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Potato yellow mosaic geminivirus AC2 protein is a sequence non-specific DNA binding protein

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Abstract The AC2 protein of potato yellow mosaic geminivirus (PYMV) is by analogy with related geminiviruses thought to be a transcriptional activator protein. We have over-expressed the AC2 open reading frame in *E. coli* and purified the protein from bacterial extracts to near homogeneity. We have studied the interaction of the AC2 protein with DNA and from gel retardation assays shown that it binds both double-stranded (ds) and single-stranded (ss) DNA non-specifically. The binding to PYMV intergenic region ds DNA appeared to be independent of the presence of zinc ions and did not require the protein to be phosphorylated.

Key words: Protein expression; DNA-binding protein; Transactivation; Potato yellow mosaic geminivirus

1. Introduction

Geminiviruses are single-stranded (ss) DNA plant viruses with a characteristic paired morphology that replicate *via* double-stranded (ds) DNA intermediates in infected cell nuclei [1]. Potato yellow mosaic virus (PYMV) belongs to the bipartite *Geminivirus* genus in subgroup III [2] and is the only geminivirus known to infect potato naturally [3]. The PYMV genome consists of two DNA components designated A and B [2] both of which are required for infectivity [4,5]. The PYMV genome contains six or seven open reading frames (ORFs) which are transcribed bidirectionally and are separated by an intergenic region, termed the common region (CR), which is almost identical in both DNAs [2]. The CR contains the origin of DNA synthesis as well as transcriptional and regulatory sequences [1]. Recent genetic analysis has shown that the organisation and the function of the ORFs in both PYMV [6] and tomato golden mosaic virus (TGMV) [7] are similar and places the two viruses in the same phylogenetic cluster. These proteins are involved in virus DNA replication, cell-to-cell movement, transcriptional regulation or particle structure [6,8,9].

Mutagenesis of TGMV illustrated that the AC2 ORF was required for symptom formation in whole plants but not viral DNA replication in agroinoculated leaf discs [8] and the same result was found for PYMV [6]. The AC2 gene product of subgroup III geminiviruses can complement mutations in heterologous viral genomes both in protoplasts [10] and in plants [11,12]. The TGMV AC2 gene product is a transcriptional activator protein that is necessary for the efficient expression of virion-sense AV1 (coat protein) and BV1 (movement protein) genes [9].

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All of the AC2 proteins in the subgroup III geminiviruses including PYMV contain a conserved basic domain close to the N-terminus and a conserved acidic domain near the C-terminus [9]. Conserved cysteine and histidine residues immediately downstream of the basic region in AC2 have some similarities with zinc-binding proteins and these residues are arranged as Cys-X1-Cys-X4-His-X2-Cys-X6-His-X4-His-Cys in both TGMV and PYMV. Several different permutations of these residues can be envisaged, some of which are very similar to other well-known transcription factors [13]. Indeed, the CCHC motif above has also been identified in several proteins known to bind either ssDNA or RNA from both animal [14] and plant viruses [15]. Here we report the over-expression of the PYMV AC2 protein in *Escherichia coli* and its purification to near homogeneity. Using gel retardation assays we demonstrate that AC2 binds both ds and ssDNA in a non-specific fashion, the former cooperatively, and the significance of these results regarding transcriptional control of either, or both viral and host genes by AC2 is discussed.

