Foxtail Mosaic Virus-induced Flowering Assays in Monocot Crops

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Highlight: We have demonstrated that expression of *FT* genes from FoMV triggers early flowering in millet and wheat, therefore establishing a virus-induced flowering assay in cereal crops.

Abstract

Virus-induced flowering (VIF) exploits RNA or DNA viruses to express flowering time genes to induce flowering in plants. Such plant virus-based tools have recently attracted widespread attention for their fundamental and applied uses in flowering physiology and in accelerating breeding in dicotyledonous crops and woody fruit-trees. We now extend this technology to a monocot grass and a cereal crop. Using the Foxtail mosaic virus-based VIF system, dubbed FoMViF, we showed that expression of florigenic *Flowering Locus T* (*FT*) genes can promote early flowering and spikelet development in proso millet, a C4 grass species with potential for nutritional food and biofuel resources, and in non-vermalized C3 wheat, a major food crop worldwide. Floral and spikelet/grain induction in the two monocot plants was caused by the virally expressed untagged or FLAG-tagged *FT* orthologues, and the florigenic activity of rice *Hd3a* was more pronounced than its dicotyledonous counterparts in proso millet. The FoMViF system is easy to perform and its efficacy to induce flowering and early spikelet/grain production is high. In addition to proso millet and wheat, we envisage that FoMViF will be also applicable to many economically important monocotyledonous food and biofuel crops.

Keywords: FoMV; Flowering time genes, VIF; Monocots, Proso millet; Wheat

Abbreviations: ALSV, *Apple latent spherical virus*; BMV, *Brome mosaic virus*; BSMV, *Barley stripe mosaic virus*; CLBV, *Citrus leaf blotch virus*; CLCrV, *Cotton leaf crumple virus*; CP, coat protein; FoMV, *Foxtail mosaic virus*; FoMViF, FoMV-induced flowering; *FT*, *Flowering Locus T*; LD, long-day; PVX, *Potato virus X*; RdRP, RNA-dependent RNA polymerase; SD, short-day; VIF, virus-induced flowering; VIGS, virus-induced gene silencing; ZYMV, *Zucchini yellow mosaic virus*

Introduction

Virus-induced flowering (VIF) uses RNA or DNA viruses as vectors to express flowering time genes such as *Flowering Locus T* (*FT*) (Kardailsky et al., 1999; Srikanth and Schmid, 2011) to induce flowering in plants (McGarry et al., 2017). This approach has recently attracted wide interest for its practical applications in accelerating breeding in crops and woody fruit-trees (McGarry et al., 2017; Qin et al., 2017). The first VIF was established in *Cucurbita moschata* through ectopic expression of *Arabidopsis thaliana* FT (AtFT) proteins from *Zucchini yellow mosaic virus* (ZYMV) to induce early flowering (Lin et al., 2007; Yoo et al., 2013). Subsequently, a *Potato virus X* (PVX)-based VIF was developed to express AtFT protein or mRNA to promote flowering in short-day (SD) tobacco (*Nicotiana tabacum* Maryland Mammoth, MM) under non-flowering long-day (LD) conditions (Li et al., 2009; 2011). *Apple latent spherical virus* (ALSV), *Citrus leaf blotch virus* (CLBV) and *Cotton leaf crumple virus* (CLCrV) were also later used to express *FT* for floral induction in soybean, apple, pear, gentian and lisianthus plants (Yamagishi and Yoshikawa, 2011; Yamagishi et al., 2011; 2014; 2016; Fekih et al., 2016), cotton (McGarry and Ayre, 2012; McGarry et al., 2016) and citrus (Velazquez et al., 2016). More recently, we further characterized and developed the PVX-based VIF approach to assess FT protein function including investigations into the impact of single amino-acid mutations on the floral inducing function of the AtFT protein; the influence of various polypeptide tags on the AtFT activity; and the function of mono- and dicotyledonous *FT* and *FT-like* genes to induce flowering in MM tobacco under non-inductive conditions (Qin et al., 2017). These findings demonstrate that VIF represents an efficient system for functional analysis of proteins in flowering and a potential strategy to speed up crop breeding programme.

To date, VIFs have only been developed in dicotyledonous plants (dicots) (McGarry et al., 2017; Qin et al., 2017), but not for monocotyledonous species (monocots) including any economically important cereal crops. *Barley stripe mosaic virus* (BSMV) has, however, been modified as a functional genomics tool for virus-induced gene silencing (VIGS) in barley (*Hordeum vulgare* L.) and wheat

(*Triticum aestivum*) (Holzberg et al., 2002; Meng et al., 2009; Pacak et al., 2010; Yuan et al., 2011), while VIGS platforms based on *Brome mosaic virus* (BMV) in rice (*Oryza sativa* L.), barley, and maize (*Zea mays* L.; Ding et al., 2006); *Bamboo mosaic virus* in *Brachypodium distachyon* (Liou et al., 2014); *Rice tungro bacilliform virus* in rice (Purkayastha et al., 2010) and *Cucumber mosaic virus* in maize have also been reported (Wang et al., 2016). Recently, *Foxtail mosaic virus* (FoMV) was engineered as an effective VIGS system to induce gene silencing in barley, wheat, sorghum (*Sorghum bicolor*), foxtail millet (*Setaria italica*), green foxtail (*Setaria viridis*) and the sweet corn line Golden 3 Bantam (Liu et al., 2016; Mei et al., 2016). Moreover, BSMV and FoMV have been exploited to allow over-expression of endogenous and exogenous proteins in both dicots and monocots (Zhang et al., 2019; Liu and Kearney, 2010; Bouton et al., 2018; Cheuk and Houde, 2018), indicating these viruses have the prospective for being modified as valuable VIF tools in monocots.

FoMV, like PVX, is a positive-sense single-stranded (ss) RNA potexvirus. FoMV can infect 56 Poaceae species and at least 35 dicot species (Paulsen and Niblett, 1977; Short and Davies, 1987). The FoMV genome consists of a 5'-methylguanosine cap and a 3'-polyadenylated tail. It encodes an RNA-dependent RNA polymerase (RdRP), three movement proteins expressed from the triple-gene block, coat protein (CP) and a small unique 5A polypeptide (Robertson et al., 2000; Bruun-Rasmussen et al., 2008). Similar to PVX, FoMV has been used to silence (Ruiz et al., 1998; Lin et al., 2008; Zhou et al.; 2012; Chen et al., 2015a; 2015b Zhao et al., 2016) or over-express genes in plants (Liu et al., 2016; Mei et al., 2016; Bouton et al., 2018). To test whether FoMV can be used for VIF (designated FoMViF hereafter) in monocots, we took the advantage of the FoMV vector previously developed in our groups (Liu et al., 2016) to express four flowering time genes including *AtFT*, rice *Hd3a*, tomato (*Solanum lycopersicum*) *SFT* and tobacco *NtFT4* (Kojima et al., 2002; Lifschitz et al., 2006; Harig et al., 2012) in proso millet (*Panicum miliaceum* L.) and wheat. We have demonstrated that expression of *FT* genes from FoMV was able to trigger early flowering in monocot crops, therefore successfully established a virus-induced flowering assay FoMViF in cereal crops.

Materials and Methods

Plant Materials and Growth Conditions

Plants of *N. benthamiana*, proso millet (*Panicum miliaceum* L. CGRIS 00000390) and winter wheat (*Triticum aestivum* L, 2n=6x=42, AABBDD, originated from the elite variety YZ4110) were grown under long-day (LD, 16 h light/8 h dark) or short-day (SD, 8 h light/16 h dark) conditions at 23°C. For vernalization, wheat were sown, germinated and grew at 4°C for 4 weeks before being transferred to grow at 23°C under LD.

