



Functional characterization of the replication-associated protein (Rep) promoter of an alpha-satellite associated with *Ageratum* enation virus

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Abstract *Ageratum* enation virus (AEV) (*Begomovirus agerati*), belonging to the genus *Begomovirus*, is associated with DNA components known as alphasatellite and betasatellites. The alphasatellite encodes the replication-associated protein (alpha-Rep), which is essential for self-replication but relies on helper viruses for transmission between plants. In this study, we propose that the alpha-Rep promoter exhibits bidirectional promoter activity and can drive the expression of heterologous genes in plants. Using Agrobacterium-mediated transient and stable transgenic

techniques, we identified the minimal promoter sequence of the Rep gene from the *Ageratum* enation virus-associated alpha satellite (AEA) (*Coleus-atellite agerati*) that initiates β -glucuronidase (GUS) reporter gene transcription in *Nicotiana benthamiana* plants. Promoter deletion analysis showed varying promoter activities. Especially, all essential cis-elements for strong promoter activity were located within the 331 nucleotides upstream of the translation start codon, displaying robust and consistent transient GUS expression compared to the control promoter (CaMV35S). The findings lay the groundwork for developing a versatile viral vector system suitable for reverse genetics and genome editing across a broad host range, enhancing our understanding of viral-host interactions and their applications in biotechnology.

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Introduction

Begomovirus, the largest and most widespread genus of plant-infecting DNA viruses in the *Geminiviridae* family, is transmitted predominantly by whiteflies (*Bemisia tabaci*) and affects various crops (Pandey et al., 2021). The begomovirus genome comprises circular, single-stranded (ss) DNA that is approximately

2.6–2.7 kb in size, encapsulated in a twinned quasi-icosahedral particle, and encodes multiple overlapping transcripts with 5 to 8 open reading frames (ORFs) (Borah et al., 2016; Stenger et al., 1992). These viruses can be classified as either bipartite, consisting of DNA-A and DNA-B, predominantly found in the New World, or monopartite containing only DNA-A and mainly identified in the Old World (Briddon et al., 2010; Villegas et al., 2019).

Ageratum enation virus (AEV) is a whitefly-transmitted monopartite begomovirus first isolated from *Ageratum conyzoides* in Nepal (Briddon et al., 2003) and has since been identified in various economically important plants in India, including *Cleome gynandra* (Raj et al., 2010), *Trichosanthes dioica* (Raj et al., 2011), *Crassocephalum crepidioides* (Y. Kumar et al., 2011), *Solanum lycopersicum* (Swarnalatha et al., 2013), *Amaranthus spp.* (Srivastava et al., 2013a, 2014), *Tagetes patula* (Marwal et al., 2014), *Calendula officinalis* (Jaidi et al., 2015), *Papaver somniferum* (Srivastava et al., 2017) and *Trigonella foenum-graecum* (Swarnalatha et al., 2019). They are associated with alphasatellites and betasatellites, which are circular ss-DNA molecules about 1.4 kb in size, each encodes a single gene in the complementary strand. These satellites influence viral symptoms, pathogenesis, and act as silencing suppressors (Zhou, 2013). Alphasatellites possess three key features: a gene encoding a rolling-circle replication (RCR) associated protein (Rep), an adenine-rich region, and a conserved intergenic region (IR) essential for replication and transcription regulation (Zhang et al., 2012a). The IR contains a bidirectional promoter with essential regulatory elements, transcription factor binding sites, and transcription start sites for controlling gene expression and replication (Borah et al., 2016). Although alphasatellites can self-replicate in the host plants using alpha-Rep protein, but rely on helper viruses for movement and transmission within and between plants (Xie et al., 2010).

Several members of the *Begomovirus* have been intensively studied to elucidate the structure and function of their promoter elements, highlighting the potential of geminiviral promoters for heterologous gene expression in plants (Xie et al., 2003). These promoters can drive constitutive gene expression in transgenic plants, as documented in prior studies (Zhang et al., 2012b). For example, the first alphasatellite promoter characterized in tobacco curly shoot

alpha-satellite (TbCSA), which exhibited low promoter activity (13%) as compared to the widely used CaMV 35S promoter, limits its effectiveness for stable and transient gene expression in transgenic plants (Zhang et al., 2012a).

