Studies on the development of a novel seed production technology for cabbages using the grafting-induced flowering with radish rootstocks

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Ko Motoki

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General Introduction

Grafting-induced flowering in basic research and plant breeding

Grafting-induced flowering has been intensively studied in various plant species as a key phenomenon for systemic floral regulation in plants (Chailakhyan, 1936; Zeevaart, 1976). Plants under non-inductive condition can be induced to flower with grafting to florally induced donor plants. This phenomenon evoked the concept of "florigen", a transmissible floral inducer common to a wide range of plant species, which is synthesized in the leaves under inductive condition and transported to the shoot apex to induce flowering (Chailakhyan, 1936). The entity of florigen had been unknown for a long time, but only in recent years it was finally revealed that a small globular protein FLOWERING LOCUS T (FT) and its orthologs were the major component (Abe et al., 2005; Corbesier et al., 2007; Kobayashi et al., 1999; Lin et al., 2007; Notaguchi et al., 2008; Tamaki et al., 2007). Several grafting experiments confirmed that FT is translocated from induced donor leaves to recipient shoot apical meristem (SAM) through the graft junction, and is a causal factor of the grafting-induced flowering (Corbesier et al., 2007; Lin et al., 2007; Notaguchi et al., 2008).

FT protein has been expected to be applied to the floral induction technology in agriculture. However, unlike other low molecular weight plant hormones, the application of FT to the floral induction of plants by external treatment has not been realized. This is because FT protein is a high molecular weight (20 kDa) and cannot penetrate cell membranes. Therefore, newly developed direct applications of FT in agriculture are limited to the transient expression using viral vectors or the transgenic introduction (Putterill and Varkonyi-Gasic, 2016; Yamagishi et al., 2014). Along with these transgenic based techniques, grafting remains a classic but important method to induce flowering by introducing FT protein into plants externally in a non-transgenic manner. Thus, grafting-induced flowering has potential to be applied to the plant breeding and seed production, to shorten the time needed for floral induction, and to expand the environment in which seed production is possible. In fact, grafting-induced flowering been applied for the floral induction of several crop species which have long generation time or difficulty in flowering under natural conditions, such as sweet potato (*Ipomoea batatas*; Sunakawa, 1966), citrus (*Citrus sinensis*; Soares et al., 2020), jatropha (*Jatropha spp.* ; Tang et al., 2022; Ye et al., 2014).

Failure of grafting-induced flowering in several plant species

However, the use of this technique is currently limited to specific plant species. One of the reason would be that there are plant species that do not flower even when grafted onto florally induced plants (van de Pol, 1972). Factors such as differences in the sensitivity of the recipient plants to florigen, the amount and duration of the production of florigen in the donor plants, and the transmission efficiency of florigen in both donor and recipient plants have been postulated as reasons for the unsuccessful induction of flowering

by grafting in certain plants (Putterill and Varkonyi-Gasic, 2016; Zeevaart, 1976). The sensitivity of the receptor plants to florigen has not been studied in detail at the molecular level. Although genes that antagonize FT and suppress its action at SAM, such as the TERMINAL FLOWER 1 (TFL1) homologues, are known to exist (Bradley et al., 1997; Ohshima et al., 1997; Zhu et al., 2020), its relationship to the unsuccessful floral induction by grafting is unknown. With respect to the production of florigen in the donor plants, previous studies have reported that the flowering responses of recipient plants were associated with *FT* transcription levels in donor plants (Notaguchi et al., 2008; Tang et al., 2022; Yoo et al., 2013b). On the other hand, several studies reported a failure of floral induction in the recipient plants even when *FT*-overexpressing transformant was used as a donor rootstock (Bull et al., 2017; Odipio et al., 2020; Tränkner et al., 2010; Wenzel et al., 2013; Zhang et al., 2010). In these cases, the rapid floral induction of *FT*-overexpressing transformant itself was observed, thus confirming that FT itself is capable of inducing flowering alone in these plants. Several scenarios have been considered in these studies as a possible cause such that FT itself is not translocatable in the plant species (Tränkner et al., 2010), that the scion is not capable of transporting FT (Tränkner et al., 2010), that the lack of leaves on the rootstock which can supply FT to the scion (Odipio et al., 2020), or that the dilution or attenuation of FT occurs when moving across the graft junction and into the receptor scion (Zhang et al., 2010). However, these scenarios were not examined with the actual amount of FT protein accumulated in the scion in these studies. Recently, a study quantifying FT proteins in jatropha reported that the longer the distance from the graft junction in the scion was, the less efficient the transport of FT from the *FT*-overexpressing rootstock was, and the less floral induction occured in the buds (Tang et al., 2022). It was reported in another study which failed to induce the flowering of trifoliate orange (*Citrus trifoliata*) by grafting onto FT-overexpressing rootstocks that the amount of FT protein accumulated in the scion was lower than the amount of FT protein accumulated in the rootstock (Wu et al., 2022). These studies are supporting the above hypothesis that insufficient FT protein accumulation in the scion may be the reason for the failure of grafting. However, in these studies, the factors required for the rootstock to supply enough amount of FT protein to the scion were not examined. It will be important to clarify such rootstock factors to apply the grafting method to the practical breeding and seed production in wider range of plant species.

Flowering characteristics of cruciferous crops in relation to their economic production and breeding

In cruciferous crops (Brassicaceae), which include major vegetable and oil crops, the timing of flowering is an important determinant of harvest time and yield potential (Leijten et al., 2018). Most cruciferous crops are induced to flower after exposure to prolonged low temperature, a phenomenon known as vernalization. In crops where the reproductive organs are harvested, such as broccoli (*Brassica oleracea* L. var. *italica*), cauliflower (*B. oleracea* L. var. *botrytis*), and rapeseed (*Brassica. napus* L.), flowering time directly determines the timing of harvest initiation. Therefore, breeding of the flowering time in these crops

are focused on the adaptation to the local and seasonal environment to ensure the floral induction at the preferable timing for the economic production. In contrast, in crops where the vegetative organs are harvested, such as Chinese cabbage (*Brassica. rapa* L. var. *pekinensis*), cabbage (*B. oleracea* L. var. *capitata*), and radish (*Raphanus sativus* L.), repression of the floral induction during the cultivation period is important because stem elongation followed by flowering (bolting) spoils the quality of the harvesting site and limits the harvestable period. Thus, breeding of late-bolting cultivars has been intensively pursued in these crops to enable the year-round production (Kitamoto et al., 2017; Leijten et al., 2018).

In some situations, vernalization requirement leads to a delay in the breeding cycle because of the increase in time required for flowering. Especially in cabbage, the delay in the breeding cycle due to the vernalization requirement is particularly remarkable. This is because cabbage is a plant-vernalization-type plant, which becomes sensitive to low temperatures only after developing to a certain size by expanding many true leaves, and it also requires long-term low-temperature exposure for flowering (Ito and Saito, 1961; Miller, 1929). Cabbage takes about six months or more for one generation, which is much longer than other cruciferous crops. In extreme cases, there exist a cabbage accession which cannot flower even after overwintering (Kinoshita et al., 2021). Furthermore, cabbage is a polycarpic plant, which has ability to turn back to vegetative growth after flowering; thus, reversion from reproductive growth to vegetative growth tends to occur when vernalization is insufficient (Ito and Saito, 1961). The vernalization requirement also limits regions suitable for seed production of cabbage. Research on floral induction by artificial low-temperature treatment in the climate chamber (Wang et al., 2000), gibberellin treatment (EL-Eslamboly and Hamed, 2021; Nyarko et al., 2007) and drought shock treatment (EL-Eslamboly and Hamed, 2021) has been conducted with the intention of the seed production in tropical and subtropical regions where cabbage is difficult to flower under natural conditions. However, these treatments cannot completely substitute the low-temperature exposure necessary for the floral induction of cabbage. If a method enabling cabbage to flower without low-temperature exposure, it will shorten the time and will remove the regional limitations for cabbage breeding and seeds production.

Potential and limitation in the usage of grafting-induced flowering for the floral induction in cabbage

Kagawa (1957) reported that he could induce cabbage flowering without low-temperature treatment by grafting it on a flowering radish (*R. sativus*) rootstock. To my knowledge, this is the only report where cabbage has successfully flowered without low-temperature treatment. Interestingly, in this study by Kagawa (1957), when grafted onto florally induced radish rootstocks, one in five cabbage scions flowered without low-temperature treatment; however, when grafted onto florally induced cabbage, no cabbage scions flowered. Kagawa discusses that this difference in floral induction of the grafted cabbages may be caused by the difference in transmissibility of floral stimulating agents between radish and cabbage

used as a rootstock. However, his findings have not been reproduced thereafter. A different publication reported that cabbage did not flower at all when they were grafted onto florally induced radish (Hamamoto and Yoshida, 2012). These results suggest that it may be possible to induce flowering of cabbage without low-temperature treatment by grafting onto florally induced plants, but success may vary depending on the plant species used as a rootstock, or other conditions during the grafting procedure.

Genes related to the vernalization flowering pathway of cruciferous crops

Genes related to the vernalization flowering pathway have been identified in *Arabidopsis thaliana*, which belongs to the same plant family as cabbage, Brassicaceae. The floral repressor gene *FLOWERING LOCUS C* (*FLC*), which acts as an integrator of the vernalization pathway, encodes a MADS-box protein that represses the expression of the floral promoter gene *FT* and floral integrator gene *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) by directly binding to their first intron and promoter, respectively (Helliwell et al., 2006; Michaels and Amasino, 1999). Before low-temperature exposure, *FLC* is highly expressed and suppresses flowering. After the epigenetic silencing of *FLC* by prolonged low-temperature exposure, *FT*, *SOC1*, and other flowering-related genes are activated to promote flower bud differentiation (Bastow et al., 2004; Helliwell et al., 2006; Searle et al., 2006). The function of *FLC* is also conserved in *B. oleracea*, and *FLC* homologs are known to be involved in flowering time determination of several *B. oleracea* vegetables. *BoFLC4* (also known as *BoFLC2*) is one of the *FLC* homologs in *B. oleracea*, and was shown to be strongly associated with the vernalization requirement by quantitative trait locus analysis in broccoli (Irwin et al., 2016; Okazaki et al., 2007), cabbage (Okazaki et al., 2007), and cauliflower (Ridge et al., 2015). Meanwhile, *BoFLC3* was recently reported to be involved in curd induction variation in the subtropical broccoli breeding lines under the subtropical environment (Lin et al., 2018). *FT* homologs in *B. oleracea* increase their expression level after low-temperature exposure in similar to *A. thaliana* (Irwin et al., 2016; Lin et al., 2005; Ridge et al., 2015), and two *FT* loci (*BoFT.C2* and *BoFT.C6*) have been reported (Wang et al., 2012). *SOC1* homologs in *B. napus*, a closely related species of *B. oleracea*, have also similar expression patterns to their ortholog in *A. thaliana* (Guo et al., 2014). In radish, several transcriptome analysis suggested the involvement of the homologs of *FLC*, *FT*, and *SOC1* in the vernalization flowering pathway (Jung et al., 2016; Nie et al., 2016a, 2016b).

Effects of the environmental condition during the seed production on the traits of the progenies

Environmental conditions during the seed production have been shown to affect the traits of the progenies in several plant species. The temperature encountered by the mother plant has been reported to affect the flowering response of the progenies in radish (Shinohara, 1959), *Eustoma grandiflorum* (Imamura et al., 2009), *Bupleurum rotundifolium* L. (Goto et al., 2011), and chicory (*Cichorium intybus*) (Wiebe, 1989). In *A. thaliana*, even the temperature condition prior to flowering of mother plant has been

shown to affect the seed traits (Chen et al., 2014). It was also reported that differences in seed harvesting methods had significant effect on the traits of the obtained seeds such as seed size, the initial growth and the flowering response (Hagiya, 1949, 1950). Additionally, while the possibility of the direct horizontal gene transfer from the rootstock to the progenies of the grafted plants is thought to be extremely low (Bock, 2017), transgenerational trait change due to epigenetic alteration in the progenies of the grafted plants can be induced by mobile small RNAs from the rootstock in some cases (Kundariya et al., 2020). These previous studies raise the possibility that cabbage seeds obtained by the grafting-induced flowering method, where mother plants do not experience the low temperature at all, and grow on the radish rootstocks during the seed formation, may exhibit different traits in flowering and other agronomic traits compared to the seeds produced by the conventional vernalization-induced flowering method. Clarification of this point is important to predict the traits of the obtained seeds for the application of grafting-induced flowering to breeding and seed production technologies.

Objective of this study

The main objective of this study was to establish the basis of the floral induction technique of cabbage by grafting for the development of novel seed production technologies. This study also aimed to elucidate the required characteristics of the rootstocks for the successful floral induction at the molecular level via the quantitative analysis of FT protein, and to obtain knowledge applicable not only to cabbage, but also to other crops. In Chapter 1, I reproduced the grafting-induced flowering of cabbage, and searched radish accessions which can stably induce the flowering of cabbage. Furthermore, through the expression analysis of flowering related genes, the mechanism of floral induction in cabbage by grafting onto radish was inferred. In Chapter 2, I developed an antibody to detect FT protein in radish and cabbage, and established a quantitative analysis method for FT protein. In Chapter 3, I applied the method of Chapter 2 to elucidate the relationship between FT protein accumulation and the flowering response in the grafted cabbage. Traits of the rootstock associated with the FT protein accumulation in the grafted cabbage was also investigated. In Chapter 4, I evaluated the seed traits and field performance of cabbage seeds obtained by the grafting-induced flowering to confirm the applicability of the grafting method to breeding and seed production of cabbage. Finally, the findings from Chapters 1 to 4 were integrated to discuss the conditions required for stable induction of flowering by grafting in cabbage and other crops from the point of view of the quantitative effects of FT protein, and to discuss issues for practical application of the grafting method to seed production technologies.

Chapter 1.

Reproduction of the floral induction of cabbage by grafting onto radish rootstocks and the estimation of its mechanism

1.1. Introduction

In the pioneering work by Kagawa (1957), he could induce flowering of cabbage without vernalization treatment by grafting onto radish rootstocks. However, the number of cabbages induced to flower was as low as 1 out of 5 grafted plants, and this phenomenon was not reproduced thereafter. I hypothesized that there is a difference in the ability to induce flowering of the grafted cabbage not only between *B. oleracea* and *R. sativus*, but even within *R. sativus* accessions. *R. sativus* is broadly cultivated in the world, including East Asia, Southeast Asia, Middle East and Occident (Kobayashi et al., 2020), and has diverse flowering characteristics (Kitashiba and Yokoi, 2017). Therefore, I expected that there exist *R. sativus* accessions with superior ability to induce flowering of the grafted cabbage.

In this chapter, to identify rootstocks enabling the stable reproduction the grafting-induced flowering of cabbages, the flowering response of cabbages grafted onto several *B. oleracea* and *R. sativus* rootstocks was investigated under controlled environmental conditions. At the same time, I estimated the mechanism of the grafting-induced flowering of cabbage, by comparing the expression pattern of flowering-related genes of the cabbage plants which were induced to flower by exposing to vernalization treatment and by grafting onto radish rootstocks. The expression level of *FT* homolog in the rootstocks were also investigated to understand the difference in the ability to induce flowering of the grafted cabbage.

1.2. Materials and Methods

Plant material and growth condition

All plants were grown in a growth room maintained at 22 ± 2°C, PPFD 80 μmol m^{-2} s^{-1}, irradiated with fluorescent lamps (NEC Lighting, Ltd., Japan), under long-day [16/8, light (L)/dark (D)] conditions. Japanese commercial cabbage cultivar, ‘Watanabe-seiko No.1’ (Genebank Project, NARO, Japan, accession No. 25974), ‘Kinkei No.201’ (Sakata Seed Corp., Japan), and ‘Red cabbage’ (Nakahara Seed Product Co., Ltd., Japan) were used as scion plants for grafting experiment. It was previously reported that ‘Watanabe-seiko No.1’ requires vernalization at 4–9°C for 6–7 weeks to induce flower bud differentiation (Ito and Saito, 1961). The two other cultivars require vernalization for flower bud differentiation as well. To reduce the effect of juvenility of the scion on its flowering response, cabbage seedlings grown for more than 10 weeks after sowing were used for collecting scions. The terminal shoots of the cabbage seedlings were pinched to elongate the lateral shoots, which were used as scions. For the grafting experiments using ‘Watanabe-seiko No.1’ as a scion, two

cultivars of Chinese kale ‘Kairan #1’ (Tsurushin Seed Ltd., Japan) and ‘Kairan #2’ (Kuragi Seed Ltd., Japan); four cultivars of radish for root vegetalbes (*R. sativus* L. var. *longipinnatus*) ‘Osaka-shijunichi’ (Noguchi Seed, Japan), ‘Wakayama’ (Noguchi Seed), ‘Shinshu-jidaikon’ (Shinshu-sankyo Seed Co., Japan), and ‘Hayabutori-shogoin’ (Takii Seed Co., Japan); and three accessions of radish for seed-pod vegetalbes (*R. sativus* L. var. *caudatus*) ‘Rat’s tail-CH’ (Chiltern Seeds, United Kingdom), ‘Rat’s tail-G2’ (Genebank Project, NARO, Japan, accession No. 76703), and ‘Rat’s tail-G4’ (Genebank Project, NARO, Japan, accession No. 86212) were used as rootstocks (Table 1-1). These plants were chosen because flowering induction is easy; Chinese kale does not need low-temperature treatment for flowering, and radishes are seed vernalization plants that can be vernalized even at the seed stage. As a control, non-vernalized ‘Watanabe-seiko No.1’ [‘Watanabe-seiko No.1’ (NVstock)] were also used as rootstock plants. For the expression analysis of *RsFT* in radish exposed to different periods of seed vernalization treatment, in addition to the radish cultivars listed above (except for ‘Rat’s tail-G4’), ‘Utsugi-gensuke’ (Noguchi Seed), ‘Comet’ (Noguchi Seed), ‘Kuronaga-kun’ (Noguchi Seed), and ‘Tokinashi’ (Noguchi Seed) were also used. For the low-temperature treatment of rootstocks, seeds were sown on wet filter paper and germinated in dark condition at 22°C for 1–2 days. The germinated seeds were incubated at 2°C in the dark for various, predefined periods of time (Table 1-1, Table 1-3). The vernalized seeds were transplanted into 7.5 cm diameter plastic pots filled with granular rockwool (Nippon Rockwool Corp., Tokyo, Japan) and cultivated until bolting occurred. All plants were irrigated and fertilized from underneath the bottom of the pots using a half-strength nutrient solution (Enshi-shoho, formulated by the National Horticultural Research Station, Japan). For the grafting experiments using ‘Kinkei No.201’ and ‘Red cabbage’ scions, non-vernalized ‘Rat’s tail-CH’ seedlings were grown in 6.0 or 7.5 cm plastic pots for use as rootstocks as described above.

Grafting of cabbage scions to rootstock plants

For radish rootstocks, seedlings that had bolted to a height of 5–8 cm from the top of the hypocotyl were used as rootstocks (Fig. 1-1A). For two cultivars of Chinese kale rootstocks, seedlings at 5–8 weeks were used as rootstocks. For ‘Watanabe-seiko No.1’ (NVstock), seedlings 6–7 weeks after sowing were used as rootstocks. The stem of the rootstock was cut at a height of 3–5 cm from the top of the hypocotyl, and the cabbage scion with 2–3 expanded leaves was grafted onto the stem of the rootstock by cleft grafting (Fig. 1-1B). After grafting, the scion and a portion of the leaves of the rootstock plant were covered with a clear polyethylene bag to maintain high humidity, and the plants were grown under light conditions in the growth room. One to two weeks after grafting, once the scion and rootstock were fully connected, the polyethylene bag was removed. To promote translocation of assimilates from the rootstock to the scion, all the lateral shoots of the rootstock were removed and new leaves on the scion were removed with only 3–4 newly expanded leaves remaining, so that scion can keep sink activity.

Self-pollination of cabbage flowers

Cabbage scions that flowered after grafting were self-pollinated by CO_2 treatment (Nakanishi and Hinata, 1975). Cabbage flowers were pollinated with self-pollen, and the inflorescence was immediately covered by two-layered clear polyethylene bags. Then, 120 mL of CO_2 gas (equal to almost 20% of the volume of the polyethylene bag) was injected into the bag, with the opening tied. The polyethylene bags were removed 12–24 h after pollination.

Measurements of the growth and flowering parameters of the rootstock and scion

The number of leaves on the rootstock plants (L_n) and the length (L_l) and the width (L_w) of the biggest leaf of the rootstocks were measured immediately after grafting. Then, the total leaf area of the rootstock (L_a) was estimated using a preliminary calculated regression equation (Chinese kale: $L_a = 0.2357 \times L_n \times L_l \times L_w + 128.65$, radish: $L_a = 0.2407 \times L_n \times L_l \times L_w + 65.609$, Supplementary Fig. 1-S1 and Table 1-S1). Plant growth continued until 60 days after grafting (DAG), and the appearance of flower buds was evaluated by daily visual inspection. On 60 DAG, the total number of expanded leaves of the scion (2 cm length or more) was counted, and scion flower bud differentiation was evaluated under a microscope if obvious flower buds were not visible. If the number of leaves of the scion at 60 DAG was less than 10, the grafting was regarded as a failure and the data were excluded from the calculation for the days to grafting after sawing, L_a, number of expanded scion leaves, and percentage of scions with flower buds. The percentage of scions with flower buds was calculated as follows: (the number of individuals with differentiated flower buds at 60 DAG)/(the number of the successful grafts) × 100.

Gene expression analysis

For gene expression analysis of flowering-related genes in the grafted rootstocks and scions, leaves were sampled as follows. The tip of third expanded leaf from the top of 'Watanabe-seiko No.1' scions, which had flowered following grafting onto 'Rat's tail-CH' and 'Rat's tail-G2', were sampled twice: at 7 DAG and within 1 week from flower bud appearance. As a control, the tip of third leaf from the top of 'Watanabe-seiko No.1' scions grafted onto 'Watanabe-seiko No.1' (NVstock) were sampled at 7 and 60 DAG. For the analysis of rootstocks, the tip of healthy and non-yellowing fresh leaves at the lowest position were sampled at 30 DAG. For comparison with 'Watanabe-seiko No.1' which had flowered by grafting, expression analysis of flowering-related genes was also performed on 'Watanabe-seiko No.1' which had been subjected to low-temperature treatment. The seedlings of 'Watanabe-seiko No.1' were grown in the growth room for 10 weeks after sowing. Then, the seedlings were vernalized for 8 weeks in the growth cabinet, at a temperature of 6 ± 1°C, under LED light (660 nm:450 nm = 8:2) with an output of 90 μmol m^{-2} s^{-1} PPFD in long-day (16L/8D) conditions. After

low-temperature treatment, the seedlings were returned to the growth room and continued to grow for 2 weeks. The eighth expanded leaf from the top was collected at the beginning and the end of the 8 weeks low-temperature treatment and at 2 weeks after returning to the growth room. For the expression analysis of *RsFT* in radish cultivars exposed to different periods of seed vernalization treatment, the tip of the largest leaf was sampled at the day when the plant had bolted to a height of 3 cm from the top of the hypocotyl. All leaf samples were collected at 1 h before the end of the light period. Sampled leaves were immediately frozen with liquid nitrogen and stored at -80°C until RNA extraction.

Total RNA was extracted from leaves using Sepasol RNA I Super G (Nacalai Tesque, Kyoto, Japan), purified using a high-salt solution for precipitation (Takara Bio Inc., Ohtsu, Japan), and reverse transcribed with ReverTra Ace® (Toyobo Co., Ltd., Osaka, Japan), following which 1 μL of 10-fold diluted reverse transcription (RT) product with pure water was used as a template for quantitative RT-PCR (RT-qPCR). RT-qPCR was performed using the THUNDERBIRD® SYBR® qPCR Mix (Toyobo Co., Ltd.) according to the manufacturer's instructions using the LightCycler® 480 system (Roche Diagnostics K.K., Tokyo, Japan). The RT-qPCR cycling was performed as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Single-target product amplification was evaluated using a melting curve. All RT-qPCR primers used in this study are listed in Supplementary Table 1-S2. The primer sequences were designed on the basis of structural gene sequences published in the NCBI databases1 and the report of Irwin et al., (2016). For primers analyzing individual homologous transcripts of *FLC* (*BoFLC3* and *BoFLC4*) and *FT* (*BoFT.C2* and *BoFT.C6*), particular caution was used to design primers for the different sequences between the two homologous genes. For the expression analysis of cabbage scion and vernalized 'Watanabe-seiko No.1', primer sets *BoFLC3, BoFLC4, BoFT.C2, BoFT.C6, BoSOC1*, and *BoActin* were used. For the expression analysis of 'Watanabe-seiko No.1' and Chinese kale rootstocks, *BoFT.C6* and *BoActin* were used, whereas *RsFT* and *RsActin* were used for radish rootstocks.

Sequence analysis of FT homologs

The PCR was performed using a primer set (Table 1-S2) that amplifies the full CDS of the *FT* homolog mRNA. RT products of total RNA extracted from the leaves of the rootstock plants were used as templates. The CDS was determined by direct sequencing of the amplification products using Sanger sequencing. The sequence data were deposited in DDBJ with accession numbers LC414370-LC414380.

1.3. Results

Initial flowering response of rootstock plants and scion growth after grafting

Radish rootstocks bolted within approximately 20–40 days after sowing (DAS), with differences observed in the number of days until bolting among different cultivars (Table 1-1). The earlier the rootstock bolted, the smaller their leaf area tended to become, with the leaf area being the smallest in ‘Rat’s tail-G2’ vernalized at 2°C for 3 days (133.1 ± 16.3 cm^2) with the largest leaf area observed in ‘Rat’s tail-CH’ (528.5 ± 227.3 cm^2) (Table 1-1). Flower bud appearance was observed in some individuals of Chinese kale at the time of grafting (5th–8th weeks after sowing, data not shown). The leaf areas of the two Chinese kales were 425.4 ± 114.3 cm^2 and 456.7 ± 51.6 cm^2 for ‘Kairan #1’ and ‘Kairan #2’, respectively. The leaf area of ‘Watanabe-seiko No.1’ (NVstock) was 287.2 ± 67.8 cm^2. The scion and the rootstock were successfully connected at 1–2 weeks after grafting, and the percentage for grafting success was approximately 70–80%, without large differences observed between rootstock cultivars (Table 1-2). The total number of expanded scion leaves at 60 DAG was on average 21.1 ± 2.0 to 32.1 ± 10.7 (Table 1-2). There was moderate positive relationship between the leaf area of the rootstock at 0 DAG and the number of expanded leaves of the scion at 60 DAG (r = 0.54, Supplementary Fig. 1-S2).

Flowering of grafted scions and seed formation

When ‘Watanabe-seiko No.1’ was grafted onto non-vernalized ‘Watanabe-seiko No.1’, Chinese kales and some of the radish cultivars, the scions continued vegetative growth and did not form flower buds at 60 DAG (Table 1-2; Figs. 1-2A_1–C_2). On the other hand, a portion of the scions grafted onto radish cultivars ‘Wakayama’ (1 out of 17), ‘Hayabutori-Shogoin’ (3 out of 18), ‘Rat’s tail-CH’ (6 out of 12), and ‘Rat’s tail-G2’ (2°C, 3 days, 6 out of 8) formed flower buds (Table 1-2; Figs. 1-1C–E, 1-2D_1, D_2, E_1, E_2). In particular, the percentage of the scions with flower buds was high when ‘Rat’s tail-CH’ and ‘Rat’s tail-G2’ (2°C, 3 days) were used as the rootstock plants, with percentages of 50.0% and 75.0%, respectively (Table 1-2). The average number of days from grafting to flower bud appearance was 47 days, 43.3 ± 10.1 days, and 49.0 ± 6.7 days in ‘Hayabutori-Shogoin’ (only one scion formed visible flower bud before 60 DAG), ‘Rat’s tail-CH’, and ‘Rat’s tail-G2’ (2°C, 3 days), respectively (Table 1-2). The earliest flower bud appearance was observed at 29 DAG when ‘Rat’s tail-CH’ was used as a rootstock plant. The scions flowered approximately 2–3 weeks after flower bud appearance (Table 1-S3), and most flowers formed normal floral organs with pistils and stamens (Fig. 1-1F). The scions produced 2–3 inflorescences and finished blooming within approximately 1 month and returned to vegetative growth (Fig. 1-S3).

I carried out self-pollination using the successfully flowered scions by CO_2 treatment and obtained matured seeds roughly 80 days after pollination (Fig. 1-1G). These seeds germinated and grew

normally (Figs. 1-1H, I). The 'Kinkei No.201' and 'Red cabbage' scions grafted onto 'Rat's tail-CH' also flowered and produced normal seeds (Table 1-S4; Fig. 1-S4).

Expression analysis of flowering-related genes in scions

In 8 weeks vernalized 'Watanabe-seiko No.1', two out of three tested plants flowered within 1 month after the end of low-temperature treatment. This partial flowering may be because the low-temperature treatment in this study was not enough to fully induce flowering of this cabbage cultivar. On 8 weeks vernalized 'Watanabe-seiko No.1', the expression of two *FLC* homologous genes, *BoFLC3* and *BoFLC4*, decreased, relative to *BoActin*, to approximately 7 and 50% of the level from the start of treatment, respectively (Figs. 1-3A, C). Conversely, in the graft-flowered scions, the expression levels of *BoFLC3* and *BoFLC4* were the same or higher than control plants grafted onto 'Watanabe-seiko No.1' (NVstock), at both 7 DAG and flower bud appearance (Figs. 1-3B, D). The expression level of *BoFT.C2* and *BoFT.C6* increased after low-temperature treatment with large variability (Figs. 1-3E, G). This variation in the expression of *FT* homologs may reflect the inadequacy of low-temperature treatment. In contrast, those *FT* homologs were very lowly expressed in all graft-flowered scions (Figs. 1-3F, H). Further, the expression level of *BoSOC1* tended to increase after low-temperature treatment (Fig. 1-3I). In the graft-flowered scions, the expression levels of *BoSOC1* significantly increased compared with the control grafts (Fig. 1-3J).