Construction of FoMViF Vectors

The Arabidopsis, rice, tomato, tobacco *FT* homologous genes were PCR amplified from using plasmid PVX/AtFT, PVX/Hd3a, PVX/SFT, and PVX/NtFT4 (Qin et al., 2017) as DNA templates, PrimeSTAR HS DNA polymerase and four sets of primers FoMV-AtFT-F/R, FoMV-Hd3a-F/R, FoMV-SFT-F/R and FoMV-NtFT4-F/R (Table S5) respectively. The PCR products were digested with *Hpa*I and *Asc*I, and cloned into the *Hpa*I/*Asc*I sites of the FoMV-sg vector (Liu et al., 2016) to produce FoMV/AtFT, FoMV/Hd3a, FoMV/SFT and FoMV/NtFT4 (Fig. 1B). To in-frame fuse the 3xFLAG to each of the FT open reading frame, we first amplified the 3xFLAG sequence using the primer FoMV-FTs-FLAG-R along with FoMV-AtFT-FLAG-F, FoMV-Hd3a-FLAG-F/ FoMV-SFT-FLAG-F or, FoMV-NtFT4-FLAG-F (Table S5), respectively. The PCR reaction contained 0.5µl 10mM primer F/R, 0.5µl 2.5mM dNTPs, 1µl 10ng/µl template, 2µl 10X PrimeSTAR HS DNA polymerase buffer, 0.1µl PrimeSTAR HS DNA polymerase and 15.4µl ddH_2O. The amplification was carried out with 30 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 30 seconds, followed 1 cycle of 72 °C for 10 min and then at 4°C. The amplified fragments were then assembled by Seamless Cloning (pEASY-Uni Seamless Cloning and Assembly Kit, TransGen Biotech) into the AscI-linearized FoMV/AtFT, FoMV/Hd3a, FoMV/SFT or FoMV/NtFT4 to generate FoMV/AtFT-FLAG, FoMV/Hd3a-FLAG, FoMV/SFT-FLAG and FoMV/NtFT4-FLAG (Fig. 1B). All FoMViF constructs were confirmed by nucleotide sequencing. Each of the FoMViF vectors was mobilized into *Agrobacterium*

tumefaciens GV3101 as previously described (Hong et al., 1996), confirmed by PCR and *Agrobacterium* glycerol stocks were made and kept at -80°C freezer.

Enrichment of FoMV vectors in Nicotiana benthamiana

Agrobacterium tumefaciens GV3101 containing each of the FoMV vectors were freshly cultured from *Agrobacterium* stocks. Overnight cultures were then centrifuged at 8,000 rpm for 10 min and resuspended in sterile distilled water to make the final OD_{595} to 1.0. *Agrobacterium* suspension (OD_{595}: 1.0) was then infiltrated into young leaves of *N. benthamiana* at six-leaf stage through needleless 0.5-ml syringe. Plants were then grown in an insect-free growth room at 23°C under LD. FoMV infection normally caused mild leaf curling and mosaic symptoms on systemic leaves at 10-14 days after agroinfiltration. At this stage, young leaf tissues were collected and used directly for FoMViF assays or freeze-dried and stored at -80°C freezer for later use.

FoMViF in proso millet and wheat

Sap was prepared by grinding 1 gram of leaf tissues of healthy or FoMV-infected *N. benthamiana* in 1 ml TE (10mM Tris-1mM EDTA buffer, pH8.0). Young leaves of 5-8 proso millet plants or wheat plants without vernalization treatment at 2-3 leaf stage (17-26 days after sowing seeds; DASS) were mechanically inoculated with sap (Liu et al., 2016). Such inoculation should be handled with care because heavy-handed rubbing of leaves sometimes damaged young plants and led them to premature death. For mock controls in all FoMViF experiments, plants were inoculated with healthy *N. benthamiana* leaf sap prepared in TE buffer. Plants were then grown at 23°C under the non-inductive LD conditions in insect-free growth rooms. Local and systemic viral symptoms, flowering time and spikelet development in millet and wheat plants were daily examined after sap inoculation, and phenotypically recorded using a Nikon D7000 camera. Sap inoculation of proso millet or wheat often resulted in 100% plant infection. Each set of FoMViF assays was repeated at least twice. For statistical analysis, equally squared variances and two-tailed Student's ***t***-tests against FoMV were carried out to verify if changes of flowering time in these FoMViF assays would show statistically

199 significant differences.

200 *RT–PCR*

201 Total RNA was extracted from proso millet and wheat leaf tissues with obvious viral
202 symptoms at 7-14 days post-inoculation. RNA was also isolated from healthy leaves
203 that were mock-inoculated and used as controls. First-strand cDNA was synthesized
204 from DNase I-treated total RNAs by M-MLV Reverse Transcriptase according to the
205 manufacturer's instructions (Promega). RT-PCR was performed to detect virally
206 expressed *FT* RNAs using a set of primers FoMV seq-F and FoMV seq-R. The PCR
207 reaction contained 0.5μl 10mM primer F/R, 0.5μl 2.5mM dNTPs, 2μl 1/10 cDNA
208 template, 10μl 2xTaq MasterMix (CWBIO) and 8.5μl ddH_2O. The amplification was
209 carried out with 40 cycles of 95 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec.
210 The resulting RT-PCR products were isolated and directly sequenced using primer
211 FoMVseq-F (Table S5) as previously described (Qin et al. 2017). Proso millet and
212 wheat 18S rRNAs were served as the internal control (Table S5). Densitometric analysis
213 of RT-PCR bands was performed using ImageJ software (National Institutes of Health)
214 to show the stability of recombinant viruses. Intensity of the larger or small bands in
215 individual agarose gels were measured using the ImageJ software following software
216 supplier's guidance. The relative RNA levels were determined by the ratio of intensities
217 of upper (recombinant virus) band against lower (wild-type virus) band.

218 *Protein extraction and Immumoblot analysis*

219 Total protein was extracted from symptomatic young leaf tissues at 7-14 days post
220 inoculation using extraction buffer containing 50mM Tris, pH7.5, 150mM NaCl, 2mM
221 EDTA, 5% glycerol, 0.1% Tween 20, 1mM dithiothreitol, and 0.2mM PMSF (Hong et
222 al., 1996). Protein was also extracted from healthy leaves that were mock-inoculated
223 collected and used as controls. 10 μg of total proteins were separated by electrophoresis
224 on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to a
225 nitrocellulose membrane (Bio-Rad), blotted with 1:2000 mouse anti-FLAG
226 (Sigma-Aldrich) antibodies, detected by 1:5000 goat anti-mouse IgG horseradish

peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) following the ECL chemiluminescence detection protocol. Ponceau S staining was used as show to an equal loading of total protein samples.

Results

FoMV-induced flowering (FoMViF) in monocots

We have previously described the development of the FoMV-based vector to deliver hairpin double-stranded (ds) RNAs in order to silence genes in monocot plants (Liu et al., 2016). In this vector, the FoMV CP subgenomic RNA promoter was duplicated upstream of the original CP promoter. We envisaged that the duplicated promoter, as those designed in the PVX vector (van Wezel et al., 2002), could also direct the biosynthesis of an extra subgenomic RNA from the recombinant viral genome as mRNA to promote efficient translation of the protein of interest. This is how flowering time genes could be virally expressed via FoMV in monocot plants (Fig, 1). The procedure of the FoMViF assay is outlined in Fig. 1A and can be achieved as follows: (1) The *FT* gene (or any flowering gene) is cloned into the binary FoMV vector (Fig. 1B) and verified by sequencing. (2) The recombinant FoMV vector is mobilised into *Agrobacterium tumefaciens* GV3101 or LBA4404. (3) *Agrobacterium* carrying the binary FoMV vector can be directly injected into the stem tissues, a procedure called agroinjection, to inoculate monocot plants for FoMViF. Alternatively, we often include an extra step where the *Agrobacterium* carrying the FoMV vector is first infiltrated into *N. benthamiana* leaves via agroinfiltration to enrich the titre of recombinant FoMV inoculum for the subsequent infection of the monocot plants. Systemic *N. benthamiana* leaf tissues infected with FoMV are collected, and the fresh leaf materials can be either used directly or freeze-dried for storage at -80°C for later use. Sap is prepared by grinding fresh or dried leaf tissues in 10mM Tris-EDTA buffer (pH8.0); and mechanically inoculated onto young leaves of monocot plants. Plants are then grown under LD or SD conditions and monitored for floral induction (Li et al., 2009; Qin et al., 2017).

In this work we cloned both free and 3xFLAG-tagged, Arabidopsis *AtFT*, rice *Hd3a*, tomato *SFT* and tobacco *NtFT4* into the FoMV vector (Liu et al., 2016) and produced FoMV/AtFT, FoMV/Hd3a, FoMV/SFT, FoMV/NtFT4, FoMV/AtFT-FLAG, FoMV/Hd3a-FLAG, FoMV/SFT-FLAG and FoMV/NtFT4-FLAG constructs (Fig. 1B). These *FT* genes display a range of nucleotide polymorphisms (Fig. S1), although at the amino-acid level their protein products are conserved (Fig. S2). We investigated inductive and non-inductive conditions for early flowering in proso millet and the winter wheat, and then tested whether these *FT* genes could be expressed by FoMV and whether viral expression of *FT* genes could trigger early flowering in proso millet and wheat under non-inductive conditions.

