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Simultaneous Analysis of the Bidirectional African Cassava Mosaic Virus Promoter Activity Using Two Different Luciferase Genes

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Abstract. The expression of geminivirus genes is controlled by bidirectional promoters which are located in the large intergenic region of the circular DNA genomes and specifically regulated by virus encoded proteins. In order to study the simultaneous regulation of both orientations of the DNA A and DNA B promoters of African cassava mosaic virus (ACMV), they were cloned between two different luciferase genes with the firefly luciferase gene in complementary-sense and the *Renilla* luciferase gene in virion-sense orientation. The regulation of the ACMV promoters by proteins encoded by the complete DNA A, as well as by the individually expressed transactivator (TrAP) or replication-associated (Rep) proteins was assessed in tobacco and cassava protoplasts using dual luciferase assays. In addition, the regulation of the DNA A promoter integrated into tobacco genome was also assessed. The results show that TrAP activates virion-sense expression strongly both in cassava and tobacco protoplasts, but not in transgenic tobacco plants. In contrast to this, DNA A encoded proteins activate virion-sense expression both in protoplasts and in transgenic plants. At the same time they reduce the expression of the complementary-sense Rep gene on DNA A but activate the expression of the complementary-sense movement protein (MPB) gene on DNA B. The degree of MBP activation is higher in cassava than in tobacco protoplasts, indicating that the plant host also influences the promoter strength. Transient transformation experiments using linearized DNA indicate that the different regulation of the ACMV DNA A promoter in protoplasts and transgenic plants could be due to different DNA curvature in free plasmids and in genes integrated in plant genomic DNA.

Key words: African cassava mosaic virus, dual luciferase assay, promoter regulation

Introduction

African cassava mosaic virus (ACMV) is a member of the *Geminiviridae*, a diverse family of plant infectious agents characterized by their circular single-stranded DNA (ssDNA) encapsidated in twin icosahedral particles (1–3). It belongs to the *Begomovirus* genus, which comprises viruses transmitted by whitefly and having a mono- or bipartite genome (DNA A and DNA B). ACMV replicates in the nuclei of host cells through double stranded DNA intermediates via a rolling circle mechanism and it recruits most proteins of the replication machinery from its hosts. The genes for the coat protein (CP) (4), the replication-associated protein (Rep) (5) and the regulator proteins TrAP and REn (5,6), which provide the viral functions required for replication, are all located on the DNA A. Hence, DNA A is capable of autonomous replication and encapsidation but is unable to infect plants systemically (7). The nuclear shuttle protein (NSP) and the movement protein (MPB) genes residing on DNA B provide functions for virus movement (8–10). Both genomic components, DNA A and B, are required for infectivity (11).

The arrangement of the open reading frames (ORFs) of the DNA A and B (Fig. 1a) suggests that they are expressed in a bidirectional manner (4). The ORFs on both DNA A and B are arranged similarly

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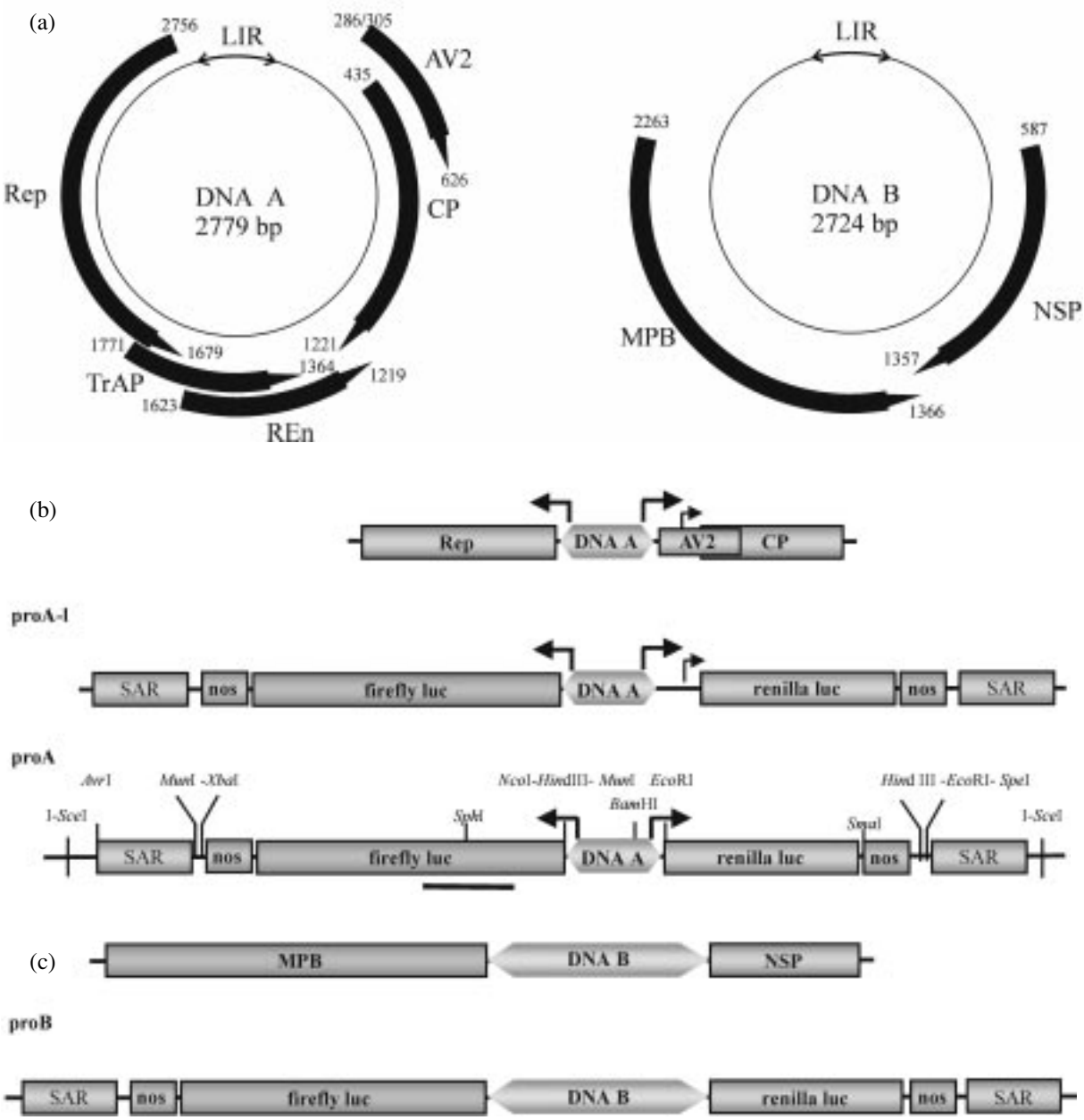

