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Cloning of the coat protein gene from beet necrotic yellow vein virus and its expression in sugar beet hairy roots

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Summary. Expression of the beet necrotic yellow vein virus (BNYVV) coat protein (CP) gene in transgenic sugar beet hairy roots was accomplished as a step towards CP-mediated virus resistance. A cDNA for the CP gene and its 5′ terminal untranslated leader sequence was prepared from BNYVV RNA, using two oligodeoxynucleotides to prime the synthesis of both strands. Second-strand synthesis and amplification of the cDNA were done by Taq DNA polymerase chain reactions. Run-off transcripts of the cloned cDNA sequence were obtained and translated in vitro, yielding immunoreactive CP. A binary vector construction containing the CP gene under the control of the 35S promoter of cauliflower mosaic virus was prepared and used for *Agrobacterium rhizogenes*-mediated transformation of sugar beet tissue. Stable integration and expression of the CP gene in sugar beet hairy roots was demonstrated by Southern, Northern, and Western blot analysis, respectively.

Key words: BNYVV – Coat protein – Hairy roots – Plant virus resistance – Sugar beet

Introduction

Beet necrotic yellow vein virus (BNYVV) is a soil-borne virus which causes a severe disease called "rhizomania" in sugar beet plants (e.g., Tamada 1975). It is transmitted to the plant roots by the fungus *Polymyxa betae*. The virus has rod-shaped particles of four different lengths, corresponding to four different single-stranded, plus-sense RNA species. The nucleotide sequences of the four RNAs have been determined (Bouzoubaa et al. 1987). The coat protein cistron of BNYVV is located on RNA-2, extending from nt 145 to 708 (Bouzoubaa et al. 1986). The 5′ terminal untranslated sequence from nt 1 to 144 may function as a translational enhancer for the contiguous open reading frame, as was shown for the 5′ leader sequences of several plant RNA viruses (Gallie et al. 1987). We therefore included the complete leader sequence in the BNYVV CP gene construction.

Protection from virus infection mediated by expression of the viral CP in transgenic plants has been demonstrated for a number of viruses of different groups (for reference, see Wilson 1989). In contrast to many other plant species, sugar beet has thus far been very recalcitrant to regeneration after transformation. Inoculation of *Beta vulgaris* with *Agrobacterium rhizogenes* gives rise to the formation of transformed hairy roots (Hamill et al. 1986) which can be isolated and cultured. Since hairy root cultures derived from single root tips probably represent clones that originated from one transformed cell (David et al. 1984), an efficient selectable marker gene to separate transformed from nontransformed cells is not required.

In this paper, we report the cloning of the CP gene from BNYVV into vectors for in vitro transcription, transient expression, and stable integration. The functional integrity of the cloned CP gene is shown by in vitro transcription/translation, and its expression in sugar beet tissue is demonstrated in transformed hairy roots as a model system.

Materials and methods

BNYVV isolate Yu2 (Burgermeister et al. 1986) and sugar beet cv Kawevera (KWS AG, Einbeck) were used in the experiments. *E. coli* JM109 was used for cloning of the vector constructions. Standard molecular biological techniques were carried out as described by Maniatis et al. (1982).

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cDNA synthesis and amplification

BNYVV was purified and viral RNA was extracted as described by Burgermeister et al. (1986), using 1% sodium dodecylsulfate, 5 m*M* EDTA in 10 m*M* TRIS-HCl (pH 7.8). cDNA for the CP gene and its leader sequence was synthesized using two synthetic oligodeoxynucleotide primers, which were synthesized on a Gene Assembler (Pharmacia-LKB, Uppsala) and purified by polyacrylamide gel electrophoresis.

cDNA first-strand synthesis was carried out employing 2 µg unfractionated BNYVV RNA, 0.12 µg (20 pmol) primer 1, 5′-CAGCTAATTGCTATTGTC-3′, 60 units AMV reverse transcriptase (Boehringer, Mannheim), and additional components of a reverse transcription reaction. Approximately 94 ng ss-cDNA was obtained, as determined from the incorporation of [α-^{32}P]dCTP-derived radioactivity. RNA was hydrolyzed with NaOH, the solution was extracted with phenol/chloroform, and ss-cDNA was precipitated with ethanol.