Flowering conditions in proso millet and wheat

In order to test the potential application of FoMViF in monocots, we selected two cereal crops – one is the lesser-studied proso millet whilst another is the major food crop wheat. Proso millet is a C4 photosynthetic annual grass species that is well adapted to many soil and climatic conditions. It produces grains rich in starch, protein, essential amino acids, health-promoting phenolic compounds and high calcium content, but lacking gluten, thus it is particularly nutritious for people who cannot tolerate wheat. Moreover, starch derived from proso millet can be readily converted to ethanol. There is an increased demand for breeding new proso millet varieties for human nutrition products and as an alternative to corn for biofuel production (Taylor et al., 2006; Saleh et al., 2012). To test how proso millets respond to photoperiods in our laboratories, we grew proso millet at 23°C under SD (8-hr light/16-hr dark), LD (16-hr light/8-hr dark) or LD/SD mixed conditions (Fig. 2). Under SD plants started to produce millet spikelet at an average of 56 days after sowing seeds (DASS) (Fig. 2A). However, at this time under LD, no spikelet developed, but development of millet spikelet was delayed by 3-5 weeks (Fig. 2B). The appearance of spikelet in proso millet occurred at approximately 88 DASS on average under LD (Fig. 2C, Table 1). We also germinated seeds and grew plants initially under LD for 4 weeks, then changed the growth condition to SD. Under such LD/SD transfer conditions, proso millet started to flower and produce spikelet at

about 63 DASS (Fig. 2D). These data show that proso millet is a facultative SD plant, consistent with previous characterization (Kumar et al., 1977).

For the hexaploid wheat, vernalization treatment under cold temperature is essential for flowering/heading (Zhang et al., 2019). In our laboratories we found that giving wheat a vernalization period of 4 weeks at 4°C and then growing at 23°C under LD conditions (16-hr light/8-hr dark) induced flowering/heading at approximately 90 DASS (Fig. 2E). Under such conditions, wheat plants with mock inoculation or treated with the empty FoMV or the four FoMViF vectors all started to flower in the same time (Fig. S3). Therefore, these conditions could not be used for our FoMViF assays in wheat. However, without vernalization, wheat remained vegetative at 23°C under LD settings and no flowering/heading was observed at 126 DASS or later. Thus, we choose the less inductive LD (for proso millet), or LD without vernalization treatments (for wheat) to examine whether FoMViF could trigger early flowering/heading and spikelet development in these monocot plants.

FoMViF induces early flowering in proso millet

Although agroinjection could be directly used to inoculate FoMV into monocot plants, we preferred to use higher titer viral inoculum enriched via agroinfiltration of *N. benthamiana* in our FoMViF assay (Fig. 1A). We mechanically inoculated young leaves of proso millet plants with virus-infected *N. benthamiana* leaf sap at the 3-leaf stage (21 DASS) and found that this led to almost 100% occurrence of FoMV infection (Fig. 3). Compared to proso millet with mock-inoculation (Fig. 3A), plants inoculated with FoMV, FoMV/AtFT, FoMV/Hd3a, FoMV/SFT or FoMV/NtFT4 developed local infections approximately 1 week post-inoculation (i.e. 28 DASS), as evidenced by development of irregular mosaic and yellowing lesions on the inoculated leaves (Fig. 3B-F). Similar viral symptoms appeared on emerging systemic young leaves and *FoMV-FT* mRNA was readily detectable by RT-PCR in these leaf tissues although we also detected a smaller band (Fig. 3G; Fig. S4; Table S1). These results suggest that some recombinant FoMViF viruses lost the inserted *FT* genes and reversed to FoMV. Systemically infected plants were often dwarfed when compared to mock-inoculated

313 healthy control plants (Fig. 3H-M). However, at an average of 63 DASS, proso millet in
314 which the rice Hd3a florigen was expressed by FoMV/Hd3a developed spikelets (Fig.
315 3K, Table 1, Table S2). Millet spikelets also appeared later in plants that were infected
316 with FoMV/AtFT (Fig. 3J) or FoMV/SFT (Fig. 3L) at 76 or 75 DASS on average,
317 respectively (Table 1; Table S2). At this time no flowering or spikelet development was
318 observed in mock-inoculated or FoMV-infected millet plants (Fig. 3H and I). These
319 control plants produced spikelets afterwards, but always later than exogenous
320 *FT*-expressing plants except for FoMV/NtFT4 which only triggered early flowering in
321 one experiment (Fig. 3M; Table 1; Table S2). Taken together, these results demonstrate
322 a successful establishment of FoMViF in proso millet, a monocot grass species.

323 *Differential activities of monocot and dicot FT genes in flowering induction in proso*
324 *millet*

325 Rice *Hd3a*, and the dicot *FT* genes to a lesser extent, when expressed from the FoMViF
326 vectors promoted early floral and spikelet development in proso millet (Fig. 3),
327 suggesting that the monocot and dicot *FT* genes had different capabilities to accelerate
328 or induce flowering in our FoMViF assays (Table 1; Table S2). We observed visible
329 FoMV/FT infection (Fig. 3C-F) and detected efficient accumulation of virally expressed
330 *FT* mRNAs in all tested plants (Fig. 3G; Fig. S4). Interestingly, *AtFT* and *SFT* showed
331 some effect on flowering induction and spikelet formation, whilst *NtFT4* had very little
332 impact on flowering and spikelet-forming time (Table 1, Table S2). In contrast,
333 expression of the rice *Hd3a* gene from FoMV/Hd3a induced floral and spikelet
334 development, markedly earlier than the three dicot counterparts and controls (Table 1;
335 Table S2). Considering the equivalent expression levels of viral *FT* transcripts (Fig. 3G)
336 but varied flowering time (Fig. 3J-M; Table 1; Table S2) in proso millets treated with
337 different FoMViF vectors, we concluded that the monocot rice *Hd3a* gene is the most
338 efficient at inducing flowering in proso millet among the four tested monocot and dicot
339 *FT* genes.

340 *Expression of FLAG-tagged FT proteins affects flowering time in proso millet*

To further investigate the correlation of *FT* gene expression with the induction of early flowering in proso millets under LD conditions, we used 3xFLAG to tag Hd3a, AtFT, SFT and NtFT4 (Fig. 1B; Fig. 4). In plants that were infected with FoMV/AtFT, FoMV/Hd3a, FoMV/SFT or FoMV/NtFT4, we were able to detect both the *FoMV-FT-FLAG* mRNA by RT-PCR (Fig. 4A) and the FLAG-tagged FT proteins probed with a specific anti-FLAG antibody (Fig. 4B and C). Expression of both *FT* transcripts and protein products was clearly linked with the viral infection of these plants (Fig. S5). No virally expressed *FT* RNA or FT protein was detected in mock-inoculated proso millets, although FoMV RNA but not FT protein was readily detectable in FoMV-infected plants (Fig. 4A-C). Expression of the FLAG-tagged monocot (Hd3a-FLAG) and dicot FT proteins (AtFT-FLAG, SFT-FLAG and NtFT4-FLAG) was able to induce early flowering when compared to mock-inoculated or FoMV-infected control plants (Fig. 5A-F; Table 2; Table S3). We also noticed a negative impact of the FLAG tag on the florigenic activity of the free rice Hd3a protein. However, the influence of the FLAG tag on the functionality of dicot FT proteins was less obvious (Table 1; Table 2). Consequently, the four FT-FLAG fusion proteins enabled plants to flower 4-11 days earlier although they did not show much difference among each other to shorten flowering time in proso millets under LD conditions (Fig. 5; Table 2; Table S3).

FoMViF in wheat

To test the feasibility of the FoMViF assay in other monocots, we mechanically inoculated young wheat plants at 2-3 leaf stage (approximately 21 DASS) with sap in 10mM Tris-1mM EDTA buffer (pH8.0) produced from healthy *N. benthamiana* (mock) or plants infected with FoMV/Hd3a (Fig. 1A; Fig. 6). Mock-inoculated controls and virus-infected wheat plants were then grown at 23°C under LD without vernalisation (*i.e.* non-flowering inductive conditions). As we previously reported, FoMV could effectively infect wheat (Liu et al., 2016). Local chlorosis and yellowing developed on sap-inoculated leaves at approximately 1-week post-inoculation (*i.e.* 28 DASS; Fig. 6A and B), and systemic infection occurred subsequently. No flowering/heading and no

grain development were observed in mock-inoculated wheat plants at 8-weeks post-inoculation (*i.e.* 84 DASS; Fig. 6C), at which time (approximately 77 DASS on average) the first heading was clearly appearing on wheat plants that were infected with FoMV/Hd3a (Fig. 6D; Table S4). At 119 DASS, control plants remained vegetative and underwent no reproductive transition (Fig. 6E) whilst wheat plants with virally expressed Hd3a developed the 2nd, 3rd and 4th heads at this stage (Fig. 6F and G). Indeed, even after the wheat grains fully matured in plants in which Hd3a was expressed from FoMV/Hd3a, no obvious heading/flowering was noticeable in all control plants, although these plants (including mock-inoculated and FoMV- or FoMV/Hd3a-FLAG infected wheats) started to develop heading at very late stage (Fig. 6G; Table S4). Moreover, the induction of flowering and development of spikelet were well-correlated with the expression of the *Hd3a* transcripts by FoMV/Hd3a in virus-infected wheat, even if the level of the *Hd3a* mRNA was relatively low (Fig. 6H; Table S1). The FLAG-tagged Hd3a fusion protein was readily detected in wheat plants infected with FoMV/Hd3a, but not FoMV or mock treatment (Fig. 6I; Fig. S6). These results show that FoMViF is functional in the economically important cereal crop wheat.

