METHODOLOGY

Development and application of a self-assembling split-fluorescent protein toolkit to monitor geminiviral movement and infection in plant

Yaqin Wang[1†], Tao Hu[1†], Hui Liu[1], Chenlu Su[1], Li Xie[2], Zhanqi Wang[3*] and Xueping Zhou[1,4*]

Abstract

Geminiviruses are a group of circular single-stranded DNA viruses that cause severe diseases in many crop plants. However, there is still no fluorescent protein tag suitable for labeling viral proteins endogenously due to the limited genomic space and structure of geminiviruses for foreign gene fragment insertion. Here, we established a split super-folder green fluorescent protein (sfGFP)-based imaging system that provides a platform to visualize the subcellular localization of geminiviral proteins in *Nicotiana benthamiana*. A short fragment of the GFP- coding sequence (GFP11) was inserted into a specific locus of the geminiviral genome, while the remainder of the GFP (GFP1–10) was transiently or constitutively expressed in *N. benthamiana*. Consequently, complementation fluorescence enables the examination of the subcellular localization of viral proteins in particular cells. Using this split sfGFP system, we examined the subcellular localization of the coat protein and BV1 protein of tomato golden mosaic virus (TGMV) and further monitored its intercellular and long-distance movement in *N. benthamiana* during viral infection. This approach allows us to study endogenously the subcellular localization and viral movement of geminiviruses in *N. benthamiana*. This new split sfGFP system may also hold the potential to provide orthogonal fluorescent proteins usable for geminiviral genome tagging in plants.

Keywords Fluorescent protein, Super-folder GFP, *Nicotiana benthamiana*, Geminivirus, Tomato golden mosaic virus, Subcellular localization, Movement, Infection

†Yaqin Wang and Tao Hu contributed equally to this article.

*Correspondence:
Zhanqi Wang
zhqwang@zju.edu.cn
Xueping Zhou
zzhou@zju.edu.cn
[1] State Key Laboratory of Rice Biology and Breeding, Institute of Biotechnology, Zhejiang University, Hangzhou 310058, China
[2] Analysis Center of Agrobiology and Environmental Sciences, Zhejiang University, Hangzhou 310058, China
[3] Key Laboratory of Vector Biology and Pathogen Control of Zhejiang Province, College of Life Sciences, Huzhou University, Huzhou 313000, China
[4] State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193, China

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Background

Viral infection is a highly dynamic process in which plant viruses shift their genomes and/or virions from the replication sites to adjacent cells via plasmodesmata and, finally, into the phloem to spread throughout the host plants (Feng et al. 2013; Rui et al. 2022; Zhang et al. 2022). Currently, the most convenient and effective approach for monitoring plant virus infection is the method based on fluorescent protein tags coupled with laser scanning confocal microscopy (Pasin et al. 2014; Wieczorek et al. 2020). For example, Wang et al. (2015a) inserted a green fluorescent protein (GFP) gene between the *N* and *P* genes of sonchus yellow net rhabdovirus (SYNV) to develop a recombinant reporter virus SYNV-GFP, which was utilized as a robust reverse genetics system for research on SYNV movement and morphogenesis. Likewise, Fang et al. (2022) subcloned a red fluorescent protein (RFP) gene between the *N* and *P* genes of the antigenome cDNA of northern cereal mosaic virus (NCMV) to construct an infectious clone that was used to monitor NCMV infection and movement in host plants and insect vectors. In addition, the GFP was also used to be fused to the C4 protein of geminiviruses to determine its subcellular localization and explore its interaction with a GSK3/SHAGGY-like kinase (NbSKη) during viral infection in *Nicotiana benthamiana* (Mei et al. 2018a, b).

Geminiviruses are a large and diverse group of circular single-stranded DNA viruses that are widely distributed all over the world (Zhong et al. 2017; Li et al. 2022). The virion of geminiviruses is a unique particle morphology of twinned (geminate) icosahedra that is approximately 22×38 nm in size (Fiallo-Olivé et al. 2021). It has been well demonstrated that the genomic DNA of geminiviruses is enclosed within viral coat proteins (CPs) to be packaged into virions that are well-ordered and finely packed. Compared with other plant viruses, the genome size of geminiviruses is always small, and insertions of foreign DNA fragments (>500 bp) into the genome of geminiviruses are sufficient to destroy their replication and/or infectivity during viral infection (Gu et al. 2014; Tuttle et al. 2015; Yang et al. 2017; Bhattacharjee and Hallan 2022). Collectively, it is impossible to investigate the geminiviral movement and/or infection by inserting fluorescent protein genes with more than 500 bp in length into geminiviral genomes. Therefore, it is imperative to develop a feasible and practical detection system to monitor geminiviral movement and/or infection in plants.