Expression and sequence analysis of FT homologs of rootstock plants

In the previous studies, two *FT* homologs in *B. oleracea*, and one *FT* homolog in *R. sativus* have been reported (Nie et al., 2016b; Wang et al., 2012). To predict the candidate *FT* homologs which mainly act as transmissible floral signal in the Chines kale and radish rootstocks, I searched genes which have high homology with *A. thaliana* FT protein (Accession No. AAF03936) in the NCBI reference sequence database of *B. oleracea* and *R. sativus* using BLAST program. As a result, I could find three and two genes without insertion or deletion within any of the exons in *B. oleracea* and *R. sativus*, respectively (Fig. 1-S5A). I also conducted BLAST genome search against reference genome database of *B. oleracea* (BOL reference Annotation Release 100) and *R. sativus* (Rs1.0 reference Annotation Release 100) and got the same result. Among those genes, two for *B. oleracea* and one for *R. sativus* were identical to the *FT* homologs reported in previous studies (XP_013619513 / BoFT.C2, XP_013590834 / BoFT.C6, XP_018470278 / RsFT). Both of the other two proteins (XP_013635334/*B. oleracea*, XP_018460008/*R. sativus*) belonged to TWIN SISTER OF FT (TSF) clade by phylogenetic analysis (Fig. 1-S5B). Although all of these genes were assumed to have *FT*-like function from their amino acid sequence, *BoFT.C6* and *RsFT* were chosen for expression analysis because they were expressed at much higher level than the other genes in the lowest leaf of Chinese kale and radish, in my preliminary experiment.

There were differences among the expression levels of *FT* homologs in each rootstock cultivar at 30 DAG (Fig. 1-4 A, B). In the leaves of 'Watanabe-seiko No.1' (NVstock), the *FT* homolog, *BoFT.C6*, was very lowly expressed. In Chinese kales, the *FT* expression levels were 2–3 times higher than 'Watanabe-seiko No.1'. Expression level differed within radish cultivars. The expression level of *RsFT*, relative to the expression of *RsActin*, was the highest in 'Rat's tail-G2' (2°C, 3 days), and its expression level was significantly higher than all other cultivars. Conversely, the average expression levels of *FT* homolog in 'Rat's tail-G2' (2°C, 0 day), 'Rat's tail-G4', and 'Rat's tail-CH' were the lowest among radish cultivars. When the expression level of *RsFT* in the rootstocks was compared by the occurrence of the flower bud differentiation of the grafted cabbage, significantly higher expression was observed in the rootstocks where the grafted cabbage had differentiated buds (Fig. 1-4 C). However, there were also individuals for which flower bud differentiation of the grafted cabbage did not occur, even though the expression level of *RsFT* of the rootstock was comparable to that of individuals for which flower bud differentiation of the grafted cabbage was induced.

Sequence analysis of the *FT* homolog CDS revealed several amino acid sequence polymorphisms within Chinese kale cultivars and between Chinese kale and radish (Fig. 1-S6). However, no amino acid polymorphism was observed in the coding region among the radish cultivars used in the grafting experiment.

The analysis of RsFT expression in several radish cultivars exposed to different period of seed vernalization treatment

To clarify the factors that cause differences in *RsFT* expression among radish cultivars, I investigated the expression level of *RsFT* in leaves of several radish cultivars that were exposed to different periods of seed vernalization treatments and then grown under long-day condition. In addition to the cultivars used in the grafting experiment, I also investigated another four radish cultivars which have relatively stronger vernalization requirement for floral induction (Table 1-3). About half of the cultivars were fully bolted by 28 DAS with 2 weeks of vernalization treatment (Table 1-3). On the other hand, in 'Hayabutori-shogoin', 'Comet', 'Kuronaga-kun' and 'Tokinashi', bolting did not occur at all with 2 weeks of vernalization treatment, and a longer period of vernalization was required for all individuals to bolt. In particular, the late-bolting cultivar 'Tokinashi' required an 8 weeks of seed vernalization treatment. Days to bolting was tended to be shortened in all cultivars with longer periods of seed vernalization treatment. The expression level of *RsFT* tended to increase with longer periods of vernalization treatment with the exception of 'Rat's tail-G2' (Fig. 1-5A). With 4 weeks or a longer vernalization treatment, those cultivars with weaker vernalization requirement showed comparable expression revel of *RsFT*. In contrast, in 'Hayabutori-shogoin', 'Comet', 'Kuronaga-kun', and 'Tokinashi', which required a longer vernalization treatment for bolting, the expression level of *RsFT*

was tended to be lower than that in the other cultivars even after 4 weeks or a longer vernalization treatment.

Next, I further examined the characteristics of the flowering response and *RsFT* expression with shorter vernalization treatments in early-flowering radish cultivars. In ‘Shinshu-jidaikon’, bolting did not occurred at all without vernalization, whereas in ‘Rat's tail-G2’ and ‘Rat's tail-CH’, bolting occurred even without vernalization (Fig. 1-5B). The expression levels of *RsFT* was suppressed in ‘Shinshu-jidaikon’, but was much higher in ‘Rat's tail-G2’ and ‘Rat's tail-CH’ with one week of vernalization treatment. Especially in ‘Rat's tail-CH’, there were individuals with high *RsFT* expression even when no vernalization treatment was given at all.

Table 1-1. Basic features of rootstock plants used for grafting.

Species	Cultiver/ Accession name	Origin	Seed vernalization treatment (days)	Days to grafting after sowing[z]	Number of leaves left on rootstock plant[z]	Estimated total area of leaves left on rootstock plant (cm^2/plant)[z]
B. oleracea var. *capitata*	Watanabe-seiko No.1 (NVstock)[y]	Genebank project, NARO, Japan, accession No.25974	0	48.1±3.2	4.6±0.8	287.2±67.8
B. oleracea var. *albograbra*	Kairan #1	Tsurushin Seed Ltd. , Japan	0	47.5±8.6	6.4±1.7	425.4±114.3
	Kairan #2	Kuragi Seed Ltd. , Japan	0	50.4±1.7	7.0±1.2	456.7±51.6
R. sativus var. *longipinnatus*	Osaka-shijunichi	Noguchi Seed Co., Japan	6-8	34.6±6.7	4.4±1.3	226.8±96.2
	Wakayama	Noguchi Seed Co., Japan	6-9	28.1±4.1	4.3±1.0	258.3±109.6
	Shinshu-jidaikon	Shinshu-sankyo Seed Co., Japan	4-8	31.1±6.8	5.0±1.4	266.1±88.2
	Hayabutori-shogoin	Takii Seed Co., Japan	18-21	23.0±1.5	3.9±0.9	149.2±22.9
R. sativus var. *caudatus*	Rat's tail-CH	Originally bought from Chiltern Seeds, UK, then seed propagated	0	41.2±6.1	6.5±2.2	528.5±227.3
	Rat's tail-G2 (2°C 0 day)	Genebank project, NARO, Japan, accession No.76703	0	27.4±0.55	5.2±1.3	342.5±148.4
	Rat's tail-G2 (2°C 3 days)	Genebank project, NARO, Japan, accession No.76703	3	19.75±1.0	3.3±0.5	133.1±16.3
	Rat's tail-G4	Genebank project, NARO, Japan, accession No.86212	3	36.5±7.1	5.9±1.3	496.9±277.4

All the data of successfully grafted plants were used for the calculation of each item (n = 5–18).

[z]Mean±SD.

[y]Non-vernalized stock as a grafting control.

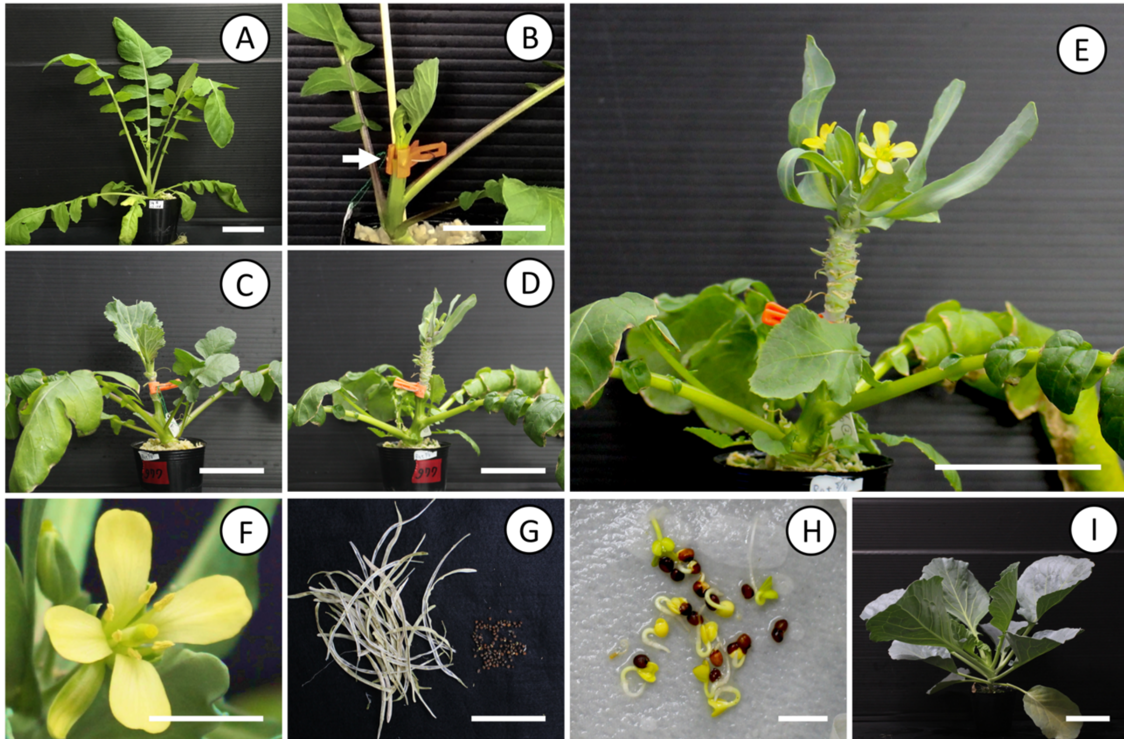

Fig. 1-1. Non-vernalization flowering and seed production of 'Watanabe-seiko No.1' cabbage by grafting onto a bolting radish rootstock. A shoot of 'Watanabe-seiko No.1' with 2–3 leaves was grafted onto a stem of bolting radish rootstock. Grafted plants were grown in a growth room maintained at 22 ± 2°C, 16L/8D. Until flower bud appearance, the leaves of the grafted scion were continuously removed so that only 3–4 leaves remained. (A) Typical bolting radish rootstock used for grafting; the cultivar is 'Hayabutori-shogoin'. (B) Rootstock and scion immediately after grafting. The white arrow indicates the grafted position. (C–F) Time-course pictures of non-vernalization flowering of 'Watanabe-seiko No.1' grafted onto 'Rat's tail-CH', (C) 25 DAG, (D) 53 DAG, (E) 57 DAG, and (F) close-up picture of (E). (G) Seed pods and seeds produced by non-vernalization flowered 'Watanabe-seiko No.1' grafted onto 'Rat's tail-CH'. Picture shows the whole seeds produced by one grafted scion. (H) Germination of the seeds of (G). (I) Normal-grown seedlings of the seeds of (G). Scale bars, 5 cm (A–E, G, I) and 1 cm (F, H).

Table 1-2. Flowering response of 'Watanabe-seiko No.1' cabbage grafted onto *B. oleracea* and *R. sativus* rootstocks.

Rootstock		Number of successful grafting (plant/plant)	Number of expanded leaves of scions[z]	Percentage of scions with flower bud (plant/plant)	Days to flower bud appearance[z] [y]
Control	Non-grafted	8/8	-	0% (0/8)	-
	Watanabe-seiko No.1 (NVstock)	7/7	21.1±2.0	0% (0/7)	-
B. oleracea var. *albograbra*	Kairan #1	12/14	32.1±10.7	0% (0/12)	-
	Kairan #2	13/18	27.1±3.9	0% (0/13)	-
R. sativus var. *longipinnatus*	Osaka-shijunichi	17/18	23.8±3.8	0% (0/17)	-
	Wakayama	17/19	21.7±4.6	6% (1/17)	-
	Shinshu-jidaikon	18/18	23.2±4.6	0% (0/18)	-
	Hayabutori-shogoin	18/22	22.9±3.4	17% (3/18)	47
R. sativus var. *caudatus*	Rat's tail-CH	12/16	29.3±8.2	50% (6/12)	43.3±10.1
	Rat's tail-G2 (2°C 0 day)	5/8	23.6±6.4	0% (0/5)	-
	Rat's tail-G2 (2°C 3 days)	8/10	22.4±2.2[x]	75% (6/8)	49.0±6.7
	Rat's tail-G4	13/19	21.2±6.2	0% (0/13)	-

Data were collected at 60 DAG. All the data of successfully grafted plants were used for the calculation of each item (n = 5–18), unless otherwise stated.

[z]Mean ± SD.

[y]The data of scions which formed visible flower bud within 60 DAG are presented (n = 1 for 'Hayabutori-shogoin', n = 6 for 'Rat's tail-CH', and n = 5 for 'Rat's tail-G2' vernalized for 2°C, 3 days).

[x]Only the data for 5 of 8 plants are presented because of the lack of measurement.

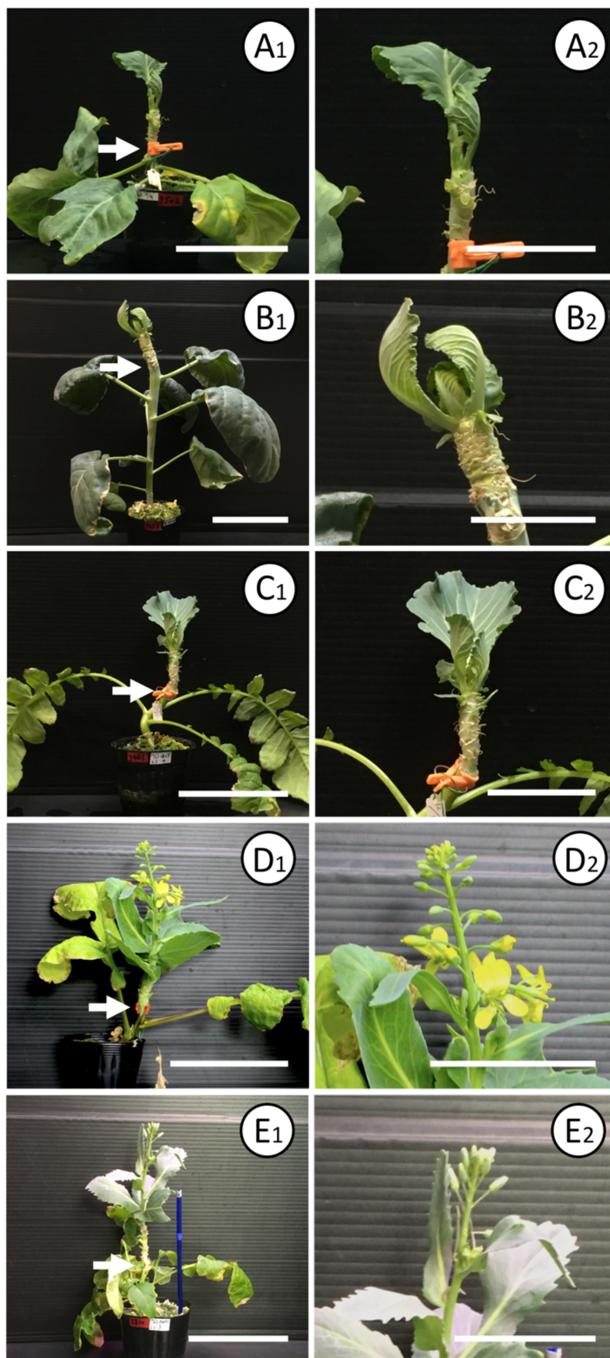

Fig. 1-2. Different flowering responses of 'Watanabe-seiko No.1' induced by different species and cultivars of rootstocks. A shoot of 'Watanabe-seiko No.1' cabbage with 2–3 leaves was grafted onto a stem of a flowering-induced rootstock. Grafted plants were cultivated in a growth room maintained at 22 ± 2°C, 16L/8D. Until flower bud appearance, the leaves of grafted scions were continuously removed so that only 3–4 leaves remained. White arrow indicates graft union. (A_1, A_2) Control grafting where the scion was grafted onto a non-vernalized cabbage, 'Watanabe-seiko No.1' at 60 DAG. The scion continued vegetative growth (A_2 is a close-up picture of A_1, and the same applies for B_1–E_2). (B_1, B_2) A scion grafted onto Chinese kale 'Kairan #1', continuing vegetative growth at 60 DAG. (C_1, C_2) A scion grafted on radish 'Shinshu-jidaikon', continuing vegetative growth at 60 DAG. (D_1, D_2) A scion grafted onto radish 'Rat's tail-CH', having several flower buds and opened flowers, at 47 DAG. (E_1, E_2) A scion grafted onto radish 'Rat's tail-G2' (2°C, 3 days), having several flower buds. Picture was taken at 60 DAG. Scale bars, 10 cm (A_1, B_1, C_1, D_1, E_1) and 5 cm (A_2, B_2, C_2, D_2, E_2).

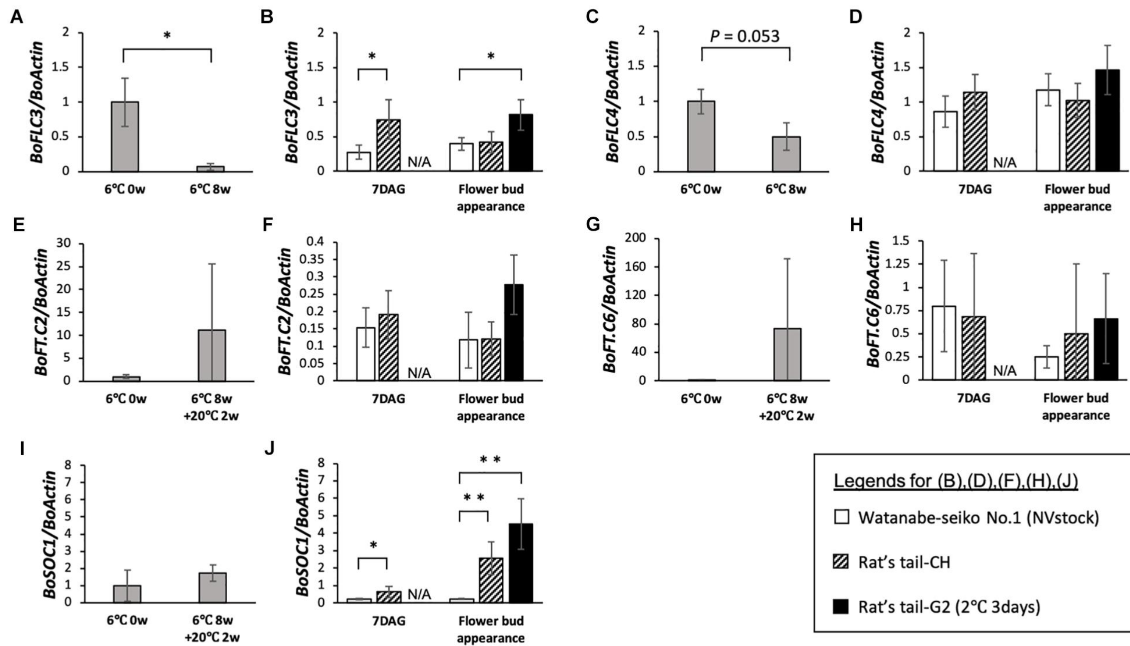

Fig. 1-3. Expression analysis of flowering-related genes in the vernalized-flowered and non-vernalized graft-flowered 'Watanabe-seiko No.1'. For each gene, expression levels normalized to the mean before vernalization (6°C 0 week) are shown. (A, C, E, G, I) Expression level of *BoFLC3, BoFLC4, BoFT.C2, BoFT.C6*, and *BoSOC1* in vernalized plants (n = 3 for each gene). (B, D, F, H, J) Expression level of *BoFLC3, BoFLC4, BoFT.C2, BoFT.C6*, and *BoSOC1* in control scions grafted onto 'Watanabe-seiko No.1' (NVstock) (n = 4) and flowered scions grafted onto 'Rat's tail-CH' (n = 6) or 'Rat's tail-G2' (2°C, 3 days) (n = 4). The leaf samples collected at the point of "Flower bud appearance" were taken at 60 DAG for the control scions grafted onto 'Watanabe-seiko No.1' (NVstock), and at within 1 week after flower bud appearance for the scions grafted onto 'Rat's tail-CH' or 'Rat's tail-G2' (2°C, 3 days). ** and * indicate statistically significant differences (Student's *t*-test, $P < 0.01$ and 0.05, respectively). N/A, data not available. Error bars are the standard deviations of the mean.

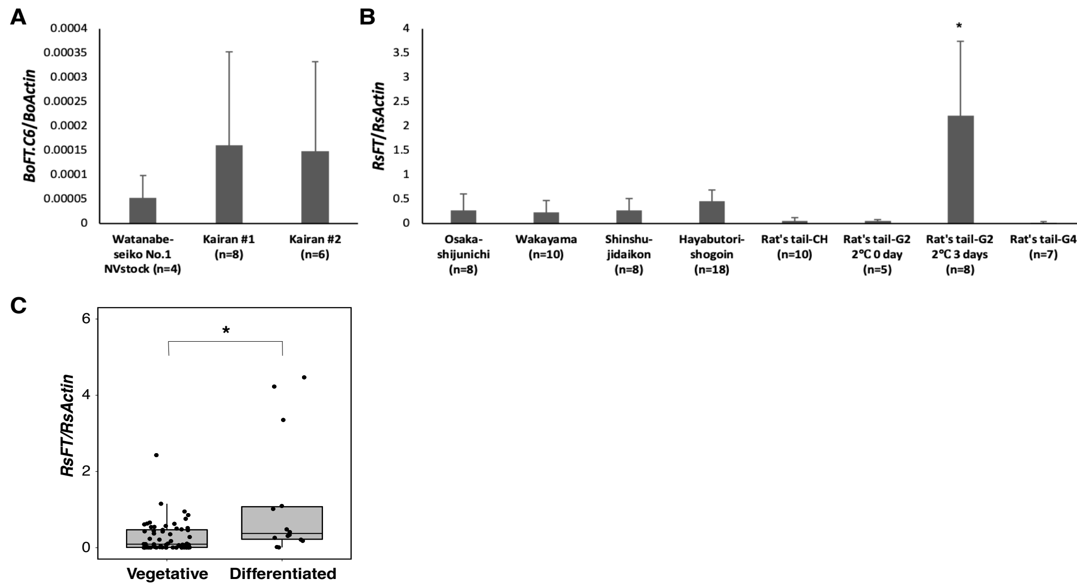

Fig. 1-4. Expression analysis of *FT* homologs in leaves of rootstock plants at 30 DAG. The lowest leaves of the rootstock were sampled at 30 DAG, and the expression level of the *FT* homolog was determined by RT-qPCR. The number of plants investigated are shown in the graph for each cultivar (n = 4–18). (A) The expression level of *BoFT.C6* in 'Watanabe-seiko No.1' and Chinese kale. *BoActin* were used as a reference gene. (B) The expression level of *RsFT* in radish cultivars. *RsActin* was used as a reference gene. * indicates significant difference against other rootstocks by the Tukey HSD test ($P < 0.001$). (C) The expression level of *RsFT* in radish rootstocks compared by the occurrence of the flower bud differentiation of the grafted cabbage scions at 60 DAG. * indicates significant difference by the Wilcoxon rank sum test ($P < 0.05$). Error bars are the standard deviations of the mean.

Table 1-3. Flowering response of several radish cultivars against seed vernalization treatment.

Cultivar	Seed vernalization treatment			
	2 weeks	4 weeks	6 weeks	8 weeks
Rat's tail-G2	5/5 (15.0±1.4)	5/5 (13.4±0.5)	5/5 (12.4±1.9)	5/5 (12.4±0.5)
Rat's tail-CH	5/5 (18.6±1.9)	4/4 (18.8±3.3)	5/5 (17.8±2.9)	5/5 (15.2±1.3)
Shinshu-jidaikon	5/5 (18.4±1.9)	5/5 (14.8±1.0)	5/5 (14.2±1.0)	5/5 (14.6±1.2)
Osaka-shijunichi	5/5 (17.6±2.3)	5/5 (13.2±0.4)	5/5 (14.4±1.0)	5/5 (13.6±1.2)
Wakayama	5/5 (16.2±1.7)	5/5 (13.8±0.4)	5/5 (12.6±1.0)	5/5 (13.8±1.5)
Utsuki-gensuke	5/5 (20.8±2.0)	5/5 (14.6±1.7)	5/5 (14.6±0.8)	5/5 (14.4±0.5)
Hayabutori-shogoin	0/5 (-)	5/5 (20.4±1.0)	5/5 (15.2±1.3)	5/5 (16.8±0.7)
Comet	0/5 (-)	2/5 (23.5±0.5)	5/5 (22.0±2.3)	3/3 (18.0±0.0)
Kuronaga-kun	0/5 (-)	4/5 (25.0±1.2)	5/5 (21.8±2.4)	4/4 (23.0±1.4)
Tokinashi	0/5 (-)	0/5 (-)	2/5 (25.5±2.5)	5/5 (24.4±2.2)

Figures indicate the ratio of the bolted plants within 28 DAS (plants/plants).
Figures in the parenthesis indicate the days to bolting after the sowing (mean±SD).

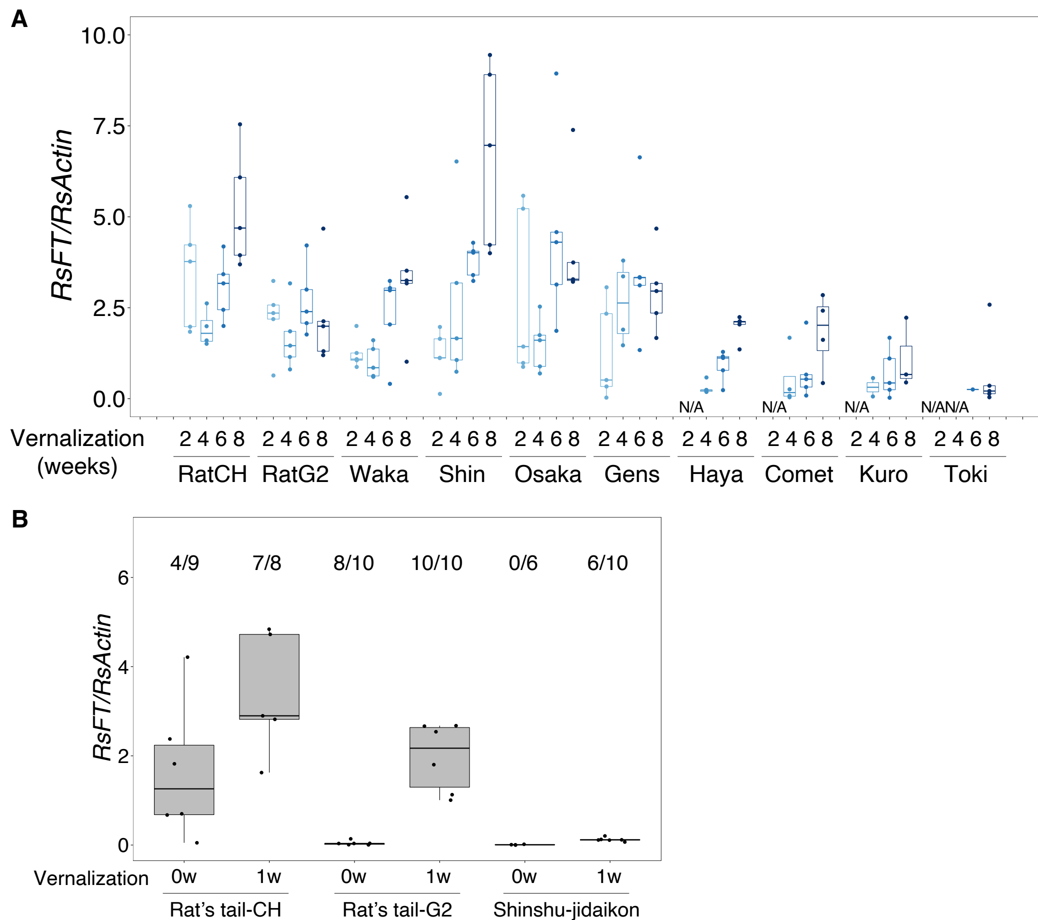

Fig. 1-5. Expression analysis of *RsFT* in several radish cultivars exposed to different periods of seed vernalization treatment. After the seed vernalization treatment at 2°C, the seeds were sown and grown in a growth room maintained at 22 ± 2°C, 16L/8D. The largest leaf of the radish plants was sampled at the day of bolting, and the expression level of the *RsFT* was determined by RT-qPCR. The day of bolting was defined as the day when the plants bolted to a height of 3 cm from the top of the hypocotyl. (A) The expression level of *RsFT* in ten radish cultivars exposed for 2–8 weeks of seed vernalization treatment. N/A indicate data not available for the treatment because no plants were bolted within 28 DAS. RatCH: 'Rat's tail-CH', RatG2: 'Rat's tail-G2', Waka: 'Wakayama', Shin: 'Shinshu-jidaikon', Osaka: 'Osaka-shijunichi', Gens: 'Utsugi-gensuke', Haya: 'Hayabutori-shogoin', Comet: 'Comet', Kuro: 'Kuronaga-kun', Toki: 'Tokinashi'. (B) The expression level of *RsFT* in 'Rat's tail-CH', 'Rat's tail-G2', and 'Shinshu-jidaikon' exposed for 0 or 1 week of seed vernalization treatment. The leaves were sampled at the day of bolting except for 0w vernalized 'Shinshu-jidaikon', where the leaves were sampled at 26 DAS. This was because no plants bolted in 0w vernalized 'Shinshu-jidaikon'. For each cultivar, sampling was conducted from the first six plants that had started bolting. Figures above the boxplot indicate the ratio of the bolted plants within 28 DAS (plant/plant).

