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Establishment of sugarcane mosaic virus-based vector for dual gene expression in maize

Huangai Li[1,3], Ke Xie[2], Xiyin Zheng[1], Meng Han[1], Asigul Ismayil[1], Na Liu[1], Xiang Wei[1], Rong Zhong[1], Yan Wang[1*] and Yule Liu[1*]

Abstract

Virus-based gene expression is a simple and powerful approach for functional genetic studies in plants. Here, sugarcane mosaic virus (SCMV, *Potyvirus sacchari*) was engineered as a dual gene expression vector for simultaneous expression of two heterologous proteins in maize plants. Inoculation of the full-length cDNA clone of SCMV from agro-infiltrated *Nicotiana benthamiana* resulted in a rapid systemic infection in maize. To assess the possibility of SCMV as the gene expression vector, the marker gene *GFP* or *GUS* was inserted into either the NIb/CP or P1/HC-Pro junction site of SCMV to produce single-gene expression vectors. The results showed that these engineered SCMV vectors permitted efficient gene expression in systemically infected leaves and had the genetic capacity of inserts of more than 1800 bp, suggesting that both junction sites are suitable for heterologous gene insertion and expression. Furthermore, two different genes *GFP* and *mCherry* could be expressed simultaneously by engineering them into either NIb/CP or P1/HC-Pro junction sites of the same vector. These results clearly demonstrate the suitability of SCMV as a transient dual gene expression vector for maize plants.

Keywords Sugarcane mosaic virus (SCMV), Maize, Virus-based gene expression vectors, Dual gene expression, Mechanical inoculation

Background

The developments of high-throughput sequencing technologies and bioinformatics have generated massive genomic and transcriptomic resources involved in plant growth, development, and host defense against biotic and abiotic stresses. A common strategy for characterizing the function of these candidates is to test the targets with altered expression levels (Gleba et al. 2007; Abrahamian et al. 2020). Plant virus-based vectors provide handy tools to overexpress the genes of interest in plants. The benefits of virus-based expression systems include rapid multiplication and high yields of foreign gene expression (Scholthof et al. 1996). In addition, foreign gene expression could be achieved in the whole plant tissues accompanied by systemic co-infections of several viruses. Within the past decades, numerous plant viruses, especially RNA-based viruses, have been exploited for transient gene expression systems (Scholthof et al. 1996; Porta and Lomonossoff 2002; Gleba et al. 2007; Lico et al. 2008; Hefferon 2014; Cody and Scholthof 2019; Abrahamian et al. 2020).

*Correspondence:
Yan Wang
wangyansdts@163.com
Yule Liu
yuleliu@mail.tsinghua.edu.cn
[1] Center for Plant Biology, Tsinghua-Peking Joint Center for Life Sciences, MOE Key Laboratory of Bioinformatics, School of Life Sciences, Tsinghua University, Beijing 100084, China
[2] Guangxi Vocational University of Agriculture, Nanning 530007, China
[3] Present Address: Research Institute of Biology and Agriculture, University of Science and Technology Beijing, Beijing 100083, China

Introducing the virus into the plant cells is one of the most crucial issues for a plant RNA virus vector to express foreign genes, which can be achieved essentially via three routes. Traditionally, the entire virus sequence was transferred into a plasmid under the control of a bacteriophage promoter which permitted the generation of full-length RNA from it via in vitro transcription. The generated RNA transcripts would become infectious when inoculated into protoplasts or tissues of whole plants (Ahlquist et al. 1984; Meshi et al. 1986; Vos et al. 1988; Domier et al. 1989; Petty et al. 1989; Hemenway et al. 1990; Dolja et al. 1992). Biolistic bombardment is a powerful method that transfers virus vector-coated metal particles into intact plant tissues and cells. Viral RNA could then be transcribed, and replicated in bombarded cells or even spread to other tissues. This approach is highly efficient and applicable to many plant species, particularly monocot plants (Gal-On et al. 1995; López-Moya and García 2000; Zhang et al. 2009; Mei et al. 2016, 2019). Agro-inoculation is another way to deliver plant viruses into their target plants via *Agrobacterium tumefaciens* harboring a binary vector. Compared with the former two methods, agro-inoculation has a significant advantage in that it provides the efficient delivery of viruses at a relatively low cost without requiring sophisticated equipment (Turpen et al. 1993; Marillonnet et al. 2004; Wang et al. 2015; Feng et al. 2020).

The genus *Potyvirus*, belonging to the family *Potyviridae*, is the largest group of RNA plant viruses, with an extensive host range of plant species encompassing both dicots and monocots. Potyvirus virions are characterized by flexuous, filamentous particles, 680 ~ 900 nm long and 11 ~ 13 nm wide, encapsidating a linear single-stranded positive RNA genome ranging from 9.0 to 11.0 kb in length. The genome of a potyvirus contains a VPg covalently attached to the 5'-terminus and a poly(A) tail at the 3' terminus. Potyviral RNA is translated to produce a large polyprotein which is proteolytically processed by three viral proteinases P1, HC-Pro and NIa-Pro, resulting in ten mature proteins. P1 and HC-Pro operate only *in cis* to cleave themselves off at their C termini, whereas NIa-Pro cuts the remaining polyprotein *in cis* and *trans* (King et al. 2012). An additional small open reading frame, '*pipo*', embedded within the P3 cistron, is translated as a trans-frame fusion with the N terminal part of P3 via ribosomal frameshifting at the conserved motif of $G_{1-2}A_{6-7}$ (Chung et al. 2008). The characteristics of potyviruses, such as broad host range, filament-shaped virions and multiple self-cleaved sites, enable them to be a favorable choice for transient gene expression in plants.

The first published virus to be developed as expression vectors in maize was the maize streak virus (MSV, *Mastrevirus storeyi*) with a DNA genome. The resulting vector allowed functional reporter gene expression in agro-infiltrated leaves but failed to spread systemically. It was rendered movement defective probably because of the size packaging constraints of viral DNAs (Shen and Hohn 1994). Later, this problem was settled by using a positive-strand RNA virus, wheat streak mosaic virus (WSMV, *Tritimovirus tritici*) in the family *Potyviridae*, which shares a similar genome organization with potyviruses. Maize seedlings inoculated with transcripts of WSMV bearing an NPTII gene expressed detectable NPTII proteins in systemically infected leaves (Choi et al. 2000). However, this method is costly and inefficient in maize. More recently, more virus-based expression vectors have been developed and utilized in maize with their advantages. Foxtail mosaic virus (FoMV, *Potexvirus setariae*)-based expression system allowed heterologous gene expression in monocots. It employed an extra step of virus propagation via agro-infiltration of *Nicotiana benthamiana* with *Agrobacterium tumefaciens* containing FoMV vector, which subsequently enabled efficient virus infection and protein production in monocots (Bouton et al. 2018). Barley yellow striate mosaic virus (BYSMV, *Cytorhabdovirus hordei*) is reported to successfully express foreign proteins in maize and several other cereal crops (Gao et al. 2019). However, the requirement of planthoppers as a transmission vector for BYSMV may limit its use in many laboratories. Two potyviruses, sugarcane mosaic virus (SCMV, *Potyvirus sacchari*) and maize dwarf mosaic virus (MDMV, *Potyvirus zeananus*), and one negative-strand RNA virus maize mosaic virus (MMV, *Alphanucleorhabdovirus maydis*) have also been exploited as heterologous gene expression vectors in maize, but their applications are still restricted by the inoculation method or low viral infection efficiency (Mei et al. 2019; Beernink et al. 2021; Xie et al. 2021; Kanakala et al. 2022).

