 doi:10.20944/preprints202011.0400.v1

Article

The AC2 protein of a bipartite geminivirus stimulates the transcription of the BV1 gene through abscisic acid responsive promoter elements

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Abstract: Geminiviruses possess single-stranded, circular DNA genomes, and control the transcription of their late genes, including BV1 of many bipartite begomoviruses, through transcriptional activation by the early expressing AC2 protein. DNA binding by AC2 is not sequence-specific, hence the specificity of AC2 activation is thought to be conferred by plant transcription factors (TFs) recruited by AC2 in infected cells. However, the exact TFs AC2 recruits are not known for most viruses. Here we report a systematic examination of the BV1 promoter (P_{BV1}) of mungbean yellow mosaic virus (MYMV) for conserved promoter motifs. We found that MYMV P_{BV1} contains three abscisic acid (ABA)-responsive elements (ABREs) within its first 70 nucleotides. Deleting these ABREs, or mutating them all via site-directed mutagenesis, abolished the capacity of P_{BV1} to respond to AC2-mediated transcriptional activation. Furthermore, ABRE and other related ABA-responsive elements were prevalent in more than a dozen Old World begomoviruses we inspected. Together these findings suggest that ABA-responsive TFs may be recruited by AC2 to BV1 promoters of these viruses to confer specificity to AC2 activation. These observations are expected to guide the search for the actual TF(s), furthering our understanding of the mechanism of AC2 action.

Keywords: Geminivirus; bipartite begomovirus; transcriptional trans-activation; abscisic acid; promoter motifs; AC2; BV1; mungbean yellow mosaic virus

1. Introduction

Plant viruses of the family *Geminiviridae* are among the most important pathogens of crop plants worldwide [1–4]. Examples of agriculturally devastating geminiviruses include tomato yellow leaf curl virus (TYLCV) and its close relatives, maize streak virus (MSV), and numerous variants of African cassava mosaic virus [5,6]. An increasing number of geminiviruses have been discovered during the last decade, with their geographic distribution steadily broadening, thanks to the global climate change that permits their insect vectors to flourish in previously hostile territories [7]. As a result, more research is urgently needed in order to inform more effective management of these viruses.

Geminiviruses all have characteristic, two-headed particles that encapsidate single-stranded (ss), covalently circularized DNA genomes, mostly under 3 kilobases (kb) in size [2]. These small ssDNA genomes nevertheless harbor multiple open reading frames (ORFs) on both the genome (or viral, V) strand and the strand complementary to the genome (C strand). Many of these ORFs overlap with each other, allowing for up to six different viral proteins per genome segment. While most geminiviruses have just one circular ssDNA genome segment, some viruses belonging to the genus Begomovirus, known as bipartite begomoviruses, have a second genome segment that encode additional important proteins [7,8]. Bipartite begomoviruses share similar coding strategies. Typically one of the genome segments, designated DNA-A, encodes five proteins, with one of them, AV1, on the V strand; and the remaining four, AC1-4, on the C strand. The other genome segment, DNA-B, encodes just two proteins: BV1 on the V strand, and BC1 on C strand. AC1, also known as

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Rep, is the earliest expressing viral protein, and the only viral protein absolutely needed for the replication of the viral genome [1,2,9,10]. It functions by coercing the host cell DNA replication machinery into replicating the viral genomes in the host cell nucleus. AC2, AC3, and AC4 are also early expressing, with AC2 serving as the transcriptional trans-activator (TrAP) of late genes. AC3 is a replicational enhancer (REn), and AC4 acts as a multifunctional symptom determinant [2,3,11,12]. AV1, BC1, and BV1 function as the capsid protein (CP), movement protein (MP), and nuclear shuttle protein (NSP), respectively.

The mechanism of AC2-mediated trans-activation of late genes, especially those encoded by DNA-B, is of great research and application interests because, once understood, it offers the potential of engineering begomovirus resistance that is activated by the invading virus itself. For example, antiviral defense factors, such as resistance genes (R genes) or silencing-inducing double-stranded (ds) RNA, can be placed under control of promoters activatable by AC2, so that they would only express when the corresponding virus replicates in the cell to produce AC2. The particular mechanism adopted by tomato golden mosaic virus (TGMV) has been extensively examined by Sunter and colleagues. Sunter and Bisaro were among the first to show that AL2 of TGMV, which corresponds to AC2 in other bipartite begomoviruses, activated BR1 (BV1 in other viruses) expression at the transcription level [13,14]. Note that although the AC2/AL2 proteins of different bipartite begomoviruses share important structural features characteristic of transcription factors (TFs), their interaction with DNA, including ssDNA, did not display any sequence specificity [15–17]. It has hence been hypothesized that AC2 exerts its trans-activation role by recruiting various TFs of the host plant, with the latter conferring the needed DNA-binding specificity. Consistent with this hypothesis, AL2 of TGMV was found to interact with the PEAPOD2 (PPD2) protein of Arabidopsis [18]. PPD2 was also shown to bind to the promoter of BR1, hence bridging AL2 and the BR1 promoter, facilitating the transcriptional activation of BR1 [19].

Mungbean yellow mosaic virus (MYMV) is a bipartite begomovirus that infect important legume crops such as mungbean, soybean, and lima bean [20]. Its two genome segments are approximately 2,730 and 2,660 nucleotides (nt) in size, respectively, encoding a similar set of viral proteins as TGMV, albeit with notable differences. Compared to TGMV, MYMV and other so-called "Old World" begomoviruses possess two additional ORFs, AV2 and BV2, although the functions of the putative AV2 and BV2 proteins remain enigmatic [21,22].