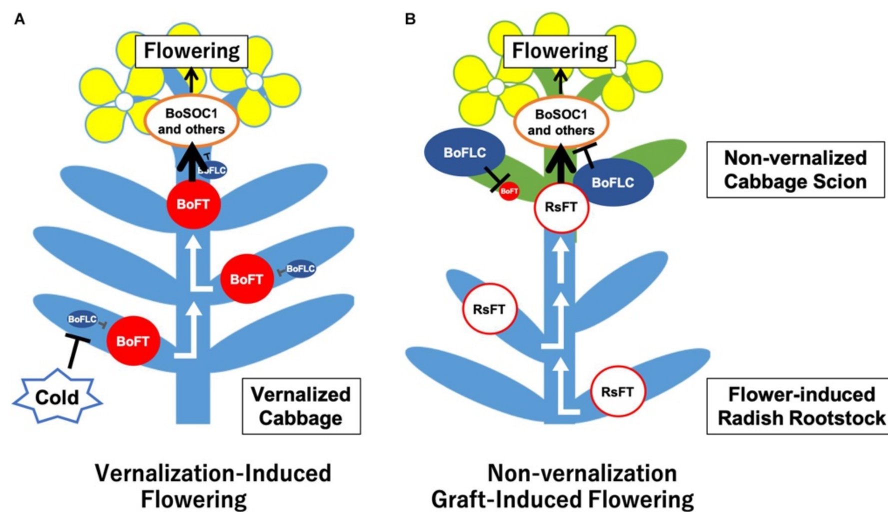

Fig. 1-6. Assumed model of cabbage non-vernalization flowering induced by grafting onto radish rootstock in this study. (A) Vernalization induced cabbage flowering. After low-temperature treatment of cabbage seedlings, *BoFLCs* are down-regulated and *BoFTs* are up-regulated in the leaves and/or shoot apical meristem (SAM). Then, FT proteins are transported to the SAM, which up-regulates *BoSOC1s* and other flowering-related genes, inducing flower bud differentiation. (B) Non-vernalization cabbage flowering induced by grafting onto radish rootstock. In the cabbage scion that has not undergone low-temperature treatment, *BoFLCs* are highly expressed and repress *BoFTs* expression. The RsFT protein and/or another transmissible signals are transported from the radish rootstock to the cabbage scion, up-regulating *BoSOC1s* and other flowering-related genes in the cabbage scion SAM, inducing flower bud differentiation by bypassing the flowering repression created by BoFLCs.

Table 1-S1. Data set for the calculation of total leaf area of rootstock plants.

Species	Cultiver/Accession name	Number of leaves left on rootstock plant (L_n)[z]	Size of largest leaf[z]		Assumed total area of leaves left on rootstock plant (L_a, cm^2/plant) [z,y]
			Length (L_l)	Width (L_w)	
B. oleracea var. *capitata*	Watanabe-seiko No.1 (NVstock)	4.6±0.8	16.1±2.9	8.7±1.4	287.2±67.8
B. oleracea var. *albograbra*	Kairan #1	6.4±1.7	19.9±2.2	9.5±1.8	425.4±114.3
	Kairan #2	7.0±1.2	20.2±2.6	9.9±0.6	456.7±51.6
R. sativus var. longipinnatus	Osaka-shijunichi	4.4±1.3	22.4±3.2	6.4±1.4	226.8±96.2
	Wakayama	4.3±1.0	23.2±4.2	7.4±1.7	258.3±109.6
	Shinshu-jidaikon	5.0±1.4	22.4±2.3	7.1±1.3	266.1±88.2
	Hayabutori-shogoin	3.9±0.9	17.4±1.7	5.1±0.6	149.2±22.9
R. sativus var. *caudatus*	Rat's tail-CH	6.5±2.2	26.6±2.3	10.7±1.9	528.5±227.3
	Rat's tail-G2 (2°C. 0 days)	5.2±1.3	21.9±2.9	9.4±1.6	342.5±148.4
	Rat's tail-G2 (2°C, 3 days)	3.3±0.5	15.9±2.3	5.4±0.4	133.1±16.3
	Rat's tail-G4	5.9±1.3	27.2±5.3	9.9±3.1	496.9±277.4

[z]Mean±SD.
[y]Leaf area of each stock plant was calculated by using the regression equation (Chinese kale: $L_a = 0.2357 \times L_n \times L_l \times L_w + 128.65$, radish: $L_a = 0.2407 \times L_n \times L_l \times L_w + 65.609$, Fig. 1-S1), and then the average of cultivers/accessions were calculated.

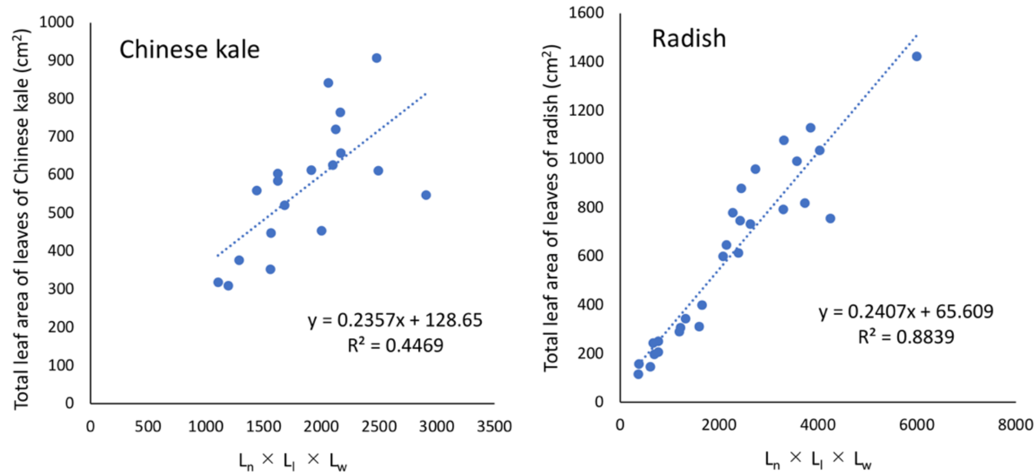

Fig. 1-S1. The relationships between leaf area and leaf parameters of rootstock plants. Bolting Chinese kale (n = 19) and radish (n = 28) were prepared with the stems cut according to the grafting method described in the Materials and Methods. The numbers of leaves remaining on the rootstock plants (L_n) were counted, and the length (L_l) and width (L_w) of the largest leaf were also measured. The total leaf areas of leaves remaining on the rootstock plants were measured by using LI-3100C Area Meter (LI-COR, Inc., USA). The leaf areas of each bolting rootstock plant were plotted against the product of $L_n \times L_l \times L_w$. Data were analyzed using regression analysis in which the dependent variable (Y) was the combined total leaf area of leaves remaining on the rootstock plant, and the independent variable (X) was the product of $L_n \times L_l \times L_w$. The results were fitted by the regression equation $Y = aX + b$ in which a and b are the regression coefficients.

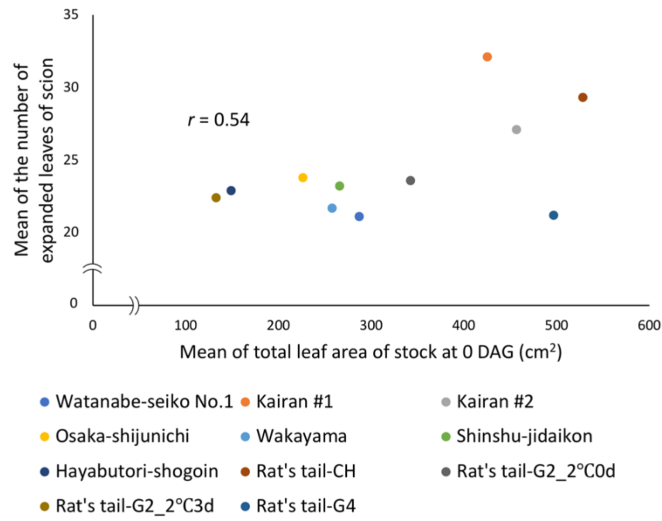

Fig. 1-S2. The relationships between total leaf area of rootstock plants at 0 DAG and the number of expanded leaves of scions.

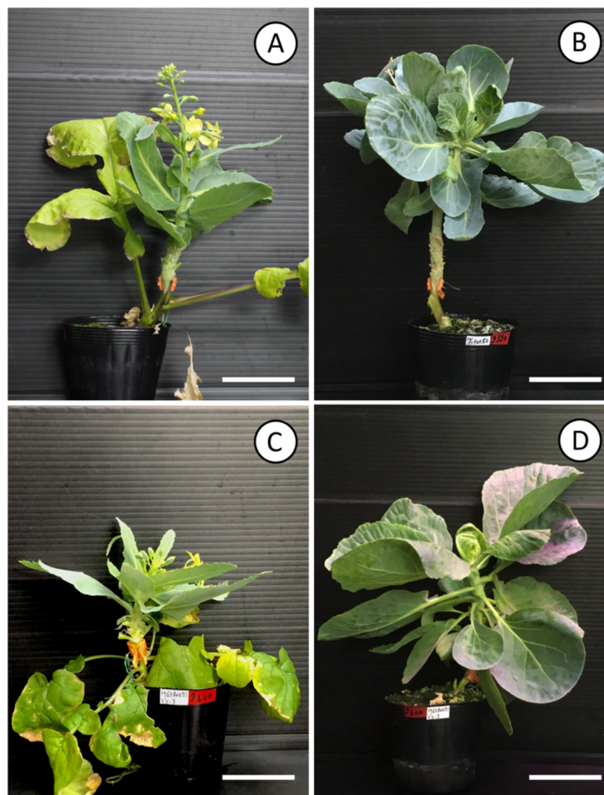

Fig. 1-S3. Reversion to vegetative growth after grafting-induced non-vernalization flowering. (A) Non-vernalization flowering of ‘Watanabe-seiko No.1’ grafted onto ‘Rat's tail-CH’ (47 DAG). (B) Reversion to vegetative growth of (A) (140 DAG). (C) Non-vernalization flowering of ‘Watanabe-seiko No.1’ grafted onto ‘Rat's tail-G2’ (54 DAG). (D) Reversion to vegetative growth of (C) (145 DAG). Scale bars, 5 cm.

Table 1-S2. Primer sets used for RT-qPCR and cDNA sequencing.

Gene	Accession Number	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)[y]	Purpose	Real-time PCR efficiency[x]
BoFLC3	XM_013773027.1	GAAGAGAATCAGGCTTTGGCT	GATTTTTAATTTCAGCCCCGTCT	163	real-time RT-PCR	100.6
BoFLC4 [z]	KU521322.1	GTAAGCTTGTGGAATCAAATTCTGA	GGCTATCAACAAGCTTCAACAATAG	129	real-time RT-PCR	100.8
BoFT.C2 [z]	XM_013764059.1	GTTACTTATGGCCAAAGAGAGGTG	GAAGATATTCTCGGAGGTGAGGATT(	172	real-time RT-PCR	109.9
BoFT.C6 [z]	XM_013735380.1	ATACGGCCAAAGAGAGGTGAC	ATGGAGATATTCTCGTAGGTGCGG	169	real-time RT-PCR	99.7
BoSOC1	XM_013774872.1	CTCTGATCATCTTCTCTCCTAAGG	TGCCTTCTCCCAAGAGTTTACG	216	real-time RT-PCR	101.4
BoActin	XM_013753106.1	GCGGACCGTATGAGCAAAGA	ACCTGCCTCATCATACTCAGC	171	real-time RT-PCR	100.3
RsFT	XM_018614776.1	GGACGAAGAAGTTAGATGAGCCTC	CATTGCTCGTATAGTAAAAGGCTTC	109	real-time RT-PCR	103.4
RsActin	XM_018637077.1	ACAACACCATGCTCAATAGG	ATCATGGTGTCATGGTTGGG	109	real-time RT-PCR	100.0
RsFT	XM_018614776.1	TCAGAAACCTCCTGTTTGTT	CCCTCGGATTTATTTAATACATATA	704	cDNA sequencing	-
BoFT.C6	XM_013735380.1	CAGAAACCACCTGTTTGTTTTAGA	GTAAAAACACTCTCATTTGTTTCCC	731	cDNA sequencing	-

[z] Primer sequences obtained from Irwin et al.(2016)

[y] Product size is based on cDNA

[x] qPCR efficiency of each primer set are the mean value of 4-6 independent standerd curve analysis. Efficiency = $\{10^{(-1/slope)}-1\} \times 100$.

Table 1-S3. Days to flower opening after flower bud appearance.

Scion	Stock	Days to flower opening after flower bud appearance[z]
Watanabe-seiko No.1	Hayabutori-shogoin	30
	Rat's tail-CH	10, 10
	Rat's tail-G2 (2°C3d)	10, 11, 13, 23

[z]Only the data of a part of flowered scions are presented because of lack of mesurement.

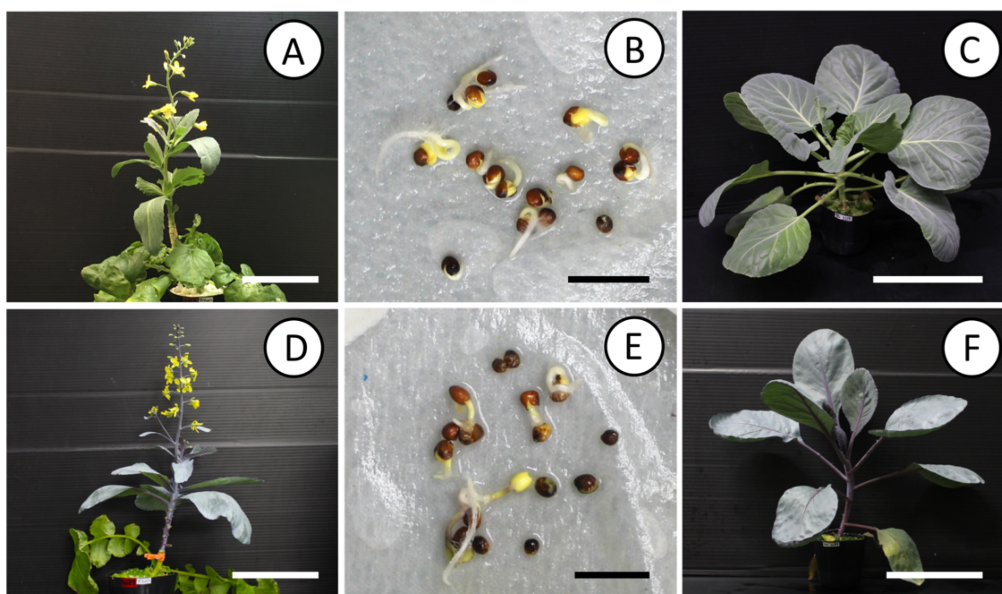

Fig. 1-S4. Non-vernalization flowering and seed production of commercial cabbage cultivars ‘Kinkei No.201’ and ‘Red cabbage’ after grafting onto the ‘Rat's tail-CH’ radish. Cabbage shoots grown in growth room (22 ± 2°C, 16L/8D) were grafted onto a stem of bolting radish rootstock. Grafted plants were grown in the same growth room. Until flower bud appearance, the leaves of grafted scions were continuously removed so that only 3–4 leaves remained. (A) Non-vernalization grafting-induced flowering of ‘Kinkei No.201’ (61DAG). (B) Germination of the seeds produced by the grafted shoot of (A). (C) Normal-grown seedlings from the seeds of (B). (D) Non-vernalization grafting-induced flowering in ‘Red cabbage’ (52 DAG). (E) Germination of the seeds produced by the grafted shoot of (D). (F) Normal-grown seedlings of the seeds of (E). Scale bars, 10 cm (A, C, D, and F) and 1 cm (B and E).

Table 1-S4. Flowering response of 'Kinkei No.201' and 'Red cabbage' cabbage grafted on 'Rat's tail-CH' rootstocks[z].

Scion	Rootstock	Number of successful grafting (plant/plant)	Percentage of scions with flower bud (plant/plant)	Days to flower bud appearance[y]
Kinkei-No.201	Rat's tail-CH	12/13	33% (4/12)	53.3±4.3
Red cabbage	Rat's tail-CH	6/6	33% (2/6)	44.0±8.0

[z]Data were collected at 60 days after grafting.

[y]Mean±SD.

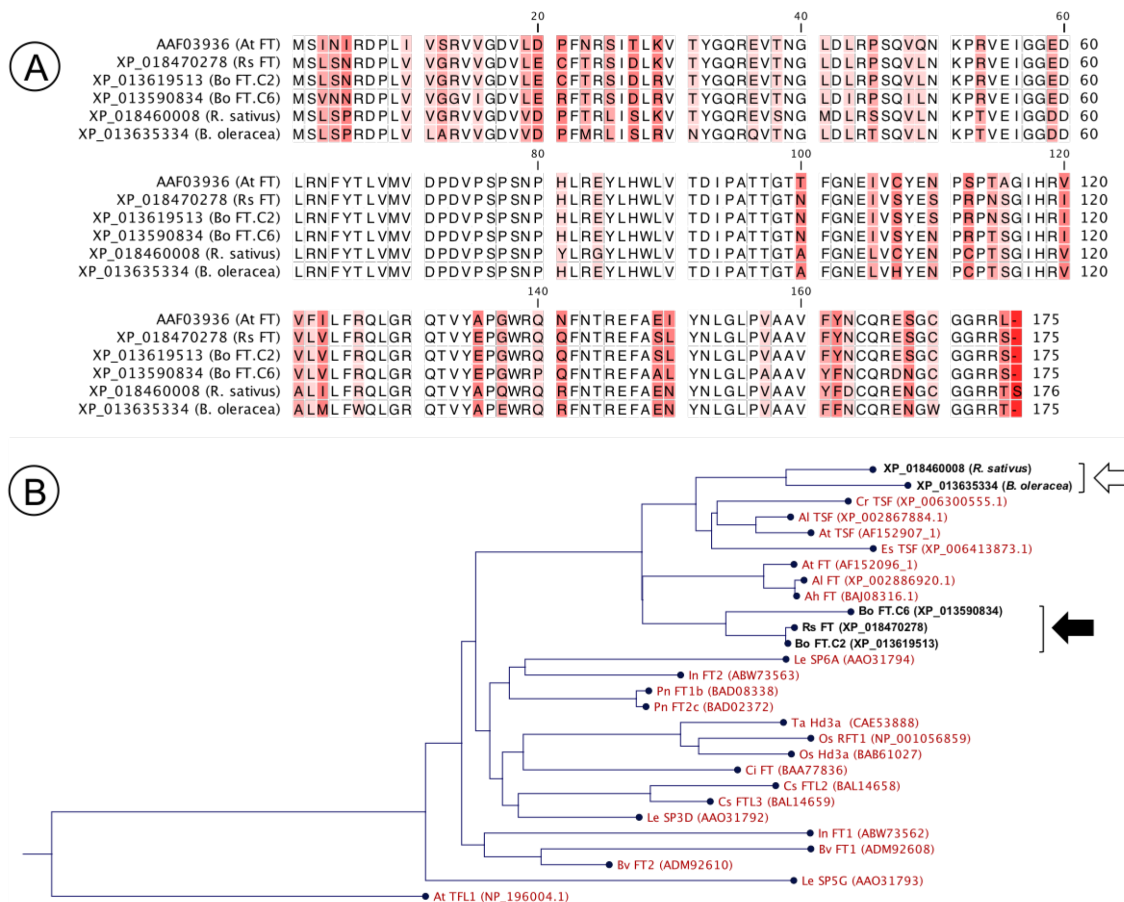

Fig. 1-S5. *FT* homologs in *B. oleracea* and *R. sativus* gemome. (A) Alignment of amino acid sequence of *A. thaliana* FT protein and FT−like proteins in *B. oleracea* and *R. sativus* genome. (B) A neighbor-joining phylogenetic tree of the FT gene family from several plant species, including FT-like genes of *B. oleracea* and *R. sativus*. The tree was constructed using the Neighbor-Joining method in CLC Sequence Viewer (https://www.qiagenbioinformatics.com/products/clc-sequence-viewer/). Black arrow and white arrow indicate the clades of FT and TSF genes, respectively, in *B. oleracea* and *R. sativus*. AtTFL1 was used as an outgroup. Ah, *Arabidopsis halleri*; Al, *Arabidopsis lyrata*; At, *A. thaliana*; Bo, *B. oleracea*; Bv, *Beta vulgaris*; Ci, *Citrus unshiu*; Cr, *Capsella rubella*; Es, *Eutrema salsugineum*; Cs, *Chrysanthemum seticuspe*; In, *Ipomoea nil*; Le, *Lycopersicon esculentum*; Os, *Oryza sativa*; Pn, *Populus nigra*; Rs, *R. sativus*; Ta, *Triticum aestivum*.

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                                          20                    40                    60
                                           |                     |                     |
Kairan#1-2          MSLNNRDPLV VGGVIGDVLE QFTRSIDLRV TYGQREVTNG LDIRPSQILN KPRVEIGGDD 60
Kairan#2-1          MSLNNRDPLV VGGVIGDVLE QFTRSIDLRV TYGQREVTNG LDIRPSQILN KPRVEIGGDD 60
Kairan#1-1          MSVNNRDPLV VGGVIGDVLE QFTRSIDLRV TYGQREVTNG LDIRPSQILN KPRVEIGGDD 60
Kairan#2-2          MSVNNRDPLV VGGVIGDVLE RFTRSIDLRV TYGQREVTNG LDIRPSQILN KPRVEIGGED 60
Osaka-shijunichi    MSLSNRDPLV VGRVVGDVLE CFTRSIDLKV TYGQREVTNG LDLRPSQVLN KPRVEIGGED 60
Shinshu-jidaikon    MSLSNRDPLV VGRVVGDVLE CFTRSIDLKV TYGQREVTNG LDLRPSQVLN KPRVEIGGED 60
Wakayama            MSLSNRDPLV VGRVVGDVLE CFTRSIDLKV TYGQREVTNG LDLRPSQVLN KPRVEIGGED 60
Hayabutori-shogoin  MSLSNRDPLV VGRVVGDVLE CFTRSIDLKV TYGQREVTNG LDLRPSQVLN KPRVEIGGED 60
Rat's tail-CH       MSLSNRDPLV VGRVVGDVLE CFTRSIDLKV TYGQREVTNG LDLRPSQVLN KPRVEIGGED 60
Rat's tail-G2       MSLSNRDPLV VGRVVGDVLE CFTRSIDLKV TYGQREVTNG LDLRPSQVLN KPRVEIGGED 60
Rat's tail-G4       MSLSNRDPLV VGRVVGDVLE CFTRSIDLKV TYGQREVTNG LDLRPSQVLN KPRVEIGGED 60
                                          80                   100                   120
                                           |                     |                     |
Kairan#1-2          LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYEN PRPTSGIHRI 120
Kairan#2-1          LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYEN PRPTSGIHRI 120
Kairan#1-1          LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYEN PRPTSGIHRI 120
Kairan#2-2          LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYEN PRPTSGIHRI 120
Osaka-shijunichi    LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYES PRPNSGIHRI 120
Shinshu-jidaikon    LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYES PRPNSGIHRI 120
Wakayama            LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYES PRPNSGIHRI 120
Hayabutori-shogoin  LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYES PRPNSGIHRI 120
Rat's tail-CH       LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYES PRPNSGIHRI 120
Rat's tail-G2       LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYES PRPNSGIHRI 120
Rat's tail-G4       LRNFYTLVMV DPDVPSPSNP HLREYLHWLV TDIPATTGTN FGNEIVSYES PRPNSGIHRI 120
                                         140                   160
                                           |                     |
Kairan#1-2          VLVLFRQLGR QTVYEPGWRP QFNTREFAAL YNLGLPAAAV YFNCQRDNGC GGRRS 175
Kairan#2-1          VLVLFRQLGR QTVYEPGWRP QFNTREFAAL YNLGLPAAAV YFNCQRDNGC GGRRS 175
Kairan#1-1          VLVLFRQLGR QTVYEPGWRP QFNTREFAAL YNLGLPAAAV YFNCQRDNGC GGRRS 175
Kairan#2-2          VLVLFRQLGR QTVYEPGWRP QFNTREFAAL YNLGLPAAAV YFNCQRDNGC GGRRS 175
Osaka-shijunichi    VLVLFRQLGR QTVYEPGWRQ QFNTREFASL YNLGLPVAAV FYNCQRESGC GGRRS 175
Shinshu-jidaikon    VLVLFRQLGR QTVYEPGWRQ QFNTREFASL YNLGLPVAAV FYNCQRESGC GGRRS 175
Wakayama            VLVLFRQLGR QTVYEPGWRQ QFNTREFASL YNLGLPVAAV FYNCQRESGC GGRRS 175
Hayabutori-shogoin  VLVLFRQLGR QTVYEPGWRQ QFNTREFASL YNLGLPVAAV FYNCQRESGC GGRRS 175
Rat's tail-CH       VLVLFRQLGR QTVYEPGWRQ QFNTREFASL YNLGLPVAAV FYNCQRESGC GGRRS 175
Rat's tail-G2       VLVLFRQLGR QTVYEPGWRQ QFNTREFASL YNLGLPVAAV FYNCQRESGC GGRRS 175
Rat's tail-G4       VLVLFRQLGR QTVYEPGWRQ QFNTREFASL YNLGLPVAAV FYNCQRESGC GGRRS 175
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Fig. 1-S6. Alignment of the *FT* homolog amino acid sequences of the rootstock plants used in the grafting experiments.

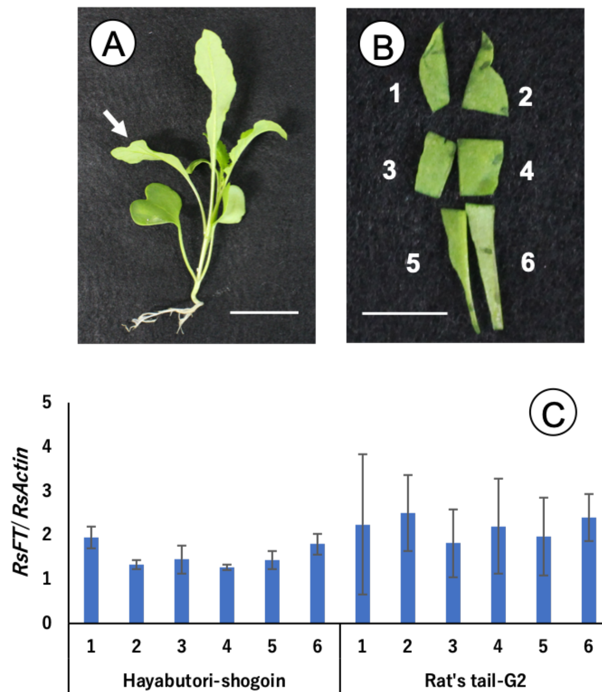


Fig. 1-S7. Expression analysis of *RsFT* in the different part of the lowest leaves of the vernalized radish. The seeds of 'Hayabutori-shogoin' and 'Rat's tail-G2' radish were vernalized at 2°C for 7 weeks, and sown in a growth room maintained at 22 ± 2°C, under long-day (16/8, light (L)/dark (D)) conditions. At the day of bolting, the lowest leaves were collected at 1 h before the end of the light period. The sampled leaves whose petiole were removed were divided into six parts, and the expression level of *RsFT* for each part was determined by RT-qPCR. For the expression analysis, primer sets for *RsFT* and *RsActin* were used. (A) Typical bolting radish used for the analysis; the cultivar is 'Rat's tail-G2' radish. The white arrow indicates the lowest leaf. Bar = 3 cm. (B) The lowest leaf in (A) which was divided into six parts. Each part was numbered as shown in the picture. Bar = 1 cm. (C) Expression level of *RsFT* in the different part of the lowest leaves. The numbers above the cultivar name indicate the position in the leaf shown in (B). For each cultivar, three plants were used for the analysis, except for position 5 in 'Hayabutori-shogoin' and position 1 and 6 in 'Rat's tail-G2', where only two plants were used because of the failure of RNA extraction. Error bars are the standard deviations of the mean. There was no significant difference in the expression levels of *RsFT* by the position within the leaf in both cultivars (one-way ANOVA, $p > 0.05$).

1.4. Discussion

Flowering mechanisms of cabbage scions without vernalization

I could successfully induce flowering in cabbage scions without low-temperature treatment by grafting them onto radish rootstocks (Figs. 1-1C–E; Figs. 1-2D$_1$, E$_1$; Table 1-2; Figs. 1-S4A, D; Table 1-S4). Expression analysis of flowering-related genes was conducted to investigate the mechanism of cabbage scion flowering without vernalization. Low-temperature treatment resulted in decreased expression of *FLC* homologs and increased expression of *FT* and *SOC1* homologs in 'Watanabe-seiko No.1' (Figs. 1-3A, C, E, G, I). Therefore, the expression pattern of *FLC, FT*, and *SOC1* observed in *A. thaliana* appears to be conserved in 'Watanabe-seiko No.1'. Conversely, in 'Watanabe-seiko No.1' plants that flowered after grafting, the expression levels of *FLC* homologs were the same as or higher than that of the control graft (Figs. 1-3B, D). Moreover, since *FT* homologs are marginally expressed in graft-flowered scions (Figs. 1-3F, H), flower bud differentiation within the cabbage scion would not be induced by transcribed *FT* originating in the scion. The expression level of *SOC1* homologs was significantly higher in graft-flowered 'Watanabe-seiko No.1' than in the control graft (Fig. 1-3J). In several plant species, the FT protein has been shown to be a major constituent of florigen, which is synthesized in leaves and transported to the shoot apex, leading to floral transition (Corbesier et al., 2007; Lin et al., 2007; Notaguchi et al., 2008; Tamaki et al., 2007). It is also known that *SOC1* is up-regulated via *FT* expression (Yoo et al., 2005). Considering that endogenous *FT* homologs are minimally expressed in the scion, *SOC1* homologs would be activated by mobile signals transmitted from the rootstocks. In *FLC*-mediated late-flowering *A. thaliana, FT* overexpression activated *SOC1* expression and strongly suppressed the late-flowering phenotype; however, it did not affect *FLC* mRNA level (Michaels et al., 2005). On the basis of this evidence, I assumed that in the graft-induced flowering of cabbage without vernalization, exogenous mobile signals like the FT protein are transported from the rootstock, inducing flowering of the cabbage by bypassing the flowering suppression created by FLC by directly activating *SOC1* and the other flowering-related genes expression (Fig. 1-6).

'Watanabe-seiko No.1', which flowered after grafting, developed a few inflorescences and then returned to vegetative growth (Fig. 1-S3). This suggests that the flowering signals transported from the rootstock were not self-amplifying, and thus the floral induction of the scion would occur in a dose-dependent manner. This reversion from reproductive growth to vegetative growth in a relatively short period could support the above hypothetical molecular mechanism of graft-induced flowering of cabbage.

Why flower bud differentiation in cabbage scions varied depending on rootstock genera or cultivars

To identify rootstocks that can induce flowering, I investigated factors of the rootstock plants related to floral induction of the scion. From the results of gene expression analysis of the graft-flowered cabbage scions, the FT protein is the hypothesized candidate for a causal mobile signal transported from the rootstock. Notaguchi et al. (2008) reported that in a grafting experiment using the *A. thaliana ft* mutant as a recipient, the higher the *FT* expression level of donor plant was, the more likely the scion was to flower. I hypothesized that the variation in successful flower bud formation was due to the difference in expression of FT proteins in the leaves of the rootstocks. Therefore, I measured the expression levels of *FT* mRNA in the rootstocks at 30 DAG, when the scion and rootstock had already been well connected and translocation between them would be actively occurring. Then, I examined the relationship between the flower bud differentiation rate of the scion and the *FT* expression level of the rootstock.

