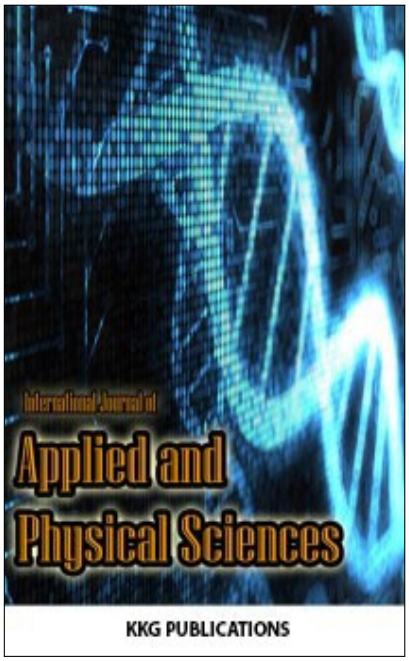


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[1, 2, 3, 4] School of Life Sciences, University of Warwick, CV4 7AL, United Kingdom

Published online: 09 November 2016

**To cite this article:** C. R. Sari, J. Taylor, Y. Hong and S. Jackson, "Development of a transient viral CRISPR expression system to manipulate flowering time in plants," *International Journal of Applied and Physical Sciences,* vol. 2, no. 3, pp. 65-70, 2016.
DOI: https://dx.doi.org/10.20469/ijaps.2.50002-3

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International Journal of Applied and Physical Sciences
2016, 2(3): 65-70

IJAPS

# DEVELOPMENT OF A TRANSIENT VIRAL CRISPR EXPRESSION SYSTEM TO MANIPULATE FLOWERING TIME IN PLANTS

CITRA RECHA SARI [1]*, JEMMA TAYLOR [2], YIGUO HONG [3], STEPHEN JACKSON [4]

[1, 2, 3, 4] School of Life Sciences, University of Warwick, CV4 7AL, United Kingdom

**Keywords:**
Flowering
FT
CRISPR-Cas9
PVX
Viral Expression

**Received:** 27 July 2016
**Accepted:** 15 September 2016
**Published:** 09 November 2016

**Abstract**. Around 180 genes have been found in Arabidopsis that control flowering time based on analysis of transgenic plants [1]. Most of these genes take part in the six main pathways controlling flowering; the photoperiod, vernalization and the ambient temperature pathways that respond to environmental conditions, while the age-dependent, autonomous, and gibberellin pathways involve a response to endogenous signals [2]. Flowering Locus T (FT) is a major gene involved in flowering in plants because FT encodes mobile florigen for floral induction. Mutation of the FT gene can result in delayed flowering. Using a transient CRISPR-Cas9 expression system mediated by virus expression, it is possible to do gene-editing in plants without incorporation of exogenous DNA. We aim to develop these approaches to manipulate flowering time in plants.



## INTRODUCTION

Flowering is one of the major developmental stages in plant growth and is essential to the reproductive success of plants. The flowering time of plants is affected by both environmental and endogenous factors that act through six major pathways to control flowering. These pathways work in different ways to enable plants to flower at the appropriate developmental stage and in the optimum environmental conditions [3]. These six pathways regulate a small number of floral integrator genes that integrate signals from multiple pathways to control flowering time. These integrator genes are Flowering Locus T (FT), Suppresor of Overexpression Constants 1 (SOC1) and Leafy (LFY) [2].

During floral induction, the shoot apical meristem changes from vegetative growth (forming leaves) to form an inflorescence meristem and flowers. In this process the meristem grows taller and more domed. The morphological changes are connected with gene expression at the apex such as the gene for the MADS box transcription factor SOC1. SOC1 activation happens quickly when Arabidopsis plants are moved from short days to the long days, but SOC1 also responds to other floral induction pathways as SOC1 is activated by GA and the age pathway, and is directly inhibited by FLC and SVP [2].