The current study investigates the transcription initiation promoter and polyadenylation signal in the IR of the *Ageratum* enation virus-associated alphasatellite (AEA) utilizing transient expression studies with the β -glucuronidase (GUS) reporter gene. Due to its broad host range, adoption of encapsidation and movement processes in association with AEV, small size, ease of manipulation, autonomous replication, and effective DNA repair template delivery, AEA is well-suited for constructing viral vectors, leading to high replication rates and elevated expression levels. Qualitative and quantitative analysis of the bidirectional promoters of the AEA Rep gene using *Agrobacterium*-mediated delivery were conducted. The expression patterns and activities of the GUS reporter gene tagged with Rep promoters were evaluated in *Nicotiana benthamiana* plants and compared to the CaMV 35S promoter. The findings indicate that the AEA Rep promoter has substantial potential as a tool for ectopic gene expression in plants and as a virus-based vector system for virus-induced gene silencing (VIGS) and genome editing (GE). Similar studies using the *Ageratum* yellow leaf curl betasatellite, adopted by wheat dwarf India virus (WDIV) (*Mastrevirus tritici*), have demonstrated its potential to develop a single-vector system for high-throughput genome editing across various plant species (Kumar et al., 2023). This approach may serve as an alternative to generating stable transgenic plants for functional gene studies in plant biotechnology applications.

Materials and methods

To characterize and map the regulatory elements within the Rep gene promoter of the alphasatellite (AEA), an analysis of its putative promoter sequence was conducted using the Plant Cis-Acting Regulatory Element (Plant CARE) program (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al., 2002). The analysis considered the entire non-coding region upstream of the Rep gene, and the first nucleotide of the translation start site of the Rep

gene was designated as +1 (Online Resource 1a). To determine the minimal functional promoter sequence, a series of bidirectional primers were designed for PCR amplification of both the longest and truncated sequences (50 bp deleted from both ends in sense and antisense orientation) of the potential promoter sequence (Table 1) (GenBank accession no. JX512905) (Srivastava et al., 2014). The PCR amplified products were cloned into the pGEM-T easy vector (Promega, Madison, USA), digested with HindIII/XbaI restriction enzymes, and subsequently inserted into the binary vector pBI121 (Chen et al., 2003) by replacing the CaMV 35S promoter. The pBI121 vector was a positive control with the CaMV 35S promoter driving the GUS reporter gene and a negative control without the GUS gene. These constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and infiltrated into 4-week-old *N. benthamiana* leaves using a needleless syringe for localized transient expression (Yang et al., 2000).

Qualitative assays involved histochemical GUS staining using X-Gluc (5-Bromo-4-Chloro-3-Indoyl- β -D-Glucuronide) after 3 days post-infiltration (dpi), enabling visualization of gene expression through microscopy, at 10X magnification (Jefferson et al., 1987). The semi-quantitative assay involved RNA isolation using the CTAB (Cetyl Trimethyl Ammonium Bromide) method (Noorani et al., 2013), cDNA synthesis via a Verso cDNA synthesis kit (Thermo Scientific, USA), and PCR amplification using GUS-specific primers (Online Resource 3), where varying

band intensities indicated the levels of GUS gene expression. The quantitative analysis included real-time PCR via 2 $\times$ iTaq Universal SYBR Green mix (Biorad, USA) using specific primers for the GUS gene and β -actin as a reference gene (Online Resource 3). The fold change in gene expression was calculated using the CT value of the samples. A fluorometric GUS assay was performed to calculate GUS activity using 4-MUG (4-methylumbelliferyl glucuronide) as a substrate. The GUS enzyme hydrolyzed 4-MUG to produce 4-MU (4-methyl-umbelliferon), which generated blue fluorescence under UV light. GUS activity was quantified in nanomoles of 4-MU per microgram of total protein per hour, thereby providing detailed quantification of GUS expression at the protein level (Jefferson et al., 1987).

Data from these experiments were analyzed statistically using GraphPad Prism software version 10.2.2 (GraphPad Software, San Diego, USA), employing one-way ANOVA and various post hoc tests to identify significant differences ($p < 0.05$) in expression levels among samples. The post hoc analysis included Tukey's test HSD (honest significant difference) and Fisher's LSD (least significant difference) tests, both individual and pairwise, for completely randomized designs (CRD) to identify statistical differences among samples. The goodness of fit was evaluated based on statistical parameters such as p-value and standard error, along with residual analysis, which includes Brown-Forsythe ANOVA test, Welch's ANOVA test, and Geisser-Greenhouse's epsilon tests.

