Expression of the beet yellows closterovirus capsid protein and p24, a capsid protein homologue, in vitro and in vivo

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The positive-sense RNA genome of beet yellows closterovirus (BYV) encompasses open reading frames (ORFs) for the viral capsid protein (CP, ORF 6) and for a CP homologue (p24, ORF 5). The sequences of the ORFs 5 and 6 were inserted into an Escherichia coli expression vector, pQE-9, under the control of the bacteriophage T5 promoter. The proteins were expressed in bacteria, purified, and used for antiserum production in rabbits. The recombinant BYV CP and p24 showed serological cross-reactions when probed with each antiserum on Western blots. The cross-reactions of the anti-p24 serum with the CP, and of the anti-CP serum with the p24, were abolished by preadsorption with the heterologous antigens, suggesting that CP and p24 share a common epitope(s) resistant to SDS denaturation. Cross-reactivity of the soluble CP and p24 was also observed in indirect plate-trapped antigen ELISA, whereas virtually none was encountered in double-antibody sandwich ELISA. Using a polyclonal anti-p24 serum preadsorbed with the recombinant CP, the p24 was detected in BYV-infected plants. Analysis of subcellular fractions of BYV-infected Tetragonia expansa indicated that both proteins are predominantly located in the soluble fraction of the host cells. Primer extension analysis of the individual double-stranded forms of the subgenomic RNAs bearing the CP and p24 genes allowed them to be mapped and their 5' start sites to be located at nucleotide positions 13588 and 12815, respectively, in the complete genome sequence. This corresponds to the 5' untranslated regions of 52 and 105 nucleotides in the subgenomic RNAs for CP and p24, respectively. The data obtained indicate that the synthesis of both subgenomic RNAs is initiated on a negative RNA strand at an adenosine residue found within the conserved sequence 5' CCAUUUPyA (shown as positive-sense), which may thus represent a core element of the subgenomic promoter. This conserved sequence also resembles the sequences at the 5' ends of the CP subgenomic RNAs of tobamoviruses and the Bromoviridae family members, the viruses evolutionarily most closely related to BYV.

Introduction

Beet yellows (BYV), the type member of the closterovirus group, is semi-persistently transmitted by aphids to a wide range of dicotyledonous hosts, and causes an economically important disease in sugar beet (Bar Joseph & Murant, 1982). The flexible filamentous particles of the virus are 1250 to 1450 nm in length. They consist of approximately 4000 protein subunits with an M_r of 22300 and a single species of positive-sense RNA of 15480 nucleotides (Bar Joseph & Hull, 1974; Carpenter et al., 1977; Agranovsky et al., 1994).

It has been proposed that the gene coding for the RNA replicase covers the 5'-terminal half of the BYV genome and is translated from the genomic RNA, whereas the other genes, including the gene for the capsid protein (CP), are expressed via a nested set of 3'-coterminal subgenomic (sg) RNAs (Dolja et al., 1990). Sequencing of the complete genome of BYV confirmed this suggestion, revealing the 5'-proximal open reading frames (ORFs) la and lb that code for the putative RNA replicase, and seven other ORFs located downstream (Agranovsky et al., 1991, 1994). In the previous work, striking amino acid sequence similarity was found between the putative p24 protein encoded in ORF 5, and the BYV CP encoded in the next downstream ORF 6, suggesting that the p24 gene evolved by duplication of, and subsequent divergence from, the CP gene (Boyko et al., 1992). Together with the CP and a putative p26 protein encoded in the similarly arranged ORFs in the genome of the related citrus tristeza closterovirus, these proteins constitute a compact lineage within the mono-
phyletic family of CPs of plant viruses with filamentous particles (Dolya et al., 1991; Boyko et al., 1992).

We report here the results of mapping the 5' ends of the sgRNAs for the BYV CP and p24. By using polyclonal antiserum to the recombinant p24 expressed in bacteria, it is demonstrated that the protein is produced in virus-infected plants.

