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Systemic monitoring of cucumber mosaic virus infection using a small fluorescent protein iLOV in plants

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Abstract

Tracking virus in plants is useful to assess plant susceptibility or resistance. The recombinant cucumber mosaic virus (CMV) expressing a 2b-fused green fluorescent protein (GFP, 25 kDa) is known to gradually lose the GFP gene in plants. Here, we constructed the CMV expressing a flavin-based small fluorescent protein (iLOV, 10 kDa). CMV-iLOV retains iLOV at 28 dpi in the upper leaves of inoculated *Nicotiana benthamiana* and allows the long-term monitoring of its distribution in whole plants. Using CMV-iLOV, we showed that *pot1*, a recessive resistant allele to potyviruses, does not confer CMV resistance in tomato.

Keywords:

iLOV, cucumber mosaic virus, in vivo monitoring viral spread

Viruses are invisible pathogens but following their accumulation and movement in plants, preferably in a non-destructive manner, is an efficient way to assess plant susceptibility or resistance. Observing virus-caused symptoms is a primary way to predict where the virus infects in the plant body, although it does not allow following accurately the plant infection. One of the most efficient ways to do so is, when possible, to develop an infectious clone expressing a reporter gene (Verchot and Carrington 1995). The accumulation of the reporter can be used as a proxy for the virus presence. Fluorescent protein-tagged viruses as such reporters are valuable research tools to monitor viral distribution in infected plants. Green fluorescent protein (GFP) is standard for this purpose. Viral pathogenicity and virulence have been successfully investigated using various GFP-tagged viruses (Abrahamian et al. 2020; Bastet et al. 2019; Yambao et al. 2008). Nevertheless, an important requirement for using this tool is that the reporter protein-encoding sequence should remain stable in the viral genome throughout the infection.

Cucumber mosaic virus (CMV) is composed of a tripartite genome: three single-stranded RNAs (RNA1, RNA2, and RNA3) are thought to be encapsidated separately in identical spherical virions. Since CMV is an important pathogen with a broad host range, infecting more than 1,000 plant species (Yoon et al. 2019), its infectious clone and derived viral vectors for transgene expression in infected plants have been developed early on. Several studies reported on recombinant fluorescent protein-expressing CMVs. GFP was initially integrated into recombinant CMVs by replacing the CMV coat protein (CP)- or movement protein (MP 3a)-encoding genes in RNA3 (Canto et al. 1997). GFP was successfully expressed in initially inoculated leaves. However, as CP and 3a are required for the systemic viral spreading, infectious CMV clones carrying GFP-replaced CP or MP 3a failed to spread in the whole plant body. Subsequently, Masuta and colleagues have developed the yellow strain (CMV-Y)-based CMV vectors by introducing the reporter gene on RNA2 either by replacing the 2b protein with a transgene (CMV-H1) (Matsuo et al. 2007) or fusing it in frame with a truncated 2b gene so that the transgene-encoding protein could be expressed as a fusion protein with the N-terminal two-thirds of 2b (CMV-A1) (Otagaki et al. 2006). Both the CMV-H1 and CMV-A1 vectors could successfully express GFP or red-fluorescent protein (dsRed2) in both the inoculated and non-inoculated upper leaves of *Nicotiana benthamiana* (Takeshita et al. 2012). Similarly, Krenz et al. (2015) independently developed CMV infectious clones based on the Fny-

CMV isolate. The authors created a GFP-expressing clone as a translational fusion protein with the truncated 2b lacking the C-terminal (2b-truncated-GFP), hence corresponding to the above-mentioned GFP-expressing CMV-A1 and another GFP-expressing clone with the intact 2b protein (2b-GFP). Both clones also systemically infected *N. benthamiana* plants and expressed GFP. However, although the CMV RNA2 encoding the 2b-truncated-GFP could be packaged in a virion and transmitted over the course of five passaging experiments, the one encoding the full-size 2b-GFP could not, probably due to its larger size. Finally, reverse transcription coupled with polymerase chain reaction (RT-PCR) revealed that both viral genomes failed to maintain the GFP open reading frame (ORF) on day 21 post-inoculation (dpi). Again, the difficulty to maintain a large coding sequence in RNA2 could be the suspected cause of this instability.

