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

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Summary

Fruit trees have a long juvenile phase. For example, the juvenile phase of apple (Malus × domestica) generally lasts for 5–12 years and is a serious constraint for genetic analysis and for creating new apple cultivars through cross-breeding. If modification of the genes involved in the transition from the juvenile phase to the adult phase can enable apple to complete its life cycle within 1 year, as seen in herbaceous plants, a significant enhancement in apple breeding will be realized. Here, we report a novel technology that simultaneously promotes expression of Arabidopsis FLOWERING LOCUS T (FT) gene and silencing of apple TERMINAL FLOWER 1 (MdTFL1-1) using an Apple latent spherical virus (ALSV) vector (ALSV-AtFT/MdTFL1) to accelerate flowering time and life cycle in apple seedlings. When apple cotyledons were inoculated with ALSV-AtFT/MdTFL1 immediately after germination, more than 90% of infected seedlings started flowering within 1.5–3 months, and almost all early-flowering seedlings continuously produced flower buds on the lateral and axillary shoots. Cross-pollination between early-flowering apple plants produced fruits with seeds, indicating that ALSV-AtFT/MdTFL1 inoculation successfully reduced the time required for completion of the apple life cycle to 1 year or less. Apple latent spherical virus was not transmitted via seeds to successive progenies in most cases, and thus, this method will serve as a new breeding technique that does not pass genetic modification to the next generation.

Introduction

The life cycle of plants is divided into several phases. The vegetative phase, which comprises the juvenile and adult vegetative phases, starts after germination. Plants in the juvenile phase are not competent to flower even under inductive conditions, while those in the adult vegetative phase can produce flowers in response to floral inductive signals under the appropriate conditions. Plants acquire reproductive competence during the adult vegetative phase and enter the adult phase (Bäurle and Dean, 2006; Huijser and Schmid, 2011; Mimida et al., 2009).

Apple (Malus × domestica) has a long juvenile phase (approximately 5–12 years) that is a constraint for genetic analysis and the creation of new apple cultivars through cross-breeding (Crosby et al., 1992, 1994). Both genetic and environmental factors are known to influence the duration of the juvenile phase in apple. Although several agrotechnical approaches, such as grafting juvenile twigs onto mature rootstocks, have been used to accelerate the phase transition in apple breeding, a period of at least a few years is still required for apple seedlings to produce flowers (Fischer, 1994; Flachowsky et al., 2009).

Arabidopsis thaliana is an annual plant, and its transition from the vegetative phase to the adult phase has been well studied. Four signalling pathways (gibberellin, autonomous, vernalization and light-dependent pathways) determine the initiation of phase transition, and a protein encoded by the FLOWERING LOCUS T (AFT) gene, namely FT protein of the phosphatidylethanolamine-binding protein (PEBP) family, serves as a signal integrator. Pivotal roles of FT-like proteins in floral induction have been demonstrated in various plant species including woody plants (Abe et al., 2005; Corbesier et al., 2007; Hecht et al., 2011; Imagura et al., 2011; Kong et al., 2010; Lv et al., 2012; Notaguchi et al., 2008; Tamaki et al., 2007; Tränker et al., 2010), while a different PEBP family protein encoded by Arabidopsis TERMINAL FLOWER 1 (TFL1) gene was shown to control vegetative meristem identity and to suppress floral induction (Bradley et al., 1997; Ratcliffe et al., 1998, 1999).

Homologues of such genes, namely MdFT1 and MdFT2 (orthologues of AFT) and MdTFL1-1 and MdTFL1-2 (orthologues of TFL1) (Esumi et al., 2005; Kotoda and Wada, 2005; Kotoda et al., 2010; Mimida et al., 2009), were discovered in apple, and up-regulation of MdFT and down-regulation of MdTFL1 genes during flower formation in apple have been reported (Hättach et al., 2008; Mimida et al., 2011).

It would be of great significance in apple breeding if the genes involved in the transition from the juvenile phase to the adult...
phase can be modified with the aim of floral induction (Flachowsky et al., 2011). Several studies have demonstrated that modification of the genes involved in floral induction by a transformation approach successfully shortens the juvenile period. For example, overexpression of AtFT-homologous genes accelerates flowering time in apple, plum, poplar, citrus and pear (Böhlenius et al., 2006; Endo et al., 2005; Hsu et al., 2006; Matsuda et al., 2008; Srinivasan et al., 2012; Tränkner et al., 2010; Zhang et al., 2010), while repression of TFL1-like genes has a similar effect in apple and pear (Flachowsky et al., 2012; Freiman et al., 2012; Kotoda et al., 2006).

