We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

6,900
Open access books available

185,000
International authors and editors

200M
Downloads

154
Countries delivered to

Our authors are among the

TOP 1%
most cited scientists

12.2%
Contributors from top 500 universities

CLARIVATE ANALYTICS
BOOK CITATION INDEX
INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

ALSV Vector Substantially Shortens Generation Time of Horticultural Plants

Ichiro Kasajima, Chunjiang Li, Noriko Yamagishi and Nobuyuki Yoshikawa

Abstract

Flowering of plants is tightly regulated by both plant maturity and seasons in the year. Now that the *Flowering Locus T* (*FT*) gene has been revealed to encode the flowering hormone florigen, researchers are seeking to regulate and modify flowering behaviours by using florigen as a genetic tool. In place of transgenic approaches, *Apple latent spherical virus* (ALSV) vector was successful in promoting flowering of both model plants (Arabidopsis and tobacco), and fruit trees (e.g. apple, pear, and loquat), vegetables (e.g. tomato and cucumber), legumes (e.g. soybean), and ornamental flowers (e.g. petunia, Japanese gentian and Eustoma). In so doing, *FT* was expressed and/or *TFL1* was suppressed by the ALSV vector. ALSV is a latent (non-pathogenic) virus isolated from an apple tree. After induction of flowering and seed production in crops, ALSV is not transferred to most of the next-generation seedlings, or it can be artificially removed from the infected plant by incubation at high temperature. Thus, the generation times of horticultural plants are approximately halved, and the generation time of apple plants is substantially shortened to within one year. Hence, ALSV technology is expected to be useful as a part of New Plant Breeding Techniques (NPBT) for agricultural application.

Keywords: early flowering, florigen, FT, generation time, virus vector

1. Introduction

Breeding of horticultural plants can take several years or more. Plant cultivars are most frequently generated by crossing between different cultivars to combine various advantageous traits together, such as fruit/flower quality, pest tolerance and vigorous growth habit. Cultivars are often crossed with each other repeatedly to generate and fix such favourable

traits. One generation time (from germination, flowering, seed set and germination of the next-generation seedling) is usually several months in herbaceous plants and several years in trees under field conditions [1]. Thus, crossing of herbaceous vegetables and ornamental flowers can be performed every year, while breeding of fruit trees may only be performed once every several years. If flowering could be accelerated in horticultural plants, we would be able to cross vegetables and flowers many times each year and cross fruit trees every year.

In order to cross vegetables several times in a year, problems related to the seasonality and day-length sensitivity of plant flowering also need to be addressed. Plants are either short-day, long-day or day-neutral (an aspect of the photoperiodism, the response to day length). Short-day plants flower in autumn and long-day plants flower in spring. Day-neutral plants flower in any growing season. Day length is now controlled by artificial lighting, but bio-technology to accelerate flowering will also solve the problem of day-length sensitivity without the need for regulating day length using lighting equipment. Alternatively, day-length sensitivity is also one of the important traits of horticultural plants. Ornamental flowers are harvested only when the plants set flowers. Fruits can be harvested only after the flowering seasons. Because of stable responses of horticultural plants to day length, farmers make great efforts to control time of flowering. Such artificial controls are possible only with lighting equipment, but the majority of crops are produced using sunlight alone. Gene modification by biotechnology such that a series of cultivars with different day-length responses are prepared for major horticultural cultivars will thus benefit agricultural production.

Basic studies using model plants have revealed the genes controlling flowering time in higher plants. After a functional gene is isolated in model plants, that gene and its homologs are often found to exert the same function in other plant species. This means that the flowering of vegetables, ornamental flowers and fruit trees can be controlled by this gene [2]. The name of the gene controlling plant flowering is '*Flowering locus T*' (*FT*). Initially, plant biologists aimed to express the *FT* gene in crops by transformation, which actually accelerated flowering [3–7]. This suggested the functional use of *FT* in controlling flowering time, but transgenic plants are not typically applicable to crop production. Plant biologists then attempted non-transgenic expression of *FT* in crops. To our knowledge, there have been no scientific papers demonstrating the control of flowering time by application of FT protein, *FT* DNA or *FT* RNA without genetic transformations or spontaneous mutations in plant genomic DNA. We used a virus vector derived from *Apple latent spherical virus* (ALSV) to deriver *FT* RNA and express FT protein in plant tissues. This system turned out to be very successful in promoting flowering in horticultural plants. This chapter explains in detail the characteristics of the ALSV vector, activities of *FT* and its related genes, and how the ALSV vector is used to promote flowering.

2. Characteristics of ALSV vector

ALSV is a rare, naturally occurring plant virus isolated from an apple tree. ALSV has only been detected in a single apple tree, excluding experimentally infected plants. The

infected tree was grown in the orchard of a Japanese fruit tree research institute (Morioka, Iwate, Japan). The cultivar of this apple is 'Indo', named after Indiana State in the United States of America. The Indo apple used to be popular in Japan, but has now been largely replaced with other cultivars such as Fuji and Orin. ALSV does not naturally spread to nearby apple trees in the orchard [8], but it can infect the seedlings of apple cultivars in the experiments [9–11]. Moreover, ALSV is a 'latent' virus. This means that apple plants (and fruits) infected by ALSV do not show any discernible changes, such as yellow spots, inferior growths or twisted leaves. Thus, ALSV does not induce disease, but such latent characteristics are advantageous for plant biotechnology. With the ALSV vector, we can specifically upregulate (activate) or downregulate (inactivate) functions of target genes, for example, among the 57,000 genes in the apple genome [12], with the ALSV vector. There remain other advantages of the ALSV vector. ALSV evenly infects plant tissues and then enables even silencing (inactivation) of apple genes [11, 13]. ALSV can also infect other plants and upregulate/downregulate specific genes. For instance, ALSV can infect to soybean, petunia, pea, cucumber, pear, rose, Eustoma and Japanese gentian. ALSV is latent in many plant species [14, 15]. Unlike genetic transformation, whose infection rates greatly differ between cultivars of the same crop, ALSV does not typically select cultivars. Exceptions are tomato and almond, where ALSV can infect only part of the cultivars.

The protocol for preparation and infection of the ALSV vector is precisely described in our recent publication [15]. Briefly, plasmid DNAs for expression of ALSV RNAs are prepared by ordinary procedures, introduced into *Agrobacterium*, and 'Agro-inoculated' into ben-thamiana tobacco (*Nicotiana benthamiana*), which is readily infected by plant viruses. Virus particles are extracted from infected leaves; viral RNAs are extracted and introduced into horticultural plants by 'particle bombardment'. The precise structures of ASLV and ALSV vector are explained below.

ALSV consists of two genomic RNAs: ALSV-RNA1 and ALSV-RNA2. **Figure 1** shows the whole sequences of ALSV-RNA1 and ALSV-RNA2. The lengths of ALSV-RNA1 and ALSV-RNA2 are 6812 bases and 3384 bases, respectively. Similar to genomic RNAs of other plant viruses [16], genomic RNAs of ALSV encode single polyproteins. Polyproteins are translated as fused proteins and are digested into individual units by protease. ALSV-RNA1 encodes enzymes such as protease, helicase and RNA polymerase. ALSV-RNA2 encodes a movement protein and capsid proteins. The cleavage sites of the polyprotein were confirmed for ALSV-RNA2 by peptide sequences, whereas the cleavage sites of the polyprotein encoded by ALSV-RNA1 were just deduced from the peptide sequence [17]. The movement protein and the three capsid proteins are all necessary for cell-to-cell movement of ALSV within plant tissues [18].

ALSV is expected to be formed in plants such as *N. benthamiana* if ALSV-RNA1 and ALSV-RNA2 sequences are expressed simultaneously. Expression should not necessarily be performed by genetic transformation: faster and easier protocols for transient expression are usually adopted. Once ALSV is formed in the cells of *N. benthamiana*, it will regenerate and spread autonomously. Three sets of different plasmid vectors have been developed for ALSV vectors (**Table 1**).

ALSV-RNA1 (AB030940.1, 6812 bases)

TCCGTTTTAGAGCTTGTGAGTTGTTATTACGTTTTGTTGTTTGAATCTCTGTTTTGTTACTCTGATTTCCTTCACGTTTGCACCTTACACTCTCTACCGATAAACATTATCATCTTTTATTTTGAATT
TTATTTGAAAAATTTTTATATTTTCAATGTCAATCACTACTCCTGTAGTGATTCGCTTTGGGAGTTTTGACTCCCCTGCTGCGGCACAGATTGCCGATACTAAGGAGGAGGAAACTCCTCTCTCTC
GCCTTGCAGCTTTAAATCGCAAGGCTCAAATTGCTGCTCGTTCAAGAGTAGCAGCTGCTGCGGCACAGTATGCCGAAGAGCAGGAGACACTAGTTCTCACTGCTCAATCCATGAGGGATGCTCT
CCCTTT...