**Methods**

**Oligonucleotides.** The following oligodeoxynucleotides were used in this study. Negative-sense primers were oligo 6' dTGAAAGTGGAA-TTAAGTC, oligo 8' dCGTCGGTTTCTCTCCG, oligo 9' dGC-AGTCCTCTCCGTGC and oligo 1' dATACGGCCGCCCT-TTATTTTTC, complementary to nucleotides (nt) 12943 to 12958, 13045 to 13060, 13711 to 13726 and 15464 to 15480, respectively [Fig. 1 (a); hereinafter the numbering is as in the complete BYV genome sequence (accession no. X73476 in the EMBL database; Agranovsky et al., 1994)]. Positive-sense primers were oligo 24' dTTTACTCTTT-GGGCCCAGGAGCAG and oligo 22' dTTTAGATCTGTG-GATCCTGAACC (underlined is the foreign sequence with BgII restriction site), represented the genome sequence from nt 12922 to 12938 and nt 13640 to 13656, respectively.

**Isolation of virus-specific RNAs.** The Ukrainian and German isolates of BYV were used throughout this work. Virion RNA was extracted from purified virus with phenol in the presence of bentonite (Karasev et al., 1989). Total RNA was isolated from BYV-infected Tetratonia expansa leaves by the method of Verwoerd et al. (1989); total dsRNA was isolated from the infected tissue using two cycles of CF-11 cellulose column chromatography (Morris & Dodds, 1979).

**Primer extension.** To map the 5' ends of subgenomic RNAs, two methods were used. In the first method, total dsRNA from BYV-infected plants was size-separated in 1% agarose gel, followed by excision of the individual dsRNA bands from the gel and isolation of dsRNA with a QIAEX gel extraction kit (Qiagen) using the manufacturer's protocol recommended for DNA isolation. Methylmercuric hydroxide was added to achieve a concentration of 20 mM to 20 to 100 ng dsRNA in 20 μl of double-distilled water, and the mixture was incubated for 20 min at room temperature to denature the dsRNA (Shelbourn et al., 1988). The reaction was stopped by adding 2 μl of 0.7 m-mercaptoethanol with 1 μl RNasin (Boehringer). The template thus prepared was mixed with 30 ng of a primer in 60 mM-Tris-HCl pH 8.3, 75 mM-NaCl, 7.5 mM-MgCl₂ (final volume 18 μl) and annealed for 10 min at room temperature. The labelling reaction was performed by adding non-labelled dATP, dCTP and dGTP (2 μM of each), 20 μCi [α-32P]dCTP and 25 units of avian myeloblastosis virus reverse transcriptase (Boehringer), followed by incubation at room temperature for 5 min. This was followed by a chase step, where 0.6 mM of each of the four non-labelled dNTPs was added before 15 min incubation at 42 °C. The samples were mixed with formamide dye, heated at 95 °C for 5 min, and analysed on a 6% acrylamide, 7 M-urea sequencing gel. A sequencing ladder was produced using the same primer and the virion BYV RNA as a template; the dioxydeoxynucleotide mixes and incubations were essentially as described by Fichot & Girard (1990).

In the second method, primer extension analysis was performed on both total ssRNA and dsRNAs templates isolated from infected plant tissue, with the oligonucleotide primers 5'-labelled with [32P]ATP. When total ssRNA was used, the reverse transcription procedure exactly followed the conventional protocol (Sambrook et al., 1989). Total dsRNA was denatured with methylmercuric hydroxide prior to the reverse transcription reaction (Shelbourn et al., 1988).

