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Development of an efficient expression system with large cargo capacity for interrogation of gene function in bamboo based on bamboo mosaic virus

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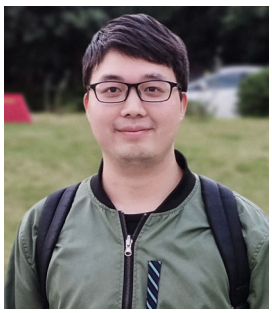

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ABSTRACT

Bamboo is one of the fastest growing plants among monocotyledonous species and is grown extensively in subtropical regions. Although bamboo has high economic value and produces much biomass quickly, gene functional research is hindered by the low efficiency of genetic transformation in this species. We therefore explored the potential of a bamboo mosaic virus (BaMV)-mediated expression system to investigate genotype–phenotype associations. We determined that the sites between the triple gene block proteins (TGBps) and the coat protein (CP) of BaMV are the most efficient insertion sites for the expression of exogenous genes in both monopodial and sympodial bamboo species. Moreover, we validated this system by individually overexpressing the two endogenous genes *ACE1* and *DEC1*, which resulted in the promotion and suppression of internode elongation, respectively. In particular, this system was able to drive the expression of three 2A-linked betalain biosynthesis genes (more than 4 kb in length) to produce betalain, indicating that it has high cargo capacity and may provide the prerequisite basis for the development of a DNA-free bamboo genome editing platform in the future. Since BaMV can infect multiple bamboo species, we anticipate that the system described in this study will greatly contribute to gene function research and further promote the molecular breeding of bamboo.

Keywords: *ACE1*, bamboo, BaMV-mediated overexpression, *DEC1*, expression, virus-mediated genome editing

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INTRODUCTION

Bamboo is widely planted in tropical and subtropical forest plantations and is one of the fastest growing plants among monocotyledonous species (Tanaka et al., 2003). Moso bamboo is one of many bamboo varieties and can grow 1 m each day under suitable conditions (Peng et al., 2013). Due to this fast growth, bamboo-based products have high economic value and wide applications as building materials or in papermaking (Qiao et al., 2013). In fact, the international trade value of bamboo reached $68.8 billion in 2018 (Ye et al., 2020). In addition to its economic value, bamboo also plays an important role in ecological protection by mitigating greenhouse gases due to its excellent carbon fixing capacity (Lobovikov et al., 2012).

Currently, most molecular research studies of bamboo use high-throughput sequencing technologies (Wang et al., 2017; Wei et al., 2017; Li et al., 2018; Guo et al., 2019;

Zhang et al., 2021b; Li et al., 2022; Zheng et al., 2022). However, transient transformation has recently been reported for bamboo in many studies, which should pave the way for functional gene studies. For example, carbon nanotube technology has been applied for the transient transformation of moso bamboo to express transgenes (Papolu et al., 2021). Protoplast transfection also has been used to investigate gene function in bamboo (Wang et al., 2019; Zhang et al., 2021a). However, the above methods based on the transient expression of genes of interest are not easily amenable to the observation of phenotypes in whole bamboo plants. *Agrobacterium tumefaciens*-mediated stable transformation have remarkable advantages for the dissection of phenotypes when expressing genes of interest in transgenic plants. Notably, among diverse bamboo species, only two varieties have been successfully transformed to date (Qiao et al., 2014; Ye et al., 2017; Huang et al., 2022). In particular, the low efficiency of Agrobacterium-mediated transformation presents the greatest obstacle to functional research and molecular breeding in bamboo.

Expression vectors based on plant viruses have been deployed in plants as an alternative to stable transformation (Seo et al., 2016; Choi et al., 2019; Fang et al., 2021). Indeed, a gene of interest can be inserted within the plant viral genome to help deliver the target gene into the host for expression. During virus infection, the target gene can be expressed throughout the entire plant, and its encoded protein can accumulate to large amounts in a short time (Gleba et al., 2007; Seo et al., 2016). For example, bean pod mottle virus (BPMV), soybean mosaic virus (SMV), and cucumber mosaic virus (CMV) have been successfully modified to allow the production of recombinant proteins in dicotyledonous plants (Zhang and Ghabrial, 2006; Seo et al., 2009). In Cucurbitaceae, *GFP* (*green fluorescent protein*) was expressed in *Luffa aegyptiaca* leaves using a vector based on tobacco ringspot virus (TRSV) (Fang et al., 2021). However, the use of plant viral vectors has not been reported for the expression of foreign genes or the overexpression of endogenous genes in bamboo. The investigation of plant virus vectors would thus have great applied value for gene functional discoveries in bamboo.

Bamboo mosaic virus (BaMV) is the most extensively studied virus to infect bamboo plants (Lin et al., 2004; Cheng et al., 2013; Hung et al., 2014; Lee et al., 2015; Huang et al., 2017; Fei et al., 2021; Lee et al., 2022). BaMV is a single-stranded positive-sense RNA, with five open reading frames (ORFs), in addition to a 5′ cap structure and a 3′ poly(A) tail (Yang et al., 1997). *ORF1* encodes a 155 kDa viral replicase (Huang et al., 2004). ORF2, ORF3, and ORF4 are three overlapping genes encoding the triple gene block proteins (TGBps): TGBp1 of 28 kDa, TGBp2 of 13 kDa, and TGBp3 of 6 kDa (Lin et al., 1994). ORF5 encodes the viral coat protein (CP), which is involved in BaMV genome encapsidation and virus movement (Lan et al., 2010; Lee et al., 2011). In a previous study, a small segment of a chlorophyll biosynthesis gene was cloned into the BaMV genome to infect *Nicotiana benthamiana* and purple false brome (*Brachypodium distachyon*) (Liou et al., 2014). In other work, a fragment of the BaMV CP was replaced with 37 amino acids from the virus capsid protein VP1 of foot-and-mouth disease virus (FMDV) and successfully produced chimeric virus particles displaying FMDV-VP1 epitopes in plants (Yang et al., 2007). Importantly, all of these studies inserted very short gene segments into the BaMV genome. One major disadvantage of viral vectors is their capacity to accommodate foreign DNA fragments, termed cargo (Ma et al., 2020). Therefore, developing a BaMV-based vector with large cargo capacity (more than 4.5 kb) will be instrumental to its wide use. Moreover, previous studies of BaMV-based vectors only tested their use in model plants, but assessing the potential of BaMV for application in its native host is urgently needed for interrogation of gene function in bamboo.

In this study, we explored the ability of BaMV to express both foreign and endogenous genes in bamboo. Indeed, we established that BaMV can successfully drive the expression of large foreign genes in both monopodial bamboo (*Phyllostachys edulis*) and sympodial bamboo (*Dendrocalamus latiflorus* Munro), indicating the high cargo capacity of our BaMV-based vectors for dissemination and expression in different bamboo species. Moreover, we individually overexpressed the two bamboo genes *ACCELERATOR OF INTERNODE ELONGATION 1* (*ACE1*) and *DECELERATOR OF INTERNODE ELONGATION 1* (*DEC1*) using this system and described the resulting phenotype. The viral vector presented in this study should help dissect gene function in bamboo. Especially, the entire procedure did not exceed 45 d with BaMV-based vectors, which was faster than with *Agrobacterium*-mediated methods. This study provides a high efficiency BaMV-mediated expression system to systematically investigate the function of genes with sizes of up to 4 kb in bamboo. In particular, these BaMV-based vectors were able to express the nuclease Cas9, providing a prerequisite tool to develop a virus-induced genome editing (VIGE) platform in bamboo (Oh et al., 2021). In summary, the BaMV-based expression system of this study successfully expressed both foreign and endogenous genes, which will greatly contribute to the research of genotype–phenotype associations and promote the molecular breeding of bamboo in the future.