I found that the expression level of the *FT* homolog tended to be high in the rootstock plants that had induced flower bud differentiation in the scion (Fig. 1-4). The *FT* homolog was minimally expressed in 'Watanabe-seiko No.1' (NVstock). In addition, in the two cultivars of Chinese kale that did not induce flower bud differentiation, the expression level of *FT* homolog was approximately 30 times lower than 'Watanabe-seiko No.1' that had been vernalized for 8 weeks (Figs. 1-3G; Figs. 1-4A). Considering that the Chinese kale cultivars used in this study do not require low-temperature treatment for flowering, it seemed that the Chinese kales may be induced to flower by a relatively small amount of FT protein or that the contribution of FT protein expressed in the lower leaves is small. The expression level of the *FT* homolog in 'Rat's tail-G2' (2°C, 3 days), which induced the highest percentage of cabbage scions to flower, was significantly higher than all other rootstocks (Fig. 1-4B). Since there was no significant difference in the expression level of *RsFT* by the position within a leaf in the preliminary experiment (Fig. 1-S7), the leaf tip I used for the analysis would represent the trend of *RsFT* expression level of the whole leaf. I presume that the total amount of *RsFT* mRNA/protein expressed in a whole plant would also be the highest in 'Rat's tail-G2' (2°C, 3 days), considering the relatively small difference in the total leaf area between radish cultivars (Table 1-1). Moreover, 2°C, 0 day vernalized 'Rat's tail-G2' rootstock, which showed scares *RsFT* expression, did not induced flower bud differentiation of the grafted cabbage (Table 1-2; Fig. 1-4B). For 'Rat's tail-CH', which highly induced scion flower bud differentiation, *RsFT* was lowly expressed in the leaf of the rootstock used for the grafting experiment (Fig. 1-4B). However, in the later experiment, I observed high *RsFT* expression in some individuals of this cultivar (Fig. 1-5B). I assumed that this difference might be caused by the different leaf position in the plant: the lowest leaf was sampled in the grafting experiment, and the largest leaf was sampled in the latter expression analysis experiment. The expression level of *RsFT* might be high in the grafting experiment, but was possibly underestimated due to the aging effect in the lowest leaf. Although further investigation is needed for 'Rat's tail-CH', these results indicate the contribution of rootstock leaf *FT* expression on flowering in the cabbage scion.

On the other hand, there were some individuals for which flower bud differentiation of the grafted cabbage did not occur, even though the expression level of *RsFT* of the rootstocks were comparable to that of the individuals for which flower bud differentiation of the grafted cabbage was induced (Fig. 1-4C). Therefore, the expression level of *RsFT* of the rootstock could explained partly for the difference in the flowering response of the grafted cabbage observed in this study. I should take into consideration that the *FT* mRNA expression level does not always reflect the protein expression level (Kim et al., 2016) and that the accumulation of the FT protein at the shoot apex can be affected by the efficiency of transport through phloem tissue (Liu et al., 2012). Different results may be obtained if the accumulation of the FT protein in the scion is investigated, and further investigation is needed to identify the contributing factors of the rootstocks responsible for the differences in floral induction.

Different expression pattern of RsFT in response to vernalization treatment among radish cultivars

Differences in the expression level of *RsFT* among radish cultivars were related to their vernalization requirement for the floral induction. Two early-flowering radish cultivars expressed high levels of *RsFT* even with shorter vernalization treatments (Fig. 1-5B). Conversely, in cultivars that required a longer vernalization treatment for flowering, *RsFT* expression stayed at low level even after 8 weeks of vernalization treatment (Fig. 1-5A). This was consistent with the previous studies which observed that *RsFT* expression levels tended to be higher in early-flowering radish accessions than in radish accessions that require vernalization to flower (Han et al., 2021; Jung et al., 2020) These results suggest that, at least under long-day conditions, the expression level of *RsFT* in radish is regulated by factors that confer vernalization requirements, and that this regulation of *RsFT* causes the different flowering response among radish cultivars to vernalization treatments. The ability of the two early-flowering radish cultivars to express high levels of *RsFT* even with shorter vernalization treatments might be related to their ability to highly induce the flowering the grafted cabbage.

In all cultivars, even after the vernalization requirement for the floral induction of all individuals was met, the expression level of *RsFT* tended to increase when a longer period of vernalization treatment was given (Fig. 1-5A, B). This indicated that expression level of *RsFT* increases quantitatively against vernalization treatment in radish. In 'Rat's tail-G2', which do not require vernalization treatment for the floral induction, 3-day vernalization treatment was required for the increased expression of *RsFT* and the floral induction of the grafted cabbage (Fig. 1-4A, Table 1-2). This suggested that vernalization treatment above the minimum amount required to induce the flowering of radish itself may be necessary for the induction of high expression of *RsFT* enough to induce the flowering of the grafted cabbage.

1.5. Summary

In this chapter, I successfully reproduced the grafting-induced flowering of cabbage by using radish rootstocks under a controlled environment. In consistent with the report of Kagawa (1957), *B. oleracea* rootstocks could not induce the flowering of the grafted cabbage. There was also a difference in the flowering response of the grafted cabbage among the radish cultivars used as the rootstocks. Especially, two early-flowering radish cultivars highly induced flowering of the grafted cabbage. To reveal the necessary condition for the stable floral induction of cabbage by grafting, it was considered to be important to clarify the factors different in these radish cultivars which are contributing the difference in the flowering response of the grafted cabbage. From the result of the gene expression analysis, it was assumed that floral induction in the grafted cabbage may be dependent on FT protein supplied by the rootstock, like the conventional model of the floral induction by grafting. There was significant difference in the expression level of *RsFT* in the leaf of the radish cultivars, which was related to the vernalization requirement of the cultivars. The expression level of *RsFT* was higher in the rootstocks which induced the flowering of the grafted cabbage. However, the expression level of *RsFT* could only partially explain the difference in the flowering response of the grafted cabbage caused by the type of the rootstocks. It was suggested that analysis of FT at protein level is necessary to further investigate the contribution of FT for the differences in the flowering response of the grafted cabbage.

Chapter 2.
Development of an anti-peptide antibody to detect FT proteins of *B. oleracea* and *R. sativus*

2.1. Introduction

In Chapter 1, I revealed that the flowering response of the grafted cabbage was different not only between *B. oleracea* rootstocks and *R. sativus* rootstocks, but also among *R. sativus* rootstocks. This indicated that comparison of the rootstocks which induce different flowering response in the grafted cabbage will clarify the rootstock factors necessary for the stable floral induction of cabbage by grafting. Because gene expression analysis suggested that differences in the expression level of *FT* in the rootstock may be responsible for the different flowering responses of the grafted cabbage, I considered it important to first clarify the relationship between the flowering response of the grafted cabbage and the amount of FT protein transferred to the cabbage. Therefore, in Chapter 2, I developed a method for the quantitative detection of FT proteins in *B. oleracea* and *R. sativus*.

Quantitative detection of native FT protein has been performed by mass spectrometry of pumpkin phloem sap (*Cucurbita moschata*) (Lin et al. 2007) and immunoblotting in *A. thaliana,* trifoliate orange (*Citrus trifoliata*) and jatropha (*Jatropha curcas*) (Kim et al., 2016; Tang et al., 2022; Wu et al., 2022). Here, I focused on immunoblotting as a simple and inexpensive method suitable for the analysis of many samples. Kim et al. (2016) reported in detail that they were able to quantitatively examine FT protein accumulation using a peptide antibody raised against a partial sequence (15 amino acid peptide sequences) of FT protein in *A. thaliana*. When I compared the peptide sequence used for the antibody raised in Kim et al. (2016) to the corresponding peptide sequence of *Brassica* and *Raphanus* FT proteins, I found two mismatches, and so I determined that another antibody should be raised for these crop species. Therefore, I raised several peptide antibodies and examined their applicability for the quantitative detection of native FT proteins in *Brassica* and *Raphanus* crop species.

2.2. Materials and Methods

Plant material and growth condition

Plants were grown in a growth room maintained at 22°C ± 2°C for LD (16h/8h, light/dark) conditions. Light was provided by fluorescent lamps (PPFD 120 μmol m^{-2} s^{-1}, NEC Lighting, Ltd.). Seeds of *B. oleracea* var. *oleracea* TO1000 (cultivar 'TO1000DH3', Arabidopsis Biological Resource Center, USA, stock number CS29002), *R. sativus* L. var. *caudatus* G2-IL1 (An inbred line isolated from the cultivar 'Rat's tail-G2' in Chapter 1), *R. sativus* L. var. *longipinnatus* Haya (cultivar 'Hayabutori-shogoin', Takii Seed Co.) were sown on wet filter paper and germinated in the dark at 22°C for 1–2 days. Germinated seeds were then vernalized at 2°C in the dark for 0–14 days depending on the experiment. Vernalized seeds were then transplanted into 7.5 cm diameter plastic pots filled with

granular rockwool (Nippon Rockwool Corp.) and cultivated further in the growth room. All plants were irrigated and fertilized from below using a half-strength nutrient solution (Enshi-shoho).

Identification of FT and TSF genes in Brassica and Raphanus crop species

Coding sequences (CDS) of *FT*-like genes in *B. napus*, *B. oleracea*, *B. rapa*, and *R. sativus* were obtained from the NCBI Reference Sequence Database (https://www.ncbi.nlm.nih.gov/refseq/) by searching for genes with high similarity (Blastn, percent identity >70%, query cover >50%) to CDS of the *A. thaliana FT* gene (NM_105222:83-610). Among them, genes without large indels (>50 bp) within the exons were aligned with *A. thaliana* and *A. lyrata* genes (*AtFT*: NM_105222, *AtTSF*: NM_118156.2, *AlFT*: XM_002886874.2, and *AlTSF*: XM_002867838.2), and a phylogenetic tree was constructed using the Create Alignment and Create Tree (Algorithm = Neighbor Joining, Bootstrap = 1,000 replicates) function in CLC Genomics Workbench 12.0 (https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-clc-genomics-workbench/). *AtTFL1* was used as an outgroup.

Production of antibodies against FT proteins of B. oleracea and R. sativus

Three partial peptides of RsFT (Pos1: CFTRSIDLKVT, Pos2: CNPHLREYLHWLVTDIPAT, Pos3: CLFRQLGRQTVYEPGWR, underbar indicates a cysteine residue added to stabilize the peptides) were selected as antigens based on the sequence comparison of FT and TSF homologs in *B. oleracea* and *R. sativus* as well as epitope prediction. Pos1 peptide sequence is specific to RsFT, Pos2 peptide sequence is common to all FT homologs and some TSF homologs, and Pos3 peptide sequence is common to all FT homologs and different from all TSF homologs. The peptide sequences were conjugated to a carrier protein (KLH) and used to immunize rabbit. Sera were obtained, purified by peptide affinity column, and used for the following experiments. Rabbit was immunized with those peptides conjugated to keyhole limpet hemocyanin protein. The resulting polyclonal antibodies were purified with a peptide affinity column and used for following analysis.

Total RNA extraction, cDNA synthesis, and quantitative reverse transcription-PCR

Total RNA was extracted from tissues using Sepasol RNA I Super G (Nacalai Tesque, Inc.), purified using Econospin™ for RNA (Epoch Life Sciences, Missouri, TX, USA) and reverse transcribed with ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd.) according to the manufacturer's instructions. Subsequently, 1 μL of 20-fold diluted RT product diluted with pure water was used as a template for RT-qPCR. RT-qPCR was performed using the THUNDERBIRD® SYBR® qPCR Mix (Toyobo Co., Ltd.) according to the manufacturer's instructions and the LightCycler® 480 system (Roche Diagnostics K.K.). RT-qPCR cycling was performed as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Single-target product

amplification was evaluated using a melting curve. Two primer sets for *RsFT* and *RsActin* (RsFT-F: GGACGAAGAAGTTAGATGAGCCTC; RsFT-R: CATTGCTCGTATAGTAAAAGGCTTC; RsActin3'-F: TTCCCTCAGCACATTCCAGC; RsActin3'-R: TTTAGCCTTCTCCTTGATACAGCTT) were used for RT-qPCR.

Vector construction and recombinant protein expression

CDS of *RsFT* and *RsTSF* were cloned from G2-IL1, and CDS of *BoFT.C2*, *BoFT.C6*, and *BoTSF.C4* were cloned from TO1000 using primer sets shown in Table 2-S1. All CDS were Sanger sequenced, and those with translations identical to ones of corresponding genes in the NCBI Reference Sequence Database were incorporated into the pET6xHN-C vector and expressed in *Escherichia coli* BL21 (DE3) strain. Recombinant proteins were extracted from bacterial cells and purified using His60 Ni Superflow Resin & Gravity columns (Clontech Laboratories, Inc., CA, USA). The recombinant proteins were separated by 14% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The relative concentration of each recombinant protein was determined by silver staining of the gel, and the same amount of protein was used for immunoblotting analysis.

Protein extraction and quantification

Total protein was extracted from leaves according to the method of Wang et al. (2010) with minor modification. Briefly, leaf petioles were frozen in liquid nitrogen in microfuge tubes, ground by bead beating using TissueLyser II (Qiagen, Valencia, CA, USA), and homogenized in approximately 5 × volume of extraction buffer [50 mM Tris–HCl, pH 9.0, 2% (w/v) SDS, 5 mM ascorbic acid, 0.1% (v/v) 2-Mercaptoethanol, 1 × protease inhibitor cocktail (P9599; Sigma-Aldrich Co. LLC, St. Louis, MO, USA)]. The homogenates were subsequently centrifuged twice for 10 min at 16,100 g. The supernatant was used for further analysis. Total soluble protein was quantified using XL Bradford assay (Intégrale Co., Ltd, Tokushima, Japan).

Immunoblotting analysis

A 15 µg of extracted protein was diluted with equal volume of 2× SDS sample buffer [125 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 10% (w/v) sucrose, bromophenol blue, 10% (v/v) 2-Mercaptoethanol] and incubated at 95°C for 3 min. Protein was loaded on a 14% or 16% (w/v) SDS–polyacrylamide gel, separated by electrophoresis, and transferred onto a nitrocellulose membrane (Immobilon-P; Cytiva, MA, USA). After transferring, the membrane was stained with Ponceau S staining solution (Beacle, Inc., Kyoto, Japan), and a picture of the membrane was taken by digital camera or scanner. After blocking with PBST buffer containing 1% skim milk at room temperature of 20–25°C, the membrane was incubated in primary antibody diluted to 300 ng/mL with Can Get Signal® Immunoreaction Enhancer Solution 1 (Toyobo Co., Ltd.) at room temperature. Then, the membrane was incubated in

1:20,000 diluted anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, Inc., Danvers, MA, USA) with Can Get Signal® Immunoreaction Enhancer Solution 2 (Toyobo Co., Ltd.) at room temperature. Detection was performed with TMB Solution for Western Blotting (Nacalai Tesque, Inc.) for 15 min at 23°C. After washing and drying, a picture of the membrane was taken by digital camera or scanner, and the intensity of the target band was quantified with Image Lab Software (Version 6.1; BioRad, Hercules, California, USA). The FT protein band intensity was normalized for variation in total protein loading in the corresponding lane, as quantified by the Ponceau-S stained membranes (Romero-Calvo et al., 2010)

Immunoprecipitation mass spectrometry

For immunoprecipitation mass spectrometry (IP/MS), Haya (0 day vernalized) and G2-IL1 (vernalized immediately after germination at 2°C for 14 days) were grown for more than 2 weeks in the growth room as described above. The petioles of mature leaves were collected at ZT 15.5–16.5, and protein was extracted as described above. Buffer exchange of the supernatant with PBST (0.02% Tween 20) was performed twice using PD-10 desalting columns (Cytiva). Dynabeads protein A (Thermo Fisher Scientific, Waltham, MA, USA) was incubated with Pos2 antibody diluted with PBST (0.02% Tween 20) and cross-linked by dimethyl pimelimidate dihydrochloride. The cross-linked beads were added to the buffer exchanged protein solution (diluted to a concentration of 900 μg/mL) at a ratio of 1:20, and incubated at 23°C for 1 h with gentle inversion. After washing with PBS, the beads were suspended in 1× SDS sample buffer without 2-Mercaptoethanol and incubated at 70°C for 10 min. After removing the beads, the supernatant was incubated at 95°C for 3 min with 2-Mercaptoethanol [5% (v/v) at final volume]. The denatured protein was separated by 14% (w/v) SDS-PAGE. Gel at ~20 kDa was excised and subjected to trypsin digestion using the In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The digests were resuspended in 0.1% formic acid and separated using Nano-LC-Ultra 2D-plus equipped with cHiPLC Nanoflex (Eksigent, Dublin, CA, USA) in trap-and-elute mode with trap column [200 μm × 0.5 mm ChromXP C18-CL 3 μm 120 Å (Eksigent)] and analytical column [75 μm × 15 cm ChromXP C18-CL 3 μm 120 Å (Eksigent)]. The binary gradient used for the separation was as follows: A98%/B2% to A66.8%/B33.2% for 125 min, A66.8%/B33.2% to A2%/B98% for 2 min, A2%/B98% for 5 min, A2%/B98% to A98%/B2% for 0.1 min, and A98%/B2% B for 17.9 min. Subsequently, 0.1% formic acid/water and 0.1% formic acid/acetonitrile were used as solvents A and B, respectively. The flow rate was 300 nL/min. The analytical column temperature was set to 40ºC. The eluates were directly infused to a mass spectrometer [TripleTOF 5600+ System with NanoSpray III source and heated interface (SCIEX, Framingham, MA, USA)] and ionized in electrospray ionization-positive mode. Data acquisition was carried out by an information-dependent acquisition method. The acquired datasets were analyzed using ProteinPilot version 5.0.1 (SCIEX). The database used was the *R. sativus*

protein library extracted from the NCBI protein database (May 2021), appended with a known common contaminants database (SCIEX). The quality of the database search was confirmed by false discovery rate analysis in which reverse amino acid sequences were used as decoys. Peptides identified with a confidence of at least 95% were considered significant.

2.3. Results

Identification of FT and TSF genes in Brassica and Raphanus crop species and anti-body raising against FT proteins of B. oleracea and R. sativus

As a result of phylogenetic analysis, 4, 2, 2, and 1 *FT* homologs were identified for *B. napus*, *B. oleracea*, *B. rapa*, and *R. sativus*, respectively (Figs. 2-1, 2-2). Additionally, 4, 2, 1, and 1 *TSF* homologs were found for *B. napus*, *B. oleracea*, *B. rapa*, and *R. sativus*, respectively. Genes were named according to their clade in the phylogenetic tree (*FT* or *TSF*) and their chromosome position in the reference genome ("U" means unplaced scaffold). Three partial peptides of RsFT (Pos1, Pos2, and Pos3) were selected as antigens based on the sequence comparison of FT and TSF homologs in *B. oleracea* and *R. sativus* as well as epitope prediction (Fig. 2-3). Antibodies raised against these peptides were used in the following experiments.

Validation of anti-peptide polyclonal antibodies by immunoblotting

Equal amounts of recombinant proteins of FT and TSF proteins of *R. sativus* and *B. oleracea* were subjected to immunoblotting analysis using the above mentioned three antibodies as a primary antibody. While Pos2 and Pos3 antibodies detected all RsFT, BoFT.C2, and BoFT.C6 recombinant proteins at the predicted molecular weight (~24.0–24.8 kDa), Pos1 antibody failed to detect any of the proteins (Fig. 2-4A). Pos2 antibody also weakly detected RsTSF and strongly detected BoTSF.C4, whereas Pos3 antibody did not detect any TSF homologs. These results suggest the possibility that Pos2 and Pos3 antibodies can detect FT homologs of *R. sativus* and *B. oleracea*, and that Pos2 antibody also has weak to strong cross-reactivity against TSF homologs.

Next, I investigated whether Pos2 and Pos3 antibodies can detect native FT protein extracted from *R. sativus* leaf. In G2-IL1 radish, *RsFT* expression level gradually increases with seed vernalization treatment (Fig. 2-4B). For immunoblotting analysis, I used protein extracted from leaf petioles of G2-IL1 plants exposed to different periods of vernalization. While Pos3 antibody could not detect obvious signal in both non-vernalized and vernalized samples, Pos2 antibody detected protein at the predicted molecular weight (~19.8 kDa) in the vernalized samples (Fig. 2-4C). Furthermore, the mRNA expression level of *RsFT* in leaf lamina and the intensity of the ~19.8 kDa band in the immunoblotting analysis of leaf petiole protein were correlated (Fig. 2-4D), further supporting the possibility that Pos2 antibody detected RsFT protein.

Validation of Pos2 antibody by immunoprecipitation mass spectrometry

Finally, the specificity of Pos2 antibody was examined by IP/MS (Fig. 2-5A). As a result, detected peptides were mostly overlapping between the two accessions (Fig. 2-5B). Peptides derived from RsFT protein were only detected in G2-IL1 (49 peptides in total) and not in Haya (Fig. 2-5C). A small number of peptides derived from RsTSF (4 peptides in total) were also detected only in G2-IL1. Together with the immunoblotting analysis, I concluded that Pos2 antibody mainly detects FT protein and also has weak~strong cross-reactivity to TSF protein depending on the species.

Quantitativity of FT protein in immunoblotting analysis using Pos2 antibody

To investigate the quantitativity of immunoblotting analysis of FT protein using Pos2 antibody, I performed immunoblotting analysis on a dilution series of total protein extracted from G2-IL1 radish leaf petioles after 14 days vernalization. The dilution series consisted of seven concentrations between 0 and 30 µg per lane. To load equal amount of protein in each lane, the total protein amount was standardized to 30 µg per lane by adding protein extracted from the leaf petiole of 0-day vernalized Haya radish, which presumably does not affect the amount of FT in the lane that is being probed (Fig. 2-5). I observed a linear relationship between the intensity of the band and the quantity of loaded protein ($R^2 = 0.9997$) between 0.6 and 12 µg for G2-IL1 radish (Fig. 2-6). Thus, I confirmed that Pos2 antibody can quantitatively detect native FT protein in the tissue expressing the protein at physiological concentrations.

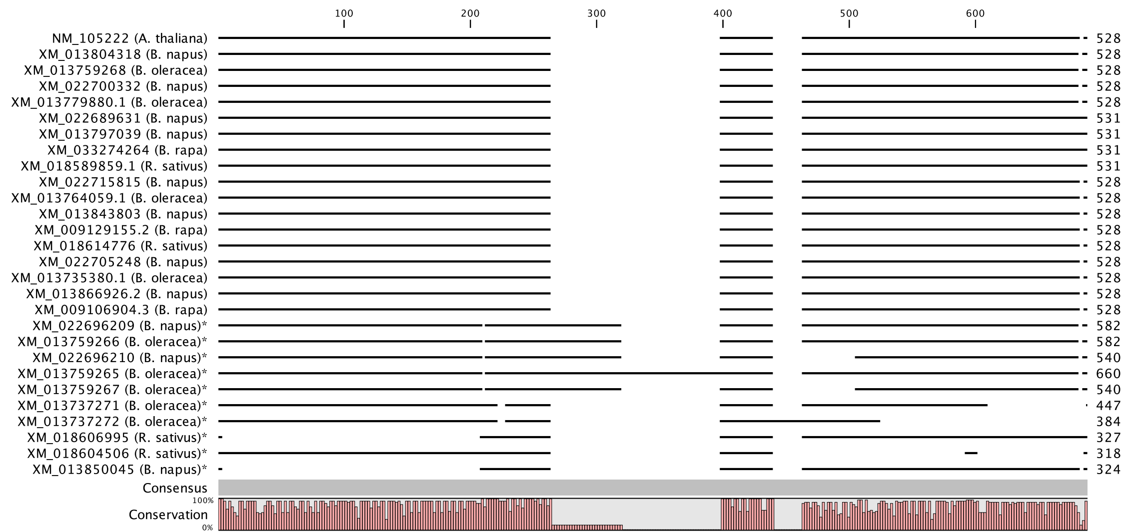


Fig. 2-1. Alignment of coding sequences (CDS) of *FT*-like genes in *Brassica* and *Raphanus* crop species. CDS of *FT*-like genes of *B. napus*, *B. oleracea*, *B. rapa*, and *R. sativus* were obtained from the NCBI Reference Sequence Database (https://www.ncbi.nlm.nih.gov/refseq/) by searching for genes with high similarity (Blastn, percent identity >70%, query cover >50%) to CDS of the *A. thaliana FT* gene (NM_105222:83-610). Sequences that met the search criteria were aligned with each other using the Create Alignment function in CLC Genomics Workbench 12.0 (https://digitalinsights.qiagen.com/products-overview/discovery-insights-portfolio/analysis-and-visualization/qiagen-clc-genomics-workbench/). Asterisk indicates the genes with large indels (>50 bp) within exons. These genes were excluded in subsequent analyses.

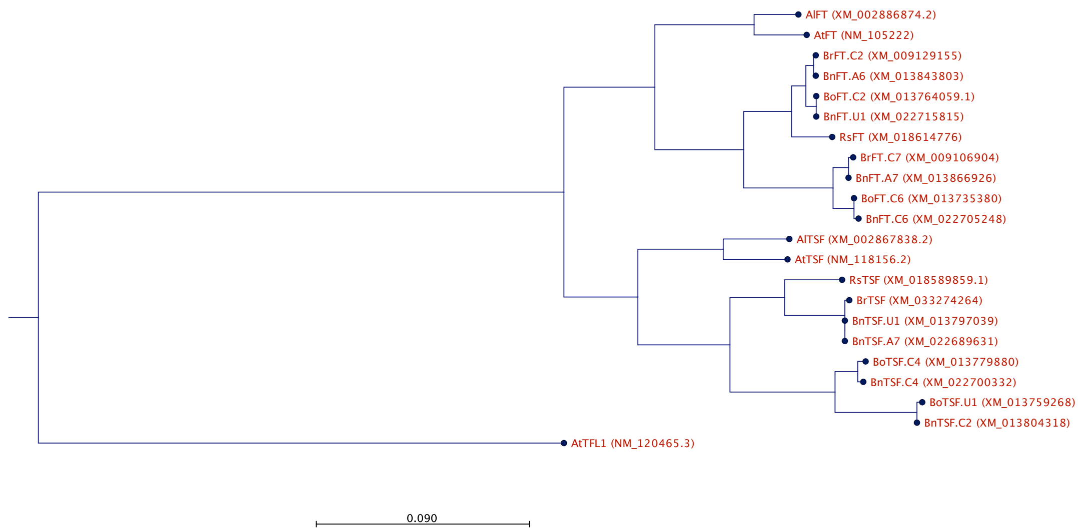


Fig. 2-2. Phylogenetic tree of *FT* and *TSF* homologs of Brassicaceae species. Coding sequences (CDS) of *FT*-like genes of *B. napus*, *B. oleracea*, *B. rapa*, and *R. sativus* were obtained from the NCBI Reference Sequence Database (https://www.ncbi.nlm.nih.gov/refseq/) by searching for genes with high similarity (Blastn, percent identity >70%, query cover >50%) to the CDS of the *A. thaliana FT* gene (NM_105222:83-610). Genes without large indels (>50 bp) within their exons that aligned with *AtFT* (NM_105222), *AtTSF* (NM_118156.2), *AlFT* (XM_002886874.2), and *AlTSF* (XM_002867838.2) were used to construct a phylogenetic tree. *AtTFL1* was used as an outgroup. Genes were named according to the clade in the phylogenetic tree (*FT* or *TSF*) and numbered with the chromosome position in the reference genome ("U" means unplaced scaffold) when two or more homologs were present in the same species. At, *A. thaliana*; Al, *A. lyrata*; Bn, *B. napus*; Bo, *B. oleracea*; Br, *B. rapa*; Rs, *R. sativus*.

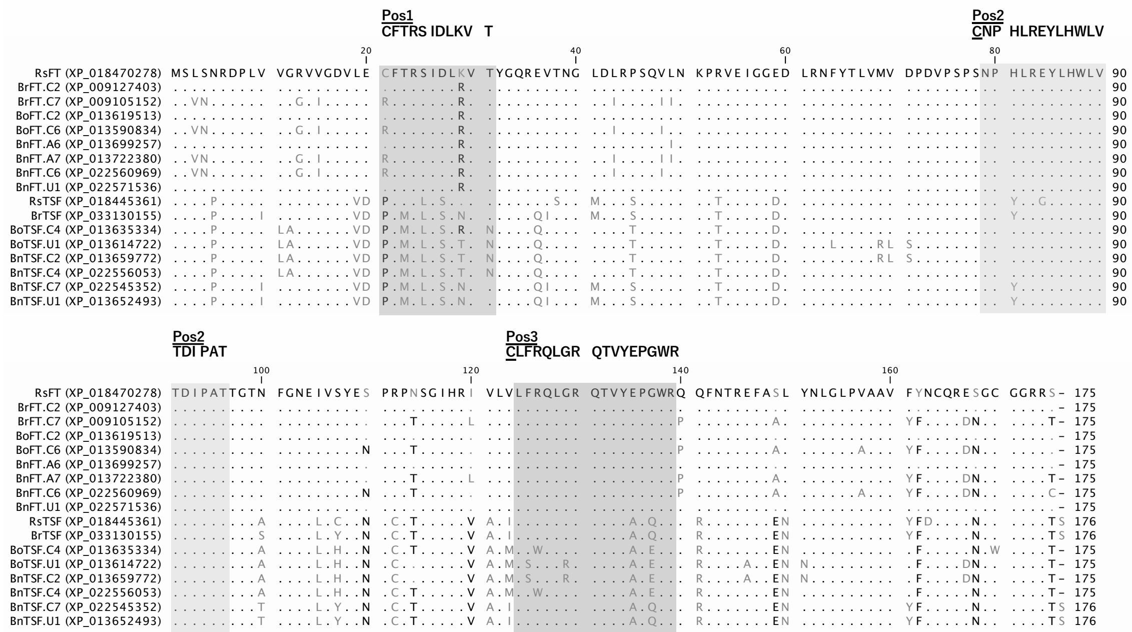


Fig. 2-3. Peptide sequences used for antibody raising. The three peptide sequences used as antigens for antibody raising are shown in the above part of the FT and TSF protein sequence alignment for *Brassica* and *Raphanus* crop species. Underbar indicates a cysteine residue added to stabilize the peptides. Matching residues are indicated as dots, and differing residues are indicated as letters (black: major residue, gray: minor residue).