Other useful floral induction approaches include plant virus vector-based methods, such as those that promote expression of endogenous genes, and virus-induced gene silencing (VIGS). Plant virus vector system can be used to add new traits to plants without altering the host genome (Giebink et al., 2004; Purkayastha and Dasgupta, 2009). We previously reported that approximately 30% of apple seedlings inoculated with an Apple latent spherical virus (ALSV) vector containing the AtFT produced flowers 1.5–2 months after inoculation (7–9 leaf stage) (Yamagishi et al., 2011) and that approximately 10% of apple seedlings produced early flowers when MdTFL1-1 was silenced by VIGS using an ALSV vector (Sasaki et al., 2011). In this study, we simultaneously expressed AtFT and silenced MdTFL1-1 in apple through an ALSV vector-based approach. We found that more than 90% of apple seedlings started flowering 1.5–3 months after inoculation with this ALSV vector and that almost all early-flowering seedlings continuously produced flower buds on the lateral and axillary shoots. In addition, cross-pollination between early-flowering apple plants produced seed-bearing fruit, indicating that our approach can shorten the protracted life cycle (sometimes longer than 10 years) of apple to 1 year or less. To the best of our knowledge, this study is the first to demonstrate successful shortening of the life cycle in apple to 1 year or less without genome modification. We also examined seed transmission of ALSV and confirmed the absence of ALSV in most progenies. Thus, despite utilization of a genetically modified virus, our technique does not pass genetic modification to the next generation and will serve as a new plant-breeding technique. Moreover, our technique can be expected to significantly reduce the breeding time in both apple and other fruit-producing plants and crops.

**Results**

Expression of FT genes derived from various plant species by means of an ALSV vector and the inductive effect on flowering

We previously showed that inoculation of apple cotyledons with an ALSV vector bearing AftF (ALSV-AftF) immediately after germination resulted in the formation of flower buds and early flowering in approximately 30% of infected seedlings (Yamagishi et al., 2011). Here, we tested 18 orthologues of FT found in the genomes of the following 13 plant species: *Arabidopsis thaliana*, satsuma (*Citrus unshiu*), cucumber (*Cucumis sativus*), pumpkin (*Cucurbita moschata*), soybean (*Glycine max*), Japanese gentian (*Gentiana triflora*), white edge morning-glory (*Ipomoea nil*), tomato (*Solanum lycopersicum*), apple (*Malus* × domestica), Japanese apricot (*Prunus mume*), pea (*Pisum sativum*), poplar (*Populus tremula*) and grapevine (*Vitis vinifera*). Briefly, we constructed ALSV vectors bearing an FT orthologue from the above species by inserting each orthologue into the XSB site of the ALSV vector (Figure 1), and we inoculated apple seedlings with each construct to examine its inductive effect on flowering. All tested FT orthologues accelerated flowering when expressed in *A. thaliana*, albeit to different extents (Table S1 and Figure S1). When expressed in tobacco plants, constructs were classified into four groups according to the extent of flowering induction (high, moderate, low and none) (Table S1 and Figure S1). On the other hand, only four orthologues, namely, *AtFT*, *A. thaliana TWIN SISTER OF FT* (AtTSF), *G. max* (GmFT2a) and *G. triflora* orthologue (*Gtf1*) orthologue, accelerated flowering when expressed in apple (Table 1). Flowering of apple seedlings infected with an ALSV vector bearing AtTSF (ALSV-AtTSF) and GfT1 (ALSV-GfT1) were shown in Figure 2a,b. Expression of other orthologues, including apple FT-like genes (MdFT1 and MdFT2) did not show floral induction (Table 1). Inoculation with ALSV-AftF or ALSV-AIf yielded a flowering rate (number of seedlings with flowers/number of infected specimens) of approximately 30%, while inoculation with an ALSV vector bearing GmFT2a (ALSV-GmFT2a) or ALSV-GfT1 yielded a flowering rate of approximately 10% (Table 1). Seedlings inoculated with ALSV-AIfS, ALSV-GmFT2a or ALSV-GfT1 (Figure 2b,c) developed only one terminal flower at the 7–9 leaf stage before entering the vegetative growth stage. Flowering-associated characteristics in these seedlings were similar to those in ALSV-AIfT-infected early-flowering seedlings (Table 2).