RESULTS

Inserting foreign DNA after the ORF4 stop codon in the BaMV genome offers the fastest transmission

To design a virus vector suitable for expression in bamboo, we extracted BaMV genomic RNA from BaMV-infected leaves of wild Ma bamboo (*Dendrocalamus latiflorus* Munro) exhibiting mosaic symptoms and converted it to DNA. We deposited the BaMV genome sequence in GenBank under the accession number OP493850. We then cloned the 2×35S promoter-driven initiation of BaMV genomic DNA in the binary vector pCAMBIA1302. To assess the potential for BaMV-based vectors to express genes, we introduced three separate cloning sites in the BaMV genomic DNA clone (Figure 1A). At each site, we added a copy of the ORF5 (*CP*) subgenomic promoter. Both RNA-DEPENDENT RNA

POLYMERASE (RDR) and DICER-LIKE (DCL) proteins are major components involved in antiviral defense, which is associated with host resistance and the speed of virus transmission (Andika et al., 2013; Sabbione et al., 2019; Perez-Canamas et al., 2021). In addition to wild-type (WT) *Nicotiana benthamiana* plants, we also used NbRDR6 and NbDCL2/4 RNA interference (RNAi) lines, designated NbRDR6i and NbDCL2/4i hereafter. We individually infiltrated Agrobacterium (*Agrobacterium tumefaciens*) harboring BaMV clones with the enhanced GFP (*EGFP*) gene inserted at one of three different insertion sites in the leaves of WT, NbRDR6i, and NbDCL2/4i *N. benthamiana* plants. We detected EGFP fluorescence in leaves at 7 d after Agrobacterium infiltration with BaMV-EGFP-2 and BaMV-EGFP-3 (Figure S1). Notably, the fluorescence signal produced from leaves infiltrated with BaMV-EGFP-3 was stronger than that with BaMV-EGFP-2. Moreover, we observed stronger fluorescence in the NbRDR6i and NbDCL2/4i lines than in the WT (Figure S1).

We waited another 30 d and investigated the fluorescence intensity and spread in infiltrated *N. benthamiana* leaves (Figure 1B). We observed much stronger EGFP signals in NbDCL2/4i plants than in the WT or NbRDR6i (Figure 1B).

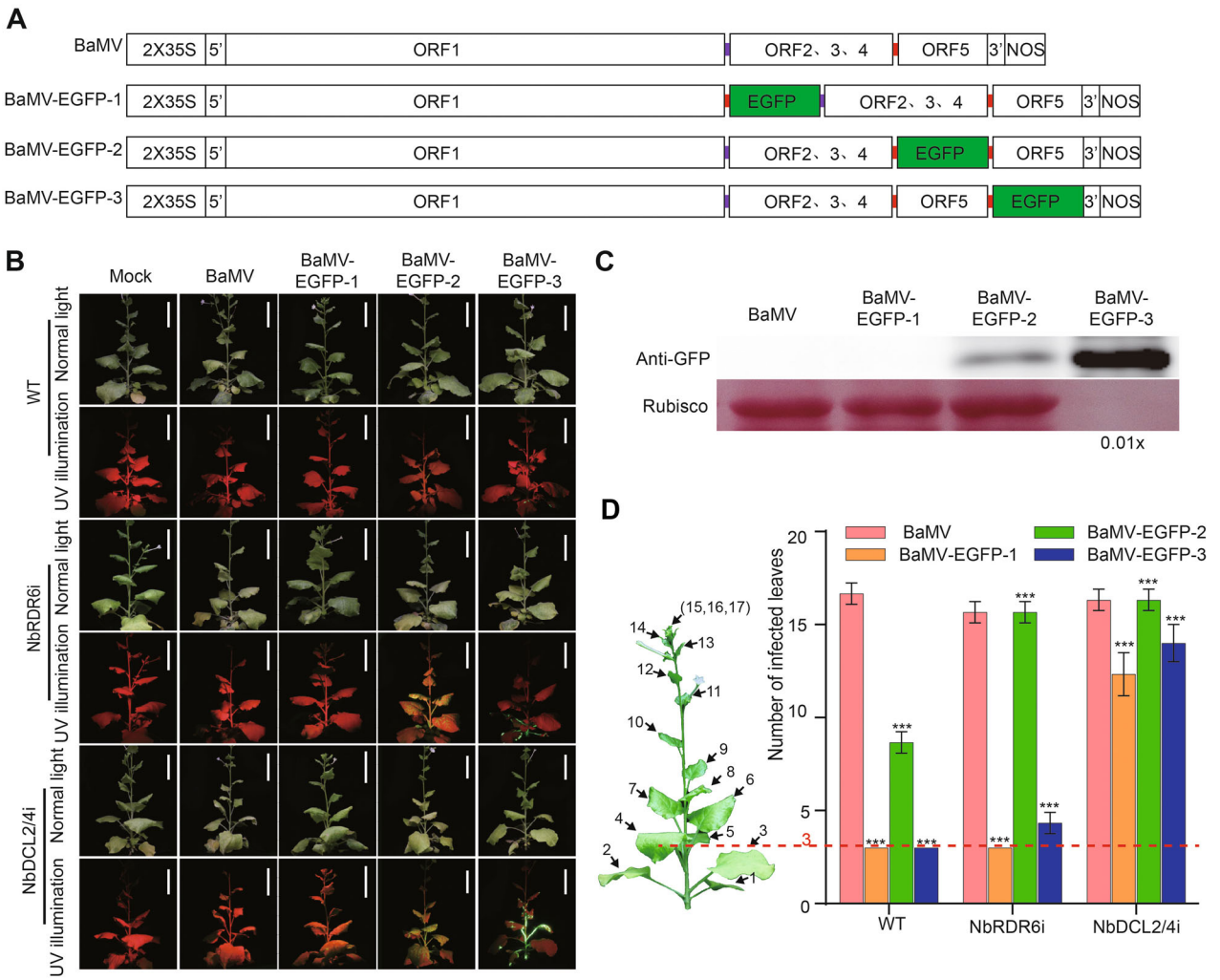

Figure 1. Testing virus propagation and expression of *EGFP* in three bamboo mosaic virus (BaMV)-based expression constructs differing in the insertion site of the foreign gene

(A) Schematic diagrams of the BaMV infectious clone and its three variants harboring *EGFP* at different insertion sites. The full genomic DNA of BaMV was inserted between two copies of the cauliflower mosaic virus (CaMV) 35S promoter (2×35S) and the NOS terminator. *EGFP* was driven by the ORF5 subgenomic promoter. *EGFP* driven by the ORF5 subgenomic promoter was cloned downstream of the stop codon of ORF1 (BaMV-EGFP-1), *ORF4* (BaMV-EGFP-2), and *ORF5* (BaMV-EGFP-3). Purple and red thin lines represent the *ORF1* and ORF5 subgenomic promoters, respectively. **(B)** Detection of EGFP fluorescent signal upon mock inoculation or infection with BaMV or BaMV-derived clones shown in **(A)** of wild-type *Nicotiana benthamiana* or transgenic NbRDR6i and NbDCL2/4i plants. Scale bars, 5 cm. **(C)** Immunoblot analysis of EGFP accumulation. 0.01× represents a 100-fold dilution of proteins extracted from leaves infected with BaMV-EGFP-3. **(D)** Transmissibility of BaMV or BaMV-derived clones shown in **(A)** in wild-type *N. benthamiana* or transgenic NbRDR6i and NbDCL2/4i plants. Leaves below the dashed line (leaves #1-3) were infiltrated with Agrobacterium cell suspensions. The y-axis represents the position of infected leaves 30 d after Agrobacterium infiltration. The experiment was performed three times. Asterisks indicate statistical significance (***, $P < 0.0001$) between BaMV and BaMV-derived clones. Data are means ± standard error (*SE*).

Among the three clones tested, fluorescence signals increased from BaMV-EGFP-1 to BaMV-EGFP-2 and BaMV-EGFP-3 (Figure 1B). Immunoblotting using protein extracts from NbDCL2/4i leaves also revealed the accumulation of EGFP in leaves infiltrated with BaMV-EGFP-3 and BaMV-EGFP-2, while we failed to detect EGFP in leaves infiltrated with BaMV-EGFP-1 (Figure 1C), which was consistent with the fluorescence detected above (Figure 1B). Therefore, infiltration with the BaMV-EGFP-3 clone was associated with the highest protein abundance of all clones tested (Figure 1C). We also determined the systemic transmission of BaMV constructs from infiltrated leaves to distant leaves. We noticed that different transmission speeds of BaMV systemic infection among the four clones assessed (BaMV as control and three BaMV-EGFPs), as indicated by EGFP fluorescence and the detection of the BaMV genomic RNA by RT-PCR. The BaMV-EGFP-2 clone showed the fastest transmission speed based on molecular detection of the BaMV genomic RNA in each leaf (Figure 1D). These results suggest that the insertion site in the BaMV genome influences virus transmission ability and gene expression. Of the three clones tested, BaMV-EGFP-1 displayed the weakest transmission and expression ability, while BaMV-EGFP-2 had the highest transmission efficiency and BaMV-EGFP-3 resulted in the highest protein accumulation. In WT *N. benthamiana* plants, the intact BaMV infectious clone (without *EGFP*) showed a faster transmission speed than any of the BaMV clones harboring *EGFP* (Figure 1D), suggesting that the speed of BaMV spread is affected by an insertion in the genome.

