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A New Plant Breeding Technique Using ALSV Vectors to Shorten the Breeding Periods of Fruit Trees

Noriko Yamagishi and Nobuyuki Yoshikawa

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Abstract

Fruit trees have a long juvenile phase. For example, the juvenile phase of apple lasts for 6–12 years and is a serious constraint for creating new varieties by breeding based on crossing and selection. In this chapter, we report a novel technology using the *apple latent spherical virus* (ALSV) vector to accelerate flowering time and life cycle in apple and pear seedlings. Inoculation of apple and pear cotyledons immediately after germination with ALSV-AtFT/MdTFL1 concurrently expressing *Arabidopsis FLOWERING LOCUS T* (*AtFT*) gene and suppressing apple *TERMINAL FLOWER 1-1 (MdTFL1-1)* gene can shorten the period from seeding to flowering to 1.5–3 months after germination and generation times in order to obtain next-generation seeds in 1 year or less. Most next-generation seedlings obtained from ALSV vector–infected plants were free of the virus. We also developed a method for eliminating ALSV vectors from infected apple and pear plants by only high-temperature treatment. A method combining the promotion of flowering in apple and pear by ALSV vector with an ALSV elimination technique is expected to see future application as a new plant breeding technique that can significantly shorten the breeding periods of apple and pear.

Keywords: apple latent spherical virus (ALSV) vector, apple, pear, promotion of flowering, elimination of ALSV

1. Introduction

Woody fruit trees have a long juvenile phase—the period between germination and flowering of plants. In apple and pear, the vegetative growth (juvenile phase) generally lasts for 6–12 years with no flowering and fruiting. After transition from the juvenile phase to the adult phase, the trees flower/fruit every year [1–3].

Several apple varieties have been bred to in order to impart resistance to diseases and insect pests, as well as for quality improvement of the fruit. Breeding of fruit trees is conventionally conducted by crossing and selection [4, 5]. So, it is necessary to cultivate many hybrid seedlings to examine their characteristics for breeding new varieties. For example, "Fuji," an apple variety that currently has the world's highest production, was selected from 787 hybrid seedlings obtained by crossing "Kokkou (Ralls Janet)" with "Delicious" apples. This variety flowered and fruited approximately 12 years after seeding [6]. Since apple fruits are produced continuously on the same tree over dozens of years, breeding of a high-quality variety is very important for apple production.

A long juvenile period of apple seedlings is a major barrier to the breeding of new varieties. Moreover, breeding of fruit trees requires large fields for cultivation of seedlings and considerable labor for their management. Technologies for shortening the juvenile phase, including grafting onto dwarfing rootstocks, have been developed; however, despite the use of these technologies, several years are required for flowering/fruition [1, 2].

In recent years, global warming is advancing owing to an increase of greenhouse gas concentrations in the atmosphere. Fruit trees are susceptible to global warming because their important physiological phenomena, such as flowering and dormancy, are dependent on environmental climates. In Japan, the influences of global warming on the production of apples have already begun to appear, with poor coloration of fruit, increased frost injury due to early flowering phase, and damage by harmful insects reported [7]. It is presumed that a further shift of land suitable for cultivation of fruit trees and changes in the distribution of diseases and pests caused by global warming will be accelerated in the future; therefore, it is necessary to implement rapid fruit breeding technologies. The improvement in efficiency of fruit tree breeding, particularly shortening of the juvenile phase, is gaining a great deal of attention.

Many genes involved in plant flowering have been identified in the past 20 years in model plants such as *Arabidopsis thaliana* and rice. The most important gene among them is the *FT* gene, which encodes the flowering hormone, "florigen." Although it has been known since its discovery that florigen is a mobile signaling substance which is synthesized in leaves and transferred to the shoot apical meristem, it is sensitive to photoperiod. Interestingly, it took approximately 70 years to identify this molecule [8]. The *FT* gene encodes an approximately 20-kDa water-soluble protein belonging to the phosphatidylethanolamine-binding protein (PEBP) family, and was reported for the first time in *A. thaliana* (*FLOWERING LOCUS T of A. thaliana* (*AtFT*) gene) and rice (*Heading gate3a* (*Hd3a*) gene) [9–12]. The *FT* protein is conserved widely in angiosperms and has a common function of promoting flowering [8]. In fruit trees, it has been reported that flowering was promoted in genetically modified orange and pear expressing the citrus florigen-like gene, *CiFT* [13, 14]. Early flowering also occurs in genetically modified apples where the florigen-like gene, *MdFT*, is expressed [15, 16].