**Table 1** Promotion of flowering in apple seedlings infected with Apple latent spherical virus (ALSV) vectors expressing a FT orthologue from various plants

<table>
<thead>
<tr>
<th>Plant species</th>
<th>FT orthologue</th>
<th>Accession No.</th>
<th>ALSV vector</th>
<th>Flowering rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>AtFT</td>
<td>AF152096</td>
<td>ALSV-AtFT</td>
<td>29.3 (17/58)*</td>
</tr>
<tr>
<td><em>A. thaliana</em></td>
<td>AtTSF</td>
<td>NM118156</td>
<td>ALSV-AtTSF</td>
<td>30.8 (8/26)</td>
</tr>
<tr>
<td><em>Citrus unshiu</em></td>
<td>CfFT</td>
<td>AB027456</td>
<td>ALSV-CfFT</td>
<td>0 (0/28)</td>
</tr>
<tr>
<td><em>Cucumis sativus</em></td>
<td>CsFT</td>
<td>AB383152</td>
<td>ALSV-CsFT</td>
<td>0 (0/9)</td>
</tr>
<tr>
<td><em>C. moschata</em></td>
<td>ComFT2</td>
<td>EF462212</td>
<td>ALSV-ComFT2</td>
<td>0 (0/9)</td>
</tr>
<tr>
<td><em>Glycine max</em></td>
<td>GmFT2a</td>
<td>AB550126</td>
<td>ALSV-GmFT2a</td>
<td>10 (1/10)</td>
</tr>
<tr>
<td><em>G. max</em></td>
<td>GfT5a</td>
<td>AB550126</td>
<td>ALSV-GfT5a</td>
<td>0 (0/8)</td>
</tr>
<tr>
<td><em>Gentiana triflora</em></td>
<td>G. triflora</td>
<td>AB650176</td>
<td>ALSV-GfT1</td>
<td>7.7 (1/13)</td>
</tr>
<tr>
<td><em>Ipomoea nil</em></td>
<td>Ift1</td>
<td>EU178859</td>
<td>ALSV-InfT1</td>
<td>0 (0/11)</td>
</tr>
<tr>
<td><em>I. nil</em></td>
<td>IfT2</td>
<td>EU178860</td>
<td>ALSV-InfT2</td>
<td>0 (0/5)</td>
</tr>
<tr>
<td><em>Solanum lycopersicum</em></td>
<td>LeFT</td>
<td>AY186735</td>
<td>ALSV-LeFT</td>
<td>0 (0/11)</td>
</tr>
<tr>
<td><em>Malus</em> × <em>domestica</em></td>
<td>MdFT1</td>
<td>AB161112</td>
<td>ALSV-MdFT1</td>
<td>0 (0/31)</td>
</tr>
<tr>
<td><em>M. x domestica</em></td>
<td>MdFT2</td>
<td>AB458504</td>
<td>ALSV-MdFT2</td>
<td>0 (0/11)</td>
</tr>
<tr>
<td><em>Prunus mume</em></td>
<td>PfMt</td>
<td>AMH49379</td>
<td>ALSV-PmMt</td>
<td>0 (0/8)</td>
</tr>
<tr>
<td><em>P. sativum</em></td>
<td>PsfTa1</td>
<td>HQ538822</td>
<td>ALSV-PsfTa1</td>
<td>0 (0/9)</td>
</tr>
<tr>
<td><em>P. sativum</em></td>
<td>PsFT</td>
<td>HQ538826</td>
<td>ALSV-PsFT</td>
<td>0 (0/7)</td>
</tr>
<tr>
<td><em>Populus tremula</em></td>
<td>PfT1</td>
<td>DQ387859</td>
<td>ALSV-PfT1</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td><em>Vitis vinifera</em></td>
<td>VfFT</td>
<td>EF157728</td>
<td>ALSV-VfFT</td>
<td>0 (0/9)</td>
</tr>
</tbody>
</table>

*Figures in parenthesis indicate number of flowered plants/Number of infected plants.