The RNA silencing suppressor P19 promotes BaMV infection and transmission

We hypothesized that suppressing PTGS from the host might improve BaMV infection. Therefore, we selected the RNA silencing suppressor P19 from tomato bushy stunt virus (TBSV) (Figure 2A). First, we co-infiltrated BaMV-EGFP-3 and the pCAMBIA1302 binary vector harboring the P19 coding sequence into *N. benthamiana* leaves. We observed stronger fluorescence signals from co-infiltrated leaves at 3 d after Agrobacterium infiltration compared to leaves only infiltrated with BaMV-EGFP-3 (Figure 2B), suggesting that P19 promoted EGFP expression from the BaMV-based clone. We also detected enhanced fluorescence signal from BaMV-EGFP-2 co-infiltrated with P19 at 7 d after Agrobacterium infiltration (Figure S2). To facilitate the use of P19, we cloned the P19 and EGFP sequences in-frame and separated by a sequence encoding the self-cleaving peptide T2A and cloned the resulting cassette at each of the three insertion sites (Figure 2A). We then infiltrated leaves from WT *N. benthamiana* and transgenic NbRDR6i and NbDCL2/4i plants with these P19-carrying clones and assessed EGFP fluorescence at 7 d after Agrobacterium infiltration (Figure S3). Fluorescence intensity was stronger in NbDCL2/4i leaves infiltrated with BaMV-P19-EGFP-3 than those infiltrated with BaMV-P19-EGFP-2 (Figure S3). We did not detect fluorescence signals in leaves infiltrated with BaMV-P19-EGFP-1 (Figure S3).

We waited another 30 d before investigating EGFP fluorescence in the same plants (Figure 2C). We determined that BaMV-P19-EGFP-1 produces the weakest fluorescence out of all P19-carrying clones tested, with detectable fluorescence only observed in NbDCL2/4i leaves. Plants infiltrated with BaMV-P19-EGFP-2 showed clear fluorescence signals in both NbRDR6i and NbDCL2/4i in the stem. Especially, NbDCL2/4i plants presented a severe phenotype with curved stems (Figure 2C). We detected EGFP fluorescence near the infiltration sites for NbRDR6i and WT leaves infiltrated with BaMV-P19-EGFP-3 (Figure 2C), while we observed EGFP fluorescence in the stems of NbDCL2/4i plants infiltrated with BaMV-P19-EGFP-3. Notably, we did not observe curved stems in WT or NbRDR6i plants infiltrated with BaMV-P19-EGFP-3 (Figure 2C).

We also detected RNA from BaMV or BaMV-derived clones in different leaves, which revealed that BaMV-P19-EGFP-2 can spread to the whole plant in both RNAi lines and the WT (Figure 2D). The BaMV-P19-EGFP-2 clone with the p19-EGFP cassette inserted site between ORF4 and ORF5 showed the highest efficiency of systemic transmission. We conclude that the insertion of P19 between ORF4 and ORF5 is efficient in promoting the transmission of BaMV. The transmission ability of BaMV-P19-EGFP-1 (Figure 2D) did not change much compared to that of BaMV-EGFP-1 (Figure 1C). The propagation speed of BaMV-P19-EGFP-3 in NbDCL2/4i plants (Figure 2D) was slower relative to that of BaMV-EGFP-3 (Figure 1C).

BaMV-based vectors have high cargo capacity to express long gene fragments in *N. benthamiana*

Inserting long coding sequences usually decreases the efficacy of viral replication (Cheuk and Houde, 2018). The *RUBY* reporter system consists of one expression cassette encoding cytochrome P450 76AD1 (CYP76AD1), L-DOPA 4,5-dioxygenase (DODA), and a glucosyltransferase, separated by the self-cleaving peptide 2A; together, these three enzymes convert tyrosine to bright red betaine (He et al., 2020). To assess the potential of our modified BaMV-based expression vectors for the expression and transmission of large genes, we cloned the *RUBY* cassette together with P19 (4,518 bp) between ORF4 and ORF5 in the BaMV genome (Figure 3A). We then infected *N. benthamiana* leaves with Agrobacterium cultures carrying the resulting modified BaMV clone. We detected the clear accumulation of the red pigment betalain at 30 d after Agrobacterium infiltration (Figure 3B). In the WT, betalain accumulation was mainly limited to veins and stems, and plant growth was not affected. NbDCL2/4i plants had a dwarf stature and accumulated betalain throughout the plant, including in stem and leaves. NbRDR6i plants displayed an intermediate state between WT and NbDCL2/4i plants. We also observed the accumulation of the red pigment in roots (Figure 3C). We confirmed the expression of the *RUBY* cassette by end-point RT-PCR in aboveground (Figure 3D) and underground tissues (Figure 3E). These results demonstrate that the modified BaMV-based vector can carry large genes and expresses them; moreover, the same vector allows systemic spread to aboveground and underground tissues.

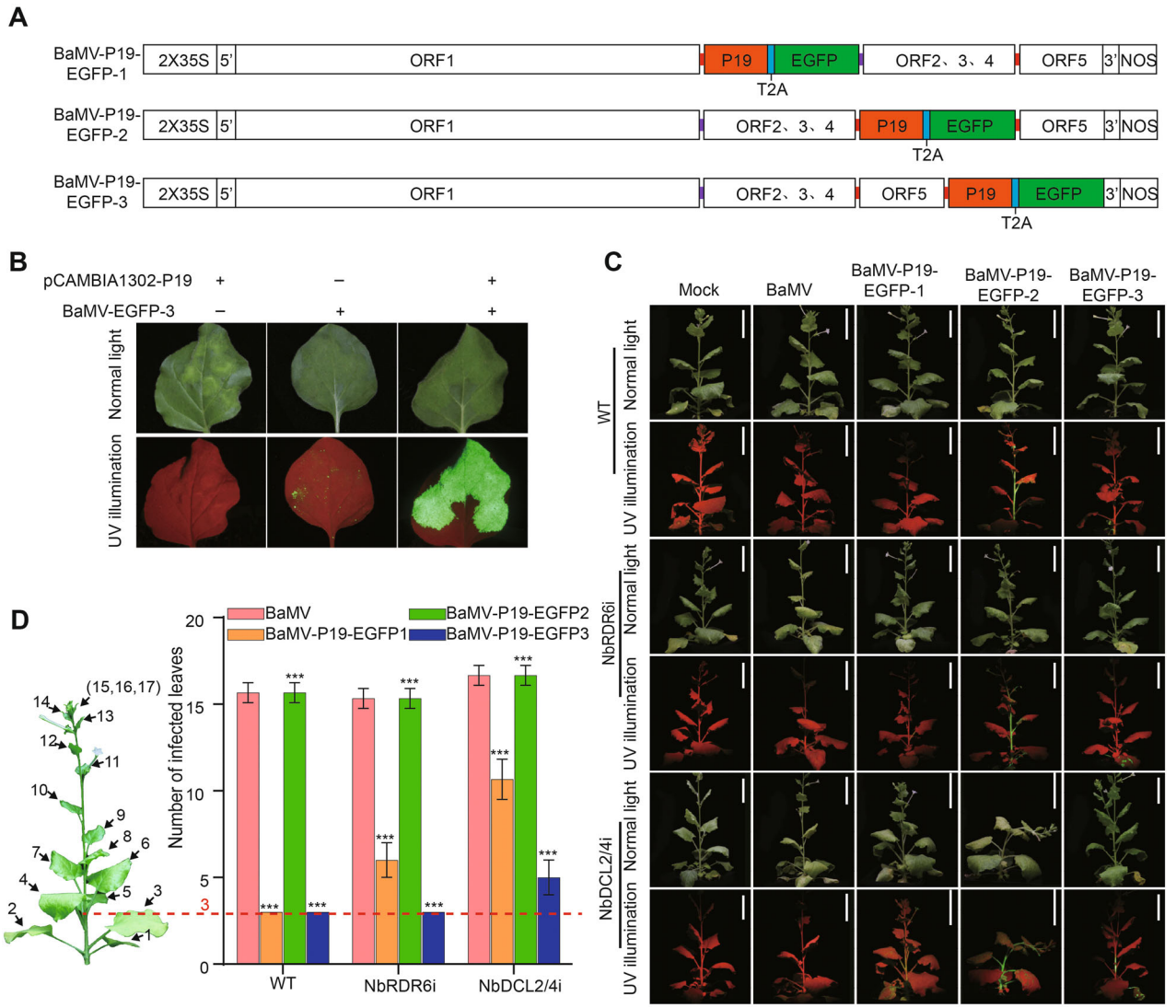

Figure 2. Effect of the virus silencing suppressor P19 on bamboo mosaic virus (BaMV)-driven expression and virus spread