Proteins belonging to the PEBP family contain the *A. thaliana TFL1* gene [17–19]. The *TFL1* gene is highly homologous with the *AtFT* gene but has an adverse function that it suppresses

flowering. Fruit trees have a gene homologous to the *TFL1* gene; the suppression of *MdTFL1-1*, an apple *TFL1*-like gene, was reported as leading to early flowering [20, 21]. Similarly, in pear, suppression of *PcTFL1-1* and *PcTFL1-2* led to the induction of early flowering [22]. In research using other genes, Flachowsky et al. reported that an early flowering apple line T1190 expressing *BsMADS4*, a transcription factor involved in the initiation of flower bud formation, could be used for breeding a disease-resistant variety via a rapid cycle breeding system where a generation was completed in 1 year [23].

Viral vector technology is a tool to express or suppress the target gene in the virus-infected plant [24, 25]. Infection of a plant by a plant virus vector integrated with the target gene for expression results in the occurrence of expression of the gene in the infected plant. Conversely, when attempting to suppress the expression of a gene, infection of a plant by a viral vector with a part of the target gene leads to induction of suppression of the target gene in the infected plant by virus-induced gene silencing (VIGS). Viral vectors have the advantage of allowing us to evaluate phenotypes rapidly. Recently, we constructed apple latent spherical virus (ALSV) vectors by adding cloning sites to the ALSV genome. The ALSV vector system can be used for the expression of a foreign gene and VIGS in various plant species [26–38].

In this chapter, we introduce an ALSV vector-based technology for early flowering and shortening of a generation time in the apple and pear. Use of this technology allows apples and pear to complete a generation within 1 year, which reduces a breeding term of fruit trees substantially. Moreover, because the viral vectors can easily be removed from both next-generation seedlings and infected plants, this technology is considered not applicable to regulations of the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Cartagena Protocol).

2. Apple latent spherical virus (ALSV) vectors

ALSV is a spherical virus with a diameter of approximately 25 nm originally isolated from an apple tree and is composed of two RNA genome segments (RNA1 and RNA2) and three types of coat proteins (Vp25, Vp20, and Vp24) [39–41]. The apple is the only natural host of ALSV; however, ALSV has a relatively wide range of hosts, and it can experimentally infect not only herbaceous plants such as *Solanaceae*, *Cucurbitaceae*, *Fabaceae*, and *Gentianaceae* but also fruit trees belonging to *Rosaceae* [26–38]. ALSV is characterized by latent infection causing no symptoms in almost all host plants and invasion of the shoot apical meristem of infected plants.