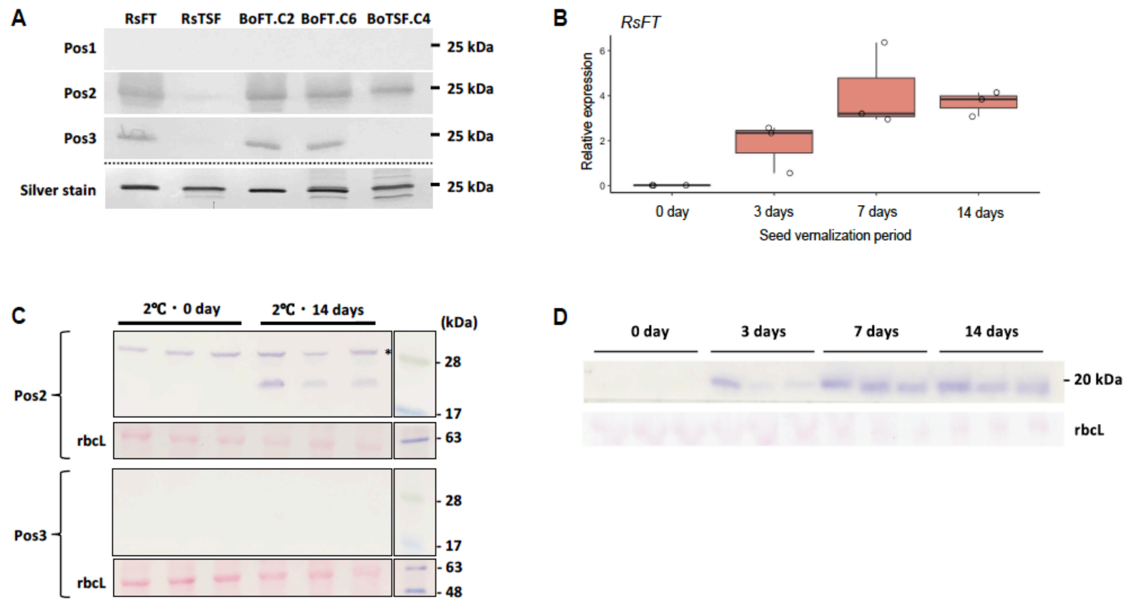
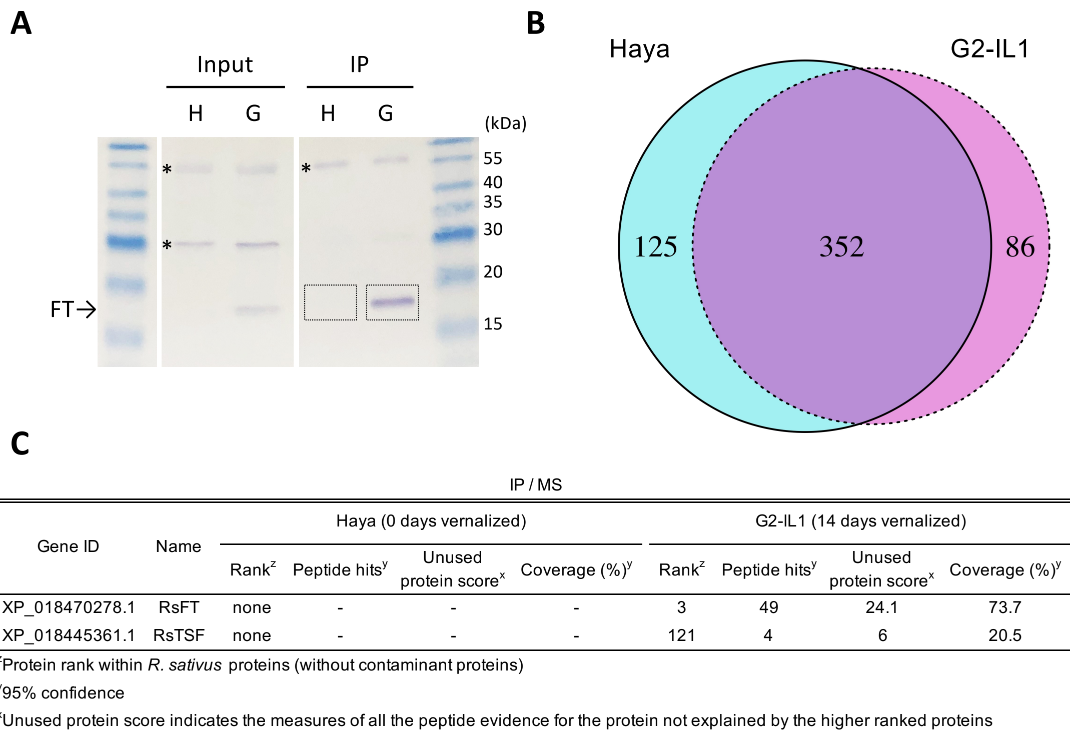


Fig. 2-4. Validation of peptide antibodies by immunoblotting. (A) Immunoblotting analysis of recombinant FT and TSF proteins using the three antibodies. Equal amounts of the recombinant proteins RsFT (XP_018470278), RsTSF (XP_018445361), BoFT.C2 (XP_013619513), BoFT.C6 (XP_013590834), and BoTSF.C4 (XP_013635334) were used for immunoblotting analysis. The top three panels show the results of immunoblotting analysis using Pos1, Pos2, or Pos3 antibody as a primary antibody, respectively. The bottom picture shows silver staining of the same proteins. (B) Expression level of *RsFT* transcript in the leaf of G2-IL1 radish with different period of seed vernalization treatment. Leaf disks were sampled from the largest leaf at14 DAS for RNA extraction, and the expression level of *RsFT* were determined by RT-qPCR (n=3). *RsActin* was used as an internal control. (C) Immunoblotting analysis of protein extracted from G2-IL1 leaf petiole treated with either 0 or 14 days vernalization (n = 3). Pos2 or Pos3 antibody was used as primary antibody. 15 µg of total protein was loaded in each lane. Upper panels show the membrane after TMB staining, and lower panels show the membrane after Ponceau S staining. The signal of Rubisco large subunit (rbcL) is shown as a loading control. Asterisk in the panel showing Pos2 antibody results indicates the non-specific band. (D) Immunoblotting analysis of the proteins extracted from the leaf petiole of the same G2-IL1 radish plants in (C) with Pos2 antibody (n=3). 15 µg of total protein were loaded for each lane.



IP / MS

Gene ID	Name	Haya (0 days vernalized)				G2-IL1 (14 days vernalized)			
		Rank[z]	Peptide hits[y]	Unused protein score[x]	Coverage (%)[y]	Rank[z]	Peptide hits[y]	Unused protein score[x]	Coverage (%)[y]
XP_018470278.1	RsFT	none	-	-	-	3	49	24.1	73.7
XP_018445361.1	RsTSF	none	-	-	-	121	4	6	20.5

[z]Protein rank within *R. sativus* proteins (without contaminant proteins)

[y]95% confidence

[x]Unused protein score indicates the measures of all the peptide evidence for the protein not explained by the higher ranked proteins

Fig. 2-5. Validation of Pos2 antibody by immunoprecipitation mass spectrometry (IP/MS). (A) Immunoblotting analysis with Pos2 antibody of the immunoprecipitated proteins. Input proteins were extracted from petioles of Haya radish without vernalization (H) and G2-IL1 radish after 14 days vernalization (G). IP indicates immunoprecipitated protein of input protein with Pos2 antibody. Asterisk indicates the non-specific bands. A dashed rectangle shows the area used for mass spectrometry. The corresponding area of another gel was sampled. (B) Venn diagram of *R. sativus* proteins detected in Haya and G2-IL1 radish by IP/MS. (C) Summary of FT homolog peptides detected by IP/MS.

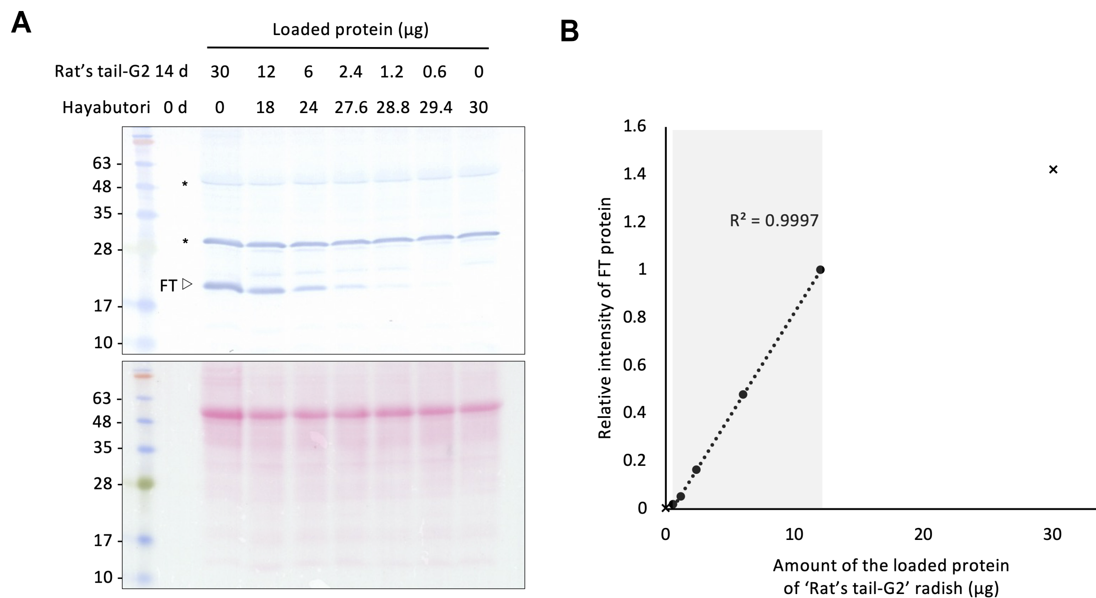


Fig. 2-6. Quantitative immunoblotting analysis of *R. sativus* FT protein using Pos2 antibody. (A) Immunoblotting analysis of the dilution series of the protein extracted from the leaf petiole of 14 days-vernalized G2-IL1 radish using Pos2 antibody. To make a same background level, protein extracted from the leaf petiole of 0 day-vernalized Haya radish was added to each lane so that the total protein amount become 30 μg per lane. Upper panels show the membrane after TMB staining and lower panels show the membrane after ponceau staining. Asterisks in the picture indicate the non-specific band. (B) Regression analysis of the amount of loaded protein of G2-IL1 radish and the signal intensity of FT protein band obtained in the immunoblotting analysis. Gray shadow shows the linear range. Coefficient of determination was calculated after excluding the two data points (data points of the lane loaded 0 and 30 μg of G2-IL1 radish, indicated as a black cross) which were out of the linear range.

Table 2-S1 Primer sets used in the cloning for recombinat protein expression, and sequencing of the plasmid.

Gene	Accession Number	Primer name	Primer (5'-3')	Product Size (bp)[z]
BoFT.C2	XM_013764059.1	pET-6HN-C_BoFT.C2_1-F	TAAGGCCTCTGTCGAGCCGAAGATGTCTTTAAGTAAT	563
		pET-6HN-C_BoFT.C2_1-R	CAGAATTCGCAAGCTTACTTCTTCGTCCTCCGCAG	
BoFT.C6	XM_013735380	pET-6HN-C_BoFT.C6_1-F	TAAGGCCTCTGTCGAATCAGAGATGTCTGTAAATAAC	563
		pET-6HN-C_BoFT.C6_1-R	CAGAATTCGCAAGCTTACTTCTTCGTCCTCCGCAG	
BoTSF.C4	XM_013779880	pET-6HN-C_BoTSF_1-F	TAAGGCCTCTGTCGAAAAGATCATGTCTTTAAGTCCG	581
		pET-6HN-C_BoTSF_1-R	CAGAATTCGCAAGCTTGGGAGGCATGGATATCTTTG	
RsFT	XM_018614776.1	pET-6HN-C_RsFT_1-F	TAAGGCCTCTGTCGAATCAAAGATGTCTCTAAGTAAT	581
		pET-6HN-C_RsFT_1-R	CAGAATTCGCAAGCTTGAAGAGGCTCATCTTAC	
RsTSF	XM_018589859.1	pET-6HN-C_RsTSF_1-F	TAAGGCCTCTGTCGAAAAGATCATGTCACTAAGTCCA	581
		pET-6HN-C_RsTSF_1-R	CAGAATTCGCAAGCTGGCATATATATATCGACGAGG	

[z] Product size is based on cDNA

Table 2-S2. Protein blast result of Pos2 peptide sequence (NPHLREYLHWLVTDIPAT, terminus cystein residue was excluded) against NCBI Reference proteins database (refseq_protein) of *B. oleracea* and *R. sativus* .

Description	Scientific Name	Query Cover	E value	Percent identity score	Accession
PREDICTED: protein FLOWERING LOCUS T	*R. sativus*	100%	8.00E-14	100	XP_018470278.1
PREDICTED: protein TWIN SISTER of FT-like isoform X4	*B. oleracea*	100%	8.00E-14	100	XP_013614722.1
PREDICTED: protein FLOWERING LOCUS T-like	*B. oleracea*	100%	8.00E-14	100	XP_013590834.1
PREDICTED: protein FLOWERING LOCUS T-like	*B. oleracea*	100%	8.00E-14	100	XP_013635334.1
PREDICTED: protein TWIN SISTER of FT-like isoform X1	*B. oleracea*	100%	5.00E-12	94.44	XP_013592725.1
PREDICTED: protein TWIN SISTER of FT-like isoform X2	*B. oleracea*	100%	5.00E-12	94.44	XP_013592726.1
PREDICTED: protein TWIN SISTER of FT-like	*R. sativus*	100%	5.00E-12	94.44	XP_018460008.1
PREDICTED: protein TWIN SISTER of FT-like	*R. sativus*	100%	3.00E-10	88.89	XP_018445361.1
PREDICTED: protein FLOWERING LOCUS T-like	*R. sativus*	100%	3.00E-10	88.89	XP_018462497.1
PREDICTED: protein BROTHER of FT and TFL 1	*B. oleracea*	100%	5.00E-09	77.78	XP_013621853.1
PREDICTED: protein BROTHER of FT and TFL 1	*R. sativus*	100%	5.00E-09	77.78	XP_018491729.1
PREDICTED: protein BROTHER of FT and TFL 1-like	*R. sativus*	100%	5.00E-09	77.78	XP_018486707.1
PREDICTED: protein CENTRORADIALIS-like isoform X1	*R. sativus*	100%	8.00E-07	72.22	XP_018475061.1
PREDICTED: protein CENTRORADIALIS-like isoform X1	*B. oleracea*	100%	8.00E-07	72.22	XP_013635230.1
PREDICTED: protein CENTRORADIALIS-like isoform X2	*R. sativus*	100%	8.00E-07	72.22	XP_018475062.1
PREDICTED: protein CENTRORADIALIS-like	*B. oleracea*	100%	8.00E-07	72.22	XP_013635092.1
PREDICTED: protein CENTRORADIALIS-like	*B. oleracea*	100%	8.00E-07	72.22	XP_013627274.1
PREDICTED: protein CENTRORADIALIS-like isoform X2	*B. oleracea*	100%	8.00E-07	72.22	XP_013635231.1
PREDICTED: protein CENTRORADIALIS-like	*R. sativus*	100%	8.00E-07	72.22	XP_018437820.1
PREDICTED: protein CENTRORADIALIS-like	*R. sativus*	100%	8.00E-07	72.22	XP_018479813.1
PREDICTED: protein CENTRORADIALIS-like	*R. sativus*	100%	8.00E-07	72.22	XP_018464205.1
PREDICTED: protein CENTRORADIALIS-like	*R. sativus*	100%	8.00E-07	72.22	XP_018463888.1
PREDICTED: protein TERMINAL FLOWER 1	*B. oleracea*	100%	3.00E-04	61.11	XP_013614451.1
PREDICTED: protein TERMINAL FLOWER 1-like	*B. oleracea*	100%	3.00E-04	61.11	XP_013621108.1
PREDICTED: protein TERMINAL FLOWER 1	*R. sativus*	100%	8.00E-04	61.11	XP_018446603.1
PREDICTED: protein TERMINAL FLOWER 1-like	*B. oleracea*	100%	0.009	61.11	XP_013610369.1
PREDICTED: protein TERMINAL FLOWER 1	*R. sativus*	72%	0.012	69.23	XP_018474870.1
PREDICTED: protein MOTHER of FT and TF 1 isoform X2	*B. oleracea*	83%	0.035	60	XP_013603338.1
PREDICTED: protein MOTHER of FT and TFL1	*R. sativus*	83%	0.035	60	XP_018437997.1
PREDICTED: protein MOTHER of FT and TF 1 isoform X1	*B. oleracea*	83%	0.035	60	XP_013603337.1
PREDICTED: protein MOTHER of FT and TF 1	*B. oleracea*	83%	0.035	60	XP_013585084.1
PREDICTED: protein TWIN SISTER of FT-like isoform X3	*B. oleracea*	44%	0.79	100	XP_013614721.1
PREDICTED: protein TWIN SISTER of FT-like isoform X2	*B. oleracea*	44%	0.79	100	XP_013614720.1
PREDICTED: protein TWIN SISTER of FT-like isoform X1	*B. oleracea*	44%	0.79	100	XP_013614719.1

Table 2-S3. The result of BLASTP search of Pos2 peptide sequence against refseq protein database of Brassicaceae species

Description	Scientific Name	Family name	Query Cover	E value	Percent identity score	Accession
PEBP (phosphatidylethanolamine-binding protein) family protein	*Arabidopsis thaliana*	Brassicaceae	100%	7.00E-11	100	NP_001320342.1
PREDICTED: protein FLOWERING LOCUS T	*Camelina sativa*	Brassicaceae	100%	7.00E-11	100	XP_010511444.1
PREDICTED: protein FLOWERING LOCUS T-like	*Camelina sativa*	Brassicaceae	100%	7.00E-11	100	XP_010415085.1
PREDICTED: protein TWIN SISTER of FT	*Camelina sativa*	Brassicaceae	100%	7.00E-11	100	XP_010451336.1
PREDICTED: protein FLOWERING LOCUS T-like	*Tarenaya hassleriana*	Brassicaceae	100%	7.00E-11	100	XP_010551361.1
protein TWIN SISTER of FT	*Brassica rapa*	Brassicaceae	100%	7.00E-11	100	XP_009105152.1
PREDICTED: protein FLOWERING LOCUS T	*Raphanus sativus*	Brassicaceae	100%	7.00E-11	100	XP_018470278.1
protein TWIN SISTER of FT	*Capsella rubella*	Brassicaceae	100%	7.00E-11	100	XP_006285929.1
protein FLOWERING LOCUS T	*Brassica rapa*	Brassicaceae	100%	7.00E-11	100	XP_009127403.1
PREDICTED: protein FLOWERING LOCUS T	*Camelina sativa*	Brassicaceae	100%	7.00E-11	100	XP_010470415.1
protein TWIN SISTER of FT	*Eutrema salsugineum*	Brassicaceae	100%	7.00E-11	100	XP_006390144.1
PREDICTED: protein TWIN SISTER of FT	*Camelina sativa*	Brassicaceae	100%	7.00E-11	100	XP_010439584.1
PREDICTED: protein FLOWERING LOCUS T-like	*Tarenaya hassleriana*	Brassicaceae	100%	7.00E-11	100	XP_010553859.1
PEBP (phosphatidylethanolamine-binding protein) family protein	*Arabidopsis thaliana*	Brassicaceae	100%	7.00E-11	100	NP_176726.1
PREDICTED: protein TWIN SISTER of FT-like isoform X4	*Brassica oleracea var. oleracea*	Brassicaceae	100%	7.00E-11	100	XP_013614722.1
protein FLOWERING LOCUS T-like	*Brassica napus*	Brassicaceae	100%	7.00E-11	100	XP_022560969.1
protein FLOWERING LOCUS T	*Arabidopsis lyrata subsp. lyrata*	Brassicaceae	100%	7.00E-11	100	XP_002886920.1
protein FLOWERING LOCUS T-like	*Brassica napus*	Brassicaceae	100%	7.00E-11	100	XP_022556053.1
PREDICTED: protein FLOWERING LOCUS T-like	*Brassica oleracea var. oleracea*	Brassicaceae	100%	7.00E-11	100	XP_013590834.1
protein TWIN SISTER of FT	*Arabidopsis lyrata subsp. lyrata*	Brassicaceae	100%	7.00E-11	100	XP_002867884.1
protein FLOWERING LOCUS T	*Capsella rubella*	Brassicaceae	100%	7.00E-11	100	XP_006300555.1
protein FLOWERING LOCUS T	*Brassica napus*	Brassicaceae	100%	7.00E-11	100	XP_013699257.1
protein TWIN SISTER of FT	*Eutrema salsugineum*	Brassicaceae	100%	7.00E-11	100	XP_006413873.1
protein TWIN SISTER of FT-like isoform X3	*Brassica napus*	Brassicaceae	100%	7.00E-11	100	XP_013659772.1
PREDICTED: protein FLOWERING LOCUS T-like	*Brassica oleracea var. oleracea*	Brassicaceae	100%	7.00E-11	100	XP_013635334.1
protein FLOWERING LOCUS T	*Eutrema salsugineum*	Brassicaceae	100%	7.00E-11	100	XP_006391553.1
protein FLOWERING LOCUS T	*Brassica napus*	Brassicaceae	100%	8.00E-11	100	XP_013705499.1

2.4. Discussion

Specificity of the generated anti-peptide polyclonal antibodies against FT

Of the three antibodies generated in the present study, only the Pos2 antibody was able to detect FT homologs in *R. sativus* and *B. oleracea*. The Pos3 antibody was generated using a peptide sequence that overlaps with the target region of a previously published peptide antibody against *A. thaliana* FT protein (Kim et al., 2016). Although Pos3 antibody was able to detect recombinant FT protein when expressed in *E. coli*, it was unable to detect native FT protein in *R. sativus* (Fig. 2-4A). Possible reasons for this are that the peptide sequence used to raise Pos3 antibody differed in two amino acids from that used by Kim et al. (2016), and that Pos3 antibody may bind non-specifically to many proteins in *R. sativus*. In the recombinant protein detection experiments, Pos2 antibody detected FT protein with higher sensitivity than TSF protein in *R. sativus*, whereas TSF protein was detected with the same sensitivity as FT homologs in *B. oleracea* (Fig. 2-4A). This difference in detection level by immunoblotting was consistent the degree of mismatch between the Pos2 peptide and the target sequence (Fig. 2-3). The results of IP/MS showed that Pos2 antibody binds mainly to RsFT with weak interaction against RsTSF (Fig. 2-5), supporting the idea that the degree of mismatch affects affinity. Considering the even higher number of mismatches between Pos2 peptide and the corresponding sequences of other FT family proteins (Table 2-S2, percent identity score), Pos2 antibody is unlikely to detect FT family proteins other than FT and TSF.

The homologous region of the Pos2 peptide is highly conserved in Brassicaceae species; the FT/TSF homologs of Brassicaceae species, including the model plant *A. thaliana*, do not contain any amino acid polymorphism in the homologous region of the Pos2 peptide (Table 2-S3). Although it was not investigated in the current study, it appears likely that Pos2 antibody could be widely used for the detection of FT/TSF homologs in Brassicaceae species.

Quantitative detection of FT protein using the developed antibody

The immunodetection of FT in the dilution series of protein extracted from *R. sativus* confirmed that Pos2 antibody can quantitatively detect native FT protein at physiological concentrations (Fig. 2-6). The linear range was 1.6 orders of magnitude, which is comparable to the dynamic range described by the manufacturer of the colorimetric detection reagent used in this study. Because the colorimetric detection method has a relatively low sensitivity and narrow dynamic range, I suppose that quantitativity of immunoblotting using Pos2 antibody can be improved by employing other detection methods, such as chemiluminescent and fluorescent detection. As a future work, it will be interesting to investigate the suitability of Pos2 antibody in Enzyme-Linked Immunosorbent Assays (ELISA), a high throughput method for quantitative detection of target proteins. As I observed several non-specific bands in immunoblotting analysis (Fig. 2-6), it may be difficult to use Pos2 antibody for direct

or indirect ELISA detection methods that use a single antibody and require higher antibody specificity. A double sandwich ELISA using the Pos2 antibody and another antibody developed for a different epitope of the FT protein may enable specific detection of FT protein by ELISA.

2.5. Summary

In Chapter 2, I developed an anti-peptide polyclonal antibody which can detect native FT protein in *B. oleracea* and *R. sativus*. I confirmed the quantitativity of the immunoblotting analysis using this antibody for native FT protein of *R. sativus* at physiological concentrations. Therefore, the developed antibody was considered to enable the investigation of the FT protein accumulation in the grafted cabbage. Also, this is the first report where the native FT protein is detected by immunoblotting analysis in cruciferous crops. The developed anti-FT antibody will make it possible not only to elucidate the necessary condition for the grafting-induced flowering of cabbage, but also to further understand the molecular mechanisms of flowering regulation in cruciferous crops.

Chapter 3.

Identification of variation factors in grafting-induced flowering of cabbage through the quantitative analysis of FT protein

3.1. Introduction

In Chapter 2, anti-FT antibody for *R. sativus* and *B. oleracea* was successfully developed. This antibody makes it possible to examine the contribution of FT protein to the grafting-induced flowering of cabbage. I hypothesized that differences in the accumulation of FT in grafted cabbage plants causes variability in the flowering response. This kind of quantitative action of FT has been previously observed in grafted plants (Tang et al., 2022) as well as in intact plants (Endo et al., 2018; Liu et al., 2012; Susila et al., 2021; Yoo et al., 2013a). This hypothesis is also supported by the observation in Chapter 1 that *RsFT* expression level was significantly different among the *R. sativus* cultivars.

In Chapter 3, I first investigated the relationship between the amount of FT protein and the flowering response of the cabbage plants which were grafted onto early-flowering accessions of *B. oleracea* and *R. sativus* (Experiment 1). Then, I explored the rootstock factors that contribute to the variable FT protein accumulation in the grafted cabbage, by changing the rootstock conditions with vernalization treatment (Experiment 2), daylength treatment (Experiment 3), and leaf trimming treatment (Experiment 4).

3.2. Materials and Methods

Plant materials and growth conditions

Plants were grown in a growth room maintained at 22°C ± 2°C for LD (16h/8h, light/dark) conditions, or at 23°C ± 2°C for MD (12h/12h, light/dark) conditions. Light was provided by fluorescent lamps (PPFD 120 μmol m^{-2} s^{-1}, NEC Lighting, Ltd.). I used the following accessions as rootstock plants: *B. oleracea* var. *oleracea* TO1000, *B. oleracea* var. *albograbra* Kairan, *R. sativus* var. *caudatus* G2-IL1, G3-IL1, CH-IL1, G2xCH F_1 (F_1 progeny derived by crossing G2-IL1 and CH-IL1), G2xG3 F_1 (F_1 progeny derived by crossing G2-IL1 and G3-IL1). The origin of each accession is listed in Table 3-S1. Seeds were sown on wet filter paper and germinated in the dark at 22°C for 1–2 days. Germinated seeds were then vernalized at 2°C in the dark for 0–28 days depending on the experiment (Table 3-S2). Vernalized seeds were then transplanted into 7.5 cm diameter plastic pots filled with granular rockwool (Nippon Rockwool Corp.) and cultivated further in the growth room. I used seeds of ‘Matsunami’ (Ishii Seed Growers Co., Ltd., Shizuoka, Japan) cabbage as scion plants. These were grown in Jiffy-7® 42 mm peat pellets (Jiffy Products of America, Inc. Batavia, IL, USA) in a growth room under the LD conditions described above for 3–4 weeks. All plants were irrigated and fertilized

from below using a half-strength nutrient solution (Enshi-shoho).

Grafting of cabbage scions to rootstock plants

Grafting was conducted according to the method described in Chapter 1 with some modification. For *R. sativus* rootstocks, seedlings that had bolted to a height of 7 cm from the top of the hypocotyl were used as rootstocks. For two accessions of *B. oleracea*, seedlings at 30 DAS were used as rootstocks. The stem of the rootstock was cut at a height of 4–5 cm from the top of the hypocotyl. A cabbage scion was then cut to be wedge-shaped at the stem with 2–3 expanded leaves remained and was grafted onto the stem of the rootstock by cleft grafting. After grafting, the scion was covered with a clear polyethylene bag to maintain high humidity. Two different curing methods described in Fig. 3-S1 were used depending on the experiment (Table 3-S2). In most cases, the scion and the rootstock were fully connected 1–2 weeks after grafting. To promote translocation of assimilates from the rootstock to the scion, all lateral shoots of the rootstock were removed. New leaves on the scion were also continuously removed until only 5 newly expanded leaves (2 cm in length or more) remained; this was to ensure that the scion could preserve sink activity. After the opening of the first flower, all leaves longer than 2 cm in length were continuously removed. Plant growth continued until 63 DAG.

Measurements of the vegetative and reproductive growth of the rootstock and scion

The total leaf area of the rootstock was estimated from the length and width of the largest leaf and the number of leaves left on the rootstock using a regression equation as described in Chapter 1. Grafting was regarded as failed if the scion plant wilted without the polyethylene bag at 21 DAG. The appearance of flower buds was evaluated by daily visual inspection. On 35 DAG, the maximum stem diameter of the scion was measured using a caliper. On 63 DAG, the total numbers of leaves and flower buds of the scion and the total number of opened flowers were counted, and scion flower bud differentiation was evaluated under a microscope if an obvious flower bud was not visible.

Total RNA extraction, cDNA synthesis, and RT-qPCR

Leaf disks were collected from the tip of the largest leaf of the rootstock on 14 DAS, 21 DAS, or 35 DAG at 0–0.5 h before the end of the light period. Total RNA was extracted from the leaf samples using Sepasol RNA I Super G (Nacalai Tesque, Inc.), purified using an Econospin™ for RNA (Epoch Life Sciences, Missouri, TX, USA), and reverse transcribed using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd.) according to the manufacturer's instructions. Subsequently, 1 μL of 20-fold diluted RT product was used as a template for RT-qPCR. RT-qPCR was performed using a THUNDERBIRD® SYBR® qPCR Mix kit (Toyobo Co., Ltd.) according to the manufacturer's instructions. The reaction was performed using a LightCycler® 480 system (Roche Diagnostics K.K.). RT-qPCR cycling was performed as follows: 95°C for 5 min, followed by 40 cycles

at 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. Single-target product amplification was evaluated using a melting curve. RT-qPCR primers for *BoFT.C6* and *BoActin* are the same ones used in Chapter 1, and RT-qPCR primers for *RsFT* and *RsActin* are the same ones used in Chapter 2.