(A) Schematic diagram of BaMV vectors harboring P19 at three different insertion sites. The sequence encoding P19 (516 bp) was cloned upstream of *EGFP* and separated by a sequence encoding the self-cleaving peptide T2A (63 bp). **(B)** Detection of EGFP fluorescent signal after co-infiltration of wild-type *N. benthamiana* leaves with both BaMV-EGFP-3 and pCAMBIA1302-P19. **(C)** Detection of EGFP fluorescent signal and propagation ability of BaMV or BaMV-derived clones shown in **(A)** harboring P19 in wild-type *N. benthamiana* or transgenic NbRDR6i, and NbDCL2/4i plants. Scale bars, 5 cm. **(D)** Transmissibility of BaMV or the BaMV-derived clones carrying P19 shown in **(A)** in wild-type *N. benthamiana* or transgenic NbRDR6i and NbDCL2/4i plants. Leaves below the dashed line were infiltrated with Agrobacterium cell suspensions. The y-axis represents the position of infected leaves 30 d after Agrobacterium infiltration. The experiment was performed three times. Asterisks indicate statistical significance (***, $P < 0.0001$) between BaMV and BaMV clones harboring *P19-EGFP* at different sites. Data are means ± standard error (*SE*).

BaMV-based vectors have high cargo capacity to express exogenous genes more than 4 kb in length in sympodial bamboo species.

We wished to test whether the BaMV-based vector would allow the expression of foreign genes and sustain virus transmission in bamboo as a host. To this end, we first infiltrated BaMV-P19-EGFP-2 into NbDCL2/4i leaves by Agrobacterium-mediated infiltration. Seven days later, we ground the infiltrated leaves into an infectious juice that we mechanically inoculated onto the leaves from 2-week-old Ma bamboo seedlings with emery. The inoculated seedlings grew new leaves that showed typical mosaic symptoms after 20 d of inoculation (Figure 4A). We detected EGFP fluorescence on leaves infected with BaMV-P19-EGFP-2 (Figure 4A). Importantly, we observed EGFP fluorescence in new tillering seedlings at 30 d after BaMV inoculation (Figure 4B), indicating that BaMV can be transmitted and spread to new tillering seedlings. We detected EGFP fluorescence even 5 months after the initial inoculation (Figure S4).

We also investigated the transmission and expression ability of BaMV clones carrying large genes in bamboo by

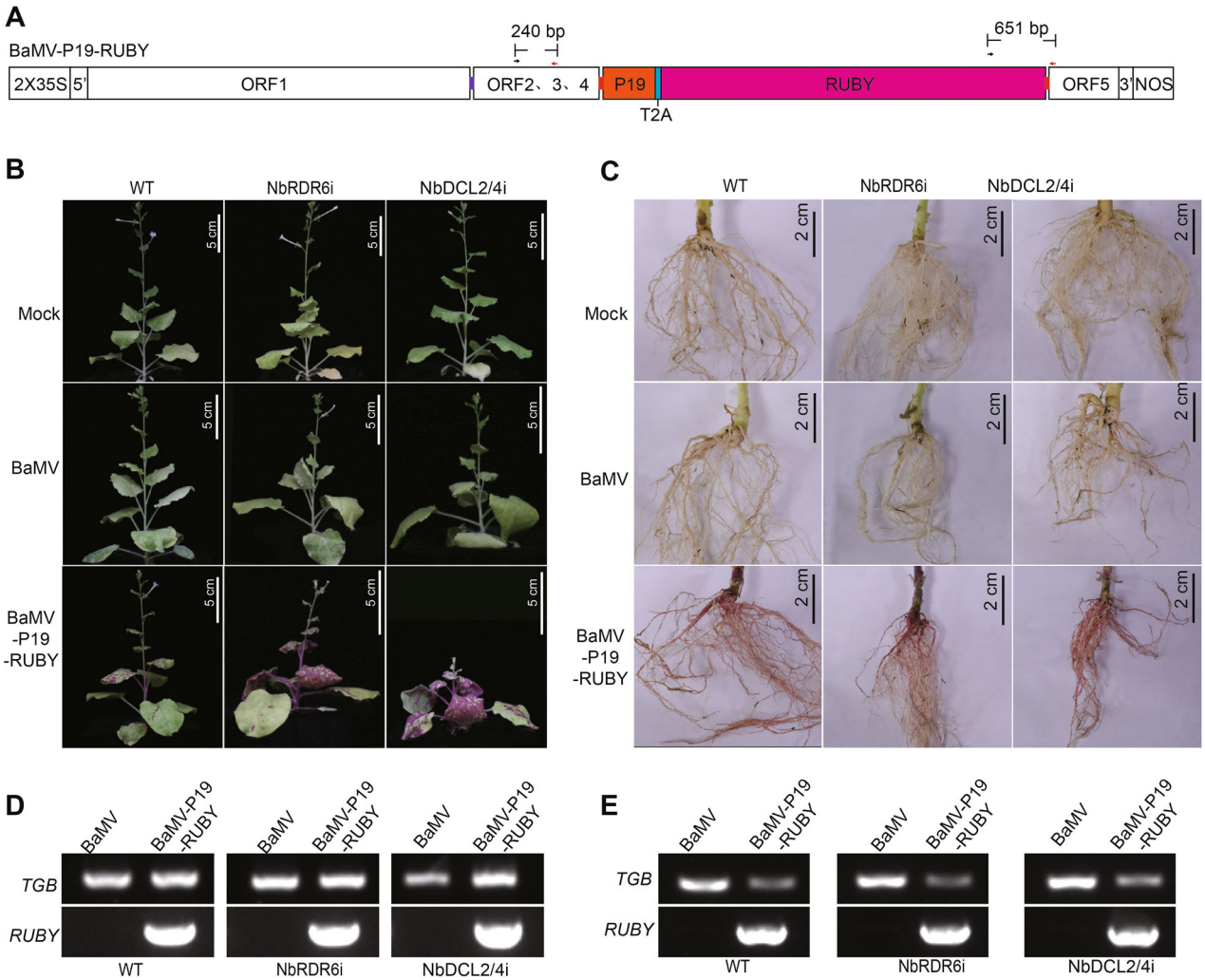

Figure 3. Expression of the large *RUBY* reporter system using bamboo mosaic virus (BaMV)-mediated expression in wild-type *N. benthamiana* and transgenic NbRDR6i and NbDCL2/4i plants

(A) Schematic diagram of the BaMV clone harboring the *RUBY* reporter (encoding three 2A-linked betalain biosynthesis enzymes). The primers used for RT-PCR validation of BaMV and *RUBY* expression are indicated above the diagram. **(B, C)** Betalain accumulation in stems and leaves **(B)** and roots **(C)** of wild-type *N. benthamiana* and transgenic NbRDR6i and NbDCL2/4i plants. **(D, E)** RT-PCR assessment of BaMV and *RUBY* expression in leaves **(D)** and roots **(E)** of wild-type *N. benthamiana* and transgenic NbRDR6i and NbDCL2/4i plants.

repeating the above procedure with NbDCL2/4i leaves infiltrated with Agrobacteria harboring BaMV-P19-RUBY. Seven days later, we ground the infiltrated leaves into an infectious juice to mechanically inoculate Ma bamboo leaves. After 45 d of growth, we detected the accumulation of betalain in noninoculated leaves (Figure 4C). In fact, almost the entire seedling derived from the new tiller was red (Figure 4C). The red pigment also accumulated in the roots of inoculated Ma bamboo seedlings (Figure 4D), indicating that the BaMV-based clone carrying the *RUBY* reporter can be transmitted and expressed in the underground tissues of Ma bamboo. We confirmed the expression of *GFP* and *RUBY* by RT-PCR and the accumulation of GFP in these seedlings by immunoblotting (Figure S5). These results suggest that the modified BaMV expression vector can carry long foreign genes, which can be expressed and spread in sympodial bamboo species.