†Effect of a wild-type (wT)ALSV vector with no FT gene.
Simultaneous expression of AtFT and silencing of MdTFL1 efficiently accelerated flowering in apple

Only limited types of FT orthologues accelerated flowering with low efficiency (10%–30%) when expressed in apple seedlings. We previously reported that silencing of MdTFL1-1 by the ALSV-based method introduced early- and perpetual-flowering traits to apple seedlings, albeit with low efficiency (Sasaki et al., 2011). In this study, we constructed an ALSV vector to express the AtFT gene as well as to silence endogenous MdTFL1-1 in apple. Briefly, the ALSV-RNA1 vector bearing a partial sequence of MdTFL1-1 (201-nt) in the 3' untranslated region (SM site immediately downstream of the end of open reading frame) and the RNA2 vector bearing the full length of ALSV in the XSB site were combined (Figure 1). When the resulting ALSV-AtFT/MdTFL1 were used for inoculation of seedlings immediately after germination, more than 90% of infected seedlings developed their first flower at the 7–22 leaf stage (1.5–3 months after inoculation), and most continued flowering (Table 2 and Figure 2d–f). Real-time polymerase chain reaction (PCR) analysis revealed that the expression of MdTFL1 mRNA in the tip of the stems in ALSV-AtFT/MdTFL1-infected seedlings approximately 2 weeks after inoculation was approximately 25% that of seedlings without inoculation (Figure 3). Floral organs, such as petals, sepals and stamens, were morphologically normal in most of the early-flowering apple seedlings, but an increase in the number of petals and a lack of pistils were occasionally observed (data not shown).

Following the findings that the simultaneous alteration of expression of two genes, more precisely, the expression of AtFT orthologue gene (MdFT1 and MdFT2) and MdTFL1-2, a MdTFL1-1 homologue, were useful in a similar strategy. When MdTFL1-2 was silenced simultaneously with the expression of AtFT, flowering was accelerated in only 1 of 7 infected seedlings (Table 3). Simultaneous silencing of MdTFL1-1 and expression of an apple FT orthologue gene (MdFT1 or MdFT2) did not accelerate flowering in any of the infected seedlings (Table 3).

**Table 2** Comparison of flowering aspects in apple seedlings infected with ALSV-AtFT, ALSV-MdTFL1 or ALSV-AtFT/MdTFL1

<table>
<thead>
<tr>
<th>ALSV vector</th>
<th>Expression gene</th>
<th>Suppression gene</th>
<th>Flowering rate (%)</th>
<th>Leaf stage of first flowering</th>
<th>No. of flowers</th>
<th>Growth after first flowering</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALSV-AtFT†</td>
<td>AtFT</td>
<td>–</td>
<td>30</td>
<td>7–9</td>
<td>1</td>
<td>Vegetative</td>
</tr>
<tr>
<td>ALSV-MdTFL1†</td>
<td>–</td>
<td>MdTFL1-1</td>
<td>10</td>
<td>8–14</td>
<td>Multiple</td>
<td>Continuous flowering</td>
</tr>
<tr>
<td>ALSV-AtFT/MdTFL1</td>
<td>AtFT</td>
<td>MdTFL1-1</td>
<td>&gt;90</td>
<td>7–22</td>
<td>Multiple</td>
<td>Continuous flowering</td>
</tr>
</tbody>
</table>

*Data from Yamagishi et al. (2011).
†Data from Sasaki et al. (2011).

**Figure 1** Schematic of the ALSV-RNA1 vector (pEALS1-3SM) with a cloning site (SalI-MluI) at the 3' terminus and the ALSV-RNA2 vector (pEALS2mSmR5) with cloning sites (Xhol-Smal-BamHI) between MP and Vp25. P35S, enhanced CaMV 35S promoter; Tnos, nopaline synthase terminator; HEL, NTP-binding helicase; C-PRO, cysteine protease; POL, RNA polymerase; MP, 42K movement protein; Vp25, Vp20, and Vp24, capsid proteins; Q/G, protease cleavage site.
that ALSV-AtFT/MdTFL1-infected apple seedlings produced flowers with fertile pistils and pollens. After low-temperature treatment at 4 °C, all 9 seeds shown in Figure 5c germinated (Figure 5e).