Discussion

Plant viruses consist of either RNA or DNA genome. These viral genomes can be modified as RNA delivery vehicles and gene expression vectors for functional genomics in plants. For instance, VIGS has emerged as a powerful tool to inhibit gene expression either at the transcriptional, post-transcriptional or translational level for dissecting gene function in dicots and monocots, including important crops recalcitrant to classical forward or reverse genetic manipulation (Liu et al., 2002; Becker and Lange, 2009; Tang et al., 2010; Kanazawa et al., 2011; Senthil-Kumar and Mysore, 2011; Sha et al., 2014; Chen et al., 2015c; Qin et al., 2015). Using viruses to deliver small guide RNA can lead to virus-induced genome editing in plants (Baltes et al., 2014; Yin et al., 2015). Virus-based technology has also been used to study RNA signalling that are associated with different plant physiological processes such as flowering and tuberization (Li et al.,

2009; 2011; Cho et al., 2015). On the other hand, transient over-expression of exogenous or endogenous genes from engineered viruses can result in gain-of-function or virus-induced gene complementation in plant development and growth, fruit ripening, plant response to biotic stresses and viral DNA replication (Hong et al., 1997; Van Wezel et al., 2002; Hong et al., 2003; Lin et al., 2008; Zhou et al., 2012; Kong et al., 2013; Bouton et al., 2018). Moreover, VIF is the latest example of how plant viruses can be exploited for the benefit of fundamental research in the field of flower physiology and practical applications in facilitation of plant breeding (McGarry et al., 2017; Qin et al., 2017). It should be noted that VIGS is completely different from VIF. VIGS is a means to knock-down target gene expression via homologous RNA-mediated degradation of target mRNA, whilst VIF such as FoMViF involves using virus vector to transiently over-express flowering time gene to induce early flowering in plants. Thus, the two-plant virus-based technologies differ in principle.

Since the early VIF assay was reported in cucurbit (Lin et al., 2007) and tobacco (Li et al., 2009), several VIF systems have been developed in dicots including cotton and fruit trees (Yamagishi and Yoshikawa, 2011; McGarry and Ayre, 2012; Yamagishi et al., 2011; Yamagishi et al., 2014; Fekih et al., 2016; McGarry et al., 2016; Velazquez et al., 2016; Yamagishi et al., 2016; Qin et al., 2017; McGarry et al., 2017). These works reveal the mechanism about how VIF operates and the potential of VIF for the study of plant reproductive biology and in crop breeding (McGarry et al., 2017; Qin et al., 2017). However, no VIF has been yet developed for any cereal crops such as wheat, rice, maize and millets that are essential for human nutrition, global food security, agriculture, and biofuel industry. Lack of VIF in monocots may be due to a shortage of suitable viruses for development of such technology. Unlike VIGS vectors which only involve delivery of a short fragment of translatable or non-translatable RNA homologous to the target genes to plant cells, virus vectors for VIF or production of functional proteins need to have the capacity to express entire mRNA from which protein products can be translated. Only a few monocot-infecting viruses such as BSMV, *Wheat streak mosaic virus*; *Triticum mosaic virus* and FoMV have been shown to have this capacity (Tatineni et al., 2015; Bouton et al., 2018; Cheuk and Houde, 2018). Here we demonstrate that

FoMV can be exploited to express *FT* genes and induce early flowering and spikelet development in grass proso millet (Figs 1-5; Tables 1-2; Tables S2-S3) and cereal crop wheat (Fig. 6; Table S4).

Stability is essential for using recombinant viruses as toolbox for gene expression in plants. Recombinant viruses are known to be unstable and commonly undergo rearrangements to its native form during infection. Moreover, longer infection times likely undergo rearrangements to their native forms during infection. These are potential issues for using recombinant virus technology in plants. Indeed, in our FoMViF assays loss of inserted *FT* genes from recombinant FoMV genome occurred during viral infection of proso millet and wheat plants (Fig. 3G; Fig. 4A; Fig. 6H; Table S1). On the other hand, florigenic FT proteins, once expressed during the early stage of plant development, would prime and initiate the vegetative-to-reproductive transition and then lead to flowering. This implies that no persistent production of FT mRNA and protein is needed at the later stage of plant growth. In this regard, stability of recombinant viruses as such may not impose a significant challenge or weakness for VIF technology, such as FoMViF described here. This notion is supported by the finding that even if a population of FoMViF vectors converted back to FoMV in proso millet and wheat (Table S1), these plants with virally expressed FT proteins still flowered early (Fig. 3; Fig. 4; Fig. 6). Indeed, loss of recombinant sequences, and reversion to a naturally occurring structure can be argued as advantageous for containment.

Another concern associated with VIF and gene expression from recombinant viruses in general is the potential VIGS effect. Since FoMV is a ssRNA virus, it has a dsRNA intermediate which are active triggers for VIGS in plants. As such, many ssRNA viruses are commonly used for VIGS and less commonly for gene delivery and protein expression. However, this does not seem to be an issue for FoMViF. Firstly, FT proteins, and the phosphatidylethanolamine-binding protein domains particularly are conserved among different plant species (Turck et al., 2008; Fig. S2). At the nucleotide level, the overall homologies of *FT* genes and mRNAs are also notably high. However, these mRNA sequences are not identical, instead nucleotide polymorphisms are common. No

20 or longer nucleotides are identical among the *FT* mRNAs (Fig. S1; *unpublished data*). Evidently, there is insufficient homology between the millet *PmFT* and the rice or dicot *FTs* to result in silencing. Secondly, our FoMViF assays were carried out under non-flowering inductive conditions. This implies that little or no endogenous FT mRNA would be produced and thus there would be little or no target mRNA for VIGS to occur. Thirdly, viral expression of florigenic FTs could occur during the early and/or later infection stage, thus, triggering plant to flower no matter whether VIGS would be induced later or not. This is evident as viral expression of *FT* genes even native *PmFT* (*unpublished data*), can result in early flowering in proso millet.

Apart from these potential disadvantages, the FoMViF has several advantages including (1) the monopartite genome makes FoMV much easier to handle. Moreover, the enriched viral inoculum in *N. benthamiana* is highly infectious in monocot plants (this work; Liu et al., 2016; Bouton et al., 2018). (2) The broad spectrum of dicot and monocot host plants allow FoMViF and other FoMV-based technology applicable to many of the most important food and biofuel crops such as wheat, millets and maize. This is particularly useful for functional genomics in monocot crops including wheat and millets in which genetic manipulation is difficult or no transformation system is yet available. (3) As shown in our work here and those recently reported by Bouton et al (2018), FoMV can be utilized to efficiently express small and large proteins for "gain-of-function" analysis in monocots. (4) The differential activities among mono- and dicot *FT* genes, as well as impacts of an FLAG tag on FT function revealed in our work provide choices of florigenic genes for FoMViF-based cereal crop breeding programme dependent on various degrees of requirements for floral and spikelet/grain induction.

Our FoMViF demonstrate that heterologous *FT* genes from either monocot (rice) or dicot (tomato and *Arabidopsis*) plants can trigger early flowering and early spikelet development in monocotyledonous proso millet (Fig. 3), and wheat in the case of Hd3a (Fig. 6), consistent with our previous finding that these *FT* genes were also able to induce early flowering in the PVX-based VIF system in dicot tobacco plants (Qin et al., 2017). Interestingly, the rice *Hd3a* was equally efficient as *SFT*, *AtFT* and *NtFT4* to

induce flower in dicot tobacco plants under non-inductive conditions (Qin et al., 2017). By contrast, the three dicot *SFT*, *AtFT* and *NtFT4* seem much less active than *Hd3a* in monocot proso millet (Fig. 3; Table 1) although their protein products share high amino acid identities (Fig. S2). We also found that the FLAG tagging could affect the function of FT proteins (Fig. 5; Fig. 6). These results further support that the photoperiodic *FT*-mediated pathway in floral induction is conserved among different plants (Turck et al., 2008); but also imply that this pathway may have undergone evolutionary divergence in dicot and monocot species. Moreover, the FoMViF works in C3 wheat (Fig. 6), reaffirming the idea that the technology can be applied to both C3 and C4 crops.