In this study, we aimed at developing a stable CMV infectious clone based on the CMV-A1 clone to express a non-invasive reporter gene. We reasoned that introducing a smaller coding sequence could help achieve this goal. We thus focused on a small (10 kDa) flavin-based alternative to GFP (25 kDa) designed specifically to overcome size-related limitations (Chapman et al. 2008). This small fluorescent protein, developed based on an improved light, oxygen, or voltage-sensing domain (iLOV), was reportedly expressed by the tobacco mosaic virus vector in infected tobacco plants (Chapman et al. 2008). Here, we demonstrate that iLOV-expressing CMV can be used for long-time CMV infection monitoring in whole plants and utilize this tool to assess whether natural resistance alleles encoding the translation initiation factor eIF4E1 in tomato (*Solanum lycopersicum*) could trigger CMV resistance.

The iLOV ORF (Fig. S1a) (Chapman et al. 2008), the trimmed and modified LOV2 domain of *Arabidopsis thaliana* phototropin 2 (Accession NP_851211.1), followed by the nucleotide sequence encoding a Flag tag peptide sequence, DYKDDDDK, was chemically synthesized and cloned between the StuI and MluI sites in the pCY2-A1 vector (Otagaki et al. 2006). This clone, named pCY2-2bΔC-iLOV-flag, was expected to express the iLOV-Flag protein fused to the C-terminal of the truncated 2b (Fig. 1a). The pCY2-2b-iLOV-flag vector, expected to express the full length 2b-fused iLOV-Flag, was constructed as described above using the iLOV-Flag ORF following to the C-terminal nucleotide sequence of 2b (Fig. S1b). These constructs, as well as pCY2-A1 (empty) and pCY2-2bΔC-GFP, expressing GFP in the infected cells (Fig. 1a), and the CMV-Y RNA1 and RNA3 infectious clones were linearized and transcribed as described previously

(Otagaki et al. 2006). Transcript mixtures of CMV RNA1, RNA3, and either pCY2-A1 derivates were mechanically inoculated into *N. benthamiana* leaves with celite No. 545 (Fujifilm Wako Pure Chemical Co., Osaka, Japan). The fluorescence of plants inoculated with CMV with the truncated 2b-iLOV-Flag (CMV-2bΔC-iLOV-Flag), the intact 2b-iLOV-Flag (CMV-2b-iLOV-Flag), and the truncated 2b fused-GFP (CMV-2bΔC-GFP) were observed with a fluorescence stereoscopic microscope with a GFP filter, excitation BP 470/40, FT 510, (VB 7010; Keyence, Osaka, Japan). At 7 dpi (Fig. 1b), each recombinant CMV was inoculated into three distinct plants, and all inoculated plants showed not only symptoms but also fluorescence in their non-inoculated upper leaves, indicating the systemic infection of the inoculated recombinant CMVs retaining the iLOV- and GFP- encoding transgenes, showing the successful infectious nature of the iLOV constructs.