Table 1 Primers used to amplify promoter fragments

Primer Name	Sequence (5'–3')	Underlined restriction site
pRep430F_F	AAGCTTTTTTGCTCTTTAAAAAAGAAAGAATGA	HindIII
pRep430F_R	TCTAGAGTTTACTCAGATAGGAGTATTAAAGGGA	XbaI
pRep430R_F	GCTCTAGAATTTTGCTCTTTAAAAAAGAAAGAATGA	XbaI
pRep430R_R	CCCAAGCTTGTTTACTCAGATAGGAGTATTAAAGGGA	HindIII
pRep331R_F	CCCAAGCTTGGGTGAATCATATTTATTATTCTTGAGAAAATCAGG	HindIII
pRep331R_R	GCTCTAGAGCGTCTCTAACTCGGGGTCTC	XbaI
pRep331F_F	GCTCTAGAGCTTGAATCATATTTATTATTCTTGAGAAAATCAGG	XbaI
pRep331F_R	CCCAAGCTTGGGGTCTCTAACTCGGGGTCTC	HindIII

Results

Identification of regulatory motifs and cis-elements in the AEA non-coding region

The analysis of the complete non-coding region (430 nt) upstream of the AEA Rep ORF (−1028 to −79) unveiled several putative regulatory motifs and cis-elements (Online Resource 1c). This included a TATA box located approximately 20 bp upstream of the nonanucleotide sequence (TAG TATTAC) at −110 nt, along with two CAAT-box motifs (−156 nt and −196 nt), a CGTCA motif (−98 nt), an abscisic acid-responsive element (ABRE) at −100 nt, and two light-responsive elements, the G-box and AE-box, located at −99 nt and −312 nt, respectively. Additional motifs with unknown functions, such as STRE, unnamed_4, and as-1, were also predicted (Fig. 2a & Online Resource 4). Two key binding sites for host transcription factors, including a TATA box element adjacent to the Rep-binding site and a G-box motif near the hairpin, both crucial for geminivirus promoter activity were identified in the IR region, indicating the potential of this non-coding sequence as a robust promoter for transgenic gene expression (Eagle & Hanley-Bowdoin, 1997; Eini et al., 2009; Zhu et al., 2002).

Evaluation of AEA Rep promoter activity in *N. benthamiana*

To analyze AEA Rep promoter activity, the 430 nt full-length and 331 nt (−1076 to −29) truncated promoter fragments were cloned bidirectionally into a promoter-less pBI121 vector, resulting in pRep430F, pRep430R, pRep331F and pRep331R constructs (Online Resource 1b). These constructs were evaluated for promoter activity in *N. benthamiana* (Fig. 1a, b & Online Resource 2). The localized blue precipitate observed at 3 dpi in histochemical staining confirmed significant GUS gene expression, with the pRep331R construct showing the highest GUS activity among all variants, albeit lower than the CaMV 35S promoter (Fig. 2b). Additional validation through microscopy, MUG assay, and molecular detection via semi-quantitative PCR and Real-Time PCR using GUS-specific primers, with a ~300 bp band, further confirmed the stability of GUS expression (Fig. 2c, d & e).

Statistical analysis indicated significant differences ($p < 0.05$) in fluorescence intensity across constructs compared to the 35S promoter, as determined by Tukey's and Fisher's tests. The analysis yielded R-squared values of 0.9012 and 0.9782, F-values of 21.89 & 107.7 and $P < 0.001$ for MUG assay and Real-Time PCR, respectively, confirming significant differences among the constructs (Online Resource 5).