## CRISPR/Cas9

Efficient and reliable ways of genome editing have recently been established including Zinc Finger Nucleases (ZFNs) and TAL Effector Nucleases (TALENs) [4]. One such method utilises the CRISPR-associated protein-9 nuclease (Cas9) from Streptococcus. The Cas9 protein together with the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) RNA has an essential function in the adaptive immunity system in bacteria and archaea as it allows the organism to eliminate invading genetic material (e.g. from viral infections). The CRISPR-Cas9 mechanism involves three steps. The first is acquisition, foreign DNA from a virus inserts into the CRISPR locus in between the short palindromic CRISPR repeats. The second step is expression when the locus is transcribed to produce a short CRISPR RNA (crRNA). Then last step is interference when the crRNA then hybridises with a trans-activating crRNA (tracrRNA) and together they guide the Cas9 endonuclease to the target sequence complimentary to the crRNA in the viral DNA, where the Cas9 cuts both DNA strands to produce a Double-Stranded Break (DSB) in that target sequence [5]. A DSB can be repaired by the cellular Non-Homologous End Joining (NHEJ) pathway, but mistakes in this repair process result in insertions and/or deletions (in-dels) which disrupt the targeted locus [6]. Alternatively a DSB might also be repaired by Homology-Directed Repair (HDR), if a donor template with homology to the targeted locus is supplied then this can result in gene sequence replacement.

The targeting of the Cas9 also requires the presence of a short conserved sequence of between 2-5 nucleotides, known as a Protospacer Associated Motif (PAM), immediately 3' to the crRNA complementary target sequence [5]. There are three

*Corresponding author: Citra Recha Sari
†Email: c.sari@warwick.ac.uk

types of CRISPR that have been identified, but the type II is the most studied. Because the type II CRISPR nuclease only needs two simple components (crRNA and tracrRNA), it makes the system easy to be adapted for genome editing. In 2012, Doudna and Charpentier developed a simplified two-component system by combining the trRNA and crRNA into a single synthetic single guide RNA (sgRNA) which is able to guide the Cas9 to the DNA target sequence [7], [12], [13].

**sgRNA (Small Guide RNA)**

The design of the sgRNA plays a critical role in the CRISPR system because the sgRNA is made up of the 20 nucleotides complementary to the DNA target sequence which will guide the Cas9 to the DNA target. Without the sgRNA the Cas9 would not find the right target sequence. A PAM site (NGG) is required to be directly 3 to the target DNA site, but it is not part of the 20 nucleotides sgRNA [8].

An efficient transient delivery method of genome editing is required because the production of transgenic plants is time-consuming, and a rapid method for gene-editing which doesn't require plant transformation would be very attractive commercially. One method to transiently express genes in plants is through the use of virus expression vectors. This study aims to develop a transient CRISPR/Cas9 viral expression system for gene-editing in plants. Here we aim to delay flowering in tobacco by targeting a key flowering time gene, FT.

We have previously used Agrobacterium transformation to stably transform Arabidopsis with CRISPR constructs targetting a specific site in the Arabidopsis FT gene in order to modify this gene and alter flowering time in the resulting transgenic Arabidopsis lines. Analysis of the sequence of the FT gene in the late flowering lines obtained showed that the FT gene had been modified at the CRISPR target site, with either loss or addition of an Adenine at the cleavage site 3 nucleotides upstream from the AGG PAM motif located immediately 3 to the CRISPR target sequence (Fig 1). Other lines from the same transformation that were not late flowering did not have a modified FT gene.