Second-strand synthesis and amplification of the ds-cDNA was carried out by Taq DNA polymerase reactions. The reaction mixture (100 µl) contained 47 ng ss-cDNA, 74 pmol primer 1, 88 pmol primer 2, 5′-GCGCAAGCTTAAATTCTAACTATTATCTCC-3′, 7.5 units Taq DNA polymerase (Biolabs, Schwalbach), 1.5 m*M* each of dATP, dGTP, dCTP, and dTTP, 16.6 m*M* $(NH_4)_2SO_4$, 67 m*M* TRIS-HCl (pH 8.8), 6.7 m*M* $MgCl_2$, 10 m*M* mercaptoethanol, and 200 µg/ml bovine serum albumin. Thirty reaction cycles were carried out, each consisting of DNA denaturation for 2 min at 94 °C, primer annealing for 2 min at 50 °C, and primer extension for 6 min at 70 °C. With respect to the relatively long DNA sequence to be amplified, the first three primer extension steps were carried out for longer times, namely, 12, 10, and 8 min. Additional Taq DNA polymerase (7.5 units) was added after 10 and 20 reaction cycles, respectively. After completion of 30 cycles, the reaction mix was extracted with phenol/chloroform and DNA was precipitated with ethanol. The reaction products were separated by agarose gel electrophoresis, and the fraction from 500 to 1,000 bp was eluted from the gel, yielding 2.5 µg amplified cDNA.

cDNA cloning and in vitro transcription/translation

The vector pGEM-3zf(+) (Promega, Madison, WI) was linearized and part of its polylinker was removed by digestion with HindIII and HincII. The cDNA was digested with HindIII and ligated to dephosphorylated vector, yielding the recombinant plasmid pGC700. This plasmid was linearized with BamHI, purified by preparative agarose gel electrophoresis, and used as a template for SP6 RNA polymerase reactions (Melton et al. 1984). In-vitro-synthesized RNA was examined by formamide-agarose gel electrophoresis as described (Burgermeister et al. 1986). Capping of the transcript was done as described (Krieg and Melton 1984). Cell-free translation was carried out using rabbit reticulocyte lysate, treated according to Pelham and Jackson (Amersham, Braunschweig, code N90), according to the manufacturer's instructions. Incorporation of [^{35}S] methionine into TCA-precipitable material was stimulated tenfold compared to the blank in translations with 0.5 µg tobacco mosaic virus RNA and sixfold with 0.5 µg BNYVV RNA or 0.5 µg capped transcript. Appropriate quantities of the translation products were analyzed by SDS-polyacrylamide gel disk electrophoresis and autoradiography. The immunological reactivity of the in vitro translation products towards an antiserum against BNYVV was examined by Western blotting.

Construction of the plant expression vectors

pGC700 was digested with HindIII and SmaI. The resulting 743-bp fragment was purified by gel electrophoresis and made blunt-ended, using the Klenow fragment of DNA polymerase I. The DNA fragment was then ligated into the SmaI-linearized plant expression vector pRT103 (Töpfer et al. 1987), yielding plasmid pTCcpl. This was digested with HindIII, releasing a 1,452-bp fragment that was gel purified and ligated into the HindIII-linearized binary vector pLX222 (Landsmann et al. 1988). The resulting plasmid is termed pLTCcpL.

Generation of transformed sugar beet hairy roots

The binary vector construct pLTCcpL was transferred from *E. coli* JM109 to *Agrobacterium rhizogenes* C58C1 (pRiA4) (Petit et al. 1983) via triparental mating, using the helper strain *E. coli* RK2013. Segments 8–10 mm long from sterile sugar beet seedlings in the four-leaf stage were inoculated with *Agrobacteria*, by cutting along the midrib or along the stem with a scalpel dipped into a colony grown for 2 days on LB agar. The segments were placed on modified MS agar containing 160 mg/l instead of 1,650 mg/l NH_4NO_3 and 5 mg/l Ca-pantothenate, and incubated at 25 °C in the light (3,000 lx). After 2–3 days they were transferred to modified MS agar containing 300 µg/ml cefotaxime. Single hairy roots, which appeared at the inoculation sites after 2 weeks were cut off, transferred to liquid Gamborg's B5 medium containing 20 g/l sucrose and 300 µg/ml cefotaxime, and grown at 26 °C in the dark on a gyratory shaker at 120 rpm. Hairy roots were not selected for kanamycin resistance, which is coded for by the *NPTII* gene also present between the border sequences of pLX222.