SCMV infects numerous species restricted to the family *Gramineae*, including several important crops such as maize, sugarcane, and sorghum (King et al. 2012; Tennant et al. 2018), causing significant agricultural losses. It is naturally transmitted by aphids, such as *Rhopalosiphum maidis*, *Aphis gossypii*, *Longiunguis sacchari*, and *Myzus persicae*, in a non-persistent manner (Singh et al. 2005), as well as mechanically through plant sap and via infected planting materials, such as cuttings or seeds. SCMV reduces crop yields by impairing photosynthesis and disrupting sugar accumulations, leading to losses of up to 80% in maize (Redinbaugh and Zambrano 2014; Akbar et al. 2021a; b). The pathogenicity of SCMV varies among strains, with symptoms including mosaic or mottled patterns on leaves, chlorosis, stunting, and reduced vigor in affected plants (Wu et al. 2012; Bevers et al. 2024).

In this study, we constructed another infectious cDNA clone of SCMV and developed it for heterologous gene expression in maize. Reporter genes were successfully expressed in SCMV-infected maize leaves when they acted as a single insert situated in either NIb/CP or P1/HC-Pro site. Based on this, we further engineered SCMV as a dual-expression vector for simultaneous expression of two proteins from both sites. This SCMV-based expression vector will be helpful in heterologous protein production and gene function analysis in maize.

Results

Construction of full-length infectious cDNA clone of SCMV

In order to construct a cDNA clone, the full-length genome sequence of SCMV isolated from maize was engineered into a binary vector pCB301 to produce pSCMV, in which the complete SCMV sequence together with 60 residues of adenine fused to its 3' end and was flanked by the CaMV 35S promoter and the Nos terminator (Fig. 1a). The *Agrobacterium tumefaciens* cells transformed with pSCMV were co-infiltrated into the expanded leaves of NahG over-expressed *N. benthamiana,* with the agrobacterium strain expressing the RNA silencing suppressor p19 of tomato bushy stunt virus

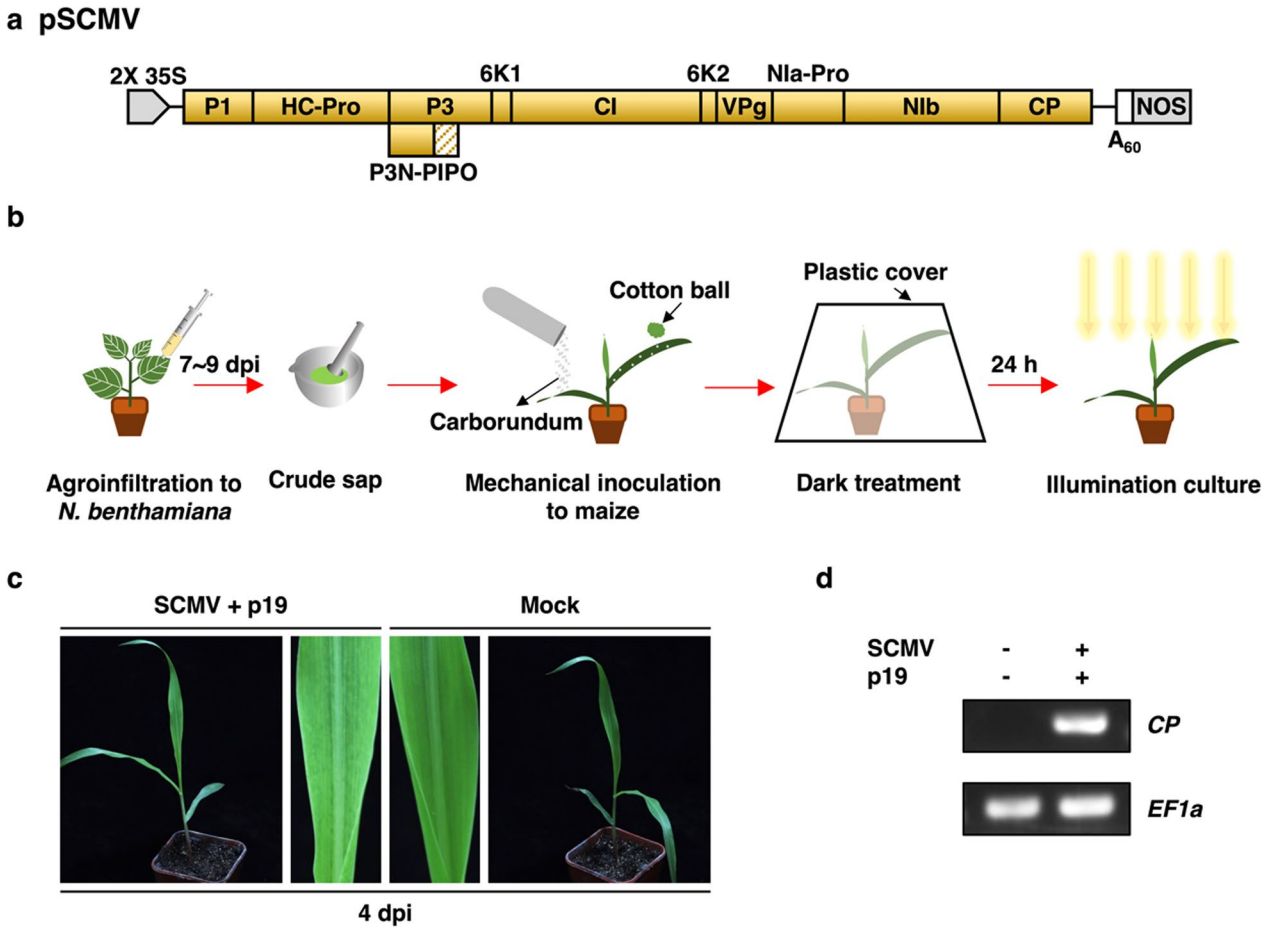

Fig. 1 Infectivity of the infectious cDNA clone of SCMV in maize plants. **a** Schematic representation of the infectious clone of SCMV. The full-length cDNA of SCMV was engineered into a binary vector pCB301 between the double CaMV 35S Promoter (2 × 35S) and the Nos Terminator (NOS). SCMV viral genome contains a long open reading frame (ORF) coding for a single major polyprotein that is proteolytically processed by three viral-encoded proteinases to form ten mature proteins: P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, Nla-Pro, Nib, and CP. The small box below the large ORF indicates the *pipo* ORF, the product of which is expressed as a fusion with the N-terminal of P3. A_{60} indicates the poly(A) tail. **b** Illustration of the procedure for virus inoculation of maize plants with SCMV-based vectors. **c** Viral infection symptoms in upper systemically infected leaves of maize plants inoculated with leaf sap from pSCMV-infiltrated *N. benthamiana*. At 4 dpi, SCMV induced yellowish spots or streaks in the young leaves, whereas mock-inoculated leaves showed no symptoms. **d** Detection of SCMV RNA in the systemically infected leaves of maize. Total RNA was extracted from upper uninoculated leaves and subjected to RT-PCR analysis. Levels of EF1a transcripts in these tissues were used as an internal control