Protein extraction and quantification

A sample of the midrib of a young leaf (1–2.5 cm in length) was collected from a grafted scion on 35 DAG at 0–0.5 h before the end of the light period. Total protein was extracted according to the method of Wang et al. (2010) with minor modification. In brief, tissue was frozen in liquid nitrogen in a microcentrifuge tube, ground by bead beating using TissueLyser II (Qiagen, Valencia, CA, USA), and homogenized in approximately 5 × volume of extraction buffer [50 mM Tris–HCl, pH 9.0, 2% (w/v) sodium dodecyl sulfate (SDS), 5 mM ascorbic acid, 0.1% (v/v) 2-Mercaptoethanol, 1 × protease inhibitor cocktail (P9599; Sigma-Aldrich Co. LLC, St. Louis, MO, USA)]. The homogenate was subsequently centrifuged twice for 10 min at 16,100 g. The supernatant was used for further analysis. The total soluble protein was quantified using an XL Bradford assay (Intégrale Co., Ltd).

Immunoblotting analysis

Extracted protein was diluted with an equal volume of 2× SDS sample buffer (125 mM Tris–HCl, pH 6.8, 4% (w/v) SDS, 30% (w/v) glycerol, bromophenol blue, 10% (v/v) 2-Mercaptoethanol) and incubated at 95°C for 3 min. 30 µg protein was then loaded on a 16% (w/v) SDS–polyacrylamide gel, separated by electrophoresis, and transferred onto a nitrocellulose membrane (Immobilon-P; Cytiva). After transferring, the membrane was stained with Ponceau S staining solution (Beacle, Inc.), and a picture of the membrane was taken using a scanner. After blocking with PBST buffer containing 1% skim milk at room temperature of 20–25°C, the membrane was incubated with an anti-FT antibody (Pos2, developed in Chapter 2) diluted to 300 ng/mL in Can Get Signal® Immunoreaction Enhancer Solution 1 (Toyobo Co., Ltd.) at room temperature. Next, the membrane was incubated with a 1:20,000 diluted anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, Inc.) with Can Get Signal® Immunoreaction Enhancer Solution 2 (Toyobo Co., Ltd.) at room temperature. Detection was performed in TMB Solution for Western Blotting (Nacalai Tesque, Inc.) for 15 min at 23°C. After washing and drying, a picture of the membrane was taken using a scanner, and the intensity of the target band was quantified with Image Lab Software (Version 6.1; BioRad). The FT protein band intensity was normalized for variation in total protein loading in the corresponding lane, as quantified by the Ponceau-S stained membranes (Romero-Calvo et al., 2010), and was then normalized using a positive control (a mixture of protein extracted from the leaf petiole of a 14 day-vernalized G2-IL1 and a 0-day vernalized Haya radish) on the same blot.

3.3. Results

Differences in the flowering responses of cabbage scions grafted onto various early-flowering B. oleracea and R. sativus rootstocks

To reconfirm that the variability of the flowering response of grafted scions depends on the genotype of the rootstock (as observed in Chapter 1), young cabbage seedlings (3–4 weeks after sowing) were grafted under non-vernalized conditions to rootstocks of different genotypes (Experiment 1). In a Chapter 1, I observed a higher frequency of flower bud differentiation in cabbage scions when grafted onto early-flowering *R. sativus* cultivars. Therefore I used two early-flowering accessions from *B. oleracea* and three accessions and two F_1 hybrids from *R. sativus* as rootstocks (Table 3-S1). For all radish accessions, the appearance of flower buds and the bolting of the rootstocks were observed within 30 DAS in average without vernalization treatment (Table 3-S2). For *B. oleracea* accessions, bolting cannot be used as an indicator of floral induction because stem elongation occurs regardless of floral induction in this species. Flower bud appearance is usually observed at 25–30 DAS in the TO1000 accession and at 40–50 DAS in the Kairan accession under the same conditions (data not shown). Grafting was performed at the day of bolting in *R. sativus* accessions, and at 30 DAS in *B. oleracea* accessions. Grafting success rates ranged from 78% to 100% (Table 3-S2). The flowering response of grafted cabbage plants varied greatly depending on rootstock genotype, with the values of the percentage of scions with differentiated flower buds ranging from 0% to 100% by the end of the grafting experiment (Fig. 3-1A, B). Neither of the two *B. oleracea* accessions induced flowering of grafted scions (Fig. 3-1A, B), which was consistent with the results of Chapter 1 and the study of Kagawa (1957). On the other hand, I also observed substantial differences in the percentage of scions with differentiated flower buds among the five *R. sativus* accessions in consistent with the observation in Chapter 1 (Fig. 3-1A, B). The number of days to flower bud appearance and the number of flowers opened by 63 DAG in the scion also differed among *R. sativus* accessions (34.8–60.6 days and 0–95.4 flowers, respectively, Table 3-S2). Taken together, these results suggest that there are quantitative differences among *R. sativus* accessions with respect to the ability to induce flowering in grafted cabbage.

In addition to reproductive growth, I also examined the vegetative growth of the grafted cabbage plants. In cabbage, the maximum stem diameter is used as a proxy for vegetative growth and physiological age. This is because there is a correlation between the maximum stem diameter and vegetative growth parameters such as above-ground fresh weight and the number of expanded leaves, and because it can be measured non-destructively (Ito and Saito, 1961; Fig. 3-S2). Thus, the maximum stem diameter at 35 DAG was used as the parameter characterizing the vegetative growth of grafted scions in this study. I found significant differences in stem diameter of grafted cabbage plants among rootstock accessions (Table 3-S2). Moreover, the stem diameter of the grafted scion strongly correlated

with the total area of leaves left on the rootstock on the day of grafting (Fig. 3-1C). This indicated that scion growth was dependent on the translocation of assimilates from the rootstock. This is probably caused by the removal of mature leaves of the scion (see Materials and Methods), which I performed to maintain the sink activity of the scion, as used in previous studies (Hamner and Bonner, 1938; Kagawa, 1957).

Quantification of FT protein accumulation in grafted cabbage scions

To examine potential causes of the differences in the flowering response of the grafted cabbage, I investigated the relationship between the flowering response of the grafted scion and FT protein accumulation. I first checked whether FT protein could be detected in grafted cabbage scions using the anti-FT antibody developed in Chapter 2. Since previous studies have shown that FT protein also accumulates in sink tissues other than the shoot apex (Endo et al., 2018; Navarro et al., 2011), I extracted protein from the midribs of young leaves of scion plants at 5 weeks after grafting (Fig. 3-2A). I extracted protein using this part of the plant to avoid destructive effects on the growth of the scion. Immunoblotting analysis showed that a band of FT protein was detected only in the protein extracted from cabbage scions grafted onto G2xCH F_1 radish, but not in the protein extracted from non-grafted cabbages (Fig. 3-2B). Thus, I confirmed that FT protein was transmitted from *R. sativus* rootstocks to the grafted cabbage and that this FT protein could be detected by the developed antibody. Using this method, I then measured the FT protein accumulated in grafted scion plants by determining the relative band intensity of FT protein normalized against the total protein signal. As a result, I observed significant differences in the amount of FT protein accumulated by grafted scions among the rootstock accessions (Fig. 3-2C). When I examined this data along with flowering response patterns of the grafted scions, I found that the FT protein accumulation level was significantly higher in scions that had differentiated flower buds by the end of the experiment (Fig. 3-2D). In addition, there was a significant negative correlation between the number of days to flower bud appearance of the scion and the level of FT protein accumulation (Fig. 3-2E, $r = -0.59$, $p < 0.01$). These results suggested that differences in the flowering response of scion plants grafted onto different rootstock accessions were caused by differences in the level of FT protein accumulation in the scion.

To clarify the factors that cause differences in FT protein accumulation in scions, I measured *FT* transcript expression in the leaves of the rootstock. As mentioned above, I could not detect FT protein in scion plants grafted onto *B. oleracea* accessions; however, TO1000 showed significantly higher *FT* expression than Kairan (Fig. 3-2F). In the *R. sativus* accessions, I observed significant differences in *FT* expression, with a mean difference of up to 18.9-fold (Fig. 3-2G, between G2-IL1 and G2×CH F_1). There was also a significant but weak positive correlation between the level of *FT* transcription in the *R. sativus* rootstock and the level of FT protein accumulation in the grafted cabbage (Fig. 3-2H, $r = 0.47$, $p < 0.01$). Therefore, I hypothesized that the variability in FT protein accumulation and floral

induction in cabbage plants grafted onto *R. sativus* rootstocks was caused by differences in the level of transcription of *FT* in the rootstock. To examine this hypothesis, I next performed the following grafting experiment using *R. sativus* rootstocks with altered *FT* transcription levels by vernalization treatment.

Effect of seed vernalization treatments on the rootstock

As observed in Chapter 1, *FT* transcription was upregulated according to floral acceleration induced by vernalization treatment in *R. sativus*, I first investigated the effect of seed vernalization treatments of the rootstock on the flowering response of the grafted cabbage (Experiment 2). *R. sativus* accessions G2-IL1 and G2xCH F_1, whose grafted scions showed different flowering responses in Experiment 1, were used for this experiment. 0–14-day long seed vernalization treatments were applied to rootstocks, then grafting was performed on bolted rootstocks as in Experiment 1 (Experiment 2-1–2-2 in Table 3-S2). While both *R. sativus* accessions do not require low-temperature exposure for flowering, vernalization treatment significantly accelerated flowering of these *R. sativus* rootstock accessions (Fig. 3-3A). I confirmed that *FT* expression in the leaf of the rootstock had significantly increased in response to vernalization treatment (Fig. 3-3B). However, contrary to my expectation, the flowering response of the grafted scion was not promoted by vernalization treatment in either G2-IL1 or G2xCH F_1, despite increased *FT* expression in their rootstocks (Fig. 3C; Table S2). In particular, G2-IL1 did not induce flowering of grafted scions at all under any vernalization treatment (Fig. 3-3C). For G2xCH F_1, the percentage of flower bud differentiated scions was highest in the non-vernalized treatment (Fig. 3-3C), and the days to flower bud appearance was also shortest on average in the non-vernalized treatment (Table S2).

The level of accumulated FT protein in scion plants tended to increase with increasing duration of the vernalization treatment in G2-IL1, but there was considerable variation in this response (Fig. 3-3D). On the other hand, G2xCH F_1 showed the highest accumulation of FT protein in scions in the non-vernalized treatment (Fig. 3-3D). The level of accumulated FT protein in the scion was significantly higher in individuals whose scions had differentiated into flower buds by the end of the experiment (Fig. 3-3E), although FT protein was clearly detected in some individuals where flower bud differentiation did not occur (Fig. 3-3E). In summary, the vernalization treatment increased the expression of *FT* transcripts in the rootstock but had almost no or a negative effect on FT protein accumulation and flowering response in the scion. In this experiment, I found no correlation between the expression levels of *FT* transcripts in the rootstock and the level of accumulated FT protein in the scion (Fig. 3-3F). This indicated that there are factors other than *FT* expression levels in the rootstock that affect the amount of FT protein accumulated in the scion.

In this experiment, I observed that vernalization treatment also significantly reduced the leaf area of rootstocks at the day of grafting in both of the accessions (Fig. 3-3G). This was probably due to the

fact that bolting occurred earlier. As I also observed differences in leaf area among the rootstocks in Experiment1 (Fig. 3-1C), I speculated that the leaf area of the rootstock has an effect on FT protein accumulation and hence on flowering response in the grafted scion. Thus, I separately investigated the effect of *FT* transcription level and rootstock leaf area in the following experiments.

Effect of daylength before and after grafting

Taking advantage of the flowering characteristics of *R. sativus*, which is a long-day plant (Hagiya, 1951), I performed a grafting experiment using rootstocks with the same leaf area but with different levels of *FT* transcription by applying daylength treatments (Experiment 3). The following three conditions were applied to G2xCH F_1 rootstocks and its grafts: long-day (LD) conditions of 16 h light/8 h dark throughout the growing period (LD/LD), LD conditions up to grafting and middle-day (MD) conditions of 12 h light/12 h dark after grafting (LD/MD), or MD conditions up to 21 DAS and LD conditions afterward (Fig. 3-4A). The number of days to bolting, the leaf area of the rootstock, and the vegetative growth of the grafted scion were comparable between the LD/LD and LD/MD treatments (Fig. 3-4B, C, D). On the other hand, in the MD/LD condition, the bolting of the rootstock was significantly delayed, the leaf area of the rootstock was significantly greater, and the scion growth was significantly higher than in the other two treatments (Fig. 3-4B, C, D). This confirmed the repressive effect of shorter daylength on the flowering of *R. sativus* plants. As expected, the *FT* transcription levels of the rootstock at 35 DAG was significantly lower in LD/MD plants and did not differ between LD/LD and MD/LD plants (Fig. 3-4E). The flowering response of scions grafted onto these rootstocks showed that almost all the LD/LD and MD/LD scions reached the flower opening stage, whereas more than half of the LD/MD scions continued growing only vegetatively until the end of the experiment (Fig. 3-4F). Moreover, the level of accumulated FT protein in the scion was significantly lower in LD/MD plants and did not differ between LD/LD and MD/LD plants (Fig. 3-4G). Taken together, these results suggest that the transcription level of *FT* in the rootstock determines the level of FT protein accumulation and the flowering response of the scion if the leaf area of the rootstock is the same.

Effect of leaf trimming of rootstock plants

I then performed another grafting experiment using rootstocks with same *FT* transcription level, but with different leaf areas (Experiment 4). On the day of grafting, 0, 1, or 2 leaves of G2xCH F_1 radish were cut from the base of the petiole starting from the largest leaf, and cabbage scions were then grafted onto the rootstock (Fig. 3-5A). The leaf area of the rootstock decreased accordingly with the number of trimmed leaves (Fig. 3-5B). The stem diameter of the grafted cabbage also decreased significantly in response to leaf trimming (Fig. 3-5C; Fig. 3-S3), which confirmed that the rootstocks experienced a decrease in source capacity. In addition, the flowering response of the grafted cabbage

plants was repressed as the number of trimmed leaves increased (Fig. 3-5D), and the level of accumulation of FT protein in the scion also decreased in response to trimming, although not significantly (Fig. 3-5E). When the relationship between the leaf area of the rootstock and the accumulation of FT protein in the scion was examined in individual plants, I found a significant positive correlation (Fig. 3-5F). Taken together, these results showed that the leaf area of the rootstock determines the level of FT protein accumulation and the flowering response in the grafted cabbage when the level of *FT* transcription in the rootstock is the same.

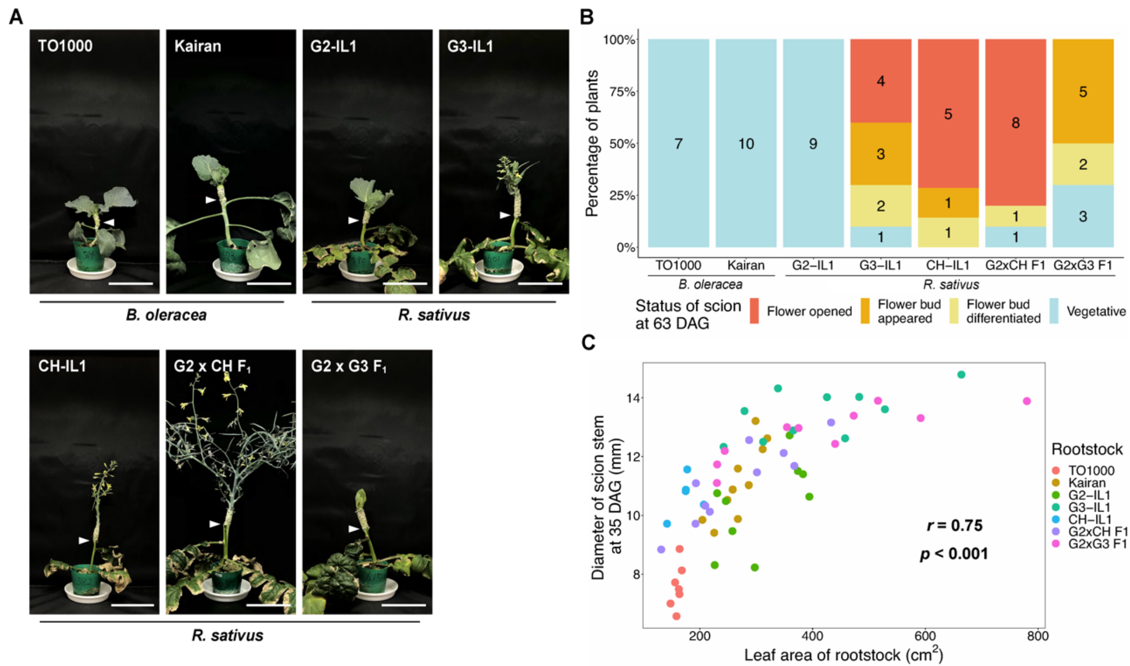


Fig. 3-1. Difference in the flowering response of cabbage scions grafted onto various early-flowering *B. oleracea* and *R. sativus* rootstocks. (A) Representative pictures of grafted plants at 63 DAG. White arrowheads indicate graft junction. Scale bars = 10 cm. (B) Floral status of the apical meristem of scions at 63 DAG. (C) Relationship between the leaf area of rootstocks at 0 DAG and the maximum stem diameter of the grafted scions at 35 DAG. The linear correlation coefficient is shown in the graph.

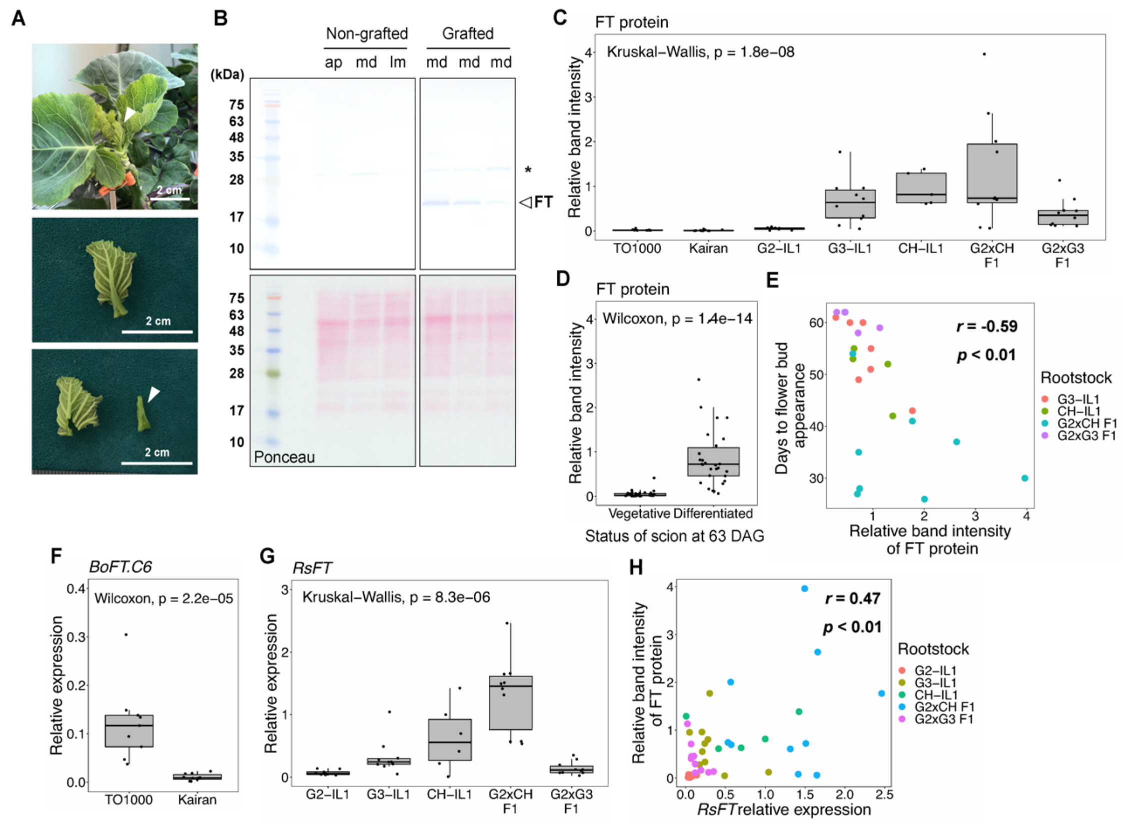

A
2 cm
2 cm
2 cm
B
Non-grafted
Grafted
ap md lm md md md
(kDa)
75
63
48
35
28
17
10
*
◁FT
Ponceau
C
FT protein
Kruskal–Wallis, p = 1.8e−08
Relative band intensity
TO1000
Kairan
G2−IL1
G3−IL1
CH−IL1
G2xCH F1
G2xG3 F1
D
FT protein
Wilcoxon, p = 1.4e−14
Relative band intensity
Vegetative
Differentiated
Status of scion at 63 DAG
E
r = -0.59
p < 0.01
Days to flower bud appearance
Relative band intensity of FT protein
Rootstock
G3−IL1
CH−IL1
G2xCH F1
G2xG3 F1
F
BoFT.C6
Wilcoxon, p = 2.2e−05
Relative expression
TO1000
Kairan
G
RsFT
Kruskal–Wallis, p = 8.3e−06
Relative expression
G2−IL1
G3−IL1
CH−IL1
G2xCH F1
G2xG3 F1
H
r = 0.47
p < 0.01
Relative band intensity of FT protein
RsFT relative expression
Rootstock
G2−IL1
G3−IL1
CH−IL1
G2xCH F1
G2xG3 F1

Fig. 3-2. FT protein accumulation in the grafted cabbage scions. (A) Sampling for the immunoblotting analysis of FT protein. Upper panel; a cabbage scion plant at 35 DAG. White arrowhead indicates sampled young leaf. Middle and lower panel; a close look of the sampled young leaf. White arrowhead in the lower panel indicates the dissected midrib which was used for the protein extraction. (B) Immunoblotting analysis of non-grafted cabbage and grafted cabbage scion. Shoot apex (ap), midrib of young leaf (md), leaf lamina of young leaf (lm) were collected from non-grafted 'Matsunami' cabbage at 52 DAS, and md was collected from 'Matsunami' cabbage grafted onto G2xCH F_1 radish at 35 DAG. 30 μg of total protein extracted from each tissue were used for the analysis. Asterisk indicates non-specific band. (C) Accumulation of FT protein in the grafted cabbage scion at 35 DAG. The band intensity of FT protein was normalized for variations in total protein loading in the corresponding lane, as quantified from Ponceau-S stained membranes, and then normalized to the positive control in the same blot. Results of the statistical test for the difference between the rootstocks are shown in the graph. (D) Difference in the accumulation of FT protein in the scion at 35 DAG by the floral status at 63 DAG. The same values of the levels of accumulated FT protein as shown in (C) are divided according to the floral status of the scion at 63 DAG. Results of the statistical test for the difference between the floral status is shown in the graph. (E) Relationship between the FT protein accumulation in the scion at 35 DAG and the number of days to flower bud appearance. Only data of the plants which developed visible flower bud by 63 DAG are shown. The linear correlation coefficient is shown in the graph. (F) *BoFT.C6* transcription level in the leaf of *B. oleracea* rootstocks at 21 DAS, and (G) *RsFT* transcription level in the leaf of *R. sativus* rootstocks at 14 DAS. *BoActin* and *RsActin* were used as an internal control, respectively. Results of the statistical test for the difference between the rootstocks are shown in the graph. (H) Relationship between the relative expression level of *RsFT* transcript in the leaf of the rootstock at 14 DAS and the FT protein accumulation in the scion at 35 DAG. Only data of *R. sativus* rootstocks are shown. The linear correlation coefficient is shown in the graph.

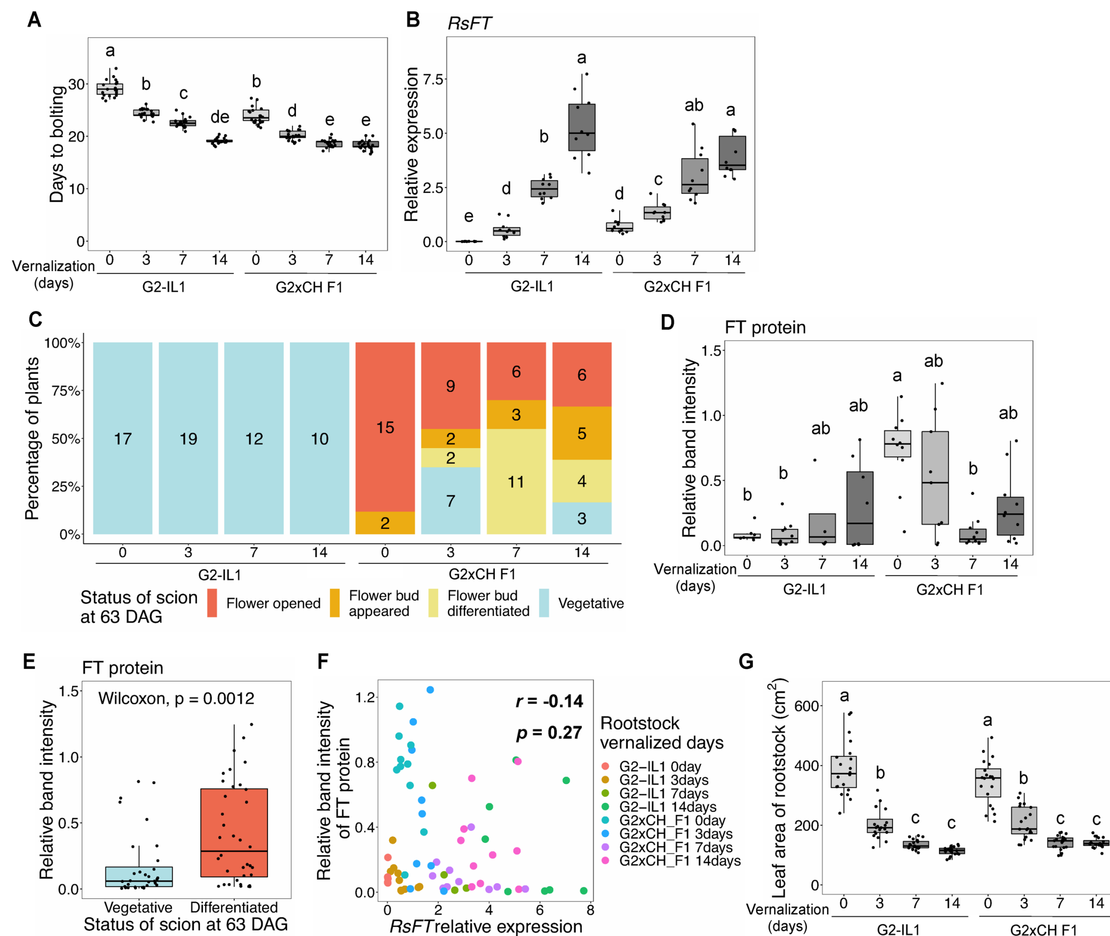


Fig. 3-3. Effect of the vernalization treatment of rootstocks on the floral induction of grafted cabbage scions. Vernalization treatment was performed by incubating the germinated seeds in 2°C under dark condition for days shown in the graph. (A) Days to bolting from sawing of rootstocks. Rootstocks were regarded as bolted when the stem reached to 7 cm in length. (B) *RsFT* transcription level in the leaf of rootstocks at 14 DAS. *RsActin* was used as an internal control. (C) Floral status of the grafted scions at 63 DAG. (D) Accumulation of FT protein in the grafted cabbage scion at 35 DAG. (E) Difference in the accumulation of FT protein in the scion at 35 DAG by the floral status at 63 DAG. Results of the statistical test for the difference between the floral status is shown in the graph. (F) Relationship between the *RsFT* transcription level in the rootstock at 14 DAS and FT protein accumulation level in the grafted scion at 35 DAG. The linear correlation coefficient is shown in the graph. (G) Total area of leaves left on rootstocks at the day of grafting. Different letters indicate statistical difference between the treatments with Tukey-Kramer test ($p < 0.05$, n = 20) in (A) and (G), and with pairwise Wilcoxon tests with the Bonferroni adjustment for multiple comparisons ($p < 0.05$, n = 4-10) in (B) and (D). Data from two repetitions of the experiment are shown together for each accession in (A), (C), and (G), and data from second repetition of the experiment are shown in (B), (D), (E) and (F).

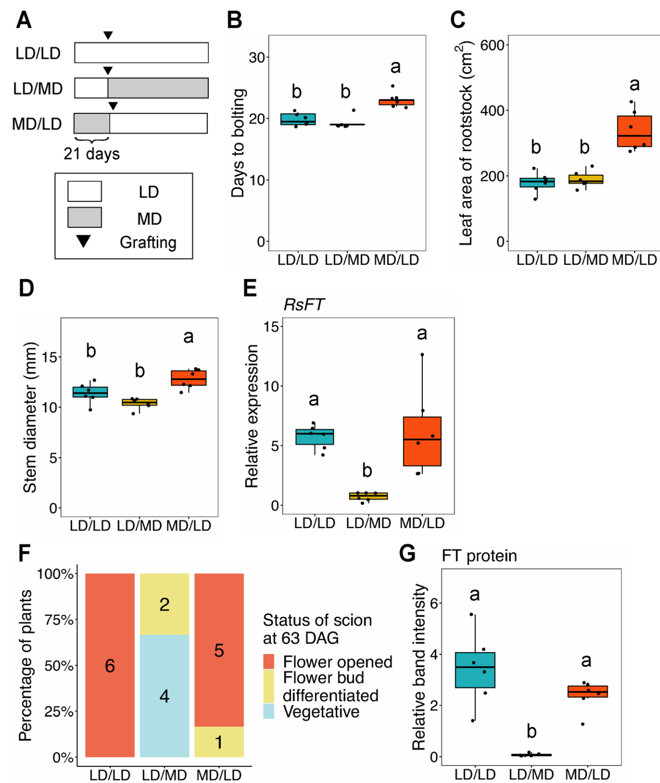


Fig. 3-4. Effect of daylength on the floral induction of cabbage scions grafted onto G2xCH F_1 *R. sativus* rootstocks. Germinated seeds of G2xCH F_1 were vernalized at 2°C for 7 days before sowing. (A) Light condition during the grafting experiment. LD, long day condition (16h/8h, light/dark); MD, middle day condition (12h/12h, light/dark). Grafting was performed ~20 DAS in LD/LD and LD/MD treatments, and ~23 DAS in MD/LD treatment when the stem of rootstocks reached to 7 cm in length. (B) Days to bolting from sawing of rootstocks. (C) Total area of leaves left on rootstocks at the day of grafting. (D) Maximum stem diameter of grafted scion at 35 DAG. (E) *RsFT* transcription level in the leaf of rootstocks at 35 DAG. *RsActin* was used as an internal control. (F) Floral status of the grafted scions at 63 DAG. (G) Accumulation of FT protein in the grafted cabbage scion at 35 DAG. Different letters indicate statistical difference between the light conditions with Tukey-Kramer test ($p < 0.05$, n = 6) in (B), (C) and (D), and with pairwise Wilcoxon tests with the Bonferroni adjustment for multiple comparisons ($p < 0.05$, n = 6) in (E) and (G).