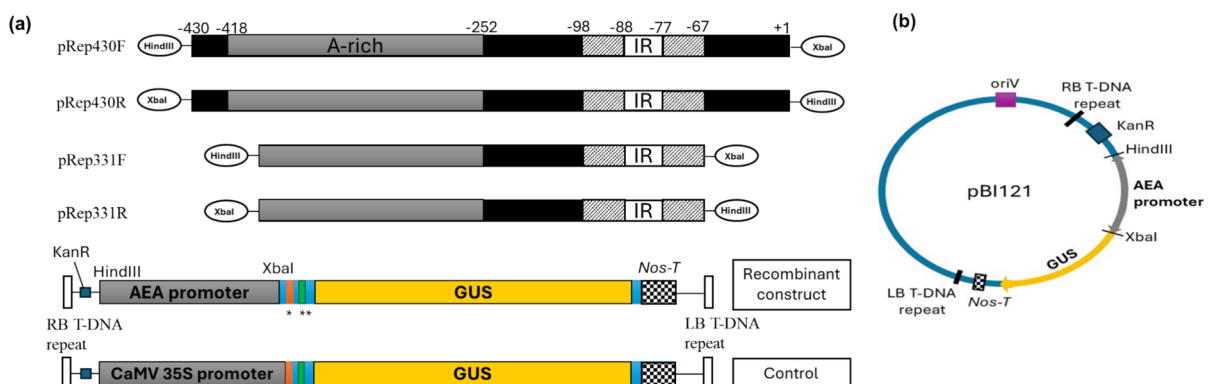


Fig. 1 (a)—Diagram of the production of Rep promoter-reporter constructs. The promoter fragment, A-rich region and intergenic region (IR) are shown in black, gray and white, respectively. The presumed translation start site of the Rep gene is assigned coordinate +1. All of the constructs were

cloned into the pBI121 binary vector for the analysis of GUS activity. Note- *-Kozak sequence, ** SV40 NLS. **(b)**—Circular recombinant pBI121 vector featuring the AEA promoter positioned upstream of the GUS gene