The current study set out to determine how expression of the BV1 gene of MYMV reacts to AC2-mediated transcriptional activation. Our study was inspired by a previous report [21], which mapped the AC2-responsive region of BV1 promoter (designated P_{BV1} hereafter) to nt positions 26 – 419 (394 nt in total) of MYMV DNA-B. Based on the results obtained by Sunter and colleagues using the TGMV system, we hypothesized that the 394-nt P_{BV1} contained conserved promoter motifs recognizable by certain plant TFs that also interacted with AC2. Here we report a systematic dissection of MYMV P_{BV1}, leading to the identification of multiple sequence motifs previously shown to be critical for plant response to abscisic acid (ABA).

ABA is a well characterized plant hormone that plays critical roles in multiple stages of plant life cycle, including maintaining seed dormancy, and spearheading plant responses to drought and cold stresses during vegetative growth [23,24]. ABA exerts these roles through multiple signal transduction pathways that enlist numerous ABA-responsive TFs. These TFs act on ABA-responsive promoters containing conserved sequence motifs known as ABA-responsive element (ABRE), coupling element 1 and 3 (CE1 and CE3), among others [25–27]. Here we report that MYMV P_{BV1} contains three ABREs within the first 70 nt region. Eliminating these ABRE motifs through either deletion or site-directed mutagenesis caused dramatic losses in the P_{BV1} responsiveness to AC2-mediated activation. ABRE, as well as CE1 and CE3, are also present in more than a dozen bipartite begomoviruses of the Old World lineage. These results strongly suggest that one or more ABA-responsive TFs are probably recruited by AC2 to confer the DNA-binding specificity needed for transcriptional activation of MYMV P_{BV1}.

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2. Materials and Methods

Constructs. The Core35S-GFP construct was based on pAI101, a binary vector modified from pCambia1300 [28,29]. An expression cassette consisting of the core 35S promoter [the last 99 nt of the cauliflower mosaic virus (CaMV) 35S promoter], the EGFP cDNA, and the poly-A signal of CaMV 35S RNA, was inserted into pAI101 at the PstI site to create Core35S-GFP. The Core35S-GFP construct was then used as the backbone to create constructs in which the Core35S promoter was replaced by the sequences of MYMV BV1 promoter, its counterpart in soybean yellow mosaic virus (SYMV), and their mutated derivatives. The corresponding DNA fragments were custom-synthesized by Integrated DNA Technologies (IDT, Coralville, IA) as gBlocks fragments, and used to replace the Core35S sequence via Gibson Assembly cloning (New England Biolabs, Ipswich, MA). The AC2-expressing construct was also made in pAI101, in which the AC2 cDNA was preceded by the CaMV 35S promoter with double enhancers (2X35S), plus a translational enhancer derived from tobacco etch virus (TEV TE) [28–30]. The identity of all constructs was verified with Sanger sequencing.

Agro-infiltration. Upon verification, the constructs were introduced into *Agrobacterium tumefaciens* strain C58C1 with electroporation [30]. In most experiments, various combinations of *Agrobacterium* suspensions were mixed together and delivered into *Nicotiana benthamiana* leaves as described [28–32]. A p19-expressing *Agrobacterium* strain was included in all combinations to alleviate RNA silencing-mediated mRNA degradation. In experiments testing the promoter responsiveness to Abscisic acid (ABA) treatment, a 50 mM ABA stock solution was prepared by dissolving ABA powder (GoldBio, St. Louis, MO) in dimethyl sulfoxide (DMSO). It was then added to the *Agrobacterium* suspensions, to a final concentration of 20 μM. Accordingly, the same Agrobacterium suspensions containing 1:2,500 diluted DMSO was used as the controls for ABA treatment.

Protein extraction and Western blotting. Proteins were extracted from the agro-infiltrated *N. benthamiana* leaves following a published protocol [28], and subjected to Western blot detection of GFP protein with a GFP antibody. The rabbit polyclonal GFP antibody was purchased from Invitrogen. The HRP-conjugated anti-rabbit secondary antibody was purchased from Abcam.

Confocal microscopy. Confocal microscopic observations were carried out using a Leica Confocal microscope (TCS SP5) available at Molecular and Cellular Imaging Center at the Ohio Agricultural Research and Development Center, The Ohio State University.

3. Results

3.1. The first 100 nt of MYMV P_{BV1} contains AC2-responsive DNA element(s).

We described in Introduction that AC2 of MYMV, similar to AL2 of TGMV, probably activated the transcription of P_{BV1} indirectly by recruiting one or more plant TFs to confer the DNA-binding specificity. Since different families of TFs are known to bind to different conserved promoter motifs, TFs that bridge AC2 and P_{BV1} could be inferred from the enrichment of a specific class of conserved DNA sequence motifs in MYMV P_{BV1}. To begin to delineate the conserved DNA motifs in MYMV P_{BV1}, we took advantage of the study of Shivaprasad and colleagues [21], which mapped MYMV P_{BV1} to a 394-nt region (nt position 26-419 of MYMV DNA-B. Fig. 1A). Since MYMV is very closely related to soybean yellow mosaic virus (SYMV), another Old World bipartite begomovirus [33], we first compared MYMV P_{BV1} with its counterpart in SYMV. Surprisingly, significant sequence similarities between these two BV1 promoters were found only within the first 103 nt (105 nt in SYMV) region, which we will call Q1 (meaning first ¼ of the P_{BV1}) from here on (Fig. 1A). By contrast, the remaining three quarters (Q234) of the promoters diverged dramatically from each other (not shown).