DNA isolation and Southern blot analysis

DNA from hairy roots was isolated using the minipreparation method of Dellaporta et al. (1983). The DNA was digested with HindIII, separated on a 0.8% agarose gel, and transferred to Amersham Hybond-N membrane by diffusion blotting overnight, with 0.25 *M* NaOH/1.5 *M* NaCl as a blotting solution. A 730-bp HindIII/SstI fragment from pGC700 was labelled with [α-^{32}P]dCTP by Nick-translation and used as a hybridization probe. The membranes were air dried, and prehybridization, hybridization, and washing of the blots was done as described by Thomas (1980), except that dextran sulfate was omitted from the hybridization solution.

RNA isolation and Northern blot analysis

Total RNA was isolated from hairy roots as described by Verwoerd et al. (1989). The RNA was electrophoresed in 1% agarose gels containing 2.2 *M* formaldehyde and transferred to nitrocellulose membranes (Schleicher & Schuell, BA85, Dassel) with 20 × SSC. The membranes were baked at 80 °C for 2 h and prehybridized and hybridized as described above for DNA blots.

Detection of BNYVV coat protein by Western blotting

Proteins were extracted from hairy roots by homogenizing 100 mg (fresh weight) of root tissue with 32 µl 0.625 *M* TRIS (pH 6.8)/10% SDS, 20 µl 85% glycerol, and 8 µl mercaptoethanol, heating at 100 °C for 5 min, and centrifuging for 10 min at 13,000 rpm in a minicentrifuge. The protein concentration of the supernatants was estimated by a dye-binding dot assay (Stegemann et al. 1973), and 50 µg of protein was applied on each lane of a 5%/15% SDS-polyacrylamide disk gel. Electrophoresis, transfer of proteins to nitrocellulose membranes, and immunological detection of BNYVV coat protein were performed as described by Burgermeister and Koenig (1984).

Results

Cloning and in vitro expression of the CP gene

cDNA was prepared from unfractionated BNYVV RNA using two oligodeoxynucleotides to prime the synthesis of the first and second strand. Primer 1 was complementary to nt 704–721 of BNYVV RNA-2. Primer 2 corresponded to nt 1–20 and had a 5′ extension of 10 nt to provide a unique HindIII site in the cDNA. Electrophoretic separation of the reaction products indicated a major fraction of the expected size of the cDNA (approximately 730 bp) and a zone of faster-migrating material, presumably unincorporated primers (Fig. 1 A). The cDNA was cut at its 5′ terminus with HindIII and inserted in defined orientation into the in vitro transcription vector pGEM-3zf(+), yielding the recombinant plasmid, pGC700. Sequence determination of the cloned cDNA (data not shown) revealed a number of base exchanges with respect to the BNYVV RNA-2 sequence published by Bouzoubaa et al. (1986).

In vitro transcription of the cloned sequence yielded about ten transcripts per molecule DNA template. The electrophoretic mobility of the transcript on formamide-agarose gels indicated a size of 950 nt (Fig. 1 B). According to our cloning procedure, the transcript is expected to have a size of 744 nt, including the 5′ terminal 721-nt sequence of BNYVV RNA-2 and 23 nt from the 5′ extension of the cDNA and the multiple cloning site of the vector. The larger apparent size estimated from the electrophoretic mobility may be due to differences in residual secondary structure and/or in nucleotide composition between the transcript and the TMV and BMV RNAs used for calibration.

Products of cell-free translation using rabbit reticulocyte lysate were separated on SDS-polyacrylamide gels (Fig. 2). Translation of natural BNYVV RNA (Yu2) gave rise to ^{35}S-labelled proteins with apparent molecular weights of approximately 76 kDa and 22 kDa (Fig. 2 A) (Ziegler et al. 1985). The in vitro transcript obtained from pGC700 directed the synthesis of a major protein comigrating with the CP (Fig. 2 A). An additional weaker protein band migrating slightly slower than the CP is also visible in the autoradiogram. This band also appeared to a lesser extent in blank translations and may therefore be unrelated to added RNA.