Fig. 1. Genome map of ACMV and the luciferase constructs. (a) ACMV DNA A and DNA B. The DNA is represented by a thin line with the two arrowheads showing the extent of the large intergenic region (LIR). The main viral ORFs are indicated by thick arrows, the numbers indicate the 5′ and 3′ positions of the individual ORFs. CP coat protein; Rep the replication-associated protein; TrAP and REn regulator proteins: NSP nuclear shuttle protein; MPB movement protein. (b) Luciferase constructs compared to original ACMV DNA A. (c) Luciferase constructs compared to original ACMV DNA B. The figures show the position of the cloned ACMV promoter (the short or the longer version of the DNA A promoter or the DNA B promoter) between the *Renilla* and the firefly luciferase genes. Both luciferase genes carry a *nos* terminator. The whole expression cassette is flanked by scaffold attachment regions (SARs). Restriction sites shown for proA are the same in proA-1 and proB.

with divergent transcription units separated by a large intergenic region that is highly conserved between the two DNAs (12). This segment also contains a $\sim$ 30 nt sequence with the potential to form a stem-loop structure which is conserved among bipartite geminiviruses and has been associated with the start of rolling circle replication (2). Fragments of DNA A (nucleotides 2759 to 282) and DNA B (nucleotides 2705 to 581) have been shown to act as regulatable promoters, here referred to as AC and AV for DNA A complementary- (c-) and virion- (v-) sense promoters and BC and BV for DNA B c- and v-sense promoters, respectively (6,13). Studies on promoter regulation have shown that AV, BV and BC promoters can be activated by TrAP while AC promoter is repressed by Rep (6,14,15). Both activation and repression occur at the level of transcription (16,17). Rep is known to bind between the TATA-box and the transcription start site, thereby probably hindering its own transcription by interfering with RNA polymerase (6,14,17). To date there is little information on the molecular mechanisms responsible for promoter activation by TrAP (6,15).

Most published data on geminivirus promoter regulation have been obtained from transient expression studies using the *uidA* reporter gene (GUS). This has the disadvantage that only one promoter can be studied in each experiment. The distinct evolutionary origins and different enzyme structures and substrates of the firefly and the *Renilla* luciferases permit the discrimination between the two reporter genes in a single extract, thus making it possible to measure two different promoter activities in one experiment (18). Moreover, both luciferases can be detected at low levels in a fast and simple assay. We report here the analysis of the activity of the ACMV promoters by quantifying transient expression of the firefly and *Renilla* luciferase genes using the Dual-Luciferase™ assay for the first time in plant cells. We examined the activity of both orientations of the ACMV DNA A and DNA B promoters and their regulation by Rep, TrAP and, simulating viral infection, the complete DNA A in transient assays using cassava and tobacco protoplasts. We also assessed the regulation of the DNA A promoter *in planta*, using protoplasts from transgenic tobacco plants. The characteristics of the regulation of the bidirectional ACMV promoter indicate that it might be a useful tool for the creation of a virus resistance strategy via induced local cell death.

Materials and Methods

Plant Material

Cassava (*Manihot esculenta* Crantz) was maintained as embryogenic suspension cultures established from friable embryogenic calli of cultivar TMS60444 (19). The suspensions were grown axenically in 25 ml of modified liquid SH medium (20,21), at 30°C under continuous low light in a growth chamber (Infors Incubator HT04) on a shaker (90 rpm). All suspensions were subcultured once a week. Tobacco, *Nicotiana tabacum* L. cv. Petit Havanna Str-r1 (SR1) (22), was maintained as axenic shoot cultures. These were grown *in vitro* at 26°C with a 16/8 h photoperiod on half-strength MS medium without growth regulators (23). SR1 tobacco seedlings used for *Agrobacterium*-mediated transformation were grown axenically on filter paper wetted with sterile water.