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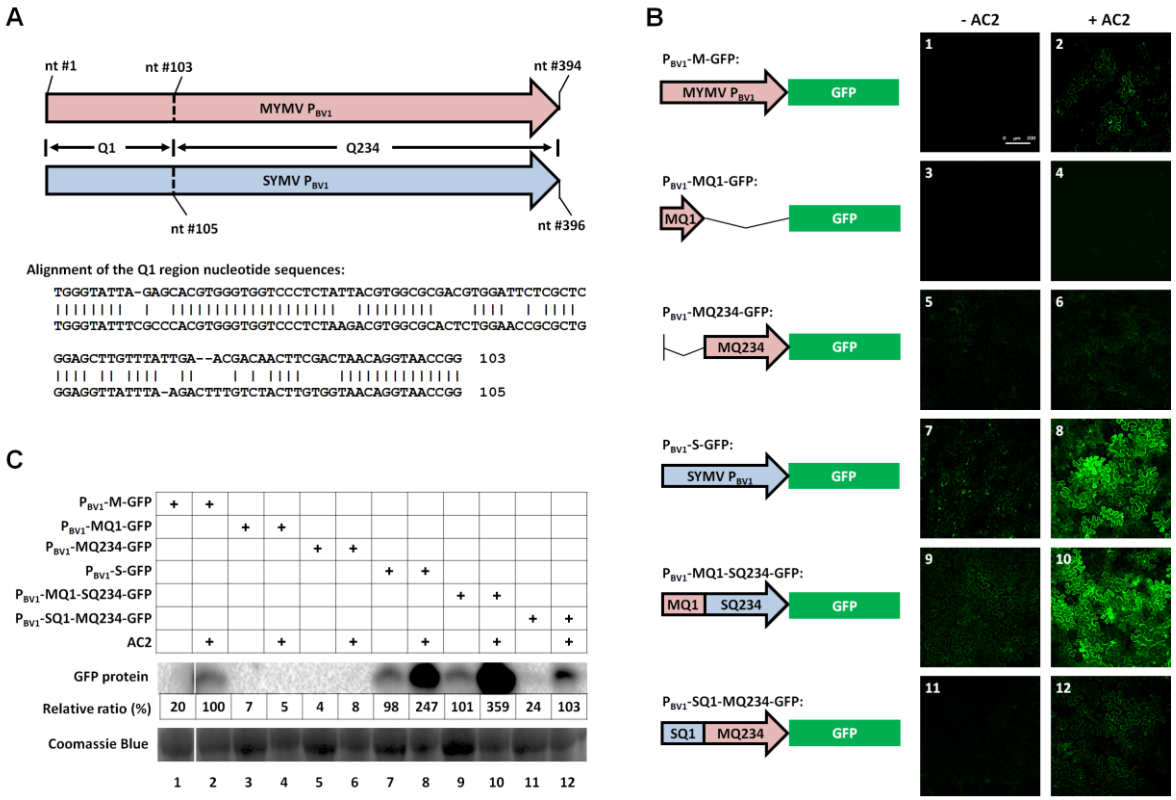

Figure 1. The first quarter of the MYMV BV1 promoter determines the specificity of AC2-mediated transcriptional activation. **A:** Schematic representation of the MYMV and SYMV P_{BV1} promoters, and the nt sequence alignment showing a high similarity between the two promoters within the first 100 nt of the approximately 400-nt promoters. Q1: first quarter, which is 103 and 105 nt in MYMV and SYMV, respectively. Q234: the remaining three quarters of the promoters that are highly dissimilar. **B:** Confocal microscope images of *N. benthamiana* cells showing the relative GFP expression levels driven by MYMV P_{BV1}, its deletion mutants (MQ1 and MQ234), SYMV P_{BV1}, and two chimeric promoters (MQ1-SQ234, SQ1-MQ234). Illustrations of the constructs are shown to the left, and the corresponding GFP expression levels in the absence (odd numbered panels) and presence (even numbered panels) of AC2 are on the right. Note that the microscope setting was the same for all images. **C.** Western blot detection of GFP protein in the agro-infiltrated leaves. The lane numbers corresponded the panel numbers in **B**. To minimize variations, each leaf sample consisted of four separate leaf sections taken from four different plants. The relative ratios were averages of two independent sets of experiments, with the band intensity value of (P_{BV1} + AC2) set at 100%.

The limited range of promoter sequence similarity, coupled with the near identity of the AC2 proteins of these two viruses (132 of the entire 135 amino acid residues are identical), led us to hypothesize that the Q1 region of MYMV P_{BV1} contained the specific sequence motif(s) needed for TF binding and hence AC2-mediated transcriptional activation. This hypothesis was tested by fusing various portions and combinations of MYMV and SYMV P_{BV1} with a GFP reporter (Fig. 1B). The resulting constructs were delivered into the cells of *N. benthamiana* plants via agro-infiltration [30]. The expression of GFP from these constructs, with or without MYMV AC2, was assessed with confocal microscopy, as well as Western blot detection of GFP protein. Note that MYMV AC2 has an additional function – suppression of RNA silencing [22]. The silencing suppression activity of AC2 can lead to mRNA stabilization, which can be mistaken as increased mRNA transcription due to AC2-mediated transcriptional activation. To neutralize this effect, we have included another construct that expresses p19, a much stronger silencing suppressor [34], in all treatments (not shown).