The immunological reactivity of the translation products obtained from in-vitro-transcribed RNA towards an antiserum raised against intact BNYVV was checked by Western blotting. A stained protein comigrating with authentic BNYVV CP was detected on the blot (Fig. 2 B). When translation products obtained from 1.5 µg RNA were loaded on the gel, the intensity of the immunostaining on the blot corresponded to about 5 ng of CP, in reasonable agreement with the amount of CP formation estimated on the basis of [^{35}S] methionine incorporation.

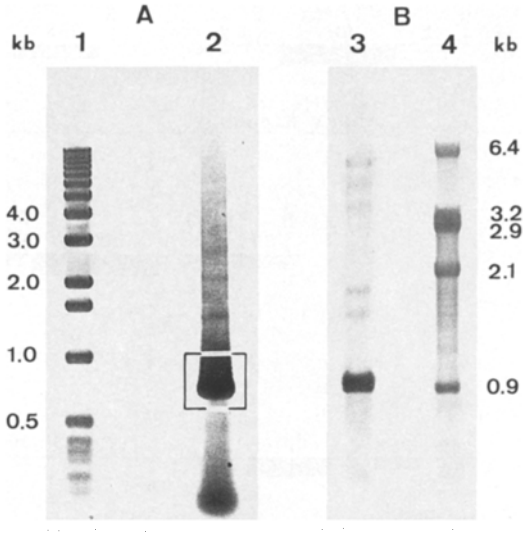

Fig. 1. **A** cDNA amplification products (*lane 2*) obtained after 30 reaction cycles, separated by electrophoresis, and stained with ethidium bromide. The fraction marked by brackets was eluted from the gel. DNA markers (1-kb ladder, BRL) were applied in *lane 1*. **B** Products of in vitro transcription (*lane 3*) obtained from 1 µg linearized pGC700, separated on a formamide-agarose gel, and stained with ethidium bromide. In *lane 4*, RNA from tobacco mosaic virus (6.4 kb) and brome mosaic virus (3.2, 2.9, 2.1, and 0.9 kb) were applied as size markers

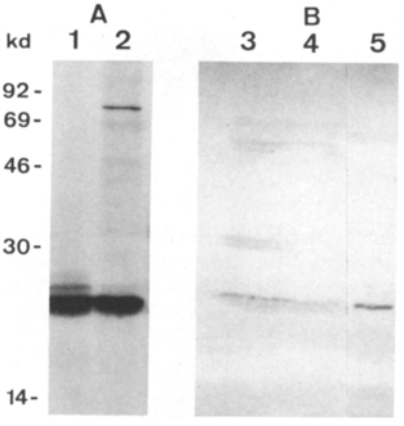

Fig. 2. **A** [^{35}S]methionine-labelled in vitro translation products of 0.25 µg in vitro transcribed RNA from pGC700 (*lane 1*) and 0.5 µg BNYVV RNA (*lane 2*), separated electrophoretically and visualized by autoradiography. ^{14}C-labelled proteins (Amersham) were used as size markers. **B** Western blot analysis of in vitro translation products from 1.5 µg (*lane 3*) and 0.5 µg (*lane 4*) synthetic transcript. A control sample of 15 ng authentic BNYVV CP was applied in *lane 5*

Expression of the CP gene in sugar beet hairy roots

Construction of the plant expression plasmids is outlined in Fig. 3. The HindIII/SmaI restriction fragment of pGC700, including the CP gene and its leader, was cloned into the plant expression vector, pRT103. The