Next, we tested the stability of the inserted reporter genes during infection. At 28 dpi, both the CMV-2bΔC-iLOV-Flag and -2b-iLOV-Flag-infected plants showed similar fluorescence, but all three CMV-2bΔC-GFP-infected plants showed less or little fluorescence despite the visible symptoms. Microperforated-leaf blotting (Murakami et al. 2016) with anti-CMV CP antibody (Japan Plant Protection Association) showed comparable CMV CP accumulation among the upper leaves and even in those infected with the CMV-2bΔC-GFP genome, ruling out the absence of CMV infection in the plants inoculated with the CMV-2bΔC-GFP genome. To assess whether this GFP fluorescence loss could be caused by the loss of the transgene in the CMV-2bΔC-GFP genome, RT-PCR-amplified the region, from upstream of the 2b ORF to the 3' noncoding end, covering the transgenes using the primer pair as follows: 5′-ATTCAGATCGTCGTCAGTGC-3′ and 5′-AGCAATACTGCCAACTCAGC-3′. Indeed, whereas all three biological replicates infected with recombinant iLOV-expressing CMVs exhibited an amplification including the transgenes both at 7 and 28 dpi, this amplification in a sample of CMV-2bΔC-GFP infected, resulted in a significantly smaller product, suggesting the loss of the transgene in the CMV-2bΔC-GFP genome (Fig. 2b). Direct sequencing of the PCR products at 28 dpi confirmed that the CMV-2bΔC-GFP lacked the C-terminal 575 nucleotides, from 146th base to the end of the GFP ORF. The broader band of that at 7 dpi indicated that the GFP transgene loss started from an early infection stage. Similar deletion of the GFP trans gene of recombinant CMVs in infected plants was reported previously (Krenz et al. 2015). The iLOV fluorescence was associated

with Flag-tagged 2b-iLOV protein and viral CP accumulation in microperforated tissue blotting using anti-Flag (Fujifilm Wako, Osaka, Japan) and CP antibodies (Fig. 2a). These results indicate that both CMV-2bΔC-iLOV-Flag and CMV-2b-iLOV-Flag retained their transgene in infected *N. benthamiana* plants, and we can monitor the distribution of these viruses during infection in *N. benthamiana* plants by observing the iLOV fluorescence for at least 4 weeks.

We then examined whether iLOV-expressing CMV works for monitoring its infection and spread in a crop plant. Tomato plants (cv. S8), used in the previous study (Atarashi et al. 2020), were mechanically inoculated with transcripts of pCY2-2b-iLOV-Flag, CMV-Y RNA1, and RNA3 genome clones as an inoculum. Their upper leaves showed yellowing and chlorosis symptoms and the iLOV fluorescence correlated with these symptoms (Fig. 3a and c). Moreover, we investigated the role of CMV resistance of a natural allele encoding an eIF4E1 variant, *pot1* that has been selected in wild tomato (*Solanum habrochaites* PI24). *pot1* is associated with a large resistance spectrum to potyviruses, but it is not known if, as in *Arabidopsis thaliana*, if the eIF4E1-based resistance could extend to CMV (Gauffier et al. 2016; Yoshii et al. 2004). As we recently showed that a genome-edited tomato carrying the in-frame deletion allele of an eukaryotic initiation factor 4E1 (eIF4E1) showed partial resistance to CMV (Atarashi et al. 2020). we were curious whether *pot1* could also render the plants CMV resistant. We inoculated the CMV-iLOV virus into tomato plants homozygote for *pot1*. No obvious CMV resistance could be detected was shown. Similar symptoms and iLOV fluorescence were observed in the upper leaves of two tomato plants carrying *pot1,* (line #1 and line #3). Consistently, no significant difference in the CMV CP accumulation in upper leaves between *pot1*-carrier and susceptible tomatoes could be detected by ELISA (Fig. 3b).

In this study, we confirmed the very fast instability of the GFP coding sequence when inserted in a CMV infectious clone. In contrast to GFP-tagged CMV, this study showed that iLOV-tagged CMV stably retained the transgene up to four weeks after inoculation. Each CMV virion contains either of the viral RNA genomes and the most extended genome, RNA1, is 3361 nt, indicating that the CMV virion can encapsidate ssRNA of this length. The RNA2 of CMV-2b-iLOV-Flag and CMV-2bΔC-iLOV-Flag share a similar length to RNA1; they would be 3410 and 3331 nt, respectively, whereas the RNA2 of CMV-2bΔC-GFP is 3687 nt-long. Although the GFP ORF-integrated RNA2 of the CMV Fny strain was experimentally shown to be encapsidated in a virion (Krenz et al.