**Construction of expression cDNA clones.** To obtain clones for the expression of CP and p24 in *Escherichia coli*, the following strategy was employed. Virion BYV RNA (2 μg) was reverse-transcribed using oligo 1, complementary to the 3' end of the BYV genome (Agranovsky et al., 1991). Approximately 30 ng of the resulting first strand cDNA was amplified by PCR using the above oligonucleotide as a negative-sense primer, and the positive-sense primers oligos 24 or 22, containing the start sequences of p24 and CP genes, respectively (Fig. 1a). For technical reasons, oligo 24 was made so that the Y-proximal AUG triplet in the p24 insert was omitted (Fig. 1b). The resulting PCR product for the p24 gene was cut with BgII and HindIII and cloned into the pQE-9 plasmid (Diagen) between the BamHI and HindIII sites. The PCR product for the CP gene was digested with BgII and XhoI and cloned into the same vector between the BamHI and SalI sites (Fig. 1 a, b). These constructions, containing BYV genome portions between nt 12919 and 13883 (p24) and between nt 13640 and 14489 (CP), were used for transformation of *E. coli* M15 cells containing the pREP4 repressor plasmid (Diagen).
Expression and purification of the virus proteins. Two ml of an overnight culture of recombinant M15 cells grown in 2 x YT medium with 100 mg/ml ampicillin and 25 mg/ml kanamycin was transferred to 23 ml of the same fresh medium and grown for 3 h at 37 °C up to a cell density 0.9 to 1.0 (A600). The expression was induced by 2 mM IPTG, followed by further growth for 3 h. The cells were collected by low-speed centrifugation and proteins were extracted by stirring the pellets for 1 h at room temperature with 2 ml of buffer A (6 M guanidine–HCl, 0.1 M Na2HPO4, 0.01 M-Tris pH 8.0). The lysates were clarified by low-speed centrifugation, applied onto a column with nitrilotriacetic acid agarose charged with nickel (Ni-NTA agarose, Diagen; Hochuli et al., 1988), and subjected to chromatography in accordance with the manufacturer’s protocol. Briefly, non-recombinant proteins were removed from the column by stepwise elution with buffer A, and then with buffers B and C (8 M-urea, 0.1 M NaH2PO4, 0.01 M-Tris pH 8.0 and 6.3, respectively), followed by elution of recombinant protein with buffer D (the same composition as buffers B and C, but with its pH adjusted to 5.9). The protein content was analysed by electrophoresis of 10 μl samples taken from 1 ml column fractions, in 8 to 20 % acrylamide gradient gels with SDS, stained with Coomasie blue (Laemmli, 1970). The protein concentration was determined using the BCA Protein Assay kit (Pierce). The peak fractions containing recombinant p24 or CP were pooled and dialysed against 6-urea, 10 mM-Tris-HCl pH 7.2, 0.2 M NaCl, 1 mM-EDTA, 8 mM-2-mercaptoethanol. To refold the proteins, urea was gradually removed from the dialysis chamber by pumping urea-free TNE buffer (10 mM-Tris-HCl pH 7.2, 0.2 M NaCl, 1 mM-EDTA, 8 mM-2-mercaptoethanol, 10 % glycerol).

Production of antisera and serological tests. Polyclonal antisera to recombinant CP and p24 of BYV were raised in rabbits by three sequential intramuscular injections at 2-week intervals. The mixture for each injection consisted of 0.3 mg of purified protein in 0.1 ml of elution buffer D, further diluted with 0.3 ml of TNE buffer (without 2-mercaptoethanol) and 0.6 ml PBS (10 mM-sodium phosphate buffer pH 7.0, 0.85% NaCl), emulsified with 1 ml of Freund’s complete adjuvant. Blood was collected 14, 28, 42, 56 and 70 days after the last injection.

Antiserum against p24 (As-p24) was preadsorbed with the recombinant CP prior to conducting some tests. Twenty μl of the antiserum, diluted at 1:50 in 0.5 % NaCl, 20 mM-Tris-HCl pH 7.4, was mixed with 70 μg of Ni-agarose-purified soluble CP and incubated for 2 h at 37 °C followed by overnight incubation at 4 °C. The mixture was clarified by low-speed centrifugation, and the supernatant was used to develop Western blots at a final dilution of the initial As-p24 of 1:500 to 1:1500. The same procedure was applied to extract the antisera against the recombinant CP (As-CP), with p24. For some experiments, the antibodies specific to CP or p24 were isolated by adsorption on blot-transferred homologous antigens and elution with glycine-HCl pH 2.8 (Harlow & Lane, 1988).

For Western blotting, proteins were transferred from the 15 % acrylamide denaturing gels into nitrocellulose membranes and the blots were treated either with anti-BYV serum (kindly provided by Josef Vetten), or with the antisera against the recombinant proteins, followed by goat anti-rabbit IgG–alkaline phosphatase conjugate (Sigma). Detection was by treatment with naphthol as-mix phosphate and 5-bromo-4-chloro-3-indolyl phosphate (Fast Red).