[Nucleotide sequence of ALSV-RNA1, 6812 bases, shown in coloured protein units]

ALSV-RNA2 (AB030941.1, 3384 bases)

TCGTTTTAGAGTTTATGAGAACTGTGCGTTGTTTGTTTCAAGTTCCTTTTGTTCTGTTTCTTGCCATCATCTCACCTTTTCTCATATCCTCTACCGATAAACACCCAATCCTTTCTTCTTTAAACAA
TCTTTGTATTGTTTTCTTATTTTTTATATTTTCTTATCTTGATACTCATCTCTCGGGTATCCTCTTTGACCTTCGCAAGAAGGCCATCTTTGATCGCTTGGATCGTTACCAAGCCATCCCTCTTCCTC
CTCCATTGCCAATCTTGGCAATTTCTGTTTTCCCTCTTTCCTCCCCTTCTTCTCAA...

[Nucleotide sequence of ALSV-RNA2, 3384 bases, shown in coloured protein units]

Figure 1. Nucleotide sequences of ALSV genomic RNAs. Protein units are shown with different colours, between the non-coding sequences at the heads and the tails of ALSV genomic RNAs. The following proteins are encoded in ALSV-RNA1 in this order: PRO-co (protease cofactor), HEL (NTP-binding helicase), Vpg (viral protein genome-linked), C-PRO (cysteine protease), and POL (RNA polymerase). The following proteins are encoded in ALSV-RNA2 in this order: MP (movement protein), capsid proteins: Vp25, Vp20, and Vp24. Genbank/INSDC accessions and nucleotide lengths are shown in parentheses.

Vector	Expressed gene	Backbone	Antibiotics	Reference
pEALSR1	ALSV-RNA1	pE18PGT	Ampicillin	[9]
pEALSR2	ALSV-RNA2	pE18PGT	Ampicillin	
pBICAL1	ALSV-RNA1	pBICP35	Kanamycin	[19]
pBICAL2	ALSV-RNA2	pBICP35	Kanamycin	
pCALSR1	ALSV-RNA1	pCAMBIA1300	Kanamycin	[20]
pCALSR2	ALSV-RNA2	pCAMBIA1300	Kanamycin	
pBIN61:HC-Pro	HC-Pro (PVY)	pBIN61	Kanamycin	
pBE2113-HCPro	HC-Pro (ClYVV)	pBE2113	Kanamycin	[21]

From left column to right column are shown: vector names, expressed genes, plasmid backbone origins, antibiotics for plasmid selections, and reference papers.
PVY, *Potato virus Y*; ClYVV, *Clover yellow vein virus*.

Table 1. List of plasmid vectors.

pEALSR1 and pEALSR2 were the original vectors used for ALSV preparation. High concentrations of these vectors are prepared from colon bacteria (*Escherichia coli*) culture and rub-inoculated (inoculated with SiC, carborundum) onto the leaves of quinoa (*Chenopodium quinoa*) plants. The so-called agro-inoculation method was more convenient, and then new vectors pBICAL1 and pBICAL2 were developed, which are typically used in our experiments. These vectors are prepared in *E. coli* and transformed into Agrobacterium (*Agrobacterium tumefaciens*). *A. tumefaciens* harbouring ALSV vectors are injected into the leaves of *N. benthamiana* for transient expression of ALSV-RNAs. pCALSR1 and pCALSR2 undergo the same procedures with pBICAL1 and pBICAL2, but appear to merit higher concentrations in *E. coli*. The infection rate of 'wild-type' ALSV without modification is almost 100% in *N. benthamiana*. ALSV vectors with exogenous insertion sequences in either cloning sites suffer from much lower infection rates in *N. benthamiana*. To compensate for the reduced infection rate, 'silencing suppressor' protein HC-Pro derived from other viruses (ClYVV or PVY, [22]) is also transiently expressed when ALSV-RNAs are agro-inoculated into *N. benthamiana*. In conclusion, triple agro-inoculation of a mixture of ALSV-RNA1 clone, ALSV-RNA2 clone and HC-Pro clone enables efficient formation of ALSV vectors.

It was not easy to insert exogenous nucleotide sequences into ALSV. After many trials (Li C et al., unpublished data), three cloning sites were successfully used for nucleotide insertions (**Figure 2**) [9, 20]. One cloning site is located immediately after the stop codon of the polyprotein encoded by ALSV-RNA1 (named the SM site, after restriction sites for *Sal*I and *Mlu*I; **Figure 2A**). Another cloning site is located at the middle of the polyprotein encoded by ALSV-RNA2, between MP and Vp25 (named the XSB site, after restriction sites for *Xho*I, *Sma*I and *Bam*HI; **Figure 2B**). Nucleotide sequences around the XSB site are complicated. First, nucleotide sequences around the protease-digested site (QG site) encoding 10 amino acid residues (LLEGQGPDFT) were duplicated. Second, sequences of the restriction sites were introduced
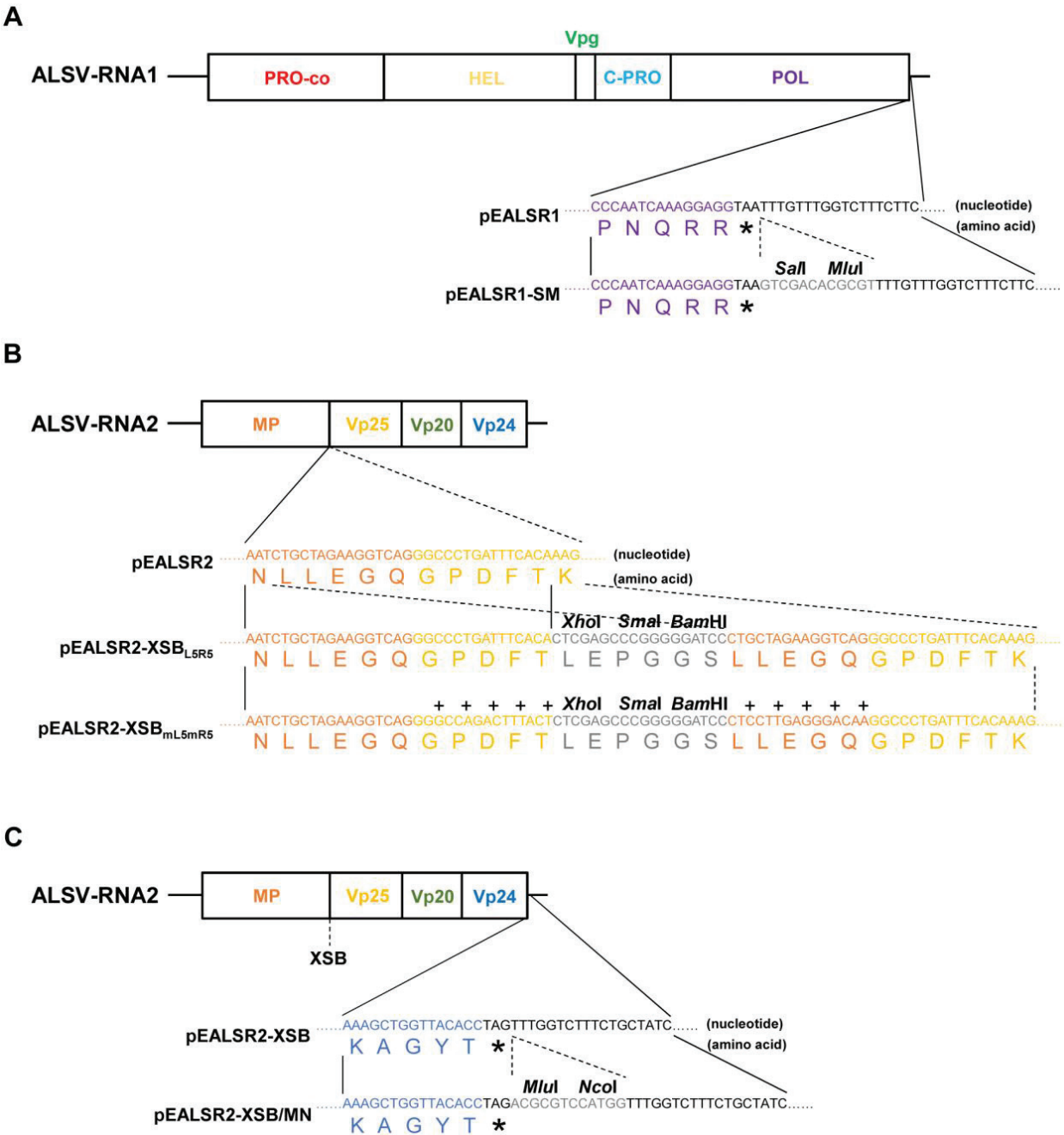
Figure 2. Structures of three cloning sites in ALSV vector. (A) The SM site near the tail of ALSV-RNA1. (B) The XSB site at the middle of the ALSV-RNA2. Introduced mutations are indicated with '+' letters. (C) The MN site near the tail of ALSV-RNA2. Introduced restriction sites are shown with grey letters in (A)–(C).