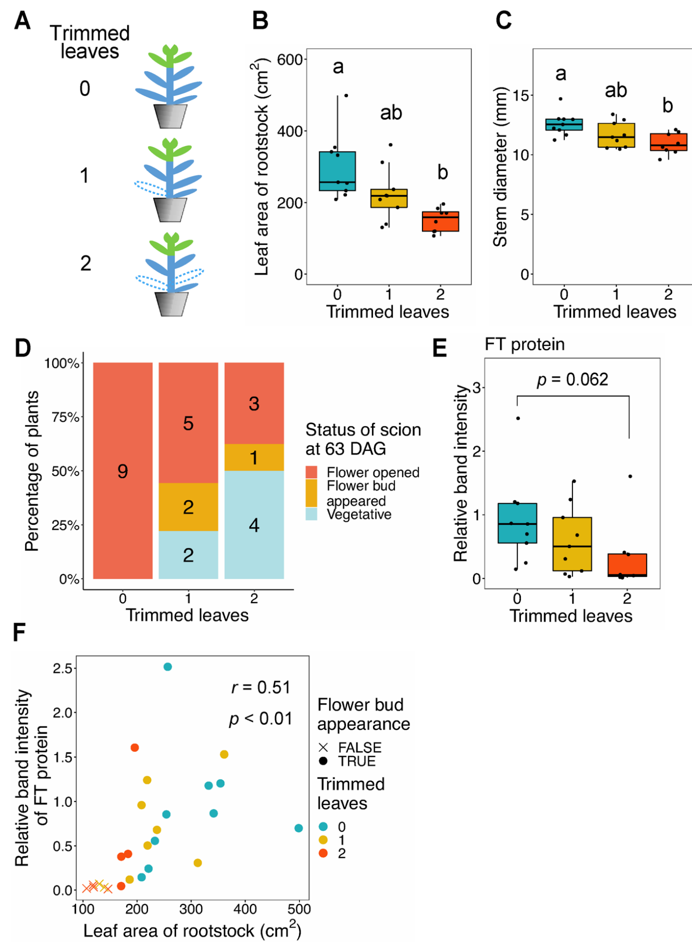


Fig. 3-5. Effect of leaf trimming of the rootstocks on the floral induction of cabbage scions grafted onto G2xCH F_1 *R. sativus* rootstocks. Seeds of G2xCH F_1 were sown without vernalization treatment. (A) Schematic illustration of the leaf trimming of the rootstocks. Dashed lined leaves indicate trimmed leaves. (B) Total area of leaves left on rootstocks at the day of grafting. (C) The maximum stem diameter of the grafted scions at 35 DAG. (D) Floral status of the grafted scions at 63 DAG. (E) Accumulation of FT protein in the grafted cabbage scion at 35 DAG. (F) Relationship between the leaf area of rootstocks at 0 DAG and the FT protein accumulation in the scion at 35 DAG. The linear correlation coefficient is shown in the graph. Different letters indicate statistical difference between the trimming conditions with Tukey-Kramer test ($p < 0.05$, n = 8-9) in (B) and (C), and with pairwise Wilcoxon tests with the Bonferroni adjustment for multiple comparisons ($p < 0.05$, n = 8-9) in (E).

Table 3-S1. *B. oleracea* and *R. sativus* accessions used in Chapter 3.

Species	Name in this study	Origin	Experiment
B. oleracea var. *oleracea*	TO1000	Seeds maintained by self pollination of cultivar ‘TO1000DH3’, Arabidopsis Biological Resource Center, USA, stock number CS29002	1
B. oleracea var. *albograbra*	Kairan	Original seeds of Chinese kale cultivar ‘Kairan’, Tsurushin Seed Ltd., Japan	1
R. sativus var. *caudatus*	G2-IL1	An inbred line of seedpod-harvesting type radish isolated from accession no. 76703, Genebank Project, NARO, Japan	1, 2
R. sativus var. *caudatus*	G3-IL1	An inbred line of seedpod-harvesting type radish isolated from accession no. 76704, Genebank Project, NARO, Japan	1
R. sativus var. *caudatus*	CH-IL1	An inbred line of seedpod-harvesting type radish isolated from cultivar ‘Rat-tailed’, Chiltern Seeds, United Kingdom	1
R. sativus var. *caudatus*	G2xCH F_1	F_1 progeny derived from the cross between G2-IL1 and CH-IL1	1, 2, 3, 4
R. sativus var. *caudatus*	G2xG3 F_1	F_1 progeny derived from the cross between G2-IL1 and G3-IL1	1

Table 3-S2. Summary of grafting experiment conducted in Chapter 3.

Experiment	Rootstock	Seed vernalization treatment (days)	Day length treatment	Number of trimmed leaves of the rootstock	Curing method after grafting	Days to bolting to 7 cm in stem length after sawing^{zyx}	Estimated total area of leaves left on the rootstock plant (cm^2/plant)^{zyx}	Number of successful grafting (plant/plant)	Maximum stem diameter of scion at 35 DAG (mm)^{yxv}	Number of scions with appeared flower bud (plant/plant)	Days to flower bud appearance^{yu}	Total leaf number of the scion to flower bud^{yu}	Number of scions with differentiated flower bud at 63 DAG (plant/plant)	Number of scions with opened flower at 63 DAG (plant/plant)	Total number of opened flower at 63 DAG^{yt}
1	TO1000	0	LD/LD	0	1	-^{w} -	161.9 ± 15.2 c	7 / 9	7.6 ± 0.8 c	0 / 7	-	-	0 / 7	0 / 7	-
	Kairan					-^{w} -	268.8 ± 36.8 bc	10 / 10	11.1 ± 1.3 b	0 / 10	-	-	0 / 10	0 / 10	-
	G2-IL1					25.6 ± 0.5 b	307.6 ± 70.0 ab	9 / 9	10.4 ± 1.5 b	0 / 9	-	-	0 / 9	0 / 9	-
	G3-IL1					28.3 ± 2.4 a	409.6 ± 128.3 a	10 / 10	13.5 ± 0.8 a	7 / 10	54.1 ± 6.8	46.1 ± 5.1	9 / 10	4 / 10	19.2 ± 20.4
	CH-IL1					27.9 ± 2.8 ab	178.5 ± 27.6 bc	7 / 7	10.7 ± 0.7 b	6 / 7	50.7 ± 5.9	35.3 ± 5.5	7 / 7	5 / 7	21.0 ± 23.5
	G2xCH F_1					22.0 ± 0.9 c	268.1 ± 94.9 bc	10 / 10	11.1 ± 1.4 b	8 / 10	34.8 ± 9.4	29.4 ± 4.7	9 / 10	8 / 10	95.4 ± 56.8
	G2xG3 F_1					26.4 ± 1.6 ab	423.5 ± 176.6 a	10 / 10	12.8 ± 0.9 a	5 / 10	60.6 ± 1.9	54.8 ± 4.6	7 / 10	0 / 10	-
2-1 (repetition1)	G2-IL1	0	LD/LD	0	1	27.9 ± 0.7 a	368.2 ± 85.4 a	8 / 10	10.9 ± 1.3 a	0 / 8	-	-	0 / 8	0/ 8	-
		3				24.5 ± 0.8 b	183.4 ± 36.6 b	9 / 10	9.4 ± 0.5 b	0 / 9	-	-	0 / 9	0/ 9	-
		7				23.0 ± 1.1 c	136.2 ± 19.8 bc	7 / 10	8.9 ± 0.8 b	0 / 7	-	-	0 / 7	0/ 7	-
		14				19.4 ± 0.5 d	113.9 ± 15.6 c	2 / 10	8.1 ± 0.8 b	0 / 2	-	-	0 / 2	0/ 2	-
2-1 (repetition2)	G2-IL1	0	LD/LD	0	1	30.4 ± 1.2 a	409.4 ± 98.5 a	9 / 10	10.4 ± 1.6 a	0 / 9	-	-	0 / 9	0/ 9	-
		3				24.3 ± 0.7 b	222.6 ± 53.2 b	10 / 10	10.0 ± 0.9 a	0 / 10	-	-	0 / 10	0/ 10	-
		7				22.3 ± 0.7 c	133.7 ± 12.7 c	5 / 10	9.6 ± 0.4 a	0 / 5	-	-	0 / 5	0/ 5	-
		14				18.9 ± 0.6 d	113.1 ± 13.3 c	8 / 10	8.8 ± 1.1 a	0 / 8	-	-	0 / 8	0/ 8	-
2-2 (repetition1)	G2xCH F_1	0	LD/LD	0	1	25.1 ± 1.3 a	343.3 ± 67.5 a	7 / 10	13.2 ± 1.0 a	7 / 7	36.6 ± 9.6	33.1 ± 5.8	7 / 7	7 / 7	73.7 ± 57.2
		3				20.7 ± 0.7 b	171.9 ± 30.6 b	10 / 10	10.4 ± 0.8 b	4 / 10	56.5 ± 5.2	42.8 ± 5.5	4 / 10	2 / 10	6.0 ± 7.1
		7				19.0 ± 0.7 c	124.1 ± 19.1 c	10 / 10	9.9 ± 0.9 b	7 / 10	51.3 ± 6.6	35.7 ± 4.8	10 / 10	5 / 10	18.0 ± 16.6
		14				18.7 ± 0.9 c	147.9 ± 15.4 bc	8 / 10	10.4 ± 0.8 b	7 / 8	47.1 ± 11.3	34.0 ± 7.8	7 / 8	5 / 8	30.2 ± 22.6
2-2 (repetition2)	G2xCH F_1	0	LD/LD	0	1	22.8 ± 0.6 a	346.5 ± 87.5 a	10 / 10	12.7 ± 1.0 a	10 / 10	45.1 ± 9.2	38.1 ± 6.5	10 / 10	8 / 10	55.4 ± 26.5
		3				19.5 ± 0.5 b	243.5 ± 49.6 b	10 / 10	10.8 ± 1.2 b	7 / 10	45.9 ± 7.5	37.1 ± 6.1	9 / 10	7 / 10	38.6 ± 37.1
		7				18.2 ± 0.6 c	158.2 ± 15.0 c	10 / 10	9.9 ± 0.8 bc	2 / 10	57.5 ± 4.9	38.0 ± 1.4	10 / 10	1 / 10	4
		14				17.9 ± 0.7 c	133.0 ± 14.4 c	10 / 10	9.3 ± 0.8 c	4 / 10	56.5 ± 4.7	38.5 ± 1.9	8 / 10	1 / 10	11
3	G2xCH F_1	7	LD/LD	0	2	19.8 ± 1.0 b	178.7 ± 31.6 b	6 / 6	11.4 ± 1.0 b	6 / 6	36.2 ± 10.7	29.2 ± 7.6	6 / 6	6 / 6	72.0 ± 58.3
			LD/MD			19.3 ± 0.8 b	189.1 ± 25.6 b	6 / 6	10.4 ± 0.6 b	0 / 6	-	-	2 / 6	0 / 6	-
			MD/LD			23.0 ± 1.1 a	337.7 ± 62.4 a	6 / 6	12.8 ± 1.0 a	5 / 6	33.0 ± 9.6	26.6 ± 6.6	6 / 6	5 / 6	103.4 ± 79.0
4	G2xCH F_1	0	LD/LD	0	2	24.7 ± 1.8 a	300.0 ± 92.4 a	9 / 9	12.6 ± 1.0 a	9 / 9	45.1 ± 4.2	34.7 ± 3.2	9 / 9	9 / 9	32.3 ± 15.9
				1		23.3 ± 1.0 a	223.6 ± 74.4 ab	9 / 9	11.7 ± 1.1 ab	7 / 9	49.6 ± 8.2	37.1 ± 4.3	7 / 9	5 / 9	34.0 ± 14.4
				2		24.4 ± 1.6 a	151.6 ± 33.4 b	8 / 8	10.9 ± 0.9 b	4 / 8	47.2 ± 12.4	35.2 ± 6.2	4 / 8	3 / 8	41.7 ± 38.0

[z] Including all the data of plants used for grafting.
[y] Mean ± SD.
[x] Different letters indicate statistical difference between each accession/treatment within the same experiment (p < 0.05, Tukey-Kramer test).
[w] Not investigated. All of the TO1000 and Kairan rootstocks were used for grafting at 30 DAS.
[v] Only the data of scions with successful grafting were used for the caluculation.
[u] Only the data of scions with appeared flower bud were used for the caluculation.
[t] Only the data of scions with opened flower were used for the caluculation.

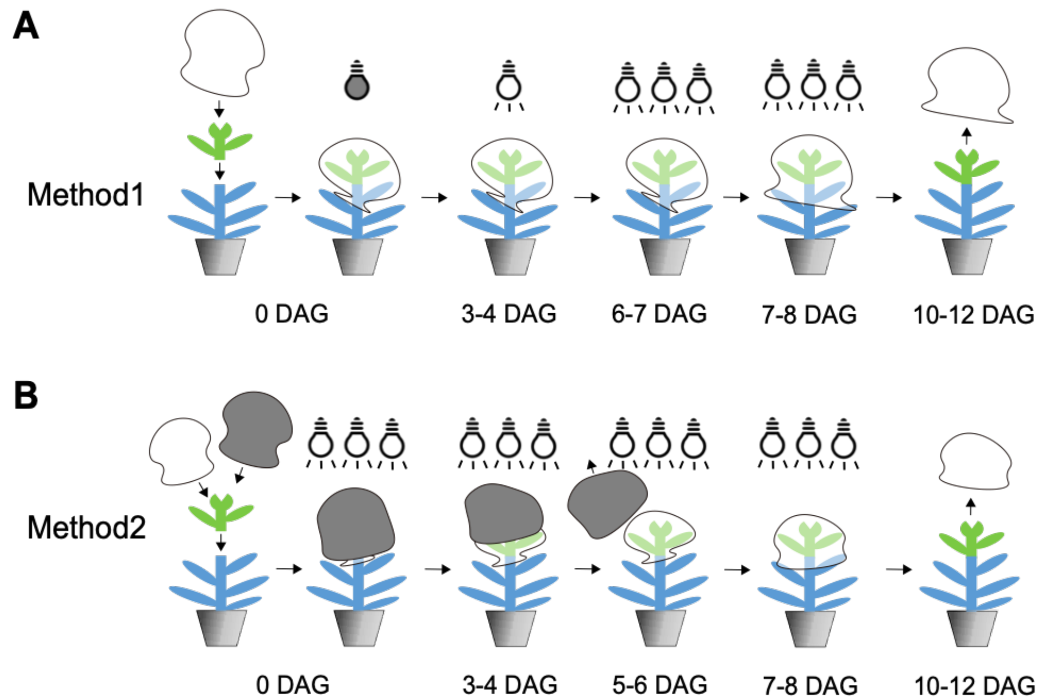


Fig. 3-S1. Two curing methods after grafting used in this study. (A) Method 1; after grafting, the scion and one leaf of the rootstock plant were covered with a clear polyethylene bag to maintain high humidity, and the plants were kept without lightning in the growth room for 3–4 days. Then the plants were grown under dim light condition (~ PPFD 40 μmol m^{-2} s^{-1}) for 3 days and moved to full light condition (PPFD 120 μmol m^{-2} s^{-1}) after that. From 7–8 DAG, the opening of the polyethylene bag was loosened and gradually widened. Once the scion and rootstock were fully connected, the polyethylene bag was removed. (B) Method 2; after grafting, only the scion was covered with a clear polyethylene bag whose inside was sprayed with water to maintain high humidity, and with a blackout bag from outside. The plants were grown under full light condition (PPFD 120 μmol m^{-2} s^{-1}) from 0 DAG. From 3–4 DAG, the blackout bag was gradually pulled up and removed on 5–6 DAG. From 7–8 DAG, the opening of the polyethylene bag was loosened and gradually widened. Once the scion and rootstock were fully connected, the polyethylene bag was removed.

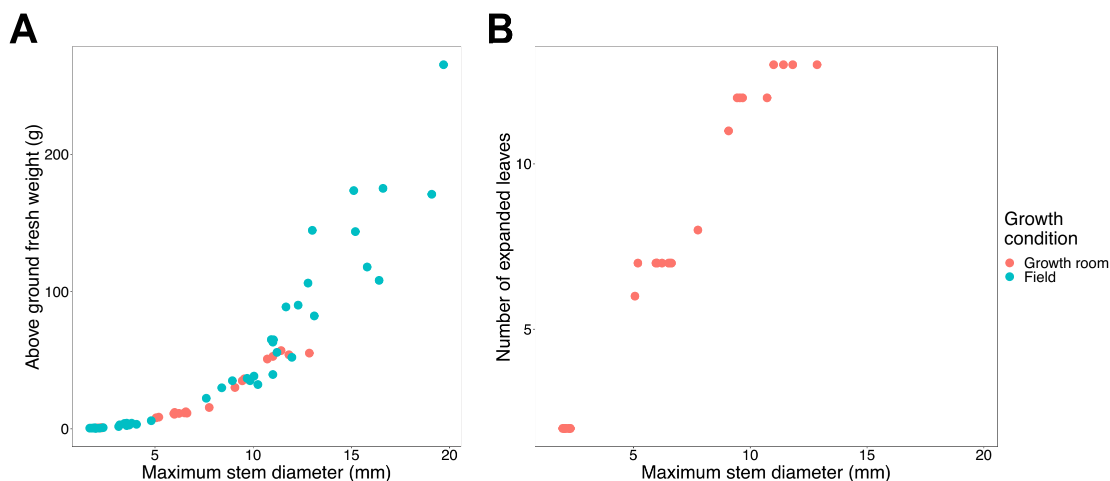


Fig. 3-S2. Relationship between maximum stem diameter and parameters of vegetative growth and age in cabbage seedlings. 'Matsunami' cabbage seedlings grown in a growth room (n = 30, 14–42 DAS) or field (n = 43, 58–156 DAS) were collected at several timepoint, and their maximum stem diameter, number of expanded leaves, and above ground fresh weight were measured. Number of expanded leaves were measured only for seedlings grown in a growth room. (A) Relationship between maximum stem diameter and above ground fresh weight. (B) Relationship between maximum stem diameter and number of expanded leaves.

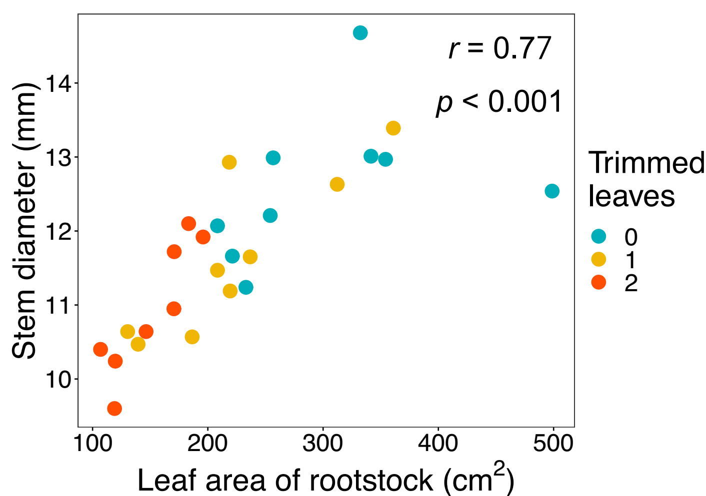


Fig. 3-S3. Relationship between the leaf area of rootstocks at 0 DAG and the maximum stem diameter of the grafted scions at 35 DAG in Experiment4. The linear correlation coefficient is shown in the graph.

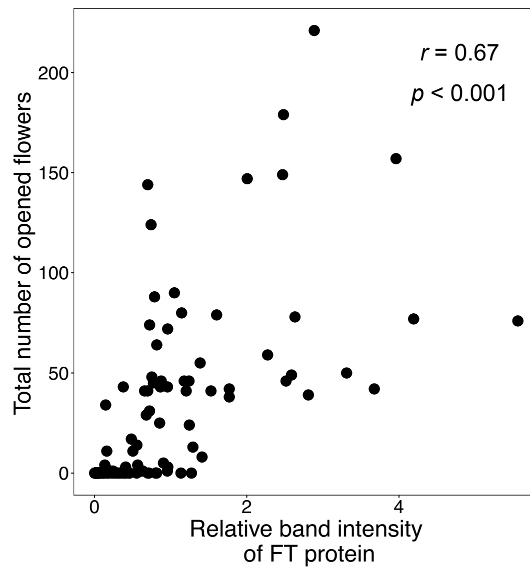


Fig. 3-S4. Relationship between the levels of accumulated FT protein in the scion at 35 DAG and the total number of opened flowers at 63 DAG in Experiment1-4. The linear correlation coefficient is shown in the graph.

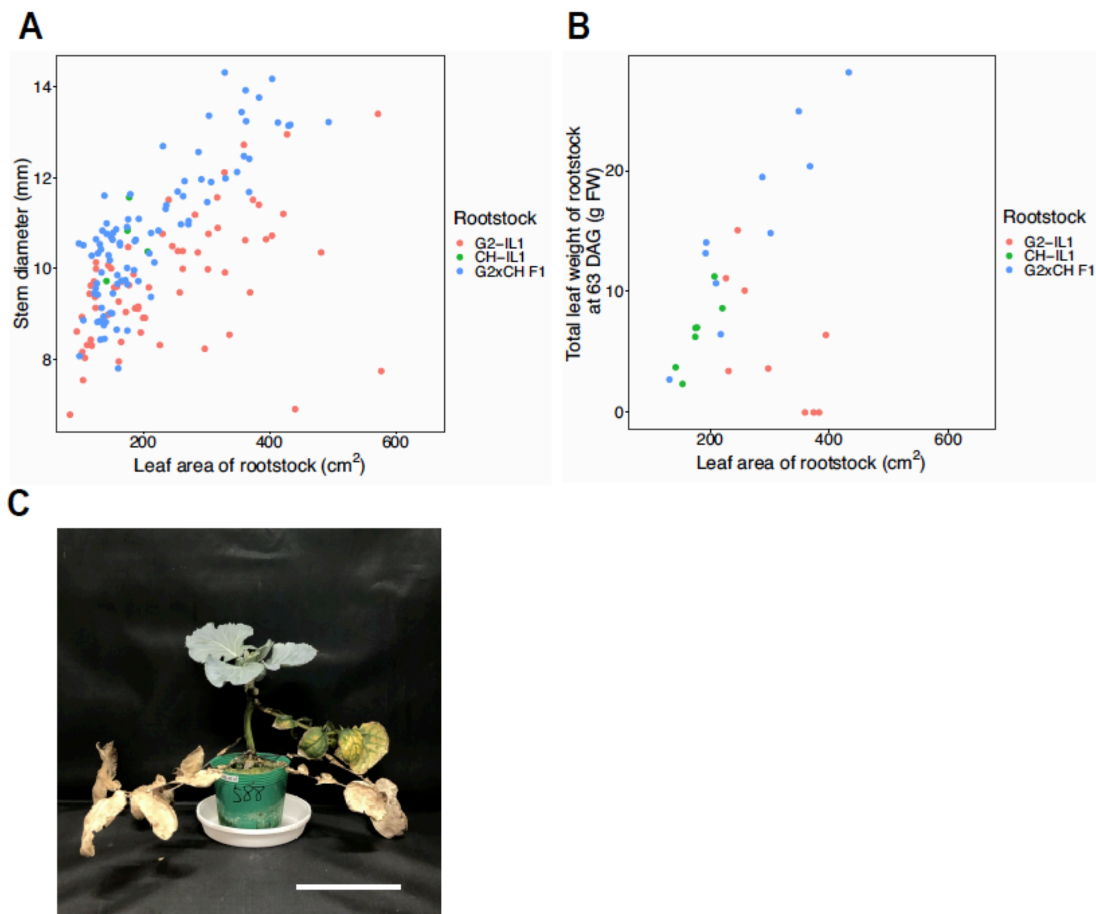


Fig. 3-S5. Difference in the vegetative growth of cabbage scion grafted onto G2-IL1, CH-IL1 and G2xCH F_1 rootstock. (A) Relationship between the total leaf area of rootstock at 0 DAG and maximum stem diameter of the scion at 35 DAG. When compared at the same leaf area, the vegetative growth of the grafted scion was less in G2-IL1 rootstock. (B) Relationship between the total leaf area of rootstock at 0 DAG and the total leaf amount of rootstock in fresh weight at 63 DAG. Linear relationship was observed in CH-IL1 and G2xCH F_1, while some individual deviating from the linear relationship were observed in G2-IL1. This was probably due to the early leaf senescence in G2-IL1. (C) Typical picture of G2-IL1 graft whose leaves of rootstock are senesced. Picture was taken at 63 DAG. Bar indicates 10 cm. Data from Experiment1 and 2 are shown in (A), and the data for Experiment1 are shown in (B).

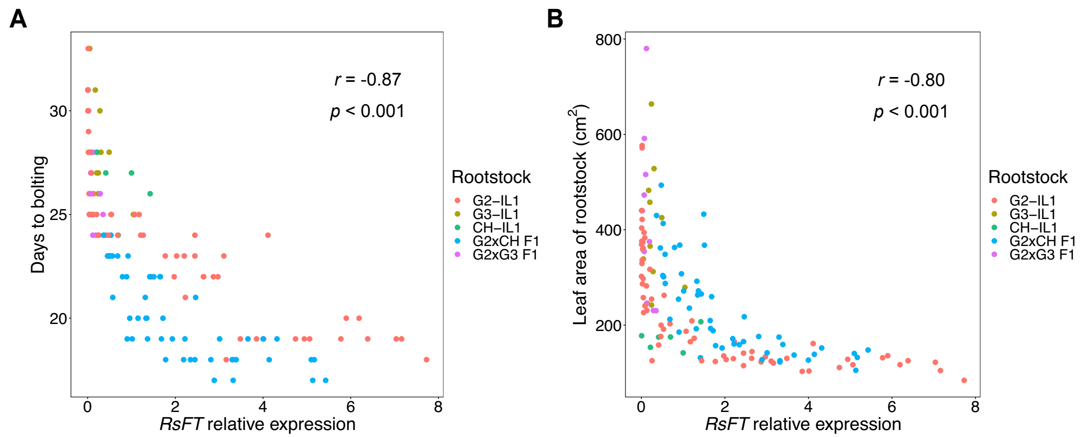


Fig. 3-S6. Relationship between *FT* transcription level at 14 DAS and vegetative and reproductive growth parameters of the rootstocks in Experiment1 and 2. (A) Relationship between *FT* transcript expression and the number of days to bolting. (B) Relationship between *FT* transcript expression and the total leaf area of the rootstock at 0 DAG. Spearman rank correlation coefficient is shown in the graphs.

3.4. Discussion

Variation in the flowering response of the grafted cabbage is quantitatively correlated with the level of accumulation of FT protein.

In this chapter, I observed that the flowering response of the grafted cabbage varied greatly depending on the genotype of the rootstock; this finding agreed with the observation in previous study (Kagawa, 1957) and Chapter 1. Moreover, even when grafted onto an early-flowering *R. sativus* accessions, whose difference in the average time to bolting was only ~6 days, the flowering response of the cabbage scions varied considerably, ranging from complete vegetative growth to complete flower bud differentiation (Fig. 3-1A, B). This finding confirmed that the donor plant itself being induced to flower is not a sufficient condition for floral induction of the grafted cabbage. To clarify the cause of this variability in flowering response, I examined the relationship between the flowering response of the scion and its level of accumulated FT protein. The results showed that the variable flowering response of the scion was consistent with the level of accumulated FT protein (Fig. 3-2D). I also confirmed this tendency in other grafting conditions where vernalization, daylength, and leaf trimming treatments were applied to the rootstock (Fig. 3-3E; Fig. 3-4G; Fig. 3-5F). This indicated that the transmission of FT protein from the donor rootstock causes floral induction in the recipient cabbage, which is in agreement with the widely accepted mechanism of grafting-induced flowering (Corbesier et al., 2007; Lin et al., 2007; Notaguchi et al., 2008).

Furthermore, I observed that the level of FT protein accumulation in the scion was correlated not only with the presence or absence of floral bud differentiation, but also with quantitative indices of floral induction, such as days to flower bud appearance and the number of opened flowers (Fig. 3-2E; Fig. 3-S4). In previous studies investigating FT transmission, the accumulation of FT protein at the shoot apex was found to be quantitatively related to the flowering response (Endo et al., 2018; Liu et al., 2012; Susila et al., 2021; Yoo et al., 2013a). Therefore, it may be that variation in the grafting-induced flowering of cabbage is caused by quantitative differences in the amount of FT protein translocated to the scion. Although the anti-FT antibody used in this study can also detect the FT protein of *B. oleracea* (Chapter 2), FT protein was not detected from cabbage scions grafted onto *B. oleracea* rootstocks (Fig. 3-2C). Since the *FT* homolog of *B. oleracea* has been shown to promote flowering in *A. thaliana* transformants (Itabashi et al., 2019), the failure of *B. oleracea* rootstocks to induce flowering in grafted cabbage in this study was considered to be due to an insufficient supply of FT protein in the scion. To clarify whether this insufficient supply of FT protein from *B. oleracea* rootstocks is caused by lower levels of gene expression or lower transmission efficiency, it is necessary to find *B. oleracea* accessions that express different levels of *FT* transcripts (or develop transformants that do) and to perform grafting experiment on them.

In contrast, in this study the threshold level of FT protein accumulation required for floral induction

of cabbage was unclear in some cases. For example, during the seed vernalization experiment, when vernalized G2-IL1 *R. sativus* plants were used as rootstocks, some individuals were not induced to flower even though FT was clearly detected in the scion (Fig. 3-3D). Functional mutations in FT protein are probably not the cause of this phenomenon since I found no non-synonymous mutations in the coding region of *FT* in G2-IL1 or any other *R. sativus* accessions (data not shown). One possible reason for differences in floral induction may be that the threshold level of FT protein required for successful induction may vary depending on the state of the scion (e.g., its physiological age). Cabbage is a plant-vernalization-type plant, which becomes sensitive to low temperatures only after developing to a size threshold characterized by the expansion of many true leaves (Miller, 1929); this property may also affect the grafting-induced flowering. In addition, I observed less vegetative growth in scions grafted onto G2-IL1 plants than in those grafted onto G2xCH F_1 plants (Fig. 3-S5A). This difference seems to be related to the fact that G2-IL1 plants show earlier leaf senescence (Fig. 3-S5B, C). Thus, it is possible that the physiological age of G2-IL1 scions did not advance sufficiently, thereby inhibiting floral induction. Since the molecular mechanisms involved in the plant-vernalization-type response of cabbage plants has not yet been elucidated, it cannot be discussed further in this study. However, I consider it to be of interest for future studies.