Double-antibody sandwich (DAS) (Clark & Adams, 1977) and indirect plate-trapped antigen (PTA) ELISA were done essentially as described by Koenig (1981). For PTA ELISA antisera were used at 1:300 dilution without the preadsorption; antigens were applied at a concentration of 2.25 μg/ml. ‘Denatured’ antigen was prepared by mixing a sample of protein in the 8 M-urea-containing buffer D, with PBS. ‘Refolded’ antigen was made by diluting soluble protein after the gradient dialysis. As a control, the recombinant 30K protein of tobacco mosaic virus (TMV) that had been expressed in bacteria in the same way as the BYV p24 and CP (kindly provided by Yuri Dorokhov), was used. Tissue print-immunoblotting was performed as described previously (Kaufmann et al., 1992). Briefly, the freshly cut leaves and leaves of BYV-infected T. expansa were pressed onto a positively charged nylon membrane (Boehringer). The membrane was blocked by incubation with PBS containing 2 % BSA, followed by development with As-p24 and goat anti-rabbit IgG–alkaline phosphatase conjugate, as for Western blots.

Fractionation of plant extracts. Subcellular fractions of the BYV-infected and healthy T. expansa plants were prepared essentially as described previously (Godefroy-Colburn et al., 1986). One gram of leaf tissue was ground in a mortar with 1.5 ml of ice-cold GB buffer (100 mM-Tris–HCl pH 8.0, 11 mM-KCl, 5 mM-MgCl2, 400 mM-sucrose, 10 % glycerol, 10 mM-2-mercaptoethanol). The extract was squeezed through a Microcloth (Calbiochem) and then subjected to centrifugation at 1000 g for 10 min; the pellet (P1 fraction) was emulsified with 100 μl of electrophoresis sample buffer (ESB; 75 mM-Tris–HCl pH 6.1, 4.5 % SDS, 9 M-urea, 7.5 % 2-mercaptoethanol). The supernatant was centrifuged at 30000 g for 30 min to yield a pellet (P230) and supernatant (S30). P30 was dissolved in 100 μl of ESB. Protein in S30 was concentrated with 75 % ammonium sulphate, followed by dialysis against distilled water, and mixing of 100 μl of the dialysed protein solution with 200 μl of ESB. The residue on the Microcloth was re-extracted with 200 μl of ESB (fraction CW). All the samples were kept for 7 min at 95 °C prior to electrophoresis.

Results

Mapping of the 5' ends of sgRNAs for CP and p24

To map the 5’ ends of the sgRNAs for p24 and CP, we have used two approaches. The method described here is an adaptation of [32P]dNTP labelling and extension RNA sequencing reactions (Fichot & Girard, 1990) for the synthesis of run-off products with non-labelled primers that are annealed to individual dsRNAs. Alternatively, a conventional protocol was used, employing the run-off reverse transcription of total ssRNA or dsRNA with 5’-labelled primers. For brevity, these methods are referred to here as ‘cool primer extension’ and ‘hot primer extension’, respectively. The primers used were complementary to the positions from nt 22 to 38 and nt 125 to 142 downstream of the first AUG codon in the p24 gene (oligos 6 and 8, respectively), and to position 72 to 87 nt downstream from the start codon of the CP gene (oligo 9; Fig. 1a). Double-stranded forms of the sgRNAs for BYV CP and p24 were identified in previous work by using in vitro translation of methylmercuric hydroxide-denatured dsRNAs isolated from low melting point agarose (Dolja et al., 1990). Here, we applied a QIAEX DNA extraction kit (Diagen) for dsRNA isolation from conventional agarose gels. In our hands, this method provided 60 to 70 % yields of an individual double-stranded template.

The run-off cDNA product of the sgRNA for CP (obtained by cool primer extension) migrated as a double band, with the lower major band coinciding with that of
Fig. 2. Primer extension mapping of the 5' termini of the BYV subgenomic RNAs for CP (b and c) and p24 (a). Run-off cDNA and dideoxynucleotide chain sequencing products were separated on a 6% sequencing gel. For 'cool primer extension' (a and b), non-labelled specific primers were annealed to the denatured individual double-stranded forms of sgRNAs, and extended with reverse transcriptase in the presence of [α-32P]dCTP and non-labelled dATP, dGTP and dUTP. (a) Subgenomic RNA for p24; the extension products of oligos 8 and 6 (E8 and E6) compared with the respective sequencing patterns. (b) Subgenomic RNA for CP; the oligo 9 extension product (E9) and the corresponding sequencing ladder. (c) 'Hot primer extension' of the [γ-32P]ATP-labelled oligo 9 with total dsRNA and ssRNA isolated from BYV-infected plants. The adenosine lane in the corresponding sequencing ladder is repeated to align A-13588 with the adjacent run-off bands. Arrowheads show the 5' starting points of the sgRNAs, with the surrounding sequence indicated.