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As seen in Fig. 1B, panel 1, GFP fluorescent cells were undetectable under a confocal microscope when the MYMV P_{BV1} (P_{BV1}-M) promoter was used to drive GFP transcription without AC2. By contrast, green fluorescent cells were numerous and easily detectable when AC2 was included (Fig. 1B, panel 2). These results indicated that AC2 activated mRNA transcription from P_{BV1}-M, and the *N. benthamiana*-based system recapitulated the transcriptional activation activity of AC2. When assessed with Western blotting, AC2-mediated transcriptional activation caused the GFP protein level to increase by approximately five folds (Fig. 1C, lanes 1 and 2). Moreover, both Q1 and Q234 portions of P_{BV1} were needed for this effect because neither alone responded to AC2 activation (Fig. 1B, panels 3-6; Fig. 1C, lanes 3-6). The need for Q234 was not surprising because it contained the essential TATA box [21]. Interestingly, P_{BV1} of SYMV (P_{BV1}-S) drove a substantially higher level of basal transcription than P_{BV1}-M (approximately five folds; Fig. 1B, panels 1 and 7, and Fig. 1C, lanes 1 and 7), but notably a smaller magnitude (2.5 times) of transcriptional stimulation by AC2 of MYMV (Fig. 1B, panels 7 and 8; Fig. 1C, lanes 7 and 8). Exchanging the Q1 and Q234 portions of the two P_{BV1} promoters strongly suggested that the increased basal transcription was attributable to Q234 of SYMV, whereas the AC2-responsive element(s) likely resided in the highly homologous Q1 portion of the two promoters (Fig. 1B, panels 9-12; Fig. 1C, lanes 9-12).

3.2. The first 73 nt of MYMV P_{BV1} is sufficient to accommodate specific AC2 activation.

In order to identify the specific DNA sequence motifs within the Q1 portion of MYMV P_{BV1}, we generated three consecutive deletions within this region (Fig. 2A), with nt #1-33 (33 nt), 34-73 (40 nt), and 74-103 (30 nt) deleted in mutants dA, dB, and dC, respectively. As shown in Fig. 2B, mutants dA and dB caused the AC2-dependent transcription to decrease by approximately 30 and 60%, respectively, whereas mutant dC retained more than 80% of the AC2-dependent transcription. These results pointed to the first 73 nt region of MYMV PBV1 as the highly critical region needed for AC2-mediated transcriptional activation.

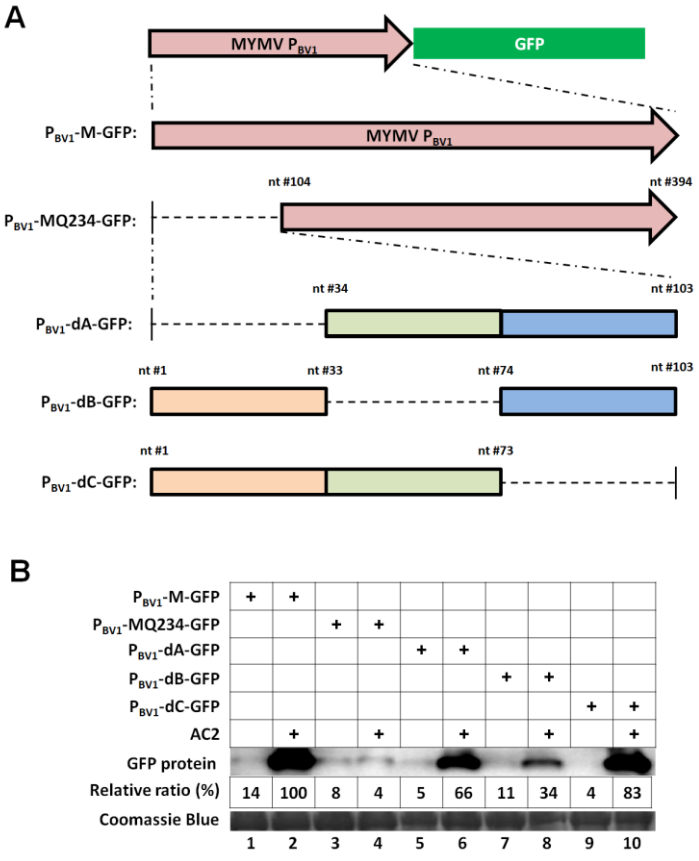

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Figure 2. The first 73 nt of MYMV P_{BV1} contains key promoter element(s) needed for AC2-mediated transcriptional activation. **A**: Diagrams of three deletion mutants of MYMV P_{BV1}. All deletions were 40 nt or smaller, with their respective boundaries shown. **B**. Western blot detection of GFP protein in the agro-infiltrated leaves harboring the various deletion mutants. Each protein sample was extracted from four separate leaf sections infiltrated with the same *Agrobacterium* suspension, taken from four different plants. The relative ratios were averages of two independent sets of experiments, with the band intensity value of (P_{BV1} + AC2) set at 100%.