Bacterial Strains

E. coli strain XL1-Blue was purchased from Stratagene (La Jolla, CA, USA). *A. tumefaciens* strain GV3101, containing helper plasmid pGV2260 (24) was kindly provided by B. Tinland, Zürich.

Plasmid Construction

The plasmid pDNA A is a partial repeat (1.3mer) of DNA A (West Kenyan isolate 844 (12) in pBS KS-. The cloning of the 1.3mer of DNA A and DNA B has been described (25). Both plasmids were kindly provided by T. Frischmuth, Stuttgart. The ACMV ORF Rep and TrAP expression vectors pRep and pTrAP (6) were kindly provided by B. Morris, New Zealand.

All DNA manipulations were performed according to standard procedures (26). Nucleotide numbering of the ACMV genome refers to pJS092 and pJS094 (27). DNA A, DNA B and names of the open reading frames are according to suggested nomenclature (28). In case the gene function is known, the name of the gene product is indicated. The bidirectional ACMV promoters were produced by PCR amplification of ACMV DNA using primers ACMVc (GGAA -2755-GC\-TTT\-TTGA\-CC\-AAG\-TCA\-ATT\-GG-2776) and ACMVs (300-GT\-GGT\-ACC\-CAC\-TATT\-GCGC\-ACTA\-GC-267) for the short version of the DNA A promoter, primers ACMVc and ACMVl

(GG\-GGTACC -440- AG\-CCC\-TGAT\-AACTGAG -425) for the longer DNA A promoter. The DNA B promoter (13) was amplified with primers ACMVBL (ATC\-CCGT -581- CAAT\-GTATA\-TACT\-TCC -564) and ACMVBR (GTT\-AG\-GCC\-ATGG -2265- TT\-CAAC\-ACT\-TTGA\-GTA\-TAA\-GC -2286). All versions of the luciferase constructs were obtained by introducing the ACMV promoters as *Kpn*I/*Mun*I fragments in between the two tobacco scaffold attachment regions (SAR, 29) of the backbone of pGluChi (30). The firefly luciferase (31, kindly provided by G. Neuhaus-Url, Basel) together with a nos terminator was introduced as an *Nco*I/*Xba*I fragment under the control of the AC or the BC promoter, while the *Renilla* luciferase from pPCV702 (32) was introduced together with a nos terminator as a blunt-ended *Hin*dIII fragment under the control of the AV or BV promoter.

A short and a longer version of the DNA A promoter were cloned. The short version of the promoter ends at the v-sense major transcription start site while the longer version includes also the minor transcription start site. Transcription from the major transcription start site of the long version gives rise to a 160 nt-long leader fragment upstream of the *Renilla* luciferase gene. The plasmids were called proA, proA-1 and proB depending on the inserted promoter (see also Fig. 1b). All promoter constructs created by PCR amplification were sequenced to ensure that no mutations had been introduced. As positive controls both the *Renilla* and firefly luciferase coding region were cloned separately under the control of a truncated 35S promoter and a 35S terminator (33, kindly provided by S. Brunner) to create plasmids pLucpos and pRenpos. To reduce the luciferase expression, thereby making measurement more convenient, the truncated 35S promoter consisted only of nucleotides 1 to 93. In order to transform plants with *Agrobacterium*, proA and proA-1 were introduced into pNC1 as I-*Sce*I-fragments. pNC1 is a pCambia1300 backbone (Cambia, Australia) with a pMCS5 (MoBiTec, Germany) polylinker containing an I-*Sce*I site. The resulting plasmids were electroporated (34) into GV3101(pGV2260).

Protoplast Isolation and Transformation

Mesophyll protoplasts were isolated from *in vitro*-grown tobacco plants and transformed with 20 µg of DNA using PEG as described (35). The constructs proA, proA-1 or proB were cotransformed either with pRep (ACMV Rep ORF under the control of a 35S promoter), pTrAP (ACMV TrAP ORF under the control of a 35S promoter), the complete DNA A or with carrier DNA. All experiments were repeated 4 to 6 times. Following transformation, the protoplasts were incubated in PCN2 (36) at RT in the dark. After 24 h protoplasts were harvested by centrifugation and resuspended in 100 µl passive lysis buffer for the Dual-Luciferase™ Reporter assay (Promega, Catalys AG, Switzerland). Extracts were stored at − 80°C for up to one month.

To prepare cassava protoplasts from embryogenic suspension cultures, approximately 1 g of TMS60444 embryogenic suspension culture was digested in the dark for 20 h at 28°C on a shaker (30 rpm). The enzyme solution and the washing solution have been described (37). The digest was filtered through a 50 µm mesh sieve and 10 ml washing solution were added through the filter. The filtrate was distributed in sterile tissue culture tubes and centrifuged at 70 g (30) for 7 min in a Hettich table centrifuge (Universal II). The supernatant was removed and the pelleted protoplasts were washed twice and resuspended in 10 ml washing solution. The protoplasts were counted, stored and transformed as described for tobacco protoplasts. The protoplasts were incubated at RT in the dark in TM2G (38) for 24 h prior to harvesting and resuspending in passive lysis buffer.

Agrobacterium-Mediated Transformation of Tobacco Seedlings

Tobacco seedlings grown for 8 days were transformed by vacuum infiltration as described (39) using an overnight culture of *Agrobacterium* grown in 2 ml YEB (40) containing the appropriate antibiotics. Resistance to hygromycin was used to select transgenic plants. The transgenic nature of 10 independent lines was confirmed by luciferase assays of leaves and by Southern blot analysis using standard procedures (41).