Table 3  Effect of *Apple latent spherical virus* (ALSV) vectors expressing *FT* and suppressing *TFL* on the promotion of flowering in apple seedlings

<table>
<thead>
<tr>
<th>ALSV vector</th>
<th>Expression gene</th>
<th>Suppression gene</th>
<th>Flowering rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALSV-AtFT/MdTFL1</td>
<td>AtFT</td>
<td>MdTFL1-1</td>
<td>90.6 (29/32)*</td>
</tr>
<tr>
<td>ALSV-AtFT/MdTFL2</td>
<td>AtFT</td>
<td>MdTFL1-2</td>
<td>14.2 (1/7)</td>
</tr>
<tr>
<td>ALSV-MdFT/MdTFL1</td>
<td>MdFT1</td>
<td>MdTFL1-1</td>
<td>0 (0/11)</td>
</tr>
<tr>
<td>ALSV-MdFT/MdTFL1</td>
<td>MdFT2</td>
<td>MdTFL1-1</td>
<td>0 (0/9)</td>
</tr>
</tbody>
</table>

*Figures in parenthesis indicate No. of flowered plants/No. of infected plants.

Taken together, these results provide evidence for possible harvesting of seeds within 8–10 months by inoculating apple cotyledons with ALSV-AtFT/MdTFL1 immediately after germination. In other words, with the ALSV-AtFT/MdTFL1-utilizing technique, apple can complete one life cycle (from the seed of the first generation to the production of seeds of the successive generation) within 1 year.
Life cycle acceleration by means of a virus vector serves as a new breeding technique with no transmission of genetic modification to successive progenies.

Plant RNA virus genomes are generally not integrated into host genomes, but some plant viruses are seed-transmitted. For example, we previously reported a seed transmission rate of a few per cent in ALSV-infected apple seedlings (‘Indo’ variety) based on the results of enzyme-linked immunosorbent assay (ELISA) and reverse transcription (RT)-PCR (Nakamura et al., 2011). In this study, we employed a more sensitive approach, real-time quantitative RT-PCR (qRT-PCR), to test for the presence of ALSV in seed embryos (cotyledons before germination) produced by ALSV-infected apple plants (‘Indo’ and ‘Golden delicious’ varieties), in cotyledons (after germination) and in true leaves grown from these seeds. The detection limit of qPCR was 500 attogram of the template per reaction, and thus, its sensitivity was 10,000–100,000-fold higher than that of ELISA and RT-PCR. When seed embryos were tested to determine levels of ALSV, 2% (2/100) of ‘Indo’ samples, as well as the positive control (ALSV-infected leaves), showed strong positive signals at around 15 cycles, indicating the high content of ALSV. On the other hand, the remaining ‘Indo’ samples and all of the 115 ‘Golden delicious’ samples showed weaker signals at around 25–35 cycles, indicating a low content of ALSV (Figure 6b). When cotyledons collected 3 weeks after germination and the third true leaves from the next-generation seedlings were tested, a high ALSV-RNA content was detected in 2.3% (2/87) of ‘Indo’ samples, but not in any of the 137 ‘Golden delicious’ samples (Figure 6c,d). In clear contrast to the results of seed embryo qRT-PCR, weak signals at 25–35 cycles, indicative of very low ALSV-RNA content, were absent in all samples harvested from seedlings 3 weeks after germination (Figure 6c,d). Thus, a trace amount of

![Figure 4](image1.png)  
Figure 4 Continuous flowering in an apple seedling infected with ALSV-AtFT/MdTFL1 at (a) 1.5 months postinoculation (mpi), (b) 3 mpi and (c) 7 mpi. Arrows in (c) show continuous flowering, even after 7 mpi.

![Figure 5](image2.png)  
Figure 5 Fruit production in apple seedlings infected with ALSV-AtFT/MdTFL1. Pollination was conducted between early-flowering apple seedlings. (a) Fruit produced on the apple seedling (9 mpi) 5 months after pollination. (b) Seedling (11 mpi) with four fruits 5 months after pollination. (c and d) Ripe fruits on apple seedlings (a) and (b) with viable seeds, respectively. (e) Seedlings germinated from seeds in (c) after low-temperature treatment for 3 months.
noninfectious virus RNA is likely to account for the weak positive signals detected in a small proportion of seed embryo samples. Further, when samples collected 2 months after germination were tested by ELISA, the detection rate was 0.98% (2/205) in ‘Indo’ seedlings and 0% (0/176) in ‘Golden delicious’ seedlings. The results of qPCR of samples collected from the corresponding seedlings 4 months after germination showed no discrepancy with the ELISA results. Thus, seed transmission of ALSV appears to have occurred at a low rate (a few per cent) in ‘Indo’ apple, but not in ‘Golden delicious’ apple.