3.3. Three ABREs within the first 73 nt of MYMV P_{BV1} cooperatively mediate transcriptional activation by AC2.

We next asked whether this 73-nt region of MYMV P_{BV1} contained any known sequence motifs of common TFs. Intriguingly, this relatively short region contained three ABREs, with the ACGTGG consensus [26,35], that were easily identifiable with a mere visual inspection (Fig. 3A). Furthermore, the fact that the first ABRE lied within the dA region, whereas the remaining two within the dB region, was consistent with Fig. 2 showing a larger impact of the dB deletion than the dA deletion. To evaluate the contribution of each of the three ABREs to AC2-mediated transcriptional activation of the P_{BV1} promoter, we then systemically mutated these ABREs, alone, and in combination, by changing the "GTGG" residues to "CCTT" (Fig. 3A). As shown in Fig. 3B, while mutating the 1st or 2nd ABRE alone appeared to have enhanced AC2-mediated activation (lanes 5-8), mutating both of them reduced the AC2 effect by 30% (lanes 11-12). Mutations within the 3rd ABRE reduced the AC2 effect by 40% (lanes 9 and 10), and combining them with mutations in 2nd ABRE further diminished the AC2 effect by 30% (lanes 13-14). While mutating both 1st and 3rd ABREs did not seem to hinder the AC2 action, it did negate the stimulative effect of mutations in 1st ABRE (lanes 15-16). Finally, simultaneously mutating all three ABREs abolished the AC2 effect to the same extend as Q234, in which the entire Q1 region was deleted. Together these results indicated that the three ABREs functioned cooperatively to accommodate AC2-mediated transcriptional activation of the P_{BV1} promoter.

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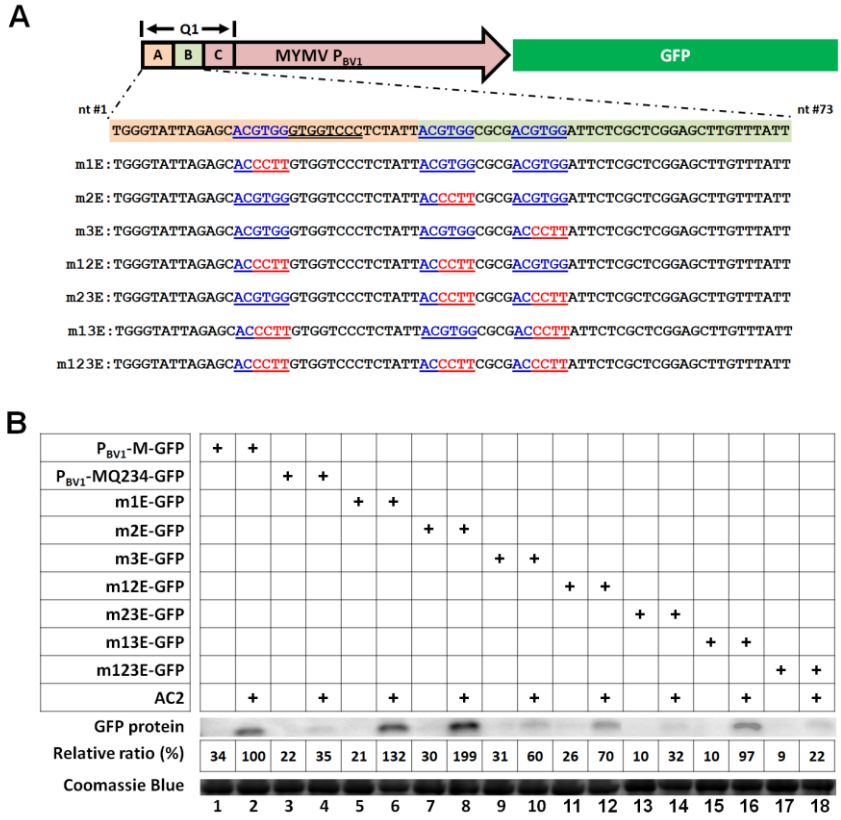

Figure 3. ABREs in MYMV P_{BV1} are needed for AC2-mediated activation. A. Top: schematic representation of the P_{BV1}-GFP construct. The boundaries of Q1 section are denoted with two vertical bars, and the three regions (A, B, C) subjected to deletions in Fig. 2 are highlighted with shades of different colors. Bottom: the sequence of the A and B regions, along with that of seven mutants in the three ABREs are variously mutated, are shown. The three ABRE motifs are underlined, and highlighted in blue fonts. The CLE motif is highlighted with double underline. The mutated nts (GTGG → CCTT) are in red fonts. B. Western blot detection of GFP protein in the agro-infiltrated leaves harboring the various mutants. Each protein sample was extracted from four separate leaf sections infiltrated with the same *Agrobacterium* suspension, taken from four different plants. The relative ratios were averages of two independent sets of experiments, with the band intensity value of (P_{BV1} + AC2) set at 100%.

3.4. BV1 promoters of many Old World bipartite begomoviruses contain ABREs and other ABA-responsive promoter motifs.

Having established that the three ABREs in MYMV P_{BV1} functioned cooperatively to confer specificity to AC2-mediated transcriptional activation, we wondered whether BV1 promoters of other bipartite begomoviruses also contained ABRE or other promoter motifs involved in plant responses to ABA. To address this question, we inspected 13 different Old World bipartite begomoviruses [8] for the presence of ABRE, as well as CE1 and CE3 motifs in their presumptive BV1 promoters. The core motif of ABRE has been determined to be ACGTGG or ACGTGT [23,25,36]. ABREs were also known to frequently overlap with the G-box motif with the consensus of CACGTG, which specifically interacts with two families of TFs known as basic helix-loop-helix (bHLH) and basic leucine zipper (bZIP), which are prevalent not only in plants, but also in yeast and animals; and to which most ABA-responsive TFs belong [23,37]. As shown in Table 1, 11 of the 13 viruses had at least one copy of ABRE (shaded light blue) in their BV1 promoters, four of them had 2 – 3 ABREs. The two viruses that lacked a fully conserved ABRE, CIGMV and KuMV, nevertheless contained the related G-box motif, or its highly conserved cores – CANNGT or ACGT [37]

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(highlighted with dotted underlines in Table 1). In summary, ABRE and the closely related G-box elements are present in the BV1 promoters of all of the 13 viruses.