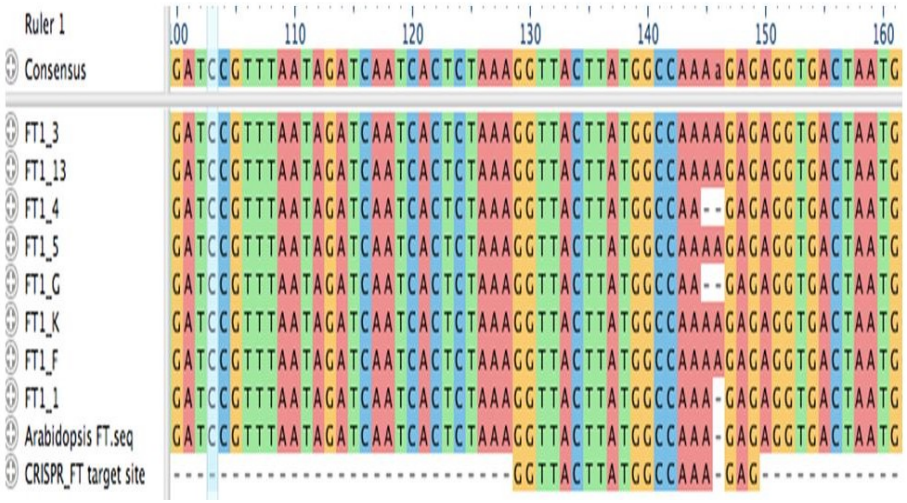


Fig. 1 . Sequence analysis of the FT gene in the late flowering transgenic arabidopsis lines

Late flowering lines FT1_3, 13, 5, K and F all contain an additional Adenine at the CRISPR target site, whereas late flowering lines FT1_4 and G have lost an Adenine compared to the WT Arabidopsis FT sequence and that of a WT-flowering transformed line (FT1_1).

We now

## METHODS

**Selection of CRISPR Target Sites**

There are four FT-like genes in tobacco; FT1, FT2, and FT3 act as floral inhibitors, whilst FT4 induces flowering and so is most likely to be the orthologue of the Arabidopsis FT gene, it is this gene that we will target in tobacco using CRISPR. There are four regions in the tobacco FT4 that can be targeted by CRISPR because they have a PAM sequence (NGG) immediately downstream, these are TobFT4_1, TobFT4_2, TobFT4_3, and TobFT4_4 (See Figure 2). TobFT4_2 and TobFT4_3 target the reverse strand sequence.

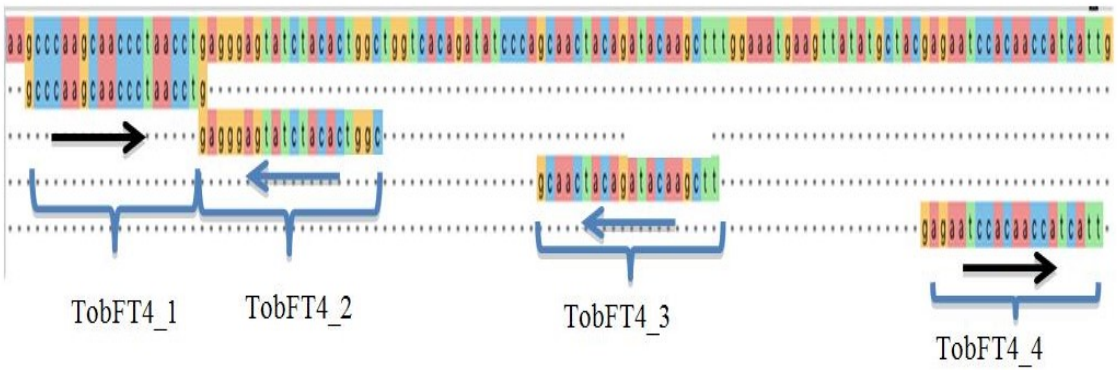


Fig. 2 . Four CRISPR target regions with a PAM site in the tobacco FT4 gene

The top line shows the sequence for TobFT4 gene. The black arrow is for CRISPR target sequences on the top strand, while the blue arrow is for those on the reverse strand.

**Making Transient Virus Expression Vectors**

The viruses that are to be used in this project are Tobacco Rattle Virus (TRV) and Potato Virus X (PVX). To make a transient CRISPR expression system using these viruses requires both Cas9 and sgRNA genes to be cloned separately into the virus vectors.