Dual-Luciferase™ Reporter Assay

Protoplast or leaf disc extracts were thawed and cell debris was collected by centrifugation. Aliquots of 50 µl of Luciferase Assay ReagentII (Promega, Catalys AG, Switzerland) were predispensed into lumino-

meter tubes before adding 10 µl of extract and mixing well with a pipette. Measurements of 5 seconds each were performed with a luminometer (Lumat LB 9507, E.G. & G. Berthold, Switzerland) with dual injectors. After measuring the luciferase activity, in relative light units (RLU), Stop and Glo™ Reagent was delivered into the tube by automatic injection. Measurement of the *Renilla* luciferase luminescence was started after a two second delay. For each experiment, background luciferase activity from protoplasts transformed with a vector without luciferase genes was subtracted throughout. Protein concentration, estimated using a Bio-Rad protein assay (Bio-Rad Laboratories, U.S.A.) as described (42), was used to normalize each measurement. All resulting values were standardized to the positive control consisting of protoplasts transformed with pLucpos and pRenpos.

Results

Luciferase Expression Under the Control of ACMV Promoters in Tobacco Protoplasts

The main objective of this work was to investigate the possibility of using the regulated ACMV promoters for designing a virus resistance strategy by engineering virus-induced local cell death. As a prerequisite towards this goal, the basal and regulated activities of the bidirectional promoters of ACMV had to be re-evaluated. Cloning the ACMV DNA A and DNA B promoters between two different luciferase genes allowed the assessment of the activation/downregulation of both orientations of the bidirectional promoters in a single experiment. The firefly luciferase gene was cloned under the control of the promoter for c-sense expression (AC/BC promoter), which in DNA A regulates Rep expression and in DNA B the expression of the protein responsible for long distance movement. The *Renilla* luciferase gene was cloned under the control of the promoter for v-sense expression (AV/BV promoter), which in DNA A is the promoter of the coat protein gene and in DNA B the promoter of the nuclear shuttle protein gene. The *Renilla* luciferase gene was cloned under the control of two different versions of the virion-sense AV promoter. The short version (AV promoter in plasmid proA) contains only the major transcription start site at position 278, while the longer version (AV-1 promoter in plasmid proA-1) allows transcription from the major and the minor (position 378) (43) transcription start sites (Fig. 1b). Transcription from the major transcription start site of proA-1 results in an RNA containing a 160 nt leader sequence upstream of the *Renilla* luciferase ORF, which in this construct replaces the ACMV coat protein ORF. In this leader two AUGs (at nucleotides 286 and 305) are upstream of the initiation codon of the coat protein gene at nucleotide 446. These additional AUGs are in frame with a small ORF AV2, which begins 8 nucleotides downstream of the major transcription start site and overlaps the ORF of the coat protein. The existence of the ORF AV2 encoded protein for ACMV has not been reported to date, but a corresponding protein has been reported for tomato leaf curl virus (TLCV) (44).

The activity of the promoters was assessed by measuring the luciferase activity in relative light units (RLU). In different experiments variable background levels of firefly luciferase (100–1000 RLU) and Renilla luciferase (1500–30,000 RLU) were observed. Within any one experiment, these background levels were quite constant and expression levels were regarded as significant only when they were at least 3 times above this background. Background levels were subtracted from all values before standardization against total protein content. The basal levels of the firefly luciferase controlled by the AC promoter from both proA and proA-1 were around 8000 RLU, independent of the promoter extension in the v-sense direction. The *Renilla* luciferase activity controlled by the AV promoter was about 50,000 RLU, while that controlled by the AV-1 promoter was higher, about 145,000 RLU. The firefly luciferase activity under the control of the BC promoter was comparable to that of the AC promoter, but the *Renilla* luciferase activity controlled by the BV promoter was on average 25 times higher (Table 1). In our experimental system the *Renilla* and firefly luciferase genes under the control of the truncated 35S promoter produced RLU values that differed maximally by a factor of 2.5 (data not shown). Similar results have been reported for mammalian cells (Promega User Manual), and therefore, similar RLU values for *Renilla* and firefly luciferase can be expected to reflect similar promoter strengths. All luciferase activities measured for any of the ACMV promoter constructs were at least 100-fold and up to 2000-fold lower than those of the same genes fused to a truncated (-1 to -93) 35S promoter.

Table 1. Basal luciferase activities of proA, proA-1 and proB in tobacco protoplasts

	proA		proA-1		proB	
	Firefly (c-sense)	Renilla (v-sense)	Firefly (c-sense)	Renilla (v-sense)	Firefly (c-sense)	Renilla (v-sense)
RLU	6600	48,200	13,000	145,000	4200	1,220,000
Standardized[a]	100%	100%	195%	300%	63%	2500%
Relative[b]	100%	730%	100%	1100%	100%	29,000%

[a]Luciferase activities of proA-1 and proB were standardized to luciferase activities of proA.
[b]V-sense *Renilla* luciferase activities were standardized to the respective c-sense firefly luciferase activities.