between the duplicated sequences. Finally, silent mutations (without amino acid changes) were introduced at the third nucleotides in each 10 codons surrounding the cloning site. These nucleotide modifications around the XSB site were necessary to insert exogenous nucleotide sequences at this site. The protease-digested site was duplicated such that both sites (before and after the inserted peptide) are cleaved by protease. Another special requirement for the virus vector is that the insertions are stably held by the virus. Nucleotide sequences in viruses, particularly repetitive sequences, are quite easily deleted (lost) probably via homologous recombination

[23–25]. To mitigate such unfavourable reactions, mutations had been introduced around the XSB site without changing the protease-digested amino acid sequence. The third cloning site is located immediately after the stop codon of the polyprotein encoded by ALSV-RNA2 (named the MN site, after the restriction sites of *Mlu*I and *Nco*I; **Figure 2C**). These cloning sites were constructed by Li C, based on pEALSR vectors, and then copied to the other vectors.

Among the three cloning sites of the ALSV vector (the SM site, the XSB site and the MN site), genes can be expressed only by their insertion into the XSB site. At the XSB site, the inserted genetic sequence is translated as a part of polyprotein, followed by digestion with protease. Cleaved proteins are expected to be attached with short peptides at both the N-terminus and the C-terminus , which derive from the protease-digested sites and the cloning site. These small attached peptides do not seem to affect the activity of inserted protein in most cases, as is experienced for transgenes introduced into transgenic plants.

All three cloning sites can be used for VIGS (virus-induced gene silencing). VIGS is a viral counterpart for gene silencing driven by antisense or inverted-repeat sequences expressed by genetic transformation [26, 27]. The virus itself is a natural target of gene silencing, then a nucleotide sequence inserted at any of the cloning sites of ALSV are silenced (degraded) by silencing mechanisms equipped in plant cells. Endogenous mRNAs harbouring the same nucleotide sequences with the inserted sequence in ALSV vector are also degraded, resulting in gene silencing. Any nucleotide sequences can be inserted at the SM site or at the MN site in principle. The nucleotide sequences inserted at the XSB site must be 'in frame' with the polyprotein, and this means the length of the inserts is a multiple of 3 and that each 'codon' encodes an amino acid without any stop codons (TAA, TAG or TGA as DNA sequences). Sizes of the inserted sequences are determined by the balance between the expected degree of silencing and potential risk of deletions (of the inserts from ALSV). Longer inserts will cause stronger silencing, but they will be more easily deleted from the ALSV vector. A length of 200 bases (or 201 bases at the XSB site) appears to be near the optimal size, with strong silencing and a relatively low possibility of deletions. Inserts shorter than 200 bases may not cause strong silencing, but the actual degree of silencing and frequency of deletions vary, depending on the inserted nucleotide sequences [14]. Insertion of nucleotide sequences at the XSB site is empirically simpler than insertions at the SM site or at the MN site. Insertions at the MN site strongly reduce the rate of viral infection, and they are also easily deleted. Insertions at the SM site have somewhat milder effects on infection rates and deletions. Insertions at the XSB site do not strongly reduce infection rate, but strongly suppress gene expression.

Model studies of silencing an endogenous gene with viral vectors often target the *Phytoene desaturase* (*PDS*) gene. **Figure 3** shows an *N. benthamiana* plant whose *PDS* gene was silenced by an ALSV vector. PDS catalyses the synthesis of carotenoids. Plants silenced in *PDS* expression become white, because they cannot accumulate both carotenoids and chlorophylls. This phenomenon (photo-bleaching) is believed to be caused by rapid 'photo-oxidation' of chlorophylls in the absence of photo-protective carotenoids in plant cells [28]. The representative mechanism of photo-protection by carotenoid would be non-photochemical quenching (NPQ) catalysed by PsbS protein and xanthophyll cycle. As Arabidopsis and rice mutants deficient in NPQ do not develop such white leaves caused by *PDS* silencing [29, 30], another major and unidentified photo-protective mechanism will be exerted by carotenoids in plant cells.
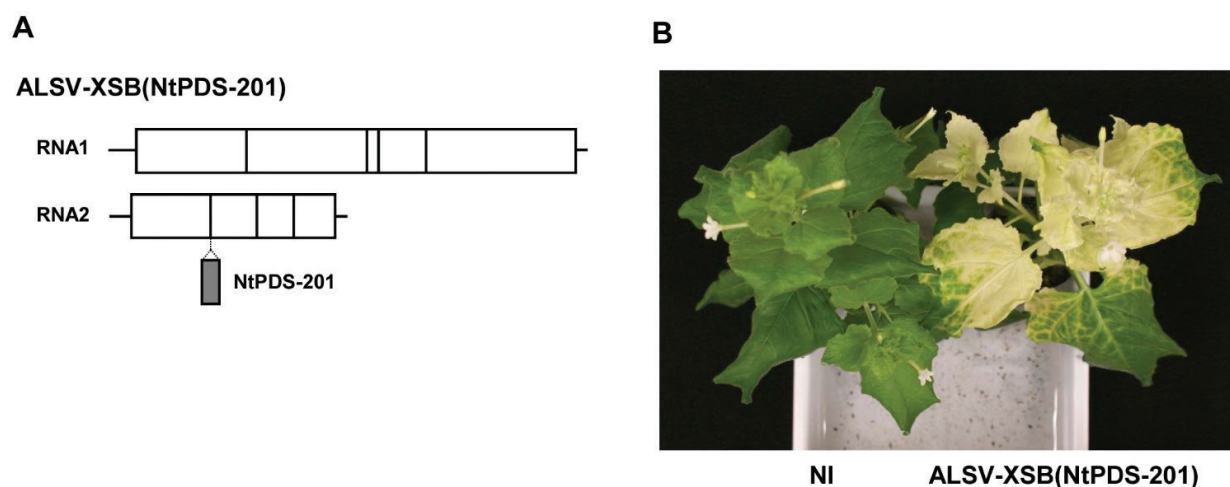
Figure 3. Silencing of *PDS* gene by ALSV vector. (A) An ALSV vector having insertion of a 201-base tobacco *PDS* gene fragment [ALSV-XSB(NtPDS-201)]. (B) The ALSV vector was infected into *N. benthamiana*. NI, non-inoculated ('healthy') plant.

Based on the degree of silencing of the apple *RubisCO small subunit* (*rbcS*) gene with the same 201-base insertion sequence, silencing effects of the three cloning sites differ from one another, and they are greater in the following order: the XSB site > the MN site > the SM site [31]. Again, insertion of a 201-base fragment of the target gene in the XSB site of the ALSV vector is recommended for efficient gene silencing with a lower risk of deletions. The SM site and the MN site are also available to silence additional target genes, by inserting different sequences at each cloning sites. Such simultaneous expression/suppression of different genes is possible, but such vectors typically show low infection rates. The MN site was also used for virus-induced transcriptional gene silencing (VITGS) in *N. benthamiana* and petunia, where upstream sequences (promoters) of the target genes are methylated by virus vectors [20]. The nucleotide sequence of an apple gene promoter was also mutated and inserted at the XSB site for infection to apple plants [15].

3. Early flowering of horticultural plants through expression of the *AtFT* gene

FT protein is a mobile signal of flowering. For example, FT induces flowering when a transgenic scion expressing the *FT* gene is grafted onto an *ft* mutant lacking *FT* gene function [32]. FT proteins produced in leaves and stems can contribute to early flowering, as well as FT proteins produced at shoot apices where flowers are formed. FT protein is originally produced in companion cells of the leaf vasculature. Then, the ALSV vector was prepared so that it expresses Arabidopsis *FT* gene (*AtFT*) at the XSB site (**Figure 4A**).