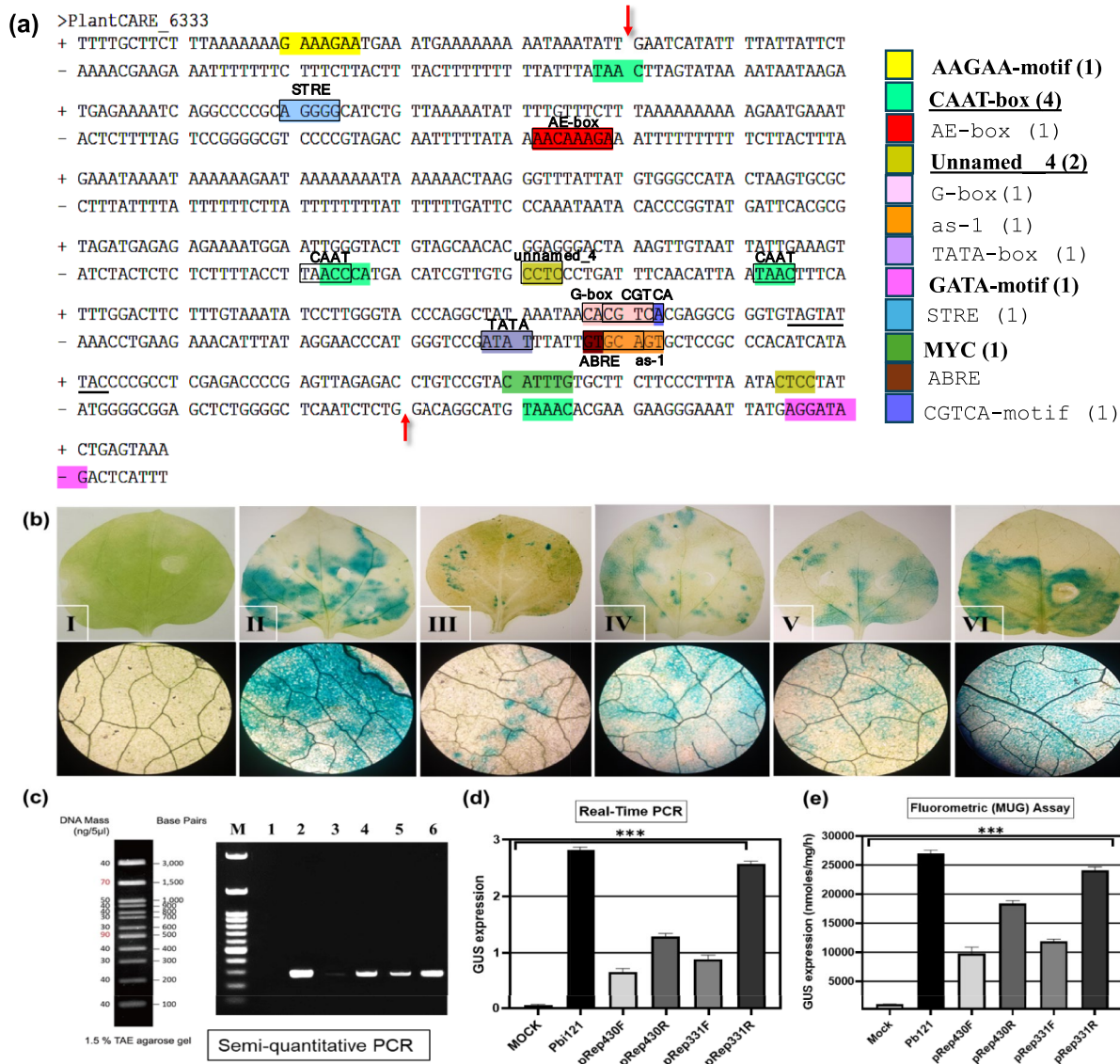


Fig. 2 (a) -Fragment contained multiple TATA-box and CAAT-box core cis-acting elements, an abscisic acid-responsive element (ABRE), MYC elements (CATTTG), an AAGAA motif (GAAAGAA), two types of MeJA (methyl jasmonate) responsiveness (CGTCA-motif and TGACG-motif), three types of light-responsive elements (G-box, GATA-motif, AE-box), followed by, STRE (AGGGG), as-1 (TGACG) and an unnamed_4 (CTCC). Note- The red arrows denote 50 bp deletion on each side and cis-acting elements in bold indicate deleted elements in promoter deletion analysis. (b)—GUS expression of the putative promoter sequence in transient transgenic systems. Histochemical staining and microscopy at 10X were performed for GUS expression in *Nicotiana*

benthamiana leaves. I—Mock, II—Positive control (pBI121), III—pRep430F, IV—pRep430R, V—pRep331F and VI—pRep331R. (c) Semi-quantitative PCR using agarose gel electrophoresis to assess GUS gene expression via different promoter constructs. M- 100 bp ladder (Thermoscientific, USA), 1- Mock, 2- positive control, 3- pRep430F, 4- pRep430R, 5- pRep331F and 6- pRep331R. (d) Real-Time PCR graph. Bars are mean ± standard errors and mean with *, **, *** are statistically different at probability values of $p \leq 0.05$ and ≤ 0.01 respectively. (e) Fluorometric GUS activity (MUG assay) analysis. The mean GUS activity from the CaMV 35S promoter of pBI121 was taken as 100%. The standard deviation in each case is shown by the error bars

The pRep430F construct showed the lowest variance, while the pRep331R promoter demonstrated superior performance. Significantly, this indicates that deleting 50 nt from both ends in antisense orientation in the pRep331R construct showed enhanced promoter activity, suggesting that the removed nucleotides may have negatively regulated promoter efficiency.

Discussion

Efficient genetic modification of plants to enhance agronomic traits relies on effective promoters to drive transgene expression. This necessitates the analysis of promoter expression patterns for developing commercially viable transgenic plants. The modified virus vectors, under suitable promoters, enable constitutive gene expression for various applications. Promoters from the *Geminiviridae* family, such as the β C1 promoter of the tomato yellow leaf curl China betasatellite have been characterized previously (Sunter & Bisaro, 2003). The cotton leaf curl Burewala virus (*Begomovirus gosypikokranense*) Rep promoter significantly enhances transgene expression in plant cells compared to CP and CaMV35S promoters (Khan et al., 2015). Although many begomoviruses' betasatellites have been characterized in terms of sequence and infectivity, studies on alphasatellite promoter studies are still limited.

The bidirectional promoter in begomoviral genomes facilitates transcription in both directions, optimizing genetic efficiency for gene expression in compact plant viruses. As recently reported, Radish leaf curl betasatellite-encoded a novel β V1 protein that induces hypersensitive response-like symptoms and interacts with the replication enhancer protein (AC3) of the helper virus (Gupta et al., 2022). Studies suggest that alphasatellites contribute to the transcription of silencing suppressor genes, promoting severe symptoms that help the virus evade host defenses and enhancing viral accumulation in plant tissues (Idris et al., 2011; Nawaz-ul-rehman et al., 2010). This bidirectional promoter activity may also support the transcription of uncharacterized ORFs in the antisense orientation of AEA-Rep, potentially encoding proteins involved in viral replication or interaction with host factors.