(TBSV, *Tombusvirus lycopersici*). No visible viral symptoms were observed in *N. benthamiana* plants, however, the SCMV RNA transcript was detected in infiltrated leaves at 2-, 5- and 7-days post-infiltration (dpi) by RT-PCR analysis (Additional file 1: Figure S1). To test virus infectivity in maize, crude saps from pSCMV-infiltrated *N. benthamiana* leaves at 7 dpi were applied to mechanical inoculation of maize inbred line B73 (Fig. 1b). Maize seedlings inoculated with leaf saps of pSCMV-infiltrated *N. benthamiana* developed typical symptoms of yellowish spots or streaks in the first systemic leaf as early as 4 dpi, whereas the mock-inoculated plants did not (Fig. 1c). All of inoculated plants (n = 16) developed viral symptoms within 4 to 7 dpi, indicating a 100% infection efficiency of this infectious cDNA clone in maize (Table 1). RT-PCR analysis revealed the presence of SCMV RNA in upper uninoculated leaves (Fig. 1d), indicating that the cDNA clone of SCMV was systemically infectious in maize. Thus, agro-infiltrated *N. benthamiana*, a non-host for SCMV, can serve as a good inoculum source of SCMV for mechanical inoculation to maize.

The pSCMV vector infects multiple inbred maize lines

To evaluate SCMV infectivity in maize inbred lines, 17 maize inbred lines including B73 were mechanically inoculated with SCMV-containing saps from *N. benthamiana*. All the inoculated plants were monitored over eight weeks for symptom development and RT-PCR analysis of viral RNA. SCMV was infectious to all tested inbred lines and caused similar symptoms at an early stage of 4 ~ 7 dpi (Table 1). According to the infection efficiency, these lines could be divided into three groups: (i) Thirteen inbred lines had a high infection efficiency over 80% including B73 (100%), Mo17 (87%), Qi319 (93%), NH60 (94%), Xin03 (81%), Jing2416 (100%), Ph6WC (88%), D1798Z (94%), Zong31 (94%), B104 (100%), H99 (93%), and Zheng58 (81%); (ii) Four inbred lines exhibited a middle infection efficiency range from 50%–80% including Hongzhouchang7 (64%), Jing724 (78%), G1783 (71%), and M54 (69%); (iii) Only one line, Huangzao4, showed a low efficiency approximately 29% (Table 1).

Table 1 Infection efficiency of SCMV in 17 maize inbred lines

Maize inbred lines	Symptom appearance	Infection efficiency[a]	
		Ratio (%)	Details
B73	4 dpi	100	16/16 (5/5, 5/5, 6/6)
Mo17	5 dpi	87	13/15 (1/2, 8/8, 4/5)
Huangzao4	5 dpi	29	4/14 (2/4, 1/4, 1/6)
Qi319	4 dpi	93	13/14 (4/4, 6/6, 3/4)
Hongzhouchang7	5 dpi	64	9/14 (1/1, 5/9, 3/4)
NH60	4 dpi	94	16/17 (5/5, 5/6, 6/6)
Xin03	5 dpi	81	13/16 (3/5, 4/5, 6/6)
Jing2416	5 dpi	100	15/15 (4/4, 5/5, 6/6)
Jing724	5 dpi	78	7/9 (2/3, 2/3, 3/3)
G1783	7 dpi	71	12/17 (4/5, 3/6, 5/6)
Ph6WC	7 dpi	88	14/16 (4/6, 6/6, 4/4)
M54	5 dpi	69	11/16 (2/2, 5/9, 4/5)
D1798Z	6 dpi	94	16/17 (6/6, 4/5, 6/6)
Zong31	4 dpi	94	15/16 (5/5, 4/5, 6/6)
B104	5 dpi	100	10/10 (1/1,4/4, 5/5)
H99	5 dpi	93	13/14 (4/4, 4/5, 5/5)
Zheng58	6 dpi	81	17/21 (4/5, 4/6, 9/10)

[a] Three independent experiments were performed with three biological replicates each

Engineering of SCMV as a viral gene expression vector in maize

To develop pSCMV as a viral vector for expressing heterologous proteins in maize, two classic proteolytic cleavage sites for potyviruses, NIb/CP and P1/HC-Pro sites, were employed for the insertion of foreign genes. The green fluorescent protein (*GFP*) gene was inserted into pSCMV to generate the GFP-expressing vectors pSCMV-GFP$^{NIb/CP}$ and pSCMV-GFP$^{P1/HC-Pro}$, GFP sequences in the former vector were located between NIb and CP proteins, while in the latter between P1 and HC-Pro (Fig. 2a). In pSCMV-GFP$^{NIb/CP}$, the artificial proteolytic site NIb/CP recognized by NIa protease were fused to both the N-terminal and C-terminal ends of GFP to promote the release of GFP from viral polyprotein, while in pSCMV-GFP$^{P1/HC-Pro}$, the P1/HC-Pro and NIb/CP cleavage sites were utilized to complete this proteolytic process.

To examine the expression of GFP from the SCMV vectors in maize, maize seedlings of inbred line B73 were inoculated with saps from *N. benthamiana* infiltrated with pSCMV-GFP constructs. Inoculation with SCMV-GFP$^{NIb/CP}$ or SCMV-GFP$^{P1/HC-Pro}$ caused systemic SCMV infection in maize plants at 4 dpi, evidenced by the appearance of typical mosaic symptoms in upper uninoculated leaves, similar to infection by wild-type pSCMV (Fig. 2b). About 88% of plants were infected with these two recombinant viruses (Table 2), slightly lower than that of wild-type SCMV (Table 1), indicating that insertion of GFP in the SCMV genome did not significantly affect virus infection and movement. GFP fluorescence was readily detected under UV-light accompanied by systemic spread of SCMV through the plant (Fig. 2b). We estimated GFP expression in the systemically infected plants by Western blotting analyses in a time-course experiment, and detected the GFP as a fully processed protein in systemic leaves even at 4 dpi (the early stage of virus infection), and increased rapidly to the highest level at 7 dpi (Fig. 2c). Hereafter, GFP expression declined but was still detectable in the fifth systemically

infected leaves at 28 dpi (Fig. 2c). There was no noticeable difference in the GFP expression of plants inoculated with SCMV-GFP$^{NIb/CP}$ and SCMV-GFP$^{P1/HC-Pro}$ (Fig. 2b, c). Therefore, SCMV-based vectors can be successfully applied in maize to express heterologous proteins that were inserted in NIb/CP or P1/HC-Pro sites.