Previously, we used ALSV vectors constructed using pUC18 plasmid. These vectors had to be inoculated to *Chenopodium quinoa* plants for virus propagation [42]. Currently, we constructed RNA1 and RNA2 vectors using Ti plasmid where cloning sites for foreign gene were introduced in both RNA vectors [43, 44]. As shown in **Figure 1a**, RNA1 vector has a cloning site in the 3'-noncoding region (SM), and RNA2 vector has two cloning sites in the ORF (XSB) and
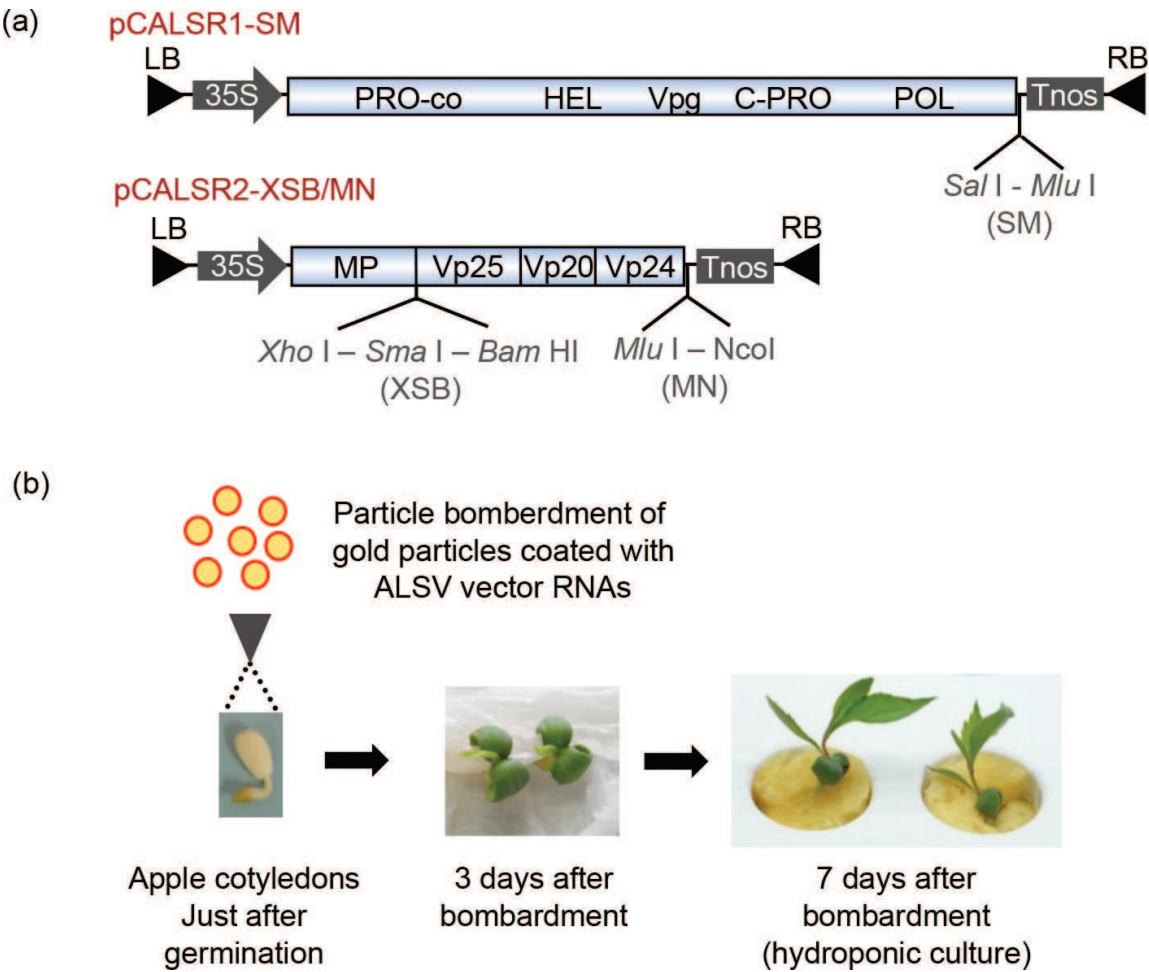
Figure 1. (a) Genetic map of ALSV binary vector (pCALSR1-SM and pCALSR2-XSB/M). LB and RB, left and right borders, respectively. 35S, CaMV 35S RNA promoter. Tnos, nopaline synthase terminator. SM, MN, and XSB, cloning sites. (b) Outline of biolistic inoculation of ALSV vectors to apple cotyledon just after germination.

the 3′-noncoding region (MN). These vectors can be inoculated to *Nicotiana benthamiana* by agro-infiltration [43, 44].

3. Efficient inoculation method of ALSV vector

Generally, it is difficult to directly inoculate cDNA clones of ALSV vector to apple and pear seedlings because cDNA clone results in a very low infection rate. Therefore, we first inoculated the clones to an experimental plant, *N. benthamiana* by agro-infiltration, which have been established to introduce many plant viruses to plants, for viral multiplication. Then, we reinoculated the virus preparation (crude sap of infected *N. benthamiana* leaves) to a propagation host *C. quinoa* for preparing the high-titer inocula for fruit trees. We established an efficient inoculation method using RNA sample extracted from the infected *C. quinoa* leaves by particle bombardment [42, 45]. Inoculation of apple seedlings immediately after germination

by this method (**Figure 1b**) allowed us to achieve 90–100% infection rate. Cotyledons immediately after germination of fruit trees appear to be highly susceptible to viral infection.