Effect of the Expression of the Viral ORFs on the Activity of ACMV Promoter

The results from the cotransformation experiments (Fig. 2a) show that the two orientations of both DNA A and DNA B promoters are regulated independently of each other and that the regulation of the DNA B promoters differs from that of the DNA A promoters. TrAP alone activates both v-sense (*Renilla* luciferase) and c-sense (firefly luciferase) expression. In cassava, the stimulation factor for the AC and AV promoters was about 7 and 14, respectively, and for the BC and BV promoters about 57 and 30 (Fig. 2a). In contrast, Rep reduced c-sense expression from the AC promoter about 35 fold and from the BC promoter about 3 fold, but did not affect v-sense expression significantly (Fig. 2a). The strongly reduced AC promoter activity in the presence of Rep was still clearly above background level, indicating that even under the most repressive conditions tested, the promoter maintained some of its activity. Cotransfections with the complete DNA A were performed to detect possible interactions between the positive and negative viral expression factors and also to detect possible additional factors. DNA A cotransfection had a very strong activatory effect on the BC promoter (36 fold) and on the AV promoter (20 fold), a moderately positive effect on the BV promoter (8 fold) and hardly any effect on the AC promoter.

In tobacco, the effects of single cotransfected

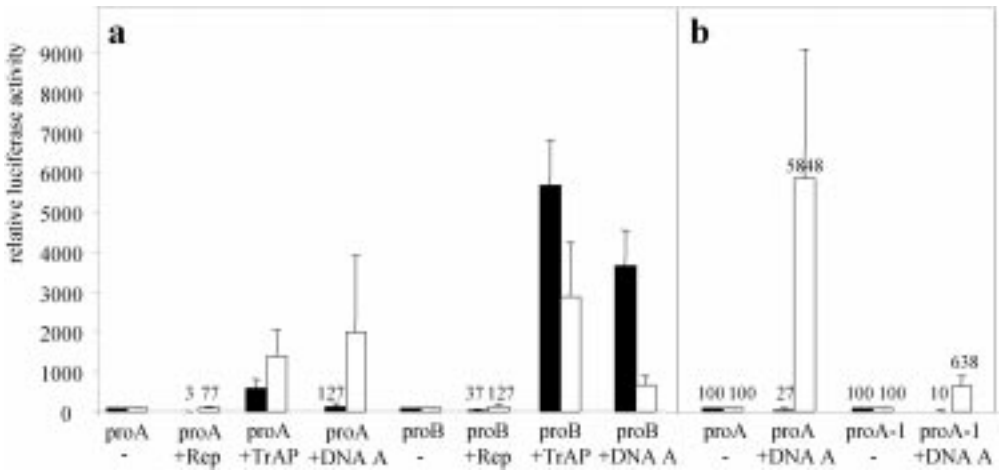

Fig. 2. Effect of the expression of ACMV proteins on the activity ACMV promoters in cassava and tobacco protoplasts. (a) Regulation of luciferase expression by ACMV DNA A (proA) and ACMV DNA B (proB) promoters in cassava protoplasts after cotransformation with pRep, pTrAP or the complete DNA A. (b) Regulation of the short (proA) and longer (proA-1) versions of the DNA A promoter in tobacco protoplasts. A value of 100 was assigned to the basal activity of each promoter construct alone (-). Columns represent the mean luciferase activity as a percentage of the basal activity of four to six independent experiments; error bars represent standard deviation. Black columns represent firefly luciferase activity (complementary sense expression), white columns represent *Renilla* luciferase activity (virion-sense expression).

genes on the AV and AC promoters were very similar to those found in cassava (Figs. 2b and 3a). However, the B promoters were more strongly repressed by Rep (results not shown). Some quantitative differences to cassava were also observed with the complete DNA A, which activated the AV promoter more than 50 fold and both B promoters about 20 fold. In addition, a strong disparity in the activation of the AV and AV-l promoters by DNA A was observed: while expression from the shorter (AV) promoter construct was activated about 60 fold, expression from the longer (AV-l) promoter construct was activated only 6 fold. Thus the AV-l promoter directed an about 3 fold higher basal expression level than AV (Table 1), but after activation by DNA A about 3 fold more luciferase was expressed from the AV than from the AV-l promoter.

The activity of the AC promoter is oppositely regulated by the two viral activator proteins Rep and TrAP, which are expressed from the same transcription control region. In order to analyze the coordinated function of these two proteins, we cotransfected proA with different ratios of TrAP and Rep expressing plasmids. Increasing amounts of Rep decreased AC promoter activity 7 fold even in the presence of TrAP concentrations that alone would activate the AC promoter about 10 fold. A small amount of Rep (one tenth of TrAP) had little effect under these conditions. In contrast, low levels of TrAP activated the AV promoter, even though higher levels were more effective. Neither the basal nor the TrAP activated AV promoter activity was affected by Rep. When equal amounts of Rep and TrAP were used, the increase of v-sense expression and the decrease of c-sense expression resembled the regulation pattern observed for the complete DNA A.

Regulation of the ACMV DNA A Promoter in Transgenic Tobacco

To test whether the results from the transient expression experiments in protoplasts could be confirmed using stably integrated genes, transgenic tobacco plants containing proA or proA-1 were produced by *Agrobacterium*-mediated transformation. Of the 10 transgenic lines tested, 6 contained the short version of the DNA A promoter and 4 contained the longer version of the promoter. All lines contained 2–10 copies of the T-DNA. Transgenic plants were grown as *in vitro* shoot cultures as well as in the greenhouse, where they produced seeds normally.