Stability of the heterologous gene expressed from the SCMV-based vectors

The stability of the GFP-expressed vectors pSCMV-GFP$^{NIb/CP}$ and pSCMV-GFP$^{P1/HC-Pro}$ was investigated by examination of the retention of GFP sequence in the infected maize plants. Total RNA was extracted from each infected plant at 4, 7, 14, 21, and 28 dpi, and analyzed for the *GFP* gene insertion in the viral genome by RT-PCR using primers flanking the insert. In the case of recombinant virus SCMV-GFP$^{NIb/CP}$, a unique band, diagnostic of viral RNA carrying intact *GFP* inserts, was detected at 4, 7, and 14 dpi (Fig. 3a). Later, these larger products decreased in abundance with a concurrent increase of smaller products, representing truncated *GFP* expression (Fig. 3a), implying that SCMV-GFP$^{NIb/CP}$ was stable until 14 dpi and the insert deletion autonomously occurred over virus replication. Similar to SCMV-GFP$^{NIb/CP}$, SCMV-GFP$^{P1/HC-Pro}$ was stable for 4 and 7 dpi without detectable truncated *GFP* (Fig. 3b). However, at 14 dpi, a small ratio of *GFP* deletion in viral genome was detected in some but not all infected plants (Fig. 3b). These results suggest that *GFP* gene inserts in the SCMV expression vectors were stably maintained for at least 2 ~ 3 weeks in maize.

GUS expression from SCMV

Next, we investigated the capacity of SCMV vectors for expressing a large protein encoded by the beta-glucuronidase gene (*GUS*) with more than 600 amino acids. The *GUS* gene was engineered into SCMV using the same *GFP* strategy to generate pSCMV-GUS$^{NIb/CP}$ and pSCMV-GUS$^{P1/HC-Pro}$. The infectivity of GUS-expressed SCMV-GUS$^{NIb/CP}$ and SCMV-GUS$^{P1/HC-Pro}$ drastically reduced compared to wild-type SCMV. Approximately 50% maize plants were infected with SCMV-GUS$^{NIb/CP}$ and 47% were infected with SCMV- GUS$^{P1/HC-Pro}$ (Table 2). Viral infection symptoms initially developed on plants infected with SCMV-GUS$^{NIb/CP}$ and SCMV-GUS$^{P1/HC-Pro}$ at 4 ~ 5 dpi (Table 2). However, 1 ~ 3-day delay of symptom appearance occurred in most of the infected plants compared with those infected with wild-type SCMV. Histochemical staining revealed vigorous GUS activity with blue spots observed in either SCMV-GUS$^{NIb/CP}$ or SCMV-GUS$^{P1/HC-Pro}$ infected maize plants (Fig. 2e) at 7 dpi. On the other hand, mock-inoculated plants or those infected with wild-type SCMV showed no staining. Western blot analysis confirmed GUS expression in the infected plants (Fig. 2f).

Simultaneous expression of two foreign proteins from SCMV-based vector in maize

As shown above, heterologous genes were successfully expressed by SCMV via insertion into the viral genome either in NIb/CP or P1/HC-Pro site. Then, we anticipated the possibility of simultaneous delivery of two genes using one vector. To test this, *GFP* and *mCherry* genes were engineered into these two proteolytic cleavage sites of pSCMV, generating the dual gene expression vectors pSCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ and pSCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$ (Fig. 4a). The resulting vectors were infiltrated into *N. benthamiana* and subsequently inoculated into maize plants. Maize plants infected with either pSCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ or pSCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$ displayed typical symptoms on the upper uninoculated leaves at 4 dpi, indicating that the dual-expression vectors were infectious to maize. Approximately 87% of inoculated plants were infected with pSCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ and 88% of those with pSCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$, similar to that caused by single-gene expression

(See figure on next page.)

Fig. 2 Expression of single foreign protein from SCMV vectors in maize plants. **a** Schematic representation of single-gene expression vectors: pSCMV-GFP$^{NIb/CP}$, pSCMV-GFP$^{P1/HC-Pro}$, pSCMV-GUS$^{NIb/CP}$, and pSCMV-GUS$^{P1/HC-Pro}$. Foreign gene GFP or GUS was inserted into NIb/CP or P1/HC-Pro junction to generate these expression vectors. In pSCMV-GFP$^{NIb/CP}$ and pSCMV-GUS$^{NIb/CP}$, the release of foreign proteins from viral polyprotein was processed by NIa-Pro, whereas in pSCMV-GFP$^{P1/HC-Pro}$ and pSCMV-GUS$^{P1/HC-Pro}$, that was accomplished by P1 and NIa-Pro. **b** Visualization of GFP expression in maize plants inoculated with mock, wide type and GFP-expressed SCMV vectors. The whole plants (left two columns) and upper uninoculated leaves (right two columns) were photographed under normal and UV light at 7 dpi. **c** and **d** Western blot analysis of GFP accumulation in leaves systemically infected with SCMV-GFP$^{NIb/CP}$ (**c**) and SCMV-GFP$^{P1/HC-Pro}$ (**d**). Total protein was extracted from infected leaves at 4, 7, 14, 21, and 28 dpi and subjected to western blot analysis with antibody against GFP. The rubisco large subunits were stained by Coomassie Brilliant Blue G-250 and used as a control for the amount of protein loaded for western blot analysis. **e** Histochemical analysis of GUS expression in leaves systemically infected with SCMV-GUS$^{NIb/CP}$ (left) and SCMV-GUS$^{P1/HC-Pro}$ (right) at 7 dpi. **f** Western blot analysis of GUS accumulation in leaves infected with GUS-expressed SCMV vectors using an antibody against GUS protein. Coomassie Blue stained Rubisco is shown as a loading control

