to detect PVY in naturally infected

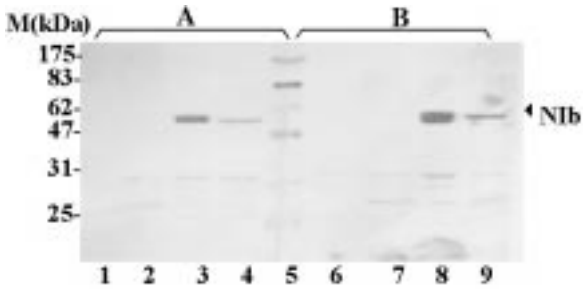

Figure 1. Detection of the recombinant NIb in Western blots. Lanes 1, 6: cells contain plasmid without insert; lanes 2, 7: crude extracts from non-induced cells; lanes 3, 8: crude extracts from induced cells; lanes 4, 9: 100 ng of purified NIb and lane 5: prestained protein marker. A: probed with MAb1G12; B: probed with MAb3G4.

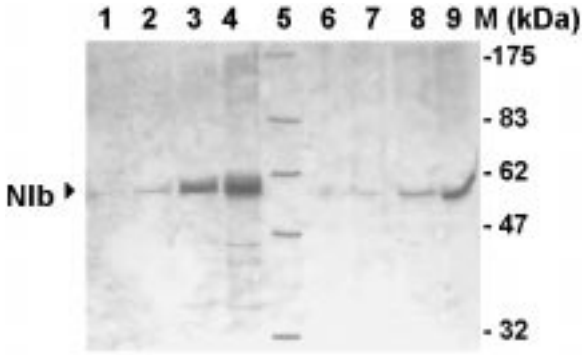

Figure 2. Effect of healthy plant extracts on the sensitivity of detection of recombinant NIb in Western blot. Lanes 1, 2, 3, 4: 27, 55, 110 and 1.100 ng of purified recombinant NIb, respectively; lane 5: prestained protein marker; lane 6, 7, 8, 9: the same amount of the recombinant NIb as in lanes 1–4, respectively, but in the healthy plant extract. Blot was probed with MAb3G4.

Solanum tuberosum L. from a green house and field. A_{405nm} values are at least 3 times higher than those of healthy controls.

The A_{405nm} values for infected plants obtained by PTA-ELISA for NIb ranged from 0.3 to 0.5 after 2–3 h substrate incubation. Comparative A_{405nm} values for CP detection reached $\geq$1.5 (data not shown). This sensitivity would not be satisfactory for the routine detection of potyvirus infection but is possibly sufficient to block RdRp activity via corresponding scFv in transgenic plants. The low values might result from

(i) the insolubility of NIb: it is incompletely bound to the surface of the ELISA-plates;
(ii) linear epitopes of viral NIb recognised by the antibody are not fully exposed and not accessible to the antibody (Milton and Van Regenmortel, 1979);
(iii) plant proteins suppressing binding of NIb to ELISA plates.

In Western blot analysis the four MAbs, 1B7, 1G12, 3C6, 3G4, were able to detect a polypeptide with the expected molecular mass of the NIb protein ($\sim$65 kDa) in potyvirus infected plants. MAb1G12 and 3G4 were specific for NIbs of potyviruses since a band is observed on the blot (Figure 3A,C). MAb3C6 and 1B7 have a similar reactivity with NIb, but cross-reacted with a $\sim$55 kDa protein (Figure 3B,D). In some cases, immunoblot assays revealed a smaller ($\sim$30 kDa) band. This band probably results from degradation of NIb, since a similar band sometimes was found in a preparation of recombinant NIb. Both MAb 1B7 and 3C6, specific for regions 1 and 2 respectively, cross-reacted with a $\sim$55 kDa protein. We speculated that it might be a kind of plant host RdRP (Schiebel et al., 1993, 1998) owning the same conserved regions as viral RdRp. It is supposed that the plant RdRPs are involved in plant defense mechanisms (Jorgensen et al., 1998; Wassenegger and Pélissier, 1998). However, these MAbs did not react with a cDNA-derived tomato RdRP expressed in *E. coli* (kindly provided by Dr. Wassenegger, data not shown). Consequently, the nature and function of this protein remains unclear.

Time course experiments revealed that NIb can be first detected in systemically infected leaves approximately 5 dpi for PVY. It remains detectable at least 28 dpi tested (Figure 4). This result is in agreement with observations of both Bounoch et al. (1991) and Hajimorad et al. (1996) in that tobacco etch potyvirus and peanut stripe potyvirus could be first detected in systemic leaves five days post infection.

In conclusion, MAb 3G4 and 1G12 raised to synthetic peptide R1 and R2, corresponding to conserved regions of potyviral NIb, can specifically recognise the native NIb protein derived from potyvirus infected plants. These antibodies can be a valuable tool for the investigation of functions of RdRp during the virus life cycle, and can be directly used in immunoaffinity purification of potyviral RdRp from infected plants. Moreover, in current experiments these MAbs serve as templates for the production of scFv, which will be used to block virus multiplication in transgenic plants.