Horticultural plants, such as soybean and Eustoma infected by the ALSV vector expressing *AtFT* generate flowers earlier than normal (**Figure 4B** and **C**) [33, 34]. Such early-flowering plants often set seeds that germinate normally to generate next-generation seedlings. The ALSV vector has therefore successfully shortened the generation times of horticultural plants. Additional example of early-flowering plants is shown in **Figure 5**. These plants flowered at juvenile phase, showing the successful induction of early flowering by the ALSV-XSB(AtFT) vector.
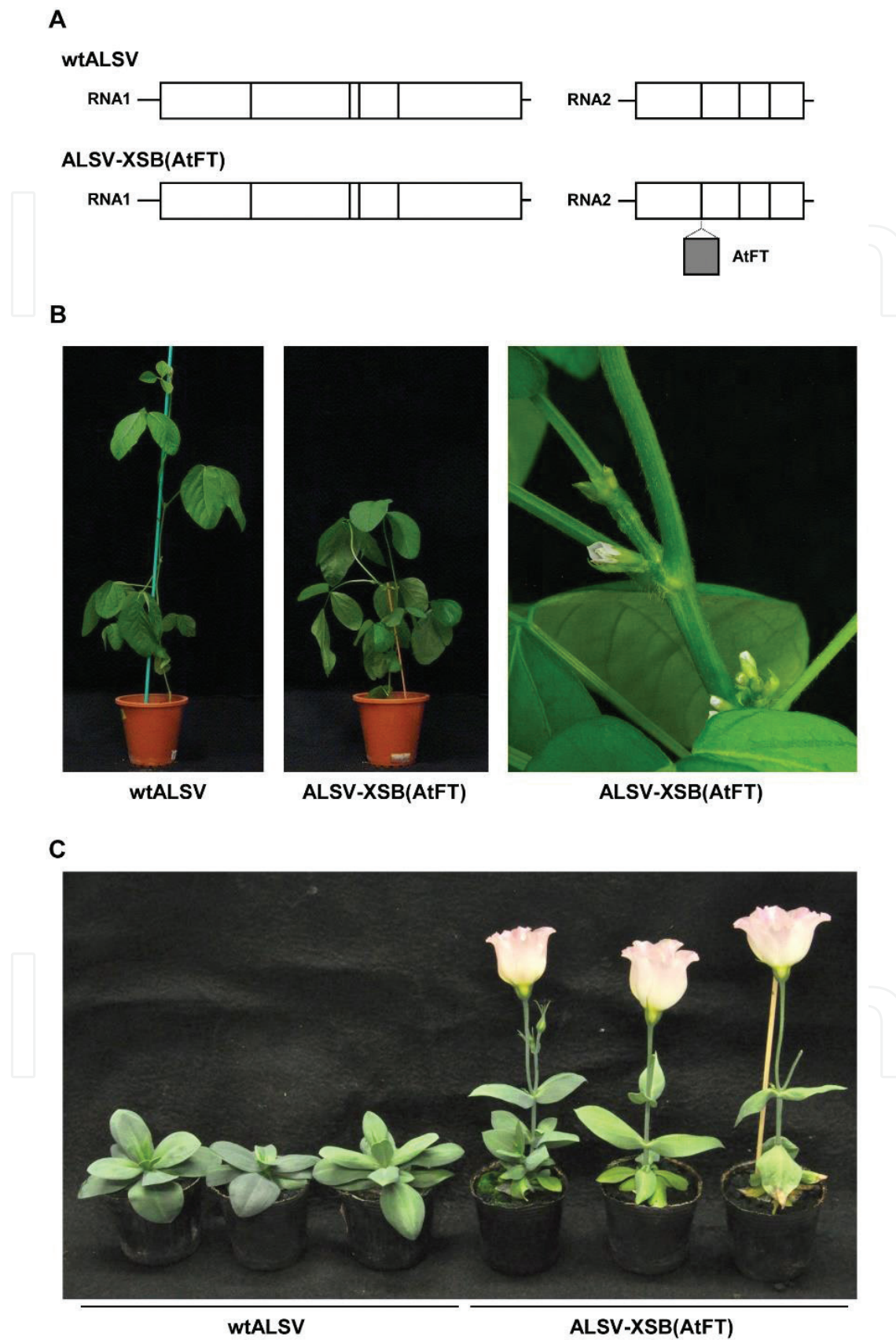
Figure 4. Early flowering of soybean and Eustoma. (A) Schematic representation of the empty vector (wtALSV) and the vector expressing *AtFT* [ALSV-XSB(AtFT)]. (B) Soybean plants (cultivar 'Tanba-Guro') 1 month after inoculation of germinated seeds. (C) Eustoma (Lisianthus) plants 2 months after inoculation of 1-month-old seedlings.

Figure 5. Additional example of early-flowering plants. Petunia seedlings which exhibit early flowering via infection by the ALSV-XSB(AtFT) vector. Left plants are infected by wtALSV. The photograph was originally reported in Ref. [10].

4. Differential activities of *FT* genes in the ALSV vector

FT genes derived from various plant species are expressed by ALSV vectors in Arabidopsis, tobacco and soybean (**Figure 6A**) [11]. These *FT* genes are designated *AtFT* and *AtTSF* (derived from Arabidopsis), *VvFT* (grapevine), *GtFT1* and *GtFT2* (Japanese gentian), *InFT1* and *InFT2* (morning glory), *CsFT* (cucumber), *PmFT* (Japanese apricot), *GmFT2a* and *GmFT5a* (soybean), *LeFT* (tomato), *CiFT* (unshu mikan/satsuma mandarin), *PsFTa1* and *PsFTc* (pea), *MdFT1* and *MdFT2* (apple), *ComFT2* (squash) and *PtFT1* (aspen). Sizes of these FT proteins range from 172 to 184 amino acid residues, but their sizes have no correlation with their activities. A previous report described the degrees of inductions of Arabidopsis and tobacco flowering by these ALSV vectors [11]. **Figure 6B** summarizes these data, together with unpublished data on the induction of soybean flowering.