Conclusions

Virus-induced flowering (VIF) has recently attracted extensive interest for its practical applications in accelerating breeding in dicotyledonous plants and woody fruit-trees. We now extend this technology to a monocot grass and a cereal crop. Using the FoMV-based VIF system, we showed that expression of *FT* orthologues can promote early flowering and development of spikelet in proso millet, a C4 grass species with potential for nutritional food and biofuel resources, and in wheat, a main food crop worldwide. Floral induction in the two monocot plants is caused by the virally expressed *FT* genes, and the florigenic activity of rice *Hd3a* was more pronounced than its dicotyledonous counterparts in proso millet. This system is easy to perform and the efficacy to induce flowering is high. In addition to proso millet and wheat, we envisage that FoMViF will be also applicable to many important food and biofuel monocots in terms of functional genomics as well as molecular breeding for cereal improvement.

Supporting information

Fig. S1 Comparisons of tomato *SFT*, tobacco *NtFT4*, and rice *Hd3a*, wheat *TaFT1* and Arabidopsis *AtFT* nucleotide sequences

Fig. S2 Comparisons of Arabidopsis AtFT, tobacco NtFT4, tomato SFT, rice Hd3a, and

wheat TaFT1 protein sequences

Fig. S3 Vernalization rescinds impact on FoMViF to induce early heading/flowering in wheat

Fig. S4 Sequencing confirmation of heterologous *FT* gene expression in proso millet

Fig. S5 Systemic symptoms of FoMV infection in proso millet

Fig. S6 Original blot and PAGE gel used for Fig. 6I.

Table S1 Stability of recombinant FoMViF vectors in plants

Table S2 Data for FoMViF in proso millet

Table S3 Data for FLAG-tagged FoMViF in proso millet

Table S4 Data for FoMViF in wheat

Table S5 Primers used in this work

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Data Availability

Data supporting the findings of this work are available within the paper and its Supporting information files.

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TABLE

Table 1 FoMViF in proso millet[*]

	Expt I	Expt II	Expt III	Expt I-III	P value[**]
Mock	78.2 ± 1.9	91.3 ± 2.3	92.7 ± 3.3	87.4 ± 8.0	0.3733
FoMV	74.6 ± 4.6	80.0 ± 0.0	86.5 ± 5.1	80.4 ± 6.0	n.a.
FoMV/AtFT	73.4 ± 6.8	72.8 ± 1.5	82.8 ± 3.9	76.3 ± 5.5	0.4892
FoMV/Hd3a	59.6 ± 1.1	59.5 ± 1.0	68.3 ± 1.5	62.5 ± 5.1	**0.0147**
FoMV/SFT	72.0 ± 1.6	71.0 ± 0.7	83.0 ± 5.0	75.3 ± 6.7	0.3117
FoMV/NtFT4	71.6 ± 4.3	83.4 ± 0.5	86.3 ± 2.9	80.4 ± 7.8	0.9308

[*]Raw data for flowering time (Days after sowing seeds, DASS) in each experiment are shown in Table S2. The flowering time (DASS) in each experiment and overall is represented as Mean ± SD. [**]P values generated in equally squared variances/two-tailed Student's ***t***-test against FoMV for the overall flowering time are shown. P≤0.05 is regarded to have a statistically significant difference. n.a.: not applicable. As demonstrated in individual experiments (Table S2), only viral transient expression of rice Hd3a triggered significantly early flowering.

Table 2 FLAG-tagged FoMViF in proso millet*

	Expt I		Expt II	
	DASS	P value**	DASS	P value**
Mock	80.0 ± 0.0	0.006	80.0 ± 2.3	0.08
FoMV	77.5 ± 1.7	n.a.	77.0 ± 0.0	n.a.
FoMV/AtFT-FLAG	69.5 ± 3.7	0.008	73.6 ± 1.1	0.001
FoMV/Hd3a-FLAG	66.6 ± 2.1	6.67×10^{-5}	73.4 ± 0.9	9.62×10^{-5}
FoMV/SFT-FLAG	67.8 ± 3.6	0.002	73.2 ± 1.7	0.012
FoMV/NtFT4-FLAG	70.7 ± 3.5	0.018	72.6 ± 1.8	0.005

*Raw data are available in Table S3. Flowering time is shown in days after sowing seeds (DASS; Mean ± SD) in each experiment. **P values generated in equally squared variances/two-tailed Student's ***t***-test against FoMV are shown. P≤0.05 is regarded to have a statistically significant difference. n.a.: not applicable.

FIGURE LEGENDS

Fig. 1 A FoMV-induced flowering (FoMViF) assay in monocotyledonous plants. A, Outline of the FoMViF procedure in monocots. B, FoMV vectors for expression of florigen genes. The genome of *Foxtail mosaic virus* (FoMV) encodes a 152Kd RNA-dependent RNA polymerase (RDRP), three movement proteins (26K, 11.3K and 5.8K), coat protein (CP) and the small 5A polypeptide. The duplicated CP subgenomic RNA promoters are indicated. The cDNA copy of the FoMV genomic RNA is under the control of the enhanced CaMV 35S promoter and the NOS terminator in a plant binary vector (Liu et al., 2016). The left and right borders (LB and RB) of the Ti-plasmid are indicated. Arabidopsis *AtFT*, rice *Hd3a*, tomato *SFT* and tobacco *NtFT4* and their FLAG-tagged genes were cloned downstream of the duplicated CP promoter into the *Hpa*I/*Asc*I sites of the FoMV vector. The positions of primers FoMVseqF/FoMVseq-R (Table S5) which were used for colony-PCR screening, sequencing and RT-PCR detection are indicated along the FoMV genome.

Fig. 2 Photoperiodic effects on flowering time in proso millet and wheat. A, Proso millet plant flowered at an average of 56 DASS in SD. B, Proso millet plant remained vegetative at 56 DASS in LD. C, Vegetative-reproductive transition in proso millet in LD. Plants started to flower at an average of 88 DASS in LD. D, Proso millet plant flowered at 63 DASS under its growth under LD for 4 weeks and transferred to SD. E, Flowering time/heading date in wheat. Wheat plants underwent vernalization treatment for 4 weeks, then grew under LD and started to flower (heading) at approximately 90 DASS. Plants were photographed at 56 DASS (A and B); 63 DASS (D); 84 DASS (C) and 90 DASS (E). The boxed sections of each panel were enlarged to show clear phenotypes. Bar = 3 cm.

Fig. 3 Viral expression of heterologous monocot and dicot florigen genes induces flowering and early spikelet development in proso millet. A, Healthy leaf from a mock-inoculated millet plant. B-F, Viral symptoms on millet leaves infected with FoMV (B), FoMV/AtFT (C), FoMV/Hd3a (D), FoMV/SFT (E) or FoMV/NtFT4 (F). G, RT-PCR detection of virally expressed *FT* mRNA (upper panel). The sizes of the 2000-bp DNA marker as well as the positions of *FT* mRNA and FoMV RNA are indicated. The proso millet 18S rRNA was used as internal RT-PCR control (bottom panel). H-M, Early flowering and millet spikelet development. Millet plants remained

vegetative in mock (H)- or FoMV (I)-inoculated controls. Effective *FT* expression (G) induced early flowering and development of millet spikelet in plants infected with FoMV/AtFT (J), FoMV/Hd3a (K), FoMV/SFT (L) or FoMV/NtFT4 (M). Photographs were taken at 28 DASS (A-F), or 77 DASS (H-M). The boxed section of each plant was enlarged to show clear phenotypes on the top of panels of H-M, respectively. Bar = 3cm.

Fig. 4 Analysis of FT-FLAG expression in proso millet. A, RT-PCR assay of *FT-FLAG* mRNA (upper panel). Proso millet 18S rRNA served as internal control (bottom panel). B and C, Detection of FT-FLAG proteins using a specific anti-FLAG antibody. Western blot (B) and a protein PAGE gel (C) are shown. Total RNAs or proteins were isolated from plants mock-inoculated or infected with FoMV, FoMV/AtFT-FLAG, FoMV/Hd3a-FLAG, FoMV/SFT-FLAG or FoMV/NtFT4-FLAG. Sizes and positions of the 2000-bp DNA ladder or the pre-stained protein marker, positions of viral *FT-FLAG* mRNA, FoMV RNA, 18S rRNA as well as the FT-FLAG fusion proteins are indicated.