4. Induction of early flowering of apple and pear by ALSV vector infection

We first constructed an ALSV vector expressing *AtFT* (ALSV-AtFT) and inoculated this vector to cotyledons of apple seedlings as shown in **Figure 1a**. The results indicated that approximately 30% of the infected seedlings formed flower buds and flowered 1.5–2 months after the inoculation at the stage 7–8 true leaves [30]. This was likely the result of expression of *AtFT* from ALSV-AtFT in the shoot apical meristem of the infected seedlings. The flowers induced by ALSV-AtFT infection showed an apparently normal morphology. Pollens collected from these flowers were used for pollination of apple flowers that flowered naturally, which led to the formation of fruits with normal seeds. Thus, inoculation of ALSV-AtFT allowed us to achieve early flowering in apple seedlings. However, the flowering rate was no greater than approximately 30% of the infected seedlings, and the flowered seedlings shifted to vegetative growth and flowered again only rarely. In the following experiments, we constructed ALSV-MdTFL1 which a part of *MdTFL1-1* gene was inserted in a cloning site in the XSB site of RNA2 vector. This vector suppressed the expression of an *MdTFL1-1* gene in infected apple [29], and the infection of this vector resulted in the formation of flower buds 1.5–3 months after inoculation (stage 8–19 true leaves). The infected apple seedlings showed continuous flowering where they formed flower buds on the extending auxiliary buds for several months. Unfortunately, the rate of flowering by ALSV-MdTFL1 infection was as low as approximately 10% of the infected seedlings.

Finally, we constructed an ALSV vector (ALSV-AtFT/MdTFL1) that expresses *AtFT* and suppresses *MdTFL1-1* gene concurrently [46]. Surprisingly, greater than approximately 90% of ALSV-AtFT1/MdTFL1-infected apple seedlings formed flower buds and flowered 1.5–3 months after inoculation (stage 7–22 true leaves) (**Figure 2a**), and the majority of these early flowering seedlings showed continuous flowering in which they flowered continuously over several months (**Figure 2b**). It was confirmed that their pollens were fertile and pollination between early flowering individuals led to fruit formation (**Figure 2c**). Their fruit skin color displayed green, yellow, and red coloring in the process of maturation depending on the individual (**Figure 2d**). Their fruit size was approximately 2.5–4.5 cm, and seeds formed inside, with seeds germinating and growing normally after breaking of dormancy (**Figure 2d**) [46]. In addition, the total soluble solids (TSS) determined by a refractometer was showed 7.8–13.5% in their juice. From the above, fruits formed in seedlings infected with ALSV-AtFT/MdTFL1 were likely to be used for evaluating quality regarding their skin color and sugar content.