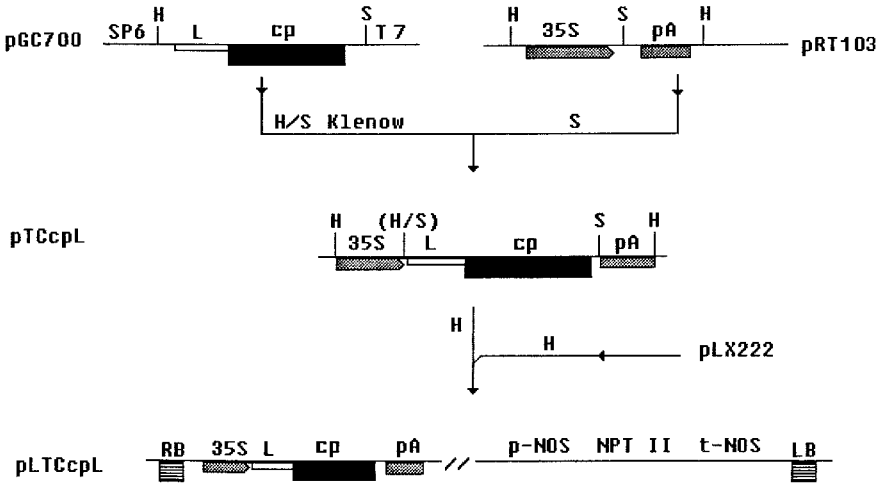

Fig. 3. Construction of the plant expression vectors pTCcpL and pLTCcpL containing the BNVYY coat protein gene under the control of the ^{35}S promoter. SP6, SP6 promoter; T7, T7 promoter; pA, polyadenylation signal; cp, coat protein gene; L, leader sequence; H, HindIII, S, SmaI; RB, right border; LB, left border; p-NOS, NOS promoter; t-NOS, NOS terminator

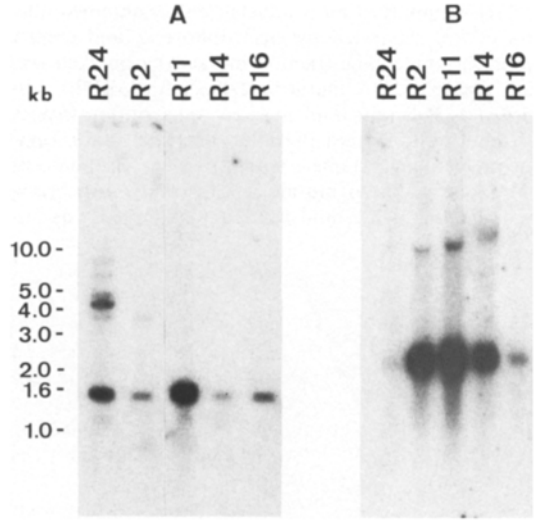

Fig. 4 A and B. Southern (**A**) and Northern (**B**) analysis of different hairy root lines as indicated above the lanes. Thirty micrograms of HindIII-digested DNA (**A**) and 20 µg of total RNA (**B**) were applied in each lane. A 730-bp fragment from pGC700 was used as hybridization probe

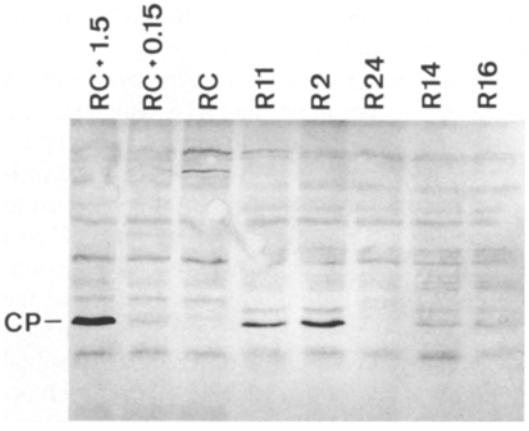

Fig. 5. Western blot detection of BNYVV coat protein (CP) in extracts from different hairy root lines as indicated above the lanes. Fifty micrograms of total protein was applied in each lane of a SDS-polyacrylamide gel. CP was detected using an antiserum made against BNYVV particles. RC: hairy root line without integrated CP gene. RC+1.5 and RC+0.15: extract from line RC plus 1.5 ng or 0.15 ng of purified BNYVV added, respectively

resulting plasmid pTCcpL carries the CP gene under the control of the ^{35}S promoter of cauliflower mosaic virus and 3′ flanked by the corresponding polyadenylation signal.