Apple latent spherical virus transmitted by seeds was already detectable in the seed embryo before germination, enabling definitive diagnosis of ALSV infection at an early stage. Taken together, ALSV was absent in most of the progenies of infected maternal plants. In other words, the genetic makeup was generally intact in the successive generation of ALSV-infected plants—the products of the present breeding technique.

**Discussion**

Perennial trees require a long juvenile phase before producing flowers. The juvenile period is 5–12 years in apple and is a serious constraint for apple breeding. We previously showed that approximately 30% of seedlings inoculated with ALSV-AtFT started flowering roughly 1.5 months after inoculation (Yamagishi et al., 2011). In this study, we first tested whether 18 FT orthologues found in various plant species, as well as AtFT, accelerate flowering by expressing each construct in apple seedlings by means of an ALSV vector. AtFT and AtTSF induced flowering more potently than other FT orthologues, including apple orthologues of FT (MdFT1 and MdFT2). In addition, early flowering was observed in only approximately 30% of apple seedlings expressing AtFT and AtTSF. Thus, we concluded that the low rate of early flowering cannot be improved solely by expression of an FT gene (Table 1).

Following these findings, we constructed a modified ALSV vector (ALSV-AtFT/MdTFL1) that expresses AtFT while silencing MdTFL1-1, and we examined its inductive effect on flowering. The first flower was observed in more than 90% of infected seedlings at 1.5–3 months after inoculation with ALSV-AtFT/MdTFL1 (Table 2 and Figure 2), and this induction was likely attributed to the increased translation of AtFT combined with VIGS of MdTFL1-1 as a result of the proliferation of ALSV-AtFT/MdTFL1 in the stem tips of infected seedlings (Figure 3) (Yamagishi et al., 2011). Hättach et al. (2008) examined the changes in gene expression in the apple stem apex upon transition from vegetative to reproductive growth states and found increases in MdFT expression and decreases in MdTFL1 expression during the formation of flower buds. Similarly, Mimida et al. (2011) examined shoot apical meristems by in situ hybridization and found increases in MdFT expression and decreases in MdTFL1-1/2 expression in the stem apex during the phase transition. It is likely that similar changes were induced in the stem apex in apple seedlings infected with ALSV-AtFT/MdTFL1. In other words, ALSV-AtFT/MdTFL1 induces changes in gene expression in the stem apex of seedlings a few months after germination, and such changes—which normally occur in the meristem of mature trees—accelerate flowering.

In contrast, the same strategy did not work with apple FT orthologues (MdFT1 and MdFT2) (Table 3). An alignment of FT

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Figure 6 Detection by quantitative real-time RT-PCR analysis of ALSV-RNA1 from apple seeds (cotyledons) before germination (a and b), from uninfected (a) and ALSV-infected (b) trees, from cotyledons (c), and from the third true leaves (d) of apple seedlings of ALSV-infected trees three weeks after germination. Samples in (a) from ‘Ourin’, and samples in (b), (c) and (d) from ‘Indo’ and ‘Golden delicious (GD)’. PC, positive control (samples from infected leaves); S, Examples of 20 samples of seeds and seedlings; Ct, cycle threshold; ΔRn, fluorescent signal of amplified by PCR.
amino acid sequences showed that the functional domain and the amino acids important for AtFT function were conserved in MdFT1 and MdFT2 (Figure S3). Probably, MdFT1 and/or MdFT2 need to be supplemented by other genetic factors. Indeed, among the 18 FT genes tested, MdFT1 induced flowering more weakly than other FT orthologues in both A. thaliana and tobacco plants. It would be intriguing to know which genetic factors control flowering in apples.