the base A-13588 in the adjacent sequencing ladder made with the same primer (Fig. 2b). This corresponds to a 52 nt 5' non-coding region in the sgRNA for CP. Essentially the same result was obtained when the 5'-labelled oligo 9 was extended using total ssRNA or dsRNA as the template. It should be noted, however, that the hot primer extension produced a single band coinciding with that of A-13588, for both types of templates (Fig. 2c).

Attempts to map the 5' end of the p24 sgRNA by the hot primer extension were unsuccessful, presumably because of the relatively low content of the corresponding individual ss- and dsRNA species in the infected plants (Dolja et al., 1990) and also an insufficient amount of the specific label being available from end-labelled primers. The cool primer extension produced the run-off products of oligos 6 and 9 visualized on autoradiograms as double bands; the lower (stronger) band in both cases
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Fig. 3. SDS–PAGE (8 to 20% acrylamide gradient) of proteins expressed in *E. coli* strain M15. Lane M, *M*<sub>r</sub> standards; lane 1, total protein from non-induced cells harbouring pQE-BYVCP expression plasmid; lane 2, the same, IPTG-induced; lane 3, recombinant BYV CP after Ni-NTA chromatography; lane 4, recombinant BYV p24 after Ni-NTA chromatography; lane 5, total protein from the IPTG-induced cells bearing the pQE-BYVp24 plasmid; lane 6, the same, non-induced.

**Correspondence of the sgRNA to the 5’ UTR region.**

The lower band, corresponding to the base A-12815, and the upper band to the base C-12814, in the sequencing patterns produced with the same primers (Fig. 2a). We suggest that the position of the lower band reflects the correct 5’ start of the sgRNA for p24, corresponding to a 5’ untranslated region of 105 nucleotides. This is compatible with the previous data indicating that the BYV-specific mRNAs are capped (Karasev *et al.*, 1989). Since the 5’ end penultimate base in capped eukaryotic messengers is purine rather than pyrimidine (reviewed in Banerjee, 1980), adenosine is a more likely candidate than the upstream cytosine to be the 5’ base in the BYV sgRNAs. The upper bands corresponding to extensions to one nucleotide beyond A-13588 and A-12815 in the sgRNAs for CP and p24, respectively, might be artefacts of reverse transcription under the cool primer extension conditions. Nevertheless, a possibility that some microheterogeneity exists in the 5’ start sequence of the p24 sgRNA cannot be completely dismissed.

**E. coli over-expression and purification of p24 and CP**

The plasmid pQE-9 used for over-expression of the BYV proteins contains a sequence cassette of the bacteriophage T5 promoter, ribosome binding site, and a short ORF coding for Met–Lys–Gly–Ser–6×His, followed by a polylinker (Fig. 1b). The oligonucleotide primers used in this study were designed so that the BYV ORFs 5 and 6, present in their entirety in the respective PCR products, could be cloned in-frame with this sequence, using identical or compatible restriction sites in the polylinker and the PCR product (Fig. 1a, b). The resulting recombinant proteins that are over-expressed in *E. coli* should have the authentic C termini and the above foreign sequence at their N termini. The presence of a tail of six histidines having a weak positive charge allows purification of such proteins by affinity chromatography on Ni–NTA agarose (Hochuli *et al.*, 1988).

We have obtained the recombinant plasmids pQE-BYVCP and pQE-BYVp24 bearing the inserts of ORFs 6 and 5, respectively, with portions of the downstream virus sequence (Fig. 1b). The IPTG-induced expression in *E. coli* cells bearing these plasmids resulted in production of the CP and p24 at levels of 25% to 30% of total cell protein (Fig. 3). Both the CP and p24 that were expressed in bacteria had electrophoretic mobilities closely corresponding to the *M*<sub>r</sub> values expected for the ORF 6 and ORF 5 products; the anomalous mobility of p24 migrating faster than BYV CP which has a deduced *M*<sub>r</sub> of 22300, has been mentioned previously (Boyko *et al.*, 1992; Fig. 3).