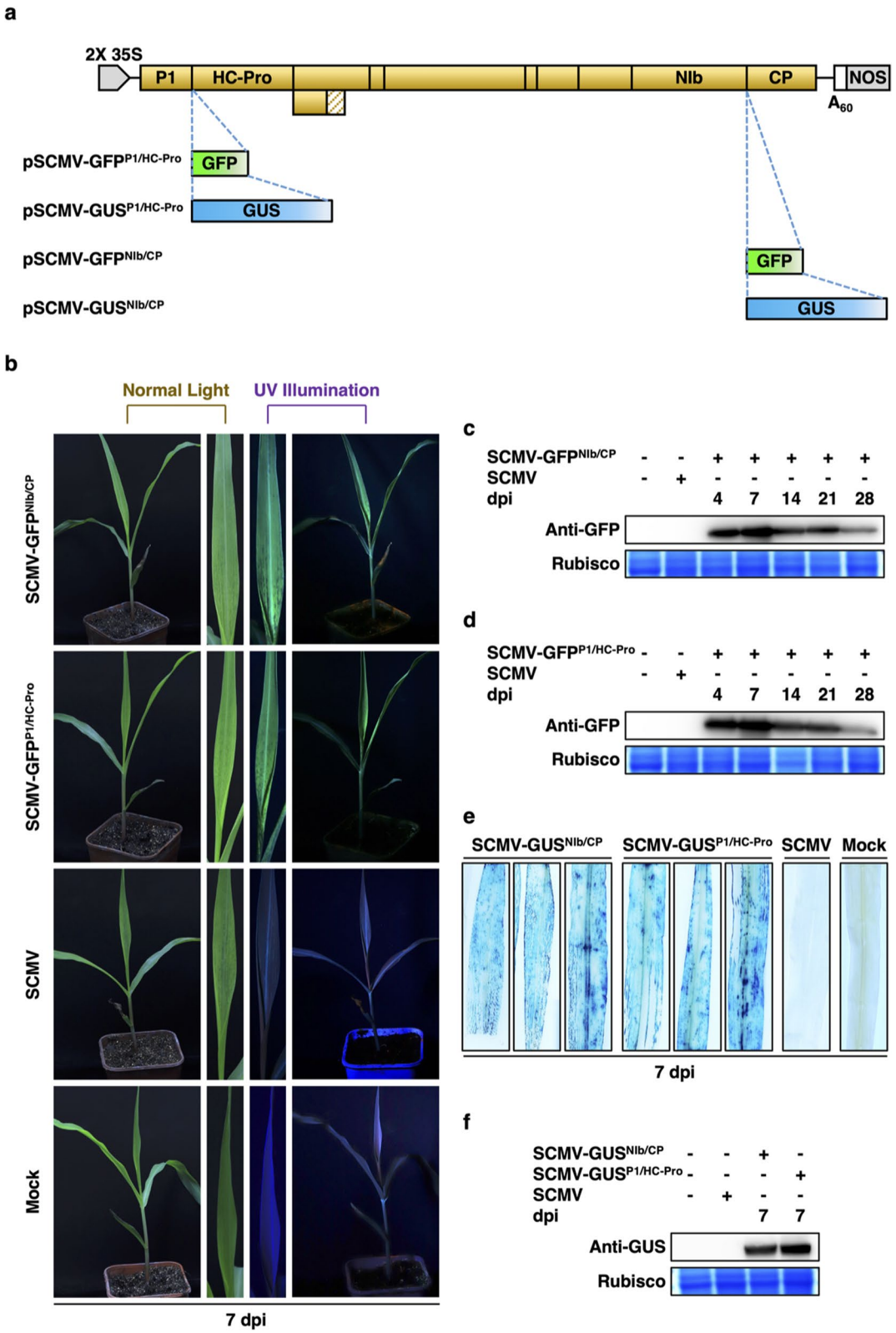

Fig. 2 (See legend on previous page.)

Table 2 Infection efficiency of heterologous gene-expressed SCMVs in maize

Vectors	Symptom appearance	Infection efficiency	
		Ratio (%)	Details (%)
pSCMV-GFP$^{NIb/CP}$	4 dpi	88	15/17 (5/5, 6/6, 4/6)
pSCMV-GFP$^{P1/HC-Pro}$	4 dpi	88	14/16 (4/5, 4/5, 6/6)
pSCMV-GUS$^{NIb/CP}$	5 dpi	50	8/16 (2/4, 4/6, 2/6)
pSCMV- GUS$^{P1/HC-Pro}$	4 dpi	47	7/15 (2/4, 3/6, 2/5)
pSCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$	4 dpi	87	13/15 (5/6, 5/5, 3/4)
pSCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$	4 dpi	88	14/16 (5/6, 4/5, 5/5)

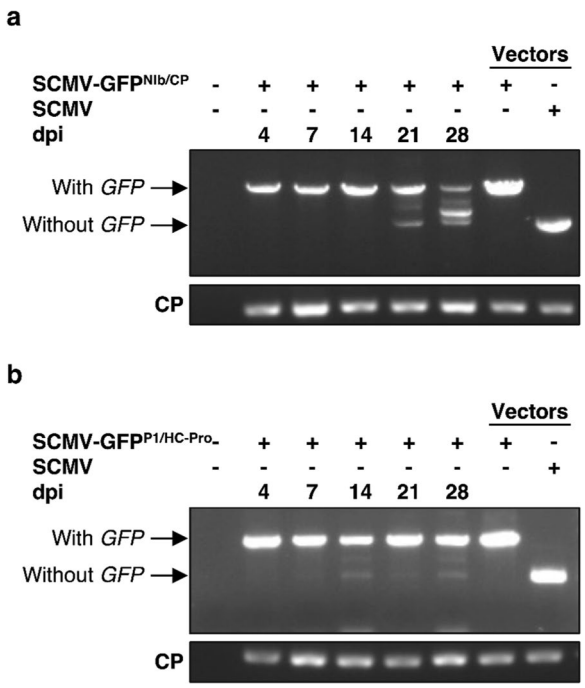

Fig. 3 Stability of the *GFP* gene insertion in SCMV-based single-gene expression constructs. Total RNA isolated from leaves infected with GFP-expressed SCMV vectors of SCMV-GUS$^{NIb/CP}$ (**a**) and SCMV-GUS$^{P1/HC-Pro}$ (**b**) were analyzed at 4, 7, 14, 21, and 28 dpi by RT-PCR amplification with primers flanking the insertion of *GFP* (upper panels). Plasmids pSCMV and pSCMV-GUS$^{NIb/CP}$ (**a**) or pSCMV-GUS$^{P1/HC-Pro}$ (**b**) were amplified as controls. Levels of CP transcript (lower panels) amplified from tested samples were served as internal controls

vectors pSCMV GFP$^{NIb/CP}$ and pSCMV-GFP$^{P1/HC-Pro}$ (Table 2).

Analysis of the fluorescent proteins by confocal microscopy in the infected cells revealed that both GFP and mCherry proteins were expressed throughout the cytoplasm and nucleus, fully colocalized in the infected cells (Fig. 4b). Symptomatic leaves were collected at different dpi and the expression of the fluorescent proteins was assayed by western blot analysis. The gene expression from the dual-expression vectors exhibited the same pattern as the single-gene expression vectors. Regardless of the fluorescent proteins expressed from either SCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ or SCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$, both GFP and mCherry expression were detectable with a relatively high intensity accompanied by the emergence of viral symptoms at 4 dpi and reached the peak at 7 dpi, thereafter gradually decreased at 14 dpi, 21 dpi and 28 dpi in the infected leaves (Fig. 4c), implying that two different genes can be simultaneously expressed from a single SCMV vector.

Stability of the heterologous gene expressed from the SCMV-based dual-expression vectors

The stability of insert in the dual-expression vectors was assessed using the same primer pairs as for the single-gene expression vectors (Fig. 5). RT-PCR analysis of viral progeny RNA verified that the *GFP* inserts were stable in the viral genome of SCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ or SCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$ at least over

(See figure on next page.)