Strong promoters are crucial for high transgene expression in crop improvement and protein production. The CaMV 35S promoter is well-known for

facilitating high-level gene expression; but it has limitations, including variable effectiveness across different tissues, inability to support multiple or bidirectional gene expression in transgenic plants, and poses risks of gene silencing when employed in multiple copies (Kishi-Kaboshi et al., 2019; Sunilkumar et al., 2002). To address these issues, this study examined the bidirectional promoter region of AEA to identify a novel promoter with a broad host range. By constructing the longest promoter construct and a series of deletion mutants, significant differences ($P > 0.05$) in GUS expression levels were observed among the different constructs. The analysis of cis-regulatory elements in the AEA bidirectional promoters contributed insights into the transcriptional regulation of both the virus and the host plant. Specifically, the TATA-box aids transcription initiation by interacting with transcription factors, recruiting RNA polymerase II for viral transcript production (Tjitro et al., 2019), while the CAAT-motif is critical for major late promoter transcription (Song et al., 1996). The CGTCA motif and G-box are significant for modulating gene expression intensity, whereas ABRE and AE-box are crucial for plant responses to environmental stimuli and circadian rhythm regulation (Hernandez-Garcia & Finer, 2014; Reppert & Weaver, 2001; Zhang et al., 2019).

Through deletion analysis of the bidirectional promoter, the optimal promoter length for activity was elucidated, demonstrating that deletions at both ends significantly increased GUS expression levels. The pRep331R promoter demonstrated activity that was twice as high as the other three promoter constructs. The pRep331R sequence harbors two key features: a conserved structure with a predicted hairpin containing a nonanucleotide sequence common to nanoviruses and geminiviruses, which is cleaved by the Rep protein to initiate virion-strand DNA replication, and an A-rich region downstream of the Rep gene with an A-content of 56.28%, distinguishing it from nanoviruses (Xie et al., 2010). Furthermore, the AEA promoter sequence demonstrates similarity with type 3 alphasatellites associated with the tobacco curly shoot virus (*Begomovirus nicotianae*) and ageratum yellow vein betasatellite (*Betasatellite ageraflavi*) complex (Xie et al., 2010). These findings suggest that Rep promoter elements are not uniformly distributed throughout the non-coding region, i.e., the 331 nt fragment in the antisense orientation (pRep331R)

spanning nt 29–1, 1378–1076 upstream of the Rep gene exhibits strong promoter activity. Deletions at the 5'- and 3'-termini of the pRep331R promoter likely enhanced GUS expression by removing cis-regulatory elements, such as AAGAA-motif and CAAT-motif, MYC, CAAT-motif, unnamed_4, and GATA-motif elements, that may function as transcriptional repressors or hinder efficient transcriptional activation. This corroborates the previous research, indicating that a smaller fragment could outperform the entire non-coding region (Srivastava et al., 2013a, 2013b; Xie et al., 2003; Zhang et al., 2012a).

Conclusion

pRep331R is potentially the minimal sequence required to drive transgene expression in *N. benthamiana*, a commonly used model plant, achieving about 87.895% of the activity of the CaMV 35S promoter. This suggests its utility as a valuable tool for high-level gene expression in plant genetic engineering, however, further validation is required to assess its broader applicability in dicot plants. AEA promoter region functions not only as a bidirectional promoter but also contains minimal sequences necessary for the origin of replication of satellite as well as recognition motifs likely involved in cell-to-cell movement, through interacting with the begomovirus genome. This research emphasizes the potential of AEA, which features key advantages including compact size for efficient vector packaging, constitutive and bidirectional transcriptional activity for co-expression of multiple genes, and possible host-specific compatibility that may enhance performance in begomovirus-susceptible plants, that may be suitable for the begomovirus genome-based vector systems. Furthermore, AEV and its associated satellites may show promise as delivery tools for genome-editing reagents, paving the way for the development of a single vector system for tissue culture-free genome editing across a wide range of plant species, further establishing its plant biotechnology applications.

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Author contributions A.S. and J.K. contributed to the study's conception and design. Material preparation was carried out by T.S. and T.G., while data collection and analysis were performed by T.S. and S.D. The first draft of the manuscript was written by T.S., A.S., and V.C. and all authors commented on previous versions. All authors read and approved the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval and consent This research was carried out solely on plant viruses that were non-pathogenic for humans. Therefore, there is no ethical issue.

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