It was revealed that infection of apple seedlings by ALSV-AtFT1/MdTFL1 allowed us to shorten one generation (from seeds of the current generation to formation of the
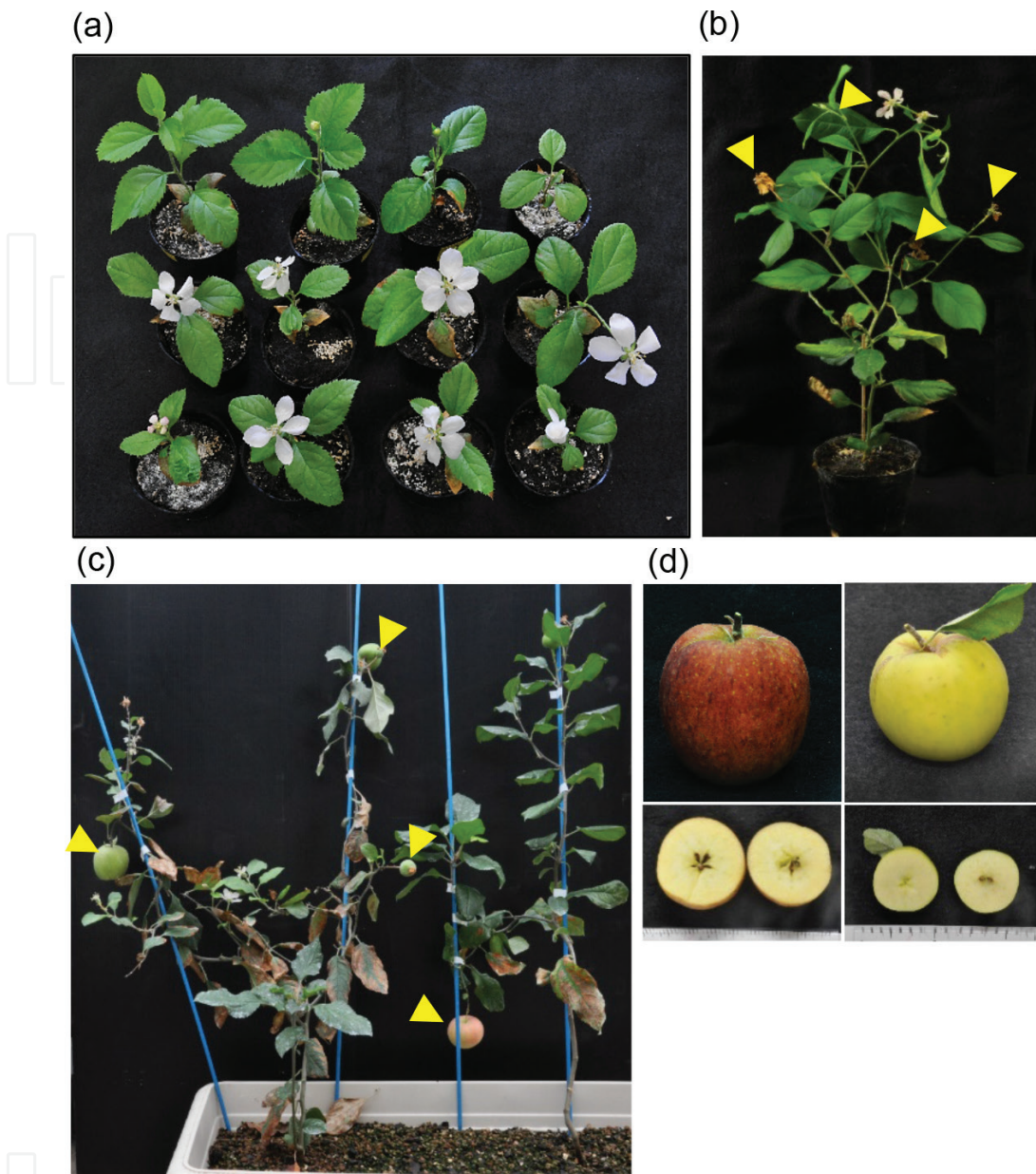
Figure 2. (a) Precocious flowering of apple seedlings infected with ALSV-AtFT/MdTFL1 (45 days postinoculation (dpi)). Eleven of 12 apple seedlings infected with ALSV-AtFT/MdTFL1 produced flower buds within 2 months. (b) A seedling (3 months postinoculation (mpi)) showing continuous flowering. Arrows indicate the flowers. (c) Apple seedlings with fruits (arrows) 8.5 mpi. (d) Ripe fruits on ALSV-AtFT/MdTFL1 infected apple seedlings (10 mpi) with viable seeds.

next-generation seeds) of apples that usually takes 5–12 years, to a year or less. This new technology is expected to be able to shorten substantially the period for breeding a new variety of apple via crossbreeding. In addition, as conventional breeding of fruit trees requires large fields, the use of this new technology enables completion of one generation in a growth chamber (**Figure 3**).

We also verified whether the technology of inducing early flowering using ALSV vector was applicable to pear [46]. We inoculated cotyledons of pear seedlings immediately

Figure 3. (a) Precocious flowering and fruit production of apple seedlings infected with ALSV-AtFT/MdTFL1 (7 mpi) in a growth chamber with a hydroponic cultural apparatus. Arrows indicate fruits. (b) Ripe fruits in seedlings shown in (a) with viable seeds. Scale bars indicate 1 cm.