2. Materials and methods

2.1. Expression and purification of the AC2 protein

The ORF of the AC2 protein was expressed in *E. coli* and purified from the bacterial lysate. The complete AC2 ORF was amplified from linearised PYMV DNA A, excised from pMAH2 [4] with *Hin*dIII, by PCR using oligonucleotides corresponding to the 5'-end of the gene (5'-ACTGGACTCTAACATATGCGGTCTTCA-3'; a *Nde*I site introduced for cloning is underlined) and to a sequence 140 nucleotides downstream of the AC2 stop codon (5'-ATGATTCGATCT-TAAATT-3'). The PCR-amplified fragment (0.56 kb) was digested with *Nde*I and the approx. 0.5 kb *Nde*I-*Nde*I fragment (one *Nde*I site being created by the oligonucleotide while the other was present in PYMV DNA A starting at nucleotide 1121) was cloned into the *Nde*I site of the expression vector pET3a [16] to generate the pETAC2 plasmid. The *Nde*I restriction site of pET3a includes the translation initiation signal so that pETAC2 expresses the AC2 protein without any change in the coding sequence and this was confirmed by sequencing. The recombinant vector was cloned into *E. coli* strain BL21(DE3) pLysE in the presence of 50 μg/ml ampicillin and 50 μg/ml chloramphenicol. Cloning techniques were as described [17]. Colonies were screened for expression of the AC2 protein after induction with isopropylthio-β-D-galactoside (IPTG).

Purification of the AC2 protein was then essentially performed as described previously for the tobacco mosaic tobamovirus movement protein with some minor modifications [18]. Liquid cultures (20 ml 2TY [17], containing 150 μg/ml ampicillin and 150 μg/ml chloramphenicol) of strain BL21(DE3) containing the pLysE and pETAC2 plasmids were grown at 37°C to a cell density of 0.7 (A_{600}). After the addition of IPTG (0.4 mM), to induce expression of the chimeric gene, the cells were grown for a further 3 h. The cells were harvested by centrifugation (7000×*g*, 10 min at 4°C) and the pellets resuspended in 3 ml buffer L (10 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 200 mM NaCl, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) [19]. The cells were lysed by sonication (MSE Soniprep 150; amplitude 15 μm, 3 min) and the lysates centrifuged (12000×*g*, 5 min at 4°C) before resuspending the pellet (con-

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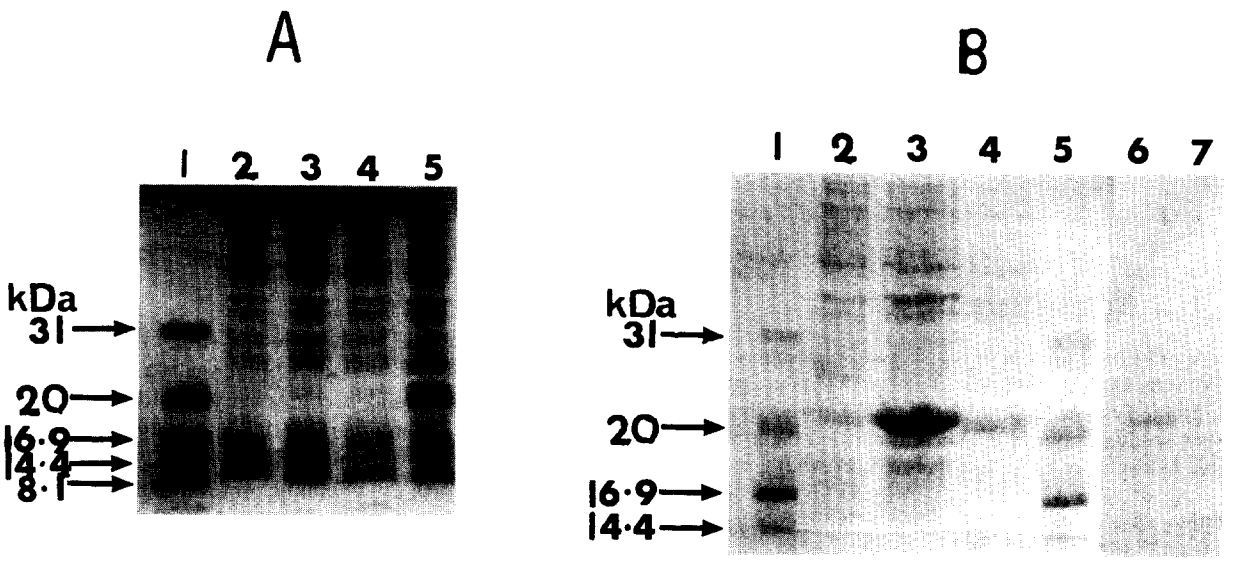