Fig. 4 Simultaneous expression of two foreign proteins by SCMV-based dual gene expression vectors in maize. **a** Schematic representation of dual-expression vectors: pSCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ and pSCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$. Foreign genes *GFP* and *mCherry* were individually inserted into either NIb/CP or P1/HC-Pro junction of the same vector to expect that GFP and mCherry could be simultaneously expressed from one SCMV vector. **b** Examination of GFP and mCherry expression in maize leaves systemically infected with SCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ and SCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$ under confocal microscope at 7 dpi. Bars represent 20 μm. **c** and **d** Western blot analysis of GFP and mCherry accumulation in leaves systemically infected with SCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ (**c**) and SCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$ (**d**). Total protein was extracted from infected leaves at 4, 7, 14, 21, and 28 dpi and subjected to western blot analysis with antibodies against GFP and mCherry. Coomassie Blue stained Rubisco is shown as a loading control

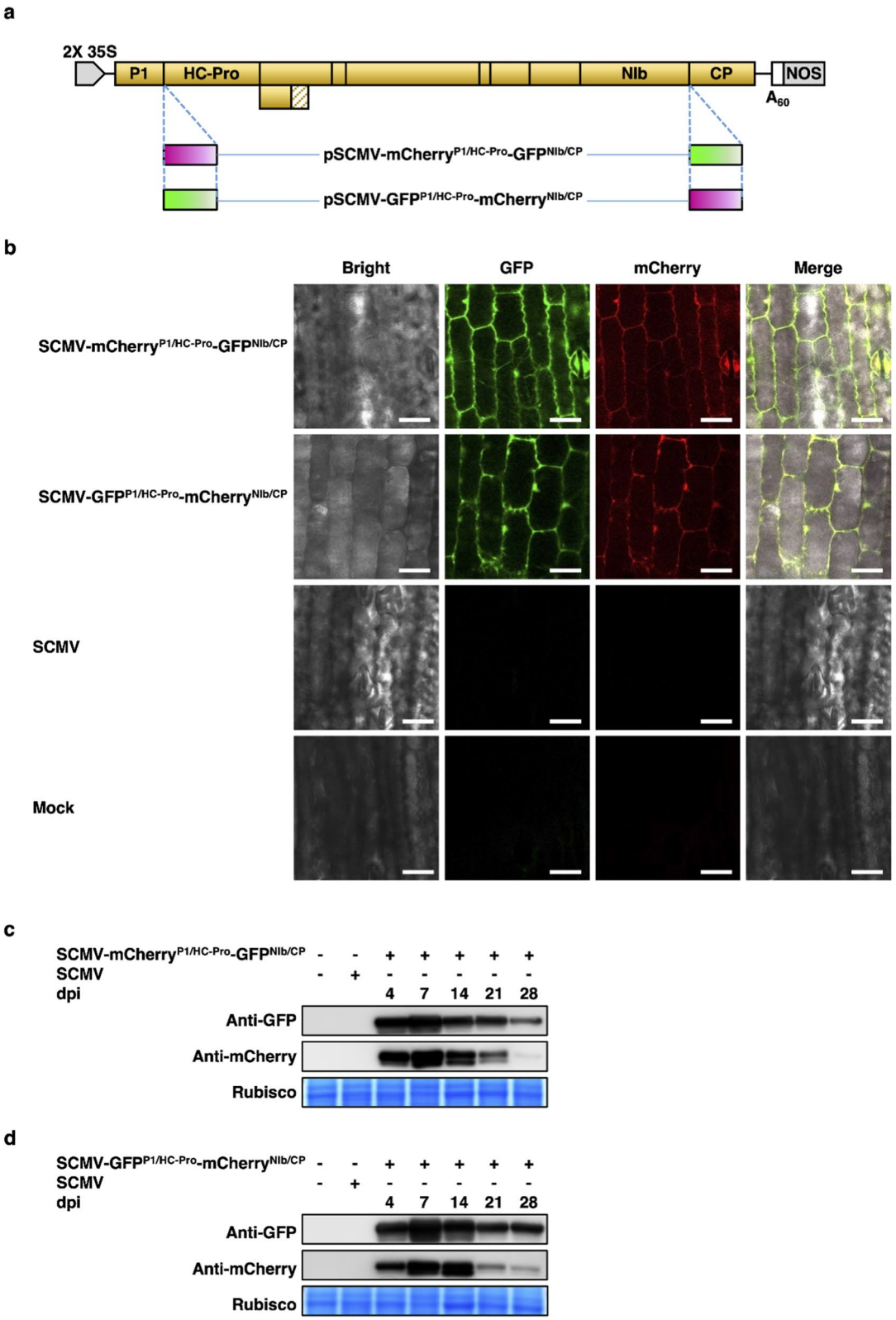

Fig. 4 (See legend on previous page.)

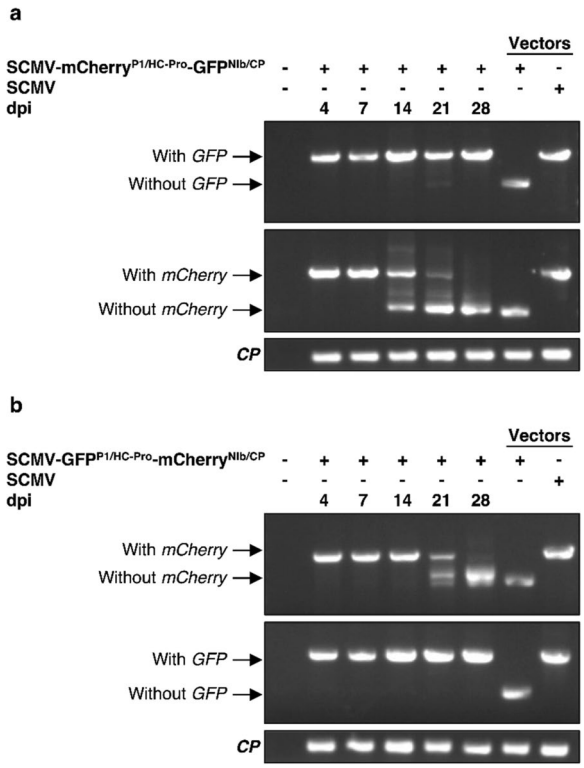

Fig. 5 Stability of gene insertions in SCMV-based dual-gene expression constructs. Total RNA isolated from leaves infected with SCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ (**a**) or SCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$ (**b**) were analyzed at 4, 7, 14, 21, and 28 dpi by RT-PCR amplification with primers flanking the insertion of *GFP* and *mCherry*, respectively (upper two panels). Plasmids pSCMV and SCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ (**a**) or SCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$ (**b**) were amplified as controls. Levels of CP transcript (lowest panels) amplified from tested samples served as internal controls

28 dpi, although truncated bands were faintly detected in one sample (Fig. 5). However, the *mCherry* gene was more unstable compared with the *GFP* gene in both constructs. For SCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$, the intact fragments of the *mCherry* inserts were uniquely amplified at 4 dpi and 7 dpi, but the smaller bands indicating *mCherry* deletions appeared from 14 dpi and a complete deletion at 28 dpi (Fig. 5a). On the other hand, for SCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$, the stability of the *mCherry* inserts in the viral genome was maintained until 14 dpi, and the deletions of this gene were observed at 21 dpi and 28 dpi (Fig. 5b). These results indicated that *GFP* gene was more stable than *mCherry* gene in the dual-expression vectors, no matter where they were inserted in the SCMV genome.