In promoters of plant ABA-responsive genes, ABREs, especially when present in single copy, frequently mediate the ABA responses in coordination with other promoter elements, among them CE1 and CE3 [26,35]. The core motif of CE1 has been variously defined as CCACC, CCGCC, CACCG, or CGCCG, in different plant genes and experimental systems [23,26,35,38–40]. Importantly, CE1, and also CE3, are functional in both strands of the promoter DNA. As shown in Table 1, at least one CE1 motif (shaded purple) was identified in eight of the 13 promoters. Indeed, if we included the GGTGG (CCACC in the complementary strand) motif that overlapped with ABRE in the first five promoters, we would end up having 11 of the 13 promoters with various CE1 core motifs. Interestingly, the GGTGG element also frequently overlapped with the conserved late element (CLE; double-underlined in Table 1) found in the promoters of many geminiviral late genes, including both AV1 and BV1 [41,42], which further implicates the CE1 motifs in the transcriptional activation of BV1 genes.

We next analyzed the 13 BV1 promoter sequences for CE3 motifs, using the GTGTC and CGCGTG core motifs (painted green in Table 1) reported by others ([26,35,38,39]. Note that the GTGTC motifs that overlapped with the ACGTGT, one of the ABRE consensuses, were not counted as CE3. This analysis showed that six of the 13 promoters contained CE3 motifs. Altogether we found that all 13 BV1 promoters analyzed contained ABA-responsive promoter elements, and 12 out of 13 had more than one element. These findings are highly consistent with the involvement of ABA-responsive TFs in the activation of BV1 genes in these viruses.

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Table 1. ABA-responsive ABRE, CE1, and CE3 motifs within BV1 promoters of bipartite begomoviruses

Virus_DNA-B Accession	DNA-B nt #26-175 (150 nt), conserved promoter elements denoted
MYMV-Vigna [KA22] _AJ132574.1	TGGGTATTAGAGCACGTGGGTGGTCCCTCTATTACGTGGCGCGACGTGGATTCTCGCTCGGAGC TTGTTTATTGAACGACAACTTCGACTAACAGGTAACCGGTTTGGTTACCTTTCGTACATGGACA AATTTGTCTTTTCCTCAAAAAG
MYMV-Vigna [KA34] _AJ439057.1	TGGGTATTAGAGCACGTGGGTGGTCCCTCTATTACGTGGCGCGACGTGGATTCTCGCTCGGAGG TTGTTTATTGAACGACAACTTGGACTAACAGGTAACCGGTTTGGTTACCTTTCGTACATGGACA AATTTGTCTTTTCCTCAAAAAG
SYMV_AJ582267.1	TGGGTATTTCGCCCACGTGGGTGGTCCCTCTAAGACGTGGCGCACTCTGGAACCGCGCTGGGAG GTTATTTAAGACTTTGTCTACTTGTGGTAACAGGTAACCGGCTAAGTGACCGGTTGGAAAGCGT GCCTTTCCCACCCCTGGCAAAT
MYMIV_AY271894.1	TCGGTTTTAGAGCACGTGGGTGGTCCCTCTAGTACGTGGCGCGCTCTGGAGTCTCGCTCGAAGC TTGTTTATCGAACGACTACTTAGAGTTACAGGTAACCGGATAGGTGACCGATCGTACATGGACA AATTTGTCTTTTCCTCAAAAAG
HYMV_AJ627905.1	GACCTGGATCACTCACGTGGGTGGTCCCGCTCATCGTGGCGCAACGAGGAGTCTCGTTGGGAGT TTTTATAATGGGTTACTACTTGGGAGAAGTGGGGAAACCGGAAGCTACCGCCGCAAATCGAGAA ATTTGCTTTTAATGCGAAATTA
SLCMV_AJ579308.1	GTGGTGGCCCCCCCCACGTGGGGATGTCCCCCTCTCACAACGCTCACTAGAAGGTTCAACATGT TGGTGGCCCCACGATGTTGTTTATAACGTCTATAACGTTTGAGACTCGAAGCTTGTGATGCCAC GTATGCGTTATTAGTACTTCGT
ICMV_AJ314740.1	GTGGTGGACCCCTCCCCCACGTGGAGATGTCCCCCACTCAGAACGCTCACTAGGAGGTTCAACA TGTTGGTGGCCCCACGATGTTGTTTATAACGTCTATAACGTCTGAGACTCCAAGCTTGTGATGC CACGTATGTGTTATTTGTACTT
ClGMV_DQ641693.1	TATAGTATGTGTCATGTGTGGGTCCCATGTCACGTGATTTTGTACCTTTCTGTCCCCCAGCTAG CCGACAAAGTGGGACCCACTCACGTGATTAGCACTTGCCTTGTACCTTCCTTCTGTGTACGTTC TGGTCAAAAAGACTAGTATACC
KuMV_DQ641691.1	TGCGGTGTGGTCCCCCCGCCCATGTGCTTTCAATCTCGTCCGCACGTTTTTTTTAGTCTTCGCG CGTGGTGTGAATGCGCCGTAATGCGCTTGCCGCAAATAACGTTATAACTGCTTTAATTTGAATT TCTTTAAATTATGTGGAATACC
SLCCV_AM260207.1	CTCTAAATCTGGCCGTCTATTTTCTATAAATGCGCATCAAACGAGTGCGATGAACAACACGTGT CCTATGTGTCATCCTTTACTAAAGGAAAGGATTGTACGATATCGAAATTTGCACATGGTGGACC CCAGTGACTAGATATGCAATAT
SLCPHV_AB085794.1	CTTCAAATCTGTCCGTCTATTTTGTACAAAAGCTTATCAAGGTGCTGCGATGCACAACACGTGT CCTCTATTACATCCTTTACTAAAGTAAAGGTTTGTACGATTTCCAAGTGCCGCACATGGTGGTC CCCAGTGACTAGATATGCAATA
LYMV_AF509740.1	TCTCCCCACAGTCGTTGATTTGTTCTAAAGGCGCGCACGATTGCATGTTTTCGCAACACGTGTC CTCTTATGATTACTTTATGAAAATAAAGTTAGCTGTGTTCTGAAATTTCGCACATGGTGGTCCC CCAGATACTAGATAGAAATATC
ToLCNDV_AJ875158.1	CCCTATCCTGACCGTTGCTGTGTAATCATTGCACCAAGTTAGTCATCCGATTTGCAACACGTGT ATCCCACTAACAGACTTTATGCAAATAAATGTCCGATATCTGTGTCGACAATGCTTGTGTGTTC CCCTTATATCTTGTCGTACCCC
ToLCuGuV_AY190291.1	CCTTATCTTGACCGTTGCTGCGTAATCATTGCACCAAGTTACTCATCCGATTTGCAACACGTGT ATCCCACTAGCAGACTTTATGCAATTAAATGTCTGATATCTGTGTGTACAATGCATATGTGTTC CCCTTATATCTTGTCGTACCCC