2015), stable retaining of the iLOV-Flag transgene appears to be attributed to its shorter length than that of GFP ORF and thus the comparable length of the RNA2s with iLOV to RNA1. A defective aspect of iLOV is its weaker fluorescence than that of GFP (Fig. 1a) though the iLOV fluorescence was able to be readily detected in infected tomato plants using a fluorescence stereoscopic microscope (Fig. 3). iLOV-tagged CMV allows us monitoring CMV distribution in an infected plant body. Using this clone, we could examine the effect of the *pot1 eIF4E1* natural allele on CMV resistance. *pot1*, an allele encoding a modified eIF4E1 protein harboring 8 amino acid changes compared to the susceptible allele, reportedly show a wide resistance spectrum to potyviruses (Gauffier et al. 2016). Our study shows that *pot1* does not confer resistance to CMV. We recently showed that a genome-edited eIF4E1 allele confers partial CMV resistance but not to potyviruses (Atarashi et al. 2020), suggesting that the genome-edited eIF4E1 allele with distinct mutations from the one selected in wild tomato, have a distinct mode of action to render antiviral resistance to each other.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Figure legends

Fig. 1 Structures of recombinant CMV RNA2 infectious clones with transgenes encoding fluorescent proteins (a) and iLOV and GFP fluorescence observed in *N. benthamiana* infected with the recombinant CMVs on day 7 post-inoculation (dpi) (b). (a) Flag-tagged iLOV and GFP were fused to two-third of the 2b N-terminal gene (pCY2-2bΔC-iLOV-flag and pCY2-2bΔC-GFP) or the intact 2b (pCY2-2b-iLOV-flag) in the CMV RNA2 constructs. (b) Recombinant CMVs with these constructs (a) were transcribed and inoculated into *N. benthamiana* (upper panels) and their fluorescence was observed (lower panels) at 7 dpi. A healthy and empty vector (2bΔC-empty) infected plants were also observed as a control and no obvious fluorescence was detected.

Fig. 2 Comparison of the fluorescence of *N. benthamiana* upper leaves infected with iLOV- and GFP-expressing recombinant CMVs at a later stage of infection (28 dpi) (a). RT-PCR analysis was performed to assess whether these recombinant CMVs retained the fluorescent gene, iLOV or GFP (b). Arrows indicate the expected PCR product length if the CMVs possess the fluorescent gene. Cs were amplified from the corresponding plasmids as a control of each RT-PCR. M indicates 100 bp as a ladder marker.

Fig. 3 iLOV fluorescent images of tomato plant upper leaves following inoculation with a recombinant 2b-iLOV-flag-expressing CMV at 27 dpi (a). (b) CMV CP accumulations in the upper leaves at 33 dpi. ELISA-based comparison of tomato cultivar S8 (n = 8) and tomato plants carrying a recessive resistant gene *pot1*, *pot1-#1* (n = 6) and *pot1-#3* (n = 8) by using anti-CMV antibody. A Tukey–Kramer test ($p < 0.05$) did not show a significant difference in the CMV CP accumulation among tomato plants with or without *pot1*. A closed spot was an outlier in the box plot graph. Boxplots show 25th and 75th percentiles and median values, and whiskers show minimum and maximum values within 1.5 × interquartile range (IQR). The outlier, indicated by a closed spot, was value less than that subtracted with 1.5 × IQR from the first quartile. (c) A fluorescent image of leaves of healthy and CMV-Y-inoculated plants was shown in an upper panel. Lower panels were results of western blotting with antibody to CMV CP to confirm CMV-Y infection in an upper leaf of a CMV-Y inoculated plant in the upper panel. An image of a polyacrylamide gel stained with Coomassie brilliant blue was shown as a loading control.