BaMV-based vectors have high cargo capacity to express genes more than 4 kb in length in monopdial bamboo species.

Having validated the potential of our BaMV-based vector in one bamboo species, we turned to moso bamboo, which has great economic value and a wide planting area. We used the same method as above to inoculate moso bamboo, using infectious juice from *NbDCL2/4i* leaves infiltrated with BaMV-P19-EGFP-2 to mechanically inoculate the leaves of 2-week-old moso bamboo seedlings. As with Ma bamboo, we observed a mosaic leaf phenotype on new leaves at 20 d after inoculation, as well as EGFP fluorescence (Figure 5A). We also detected EGFP

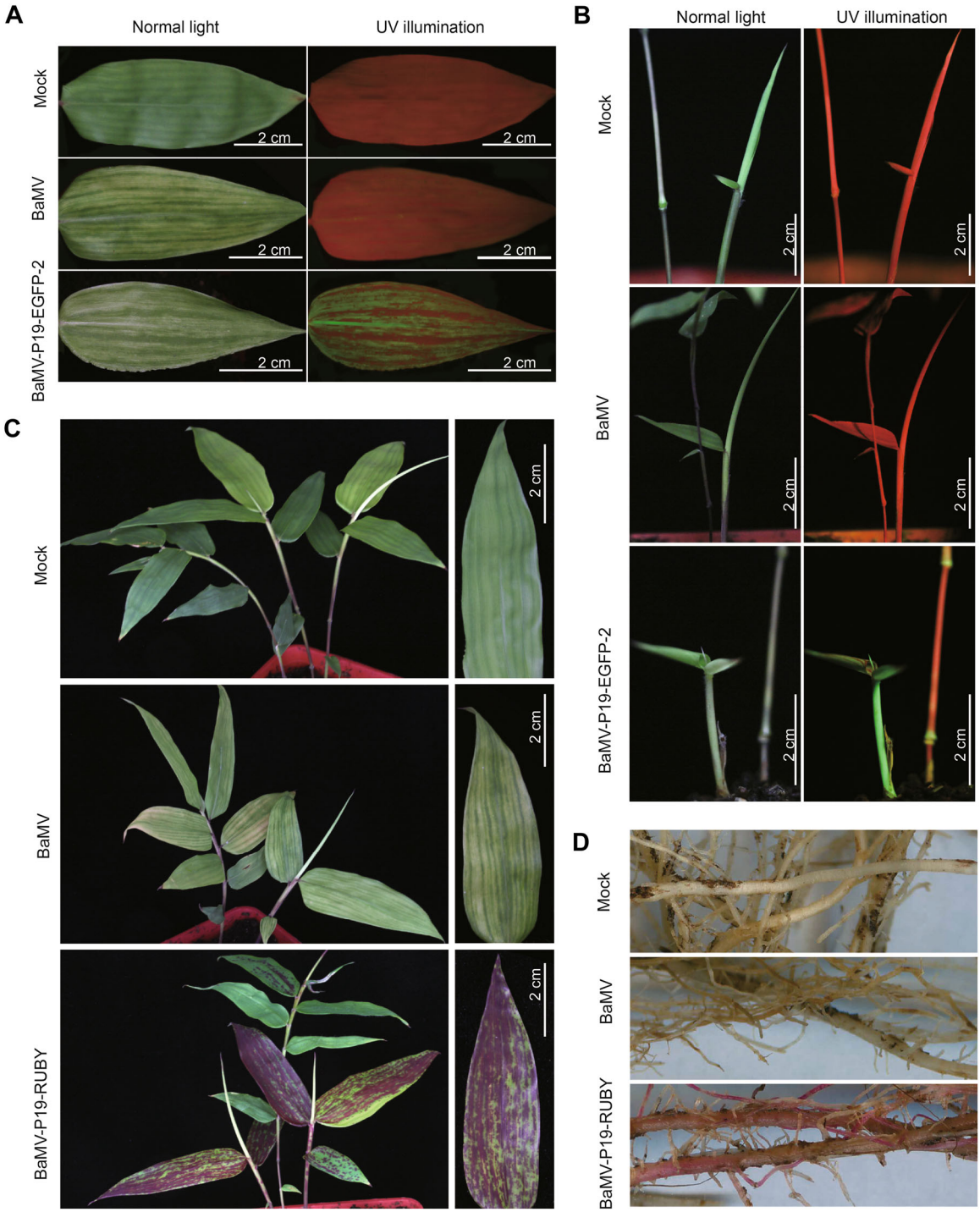

Figure 4. Expression of *EGFP* and the *RUBY* reporter system in Ma bamboo using bamboo mosaic virus (BaMV)-mediated expression
(A) Detection of EGFP fluorescence signal from Ma bamboo leaves inoculated with BaMV-P19-EGFP-2 using the BaMV clone as negative control. **(B)** Detection of EGFP fluorescence signal from Ma bamboo tillering seedlings inoculated with BaMV-P19-EGFP-2 using the BaMV clone as negative control. **(C)** BaMV-mediated expression of the *RUBY* reporter system from the BaMV-P19-RUBY clone in Ma bamboo leaves, as indicated by the accumulation of the red pigment betalain. The BaMV clone was used as negative control. Right panels show enlarged views of single leaves from the left panels. **(D)** Betalain accumulation in the roots of Ma bamboo plants inoculated with the BaMV clone (negative control) or the BaMV-P19-RUBY clone.

fluorescence in roots at 45 d after inoculation and in new tillering buds (Figure 5B). Moso bamboo seedlings inoculated with infectious juice from NbDCL2/4i leaves infiltrated with BaMV-P19-RUBY showed accumulation of the red pigment in roots (Figure 5C) and leaves (Figure 5D). We confirmed the expression of *GFP* and *RUBY* by RT-PCR and the accumulation of EGFP by immunoblotting (Figure S6). These results show that BaMV-mediated expression is effective in monopdial bamboo species.