More recently, a split super-folder GFP (sfGFP) system was established and improved by Kamiyama et al. (2016) and Feng et al. (2017) to be used for labeling proteins, visualizing protein subcellular localizations, and detecting cell–cell contacts in live cells. In this sfGFP system, the encoding sequence of GFP is split between β-strands 10 and 11, which produces GFP1-10 and GFP11, respectively (Kamiyama et al. 2021). However, neither GFP1-10 nor GFP11 by itself is fluorescent. Only when both of them exist in the same cell in which they are combined into a complete GFP structure that can emit visible green fluorescence (Kamiyama et al. 2016, 2021; Feng et al. 2017). More importantly, the GFP11 is a small protein tag that consists of a 16-aa (amino acids) peptide. Therefore, it can be flexibly fused to various proteins with a minimal impact on the targets in live cells (Feng et al. 2017; Pedelacq and Cabantous 2019). Additionally, the GFP11 can be tandemly arranged, which provides a multimerization scaffold to recruit multiple GFP1-10 proteins and significantly amplifies the fluorescence signal (Kamiyama et al. 2016, 2021). Based on these advantages, the split sfGFP system has been widely used in systems ranging from effector secretion of bacteria (Park et al. 2017) to cell-type-specific labeling of cellular proteins in *Drosophila* (Kamiyama et al. 2021) and to RNA live cell imaging in plants (Huang et al. 2022). However, to the best of our knowledge, this system has not been utilized to investigate the movement and/or infection of geminiviruses in plants.

Nicotiana benthamiana, a species in the family *Solanaceae*, is widely used as a model plant for virus research because it can be infected by various plant viruses (Goodin et al. 2008; Bally et al. 2018). Furthermore, it can also be genetically transformed and regenerated efficiently and is a good candidate for virus-induced gene silencing or transient protein expression (Goodin et al. 2008; Bally et al. 2018). Tomato golden mosaic virus (TGMV), one of the bipartite begomoviruses, systemically causes severe chlorosis and leaf curl diseases in tomato and tobacco plants (von Arnim and Stanley 1992; Shung et al. 2006). In 1998, the first report of the geminivirus-derived virus-induced gene silencing (VIGS) vector was based on TGMV (Kjemtrup et al. 1998). Subsequently, the TGMV-based VIGS vector underwent a variety of optimizations and was widely used for gene silencing in plants (Peele et al. 2001; Burch-Smith et al. 2004; Jagram and Dasgupta 2023).

In this study, we aimed to establish the GFP11 tag as an effective approach for labeling geminiviral proteins in *N. benthamiana*. Despite widespread applications of split GFP, it has not been used for monitoring geminiviral infections in *N. benthamiana*. Here, we improved the properties of a split GFP-based system and demonstrated its utility for labeling geminiviral proteins and monitoring geminiviral infections in *N. benthamiana*.

Results

Establishment of the sfGFP system in *N. benthamiana* epidermal cells

Inspired by split fluorescent proteins previously used for endogenous protein labeling in human and *Drosophila* cells (Kamiyama et al. 2016, 2021; Feng et al. 2017), we developed a general strategy for engineering a self-complementing sfGFP system. Eventually, we established a green-colored sfGFP system that has enhanced brightness compared to GFP and is suitable for monitoring geminivirus movement and infection in *N. benthamiana*. To this end, a synthetic GFP (sGFP) that has enhanced brightness was used (Wang et al. 2011). The coding region of sGFP is 693 nt in length and encodes a protein of 230 aa (Additional file 1: Figure S1). Homology modeling analysis showed that the 3D structure of sGFP is very similar to a variant GFP from *Aequorea victoria* (RCSB PDB: 1QYO) (Barondeau et al. 2003), which possesses five α-helixes and eleven β-strands (Fig. 1a). Furthermore, we chose to split between the tenth and eleventh β-strands of sGFP, which resulted in a 213-aa fragment that was called GFP1–10 and a 16-aa fragment that was called GFP11 (Fig. 1a). Thus, this designed self-complementing system based on the sGFP is named split sfGFP system.