When the luciferase activity of mesophyll protoplasts from transgenic plants transformed with pRep or pTrAP or with the complete DNA A was measured, high variation was observed between plants and experiments. A representative experiment with some of the transgenic plant lines and a wildtype SR1 tobacco (cotransformed with proA) is shown in Table 2. In contrast to previous experiments using non-

Table 2. Luciferase expression (RLU) of protoplasts from transgenic tobacco plants after transient transformation with ACMV expression cassettes

		Constructs Used for Transformation			
Plant Line	Promoter	Control	Rep	TrAP	DNA A
slr 1	AC	200,000	240,000	260,000	230,000
slr 1	AV	6000	6200	7500	31,000
slr 9	AC	1870	2300	2200	2150
slr 9	AV	3150	2900	2500	11,000
slr 11	AC	13,000	21,000	22,000	28,000
slr 11	AV	3500	3800	4000	170,000
llr 7	AC	16,300	18,000	15,000	20,400
llr 7	AV-1	3100	2800	2600	3800
llr 5b	AC	2100	2900	2300	2700
llr 5b	AV-1	1600	1300	930	1400
wt	AC[a]	660	88	4000	700
wt	AV	7800	8900	78,000	454,000

[a]Wildtype protoplasts were cotransformed with proA and the corresponding ACMV expression cassettes.

integrated promoter constructs, where v-sense expression was always stronger, in the transgenic plants the basic level of c-sense expression was either much stronger than or similar to v-sense expression. Also, contrary to the cotransformation experiments, TrAP did not function as an activator in protoplasts from transgenic plants. Only when the complete DNA A was used, an increase of v-sense expression could be found, while c-sense expression remained unaffected. The increase of v-sense expression varied from 3- to 50-fold in the same plant line in different experiments. As the variation between experiments was much higher than that between the different lines, the degree of activation could not be correlated to the gene copy number. Also, in contrast to cotransformation experiments, the expression of neither v- nor c-sense from the longer version of the DNA A promoter was notably altered by complete DNA A or TrAP in protoplasts derived from transgenic plants.

In order to see whether the different regulation of the ACMV promoter observed in transgenic plants is due the different structures of plasmid DNA and of DNA stably integrated in the plant genome, pRep, pTrAP or complete DNA A was cotransformed to tobacco protoplasts with proA linearized outside the coding region (Fig. 3b). The activation of v-sense expression from a linearized plasmid was 3-fold lower than that from a circular plasmid. Also, activation of v-sense expression by the complete DNA A was around 6-fold lower when the linearized version of proA was used.

Discussion

The intergenic region of the geminivirus genome contains *cis*-acting elements that are important for the regulation of viral gene expression and viral replication. A regulated, bidirectional promoter is situated within this intergenic region (4,6,12,45,46). Previous studies on geminivirus promoter regulation investigated mainly promoter-GUS fusions, testing the transcriptional activity of each direction of the promoter separately (6,13,14,15,17,47,48,49,50, 51,52,53). The results obtained with all studied begomovirus promoters revealed a similar regulation pattern: the TrAP protein was found to induce v-sense transcription while Rep down-regulated c-sense transcription (6). However, details of basal promoter strength and the degree of activation or repression by viral proteins varied considerably. It remained unclear whether these variations reflect differences of the respective assay systems (e.g. plant material, incubation time of transformed protoplasts, exact promoter sequences, etc.) or, alternatively, differences in the replication cycle of the respective viruses. Tomato golden mosaic virus (TGMV), for example, starts replicating after 18–24 h in protoplasts (48), while in cells infected by ACMV or beet curly top virus (BCTV), replicating DNA can only be detected 2–3 days after infection (7,54).

We intended to use the regulation mode of the ACMV promoter for a novel strategy for engineered virus-inducible virus resistance, which required a

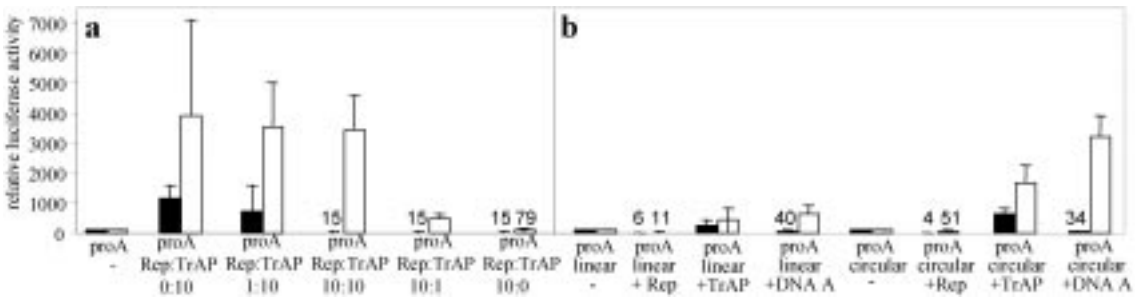

Fig. 3. Effect of the expression of TrAP and Rep on DNA A promoters in tobacco protoplasts. (a) Effect of the combined expression of TrAP and Rep on DNA A promoters. Luciferase expression of proA in tobacco SR1 protoplasts after cotransformation with different combinations of pRep and pTrAP. (b) Effect of TrAP, Rep or complete DNA A on the activity of ACMV promoters on linear and circular plasmids in tobacco protoplasts. A value of 100 was assigned to the basal activity of each promoter construct alone (-). Columns represent the mean luciferase activity as a percentage of the basal activity of four (a) and five (b) independent experiments; error bars represent standard deviation. Black columns represent firefly luciferase activity (complementary sense expression), white columns represent *Renilla* luciferase activity (virion-sense expression).