after germination with ALSV-AtFT/MdTFL1 and confirmed that approximately 33% of the infected individuals flowered and showed continuous flowering where they flowered continuously over several months as the apple seedlings did (**Figure 4a, b**). We also constructed an ALSV vector (ALSV-AtFT/PcTFL1) that simultaneously performs *AtFT* expression and suppression of *PcTFL1-1* expression and inoculated to cotyledons of pear seedlings. The results indicated that approximately 86% of the infected individuals showed continuous flowering. This effect is likely due to replacement of the apple gene (*MdTFL1-1*) by pear counterpart (*PcTFL1-1*). This confirmed that the sequence identity is important for efficient gene silencing as reported elsewhere [28, 47, 48]. In addition, the pollens of early-flowered pear seedlings were fertile and triggered fruition of the infected individuals via pollination with their pistils (**Figure 4c**). We presume that this technology is likely to be feasible for all fruit trees that are susceptible to ALSV infection, and substantially contributes to optimization of breeding by crossing of new fruit tree varieties.
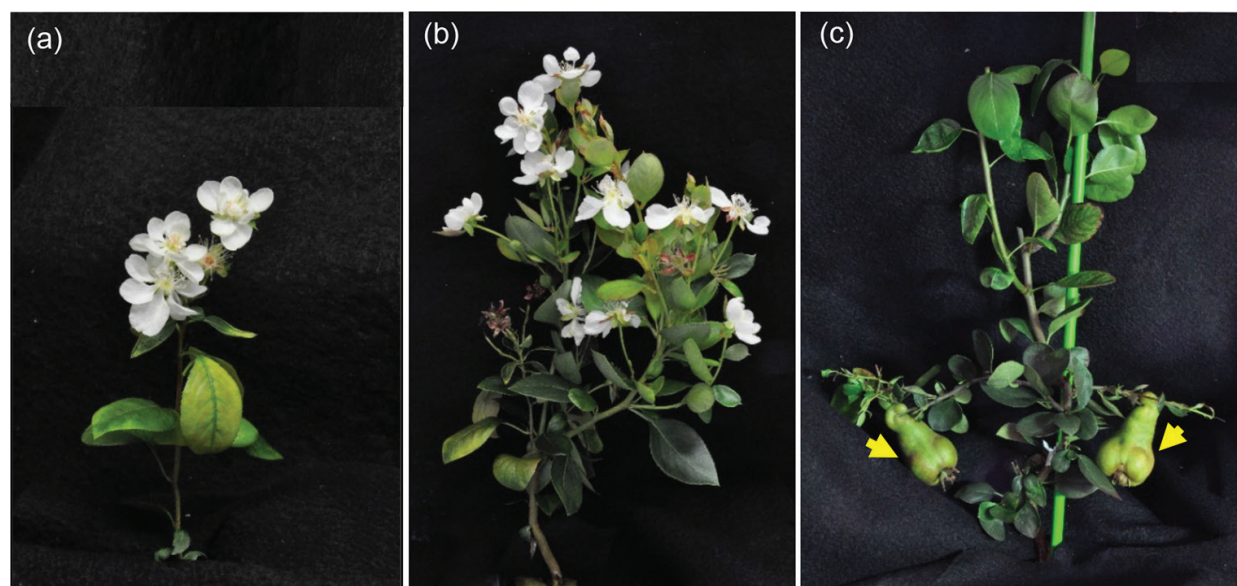
Figure 4. (a) Precocious flowering of a pear seedling infected with ALSV-AtFT/MdTFL1 (45 dpi). (b) Continuous flowering of a pear seedling infected with ALSV vector (3 mpi). (c) Fruition on a pear seedling infected with ALSV vector (7 mpi). Arrows indicate fruits on a pear seedling.

5. Elimination of ALSV from infected apple and pear trees

We tested 487 seeds obtained using pollens of ALSV-infected apple trees as the pollen parent, as well as 450 seeds from fruits on ALSV-infected apple trees, by ELISA and RT-PCR to test seed transmission. The rates of seed transmission from pollens and ovules were 0.38 and 4.5%, respectively [49]. We also investigated the rate of seed transmission from ovules using qRT-PCR, indicating that approximately 1% seedlings (two individuals out of 192 individuals) were infected with the virus [50]. Examination of 47 next-generation apple seedlings obtained from early-flowered seedlings using ALSV technology (ALSV-AtFT1/MdTFL1) showed that none of them were infected with ALSV vector, indicating that virus-free individuals can be obtained successfully [46].