To determine protein labeling using this split sfGFP system, we first expressed the CP encoded by DNA A of TGMV (TGMV A) fused with GFP11 at its N- or C-terminal in *N. benthamiana* epidermal cells (Fig. 1b). Histone 2B (H2B)-RFP (Martin et al. 2009) and fibrillarin 1 (FIB1)-CFP (Li et al. 2020) were used as nuclear and nucleolar markers, respectively. With the co-expression of GFP1–10, bright green GFP fluorescence was observed in the nucleus of *N. benthamiana* epidermal cells (Fig. 1c). Interestingly, compared with the C-terminal GFP11-tagged CP, N-terminal GFP11-tagged CP complemented with GFP1–10 showed much stronger GFP fluorescence (Fig. 1c). Furthermore, we also examined the protein labeling of sfGFP system using another viral protein BV1 which was encoded by DNA B of TGMV (TGMV B) (Fig. 1b). Similarly, the GFP11 was fused to the N- or C-terminal of BV1 and then expressed in *N. benthamiana* epidermal cells. As shown in Fig. 1d, evident green GFP fluorescence was observed in the nucleus of epidermal cells of *N. benthamiana* co-expressing N- or C-terminal GFP11-tagged BV1 together with GFP1–10. Notably, compared with the N-terminal GFP11-tagged BV1, C-terminal GFP11-tagged BV1 complemented with GFP1–10 showed much stronger GFP fluorescence (Fig. 1d), which is just the opposite of what was obtained from the CP (Fig. 1c). These results suggest that the split sfGFP system which can be used for protein labeling of viral proteins is successfully established in *N. benthamiana* epidermal cells.
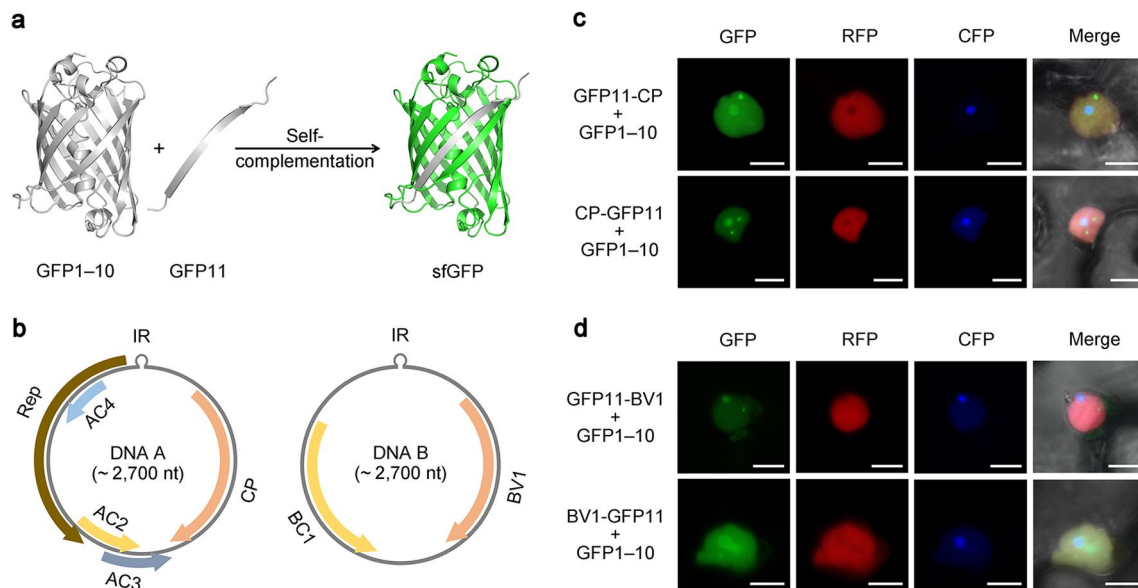

Fig. 1 Establishment of a split super-folder GFP (sfGFP) system in *Nicotiana benthamiana* epidermal cells. **a** Construct design and complementation scheme of the split sfGFP system. **b** Genomic organization of tomato golden mosaic virus (TGMV) with DNA A under GenBank accession no. K02029 and DNA B under GenBank accession no. JF694491, respectively. Fluorescence images of *N. benthamiana* epidermal cells expressing N- or C-terminal GFP11-tagged coat protein (CP) **c** or BV1 protein **d** together with GFP1–10, respectively, at 48 h post-infiltration (hpi). Histone 2B (H2B)-RFP (RFP) and fibrillarin 1 (FIB1)-CFP (CFP) were used as nuclear and nucleolar markers respectively. GFP, GFP fluorescence; RFP, RFP fluorescence; CFP, CFP fluorescence. Scale bars: 10 μm

Endogenous labeling of viral proteins with GFP11 in the genome of TGMV and intercellular viral movement assay in *N. benthamiana* leaves