ALSV-AtFT/MdTFL1 introduced an early-flowering trait into a much larger proportion of apple seedlings while also introducing a perpetual-flowering trait (Figure 2E and Figure 4b,c). The perpetual-flowering trait was observed in pear when PtTFL1-1 (TFL1-1 orthologue) and PtTFL1-2 (TFL1-2 orthologue) were silenced by a transformation approach (Freiman et al., 2012) and also in plum transformed with poplar FT1 (Srinivasan et al., 2012). It is likely that ALSV-AtFT/MdTFL1 creates conditions that induce perpetual flowering in the stem apex through a mechanism involving both AtFT expression and MdTFL1 repression. It is noteworthy that AtFT was not maintained in approximately 40% of infected seedlings 6 months after inoculation with ALSV-AtFT/MdTFL1 and that the repetitive expression of flowering and vegetative growth, characteristic of ALSV-AtFT/MdTFL1-infected seedlings, was gradually replaced by continuous vegetative growth in infected seedlings (Figure S2). This suggests that the balance between AtFT expression and MdTFL1 repression is important for floral differentiation from the shoot apical meristem, an early-flowering trait is transient, and the transition to the reproductive phase is incomplete in ALSV-AtFT/MdTFL1-infected seedlings. Recently, involvement of micro RNA (miRNA) in phase transition was reported in both annual and perennial plants (Huijser and Schmid, 2011; Wang et al., 2011). Future advances in apple miRNA research may lead to development of a new technique that enables the induction of phase transition.

ALSV-AtFT/MdTFL1-infected seedlings kept producing early flowers while growing for at least 6 months, and thus, successfully produced fruits (Figure 5). Although regulation of genes related to flower bud formation through transformation was shown to accelerate breeding of fruit trees within a 1-year cycle (Flachowsky et al., 2009, 2011; Freiman et al., 2012; Le Roux et al., 2012), the present study is to the best of our knowledge the first to demonstrate a technique that enables harvesting of seeds for the next-generation progenies within 1 year without genome modification. At present, ALSV vector techniques could be effectively applied to only cotyledons from seedlings, not to apple varieties of well-known genotype. However, this method is very useful for promotion of a breeding cycle that starts with apple seeds.

The present study also showed that the rate of seed transmission of ALSV is extremely low and that the transmission can be diagnosed in a definitive manner. We previously reported that ALSV had not spread from the infected apple trees to the neighbouring apple trees as the virus was first detected in 1984, indicating that there is no vector(s) for transmission of ALSV in the orchard tested and that horizontal transmission of ALSV via pollen has not occurred among these apple trees (Nakamura et al., 2010). Thus, ALSV vector is easy to manage safely in terms of biocontainment. Taken together, our rapid breeding technique using an ALSV vector instills apple seedlings with an early-flowering trait that enables life cycle completion within 1 year, while eliminating transmission of the ALSV vector to successive progenies in most cases. In other words, successive progenies cannot be distinguished from genetically unmodified apple seedlings, and thus, we believe the products of this technique (the successive progenies) are not subject to the regulations of genetically modified organisms. Our ALSV vector-based technique, which accelerates the plant life cycle, is distinguishable from conventional transgenic approaches and therefore needs to be evaluated from a new perspective.

**Experimental procedures**

**Construction of ALSV vectors expressing each of several FT orthologues from various plant species**

Eighteen FT orthologues from 13 species (A. thaliana, Citrus unshiu, Cucumis sativus, Cucurbita moschata, Glycine max, Gentiana triflora, Ipomoea nil, Solanum lycopersicum, Malus x domestica, Prunus mume, Pisum sativum, Populus tremula and Vitis vinifera) were synthesized and inserted into the XSB site of pEALS2RmL5mR5 (Figure 1) (Li et al., 2004). Chenopodium quinoa was infected with each of the resulting FT-bearing vectors plus PEALSR1 to obtain ALSV vectors that promote expression of their respective FT orthologue (Igarashi et al., 2009).

**Construction of ALSV vectors that simultaneously promote expression of AtFT and silencing of MdTFL1-1**

A partial sequence of MdTFL1-1 (accession no. AB052994) (Sasaki et al., 2011) was inserted into the SM site of pEALSR1SM. Chenopodium quinoa was infected with the resulting construct plus AtFT-bearing pEALSR2mL5mR5 to obtain ALSV-AtFT/MdTFL1 that simultaneously promotes the expression of AtFT and silencing of MdTFL1-1.