The average number of leaves at flowering was 30 in plants infected by the control wtALSV vector. The number of leaves was more than halved (less than 15) when plants were infected by the ALSV vectors expressing 10 *FT* genes, whereas, the number of leaves was more than 15 when plants were infected by the ALSV vectors expressing the remaining 9 *FT* genes. Among the 19 *FT* genes examined, *AtFT* and *VvFT* most strongly induced flowering, whereas *GtFT2* and *MdFT1* scarcely induced flowering. What determines the differences in the degree of flowering induction by these *FT* genes? **Figure 6C** shows the phylogenetic tree of the 19 FT proteins. The five FT proteins most strongly inducing flowering (indicated by circles) and the five FT proteins most weakly inducing flowering (indicated by triangles) are evenly distributed within the tree. This suggests that the activities of FT proteins were modified after species differentiation, rather than that their activities were evolutionarily fixed before species
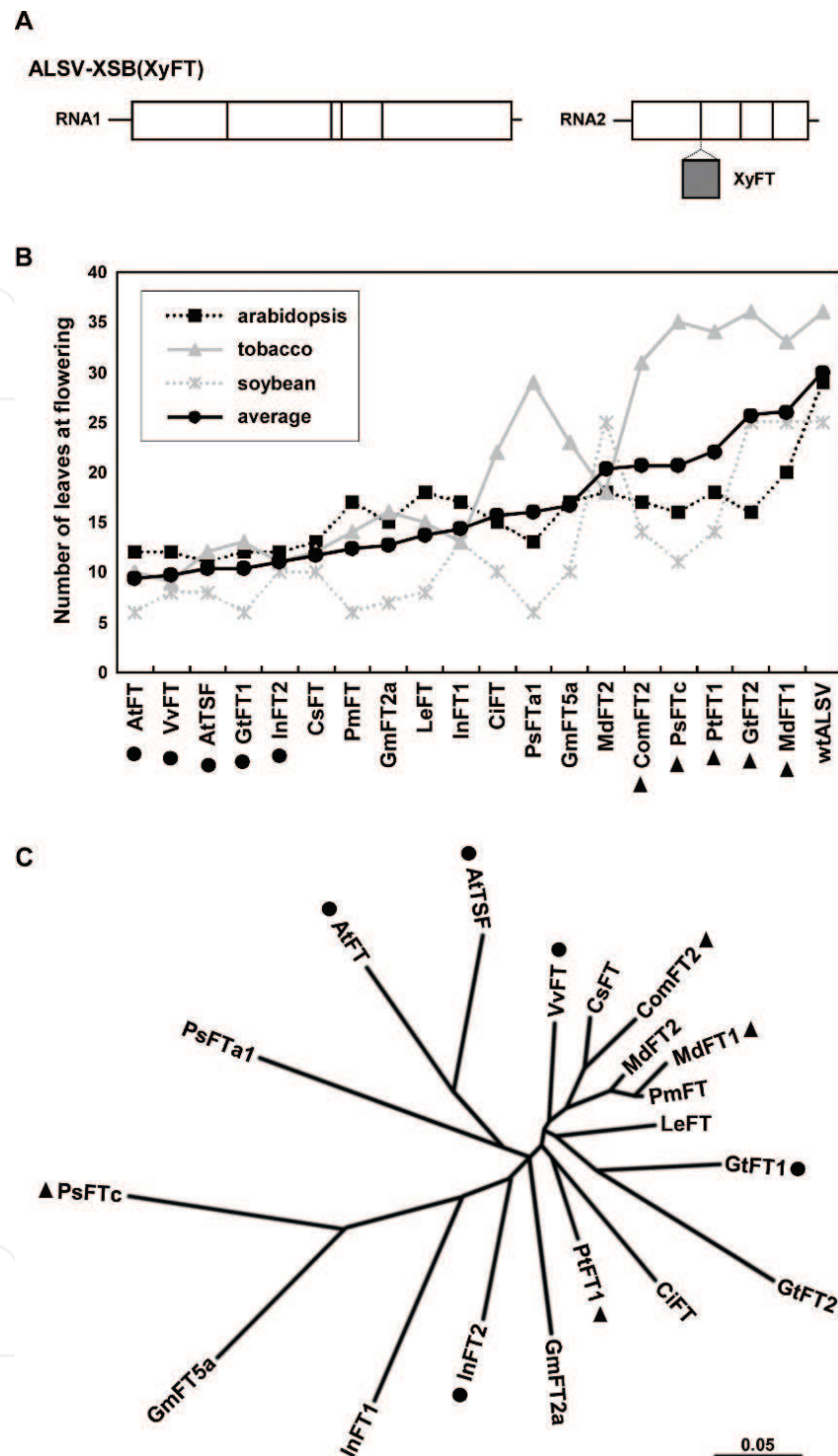
Figure 6. Induction of early flowering with various *FT* genes. (A) ALSV vectors used in this analysis. 'XyFT' represents *FT* genes derived from various plant species (such as *AtFT* and *MdFT1*). Respective *FT* genes are inserted at the XSB site in independent vectors. (B) Flowering times of ALSV-infected plants (Arabidopsis, tobacco or soybean) counted by the number of true leaves at flowering. Arabidopsis was grown at 25°C under a short-day condition (8 h:16 h light/dark photoperiod), and tobacco and soybean were grown at 25°C under a long-day condition (16 h:8 h light/dark photoperiod). 'Average' represents the average values of the numbers of leaves at flowering in Arabidopsis, tobacco and soybean. 'wtALSV' represents the wild-type ALSV vector without any insertion. MdFT2, GtFT2, MdFT1 and wtALSV did not induce soybean flowering, then the numbers of leaves are conveniently set as 25. (C) Phylogenetic tree of the FT proteins expressed by the ALSV vectors in this analysis. In (B) and (C), the five FT proteins most strongly inducing flowering are indicated by filled circles, and the five FT proteins most weakly inducing flowering are indicated by filled triangles.
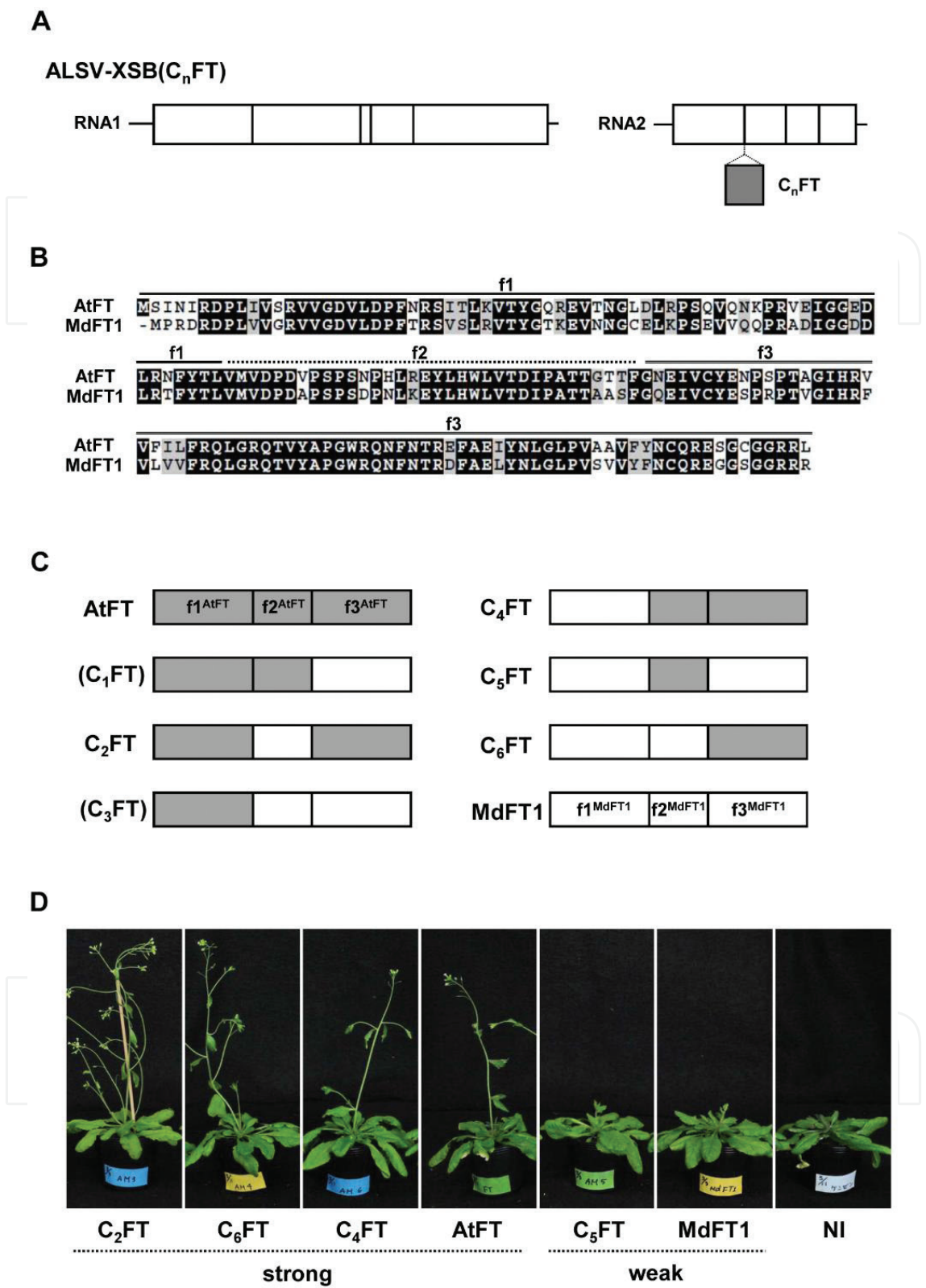
Figure 7. Activities of chimeric *FT* genes in Arabidopsis. (A) ALSV vectors used in this analysis. CₙFT, chimeric *FT* genes. (B) Amino acid sequences of AtFT and MdFT1, and the three fragments (f1, f2 and f3) defined in this analysis. (C) Combinations of FT fragments in chimeric FT proteins. The fragments derived from AtFT are grey, and the fragments derived from MdFT1 are white. (D) Arabidopsis plants infected by ALSV vectors. NI, non-inoculated plant. Juvenile plants with three true leaves were inoculated by particle bombardment of viral RNA. Viral infection was confirmed by RT-PCR (reverse transcription-polymerase chain reaction) analysis. Photographs were taken 30 days after inoculation.

differentiation. Even the *MdFT1* gene, the weakest *FT* gene in this analysis, more or less shortened flowering time when expressed in transgenic Arabidopsis and apple [35], confirming its activity as florigen. The activities of *FT* genes examined in our analysis are inconsistent with grafting experiments using transgenic Arabidopsis plants ectopically expressing *AtFT* or *AtTSF* [36]. In this paper, they found that AtTSF is scarcely mobile from rootstock to scion, then fails to induce early flowering. In contrast, AtTSF was highly active when expressed by the ALSV vector in **Figure 6**. The activity of FT proteins as the regulator of gene expressions may matter when expressed by ALSV vectors, rather than their mobility along the phloem.