To further examine the protein labeling using the split sfGFP system, we directly cloned the coding sequence (CDS) of GFP11 into the N- or C-terminal of CP or BV1 of TGMV in the genome of TGMV and produced a variety of GFP11-tagged infectious clones. Subsequently, the resulted GFP11-tagged infectious clones were agroinfiltrated into the left side of *N. benthamiana* leaves, and GFP1–10 and FIB1-CFP were agroinfiltrated into the right side of *N. benthamiana* leaves (Fig. 2). At 72 h post-infiltration (hpi), leaf discs obtained from the right side of agroinfiltrated *N. benthamiana* leaves were
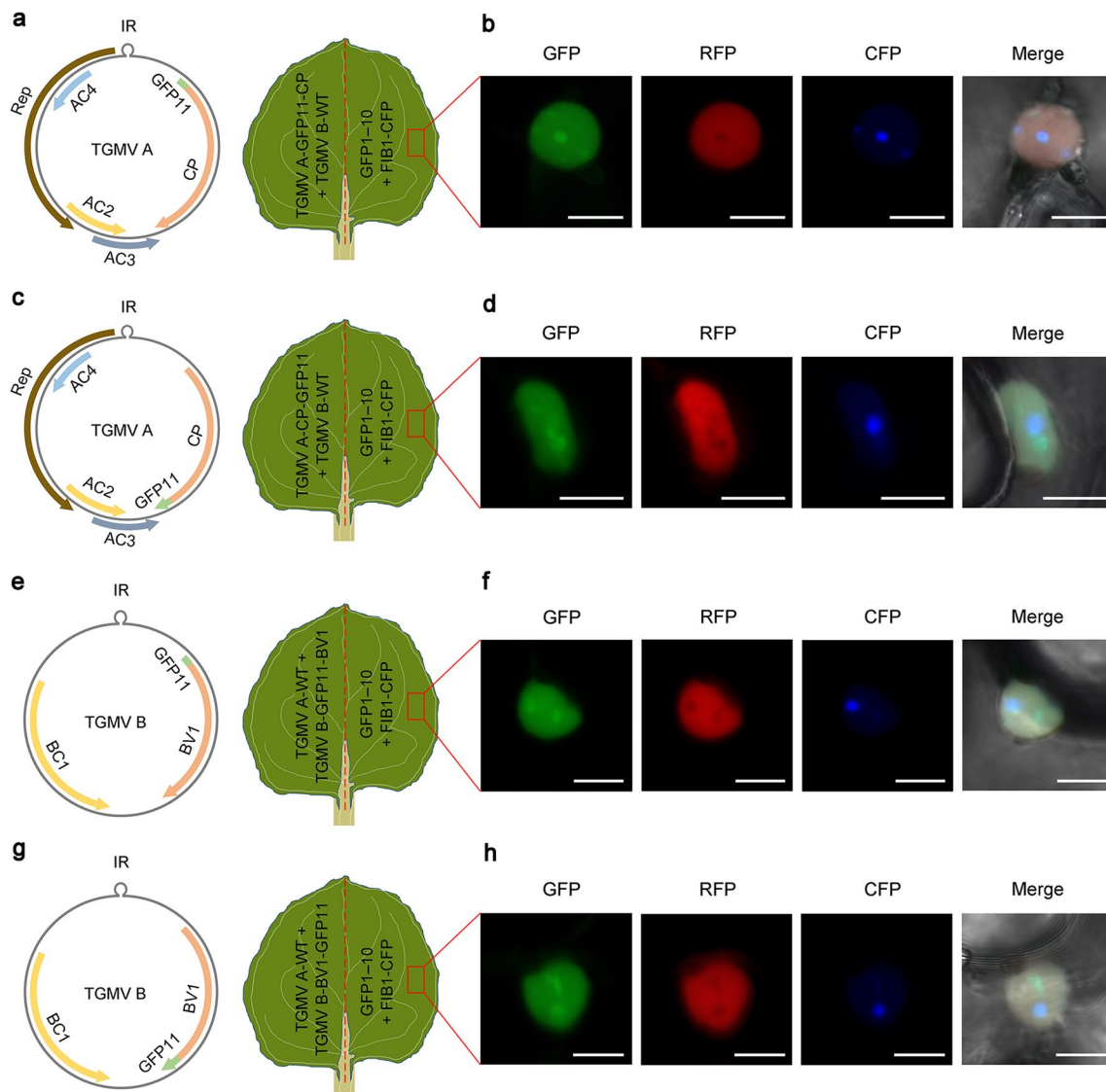

Fig. 2 Viral protein labeling with GFP11 in the genome of tomato golden mosaic virus (TGMV) and viral movement assay in *Nicotiana benthamiana* leaves. **a** Labeling method with GFP11 at the N-terminal of coat protein (CP) in the genomic DNA A of TGMV (TGMV A) and viral inoculation strategy with GFP11-tagged infectious clones. **b** Confocal microscopy examination of the *N. benthamiana* epidermal cells shown in Fig. 2a. **c** Labeling method with GFP11 at the C-terminal of CP in the DNA A of TGMV and viral inoculation strategy with GFP11-tagged infectious clones. **d** Confocal microscopy examination of the *N. benthamiana* epidermal cells shown in Fig. 2c. **e** Labeling method with GFP11 at the N-terminal of BV1-encoded protein in the genomic DNA B of TGMV (TGMV B) and viral inoculation strategy with GFP11-tagged infectious clones. **f** Confocal microscopy examination of the *N. benthamiana* epidermal cells shown in Fig. 2e. **g** Labeling method with GFP11 at the C-terminal of BV1 protein in the genome of TGMV B and viral inoculation strategy with GFP11-tagged infectious clones. **h** Confocal microscopy examination of the *N. benthamiana* epidermal cells shown in Fig. 2g. Histone 2B (H2B)-RFP (RFP) and fibrillarin 1 (FIB1)-CFP (CFP) were used as nuclear and nucleolar markers, respectively. GFP, GFP fluorescence; RFP, RFP fluorescence; CFP, CFP fluorescence. Scale bars: 10 μm

dissected and examined using a laser scanning confocal microscope as described previously (Wang et al. 2022). As shown in Fig. 2b, d, noticeable green GFP fluorescence was observed in the nucleus of epidermal cells of *N. benthamiana* co-agroinfiltrated N- or C-terminal GFP11-tagged CP directly in the genome of TGMV with GFP1–10. These observations indicated that infectious clones of TGMV with N- or C-terminal GFP11-tagged CP in its genome could move from agroinfiltrated cells to neighboring cells freely. Similarly, intense green GFP fluorescence was detected in the nucleus of epidermal cells of *N. benthamiana* co-agroinfiltrated N- or C-terminal GFP11-tagged BV1 directly in the genome of TGMV with GFP1–10 (Fig. 2f, h), suggesting that the infectious clones of TGMV with N- or C-terminal GFP11-tagged BV1 in its genome also could move from the agroinfiltrated cells to nearby cells without boundaries. Collectively, these results suggest that the sfGFP system is capable of labeling the viral proteins of TGMV endogenously and is a useful tool for investigating intercellular viral movement.