Both ELISA and western blotting are the antibody-based detection methods with similar detection sensitivity. The 2b-iLOV-flag-expressing CMV-, but not CMV-Y-infected, leaves showed fluorescence, indicating that the fluorescence was not caused by virus infection.

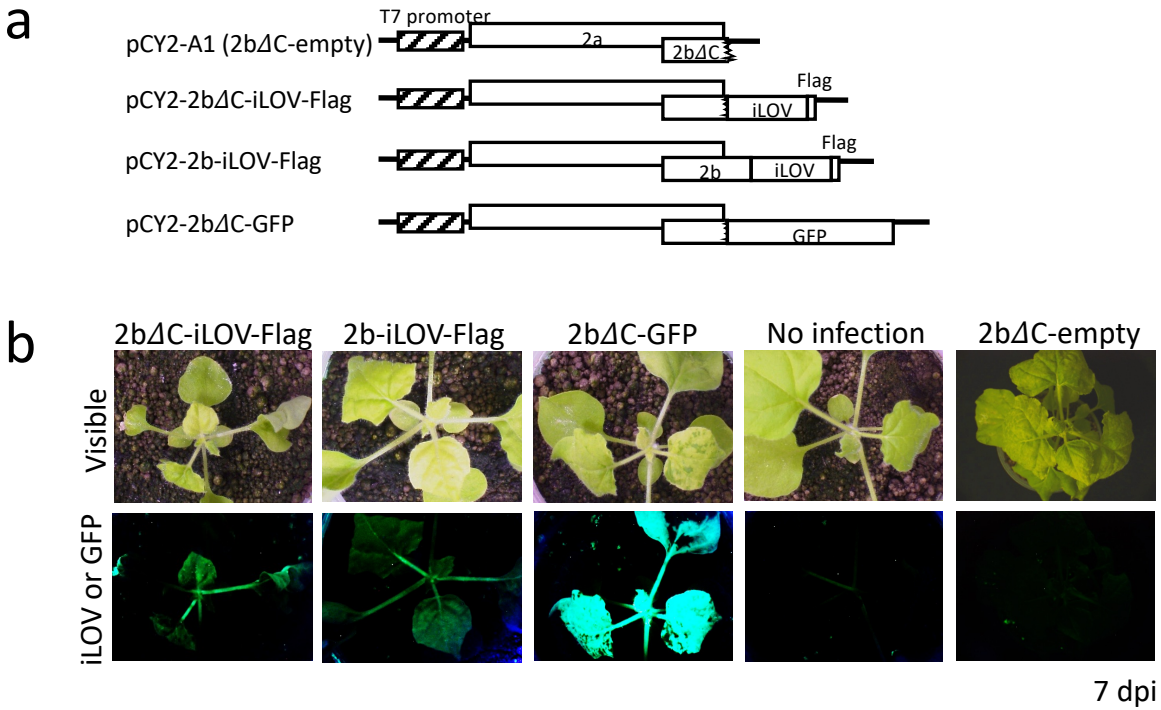

a
T7 promoter
pCY2-A1 (2bΔC-empty)
2a
2bΔC
pCY2-2bΔC-iLOV-Flag
Flag
iLOV
pCY2-2b-iLOV-Flag
Flag
2b
iLOV
pCY2-2bΔC-GFP
GFP
b
2bΔC-iLOV-Flag
2b-iLOV-Flag
2bΔC-GFP
No infection
2bΔC-empty
Visible
iLOV or GFP
7 dpi