The 1.5-kb HindIII fragment from pTCcpL, consisting of the ^{35}S promoter, the CP gene, and the polyadenylation signal, was cloned into the binary vector pLX222; this provides the left and right T-DNA border sequences required for transfer of the inserted sequences, including an *NPTII* gene for kanamycin resistance, into the nuclear genome of plants. The binary vector construct pLTCcpL was transferred to *Agrobacterium rhizogenes* via triparental mating. Sugar beet seedlings were inoculated with the transformed *Agrobacteria*, yielding single hairy roots.

Hybridization experiments with DNA from ten hairy root lines that had not been selected for kanamycin resistance indicated successful integration of BNYVV CP genes into five of these lines. Total DNA from the five positive hairy root clones was digested with HindIII, separated on an agarose gel, and hybridized with a ^{32}P-labelled 730-bp fragment from pGC700 (Fig. 4). A hybridization band of about 1.5 kb was detected. This fragment contains the ^{35}S promoter, the CP gene, and the polyadenylation signal. Bands larger than 4.0 kb with root line R24 may be the result of unspecific hybridiza-

tion. Differences in the intensity of the hybridization signals can be explained by different copy numbers of the inserted gene.

The expression of the CP gene in hairy roots was demonstrated by Northern blot hybridization and immunological detection of the protein. Hybridization of total RNA shows the presence of CP-mRNA of the expected size (Fig. 4 B). Since the different amounts of CP-mRNA in the five hairy root lines are not correlated with the intensities of the DNA hybridization signals, they are apparently not caused by different copy numbers of the integrated gene, but by position effects and/or DNA rearrangements during integration. Synthesis of the coat protein in hairy roots was shown by Western blotting of root extracts and reaction with an antiserum raised against complete BNYVV particles (Fig. 5). Expression varied from 5 ng (R14 and R16) to 20 ng (R2) of CP per milligram of total protein. The two root lines giving the strongest RNA hybridization signals (R2 and R11) also produced the highest amounts of CP. In line R24, which contained a barely detectable amount of mRNA, no CP was found.

Discussion

Transformation of sugar beet tissue with a BNYVV CP gene and expression of 5–20 ng of CP per milligram of total protein in hairy roots could be demonstrated. In general, the CP expression level required to achieve resistance in transgenic plants varies with different plants and different viruses. With alfalfa mosaic virus, high expression levels (400–800 ng of CP per milligram of total protein) were shown to be necessary to confer resistance in transgenic tobacco plants (Loesch-Fries et al. 1987). The level of resistance depended on the amount of CP.

The same holds for potato virus X resistance in transgenic potato plants (Hoekema et al. 1989). However, tobacco plants expressing only low levels (13–19 ng of CP per milligram of total protein) of cucumber mosaic virus CP were also resistant to the virus (Cuozzo et al. 1988). Other factors, e.g., consistency of expression of CP in cells important for virus multiplication or spread, may be even more significant for a high level of resistance (Stark and Beachy 1989).

To test whether the expression level of BNYVV CP in our hairy roots was sufficient to confer virus resistance, we tried to infect transgenic and nontransgenic hairy roots with BNYVV, by mechanical inoculation (Koenig and Ehlers 1989) and by using a *Polymyxa betae* zoospore suspension carrying the virus. Although roots of sugar beet seedlings can be readily infected by both methods, our attempts to infect hairy roots failed, perhaps due to physiological differences between normal and transformed roots. During the preparation of this manuscript, Kallerhoff et al. (1990) have described expression of BNYVV CP in a cell suspension culture of sugar beet transformed with *Agrobacterium tumefaciens*. Using protoplasts prepared from these cells, they demonstrated CP-mediated protection against BNYVV infection.

Sugar beet transformation has been impeded up to now by the lack of an efficient method for regeneration of whole plants following the selection of transformed cells. While sugar beet hairy roots cannot be regenerated to plants, a method for the regeneration of transformed plants from shoot-base tissues inoculated with disarmed *Agrobacterium tumefaciens* has recently been published (Lindsey and Gallois 1990). This opens up a way to test whether the CP-mediated protection against BNYVV is effective in whole plants using the CP gene constructs described in this paper.

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