more detailed knowledge of the relative activity of the two promoters in both orientations in the absence and presence of viral gene products. In order to study simultaneous transcription from both sides of the promoter, the promoter fragments were fused to the firefly luciferase gene in c-sense orientation (AC and BC promoters) and to the *Renilla* luciferase gene in v-sense orientation (AV and BV promoters). Since several start sites have been reported (43) for DNA A v-sense transcription, we tested both a short version of the promoter, covering only the start site upstream of the AV2 gene, and a longer version, covering all the start sites upstream of the coat protein gene (Fig. 1a). The two different luciferases can be tested in the same reaction mixture and should, therefore, allow a precise comparison of promoter activities. Using reference constructs containing either luciferase under the control of a truncated (-1 to -93) 35S promoter, we show that similar amounts of light units are produced by both luciferases from these constructs, indicating that light units can be directly compared and do indeed reflect the respective promoter activities. An additional advantage of the luciferase reporter genes are the short half-lives of both proteins (and possibly mRNAs) compared to the previously used GUS reporter gene (55,56), allowing more accurate measurement of gene induction or repression.

Both in cassava and tobacco, the strongest expression of the luciferase genes under the control of an ACMV promoter was still considerably lower than the expression obtained with a truncated 35S promoter, which again was about 3-fold weaker than the full-length promoter (data not shown). Thus, in our assay system, the ACMV promoters are about 300- to 6000-fold weaker than the CaMV promoter, in contrast to previous reports, which stated a 10- to 40-fold lower activity of the ACMV promoter (13,14). The activity of TGMV AV promoter on a replicon was reported to be 60 to 90-fold stronger than and without replication still comparable to that of the 35S promoter (48), whereas the activity of the TLCV promoter was equal to 35S in GUS-V2 fusions, but 4-fold lower in GUS-V1 fusions (53). In contrast to earlier reports, where the basal expression from AC promoter was considerably higher than from AV or DNA B promoters, we find that in transient assays c-sense expression is always lower than v-sense expression (see table 1). In transgenic plants, on the other hand, the AC promoter is stronger than the AV promoter, although the luciferase activities in separate plant lines and experiments vary strongly.

For the repressing effect of Rep and the activating effect of TrAP we found quantitative differences to published data. Both the 35-fold repression of c-sense expression by Rep and the 8- to 15-fold stimulation of v-sense expression by TrAP are more similar to values previously found for TGMV (17,49) and TLCV (53) than to the much smaller values reported for ACMV (6,14). The results are in agreement with a model of early/late gene regulation: since Rep is needed for replication, it should be expressed early in the infection cycle but may not be required later. On the other hand, expression of the coat protein, which will be required in high amounts later in the infection cycle once the replicated DNA is ready for encapsidation, is activated by TrAP (57). Rep also repressed c-sense expression of the DNA B promoter 2- to 5-fold, but, as expected, had little or no effect on v-sense expression. Unexpectedly, TrAP activated also the c-sense expression of DNA A promoter by about 8-fold, a property not previously recognized. Both orientations of the DNA B promoter were strongly activated by TrAP in tobacco, but in cassava a high disparity of activation between the two DNA B promoters was observed: the activation of the BC promoter was much stronger than that of the BV promoter. Otherwise, expression in the two plant species was similar. The regulation of the DNA B promoter in cassava is in accordance with the role of the two DNA B proteins: NSP has been reported to bind ssDNA and to move the DNA out of the nucleus (58) while MPB forms endoplasmatic reticulum-derived tubules which extend through the cell wall to the next cell (58,59,60). Individually expressed NSP is localized in the nucleus, but when coexpressed with MPB, it is relocalized to the cell periphery (8,61). The results obtained with cassava thus suggest a regulated targeting of NSP. Early in the infection cycle more NSP will be produced which will favor its localization in the nucleus. Later, after activation of c-sense expression, MPB will redirect NSP together with the bound viral DNA to the periphery of the cell and facilitate the movement across the cell wall. Since the regulation of the DNA B promoter is different in cassava and tobacco it follows that not only the viral proteins but also cellular factors play a role in the regulation of the ACMV promoters. Species-specific variation in the availability of such factors could

account for the lower activation of the BV promoter in cassava compared to tobacco.

In the experiments designed to mimic a viral infection, the luciferase constructs were cotransformed with the complete ACMV DNA A, which should express regulatory proteins under viral expression signals and viral control. DNA A proved to be a more efficient inducer of v-sense expression than TrAP and a less efficient repressor of c-sense expression than Rep for the DNA A promoters. The strength of the activation of v-sense expression by DNA A is surprising since in the cotransformation experiments Rep and TrAP genes were under the control of the 35S promoter, which should produce much higher protein levels than the DNA A construct. It is possible that the DNA A contains additional activating sequences, which either allow more efficient TrAP production or which produce additional proteins or protein variants with activation potential. Another possibility is that the DNA A induces cell cycle factors that could increase the expression of the reporter gene (1,52). In contrast to the differential effect on the DNA A promoter, both orientations of the DNA B promoter were strongly stimulated by DNA A, suggesting that the stimulatory effect is dominant for both the BC and BV promoters.