Fig. 1. Analysis of the recombinant AC2 polypeptide prepared from *E. coli* by SDS-PAGE in 10% gels. The AC2 protein was not present in uninduced (A, lane 2) or IPTG induced cultures of pET3a (A, lane 3) but was induced from the recombinant plasmid pETAC2 by IPTG (A, compare lane 4, uninduced and lane 5, induced). Most of the recombinant protein was present in the pellet fraction of the bacterial lysate (B, lane 3) and not in the supernatant fraction (B, lane 2). The insoluble pellet fraction above after resuspension in 1 M NaCl and centrifugation is shown in (B) lane 4. Lane 6 (B) contains a sample of the purified AC2 protein in L buffer obtained after solubilisation, centrifugation and dialysis as described in the text. Marker proteins are shown in (A) lane 1 and (B) lanes 1 and 5. Lane 7 (B) contains a protein fraction corresponding to the fraction in lane 6 (B) isolated from bacteria transformed with pETAC2 but not induced with IPTG.

taining most of the AC2 protein) in 1 ml buffer L containing 1 M NaCl. The resultant protein suspensions were centrifuged as above and the pellet resuspended in 0.5 ml buffer L containing 1 M NaCl and 3 M urea, incubated at 70°C for 10 min and re-centrifuged. The supernatant was dialysed extensively against buffer L and used for binding experiments. The concentration of the purified protein was estimated by SDS-PAGE [20] of an aliquot in parallel with known concentrations of bovine serum albumin and densitometry of the Coomassie brilliant blue-stained gel at 600 nm.

2.2. DNA

Plasmid pMAH2 [4] (containing PYMV DNA A) was double digested at unique restriction sites *Xho*I and *Nco*I (located at nucleotide 2332 and 339, respectively [2]) and 3′ recessed ends were end-filled using Klenow fragment (5 U) in the presence of 20–30 μCi [α-^{32}P]dCTP (3000 Ci/mmol). The 700 bp restriction fragment, which contained the intergenic region DNA, was purified from an agarose gel, dissolved in water and used directly in binding assays. Labelled PYMV ssDNA was produced by heating the PYMV dsDNA fragment above at 100°C for 5 min and snap-cooling on ice. Both an unlabelled 1 kb *Hin*dIII-*Xho*I fragment of plasmid pBR328 [21], which was produced as above in the presence of a mixture of cold dNTPs in the reaction, and salmon sperm DNA were used in separate competition binding assays.

2.3. DNA binding assays

Mixtures of ^{32}P-labelled DNA (0.5 μl in water; 2000 cpm) and AC2 protein (volumes up to 19.5 μl in buffer L) were combined, incubated at O°C for 1 h, mixed with gel loading buffer and electrophoresed in non-denaturing 4% polyacrylamide gels in TBE [17]. The effect of zinc ions on binding was investigated by including $ZnSO_4$ (50 mM) in the reaction mixture. Incubation of the PYMV dsDNA-AC2 protein complex with proteinase K (1 mg/ml for 30 min at 37°C) before PAGE was also carried out to confirm that any retardation effect was exclusively caused by the protein and not by other factors. Competition assays included 1000-fold excess of unlabelled salmon sperm DNA or the pBR328 fragment from above in the reactions. To investigate whether phosphorylation of the AC2 occurred in *E. coli* and was concerned with binding, a purified sample of AC2 protein was incubated with calf intestinal phosphatase (CIP; 1 U for 30 min at 37°C) prior to incubation with labelled DNA. All samples were electrophoresed until the bromophenol blue dye had reached the end of the gels which were then dried and autoradiographed.

3. Results and discussion

3.1. Expression of PYMV AC2 in E. coli

From SDS-PAGE and Coomassie blue-staining of the gels the PYMV AC2 protein was expressed in reasonably large amounts from the induced pETAC2 plasmid (Fig. 1A, lane 5). The expressed protein migrated in SDS-containing gels with an M_r of approx. 21 kDa (Fig. 1A, lane 5) when compared to molecular weight markers (Fig. 1A, lane 1). The M_r of the PYMV AC2 protein is greater than the value predicted from the sequence of the gene of 15 kDa [2]. The reason why it migrates anomalously is not known but a similar observation was made for the *E. coli*-produced TGMV AC2 protein [22]. The protein was solubilised in 3 M urea containing 1 M NaCl, refolded and was at least 95% pure, as judged by SDS-PAGE and photodensitometry of the Coomassie blue stained gels (Fig. 1B, compare lanes 6 and 7).