Insertion site selection of the GFP11 in the genome of TGMV-based infectious clones

Previous studies have shown that insertions of foreign DNA fragments into the genome of geminiviruses are sufficient to destroy their infectivity during viral infection (Gu et al. 2014; Tuttle et al. 2015). Therefore, we examined the effects of insertion sites of GFP11 on the viral pathogenicity of TGMV using the infectious clones described above. Compared with the mock-agroinfiltration control, *N. benthamiana* plants agroinfiltrated with infectious clones of wild-type (WT) TGMV A and TGMV B (TGMV A-WT + TGMV B-WT) developed severely curled leaves and yellow veins at 14 days post-inoculation (dpi) (Fig. 3a). Similarly, *N. benthamiana* plants agroinfiltrated with infectious clones of TGMV A with N-terminal GFP11-tagged CP (TGMV A-GFP11-CP) and TGMV B-WT (TGMV A-GFP11-CP + TGMV B-WT) showed moderate disease symptoms at 14 dpi (Fig. 3a). However, *N. benthamiana* plants agroinfiltrated with infectious clones of TGMV A with C-terminal GFP11-tagged CP (TGMV A-CP-GFP11) and TGMV B-WT (TGMV A-CP-GFP11 + TGMV B-WT) showed non-disease symptoms at the same time point (Fig. 3a). Western blot (WB) analysis confirmed that severe and moderate disease symptoms observed in *N. benthamiana* plants agroinfiltrated with TGMV A-WT + TGMV B-WT or TGMV A-GFP11-CP + TGMV B-WT resulted from high and middle levels of viral accumulation of TGMV, respectively (Fig. 3b). Consistently, there was no TGMV CP accumulation in systemically infected leaves of *N. benthamiana* plants agroinfiltrated with TGMV A-CP-GFP11 + TGMV B-WT (Fig. 3b). These data indicated that the insertion
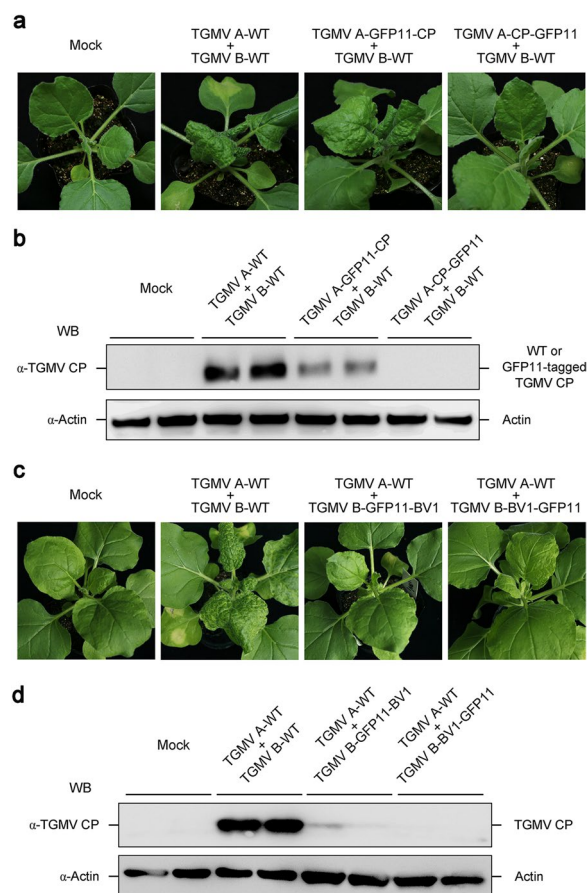

Fig. 3 Effects of the insertion sites of GFP11 on the viral pathogenicity of tomato golden mosaic virus (TGMV). **a** Disease symptoms detected in *Nicotiana benthamiana* plants agroinfiltrated with infectious clones of wild-type (WT) TGMV A (TGMV A-WT) or N- or C-terminal GFP11-tagged coat protein (CP) in TGMV DNA A together with WT TGMV DNA B (TGMV B-WT) at 14 days post-inoculation (dpi). **b** Western blot (WB) analysis of the infected leaves of *N. benthamiana* plants shown in Fig. 3a. **c** Disease symptoms detected in *N. benthamiana* plants agroinfiltrated with infectious clones of TGMV A-WT and TGMV B-WT or N- or C-terminal GFP11-tagged BV1 in TGMV DNA B at 14 dpi. **d** WB analysis of the infected leaves of *N. benthamiana* plants shown in Fig. 3c. In Fig. 3a, b, mock represents *N. benthamiana* plants agroinfiltrated with the empty vector pBINPLUS. In Fig. 3c, d, the TGMV CP protein was examined using an antibody against TGMV CP (α-TGMV CP) (top). WB analysis using an antibody to actin (α-Actin) served as a loading control (bottom)

of GFP11 in the N-terminus of CP had less impact on viral infectivity of TGMV than that in the C-terminus of CP. Surprisingly, infectious clones of TGMV with the insertion of GFP11 neither in the N-terminus nor in the C-terminus of BV1 could induce disease symptoms in *N. benthamiana* plants compared with TGMV A-WT + TGMV B-WT at the same time point (Fig. 3c). Consistently, there was no TGMV CP accumulation in

systemically infected leaves of *N. benthamiana* plants agroinfiltrated with TGMV A-WT + TGMV B-GFP11-BV1 or TGMV A-WT + TGMV B-BV1-GFP11 (Fig. 3d). These results suggest that N-terminus of CP is an appropriate site for the GFP11 insertion and that the infectious clones of TGMV A-GFP11-CP + TGMV B-WT can be used to investigate long-distance viral movement and live viral localization.