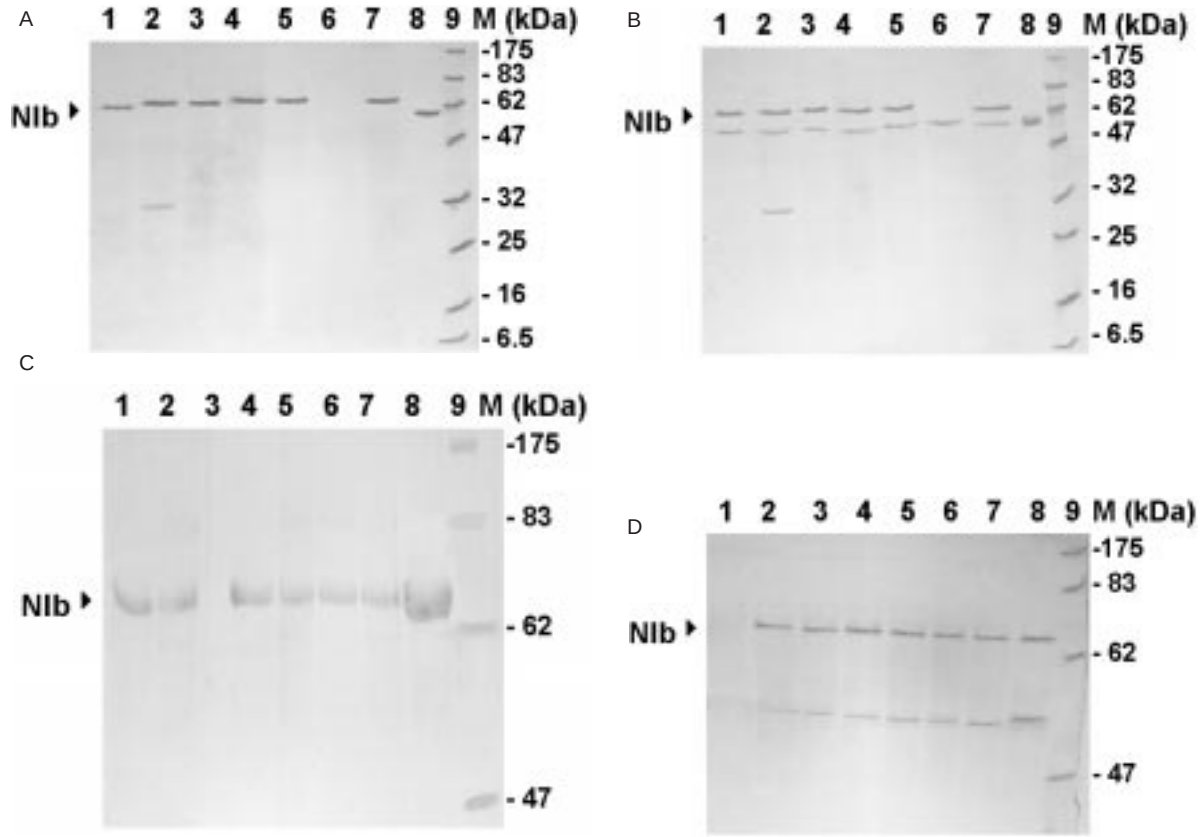

Figure 3. Detection of native NIb proteins in potyvirus infected plants in Western blots. A: probed with MAb3G4. B: probed with MAb1B7; Lane 1: PVY D884, lane 2: TuMV, lane 3: PVA, lane 4: PPV, lane 5: PVV, lane 6: healthy control, lane 7: PVY CH605, lane 8: purified recombinant NIb, lane 9: prestained protein marker. C: probed with MAb 1G12; Lane 1: PVY-D884, lane 2: PVY-CH605, lane 3: healthy control, lane 4: TuMV, lane 5: PVA, lane 6: PPV, lane 7: PVV, lane 8: PVY-NTN, lane 9: prestained protein marker. D: probed with MAb 3C6; Lane 1: healthy control, lane 2: PVY-D884, lane 3: PVY-CH605, lane 4: TuMV, lane 5: PVA, lane 6: PPV, lane 7: PVV, lane 8: PVY-NTN, lane 9: prestained protein marker.

Figure 4. Time course of NIb synthesis in systemically PVY infected leaves of *Nicotiana* plants. Lanes 1–8: healthy, 1, 3, 5, 7, 14, 21, 28 dpi, respectively, lane 9: prestained protein marker; probed with MAb3G4.

Acknowledgements

Authors thank Drs. T. Kühne, F. Rabenstein and A. Ziegler for helpful discussion, Mrs. M. Nielitz and H. Mühlheim for technical assistance. F.L. was supported by a grant of the Ministry of Culture, Sachsen-Anhalt, FRG, and both E.S and T.E. by the Federal Ministry of Science and Technology, FRG.

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