The affinity chromatography on Ni–NTA agarose yielded approximately 5 mg of recombinant BYV CP or p24 with greater than 95% purity (Fig. 3) from 25 ml of the initial growth medium. Both proteins had the same chromatographic elution profiles, being completely eluted from the column at pH 5.9.

**p24 and CP share common SDS-stable epitope(s)**

When tested on Western blots with the polyclonal serum raised against purified BYV virions, the recombinant BYV CP and p24 gave strong and weak reactions, respectively (Fig. 4d). This result is in agreement with the previous data on immunoprecipitation by the anti-BYV serum of the ORF 6 and ORF 5 products translated in a cell-free system from the respective T7 transcripts (Boyko *et al.*, 1992). To verify that a serological relationship exists between these proteins, we raised polyclonal antisera against the Ni-agarose-purified recombinant CP and p24 (As-CP and As-p24, respectively). As-p24 gave a strong reaction with p24 and also recognized CP (Fig. 4a). Likewise, As-CP strongly reacted with the homologous antigen and also showed a weaker reaction with p24 (Fig. 4b). Preadsorption of As-p24 with the recombinant CP, and As-CP with p24, abolished their ability to react with the heterologous antigens (Fig. 4c and data not shown). The antibodies from As-CP purified by adsorption on the nitrocellulose-transferred
Fig. 4. Western blots of the BYV CP and p24 expressed in bacteria and in virus-infected plants. Proteins were separated on a linear (15% acrylamide) SDS gel. The blots were developed with rabbit antisera raised against the purified p24 (As-p24), or to the CP (As-CP), or to purified BYV particles (As-BYV), as indicated. The blots (c) and (e) were developed with As-p24 preadsorbed with the recombinant CP, and the blot (f) was developed with As-CP preadsorbed with the recombinant p24. (a to c) Lanes 1, 2 and 3, purified recombinant CP (pr. CP) at 1, 0.1, and 0.01 μg, respectively; lanes 4, 5 and 6, purified p24, the same amounts. (d) Recombinant p24 and CP, 1 μg each. (e and f) Subcellular fractions extracted from BYV-infected (i) and healthy (h) *T. expansa* plants. Lane CW, cell wall debris; lane P1, 1000 g pellet; lane P30, 30,000 g pellet; lane S30, 30,000 g supernatant; lanes p24 and CP, 1 μg of the recombinant BYV p24 or CP mixed with the S30 fraction of healthy plants.

*p24 is expressed in infected plants*

To monitor p24 and CP distribution in the BYV-infected plant tissue, the subcellular fractions S30 (cytoplasm), P1 (nuclei and large organelles), P30 (membranes) and homologous antigen, and elution by a shift in pH, still showed cross-reaction with p24 and this was indistinguishable from that observed on blots treated with the initial As-CP; the same was true for the antibodies from the As-p24 thus purified (not shown). These data clearly indicate that the BYV CP and p24 do share some common epitope(s) that are resistant to SDS denaturation.

Cross-reactivity between the recombinant p24 and CP was also observed in indirect PTA ELISA, especially when the As-p24 was tested with the recombinant CP (Fig. 5). In DAS ELISA, however, there was almost no cross-reactivity between the two proteins (Fig. 5); this is in line with previous observations of a narrower specificity of this type of ELISA in heterologous reactions (e.g. Koenig, 1981; van Regenmortel & Burckardt, 1980). The much higher reactivity of As-p24 with the CP in indirect PTA ELISA as compared with that occurring in DAS ELISA might be because of low avidity of the p24 antibodies for the CP. The direct labelling with enzyme could lead to some conformational changes in these antibodies resulting in the loss of their reactivity with the heterologous antigen in DAS ELISA.