Development of stable genetic split sfGFP system in *N. benthamiana*

To demonstrate that the split sfGFP system has high enough performance to monitor viral movement and localization at the endogenous level in plants, we generated GFP1–10 transgenic *N. benthamiana* lines (Fig. 4a). WB analysis showed that the GFP1–10 protein could be stably expressed in transgenic *N. benthamiana* plants

(Fig. 4b). Compared with the mock agroinfiltration controls, when agroinfiltrated with infectious clones of TGMV A-GFP11-CP + TGMV B-WT, the GFP1–10 transgenic *N. benthamiana* plants displayed a similar phenotype to the WT plants (Fig. 4a). Consistently, *N. benthamiana* plants agroinfiltrated with infectious clones of TGMV A-GFP11-CP + TGMV B-WT showed a strong immunoblot signal of GFP11-CP in WB assays with an α-TGMV CP (Fig. 4b). These results suggest that the split sfGFP system can work successfully in GFP1–10 transgenic *N. benthamiana* lines.

Subsequently, to illustrate its usage to visualize the subcellular localization of viral proteins in the GFP1–10 transgenic *N. benthamiana* lines, we examined the developed GFP fluorescence signal using a laser scanning confocal microscope. As shown in Fig. 4c, strong GFP fluorescence was observed in the nucleus of leaf
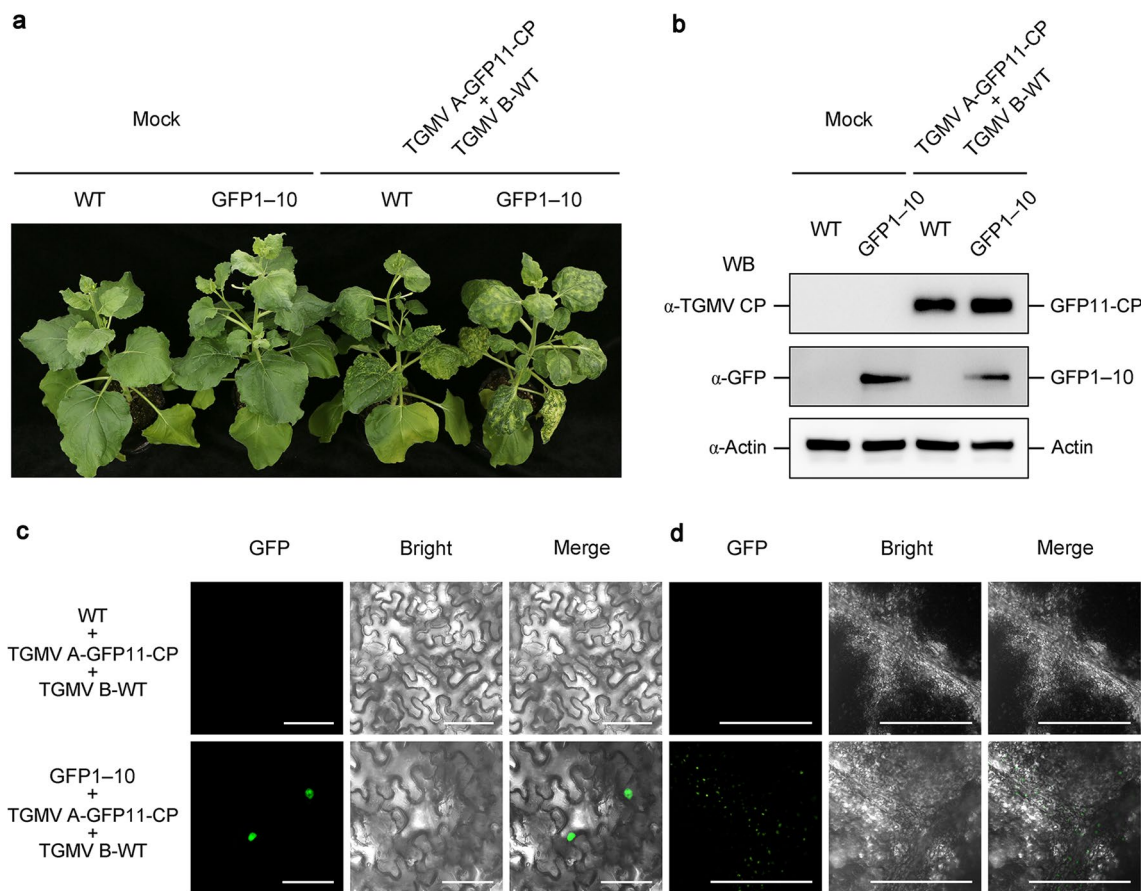