3.2. Nucleic acid binding properties of the recombinant AC2 protein

Increasing amounts of AC2 protein caused the electrophoretic retardation of fixed amounts of ^{32}P-labelled PYMV intergenic region dsDNA (Fig. 2A) and ssDNA (Fig. 2B). In the case of dsDNA a slight mobility shift at 50 and 100 ng of protein per assay (Fig. 2A, lanes 4,5) was occasionally seen. This was considered artifactual. This indicates that AC2 binds as a single discrete complex, but of unknown stoichiometry. This binding could be highly cooperative as no intermediate mobility bands were seen. For ssDNA binding the situation was less clear, particularly with lower concentrations of AC2 protein. For instance, with 20 ng AC2 protein (Fig. 2B, lane 3) unbound DNA behaved erratically during electrophoresis and the prominent slower migrating band corresponded to the lower part of smeared bands found with higher concentrations of AC2 (lanes 6–8; 200–1000 ng of AC2). This result

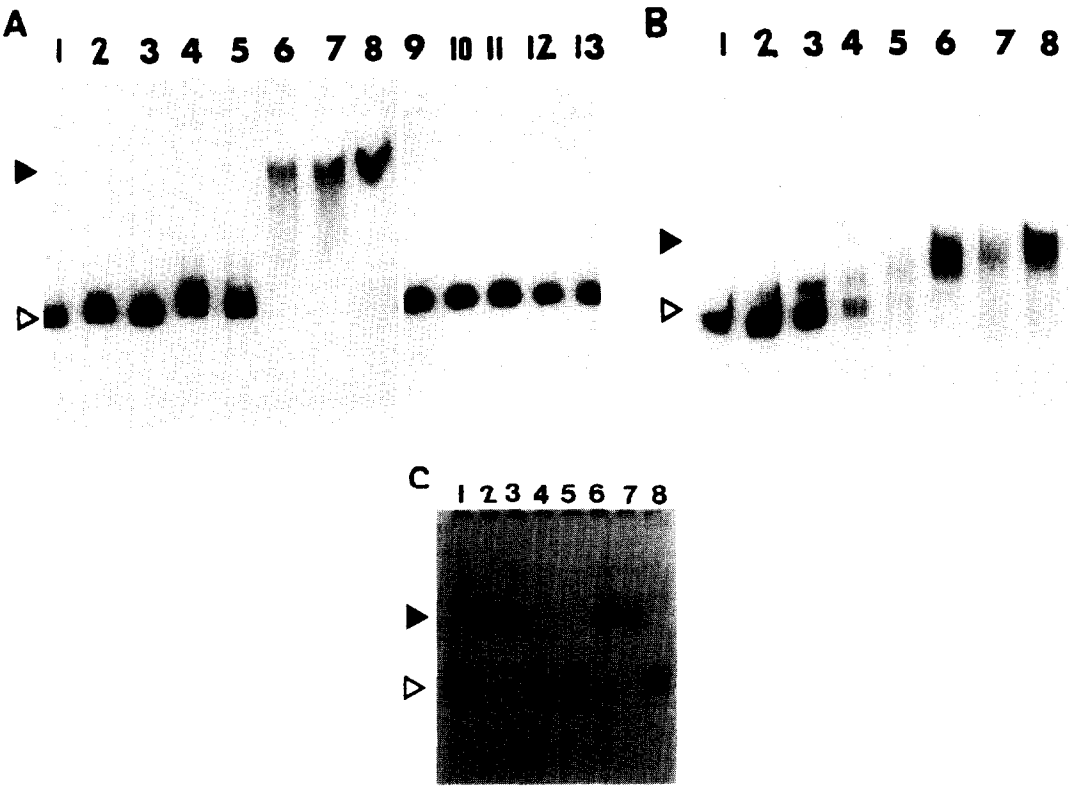

Fig. 2. Electrophoretic retardation of ^{32}P-labelled ds (A) and ss (B) PYMV intergenic region DNA by increasing amounts of AC2. The 32P-labelled probes were incubated with 0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 or 1.0 μg AC2 (respectively in both A and B, lanes 1–8). Treatment of the AC2-dsDNA complex with proteinase K (A, lane 9), or competition with 1000 fold excess of unlabelled salmon sperm DNA (A, lane 10) or pBR328 (A, lane 11) in the binding reactions as described, prevented retardation of dsDNA. Control assays with IPTG induced pET3a (A, lane 12) and uninduced pETAC2 (A, lane 13) are also shown. Neither the presence of zinc ions in the binding reaction buffer, together with AC2 (0.5 μg) (C, compare lane 2, without zinc ions and lane 3, with zinc ions) nor pre-treatment of AC2 (0.5 μg) with CIP (C, compare lane 6, incubated with CIP and lane 7, incubated without CIP) as described influenced retardation of PYMV intergenic region dsDNA. In panel C lanes 1 and 4 are respectively control samples of the probe incubated in binding buffer with and without zinc ions. Lanes 5 and 8 are respectively control samples of probe incubated with a mixture of binding buffer and CIP buffer or incubated with CIP alone. The open and closed triangles indicate, respectively the positions of free and retarded DNA.