Fig. 1

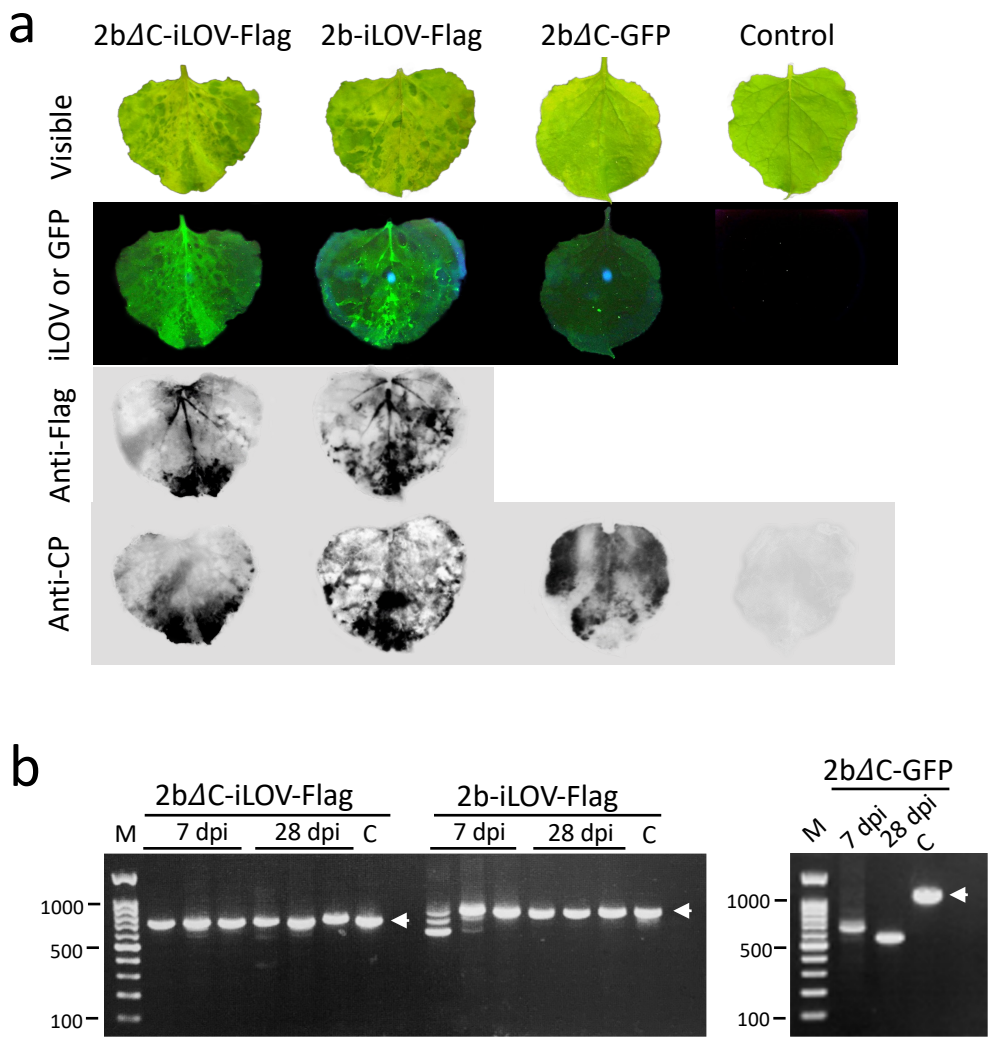

a
2bΔC-iLOV-Flag
2b-iLOV-Flag
2bΔC-GFP
Control
Visible
iLOV or GFP
Anti-Flag
Anti-CP
b
2bΔC-iLOV-Flag
2b-iLOV-Flag
2bΔC-GFP
M
7 dpi
28 dpi
C
1000
500
100