Fig. 5 Foral and spikelet induction by virally expressed FLAG-tagged FT proteins in proso millet. Proso millet plants were mock-inoculated (A), or infected with FoMV (B), FoMV/AtFT-FLAG (C), FoMV/Hd3a-FLAG (D), FoMV/SFT-FLAG (E) or FoMV/NtFT4-FLAG (F). Flowering and spikelet development under LD conditions was photographed at 67, 74 and 81 DASS.

Fig. 6 Expression of rice *Hd3a* triggers early flowering and grain production in wheat. A and B, Development of viral infection in wheat. Wheat with mock inoculation was symptomless (A). Infection by FoMV/Hd3a led to development of yellowing and chlorosis in wheat leaves (B). C-F, FoMViF in wheat. Wheat plants mock-inoculated remained vegetative and showed no sign of reproductive growth (C and E). Plants with FoMV/Hd3a infection produced early heading/flowering at 11 weeks after sowing seeds (D), and more heads were developed later on (F). Plants were photographed at 28 DASS (A and B), 84 DASS (C and D) or 119 DASS (E and F). The boxed section of plants in panels C and D was enlarged to show clear phenotypes on the top of each panel, respectively. Bar = 3cm. G, Summary of FoMViF in wheat. Flowering time (DASS) is represented as Mean±SD. Raw data for the flowering time are shown in Table S4. P values generated in equally squared variances/two-tailed Student's ***t***-test against FoMV are shown. P≤0.05 is regarded to have a statistically significant difference. n.a.: not

applicable. H, RT-PCR detection of virally expressed Hd3a transcript in wheat. Total RNA samples were isolated from plants with mock inoculation (lane 1) or plants infected with FoMV/Hd3a (lane 2). The positions of *Hd3a* mRNA and FoMV RNA as well as the 2000-bp DNA marker are indicated (upper panel). Wheat 18S rRNA served as internal control (bottom panel). I, Detection of Hd3a-FLAG proteins using a specific anti-FLAG antibody. Western blot (upper panel) and a protein PAGE gel (bottom panel) are shown (Fig. S6). Total proteins were isolated from wheat plants mock-inoculated or infected with FoMV or FoMV/Hd3a-FLAG. Three individual plants (No.1, 2 and 3) were used in case of FoMV/Hd3a-FLAG infection. Sizes and positions of the pre-stained protein marker as well as positions of the Hd3a-FLAG fusion proteins are indicated.

Fig 1

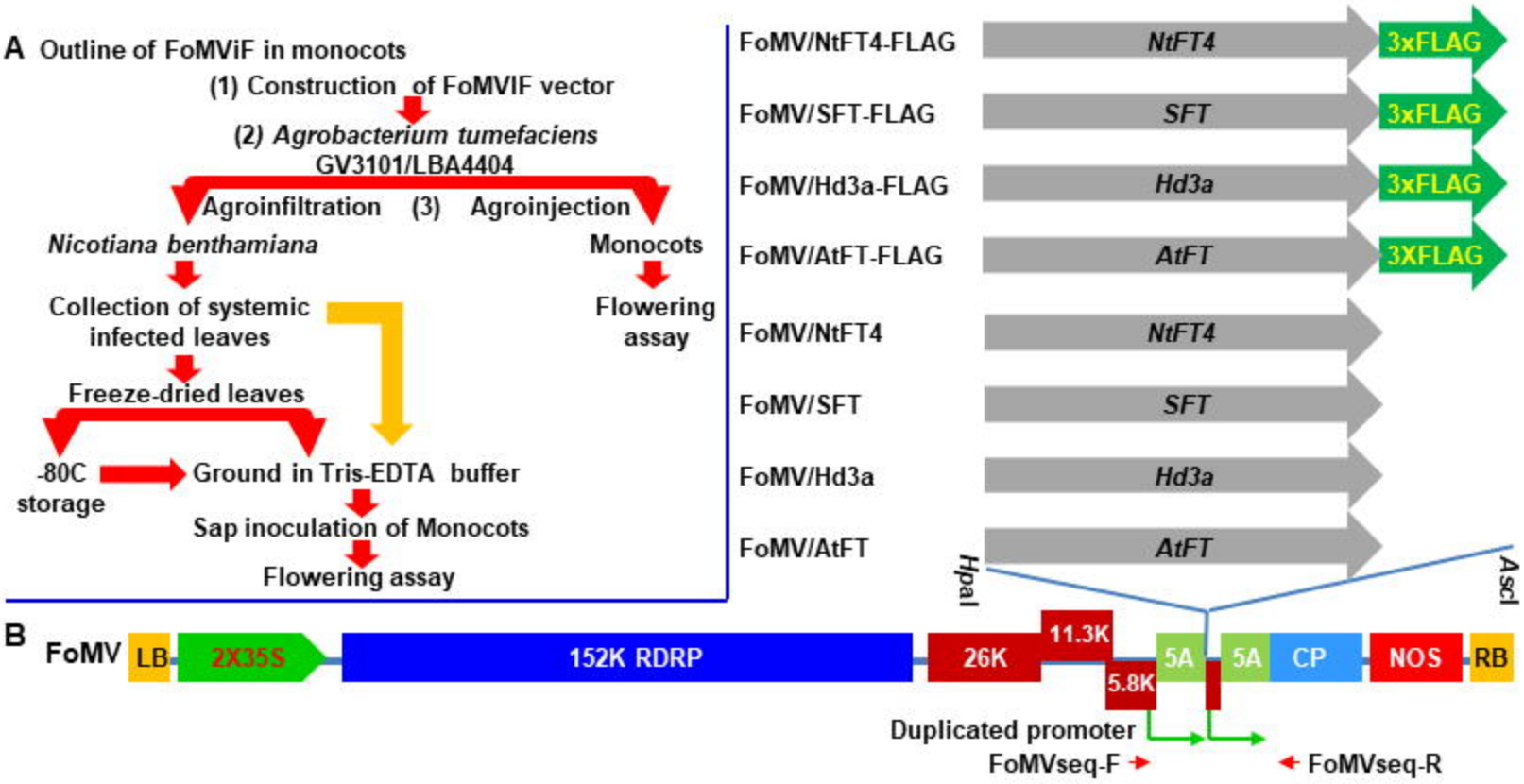

A Outline of FoMViF in monocots
(1) Construction of FoMVIF vector
(2) Agrobacterium tumefaciens GV3101/LBA4404
Agroinfiltration
(3) Agroinjection
Nicotiana benthamiana
Monocots
Collection of systemic infected leaves
Flowering assay
Freeze-dried leaves
-80C storage
Ground in Tris-EDTA buffer
Sap inoculation of Monocots
Flowering assay
FoMV/NtFT4-FLAG
NtFT4
3xFLAG
FoMV/SFT-FLAG
SFT
3xFLAG
FoMV/Hd3a-FLAG
Hd3a
3xFLAG
FoMV/AtFT-FLAG
AtFT
3XFLAG
FoMV/NtFT4
NtFT4
FoMV/SFT
SFT
FoMV/Hd3a
Hd3a
FoMV/AtFT
AtFT
HpaI
AscI
B
FoMV
LB
2X35S
152K RDRP
26K
11.3K
5.8K
5A
5A
CP
NOS
RB
Duplicated promoter
FoMVseq-F
FoMVseq-R