**Cloning of Cas9**

The Cas9 gene was amplified by PCR using primers with additional MluI restriction sites, digested using MluI and ligated into the MluI site of the PVX vector cDNA clone (Fig. 3).

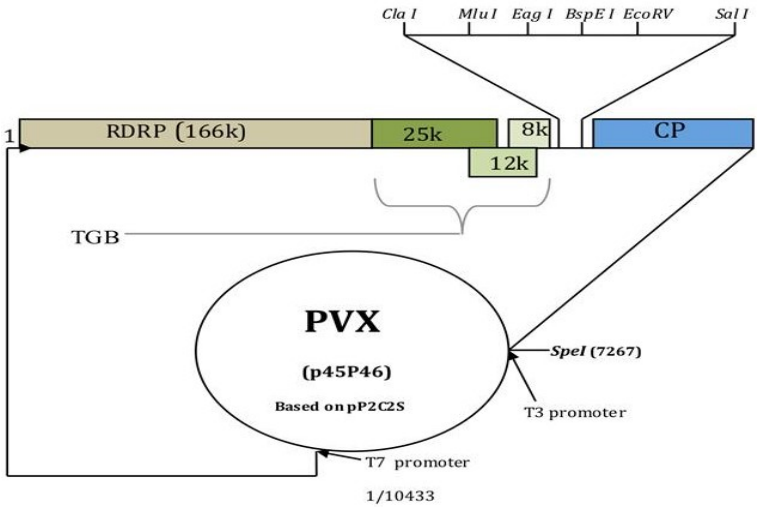


Fig. 3 . PVX virus expression vector

A Schematic representation of the plasmid vector (PVX). The RdRp (166K) is involved in promoting PVX replication while the TGB subunits (25K, 12K and 8K) are involved in PVX transportation. The restriction sites within PVX include ClaI, MluI, EagI, BspEI, EcoRV and SalI. The PVX (p45P46) vector is a modified version of pP2C2S [9].

**Cloning of sgRNA**

Four CRISPR target sites next to PAM site sequences in the Tobacco FT4 gene were identified (see Fig 3 for the previously identified Arabidopsis target sequences). Oligos for the 20bp target regions of the Tobacco FT4 gene will be made and joined to the tracrRNA to form the sgRNA gene as was done for the Arabidopsis CRISPR sgRNAs. To clone both the Arabidopsis and Tobacco sgRNAs into the PVX and TRV vectors, they will be amplified with primers containing restriction sites at each end that will enable the PCR fragments to be cloned into the PVX and TRV vectors.

**Plant Growth**

Tobacco plants are grown in Sanyo MLR growth cabinets with 8h short day conditions and light levels of 100$\mu$ molm$^{-2}$s$^{-1}$. Tobacco seeds are grown in F2+S soil media. These were put in cold room for 3 days to break the seed dormancy before being moved to the cabinets.

**Virus Onoculation of Plants**

The PVX cDNA plasmid clone is linearized and in vitro transcribed to make infectious viral RNA. The in vitro reaction was carried out in 25$\mu$l as follows: 2.5$\mu$l of 10x Buffer (Biolabs), 2$\mu$l of each ntp4 (100mM ATP, CTP, UTP, and 10mM GTP), 1.5$\mu$l 10Mm Cap , and 9$\mu$l of linearized plasmid DNA. The reaction was put at 37°C for 5 minutes then added with 2$\mu$l of T7 RNA Pol (Biolabs) then put at 37°C for 30 minutes. The reaction mixture was added with 2$\mu$l of 100mM GTP and put at 37°C for 60 minutes. The reaction was added with 45$\mu$l Phenol: chloroform: isoamyl alcohol 25:24:1 and vortexed. The upper phase (100$\mu$l) was transferred into the new 1.5ml tube and put at -20°C for 1 hour. The reaction was then added with 10$\mu$l of Sodium acetate and 100$\mu$l of 100% Ethanol and centrifuged at 15000 rpm for 18 minutes. The mixture was then washed with 70% Ethanol and air-dried. The pellet was dissolved in 20$\mu$l and equal amounts of PVX vector expressing the sgRNA (PVX-FT4_1 to 4) and PVX vector expressing the Cas9 gene were co-inoculated into 2 or 3 plants by rubbing onto leaves with carborundum powder.