Fig. 2

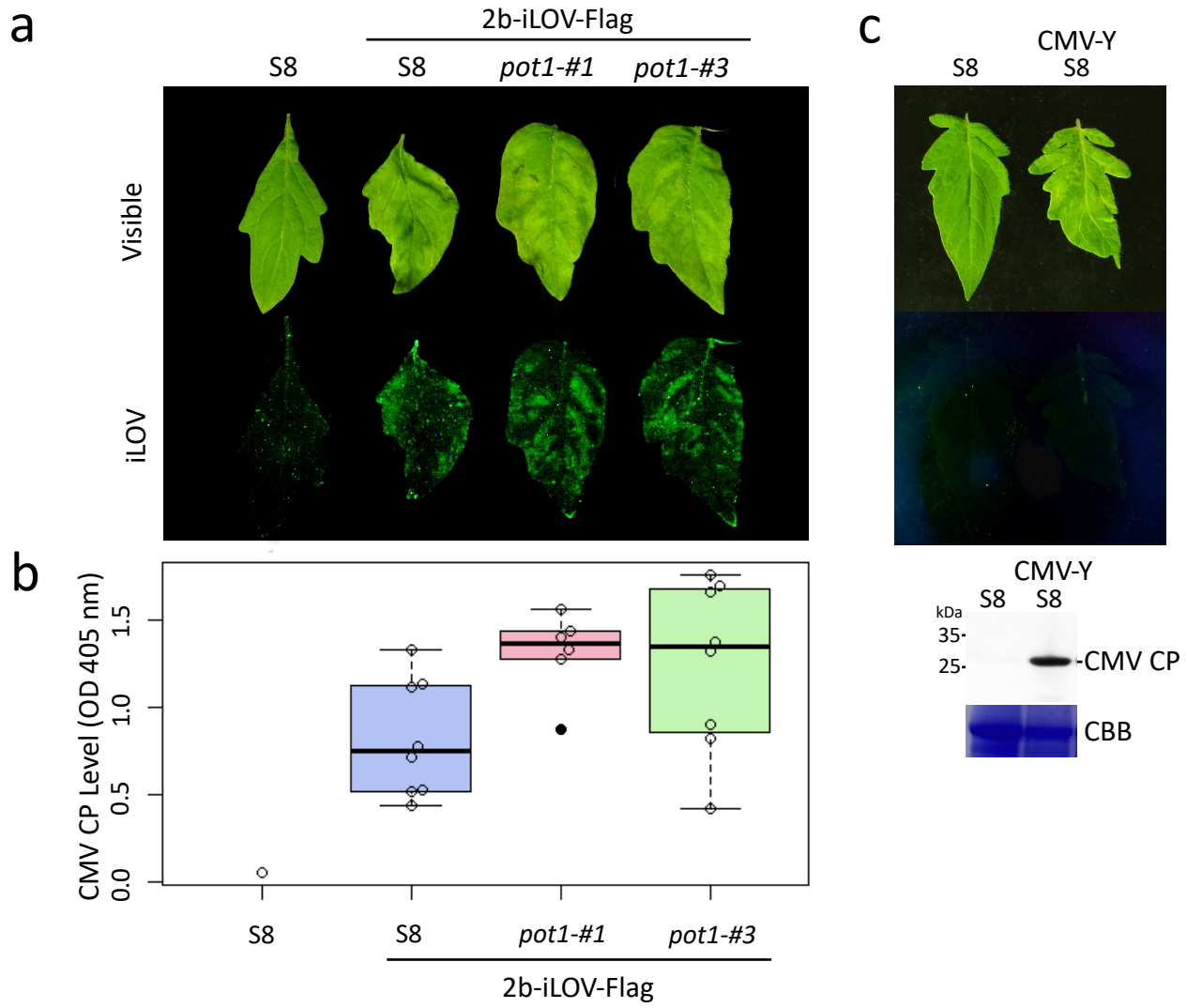

a
2b-iLOV-Flag
S8
S8
pot1-#1
pot1-#3
Visible
iLOV
b
CMV CP Level (OD 405 nm)
0.0
0.5
1.0
1.5
S8
S8
pot1-#1
pot1-#3
2b-iLOV-Flag
c
CMV-Y
S8
S8
CMV-Y
S8
S8
kDa
35-
25-
CMV CP
CBB

Fig. 3



Fig. S1 iLOV-Flag ORF with StuI and MluI sites (a) and C-terminal part of 2b followed by iLOV-Flag ORF with StuI and MluI sites (b)