In DAS ELISA, the As-p24 reacted more efficiently with the denatured than with the refolded form of this protein suggesting that many of its epitopes are cryptotopes (Fig. 5). In indirect PTA ELISA, the difference in reactivity between the denatured and refolded p24 was much less, probably because direct attachment of the refolded p24 to plates led to its partial denaturation resulting in the exposure of hidden epitopes. Denatured and refolded forms of the recombinant BYV CP showed no pronounced difference in their reactivity in DAS and indirect PTA ELISAs. The reactivity of the As-CP with the purified virus was, however, increased in indirect PTA ELISA (Fig. 5). This could be due to release of the free CP from the particles in the alkaline coating buffer (van Regenmortel & Burckardt, 1980) and might suggest that a number of epitopes that are recognized on the free CP that had been used for immunization are not accessible on the assembled virus particles. As-p24 and As-CP failed to react with the purified TMV 30K protein in both types of ELISA (not shown).
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DAS 0.45
0-4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
A B CD E

PTA
0.25
0.2
0.15
0.1
0.05
A B C

Fig. 5. Reactivity of the denatured and refolded BYV p24 (A and B, respectively) and CP (C and D, respectively), and of purified BYV particles (E), in DAS (a and c) and PTA (b and d) ELISA with antisera to the p24 (a and b) and CP (c and d).

CW (cell walls) were obtained from virus-infected and healthy T. expansa leaves (Godefroy-Colburn et al., 1986). In controls, purified recombinant p24 or CP was mixed with the S30 fraction of healthy plants to provide equivalent electrophoresis conditions. The proteins in different fractions were separated on an SDS gel, blotted onto nitrocellulose and probed with the As-CP or As-p24, each preadsorbed with the heterologous antigen. A parallel blot was treated with preimmune serum, which showed a fairly low level of the background reaction with plant proteins and did not produce bands corresponding to any protein specific to the virus-infected plant tissue (not shown).

Despite some non-specific reaction of the As-p24 with cellular proteins, a clear major band was visible in the lanes corresponding to different subcellular fractions of the BYV-infected, but not healthy, plants (Fig. 4e). The electrophoretic mobility of the protein from the infected plants was very close to that of the recombinant p24 run in a parallel lane (Fig. 4e); a slight difference could be explained by the presence of an N-terminal foreign sequence of 10 amino acids in the recombinant p24. The preadsorbed As-p24 apparently failed to recognize recombinant CP (Fig. 4e, lane CP) and the bands were thus very unlikely to be a result of the cross-reaction with the CP that was expressed in plants. In addition, p24 was detected in vivo by using two alternative approaches, ELISA and tissue printing; particularly on tissue prints of BYV-infected plants developed with the As-p24 and goat anti-rabbit IgG–alkaline phosphatase conjugate, the immunospecific stain concentrated at sieves (data not shown). This is compatible with the (semi-)phloem-limited nature of the closterovirus infection (reviewed in Bar-Joseph & Murant, 1982; Coffin & Coutts, 1993). Taken together, these results prove that p24 is indeed expressed in plants infected with BYV.

The p24-specific band was detected in lanes corresponding to all the subcellular fractions tested; the bulk of the protein was in the soluble fraction, whereas the other fractions showed roughly equal amounts of p24 (Fig. 4e). A very similar distribution was observed for the BYV CP (Fig. 4f). The fractionation method used allows only very rough separation of the cellular compartments and this could, at least in part, explain the observed distribution of the virus proteins in subcellular fractions.

Discussion

A unique feature of the closterovirus genome, thus far demonstrated for BYV and CTV, is the presence of a gene which probably arose by duplication and subsequent divergence of the viral CP gene (Boyko et al., 1992). The p24 protein, a homologue of the BYV CP, fits well the general alignment of capsid proteins of filamentous plant viruses (Boyko et al., 1992; Dolja et al., 1991). The amino acid residues conserved in the p24 and the filamentous virus CPs are mostly hydrophobic; together with the α-helical type of structure predicted for p24, this implies that this protein might also have a CP-like structural fold (Boyko et al., 1992). Our data on the presence of common epitopes in the recombinant BYV CP and p24, which are better recognized by the heterologous antisera in the denatured rather than in the refolded proteins, do not contradict these structural comparisons. The p24 product was efficiently expressed upon in vitro translation of the T7 RNA transcripts bearing ORF 5 (Boyko et al., 1992). Here, we have detected p24 in BYV-infected plants using a polyclonal antiserum raised against the recombinant protein expressed in bacteria. Analysis of the distribution of BYV p24 and CP in subcellular fractions indicates that both proteins are more abundant in cytoplasm but may also be located in other compartments of the infected cell. A similar distribution was reported for the CPs of beet necrotic yellow vein virus (reviewed in Richards & Tamada, 1992) and barley stripe mosaic virus (Donald et al., 1992).
Table 1. Similarity of the sequences at the 5’ ends of the subgenomic RNAs (subgenomic promoter elements) of BYV and other related plant viruses