**RT-PCR**

Infected leaves were extracted to get RNA using standard RNA extraction method and quantified the RNA. The RNA was treated with Rigorous DNAase treatment for RNA using Turbo DNA-free Kit (Ambion). The reaction was carried out in 60$\mu$l as follows: 6$\mu$l 1-x Turbo DNAase Buffer, 0.5$\mu$l Turbo DNAase, 2$\mu$g of RNA, and water. The reaction was put at 37°C for 30 minutes. Added with 12$\mu$l of DNAase inactivation buffer then mixed well and put in room temperature for 2 minutes. It was centrifuged for 1.5 minutes at 10.000 rpm. The RNA supernatant was took to the new tube and precipitated with $\frac{1}{10}$ volume of NaOAc and $2\frac{1}{2}$ volume of 100% EtOH then put at -20°C for 30 minutes. The reaction was spin at 13.000 rpm for 30 minutes and washed the pellet with 70% EtOH. It was re-suspended in 9$\mu$l water.

The cDNA was PRC with Cas9 internal 2F and Cas9 internal 1R primers using 60°C annealing temperature to check the Cas9 existence and sgRNA as well using Tracr-R and Tracr-F- specific CRISPR. The PCR product was run on the 1% agarose gel.

**Western Blot**

The protocol was adopted from [10]. The 7.5% separating gel recipes for making a gel was carried out in 20ml as follows: 9.6ml double distilled water, 5 ml 1.5M Tris-HCl pH 8.8, 200$\mu$l 100% SDS, 5ml Arcy/Bis (30% stock), 400$\mu$l 10% APS, and 20$\mu$l TEMED. The 4.0% stacking gel preparation was carried out in 10 ml as follows: 6ml double distilled water, 2.5 ml 0.5M Tris-HCl pH 6.8, 100$\mu$l 100% SDS, 2ml Arcy/Bis (30% stock), 200$\mu$l 10% APS, and 20$\mu$l TEMED [10].

**RESULTS**

**PVX Inoculation**

After 4 weeks inoculation PVX infected plants start to show the phenotypic effects of virus infection (See Fig 4).

(A)

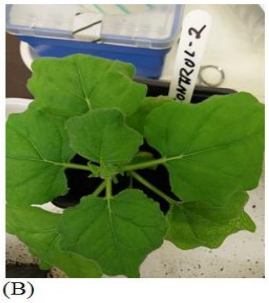
(B)

Fig. 4 . (A) Virus infection symptoms (chlorosis of the leaves) in tobacco plant co-inoculated with PVX virus vectors expressing Cas9 and FT4_1 sgRNA. (B) Control plant (inoculated with water)

**Expression of sgRNA and Cas9 Genes**

Systemic leaves from infected plants were harvested to do protein, RNA, and DNA analysis. RT-PCR was done to check if the sgRNA and Cas9 genes were being expressed in the inoculated plants, (See Fig 5). A western blot was done to verify the presence of the Cas9 protein, (See Fig 6).