Chimeric *FT* genes between *AtFT* (strongest FT) and *MdFT1* (weakest FT) were constructed and investigated in order to determine which part of the *FT* gene regulates its activity. This analysis was performed by Yamagishi N, and is presented for the first time here. The ALSV vectors in this analysis express chimeric *FT* genes at the middle of the polyprotein in RNA2 (**Figure 7A**). **Figure 7B** shows the amino acid sequences of AtFT and MdFT1. FT proteins were divided into three fragments (f1, f2 and f3); f1 corresponds to the first exon, f2 corresponds to the second and the third exons and f3 corresponds to the fourth exon of the FT genes. Chimeric FT proteins are designated C_1FT, C_2FT, C_3FT, C_4FT, C_5FT and C_6FT (**Figure 7C**). All ALSV vectors including those expressing AtFT and MdFT1 were inoculated into *N. benthamiana*, but the vectors expressing C_1FT and C_3FT did not infect *N. benthamiana*. When virus-infected Arabidopsis plants were grown under a long-day condition, C_2FT, C_6FT, C_4FT and AtFT strongly induced flowering, while C_5FT and MdFT1 only weakly induced flowering (**Figure 7D**). This shows that the C-terminus (f3) of the FT protein determines its strength as a florigen. Although we typically use the ALSV vector expressing *AtFT* to shorten generation time in horticultural plants, chimeric *FT* genes such as C_2FT many more strongly induce flowering in some plant species.

5. FT/TFL1 gene family

FT is a member of the FT/TFL1 gene family (also called PEBP, Phosphatidylethanolamine-binding protein). This gene family consists of five subgroups in plants: FT (Flowering locus T), TFL1 (Terminal flower 1), CEN (Centroradialis), BFT (Brother of FT) and MFT (Mother of FT). All Arabidopsis genes (*AtFT*, *AtTFL1*, *AtATC*, *AtBFT* and *AtMFT*) and apple genes belonging to FT/TFL1 family are displayed in a phylogenetic tree of protein sequences, together with some representative genes from other plant species (**Figure 8**). Names of apple genes and their Genbank/the International Nucleotide Sequence Database Collaboration (INSDC) accessions are as follows: MdFT1 (BAD08340.1), MdFT2 (NP_001280810.1), MdTFL1-1 (NP_001280887.1), MdTFL1-2 (NP_001280794.1), MdCENa (NP_001280813.1), MdCENb (NP_001280940.1), MdBFTa (XP_008376539.1), MdBFTb (NP_001280770.1), MdMFTa (XP_008374830.1) and MdMFTb (NP_001281044.1).

Among the five subgroups of FT/TFL1 gene family, FT and TFL1 regulates initiation (induction) of floral organ development and then regulates the time of flowering. FT protein is produced before the time of flowering and carried to shoot apices. As already described, FT positively regulates (increases) the expression of genes such as *SOC1* (*SUPPRESSOR OF OVEREXPESSION OF CO 1*) and *AP1* (*APETALA1*) to induce flowering. In contrast, TFL1 is expressed at shoot apices and negatively regulates (decreases) gene expression to suppress
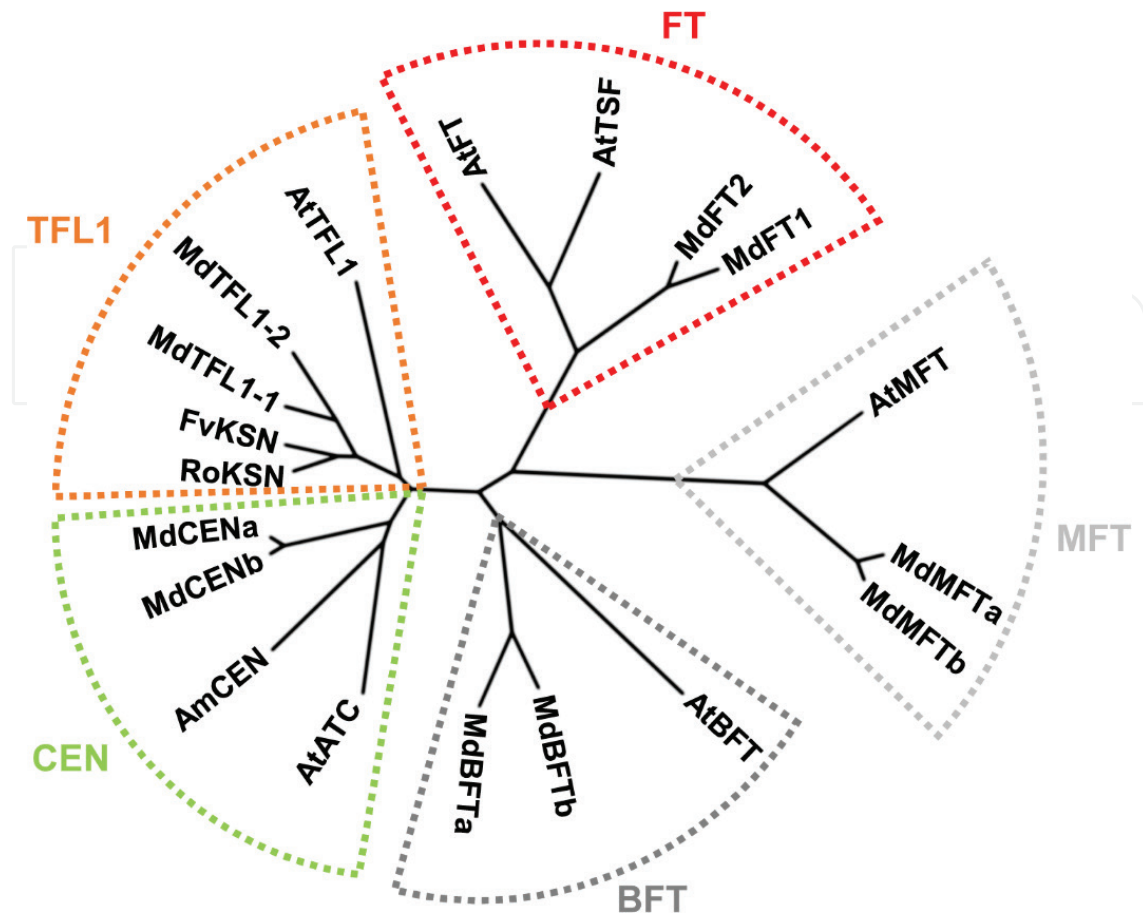
Figure 8. Phylogenetic tree of FT/TFL1 family genes. Five subfamilies are indicated by sectors.

flowering [37]. Thus, the balance between FT and TFL1 expressions have important roles in determining the time of flowering, and determining the parts of plant shoots where the flowers are generated. For example, the mutant of strawberry *FvKSN* gene and the mutant of rose *RoKSN* gene (the TFL1 family genes) can both flower in any growing season (continuous flowering), whereas ordinary strawberry and rose plants generate flowers only at one specific season of the year (seasonal flowering) [38]. The subgroup CEN is represented by the snapdragon *AmCEN* gene. This subgroup does not regulate flowering time, but does regulate the architecture of inflorescence (flower clusters at shoot apices). Snapdragon usually generates 'indeterminate' inflorescence, where many flowers are repeatedly generated on the side of the inflorescence, and the inflorescence continues to elongate without generating a flower at the very apex of the inflorescence. On the other hand, a snapdragon mutant in the *AmCEN* gene has 'determinate' inflorescence, where a limited number of flowers are generated on the side of the inflorescence, and the inflorescence stops elongating with a flower generated at the very apex of the inflorescence (called terminal flower). Thus, CEN negatively regulates flowering at the apex of the inflorescence to generate indeterminate inflorescence [39].

As apparent in the phylogenetic tree (**Figure 8**), apple has two copies of each five FT/TFL1 subgroup genes. The functions of these apple genes are not completely clear, but there are several reports on their activities and expression patterns. *MdFT1* and *MdFT2* are both expressed in

apple plants. *MdFT1* is suggested to regulate the flowering time of apple plants [35], but direct evidence has not yet been found. Expression levels of *MdCENa* are much greater than those of *MdCENb* [40]. Considering its classification into the CEN subgroup and high expression levels, *MdCENa* may regulate the inflorescence architecture of apple plants. Thus, apple plants generate clustered flowers at the apices of newly developing branches. The size of the flower clusters may be smaller in the apple mutants of the *MdCENa* gene. *MdTFL1-1* and *MdTFL1-2* share similar expression patterns. Their expression is reduced when floral buds are initiated in July [40]. According to the observations explained in the next section, *MdTFL1-1* regulates the flowering time of apple plants.