Fig 2

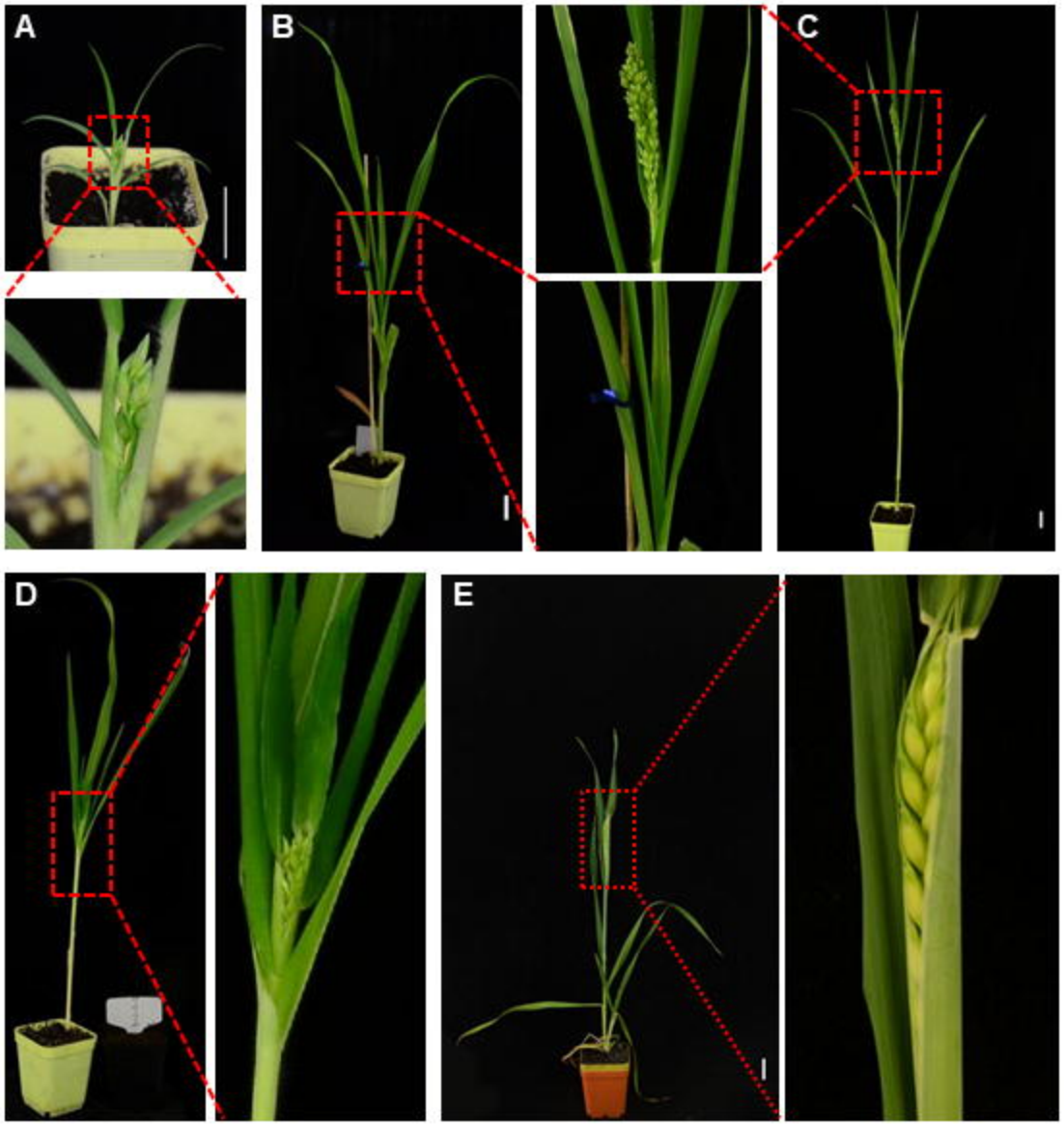


Fig 3

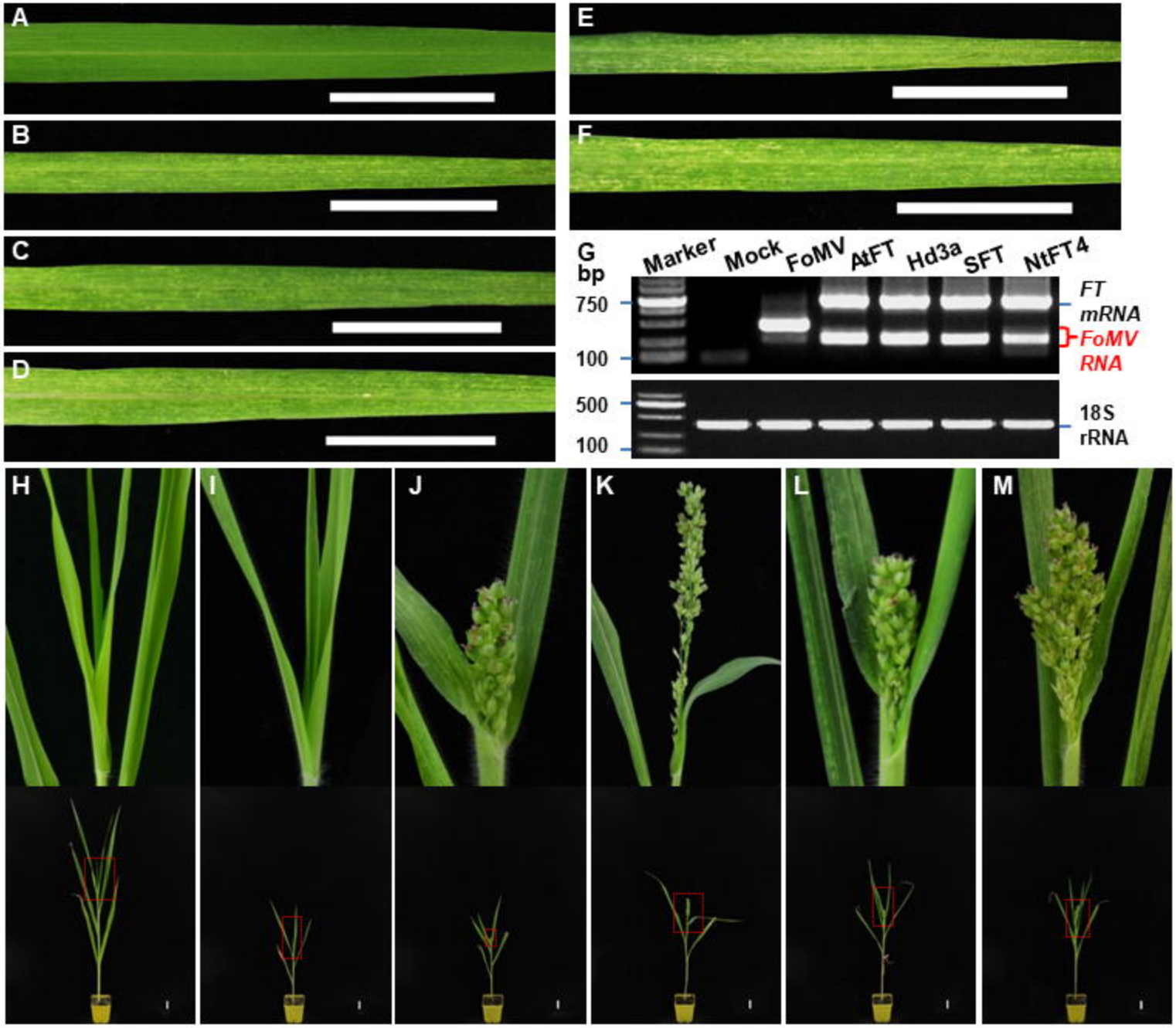

A
B
C
D
E
F
G
bp
Marker
Mock
FoMV
AtFT
Hd3a
SFT
NtFT4
750
100
500
100
FT mRNA
FoMV RNA
18S rRNA
H
I
J
K
L
M