**Plant materials and inocula**

Arabidopsis thaliana (Col) plants were grown at 25 °C under short day condition (8 h : 16 h light/dark photoperiod) in a growth chamber. Tobacco plants were grown at 25 °C under the long day condition (18 h : 6 h light/dark photoperiod) in a growth chamber. Homogenates of infected C. quinoa leaves were used as inocula for mechanical inoculation of A. thaliana and tobacco plants (Igarashi et al., 2009). Apple seed embryos were inoculated with an ALSV vector according to the method reported by Yamagishi et al. (2010). Briefly, total RNA extracted from ALSV vector-infected C. quinoa leaves was used as inocula for biolistic inoculation of apple cotyledons (seed embryos immediately after germination). Apple seeds were kept at 4 °C before use.

**RNA extraction and quantitative RT-real-time PCR**

Total RNA was extracted from shoot apices according to a previously reported method (Sasaki et al., 2011). After DNase I treatment and the following phenol/chloroform extraction, ethanol precipitation was performed and the resulting RNA pellets were dissolved in RNase-free water. First strand cDNA was synthesized using 500 ng of total RNA as a template, oligo(dT) primers and Rever Tra Ace reverse transcriptase (Toyobo, Osaka Japan). Quantitative real-time PCR was performed in a reaction volume of 20 μL using 2 μL of cDNA, SYBR Premix Ex Taq (Tli RNase H Plus) (Takara, Kyoto, Japan) and the following primer pairs (final concentrations of 0.2 μM): Mdbrc5380(+) 5′tactgatctggcagacatg3′ and Mdrbc5500(−) 5′cctgtctaccgtagaagt3′ (for amplification of MDRBC); MdTFL1-1391(+) 5′ tcaaatacagacttcc3′ and MdTFL1-1515(−) 5′gctttctagctt3′.
cagttc3' (for amplification of MdtTFL1-T). MdrbcS served as a reference gene. The conditions of real-time PCR performed with the Eco Real-Time PCR system (illumina, Inc., San Diego, CA) were 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s, annealing at 60 °C for 30 s, and a final melting step up to 95 °C for 15 s, 55 °C for 15 s and 95 °C for 15 s. Data (5 seedlings/experiment) were analysed by 2ΔΔCT data analysis.

**Determination of ALSV by quantitative RT-real-time PCR**

RNA was extracted from test specimens as described above to make 100 μg/mL RNA solutions. First strand cDNA was synthesized as described above but using 100 ng of total RNA as a template. Determination of ALSV infection in seed embryos was performed by the SYBR method. Briefly, quantitative real-time PCR was performed as described above, but using the following primer pair (final concentrations of 0.15 μM): primer4F6150 (+) 5′-ctgatagctcctctgatatg3′ and primer4R6279 (−) 5′-gagtagggtgctccgacca3′. The conditions of real-time PCR performed with the Eco Real-Time PCR system were 95 °C for 30 s followed by 35 cycles at 95 °C for 5 s, annealing at 60 °C for 30 s, and a final melting step up to 95 °C for 15 s, 55 °C for 15 s and 95 °C for 15 s. Tenfold serial dilutions of in vitro-transcribed ALSV RNA (concentration range, 500 ng/μL-500 ag/μL) were used to make a calibration curve.

**Determination of ALSV by ELISA**

The levels of ALSV in apple leaves were determined by ELISA according to the method reported by Nakamura et al. (2011).

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**References**


Reduced generation time of apple seedlings by ALSV vector

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Promotion of flowering in A. thaliana and tobacco plants infected with the ALSV vector expressing FT orthologue genes presented in Table S1.

Figure S2 (a) RT-PCR analysis of apple seedlings infected with ALSV-AtFT/MdTFL1 at 6 mpi. MdTFL1 was detected in all 17 samples, but AtFT was not detected in 7 samples No. 2, 3, 6, 9, 11, 19, or 21, indicating that MdTFL1 was stably maintained, while AtFT was deleted in 40% of samples. (b) Apple seedlings infected with ALSV-AtFT/MdTFL1. Sample No. 9, which lost AtFT, shows vegetative growth of the shoot (11 mpi). In contrast, sample No. A1 shows continuous flowering (7.5 mpi).

Figure S3 Multiple alignment of the amino acid sequences of AtFT (A. thaliana, AAF03936), MdFT1 (apple, ACL98164), and MdFT2 (apple, BAD08340).

Table S1 Effect of ALSV vectors expressing various FT orthologues on the promotion of flowering in A. thaliana and tobacco plants.