6. Early flowering by combination between *FT* expression and *TFL1* suppression in apple

As already described, early flowering of horticultural plants can be achieved by expression of highly active *FT* genes such as *AtFT* with ALSV vectors (**Figures 4–6**). AtFT induces the early flowering of apple plants at a rate of 30% when expressed by the ALSV vector, but MdFT1 does not induce early flowering in any apple plant [10]. This result reconfirms that highly active FT homologs should be expressed by ALSV vectors to achieve early flowering, rather than endogenous but weakly active FT homologs. Consistent with this idea, other highly active FT homologs (AtTSF and GtFT1) also induced early flowering in apple plants [11]. Unlike herbaceous plants such as tobacco and soybean, the early-flowering rate of apple plants was as low as 30%, even with the most highly active *FT* genes. This difference between plant species may be related to their natural intervals from germination to flowering (months in herbs and years in trees).

Early flowering of plants may be also achieved through suppression of the *TFL1* gene, the negative regulator of flowering. *MdTFL1-1* was silenced in apple plants with ALSV vector [13]. Early flowering was observed, but in only 10% of the infected apple plants. To further improve the early-flowering rate of apple plants, simultaneous expression of *AtFT* and suppression of *MdTFL1* homologs were performed [11]. The early-flowering rate was not increased when *MdTFL1-2* was suppressed simultaneously with the expression of *AtFT*, but the early-flowering rate increased to 90% when *MdTFL1-1* was suppressed simultaneously with the expression of *AtFT*. Thus, early flowering of apple plants was successful at high rates with the combination between *AtFT* expression and *MdTFL1-1* suppression. As well as early flowering at high rates, part of the early-flowering apple plants obtained by *AtFT* expression/*MdTFL1-1* suppression continuously generates flowers on branches, whereas the early-flowering apple plants obtained by only *AtFT* expression generate flowers only once. This difference in flowering traits may be also caused by negative regulation of flowering by *MdTFL1-1* in young apple plants. An example of early-flowering apple plants obtained by simultaneous *AtFT* expression and *MdTFL1-1* suppression is shown in **Figure 9**. Early-flowering apple plants set fruits and seeds after pollination with the pollen gathered from other compatible cultivars. Apple fruits typically mature at about 6 months after flowering. The next-generation seedlings germinated within 1 year, counting from the germination of the mother plant [11].

A

ALSV-XSB(AtFT)/SM(MdTFL1-1-201)

B

ALSV-XSB(AtFT)/SM(MdTFL1-1-201)
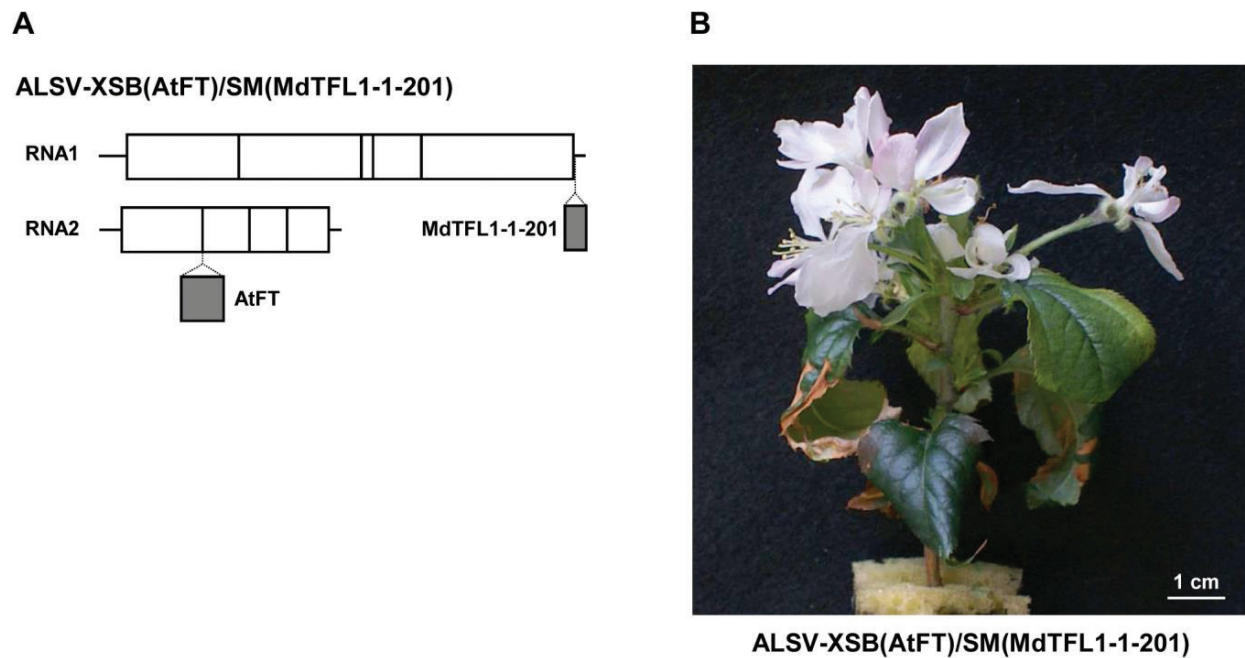
Figure 9. Early-flowering apple plant. (A) ALSV vector used in this analysis. 'MdTFL1-1-201' represents a 201-base fragment of the *MdTFL1-1* gene. (B) Early-flowering apple plant (seedling of Orin progeny: 'progeny' means 'next-generation'). Photograph was taken 67 days after inoculation of viral RNA to germinated seed.

7. Conclusion

ALSV will be among the most useful viral vectors for genetic engineering of horticultural plants, although ALSV is not presently applicable to cereals. Like other viral vectors, inserted sequences into the ALSV cloning sites are easily deleted upon infection to plants, depending on the sizes and sequences of the inserted sequences. What is more, ALSV vectors with insertions sometimes do not infect the plants. However, these problems can be managed by technical efforts and the selection of the insertion sequences. Nucleotide fragments larger than 1 kb can even be introduced into the XSB site of the ALSV vector and infected to plants. We hope that this chapter furthered general understanding of the structure and function of the ALSV vector, and promote its use in both basic and applied studies.

Early flowering through infection of ALSV vectors shortens the generation times of horticultural plants. This technique is expected to promote breeding of horticultural plants. It may not be a popular concept, but there seems to be strongly active *FT* genes (such as *AtFT*) and weakly active *FT* genes (such as *MdFT1*) according to our experiments. The analysis of chimeric *FT* genes in ALSV vector indicated that the difference in the activities of *AtFT* and *MdFT1* is determined by the C terminus, rather than the N terminus. Both types of *FT* genes will have ecological advantages in specific plants species, but highly active types of FTs are useful for early flowering of horticultural plants with ALSV vectors. In addition, simultaneous expression of *FT* and suppression of *TFL1* is beneficial for high rate of early flowering and continuous flowering. ALSV is not transferred to most of the next-generation plants, so the next-generation plants are neither transgenic nor infected by ALSV. Therefore, ALSV can be used as a new plant breeding technique (NPBT).