suggests cooperative binding of ssDNA by AC2 may also occur but further work is needed to resolve this feature. The ability of the AC2 protein (0.5 μg) to retard PYMV intergenic region dsDNA in similar assays was blocked by the addition of at least 1000 fold excess of unlabelled competitor DNA (either salmon sperm DNA or the pBR328 fragment; Fig. 2A, lanes 10 and 11, respectively). These results show that AC2 binds DNA in a non-specific fashion and suggest that AC2 may have a higher affinity with intergenic region DNA than other dsDNAs but competition assays between homologous and heterologous DNA need to performed. The binding of intergenic region dsDNA by PYMV AC2 was independent of the presence of zinc ions (Fig. 2C, lanes 1–4) although we cannot exclude the presence of minute quantities of zinc ions in the reactions. Also, an essential role for phosphorylation of AC2 in binding was discounted following treatment of the protein with CIP prior to incubation with DNA (Fig. 2C, lanes 5–8). Since bacterial kinases are generally specific for their natural substrates it was unlikely that bacterially expressed AC2 would be phosphorylated anyway but this result eliminates any effect of auto-phosphorylation. Treatment with proteinase K confirmed that the retardation effect was caused by AC2 and not other factors (Fig. 2A, lane 9).

An inspection of the sequence of the PYMV AC2 protein shows that it has the general properties expected of a transcriptional regulatory protein, including a conserved N-terminal basic and a C-terminal acidic domain. These domains are frequently concerned respectively with DNA-binding and transcription activation [23]. Our observation that the PYMV AC2 protein binds both dsDNA and ssDNA in a sequence non-specific fashion is in accord with previous studies with transcriptional regulatory proteins encoded by certain mammalian viruses. These proteins function via protein-protein interactions rather than through direct interaction with specific DNA sequences. For instance, the herpes simplex virus (HSV)1-encoded VP16 contains a potent activation domain that cannot activate transcription unless it is targeted to HSV-1 promoters by its association with the cellular DNA-binding protein Oct-1 [24]. Adenovirus E1A [25] is another example of a viral-encoded, trans-activator that requires a promoter-specific factor, possibly cellular factor ATF-2, to target it to adenoviral promoters [26] and it may also form a complex with cellular TFIID protein. In this case by interacting with both factors E1A may bridge upstream binding factors with the start site.

In geminiviruses the AC2 protein activates the promoter sequences of both the AV1 and BV1 genes by an unknown mechanism but it is likely that as with the animal virus activators above it probably exerts its influence by forming a complex with plant factors to target these promoters. Further work is required to identify the host proteins involved.

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