<table>
<thead>
<tr>
<th>Messenger RNA</th>
<th>Sequence at the 5’ start</th>
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<tbody>
<tr>
<td>BYV, sgRNA CP</td>
<td>CCAUUUUA</td>
</tr>
<tr>
<td>BYV, sgRNA p24</td>
<td>a a</td>
</tr>
<tr>
<td>TMV, sgRNA CP</td>
<td>UGUUUUA</td>
</tr>
<tr>
<td>CMV, sgRNA CP (RNA 4)</td>
<td>GGUUUAG</td>
</tr>
<tr>
<td>BMV, sgRNA CP (RNA 4)</td>
<td>Ua a UuUA</td>
</tr>
<tr>
<td>AIMV, sgRNA CP (RNA 4)</td>
<td>CCCUUUUUA</td>
</tr>
<tr>
<td>Consensus</td>
<td>XCPuUUXUFA</td>
</tr>
</tbody>
</table>

* The sequences around the starting bases (underlined) of the sgRNAs for capsid protein were from: tobacco mosaic virus, type strain (Goel et al., 1982); cucumber mosaic virus strain Q (Davies & Symons, 1988); brome mosaic virus (Ahliquist et al., 1981); alfalfa mosaic virus (Barker et al., 1983). Deviations found in other tobamoviruses (compared in Solis & Garcia-Arenal, 1990), cucumoviruses (Boccard & Baulcombe, 1993; Hayakawa et al., 1989; Karasawa et al., 1991; Owen et al., 1990) and bromoviruses (compared in Romero et al., 1992) are indicated in lower case. The Consensus is drawn allowing three exceptions in 16 compared sequences when no gaps were introduced.

which may thus represent a core element of the subgenomic promoter. In accordance with this finding the 5’ CCAUUUPyA block is also conserved in the corresponding RNA regions upstream of ORFs 5 and 6 in two other BYV isolates, German and English (Agranovsky et al., 1994; Brunstedt et al., 1991). Notably, the determined 5’-terminal sequences of both sgRNAs are similar to the 5’-most GUUUUA sequence of the BYV genomic RNA (Agranovsky et al., 1994). It is plausible that these sequences are recognized by the viral replicase on the minus RNA strand as signals for the synthesis of genomic and subgenomic messengers.

Interestingly, the conserved sequence CCAUUUPyA at the starting points of the BYV sgRNAs resembles those of the CP sgRNAs in several tobamoviruses, brome mosaic virus, cucumber mosaic virus and alfalfa mosaic virus (Table 1). Similarity between the sequences at the 5’ ends of the sgRNAs of Bromoviridae family members and those of TMV has been already noted (Goel et al., 1982). There is a tempting parallel between these data and the results of phylogenetic analysis indicating that the putative BYV RNA replicase is evolutionarily most closely related to replicases of these viruses (Agranovsky et al., 1994). It could be speculated that the as yet unidentified template-binding domain(s) in replicases of the ‘tobamo-like’ viruses (Koonin & Dolja, 1993) have been conserved along with the recognition signals in their genomic RNAs in the course of evolution.

We are grateful to Sergey Morozov for stimulating discussions and help with pilot expression tests, to Frank Niepold and Andrea Kaufmann for their help with antibody adsorption and tissue-printing techniques, to Josef Vetten and Frank Rabenstein for the anti-BYV serum and the virus-infected plant material, and to Yuri Dorkhokhov for the recombinant 30K TMV protein. Thanks are also due to Eugene Koonin for critical comments on the manuscript. We appreciate the excellent technical assistance of Anke Briske-Rode, Andrea Rusche and Carola Beier. This work was supported by a fellowship from the Alexander von Humboldt Foundation awarded to A. A. Agranovsky.

References


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(Received 14 October 1993; Accepted 14 December 1993)