Fig 4

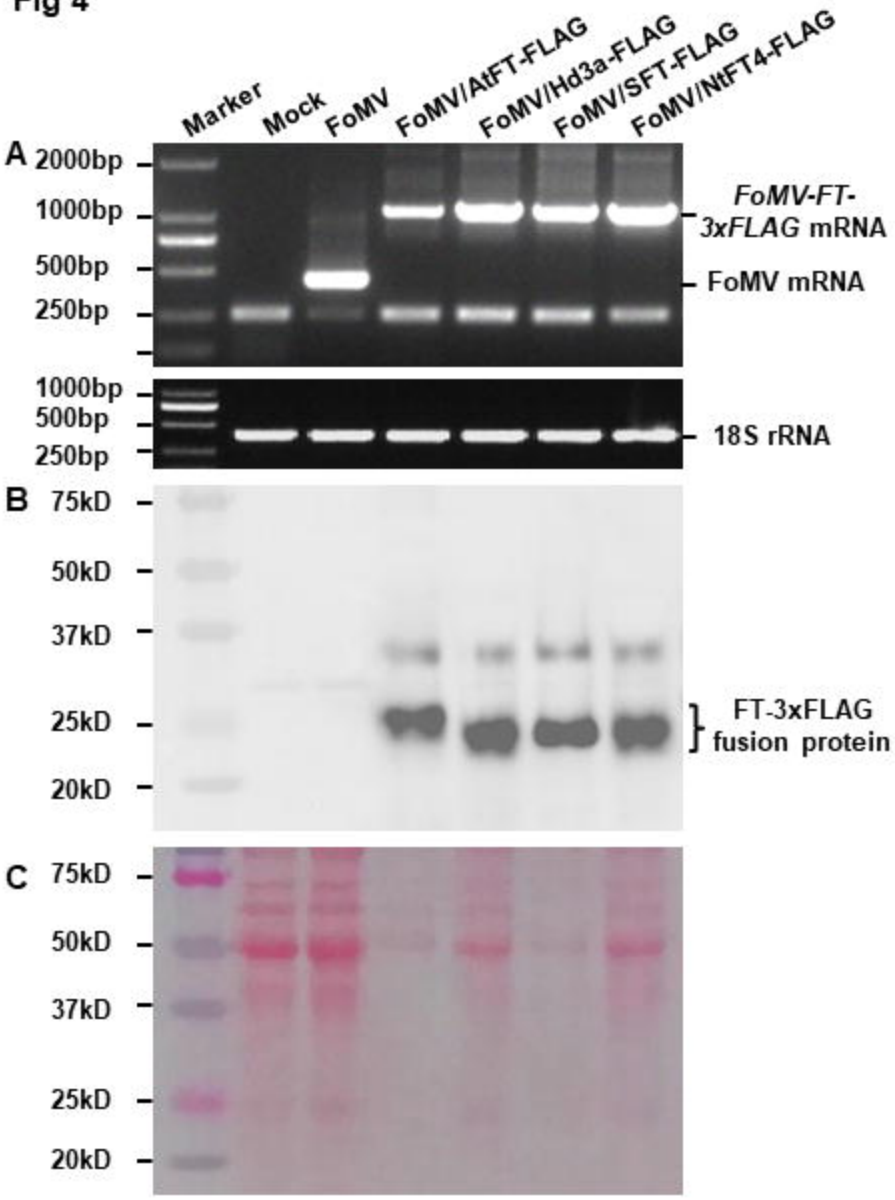

Marker
Mock
FoMV
FoMV/AtFT-FLAG
FoMV/Hd3a-FLAG
FoMV/SFT-FLAG
FoMV/NtFT4-FLAG
A
2000bp
1000bp
500bp
250bp
FoMV-FT-3xFLAG mRNA
FoMV mRNA
1000bp
500bp
250bp
18S rRNA
B
75kD
50kD
37kD
25kD
20kD
FT-3xFLAG fusion protein
C
75kD
50kD
37kD
25kD
20kD

Fig 5

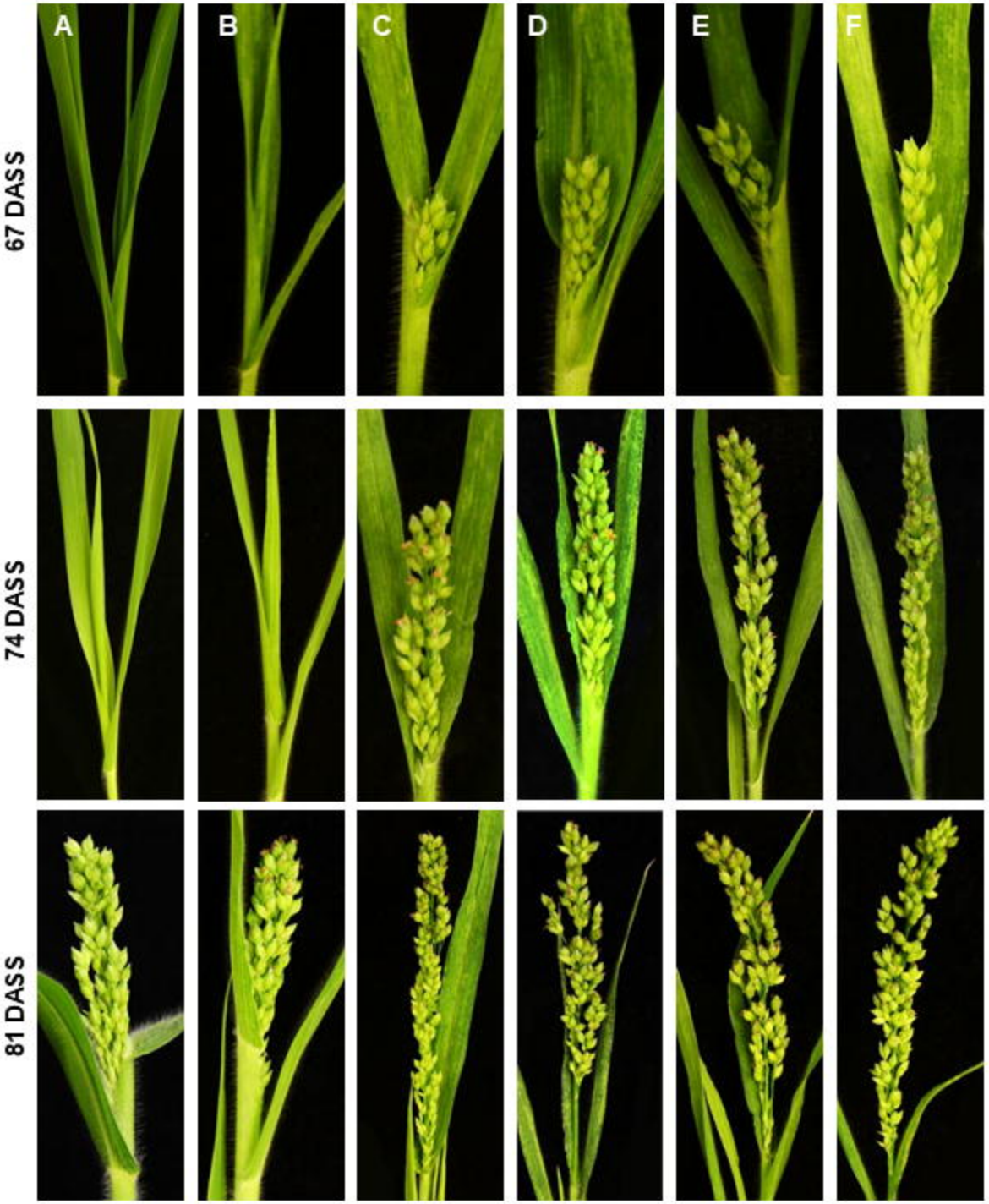
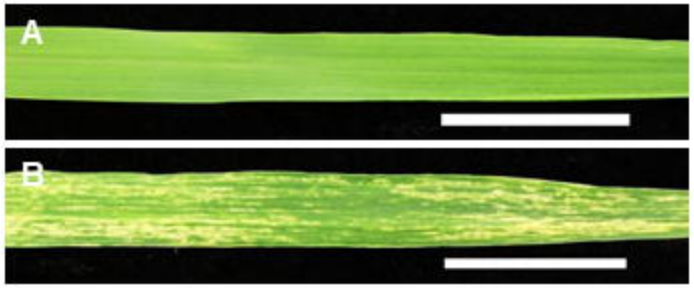
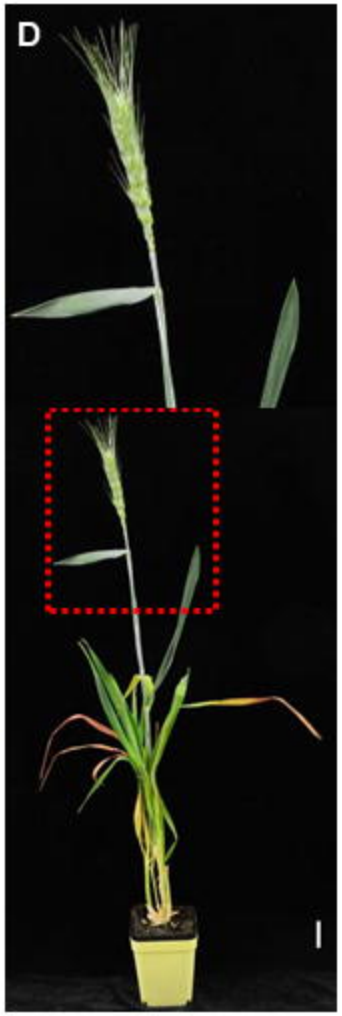
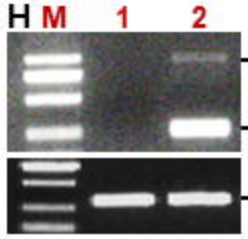


Fig 6







G

FoMViF in wheat*

	Mean ± SD	P value
Mock	134.0 ± 3.3	0.9144
FoMV	134.8 ± 13.9	n.a.
FoMV/Hd3a	76.6 ± 5.5	2.4×10^{-5}
FoMV/Hd3a-FLAG	121.0 ± 7.9	0.1731

*Raw data for flowering time (Days after sowing seeds, DASS) are shown in Table S4.