Fig. 4 Construction of the stable genetic sfGFP system in *Nicotiana benthamiana*. **a** Phenotype of wild-type (WT) and GFP1–10 transgenic *N. benthamiana* plants agroinfiltrated with infectious clones of tomato golden mosaic virus (TGMV) whose genomic DNA A tagged with GFP11 at the N-terminus of coat protein (CP) (TGMV A-GFP11-CP) together WT TGMV B (TGMV B-WT) at 14 days post-inoculation (dpi). *N. benthamiana* plants agroinfiltrated with the empty vector pBINPLUS served as the mock. **b** Western blot (WB) analysis of the infected leaves of *N. benthamiana* plants shown in Fig. 4a. TGMV GFP11-CP and GFP1–10 proteins were detected using antibodies against TGMV CP (α-TGMV CP) (top) and GFP (α-GFP) (middle), respectively. WB analysis using an antibody to actin (α-Actin) served as a loading control (bottom). **c** and **d** Subcellular localization of TGMV in epidermal cells of leaves and leaf veins of WT and GFP1–10 transgenic *N. benthamiana* plants at 7 dpi. WT *N. benthamiana* plants agroinfiltrated with the TGMV A-GFP11-CP and TGMV B-WT served as the control. GFP, GFP fluorescence. Scale bars: 30 μm

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epidermal cells of GFP1–10 transgenic *N. benthamiana* plants when agroinfiltrated with infectious clones of TGMV A-GFP11-CP + TGMV B-WT. Furthermore, GFP fluorescence was also detected in leaf veins of GFP1–10 transgenic *N. benthamiana* plants when agroinfiltrated with infectious clones of TGMV A-GFP11-CP + TGMV B-WT (Fig. 4d). Together, these results demonstrate that the split sfGFP system is a highly efficient split fluorescent protein system to monitor viral movement and localization in *N. benthamiana* plants.

Discussion

It is challenging to insert foreign DNA fragments into the genomes of geminiviruses due to the structure and space limitations (Gu et al. 2014; Tuttle et al. 2015; Bhattacharjee and Hallan 2022). In addition to the CP and replication-associated protein, geminiviral proteins are frequently low at molecular weight (Hu et al. 2020; Gong et al. 2021; Medina-Puche et al. 2021; Liu et al. 2023). Although fluorescent proteins, such as GFP and RFP, have been widely used in the field of plant virus research over the past two decades, there are still many challenges and technical difficulties in practical applications. For example, our previous study has demonstrated that the GFP fused to different terminals of a βC1 protein encoded by the betasatellite of tomato yellow leaf curl China virus (TYLCCNB-βC1) leads to different localization patterns, and the fusion of GFP at the C-terminal of TYLCCNB-βC1 protein can affect its function as a pathogenicity determinant (Cheng et al. 2011). Furthermore, a study by Wang et al. has also demonstrated that transient expression of the CP of tomato yellow leaf curl virus fused with GFP (TYLCV CP-GFP) in leaves of *N. benthamiana* mainly located in the nucleolus of epidermal cells; however, in the presence of TYLCV, TYLCV CP-GFP is observed to suffer from sequential relocalization from the nucleolus to the nucleoplasm (Wang et al. 2017). Therefore, developing an efficient tagging approach based on fluorescent proteins is essential and necessary to monitor the dynamic process and the subcellular localization of viral proteins during geminiviral infections.

Previous studies have shown that the sfGFP system is a powerful tool for labeling proteins, visualizing protein subcellular localization, and detecting cell-cell contact in live cells (Kamiyama et al. 2016, 2021; Feng et al. 2017; Pedelacq and Cabantous 2019). In this study, we described a split sfGFP system for directly labeling geminivirus proteins in *N. benthamiana*. Firstly, we showed that split GFP provides bright signals suitable for intensive imaging experiments under the laser scanning confocal microscope (Fig. 1). Secondly, by using infectious virus clones, we established a sfGFP system usable for geminiviral genome tagging to investigate intercellular

and long-distance movement of geminiviruses in *N. benthamiana* (Figs. 1, 2). Thirdly, we demonstrated that this sfGFP system can be used to monitor viral movement and localization in GFP1–10 transgenic *N. benthamiana* plants (Fig. 4).

Our platform can be easily expanded to engineer other self-complementing split fluorescent proteins with distinct colors (e.g., sfCherry, mRuby3, mRuby, mNeonGreen2, mNG2, and EBFP2) (Kamiyama et al. 2016, 2021; Feng et al. 2017; Tamura et al. 2021) to monitor geminiviral infections. Furthermore, our split sfGFP system can also be developed into a tripartite split sfGFP tool (Cabantous et al. 2013; Finnigan et al. 2016; Liu et al. 2018) for studying protein-protein interactions during geminiviral infections. Although it still needs to be improved, our split sfGFP system broadens the application of split fluorescent protein-based imaging systems in plants.

Conclusions

Here, we have established a split sfGFP system for visualizing the subcellular localization of geminiviral proteins in *N. benthamiana*. This system can be used to detect the subcellular distribution of viral proteins during geminiviral infections. We applied this system to monitor intracellular and long-distance movement and subcellular localization of TGMV proteins in *N. benthamiana*. Our method highlights the potentials of visualizing viral proteins to study the process of geminiviral infections in plants.