Discussion

Maize is not only a significantly important cereal but also a classic model organism in genetics and plant breeding. Given the importance of this staple crop, the demand for broadly applicable tools has been sustainably rising in maize research. In this study, we exploited SCMV as a vehicle for the transient expression of foreign genes in maize via simple rub inoculation. We showed that two different heterologous genes could be engineered into a single SCMV vector to get simultaneously expressed in infected plants of maize.

The development of reverse genetics systems for RNA virus genomes, especially the viruses in the family *Potyviridae* (Sánchez et al. 1998; You and Shirako 2010; Chikh Ali et al. 2011; Cheng et al. 2020), has provided inspiration for generating SCMV infectious cDNA clone. However, the delivery of transcribed viral RNA or cDNA constructs into the host cell still constraints the establishment of a reverse genetic system for SCMV. Because of the limitations of in vitro transcription and biolistic bombardment in the application, we focus on agro-infiltration approach to facilitate SCMV vector delivery due to its simplicity, convenience and versatility (Sharma et al. 2020). As expected, mechanical inoculation of maize plants with leaf sap containing viral RNA from *N. benthamiana* infiltrated with SCMV gave rise to the effective infection in maize plants, although *N. benthamiana* is outside the host range of SCMV. With this method, SCMV caused systemic infection with high efficiency in almost all tested lines, especially in lines B73, Jing2416, and B104 up to 100%. The success of SCMV infection in maize breaks our previous cognition that agro-infiltration of *N. benthamiana* was not applicable to the viruses non-infectious in this plant. Several attempts were also made to directly infiltrate the intact maize leaves with *Agrobacterium* containing vectors expressing SCMV and p19 of TBSV, however, none of them caused systemic infection by MSV, FoMV, or other strains of SCMV (Lazarowitz et al. 1989; Beernink et al. 2021).

The common strategy to express heterologous genes from a potyviral vector is to introduce foreign sequences into the viral genome at the viral protein junctions either as a fusion with viral protein or as an independent peptide proteolytically released from viral polyprotein. Our results proved that heterologous genes can be expressed from SCMV vectors in infected maize plants with a capacity for protein of more than 600 amino acids in size, and the gene expression was not obviously influenced by insertion position at either P1/HC-Pro or NIb/CP junction. It is noteworthy that the size of the gene insert appears to negatively affect SCMV infection efficiency, as evidenced by the lower efficiency observed in pSCMV-GUS$^{NIb/CP}$ and pSCMV-GUS$^{P1/HC-Pro}$ compared with

other constructs (Table 2). Larger inserts can impose a higher replication and packaging burden on the viral vector, potentially affecting its infectivity and systemic movement in host plants (Gleba et al. 2007; Willemsen and Zwart 2019; Jones et al. 2021).

Inserted heterologous sequences in the viral genome are often unstable due to the deletions caused by recombination events. It has been reported that the positions of inserted sequences strongly affect the insert stability. It seems that *GFP* sequence were more stable when inserted into NIb/CP junction than P1/HC-Pro junction of SCMV. Another important factor affecting the insert stability is the size of inserts. The expression of GUS from SCMV declined earlier than that of GFP, indicating that the *GUS* gene with a larger size was less stable than *GFP* gene in the SCMV genome. Importantly, the insert stability also varies with the nature of the inserts. In the dual-expression SCMV vectors, compared with *GFP* gene, *mCherry* was found to be inherently more unstable and prone to be deleted rapidly from the viral genome, indicating that the *mCherry* sequence were more susceptible to the recombination events. Interestingly, *GFP* sequence exhibited an enhanced stability in dual-expression vectors relative to single gene expression vectors, although the dual-expression viruses have a larger genomic size. The most possible reason for this is that simultaneous recombination rarely occurred at two insertions and the interaction between sequences may reduce the instability of GFP genes. In addition, the insert stability was affected by other factors, such as the host environment, the virus population and serial passages. The complexity of all these factors is challenging to the development of virus-based vectors.

Our previous SCMV infectious cDNA clone has been used by several research groups and proved to be a versatile tool for advancing our understanding of viral biology and improving crop protection strategies through genetic and molecular approaches (Xu et al. 2020; Xu et al. 2021; Jiang et al. 2023; Du et al. 2024; Tian et al. 2024; Xie et al. 2024). The establishment of dual-expression SCMV vectors will broaden their applications not only in plant pathology but also in functional genomics studies.

Conclusions

In summary, we developed a highly SCMV infectious clone, which can be easily and cost-effectively delivered into maize plants using inoculum from infiltrated leaves of *N. benthamiana* and can infect at least 17 inbred lines. The insertions of heterologous genes into SCMV do not interfere with the replication of the recombinant virus and enable the simultaneous expression of two proteins in maize. Although only two insertion sites of SCMV were tested in this study, there is a great potential of other insertion sites for multiple gene expression. This vector will be a powerful tool for multiple applications in elucidating gene function, protein–protein interaction and viral pathogenicity of SCMV in maize.

Methods

Plants

Maize inbred lines B73, Zheng 58, Mo17, Huangzao4, Qi319, Hongzhouchang7, NH60, Xin03, Jing2416, Jing724, G1783, Ph6WC, M54, D1798Z, Zong31, B104, and H99 were obtained from Beijing Solidwill Sci-Tech Co. Ltd (Beijing, China). *N. benthamiana* over-expressing NahG was used as a source for generating infectious viral RNA for mechanical inoculation of maize plants. All the plants were cultivated at 25°C with 60% humidity in growth rooms under a 16-h-light and 8-h-dark cycle.

Construction of SCMV infectious cDNA clones

Total RNA extracted from fresh tissues of SCMV-infected maize plants was used as a template for RT-PCR amplification of cDNA fragments. A DNA fragment containing 5'-terminal 310 bp and 3'-terminal 254 bp amplified from the SCMV genome was digested with *Stu*I/*Sac*I and cloned into a binary vector pCB301-based vector to produce the pSCMV intermediate. Two overlapping cDNA fragments of SCMV were mixed with the intermediate plasmid linearized by *Spe*I and then transformed by advanced quick assembly (AQUA) cloning (Beyer et al. 2015) to generate pSCMV. Primers used for this construction are listed in Additional file 2: Table S1.