Acknowledgements

Funding

Author details

Ichiro Kasajima, Chunjiang Li, Noriko Yamagishi and Nobuyuki Yoshikawa*

*Address all correspondence to: yoshikawa@iwate-u.ac.jp

Faculty of Agriculture, Iwate University, Iwate, Japan

References

[1] van Nocker S, Gardiner SE. Breeding better cultivars, faster: Applications of new technologies for the rapid deployment of superior horticultural tree crops. Horticulture Research. 2014;1:14022

[2] Blümel M, Dally N, Jung C. Flowering time regulation in crops—What did we learn from Arabidopsis? Current Opinion in Biotechnology. 2015;32:121-129

[3] Li WM, Tao Y, Yao YX, Hao YJ, You CX. Ectopic over-expression of two apple *Flowering Locus T* homologues, *MdFT1* and *MdFT1*, reduces juvenile phase in *Arabidopsis*. Biologia Plantarum. 2010;54:639-646

[4] Tanaka N, Ureshino A, Shigeta N, Mimida N, Komori S, Takahashi S, Tanaka-Moriya Y, Wada M. Overexpression of *Arabidopsis FT* gene in apple leads to perpetual flowering. Plant Biotechnology. 2014;31:11-20

[5] Xing W, Wang Z, Wang X, Bao M, Ning G. Over-expression of an *FT* homolog from *Prunus mume* reduces juvenile phase and induces early flowering in rugosa rose. Scientia Horticulturae. 2014;172:68-72

[6] Li C, Zhang Y, Zhang K, Guo D, Cui B, Wang X, Huang X. Promoting flowering, lateral shoot outgrowth, leaf development, and flower abscission in tobacco plants overexpressing cotton *FLOWERING LOCUS T* (*FT*)-like gene *GhFT1*. Frontiers in Plant Science. 2015;6:454

[7] Klocko AL, Ma C, Robertson S, Esfandiari E, Nilsson O, Strauss SH. *FT* overexpression induces precocious flowering and normal reproductive development in *Eucalyptus*. Plant Biotechnology Journal. 2016;14:808-819

[8] Nakamura K, Yamagishi N, Isogai M, Komori S, Ito T, Yoshikawa N. Seed and pollen transmission of Apple latent spherical virus in apple. Journal of General Plant Pathology. 2011;77:48-53

[9] Li C, Sasaki N, Isogai M, Yoshikawa N. Stable expression of foreign proteins in herbaceous and apple plants using Apple latent spherical virus RNA2 vectors. Archives of Virology. 2004;149:1541-1558

[10] Yamagishi N, Sasaki S, Yamagata K, Komori S, Nagase M, Wada M, Yamamoto T, Yoshikawa N. Promotion of flowering and reduction of a generation time in apple seedlings by ectopical expression of the *Arabidopsis thaliana FT* gene using the Apple latent spherical virus vector. Plant Molecular Biology. 2011;75:193-204

[11] Yamagishi N, Kishigami R, Yoshikawa N. Reduced generation time of apple seedlings to within a year by means of a plant virus vector: A new plant-breeding technique with no transmission of genetic modification to the next generation. Plant Biotechnology Journal. 2014;12:60-68

[12] Velasco R, et al. The genome of the domesticated apple (*Malus* x *domestica* Borkh.). Nature Genetics. 2010;42:833-839

[13] Sasaki S, Yamagishi N, Yoshikawa N. Efficient virus-induced gene silencing in apple, pear and Japanese pear using *Apple latent spherical virus* vectors. Plant Methods. 2011;7:15

[14] Igarashi A, et al. *Apple latent spherical virus* vectors for reliable and effective virus-induced gene silencing among a broad range of plants including tobacco, tomato, *Arabidopsis thaliana*, cucurbits, and legumes. Virology. 2009;386:407-416

[15] Kasajima I, Ito M, Yamagishi N, Yoshikawa N. *Apple latent spherical virus* (ALSV) vector as a tool for reverse genetic studies and non-transgenic breeding of a variety of crops. In: Rajewsky N, Jurga S, editors. Plant Epigenetics, RNA Technologies Series. Springer International Publishing AG, Cham, Switzerland; 2017. pp. 513-536

[16] Wellink J, van Kammen A. Proteases involved in the processing of viral polyproteins. Archives of Virology. 1988;98:1-26

[17] Li C, Yoshikawa N, Takahashi T, Ito T, Yoshida K, Koganezawa H. Nucleotide sequence and genome organization of Apple latent spherical virus: A new virus classified into the family *Comoviridae*. Journal of General Virology. 2000;81:541-547

[18] Yoshikawa N, Okada K, Asamuma K, Watanabe K, Igarasi A, Li C, Isogai M. A movement protein and three capsid proteins are all necessary for the cell-to-cell movement of apple latent spherical cheravirus. Archives of Virology. 2006;151:837-848

[19] Kawai T, Gonoi A, Nitta M, Kaido M, Yamagishi N, Yoshikawa N, Tao R. Virus-induced gene silencing in apricot (*Prunus armeniaca* L.) and Japanese apricot (*P. mume* Siebold

& Zucc.) with the Apple latent spherical virus vector system. Journal of the Japanese Society for Horticultural Science. 2014;83:23-31

[20] Kon T, Yoshikawa N. Induction and maintenance of DNA methylation in plant promoter sequences by apple latent spherical virus-induced transcriptional gene silencing. Frontiers in Microbiology. 2014;5:595

[21] Yaegashi H, Yamatsuta T, Takahashi T, Li C, Isogai M, Kobori T, Ohki S, Yoshikawa N. Characterization of virus-induced gene silencing in tobacco plants infected with apple latent spherical virus. Archives of Virology. 2007;152:1839-1849

[22] Csorba T, Kontra L, Burgyán J. Viral silencing suppressors: Tools forged to fine-tune host-pathogen coexistence. Virology. 2015;479-480:85-103

[23] Nagy PD, Bujarski JJ. Efficient system of homologous RNA recombination in brome mosai virus: Sequence and structure requirements and accuracy of crossovers. Journal of Virology. 1995;69:131-140

[24] Nagy PD, Simon AE. New insights into the mechanisms of RNA recombination. Virology. 1997;235:1-9

[25] Li C, Yoshikawa N. Virus-induced gene silencing of *N* gene in tobacco by *Apple latent spherical virus* vectors. In: Uyeda I, Masuta C, editors. Plant Virology Protocols: Methods in Molecular Biology. Vol. 1236. Humana Press, New York, USA; 2014. pp. 229-240

[26] Cogoni C, Macino G. Conservation of transgene-induced post-transcriptional gene silencing in plants and fungi. Trends in Plant Science. 1997;2:438-443

[27] Frizzi A, Huang S. Tapping RNA silencing pathways for plant biotechnology. Plant Biotechnology Journal. 2010;8:655-677

[28] Kumagai MH, Donson J, Della-Cioppa G, Harvey D, Hanley K, Grill LK. Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proceedings of the National Academy of Sciences of the United States of America. 1995;92:1679-1683

[29] Havaux M, Niyogi K. The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. Proceedings of the National Academy of Sciences of the United States of America. 1999;96:8762-8767

[30] Kasajima I, Ebana K, Yamamoto T, Takahara K, Yano M, Kawai-Yamada M, Uchimiya H. Molecular distinction in genetic regulation of nonphotochemical quenching in rice. Proceedings of the National Academy of Sciences of the United States of America. 2011;108:13835-13840

[31] Yamagishi N, Li C, Yoshikawa N. Promotion of flowering by *Apple latent spherical virus* vector and virus elimination at high temperature allow accelerated breeding of apple and pear. Frontiers in Plant Science. 2016;7:171

[32] Notaguchi M, et al. Long-distance, graft-transmissible action of *Arabidopsis* FLOWERING LOCUS T protein to promote flowering. Plant & Cell Physiology. 2008;49:1645-1658

[33] Yamagishi N, Yoshikawa N. Expression of *FLOWERING LOCUS T* from *Arabidopsis thaliana* induced precocious flowering in soybean irrespective of maturity group and stem growth habit. Planta. 2011;233:561-568

[34] Fekih R, Yamagishi N, Yoshikawa N. Apple latent spherical virus vector-induced flowering for shortening the juvenile phase in Japanese gentian and lisianthus plants. Planta. 2016;244:203-214

[35] Kotoda N, et al. Molecular characterization of *FLOWERING LOCUS T*-like genes of apple (*Malus* x *domestica* Borkh.). Plant & Cell Physiology. 2010;51:561-575

[36] Jin S, Jung HS, Chung KS, Lee JH, Ahn JH. FLOWERING LOCUS T has higher protein mobility than TWIN SISTER OF FT. Journal of Experimental Botany. 2015;66:6109-6117

[37] Ratcliffe OJ, Amaya I, Vincent CA, Rothstein S, Carpenter R, Coen ES, Bradley DJ. A common mechanism controls the life cycle and architecture of plants. Development. 1998;125:1609-1615

[38] Iwata H, et al. The *TFL1* homologue *KSN* is a regulator of continuous flowering in rose and strawberry. The Plant Journal. 2012;69:116-125

[39] Bradley D, Carpenter R, Copsey L, Vincent C, Rothstein S, Coen E. Control of inflorescence architecture in *Antirrhinum*. Nature. 1996;379:791-797

[40] Mimida N, Kotoda N, Ueda T, Igarashi M, Hatsuyama Y, Iwanami H, Moriya S, Abe K. Four *TFL1/CEN*-like genes on distinct linkage groups show different expression patterns to regulate vegetative and reproductive development in apple (*Malus* x *domestica* Borkh.). Plant & Cell Physiology. 2009;50:394-412