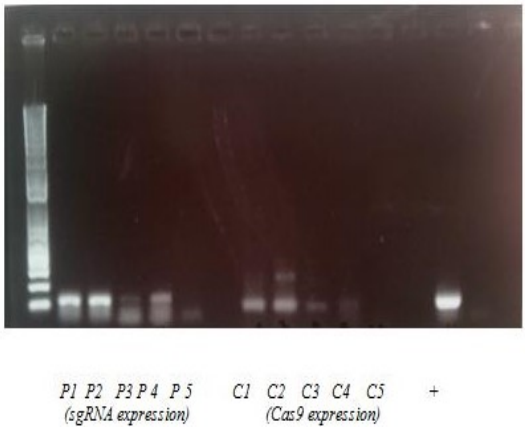


Fig. 5 . RT-PCR analysis of systemic leaves of PVX-infected plants to show expression of the sgRNA (P1-P5) and Cas9 (C1-C5) genes from the viral vector

P1:PVX-FT4_1 C1: PVX-FT4_1
P2: PVX-FT4_2 C2: PVX-FT4_2
P3: PVX-FT4_3 C3: PVX-FT4_3
P4: PVX-FT4_4 C4: PVX-FT4_4
P5:CONTROL (Non-infected) C5: CONTROL (Non-infected)
+ : Cas9 positive control

The RT-PCR shows that the various sgRNA genes and the Cas9 gene are all being expressed in the PVX co-inoculated plants, but not in the non-infected control plants.

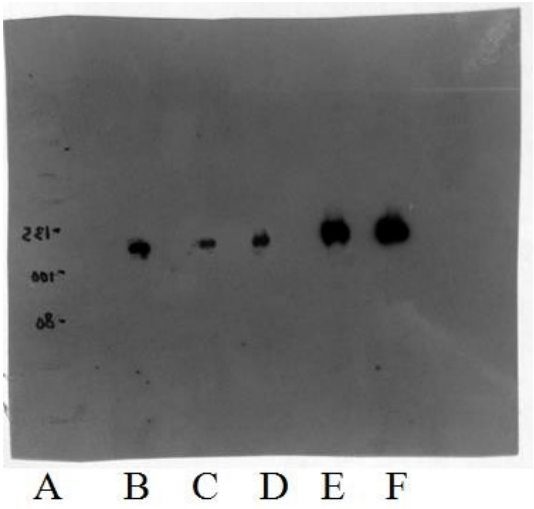


Fig. 6 . Western blot result of PVX-infected plants

PVX-TobFT4_1; (B) PVX-TobFT4_2; (C) PVX-TobFT4_3; (D) PVX-TobFT4_4; (E) PVX-Cas9 positive control; (F) Negative control. All of the samples show the presence of the Cas9 protein except sample F which is the control (uninfected plant).

The western analysis confirms the RT-PCR results and shows that the Cas9 protein is expressed in all the PVX co-inoculated plants.

## DISCUSSION

These results demonstrate that it is possible to express both sgRNA and Cas9 genes from separate PVX-based viral expression vectors which can then be co-inoculated into plants.

The RT-PCR analysis confirms that the sgRNA and the Cas9 genes are being expressed in plants that have been co-inoculated with both the PVX vector expressing the sgRNA and the PVX vector expressing the Cas9. Also the production of Cas9 protein in the inoculated plants was confirmed by western blotting. PVX is an effective virus vector for N. bethamiana [11] and PVX-infected plants showed viral infection symptoms 3 weeks following inoculation.

The detection of the CRISPR sgRNA and Cas9 protein in systemic leaves of inoculated plants indicates that the virus is able to replicate and spread throughout the plant from the inoculated leaf to other tissues where the expression of the CRISPR sgRNA and Cas9 will be able to carry out genome editing in those tissues. If new shoots develop from this gene-edited tissue then the seed from flowers developed from those shoots will also contain the gene-edited changes. As the PVX virus is not transmitted through the seed, germination of the seed will result in gene-edited plants that do not have any viral RNA, or CRISPR-Cas9 DNA inserted into the genome. Thus using this transient virus-mediated CRISPR approach, the process of gene-editing in commercial plants will be faster and without the need to create transformed plants to express the CRISPR-Cas9 genes.

**Acknowledgement**

This research was supported by Lembaga Pengelola Dana Pendidikan (LPDP) Scholarship from Indonesia Government which provided fully funded scholarship.

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