Constructions of SCMV-derived expression vectors

To generate GFP-expressed vector pSCMV-GFP$^{NIb/CP}$, overlap PCR fragment containing 436-bp upstream NIb/CP junction, GFP coding sequence fused with the NIb/CP cleavage sites at its 5' and 3' ends and 1616 bp downstream from NIb/CP junction was amplified, then doubly digested with *Avr*II/*Pme*I and cloned into *Avr*II/*Pme*I-digested pSCMV; To generate pSCMV-GFP$^{P1/HC-Pro}$, the overlap PCR fragment encompassing 2591-bp upstream from P1/HC-Pro junction, GFP sequences with P1/HC-Pro and NIb/CP cleavage sites attached to its 5' and 3' ends and 2278 bp downstream from P1/HC-Pro junction were amplified, digested with *Not*I/*Kpn*I and cloned to *Not*I/*Kpn*I -digested pSCMV. GUS-expressed vectors pSCMV-GUS$^{NIb/CP}$ and pSCMV-GUS$^{P1/HC-Pro}$ were modified from pSCMV-GFP$^{NIb/CP}$ and pSCMV-GFP$^{P1/HC-Pro}$ by replacements of *GFP* gene with *GUS* gene. The dual gene expression vectors pSCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ and pSCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$ were derived from single-gene constructs pSCMV-GFP$^{NIb/}$

^{CP} and pSCMV-GFP$^{P1/HC-Pro}$ and generated by addition of a *mCherry* gene using the similar strategy as pSCMV-GFP$^{P1/HC-Pro}$ and pSCMV-GFP$^{NIb/CP}$, respectively. The primers used for each construct are listed in Additional file 2: Table S1.

Virus inoculation of maize plants

All SCMV constructs were transformed into *A. tumefaciens* stain GV3101 or C58C1. Agrobacteria harboring each construct were cultivated overnight at 28°C in LB medium containing kanamycin (50 mg/L) and rifampicin (50 mg/L). *Agrobacterium* cultures containing the SCMV construct were mixed with those containing TBSV p19 construct at a ratio of 2:1, then pelleted by centrifugation at 2012 g for 5 min and resuspended in infiltration buffer (10 mM $MgCl_2$, and 10 mM MES pH5.6, and 200 mM acetosyringone) till $OD_{600}=1.0$. After incubation for 3 h at room temperature, *Agrobacteria* were syringe infiltrated into fully expanded leaves of 5-week-old transgenic NahG *N. benthamiana*. Crude sap inoculums were prepared by grinding agro-infiltrated leaves at 7 dpi in 20 mM Na_2HPO_4-NaH_2PO_4 (at 1:4 ratio) buffer (pH 7.2) and directly rubbed onto 2–3-seedling stage of maize that had been pre-dusted with 400-mesh carborundum (Sigma-Aldrich, USA). Inoculated plants were incubated in dark and humid conditions under plastic covers at 25°C for 48 h, then the covers were removed and the plants were grown in the greenhouse at 25°C with 16 h of illumination. All experiments were replicated at least three times.

Western blotting analysis

Total proteins from maize leaves were extracted in 2×SDS-PAGE sample buffer (2% (w/v) SDS, 20% glycerol, 120 mM Tris–HCl (pH6.8), 0.02% bromophenol blue, and 2% 2-mercaptoethanol) and denatured by boiling for 5 min. Proteins were separated by 10% SDS polyacrylamide gel electrophoresis and blotted onto Immobilon PVDF membranes (Merck Millipore, Ireland). The membranes were probed with Rat anti-GFP (1:5000, Chromotek, Germany), Mouse anti-mCherry (1:1000, abcam, UK) or Rabbit anti-GUS antibody (1:1000, abcam, UK). Following corresponding secondary antibody incubation, the blots were exposed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA) and visualized via Amersham Imager 680 (GE Healthcare, USA).

RNA extraction and RT-PCR analysis

For RT-PCR analysis, total RNA was extracted from leaves using TransZol Up reagent (Transgen Biotech, China) according to the manufacturer's instructions. RNA quality and concentration were evaluated by gel electrophoresis and Epoch Microplate Spectrophotometer (BioTek, USA). cDNA was synthesized using oligo$(dT)_{18}$ or gene-specific primers and Enzyme Mix in TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, China). After reverse transcription, PCR was conducted using primers listed in Additional file 2: Tables S1, S2.

Histochemical GUS assays

Histochemical staining was performed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc, Gold-Bio, USA) as substrate (Jefferson et al. 1987). The leaves infected with SCMV-GUS$^{NIb/CP}$ and SCMV-GUS$^{P1/HC-Pro}$ were cut into small pieces and immediately fixed in 90% acetone on ice for 2 h, rinsed three times in 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0), and then vacuum infiltrated under pressure with GUS staining solution (50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0), 10 mM EDTA, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mM X-gluc) for 30 min followed by incubation at 37°C for overnight. Plant tissues were rinsed with 50 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.0) and 70% ethanol, destained at a boiling temperature in 100% ethanol for 10 min and subsequently cleared by washing several times with 70% ethanol. Stained samples were digitally photographed using Canon EOS 6D (Canon, Japan).

Fluorescence observation and confocal microscopy

Maize leaves infected with SCMV-GFP$^{NIb/CP}$ and SCMV-GFP$^{P1/HC-Pro}$ were examined for the expression of GFP by exposure to UV irradiation supplied by Blak-Ray B-100AP High Intensity UV Lamp (UVP, USA). The fluorescence images were taken using Canon EOS 6D (Canon, Japan). Fluorescence signals emitted from leaves infected with SCMV-mCherry$^{P1/HC-Pro}$-GFP$^{NIb/CP}$ and SCMV-GFP$^{P1/HC-Pro}$-mCherry$^{NIb/CP}$ were observed using a Leica SP8 laser-scanning confocal microscope (Leica, Germany) to detect GFP and mCherry (excitation at 488 and 546 nm, respectively).

Abbreviations

AQUA	Advanced quick assembly
BYSMV	Barley yellow striate mosaic virus
FoMV	Foxtail mosaic virus
dpi	Days post-infiltration
GFP	Green fluorescent protein
GUS	Beta-glucuronidase
MDMV	Maize dwarf mosaic virus
MMV	Maize mosaic virus
MSV	Maize streak virus
SCMV	Sugarcane mosaic virus
TBSV	Tomato bushy stunt virus
WSMV	Wheat streak mosaic virus
X-Gluc	5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid

Supplementary Information

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Additional file 1. Figure S1. Detection of SCMV RNA in the agro-infiltrated leaves of *N. benthamiana*.

Additional file 2. Table S1. Primers used for construction of SCMV vectors in this study. **Table S2.** Primers used for detection of SCMV.

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Author contributions
HL, YW, and YL conceived the project and designed the experiments. HL, AI, and NL performed the experiments. KX contributed materials and reagents. HL, XZ, and MH analyzed the data and drew the figures and tables. HL, RZ, XW, and YL drafted the manuscript. All authors reviewed and approved the manuscript.

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Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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