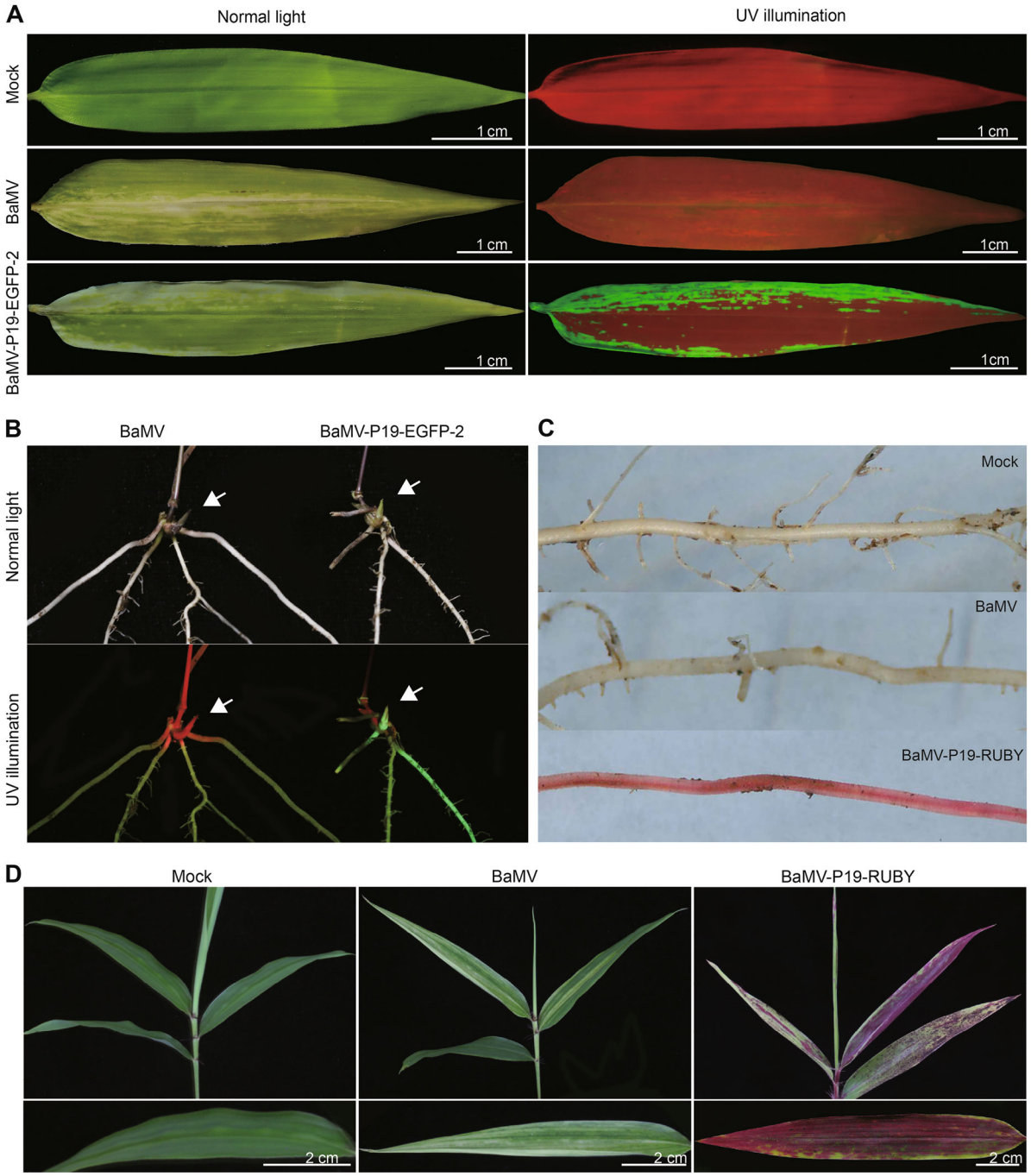

Figure 5. Expression of *EGFP* and the *RUBY* reporter system in moso bamboo using bamboo mosaic virus (BaMV)-mediated expression
(A) Detection of EGFP fluorescence signal in moso bamboo leaves inoculated with BaMV-P19-EGFP-2 using the BaMV clone as negative control. Scale bars, 1 cm. **(B)** Detection of EGFP fluorescence signal from the roots and new buds of moso bamboo plants inoculated with BaMV-P19-EGFP-2 using the BaMV clone as negative control. The arrows point to the new buds. **(C)** Accumulation of betalain resulting from the successful expression of the *RUBY* reporter in the roots of moso bamboo plants infiltrated with BaMV-P19-RUBY using the BaMV clone as negative control. **(D)** Expression of the *RUBY* reporter system in the leaves of moso bamboo plants inoculated with BaMV-P19-RUBY, as indicated by betalain accumulation; the BaMV clone served as negative control. Bottom panels show enlarged views of single leaves.

Overexpression of the bamboo genes *ACE1* and *DEC1* results in internode elongation–related phenotypes in bamboo upon GA treatment

The plant hormone gibberellic acid (GA) plays a key role in regulating the elongation of bamboo stems (Zhang et al., 2018). ACE1 and DEC1 cooperate with GA to regulate the elongation of stems in rice (*Oryza sativa*) (Nagai et al., 2020). We used the BaMV-mediated expression system to investigate the function of *ACE1* and *DEC1* in bamboo. Accordingly, we cloned the *DEC1* and *ACE1* coding sequences from

Ma bamboo based on the published genome annotation (Zheng et al., 2022) into BaMV-P19-2 (Figure 6A). We then enriched the virus by infiltrating the construct into the leaves of NbDCL2/4i via Agrobacterium-mediated infiltration, before grinding the leaves into infectious juice to mechanically inoculate the leaves of 2-week-old Ma bamboo seedlings. At 45 d after inoculation, we validated the expression of *ACE1* and *DEC1* in new tillering seedlings with mosaic symptoms in their leaves by RT-PCR (Figure S7). RT-qPCR analysis also determined that *DEC1* and *ACE1* are significantly overexpressed relative to the control group consisting of seedlings inoculated with BaMV-P19-EGFP-2 (Figure 6B). Moreover, immunoblotting indicated that DEC1 and ACE1 both accumulate (Figure 6C), demonstrating that the BaMV-mediated expression system can overexpress bamboo genes and lead to the accumulation of their encoded proteins. When the height of new tillering seedlings reached 7 cm, we sprayed them with 100 μM GA and recorded plant height every 3 d (Figure 6D). We observed clear phenotypic differences between seedlings inoculated with BaMV-P19-ACE1 and those inoculated with BaMV-P19-DEC1 after 15 d of GA treatment. In addition, Ma bamboo seedlings inoculated with BaMV-P19-EGFP-2 were shorter than BaMV-noninoculated seedlings (Figure 6E). After spraying the seedlings with GA, the seedlings overexpressing *ACE1* and *DEC1* were taller and shorter, respectively, than control bamboo seedlings (Figure 6E). These phenotypes suggest that ACE1 promotes internode elongation, while DEC1 inhibits internode elongation in concert with GA in Ma bamboo. In summary, we developed a BaMV-mediated expression system, which can be applied to infer genotype–phenotype associations for both foreign and bamboo genes in bamboo.