Elimination of ALSV vectors from infected plants may allow the use of early flowering plants as breeding materials without genetic modification. We sometimes observed a phenomenon in which ALSV multiplied in inoculated leaves but not move to upper un-inoculated leaves [38]. We incubated ALSV-infected apple and pear seedlings for four weeks in a 37°C chamber, then returned them to a 25°C, and investigated the distribution of ALSV in infected plants. It was revealed that ALSV stopped movement to new tissues after the 37°C treatment, and no ALSV multiplication was observed in new tissues developed at 25°C [38]. We attempted detection of ALSV from the shoot apical meristem tissue of ALSV-infected apple and pear seedlings after incubation at 37°C by *in situ* hybridization; however, no ALSV was detected from the shoot apical meristem tissue after incubation at 37°C. It is likely that exclusion of ALSV from the shoot apical meristem tissue by high-temperature treatment (37°C) leads to cessation of subsequent ALSV movement to newly developed tissues [38] (**Figure 5**).
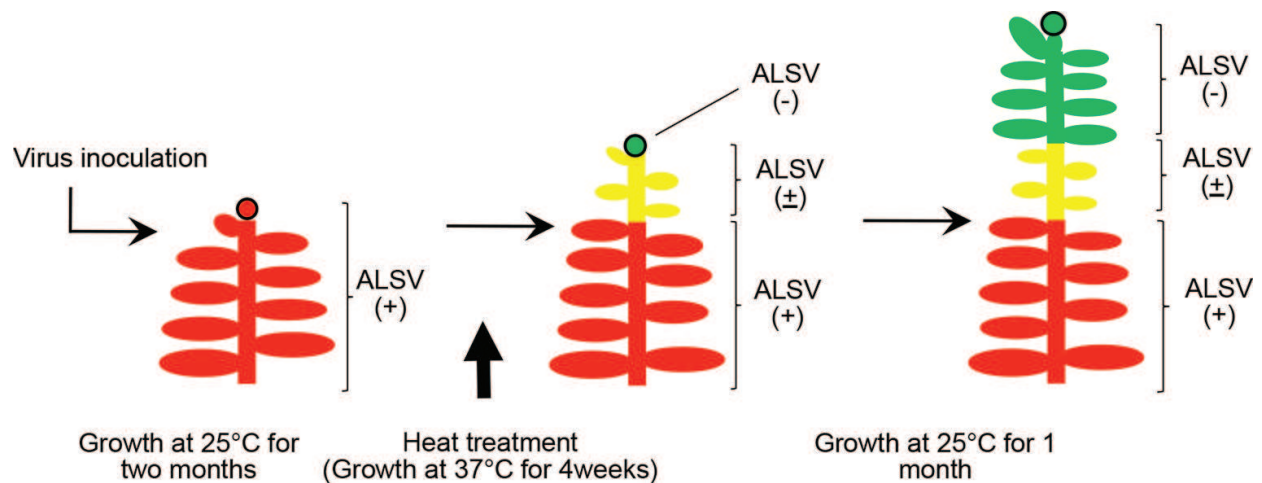
Figure 5. ALSV distribution in apple and pear seedling before and after heat treatment. *Red color* indicates the tissues which developed before heat treatment and systemically infected with ALSV. *Yellow color* indicates the tissues which developed during heat treatment and in which ALSV was weakly detected and/or not detected. *Green color* indicates the tissues which developed after heat treatment and in which ALSV was not detected. *Black circles* indicate the shoot apical meristems.

The results indicate that ALSV free tissues could be easily obtained from infected plants that flowered early by ALSV-AtFT1/MdTFL1 infection. Use of virus-free tissues as scions is likely to allow us to grow virus-free plants.

6. Discussion and perspective

The long juvenile phase of fruit trees is a significant barrier for efficient fruit tree breeding [3, 51]. The technology developed in the present study substantially shortens one generation of fruit trees via infection of the trees with an ALSV vector for promotion of flowering in fruit tree breeding. Conveniently, the majority of individuals of the obtained next-generation seedlings were free of ALSV because of low seed transmission rate of ALSV. Our ALSV vector technology, which is different from recombinant DNA technology, induces no mutation on the genome of the infected fruit tree. It is difficult to distinguish between normal plants and the plants after removal of ALSV vector. It is also possible to remove the virus easily by heat treatment from the infected materials, with these fruit trees not distinguishable from normal fruit trees. Therefore, these trees are likely to be used for breeding materials.

Velázquez et al. reported that they constructed a *clbvlNpr* vector from the *Citrus leaf blotch virus* and induced citrus in the juvenile phase to flower early via *AtFT* expression by the vector [52]. ALSV infects not only *Rosaceae* fruit trees but also citrus and grape; we can expect that ALSV will be used for the promotion of flowering in a greater variety of fruit trees in the future.

In recent years, determination of the full genome sequence of fruit trees has advanced, leading to publication of these sequences [53, 54]. This information is expected to accelerate bioinformatics, identification of molecular markers, marker selection, and omics research in fruit trees

even more [3, 51, 55–59]. The combination of virus-induced flowering technology described here with information obtained from these research is expected to lead to further optimization of fruit tree breeding in the future.

Acknowledgments

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