Notes:

1. The highly conserved core motifs of ABRE, CE1, and CE3 are: ABRE: ACGTGG, ACGTGT; CE1: CCACC, CCGCC, CACCG, CGCCG; CE3: GTGTC, CGCGTG. The well-documented G-box motif (CACGTG), as well as two variations of core motifs of G-box (CANNTG and ACGT), are highlighted with dashed underlines. Finally, the conserved late element (CLE, GTGGTCCC) found in the promoters of late genes in many begomoviruses are highlighted with double underline.
2. Virus acronyms: MYMIV – mungbean yellow mosaic India virus, HYMV – horsegram yellow mosaic virus, SLCMV – Sri Lankan cassava mosaic virus, ICMV – Indian cassava mosaic virus, ClGMV – Clerodendrum golden mosaic virus, KuMV – Kudzu mosaic virus, SLCCV – squash leaf curl China virus, SLCPHV – squash leaf curl Philippines virus, LYMV – loofa yellow mosaic virus, ToLCNDV – tomato leaf curl New Delhi virus, ToLCuGuV – tomato leaf curl Gujarat virus.

3.5. Exogenous ABA has no effect on P_{BV1} promoter activity or AC2-mediated transcriptional activation.

We have earlier revealed a definitive role for ABREs in AC2-mediated transcriptional activation of the MYMV P_{BV1} promoter. We also found that three types of ABA-responsive promoter motifs, ABREs, CE1, and CE3, were prevalent in BV1 promoters of many Old World bipartite begomoviruses. We hence wondered whether applying exogenous ABA could stimulate

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transcription from a BV1 promoter, with or without AC2. For this experiment we returned to use MYMV P_{BV1} and its m123E mutant, in which all three ABREs were mutated. We also included an additional control in which transcription of GFP mRNA was driven by the core (the last 99 nt) of the CaMV 35S promoter (Core35S) that did not contain an ABRE. Notably, transcription driven by Core35S was close to five times stronger than the AC2-activated P_{BV1}, and approximately 40 times stronger than P_{BV1} alone (Fig. 4, lanes 2, 5, and 6), verifying P_{BV1} as an extremely weak promoter in the absence of AC2 activation. Also notable is that the activity of the Core35S promoter was repressed by AC2 by approximately 40%, regardless of the presence of exogenous ABA (Fig. 4, compare lanes 7, 8 with 13, 14). Additionally, DMSO, which was used to dissolve ABA, hence included as a solvent control, appeared to stimulate Core35S transcription, but its effect seemed to be attenuated by ABA (compare lanes 7 with 8, 13 with 14).

However, ABA failed to elicit a significant stimulation of transcription from the P_{BV1} promoter, regardless of the presence of AC2. Although basal transcription experienced a slight increase for samples in lanes 15-16 (compared with lane 5), this was likely due to the stimulatory effect of DMSO. Similar conclusion could be drawn for samples in lanes 17 and 18 (compared with lane 6). In summary, although the three ABREs within P_{BV1} were clearly needed for AC2-mediated transcriptional activation, the corresponding TFs was not upregulated by ABA treatment in *N. benthamiana* leaf cells (see Discussion).