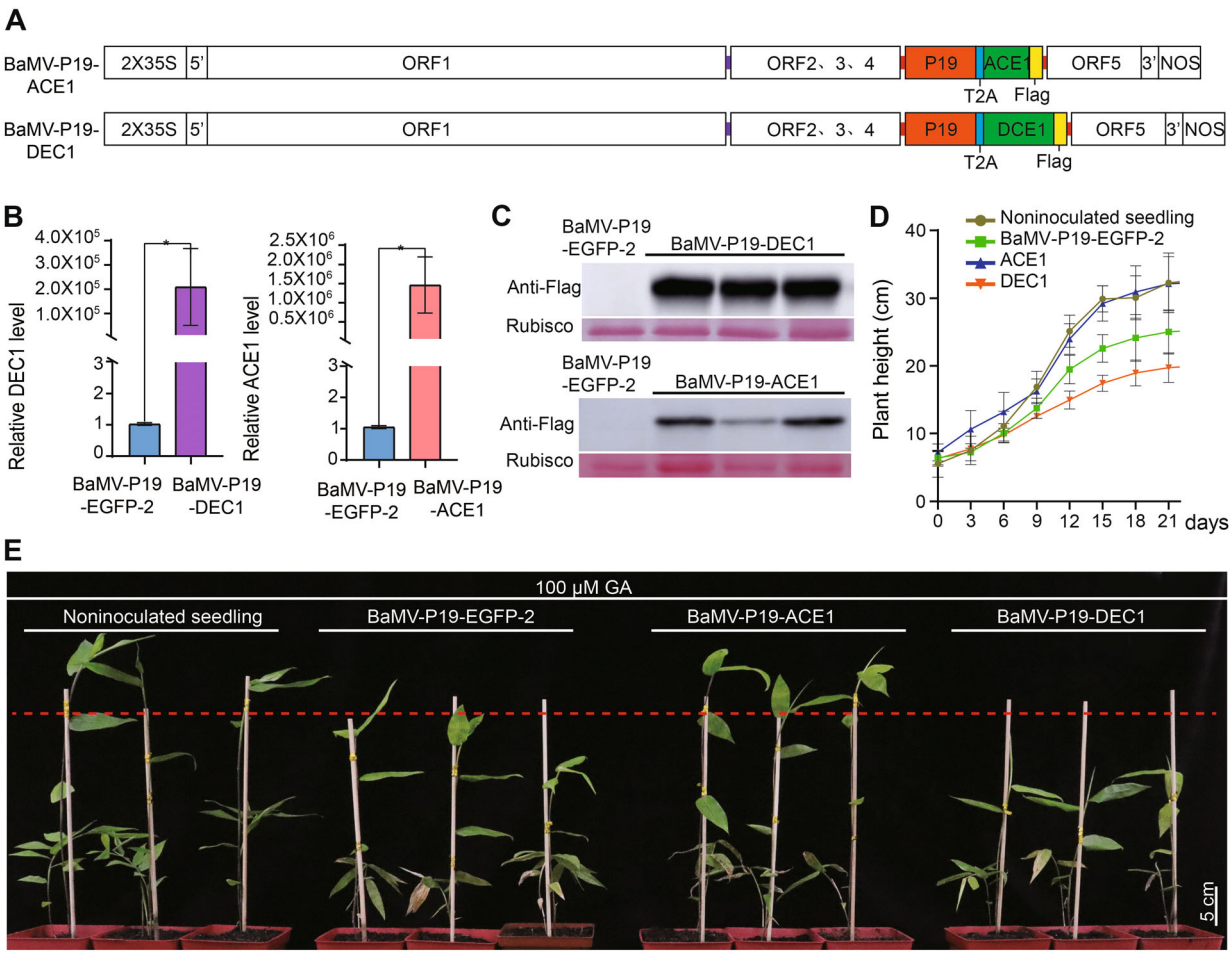

Figure 6. Overexpression of the bamboo genes *ACE1* and *DEC1* in Ma bamboo by bamboo mosaic virus (BaMV)-mediated expression
(A) Schematic diagram of the BaMV-based *ACE1* and *DEC1* expression clones. **(B)** Relative transcript levels of *DEC1* and *ACE1* in leaves of bamboo plants inoculated with BaMV-P19-DEC1 or BaMV-P19-ACE1, as determined by RT-qPCR; BaMV-P19-EGFP-2 served as control. Three replicates were performed, data are means ± *SE*. Asterisks indicate statistical significance (*, $P < 0.05$) between the controls and BaMV-P19-DEC1 or BaMV-P19-ACE1. **(C)** Immunoblot analysis of leaves collected from plants inoculated with BaMV-P19-DEC1 or BaMV-P19-ACE1 using leaves inoculated with BaMV-P19-EGFP-2 as negative control. **(D)** Progression of stem height as a function of time in seedlings treated with 100 μM GA and infiltrated with BaMV-P19-DEC1 or BaMV-P19-ACE1; noninoculated seedlings and seedlings inoculated with BaMV-P19-EGFP-2 served as controls. Seedling height was recorded every 3 d. Data are means ± *SE* with three replicates per genotype or construct. **(E)** Representative images showing stem elongation of bamboo seedlings inoculated with BaMV-P19-DEC1 or BaMV-P19-ACE1, or BaMV-P19-EGFP-2 as control; all seedlings were treated with 100 μM GA.

DISCUSSION

To date, a genetic transformation system has only been reported for two varieties of bamboo (Ye et al., 2017; Huang et al., 2022). Both studies suffered from low efficiency and long transformation periods, which constitute major obstacles for investigating gene function and phenotypes in bamboo. Expression vectors based on plant viruses have been extensively studied and exploited (Seo et al., 2016; Choi et al., 2019; Fang et al., 2021). However, there is currently no viral vector available for the functional analysis of bamboo genes. In this study, we isolated an infectious clone for BaMV from Ma bamboo plants showing typical mosaic symptoms on their leaves. We then developed BaMV-based vectors for the expression of foreign genes using EGFP as a marker. We established that inserting a gene closer to the 3′ end of BaMV between ORF4 and ORF5 had the least influence on the transmission ability of BaMV and was associated with high expression of the inserted gene. In this study, we tested the insertion sites by expressing a limited number of genes, namely, *GFP*, *RUBY*, *ACE1*, and *DEC1*, and did not rule out the possibility that other genes might have different effects at the different insertion sites. Intact BaMV without an inserted gene was highly transmissible in WT, NbRDR6i, and NbDCL2/4i *N. benthamiana* plants. However, the insertion of a foreign gene in the BaMV clone affected transmission ability, although infiltration of these BaMV-based constructs in NbDCL2/4i *N. benthamiana* plants showed greater transmission than in WT *N. benthamiana* plants, suggesting that the ability of BaMV to resist host antiviral mechanisms is affected by the insertion of genes. The underlying mechanism by which different insertion sites affect the transmission ability of bamboo mosaic virus in the host is currently unknown.

Previous studies showed that silencing suppressors can be inserted into virus genomes to enhance the transmission and gene expression of the virus (Fang et al., 2021; Zhou et al., 2021). We established here that inserting the coding sequence for the P19 suppressor between ORF4 and ORF5 greatly improved the transmissibility of BaMV-based vectors. Indeed, the resulting vector successfully expressed *EGFP* and the *RUBY* reporter in both monopodial and sympodial bamboo species. Importantly, these BaMV-based vectors carrying a foreign gene were effectively transmitted from inoculated leaves to roots and to newly tillered seedlings. This study represents the first example of a BaMV-based vector applied for the expression of foreign genes in bamboo, which should pave the way for the investigation of genotype–phenotype associations in bamboo due to the short period for BaMV-mediated expression.

One major limitation to using virus-based vectors is their restrictions on cargo capacity, which limits the size of the inserted fragment. For example, BPMV and CMV can accommodate inserted fragments of only up to 0.9 and 1.8 kb, respectively (Zhang and Ghabrial, 2006). In this study, we demonstrated that BaMV can accommodate the expression of the large *RUBY* reporter system, suggesting that BaMV-based vectors could allow the expression of *Cas9* to generate transgene-free genome-edited bamboo plants for molecular breeding studies. As a prerequisite, we confirmed that our BaMV-based vector can support the expression of *Cas9* and the production of the Cas9 protein (Figure S8). We also expressed *Cas9* using the BaMV-based vector to assess its potential use for genome editing in bamboo (Figure S8A). We confirmed the expression of the *Cas9* gene by RT-PCR (Figure S8B) 20 d after infiltration with *N. benthamiana* by Agrobacterium and the accumulation of the Cas9 protein by immunoblot analysis (Figure S8C), indicating that this BaMV vector has potential for gene editing.

The production of animal vaccines in plants can greatly improve the safety of vaccines (Fausther-Bovendo and Kobinger, 2021). Plant virus vectors have been adapted to produce the spike protein from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and prepare a vaccine against COVID-19 (Hager et al., 2022). As early as 2007, BaMV was used for the production of chimeric virus particles in plants by replacing the N-terminal 35 amino acids of the BaMV CP protein with 37 amino acids from VP1 of FMDV (Yang et al., 2007). Similarly, the same 35 amino acids of BaMV CP were replaced with 18 amino acids from VP2 of very virulent infectious bursal disease virus (vvIBDV) to generate an antigen that is displayed on the modified BaMV coat protein (Chen et al., 2012). In this study, the expression of the gene inserted after *ORF5* of BaMV was much higher than that of other insertion sites tested. Importantly, this BaMV-based clone only had high transmission ability in NbDCL2/4i plants, which should reduce the possibility of its transmission in the natural environment and ensure safe use.

In addition to the expression of foreign genes, we also individually overexpressed the two bamboo genes *ACE1* and *DEC1* using the BaMV vector described in this study. In rice, ACE1, DEC1, and GA regulate stem elongation (Nagai et al., 2020). In this study, *ACE1* facilitated the effect of GA on internode elongation in bamboo, while *DEC1* had the opposite effect. We conclude that the BaMV-based vectors described here can be used to overexpress bamboo genes to investigate the relationship between genotype–phenotype associations in bamboo.

BaMV has been reported to infect a broad spectrum of hosts, which provides promising application prospects for BaMV-mediated expression systems. Indeed, BaMV can infect the dicotyledonous model plant *Arabidopsis thaliana* (Alazem et al., 2014; Alazem et al., 2017), quinoa (*Chenopodium quinoa*) (Chen et al., 2012), and *N. benthamiana* (Seo et al., 2016; Choi et al., 2019; Fang et al., 2021). BaMV can also cause mosaic symptoms in the monocot model plant *B. distachyon* (Lin et al., 2022). At least 10 commercially valuable bamboos have been reported as hosts of BaMV (Lin et al., 1993; Chang et al., 2017). Bamboo belongs to the grass family Poaceae, which includes rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), and other cereals (Gui et al., 2010). We tested the applicability of the BaMV-P19-EGFP-2 clone by mechanically inoculating *Arabidopsis* and rice

leaves. Fourteen days after infiltration with BaMV, we observed EGFP fluorescence in *Arabidopsis* (Figure S9A) and rice (Figure S9B). RT-PCR and immunoblotting validated the expression of *GFP* and the accumulation of the encoded protein in *Arabidopsis* (Figure S9C) and rice (Figure S9D). This result demonstrates that the BaMV system described here should be compatible with other potential hosts in the Poaceae, such as rice, as well as the dicots *N. benthamiana* and *Arabidopsis*, which will further widen the application of the BaMV-mediated expression system.