Methods

Plant materials and growth conditions

In this study, WT and transgenetic *N. benthamiana* plants were used. H2B-RFP transgenetic lines (Martin et al. 2009) were kindly provided by Michael M. Goodin (University of Kentucky, USA) and propagated in our laboratory. Transgenic lines expressing the GFP1–10 of the sfGFP system were generated via *Agrobacterium*-mediated gene transfer, as described by Li et al. (2014). All experimental plants were grown in a greenhouse at 25 °C with a 16-h/8-h (light/dark) photoperiod as described by Zhong et al. (2017) and Wang et al. (2022).

Plasmid construction

For transient expression, the CDSs of *CP* or *BV1* gene of TGMV were individually cloned into the pGFP11-N and pGFP11-C vectors to generate the plasmids GFP11-CP, CP-GFP11, GFP11-BV1, or BV1-GFP11, respectively. The nucleolar marker FIB1-CFP was constructed by our colleague Dr. Fangfang Li previously (Li et al. 2020). Infectious clones of TGMV (DNA A under GenBank accession no. K02029 and DNA B under GenBank

accession no. JF694491, respectively) were gifted by Prof. Garry Sunter (Northern Illinois University, USA). N- or C-terminal GFP11-tagged CP or BV1 in the genome of TGMV were produced by overlapping PCR as described previously (Zhong et al. 2017; Mei et al. 2018a, b). For stable expression of GFP1–10 of the sfGFP system in *N. benthamiana*, the CDS of *GFP1–10* was cloned into a binary vector pCHF3 under the control of a CaMV 35S promoter (Li et al. 2014). The primers used for plasmid construction are listed in Additional file 2: Table S1.

Agroinfiltration and viral inoculation

After confirmation by sequencing, the constructs were electroporated individually into the *Agrobacterium* strains EHA105 or GV3101 as described previously (Mei et al. 2020; Gong et al. 2021). For the transient expression experiments, *A. tumefaciens* cultures containing the designed expression plasmids were adjusted to an optical density at 600 nm (OD_{600}) of 1.0 with an infiltration buffer and infiltrated into *N. benthamiana* leaves as described previously (Zhong et al. 2017; Mei et al. 2020; Wang et al. 2023). For viral inoculation, *A. tumefaciens* cultures harboring WT or GFP11-tagged infectious clones of TGMV were adjusted to an $OD_{600} = 1.0$ for each component and infiltrated into *N. benthamiana* leaves as described previously (Sunter et al. 2001; Li et al. 2014).

Laser scanning confocal microscopy

At 48 or 72 hpi, leaf discs were dissected from the agroinfiltrated leaf area of *N. benthamiana* leaves and mounted in water between two coverslips, as described previously (Feng et al. 2013; Rui et al. 2022). The slides were imaged using a laser scanning confocal microscope (Olympus, Japan) equipped with the FluoView software (FV10-ASW v3.0, Olympus) as described previously (Wang et al. 2022). GFP fluorescence was excited at 488 nm and captured at 510–550 nm, RFP fluorescence was excited at 543 nm and captured at 590–630 nm, and CFP fluorescence was excited at 458 nm and captured at 470–500 nm, as described previously (Li et al. 2018; Zhang et al. 2021; Zhou et al. 2021).

Protein extraction and WB analysis

Leaf samples (approximately 0.2 g) were ground into powders with liquid nitrogen and homogenized in 600 mL extraction buffer that contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM ethylene diamine tetraacetic acid, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate (SDS), 0.5% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 5 mM dithiothreitol, and 10% glycerol (Wang et al. 2015b; Hu et al. 2019). The homogenates were centrifuged at 15,000 g for 2×10 min at 4 °C and the resulting supernatants were subjected to SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and WB analyses as described previously (Wang et al. 2015b; Wang et al. 2022). The monoclonal antibody to β-actin was purchased from Abmart (Shanghai, China), and the monoclonal antibody against TGMV CP was produced in-house in our laboratory. After blotting and washing, immunoblotting signals were examined using a LuminataTM Forte Western HRP Substrate (MerckMillipore, United Kingdom) as described by Wang et al. (2022).

Homology modeling and visualization

The 3D structure of the sGFP protein (Additional file 1: Figure S1) was modeled using the SWISS-MODEL workspace (https://swissmodel.expasy.org/) (Bordoli et al. 2009) based on the known structure of the GFP from *A. victoria* (RCSB PDB: 1QYO, https://www.rcsb.org/) (Barondeau et al. 2003). The structure figures of the sGFP were visualized and produced with the PyMOL Molecular Graphics System (v2.5) (PyMOL 2002).

Abbreviations

CDS	Coding sequence
CP	Coat protein
dpi	Days post-inoculation
GFP	Green fluorescent protein
H2B	Histone 2B
hpi	Hours post-infiltration
NbSKη	*Nicotiana benthamiana* GSK3/SHAGGYlike kinase
NCMV	Northern cereal mosaic virus
RFP	Red fluorescent protein
sGFP	Synthetic GFP
sfGFP	Super-folder GFP
SYNV	Sonchus yellow net rhabdovirus
TYLCCNB	Betasatellite of tomato yellow leaf curl China virus
TGMV	Tomato golden mosaic virus
VIGS	Virus-induced gene silencing
WB	Western blot
WT	Wild type

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00209-6.

Additional file 1: Figure S1. Nucleotide and amino acid sequences of the synthetic green fluorescent protein (sGFP) were used in this study.

Additional file 2: Table S1. List of primers used in this study.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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