The longer version of the DNA A promoter was activated much less efficiently and even considering the 3-fold higher basal activity, the level of v-sense expression remained lower than that observed using the short version. According to published transcript analyzes, the major transcription start site is located upstream of the AUG codon of the AV2 ORF, implying that gene fusions to the downstream coat protein ORF, as found in proA-1, would be less efficiently translated due to the presence of upstream, out-of-frame start codons (62). It would therefore be expected that translation from proA transcripts is more efficient. The observed lower activation of *Renilla* luciferase expression could be explained by assuming that only transcription at the major start site is activated by DNA A. According to this model, using proA-1, more of the longer mRNA (containing the 160 bp leader sequence) is produced, while the level of the short mRNA transcribed from the minor transcription site should not be affected. On the other hand, with proA, transcription of the leaderless mRNA will be enhanced and translation can take place efficiently, resulting in a much higher level of luciferase activity. It is not clear how such an activation pattern could be incorporated into a useful model for regulation of ACMV infection. The function—if any—of the major upregulated AV2 gene is still unknown, but our data would suggest that the AV2 protein is needed later in the infection process. We have not been able to detect the corresponding mRNAs by RNase protection assays (results not shown). This is probably due to the instability of the luciferase mRNAs. It therefore remains unclear which transcripts are produced with the different expression constructs in our assay system.

For our envisaged virus resistance strategies it is necessary to use a promoter integrated in the plant genome. The studies performed with the free plasmids in protoplasts might be close to the situation of a virus infecting a cell, but the regulation of a promoter integrated in the plant chromatin could be very different. Therefore we compared the effects of either Rep, TrAP or DNA A on reporter gene expression from cotransformed or integrated copies of proA or proA-1 in parallel experiments. Interestingly, the expression levels in individual transgenic plant lines vary greatly. For example, in line slr 1 the basal c-sense expression is much higher than v-sense expression, while in line slr 9 the basal v-sense expression is slightly higher. It seems that expression of the luciferase genes is not only controlled by the ACMV promoters but that the integration site plays also a role in the regulation of gene expression, despite the presence of scaffold attachment regions (Fig. 1b), which should reduce the effects of the integration site (29,63). The observed variation in expression could also be due to the different number of integrated copies or the presence of rearranged copies of the transgenes in the transgenic plants. In protoplasts from proA transgenic plants only a moderate effect (around 5-fold) was obtained with complete DNA A, while no effect was observed with either TrAP or Rep. Only in plant line slr 11, DNA A increased gene expression 50-fold, similar to the results from experiments where episomal cotransfected proA was used. The activity of the integrated longer promoter was affected by neither TrAP, Rep nor complete DNA A. For TLCV, strong GUS expression was observed from full-length virion-sense fusion constructs in transient assays, while GUS activity from the same constructs integrated in the plant genome was reduced drastically or inhibited completely (53). Deletions or rearrangements of the transgenes due to the lethal effect of the

integrated C1 gene, could, however, explain this, but not the results obtained in our experiments.

During the many repetitions performed in the course of our experiments particularly the results involving the transactivation of reporter gene activity showed relatively large variations. Differences of DNA structure during the experiments could be one reason for this variability and possibly for discrepancies between our data and those reported previously. It is unknown how input DNA structure might be altered during transient expression experiments, but it is conceivable that DNA nicking, cutting and degradation take place and may be variable depending on protoplast features. DNA curvature has been implicated in control of replication and possibly transcription of the wheat dwarf virus (51,64), where the expression of a reporter gene from plasmid DNA was different from that from *in planta*-generated replicons. It was speculated that this could be due to the activation states or the different methylation states of the chromatin. Such features would almost certainly be different in a supercoiled plasmid and in integrated DNA, which would probably also differ in their nucleosome structure. To verify this, we transformed protoplasts with proA which was linearized outside the expression units. The result showed that expression from linear DNA was poorly activated by TrAP and complete DNA A, whereas this process was efficient from circular DNA. Different DNA curvature may also change the accessibility of the promoters to transactivating factors, as shown by the results obtained with TLCV, where integrated promoters appeared no more to be activated by C2 but by host cell transcription factors in a tissue-specific manner (53). Whether or not the integrated TLCV promoter would still have been regulated by the complete DNA A was, however, not assessed. On the other hand, a truncated TGMV coat protein promoter was shown to be activated by host transcription factors in the vascular tissues of transgenic plants, but to be dependent on AL2 in mesophyll cells (15).

A virus resistance strategy in which the unique properties of this promoter were exploited has already been published (57). In this case, the expression of a ribosome inactivating protein was induced upon virus infection. In addition to using the upregulation of the AV promoter we propose to also use the down-regulation of the AC promoter. To avoid possible negative effects of a leaky inducible AV promoter, the AC promoter could regulate the expression of the inhibitor of a toxin which is under the control of the AV promoter. As long as no infection occurs, more inhibitor than toxin will be produced. Only if the cell is infected the balance will be shifted towards toxin production. Consequently local cell death would be induced, thereby protecting the plants from viral infection. The results of our studies are of practical significance for designing new virus resistance strategies in the future.

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