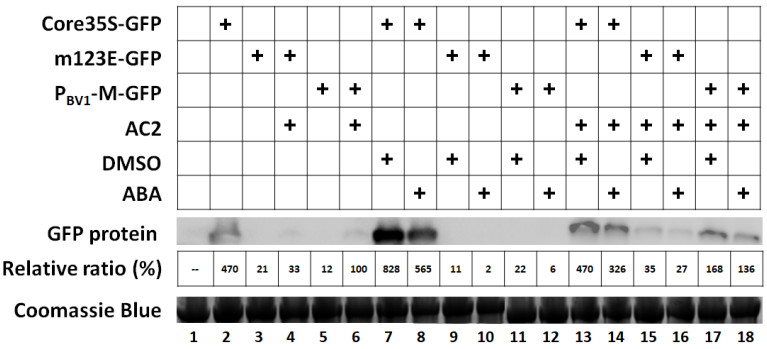

Figure 4. Exogenous ABA has no effect on PBV1 promoter activity or its responsiveness to AC2 activation. Western blotting was used to detect the levels of GFP protein in *N. benthamiana* leaves infiltrated with various combinations of Agrobacterium strains, with or without ABA (shown on top). Each protein sample was extracted from four separate leaf sections subjected to the same treatment, taken from four different plants. The relative ratios were averages of three independent sets of experiments, with the band intensity value of (P_{BV1} + AC2) set at 100%.

4. Discussion

The question of how AC2, a protein encoded on DNA-A of bipartite begomoviruses, transactivate the late genes encoded on DNA-B, a separate genome segment, is important to resolve as the answer could offer insights on how genimiviruses coordinate the expression of early and late genes to balance optimal genome replication with efficient whole plant spread. It could also inform novel strategies on controlling these viruses. Findings made using the TGMV model strongly suggest that AC2/AL2 acts on the BV1/BR1 promoter by recruiting PPD2, a putative TF of the plant host that confers the DNA-binding specificity [14,18,19]. However, whether the same TF could also be exploited by AC2 of other similar viruses was not reported. In the current study, we found that the BV1 promoters of MYMV, and multiple other bipartite begomoviruses of the Old World lineage, were enriched for conserved promoter elements characteristic of ABA responsive genes of diverse plants [23,26,38]. Furthermore, eliminating the three ABREs in the MYMV BV1 promoter led to a near complete loss of the AC2 responsiveness. Together our results strongly suggest that TFs of the

ABA response pathways could be candidates for AC2 recruitment. Since many TFs implicated in the ABA pathways are already known, this study substantially narrows down the search for TFs that interact with MYMV AC2 in future investigations.

While the importance of ABA-responsive promoter elements in BV1 expression has not been recognized before, the involvement of the closely related G-box motif in the transcription of AC1/AL1 (Rep) has been reported [43]. Separately, ABA-responsive genes were found to be induced in tomato plants infected with tomato yellow leaf curl Sardinia virus [44]. ABA has also been found to enhance plant defense against RNA viruses by inducing the expression of RNA silencing pathway genes [45]. These studies are consistent with an active role of ABA signaling in plant responses to virus infections. Conversely, it is conceivable that some geminiviruses may evolve to turn the ABA-mediated defense signaling to their own advantage by co-opting it to activate the expression of late genes of the viruses.

An unexpected finding of our experiments is that the MYMV BV1 promoter is very weak. Although we expected to see low transcription without AC2 activation, we were nevertheless surprised to realize that its activity was still many folds weaker than the Core35S promoter upon AC2 activation. Several reasons might account for this low activity. First, the activity detected with our system may only account for a fraction of BV1 transcription in actual MYMV infections. This is because in actual infections, BV1 and other late genes are expressed after viral replication, from the many progeny genomes newly synthesized in the cells. As a result, BV1 transcription is expected to occur simultaneously on many copies of DNA-B. By contrast, the amount of non-replicating DNA template in our non-replicating system is expected to be very limited. Second, optimal BV1 transcription may demand an ideal host cell environment not provided with our experimental system. MYMV is a virus of legume plants. Hence, the TFs accessible to AC2 in legumes may differ substantially from their orthologs in *N. benthamiana*, making transcriptional activation less efficient in our system. Consistent with this idea is the fact that the BV1 promoter of SYMV exhibited much higher basal transcription than the MYMV BV1 promoter in our assay, suggesting virus- and host-dependent effects.

The lack of response to ABA treatment by the MYMV BV1 promoter is also noteworthy, given the requirement of ABREs for its activation by AC2. This could be explained, if the particular ABA-responsive TF(s) were present in *N. benthamiana* leaf cells at low levels, but their corresponding genes were not responsive to ABA in these particular cells. This scenario is possible because ABA responses are known to be highly coordinated, often occurring in dividing cells at root and shoot growth points [24]. Consistent with this, geminiviruses are known to be mostly phloem limited, primarily infecting cells of vascular bundles where they induce active division of the infected cells. By contrast, the *N. benthamiana* cells we used were mostly non-dividing. Nevertheless, our system did detect a substantial level of AC2 activation, permitting the interrogation of the ABREs in the MYMV BV1 promoter. In conclusion, our study revealed a previously unrecognized class of promoter elements that mediate the AC2 transcriptional activation of a BV1 promoter of a bipartite begomovirus, and paved the way for the identification of the specific TF(s) involved in the process.

Author Contributions: Conceptualization, F.Q.; methodology, R.S., J.H.; formal analysis, R.S., F.Q.; investigation, R.S., J.H., L.Z.; writing, F.Q.; supervision, F.Q.; project administration and funding acquisition, F.Q. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported in part by a grant from the National Science Foundation (#1758912), a SEEDS grant from the Ohio Agricultural Research and Development Center, Graduate Assistantship from OARDC to R.S., as well as tuition assistances to R.S. from the Department of Plant Pathology, OSU. F.Q. is additionally supported by the HATCH project # OHO01337.

Acknowledgments: We appreciate the generosity of the labs of Drs. Lucy Stewart and Peg Redinbaugh for allowing us to share equipment. We are indebted to members of the Qu lab for stimulating discussions and material sharing.

Conflicts of Interest: The authors declare no conflict of interest.

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