In summary, this study developed an efficient virus vector tool that can express genes of interest and spread in both monopodial and sympodial bamboo species, which will substantially shorten the time needed for investigations of genotype–phenotype associations in this species. Combining this BaMV-mediated expression system with VIGE technology should greatly promote bamboo molecular breeding in the future.

MATERIALS AND METHODS

Full-length BaMV genomic clones and construction of plasmid vectors

The full-length BaMV genome was amplified from the leaves of infected Ma bamboo (*Dendrocalamus latiflorus* Munro) plants showing mosaic symptoms from the botanical bamboo garden of Fujian Agriculture and Forestry University. The sequence was deposited in GenBank under accession number OP493850. BaMV driven by a 2×35S promoter was cloned into the binary vector pCAMBIA1302 that had been linearized by digestion with XhoI and SpeI using a seamless cloning strategy.

To insert various genes at three different positions in the BaMV genome, the ORF5 subgenomic promoter and an XbaI restriction site were added after the stop codon of ORF1, ORF4, or ORF5. The *EGFP* coding sequence was then cloned at each of the three above sites separately, yielding the virus clones BaMV-EGFP-1, BaMV-EGFP-2, and BaMV-EGFP-3, respectively. A BaMV vector was also constructed harboring the P19 coding sequence from tomato bushy stunt virus (TBSV) to improve BaMV infectivity. Accordingly, the P19 stop codon was removed and cloned in-frame and upstream of a sequence encoding the T2A self-cleaving peptide and a SacI restriction site, before cloning *EGFP* at each of the above sites separately. The resulting clones were named BaMV-P19-EGFP-1, BaMV-P19-EGFP-2, and BaMV-P19-EGFP-3. The *ACE1* and *DEC1* sequences were obtained from functional annotation of the Ma bamboo genome (Zheng et al., 2022). The coding sequences for the two genes were individually cloned from Ma bamboo leaves and ligated into the SacI-digested vector using seamless cloning to generate BaMV-P19-ACE1-2 and BaMV-P19-DEC1-2.

N. benthamiana and bamboo growth conditions

Seeds from wild-type *N. benthamiana* and the transgenic lines NbRDR6-DNAi (NbRDR6i) and NbDCL2/4-RNAi (NbDCL2/4i) were sown on half-strength Murashige and Skoog medium at 28°C under a 16-h-light/8-h-dark photoperiod in a light incubator. Seedlings with three leaves were transferred to nutrient soil for culture in a greenhouse with 60% humidity under a 14-h-light/10-h-dark photoperiod.

The dehulled seeds of Ma bamboo and moso bamboo were soaked in water for 24 h and then evenly sown on the nutrient soil. The seeds were then covered with a 1-cm layer of nutrient soil and placed in a greenhouse at 25°C under a 16-h-light/8-h-dark photoperiod.

Generation of BaMV-inoculated *N. benthamiana* and bamboo

The plasmid harboring the BaMV genomic DNA was transferred into Agrobacterium (*Agrobacterium tumefaciens*) strain GV3101 by the freeze-thaw method. Single colonies were selected on YEB medium containing 50 mg/L kanamycin and 25 mg/L rifampicin, before being used to inoculate liquid YEB medium with the same antibiotics. Agrobacteria were cultured in a shaker at 28°C with shaking of 200 r/min until they reached an optical density at 600 nm of 0.5 (OD_{600}). The cells were briefly pelleted by centrifugation and washed once with infiltration buffer (10 mM MES, 10 mM $MgCl_2$, 100 μM acetosyringone, pH 5.6). Finally, Agrobacteria were resuspended in infiltration buffer and incubated at 25°C for 2 h in the dark before infiltration into *N. benthamiana* leaves. Seven days after Agrobacterium infiltration, infiltrated leaves from NbDCL2/4i were collected and macerated in 1× phosphate buffered saline (PBS) and emery to fully grind them. An appropriate amount of the resulting juice was used to mechanically inoculate bamboo leaves. After 2 d of moisturizing culture, the seedlings with BaMV-infected leaves were returned to normal growth conditions at 25°C.

RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

The indicated samples were ground to powder after quick freezing in liquid nitrogen; 1 mL of TransZol reagent was added to every 100 mg of powder. Total RNA extraction was performed according to the manufacturer's instructions (ThermoFisher Scientific, catalog number 15596026). The extracted total RNA was reverse transcribed to synthesize first-strand cDNA after removal of genomic DNA according to the protocol of the Revertaid RT kit (ThermoFisher Scientific, catalog number K1691). Oligo $d(T)_{18}$ was used as the reverse transcription primer for first-strand cDNA synthesis with a reverse transcription kit (Yeasen Biotechnology, catalog number 11141ES60). The changes in transcript levels were detected by qPCR based on the above synthesized cDNA using SYBR Green Master Mix (Yeasen Biotechnology, catalog number 11201ES03). All primers used in this study are listed in Table S1.

Protein extraction and immunoblotting

Samples from BaMV-infected bamboo and *N. benthamiana* plants were quickly frozen in liquid nitrogen and ground into

powder. An equal volume of protein loading buffer (25 mM Tris-HCl pH 6.8, 0.5 M DTT, 10% [w/v] SDS, 0.5% [w/v] bromophenol blue, and 50% [v/v] glycerol) was then added to each sample and thoroughly mixed before heating at 100°C for 10 min. The samples were centrifuged at 12,000 r/min for 10 min at room temperature; the supernatant was then separated by SDS-PAGE. The separated proteins were transferred to nitrocellulose (NC) membrane at 100 V for 90 min in transfer buffer (25 mM Tris base, 192 mM glycine, 3.5 mM SDS, and 20% [v/v] methanol). The NC membrane was washed once with Tris buffered saline (TBS, 20 mM Tris base, 150 mM NaCl, pH 7.5). The membrane was blocked in TBST (TBST: TBS and 0.05% [v/v] Tween 20) containing 5% (w/v) skimmed milk powder at room temperature for 1 h on a shaker set to 50 r/min. The blocked NC membrane was washed once with TBS before incubation in TBST containing an anti-mouse monoclonal antibody (Transgen Biotech, ProtcinFind Anti-DYKDDDDK mouse Monoclonal Antibody, catalog number HS201-01; ProtcinFind Anti-GFP Monoclonal Antibody, catalog number HT801-01) for 1 h at room temperature. The NC membrane was then washed three times with TBST for 10 min each time, followed by incubation in TBST containing a secondary antibody (goat anti-mouse IgG horseradish peroxidase [HRP] conjugate, TransGen Biotech, catalog number HS101-01) for 1 h at room temperature. The membrane was washed three times in TBST for 10 min each time and then washed once with TBS. Finally, the membrane was incubated in chemiluminescence substrate for 1 min in the dark, before being placed in a chemiluminescence instrument (Amersham Imager 600, General Electric Company) for observation.

Data availability statement

The BaMV sequence in this study has been deposited to GenBank under the accession number OP493850.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

L. G. conceived and designed the research. Y. J., B. W., M. B., and Y. L. conducted the experiments and analyzed the data with the help of S. X., Y. W., J. Z., L. Z., and H. Z. M. L. provided support in methodology. Y. J., Y. H., and L. G. wrote the article. All authors read and approved this manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.13468/suppinfo

Figure S1. EGFP fluorescence signal from *N. benthamiana* plants infiltrated with BaMV-EGFP-1, BaMV-EGFP-2, and BaMV-EGFP-3.

Figure S2. Co-infiltration of EGFP with P19 enhances EGFP expression.

Figure S3. EGFP fluorescence signal from *N. benthamiana* plants infiltrated with BaMV-EGFP-1, BaMV-EGFP-2, or BaMV-EGFP-3 harboring P19.

Figure S4. Detection of EGFP fluorescence signal from Ma bamboo plants 5 months after inoculation with BaMV-P19-EGFP-2 or BaMV as negative control

Figure S5. Validation of Ma bamboo phenotypes using RT-PCR and immunoblotting

Figure S6. Validation of moso bamboo phenotypes using RT-PCR and immunoblotting

Figure S7. Validation of DEC1 and ACE1 expression in Ma bamboo plants inoculated with BaMV-P19-DEC1 or BaMV-P19-ACE1.

Figure S8. BaMV-based vectors can express the nuclease Cas9

Figure S9. BaMV-mediated expression in model plant species

Table S1. Primer sequences for cloning the full-length BaMV DNA genomic clone, vector construction, RT-qPCR of *DEC1/ACE1*, and the BaMV full sequence