High expression of FT transcripts in the rootstock is necessary but not sufficient to cause the accumulation of FT protein and induce flowering in grafted cabbage

Previous studies have reported that the flowering responses of recipient plants were associated with *FT* transcription levels in donor plants (Notaguchi et al., 2008; Tang et al., 2022; Yoo et al., 2013b). However, in the current study, I found that the *FT* transcription levels of the donor rootstock alone could not explain the flowering response of the receptor scion. This was especially true of the vernalization-treatment experiment (Fig. 3-3). Here, the *FT* transcription level of the rootstock did not coincide with the level of FT protein accumulation in the scion (Fig. 3-3F), suggesting that the level of FT protein accumulation and floral induction in the scion may be affected by other factors. In consideration of the observed differences in both *FT* transcription levels and leaf areas of *R. sativus* genotypes (Fig. 3-1C; Fig. 3-2G), I separately investigated the effects of these two factors on FT protein accumulation in grafted scions. First, I confirmed by a daylength-manipulation experiment that the *FT* transcription level of the rootstock had a clear effect on FT protein accumulation in the grafted scion if the leaf area was the same among rootstocks (Fig. 3-4). Next, I confirmed by a leaf trimming experiment that the leaf area determined the level of FT protein accumulation in the scion if the transcription level of *FT* was comparable in all rootstocks (Fig. 3-5). This was consistent with the observations of previous grafting studies, which showed that reducing the leaf area of donor plants used for grafting suppressed the floral induction of the receptor plant (Suge, 1986; Zeevaart, 1958). Therefore, I suggest that both the level of *FT* transcription and the leaf area of the rootstock both

contribute to the level of FT protein accumulation in the grafted scion, and both *FT* transcription and leaf area need to be high to cause floral induction in grafted cabbage.

Based on these results, the discrepancy observed in the vernalization-treatment experiment between the *FT* transcription level of the rootstock and the level of FT protein accumulation in the scion seem to be related to reduced leaf area in rootstocks caused by floral acceleration (Fig. 3-3A, G). It is possible that a smaller leaf area may have counteracted the effect of the increased transcription level of *FT* brought about by vernalization treatment, and thereby reduced the total amount of FT protein produced in the whole rootstock plant. This result indicated that the leaf area of the rootstock, rather than the *FT* transcription level, may be a restricting factor for grafting-induced flowering in some cases. I suppose that previous studies where failures in grafting-induced flowering were observed despite the use of *FT*-overexpressing transformants (Bull et al., 2017; Odipio et al., 2020; Tränkner et al., 2010; Wenzel et al., 2013; Wu et al., 2022; Zhang et al., 2010) may be related to the deficiency in the amount of leaves producing FT.

Factors associated with the FT transcription level of the R. sativus rootstocks

I found large differences in *FT* transcription levels of the *R. sativus* accessions grown under the same conditions (Fig. 3-2G). This indicated that genetic factors determine the transcription level of *FT* in *R. sativus*, and it is possible that genetic selection may be performed for this trait. Previous studies observed that *FT* transcription levels tended to be higher in early-flowering *R. sativus* accessions than in *R. sativus* accessions that require vernalization to flower (Han et al., 2021; Jung et al., 2020). In this chapter, I found a correlation between the transcription level of *FT* and the days to bolting among *R. sativus* accessions (Fig. 3-S6A). Therefore, early-flowering *R. sativus* accessions seem to be suitable for preparing the rootstock to ensure high levels of *FT* transcription. In addition, I also found that vernalization treatments significantly promoted both floral induction and the transcription level of *FT* even in early-flowering *R. sativus* accessions that do not require vernalization for floral induction (Fig. 3-3A, B). Although the difference in the days to bolting was about one week between non-vernalized and 7 days-vernalized G2-IL1 plants, the difference in *FT* transcription levels between these conditions was approximately 200-fold (Fig. 3-3A, B). This shows that vernalization treatments exert significant effects on the transcriptional activation of *FT* even in early-flowering *R. sativus* accessions. I also confirmed that LD conditions were necessary to induce high *FT* expression in *R. sativus* (Fig. 3-4), which is in agreement with the fact that *R. sativus* is a LD plant (Suge and Rappaport, 1968).

Factors associated with the leaf area of the R. sativus rootstocks

When considering rootstock size, plants with larger leaf areas can induce flowering to a greater degree in grafted cabbage if rootstocks with comparable *FT* transcription levels are used (Fig. 3-5). However, because floral acceleration reduces leaf area by shortening the vegetative growth period (Fig.

3-3A, G), there is a trade-off between leaf area and *FT* expression (Fig. 3-S6B). One possible way to avoid this trade-off would be to delay the floral induction of rootstocks transiently. In the daylength-manipulation experiment, I observed that in the MD/LD treatment, the floral induction of the rootstock was delayed, the leaf area of the rootstock had increased, and the growth of the grafted scion was also greater than in the LD/LD treatment (Fig. 3-4 B, C, D). On the other hand, *FT* expression in rootstocks after grafting was similar in both the MD/LD and LD/LD treatments (Fig. 3-4E), suggesting that reducing daylength before grafting can increase the leaf area of the rootstock without hampering *FT* expression after grafting. Another approach would be to select rootstocks with genetic potential for larger leaf area and less leaf senescence. This may be possible given the observed differences in leaf area and leaf longevity among *R. sativus* accessions (Fig. 3-1C; Fig. 3-S5), as well as the morphological diversity in shape and size in both roots and leaves within extant *R. sativus* genetic resources (Yamagishi, 2017).

3.5. Summary

In this chapter, I revealed that the difference in the flowering response of the cabbage plants grafted onto different type of rootstocks can be explained by the quantitative difference in the level of FT protein accumulation in the grafted cabbage. High accumulation of FT protein in the grafted cabbage was necessary for the floral induction of the cabbage, and to achieve this, both increased level of *FT* transcription and enough leaf area was necessary for the rootstock. These two factors of the rootstocks were different among the florally induced plants of *R. sativus* accessions. This suggested the possibility of the genetic selection to develop rootstocks with higher ability to induce flowering of the grafted plants.

Chapter 4.

Characteristics of cabbage seeds formed without vernalization treatment by grafting onto *R. sativus* rootstocks

4.1. Introduction

From Chapter 1 to Chapter 3, I established the floral induction method of cabbage by grafting (Non-Vernalization grafting method; NV grafting method). I also revealed the necessary condition of the rootstocks for the floral induction of cabbage through the quantitative analysis of FT protein. In Chapter 1, I could obtain seeds of from several cabbage cultivars by NV grafting method. Additionally, I confirmed that the seeds geminated and grew into seedlings normally. However, it has not been investigated in detail on the field performance of the progenies obtained by this method. It has been reported in several crops that conditions during seed production affect the seed traits and the growth characteristics of the progenies (Hagiya, 1949, 1950; Shinohara, 1959). Seed productivity is also an important factor in the breeding and seed production. For the application of NV grafting method to the breeding and seed production of cabbage, it is important to examine if this technique has difference in the seed productivity, or the traits of the obtained progenies compared to the conventional floral induction method by vernalization treatment (Vernalization method).

In this chapter, I examined the seed productivity of the NV-grafting method and the field performance of the cabbage progenies obtained by this method.

4.2. Materials and Methods

Plant materials

Two clonal lines isolated from 'Watanabe-seiko No.1' cabbage (Genebank project, NARO, Japan, accession No. 25974) were used for the seed production experiment (line #1 and #2). Clonal lines were used to avoid the effect of the genetic heterogeneity within this cultivar. These lines were isolated from a single seed and clonally propagated *in vitro* by cuttings. Open-pollinated 'Watanabe-seiko No.1' seeds were used as a control for the field experiments. 'Rat's tail-CH' radish (originally bought from Chiltern Seeds, United Kingdom) was used as a rootstock for the grafting.

Seed production of cabbage by vernalization induced flowering

Two clonally propagated cabbage lines were transplanted into 9 cm diameter plastic pots filled with Nippi gardening soil No.1 (Nihon Hiryo Co., Ltd., Gunma, Japan) and acclimated in the plant growth room at 22 ± 2°C, 16 h daylength with a light intensity of 80–120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PPFD provided by fluorescent lamps (NEC Lighting). Seedlings with their stem diameter exceeding 1 cm were vernalized for 12–13 weeks in the growth cabinet at 6 ± 1°C, 16 h daylength with a light intensity of 90 µmol m-

^{2} s^{-1} PPFD provided by LED light (660 nm:450 nm = 8:2). After vernalization treatment, the seedlings were returned to the growth room. Self-pollination by CO_2 treatment (Nakanishi and Hinata, 1975) was performed as follows: opened flowers were pollinated with self-pollen, and the inflorescence was immediately covered by one-layered clear polyethylene bags. Then, CO_2 gas of almost 20% of the volume of the polyethylene bag was injected into the bag, with the opening tied. The polyethylene bags were removed 12–24 h after pollination. Pollination was continued until the plants ceased flowering. Mature pods were harvested after drying and before splitted. All plants were irrigated and fertilized from below using a half-strength nutrient solution (Enshi-shoho).

Seed production of cabbage by non-vernalization grafting-induced flowering

Seeds of 'Rat's tail-CH' radish were sown on wet filter paper and germinated in the dark at 22°C for a day. Germinated seeds were then vernalized at 2°C in the dark for 14 days, and then sown to 7.5 cm diameter plastic pots filled with granular rockwool (Nippon Rockwool Corp.) in the growth room described above. Grafting was conducted according to the protocol described in Chapter 1, except for the point that the lateral shoots of the rootstocks were partially left with their flower buds being removed. Two clonally propagated cabbage lines acclimated in the same growth room were used as a scion for grafting. After the first flower opened, all leaves of the cabbage scion longer than 2 cm in length were continuously removed. Self-pollination was performed in the same way of the vernalization method as described above until the plants ceased flowering. After the plants ceased flowering, removing of the leaves of scion was stopped. Mature pods were harvested after drying and before splitted. All plants were irrigated and fertilized from below using a half-strength nutrient solution (Enshi-shoho).

Growth conditions and the investigation of vegetative and reproductive growth of cabbage in the field experiments

Self-pollinated seeds of 2 clonal lines of 'Watanabe-seiko No.1' produced by the two flowering induction methods and open-pollinated seeds of 'Watanabe-seiko No.1' as control were used for the field experiments. In 2019, the sterilized seeds were directly sown in a 105-hole cell tray filled with a soil mixture composed of Nippi gardening soil No. 1 and peat moss-based growing media PRO-MIX PGX (Premier Tech Ltd., QC, Canada) at a ratio of 2:1 (v/v) on August 26th. After grown in a growth chamber (20°C, all day light condition by fluorescent lamps) for 5 days, seedlings were grown in a plastic greenhouse until transplanting. They were transplanted in the open field of the Kizu Farm, Kyoto University on September 24th. In 2021, the sterilized seeds were firstly sown on wet filter paper on September 19th and incubated in the dark at 22°C for a day. Due to the low germination rate in line #1, seed coats were removed by tweezers in all lines and then sown in a 128-hole cell tray filled with the same medium used in 2019. Seedlings were grown in a plastic greenhouse until they were

transplanted in the open field of the Kizu Farm on October 13th. In 2019, 40 seedlings were transplanted for each treatment, except for the seeds of line #1 produced by vernalization method of which I could not obtain enough established seedlings. In 2021, 24 seedlings were transplanted for each treatment. All cultivation, fertilization, and pest management were conducted according to commercial practice. After March of the following year, after transplanting, cabbage heads were divided vertically with a knife so that the main stem could bolt.

Emergence rate of the seedlings was measured 5 DAS in 2019, and 10 DAS in 2021. Length and width of the largest leaf and maximum plant width were measured on December 17th in 2019, and on December 18th in 2021. Half of the transplanted plants were harvested, and their head traits (diameter, height and weight) were investigated on January 7th in 2020, and on February 11th in 2022. Leaves covering over the half of the inside sphere were included as a head. Remained half plants were used for the investigation of the flowering time. The flowering date was defined as the day when the petals of the first flower expanded. Plants which were heavily infected by disease were removed and excluded from the flowering time measurement.

4.3. Results

Comparison of the amount and the quality of cabbage seeds obtained by vernalization method and NV grafting method

Seed production of two clonal lines of 'Watanabe-seiko No.1' was conducted for several plants in the growth room by using vernalization method and NV grafting method. These clonal lines were used to avoid the effect of genetic heterogeneity on the trait of progenies because I observed morphological variability among the individuals of 'Watanabe-seiko No.1' cabbage. In the vernalization method, cabbage plants started to open flower from ~3 weeks after the end of the vernalization treatment. In NV grafting methods, cabbage plants started to open flower from 6–8 weeks after grafting. Self-pollination of these plants by CO_2 treatment produced matured seeds in all lines and methods. The results of the seed production are summarized in Table 4-1. Flowering induction method had significant effect on the seed yield, although there was large variability depending on plants in both vernalization and NV grafting method. Amount of seeds produced by NV grafting method was comparable in line #1 and less in line #2 than vernalization method. 0.03–6.46 g of seeds was obtained per plant in vernalization method and 0.10–1.08 g of seeds was obtained per plant in NV grafting method. Flowering induction method had significant effect on the seed size, and vernalization method produced larger seeds than NV grafting method. The effect of lines was not observed for these seed traits.

Vegetative growth of the progenies

Field experiments of the progenies produced by the two flowering induction methods were conducted from summer to next spring in 2019 and 2021. The size of the seeds used for the field experiment was smaller in seeds produced by NV grafting method (Table 4-2). I observed lower emergence rate in line #1 in both 2019 and 2021, regardless of the flowering induction method (Table 4-2). The vegetative growth of each line measured in December are shown in Table 4-3. Significant effect of flowering induction method was only observed for the plant width in 2019. For the length of the largest leaf, significant effect of lines was observed in both 2019 and 2021.

The result of the measurement of the head traits are shown in Table 4-4. 'Watanabe-seiko No. 1' cabbage used in this study had tendency to produce smaller heads (less than 1 kg in average for each line) including the control line. Especially in 2021, heads were small in all lines probably due to the late transplanting. I observed no significant effect of the flowering induction methods on all of the head traits investigated in both 2019 and 2021. On the other hand, the effect of lines on the head height was significant in both 2019 and 2021, which was likely caused by the difference in the leaf length between the two lines (Table 4-3). In summary, I observed consistent effect on the vegetative growth of 'Watanabe-seiko No. 1' cabbage by the clonal lines, but not by the flowering induction methods.

Reproductive growth of progenies

After overwintering, in the field experiment, bolting and flower opening of all lines started in April in both 2019 and 2021. The results of the flowering time measurement are shown in Table 4-5. All plants flowered by the end of April, except for one individual of line #1 produced by vernalization method. Control line flowered about 10 days earlier than the other two lines in 2019, but almost same to other two lines in 2021. I did not observe significant effect of neither clonal lines nor flowering induction methods for flowering time in 2019. Significant effect of clonal lines was observed in 2021, but the difference in average days to flowering was only one day. All the flowered plant produced pollen and formed seeds when they were open pollinated in the experiment of 2021, regardless of the flowering induction methods. In conclusion, I did not observed difference in the reproductive growth of the cabbage progenies produced by the two flowering induction methods.

Table 4-1. Summary of the seed production of 'Watanabe-seiko No.1' cabbage by two different flowering induction methods.[z]

Line	Flowering induction method	n	Total seed weight per plant (g)[y]	Hundred seed weight (g)[y]
#1	Vernailzation	5	0.41 ± 0.33 (0.03 – 0.84)	0.44 ± 0.04 (0.37 – 0.48)
	NV grafting	5	0.52 ± 0.39 (0.10 – 1.08)	0.37 ± 0.10 (0.23 – 0.50)
#2	Vernailzation	5	2.62 ± 2.58 (0.31 – 6.46)	0.40 ± 0.06 (0.33 – 0.48)
	NV grafting	7	0.33 ± 0.19 (0.13 – 0.70)	0.32 ± 0.07 (0.20 – 0.42)
ANOVA	Line		n.s	n.s
	Method		*	*
	Interaction		*	n.s

[z] Seeds were obtained by self-pollination by CO_2 treatment.

[y] Mean ± SD. Figures in parentheses indicate the range of data.

Table 4-2. Traits of 'Watanabe-seiko No.1' cabbage seeds used for the experiments.[z]

Year	Line	Flowering induction method	Pollination	Hundred seed weight (g)	Emergence rate (%)[y]
2019	Control	Vernalization (Field)	Cross-pollination	0.52[x]	60.5
	#1	Vernailzation	Self-pollination	0.43	35.1
		NV grafting		0.32	35.8
	#2	Vernailzation		0.44	65.1
		NV grafting		0.36	54.9
2021	Control	Vernalization (Field)	Cross-pollination	-[x]	91.4
	#1	Vernailzation	Self-pollination	0.48	50.0
		NV grafting		0.31	48.6
	#2	Vernailzation		0.43	98.6
		NV grafting		0.41	80.0

[z] Seeds harvested from 1–2 plants in each line/methods were used for these experiments.

[y] Emergence rate was measured 5 days after sowing in 2019, and 10 days after sowing in 2021.

[x] Same seeds were used as control line in both 2019 and 2021.

Table 4-3. Vegetative growth of 'Watanabe-seiko No.1' cabbage progenies obtained by two different flowering induction methods.

Year	Line	Flowering induction method	n	Plant width (cm)[z]	Largest leaf	
					Length (cm)[z]	Width (cm)[z]
2019	Control	-	40	75.3 ± 11.9	43.5 ± 6.0	34.3 ± 5.6
	#1	Vernailzation	25	64.3 ± 8.1	45.3 ± 5.6	34.4 ± 3.9
		NV grafting	40	73.0 ± 7.9	46.2 ± 4.8	36.1 ± 4.1
	#2	Vernailzation	40	70.9 ± 10.9	43.1 ± 4.8	34.7 ± 3.9
		NV grafting	40	71.4 ± 8.4	44.5 ± 2.8	35.6 ± 3.9
	ANOVA[y]	Line		n.s	**	n.s
		Method		**	n.s	n.s
		Interaction		**	n.s	n.s
2021	Control	-	24	75.2 ± 12.4	42.8 ± 6.9	30.7 ± 5.1
	#1	Vernailzation	24	67.4 ± 10.6	41.9 ± 6.8	29.0 ± 4.9
		NV grafting	24	65.2 ± 9.3	39.7 ± 4.5	27.1 ± 3.5
	#2	Vernailzation	24	63.8 ± 6.7	38.3 ± 3.7	29.6 ± 3.0
		NV grafting	24	61.9 ± 8.5	37.0 ± 3.9	29.3 ± 3.4
	ANOVA[y]	Line		n.s	**	n.s
		Method		n.s	n.s	n.s
		Interaction		n.s	n.s	n.s

[z] Mean ± SD.

[y] Data for control line were excluded from the analysis.

Table 4-4. Head traits of 'Watanabe-seiko No.1' cabbage progenies obtained by two different flowering induction methods.

Year	Line	Flowering induction method	n	Head traits		
				Diameter (cm)[z]	Height (cm)[z]	Weight (g)[z]
2019	Control	-	18	16.4 ± 3.4	10.2 ± 1.9	448.2 ± 267.2
	#1	Vernailzation	11	15.7 ± 2.9	12.4 ± 2.3	517.7 ± 233.7
		NV grafting	20	16.7 ± 3.0	14.5 ± 2.9	673.1 ± 342.7
	#2	Vernailzation	20	15.9 ± 2.5	12.3 ± 2.3	598.0 ± 283.2
		NV grafting	20	16.5 ± 1.8	11.8 ± 1.8	580.9 ± 196.9
	ANOVA[y]	Line		n.s	**	n.s
		Method		n.s	n.s	n.s
		Interaction		n.s	*	n.s
2021	Control	-	12	6.9 ± 1.9	9.4 ± 1.9	49.7 ± 25.5
	#1	Vernailzation	12	9.2 ± 1.9	14.1 ± 2.3	119.4 ± 58.9
		NV grafting	12	9.0 ± 2.6	15.0 ± 2.6	119.7 ± 84.2
	#2	Vernailzation	12	11.0 ± 2.7	12.3 ± 3.1	176.7 ± 93.7
		NV grafting	12	10.5 ± 3.5	10.6 ± 2.6	156.3 ± 129.3
	ANOVA[y]	Line		*	***	n.s
		Method		n.s	n.s	n.s
		Interaction		n.s	n.s	n.s

[z] Mean ± SD.

[y] Data for control line were excluded from the analysis.

Table 4-5. Flowering time of 'Watanabe-seiko No.1' cabbage progenies obtained by two different flowering induction methods.

Year	Line	Flowering induction method	n[z]	Flowering ratio (%)	Days to flowering after sowing[y]
2019	Control	-	21	100	224.4 ± 4.5
	#1	Vernailzation	14	100	232.4 ± 5.7
		NV grafting	18	100	232.6 ± 2.9
	#2	Vernailzation	19	100	231.8 ± 3.9
		NV grafting	20	100	234.8 ± 3.7
	ANOVA[x]	Line		-	n.s
		Method		-	n.s
		Interaction		-	n.s
2021	Control	-	11	100	200.9 ± 0.8
	#1	Vernailzation	12	91.7	200.2 ± 1.5
		NV grafting	12	100	200.5 ± 2.0
	#2	Vernailzation	12	100	201.5 ± 1.2
		NV grafting	12	100	201.1 ± 1.0
	ANOVA[x]	Line			*
		Method			n.s
		Interaction			n.s

[z] Disease-infected individuals were excluded from the flowering time measurement.

[y] Mean ± SD. Data of non-flowered individual in 2021 was excluded from the calculation.

[x] Data for control line and non-flowered individual were excluded from the analysis.

4.4. Discussion

In this chapter, to examine the applicability of NV grafting method to the breeding and seed production of cabbage, I investigated the seed productivity of this method and the fields performance of the obtained progenies.

Seed yield of the cabbage lines tended to be smaller with NV grafting method than with vernalization method (Table 4-1). This was likely due to the difference in the number of opened flowers between the two flowering inducing methods. From a rough observation, cabbage plants tended to produce more inflorescences in vernalization method than in NV grafting method in this study. On the other hand, I observed that seed yield varied among the grafted individuals and some of the plants produced comparable amount of seeds to that of vernalization method (Table 4-1). It was shown in the previous grafting experiment that the number of flowers on receptor plants is affected by the condition of the donor plants, such as leaf area and the degree of floral induction (Zeevaart, 1958). Therefore, seed yield in NV grafting method may be increased by using radish rootstocks with larger and more florally induced leaves. Seed size of the cabbage lines also tended to be smaller with NV grafting method than with vernalization method (Table 4-1). This might be related to the difference in the source capacity of the plants between the two flowering induction methods. In the vernalization method, cabbage plants grown for 2-3 months which had many leaves were used for floral induction. In contrast, in NV grafting method, cabbage plants were grafted onto small radish rootstocks grown for less than one month which had only 3-5 leaves. This may have resulted in the difference in the ratio of source leaves to seed pods, which could lead to the different seed size.

In the field experiment, while there was difference in seed size among the tested seeds depending on the flowering induction method (Table 4-2), there was little difference in their vegetative growth (Tables 4-3, 4-4). It has been reported that in cabbage, seed size affected initial growth but had little effect on later growth and yield (Lingegowda and Andrews, 1973). Similarly in this study, the effect of different seed size seemed to be compensated during the growth period before the vegetative growth measurement in December. I also observed no difference in the reproductive growth of cabbage seeds produced by the two different flowering induction methods (Table 4-5). This suggested that the temperature history or the grafting of the mother plant may not have effect on the flowering response of the resulting seeds in cabbage. In summary, although NV grafting method has currently less seed productivity, this method will not likely to alter the trait of the progenies compared to the vernalization method.

4.5. Summary

In Chapter 4, I confirmed that NV grafting method does not alter the trait of the obtained cabbage progenies compared to the conventional vernalization method. This indicated that NV grafting method

is directly applicable to the breeding program of cabbage. On the other hand, considering its lower seed productivity, it is needed to improve the rootstock to induce more flowering of the grafted cabbage to utilize NV grafting method in broader situation in seed production of cabbage.

General Discussion

Grafting-induced flowering has potential to be applied to the seed production technologies of crops. However, it is difficult to induce flowering by grafting in some crop species, which currently limits the use of this technique to specific plant species. In cabbage, the subject of this study, Kagawa (1957) showed that it was possible to induce flowering by grafting onto a *R. sativus* rootstock. But this phenomenon has not been reproduced for more than 50 years, and it has been considered difficult to induce flowering in cabbage by grafting.

In this study, I explored the necessary condition for the stable floral induction of cabbage by grafting. I also examined the applicability of the grafting-induced flowering method to the breeding and the seed production of cabbage. In Chapter1, I found that the flowering response of the grafted cabbage differed greatly depending on the type of *R. sativus* rootstocks. It was also assumed by the gene expression analysis that the floral induction of the grafted cabbage was caused by the FT protein derived from the *R. sativus* rootstocks. In Chapter2, I developed an antibody which can detect the native FT protein of *R. sativus*. This is the first report where the native FT protein were successfully detected by immunoblotting analysis in cruciferous crops. In Chapter3, I conducted the quantitative analysis of FT protein accumulation by using the developed anti-FT antibody to explore the cause of the difference in the ability among the rootstocks to induce flowering of the grafted cabbage. The result showed that the flowering response of the grafted cabbage plants was correlated with the amount of the FT protein accumulated in them. This suggested that the difference in the ability to induce flowering of the grafted cabbage among different types of rootstocks can be explained by the quantitative difference in FT protein supply capacity of the rootstocks. It was demonstrated that the grafted cabbage plants could be stably induced flowering by using the rootstocks with high FT protein supply capacity. I revealed that the FT protein supply capacity of the rootstocks is determined by the expression level of *FT* transcript in the leaves and the total leaf area of the rootstocks. In Chapter4, I confirmed that grafting-induced flowering method do not alter the field performance of the obtained cabbage progenies compared to the progenies obtained by the conventional flowering induction method by vernalization treatment. This showed the applicability of grafting-induced flowering method to the breeding and seed production of cabbage.

For the development of the rootstocks with high FT protein supply capacity, the expression level of *FT* in the rootstock leaves is the first key factor. In Chapter 3, I confirmed the effect of the *FT* expression in leaves on the FT protein supply capacity of the rootstocks. From the result of Chapter 1, the level of *FT* expression in *R. sativus* accessions was affected by the genetic background for the vernalization requirement. The lower the vernalization requirement in the accession was, the higher the *FT* expression tended to be under the same growth condition. This was consistent with the observations in previous studies that *FT* transcription levels tended to be higher in early-flowering *R.*

sativus accessions than in *R. sativus* accessions that require longer vernalization treatment when they were grown in the same condition (Han et al., 2021; Jung et al., 2020). Future genetic analysis of the *R. sativus* accessions with regard to the vernalization requirement will identify the genes required for high *FT* expression, which will enable efficient breeding of *R. sativus* rootstocks with higher FT supply capacity. In addition, I observed the quantitative effect of the duration of the seed vernalization treatment applied to the *R. sativus* accessions on the expression level of *FT* in Chapter 1 and Chapter 3. Even in the early-flowering type *R. sativus* accessions, which do not require vernalization treatment for their floral induction, vernalization treatment increased the expression level of *FT* and shortened the time for bolting. This showed the importance of the prolonged vernalization treatment longer than the minimum duration for the floral initiation to induce high *FT* expression in *R. sativus* rootstocks.

In addition to the expression level of *FT*, I also showed that leaf area of the rootstocks has a significant effect on the FT protein supply capacity of the rootstocks in Chapter 3. In classical grafting experiments, it has been shown that the leaf area of the donor plant has a quantitative effect on the degree of floral induction of the recipient plant (Suge, 1986; Zeevaart, 1958). Considering the result obtained in Chapter 3, this phenomenon was presumably related to the FT protein supply capacity of the donor plant. Importantly, as has been reported in tomato (Shalit et al., 2009), a negative correlation between expression level of *FT* and the leaf area was observed on the *R. sativus* rootstocks in Chapter 3. This means that increased expression level of *FT* will not always accompany larger FT supply capacity of the rootstocks, because higher expression of *FT* can lead to smaller leaf area of the rootstocks, which can then cancel the effect of the increased expression of *FT*. The failure of floral induction in recipient plants grafted onto *FT*-overexpressing transformants (Bull et al., 2017; Odipio et al., 2020; Tränkner et al., 2010; Wenzel et al., 2013; Zhang et al., 2010) might be caused by this trade-off between *FT* expression level and the leaf area of the rootstock. To produce rootstocks with large FT supply capacity, it will be important to break the link between *FT* expression and the leaf area. For example, in Chapter 3, I observed that the leaf area of the rootstock became larger by the delay of the floral induction with the shorter daylength in MD/LD treatment than in LD/LD treatment. However, the *FT* expression level of the rootstock was comparable between MD/LD and LD/LD, after the rootstocks were transferred to LD condition. This suggests that enlargement of the leaf area without changing the *FT* expression level_is possible by temporarily suppressing floral induction through daylength treatments.

In agreement with the report by Kagawa (1957), cabbage could not be induced to flower at all when grafted onto the *B. oleracea* rootstocks in this study. Since FT protein could not be detected in cabbage grafted onto *B. oleracea* rootstocks, it was concluded that *B. oleracea* rootstocks used in this study were unable to induce flowering of the grafted cabbage due to their low FT supply capacity. The leaf area of the *B. oleracea* rootstocks tested in this study was comparable to that of the *R. sativus* rootstocks. This indicated that the cause of the low FT protein supply capacity could be attributed to

low *FT* expression in the leaves, or to the low transportation ability of the FT proteins in *B. oleracea*. These results indicate that the contribution of FT expressed in leaves may be low in the flowering regulation in *B. oleracea* plants themselves. In *Arabis alpina*, a perennial plant in the same Brassicaceae family as *B. oleracea*, it has been shown that vernalization-induced floral initiation depends on the activation of the floral integrator SQUAMOSA PROMOTER BINDINGPROTEIN-LIKE 15 in SAM, rather than the expression of *FT* in the leaves (Hyun et al., 2019). In *B. oleracea*, it has been reported that *FT* expression at the shoot apex was increased by low temperature encounter (Lin et al., 2005; Ridge et al., 2015). This provides the possibility that *FT* expressed in the SAM may directly induce the flowering, and that *FT* expressed in the leaves may contribute less to floral induction in *B. oleracea*. Future studies to clarify the causes of the differences in the ability of *B. oleracea* and *R. sativus* to induce flowering of grafted cabbage will not only clarify the requirements for the rootstock to induce flowering, but will also help us to understand the diversity in the mechanism of flowering regulation in cruciferous crops.

There will be several applications of the grafting-induced flowering in the practical breeding and the seed production of cabbage. It was able to stably induce flowering of young cabbage seedlings (3–4 weeks after sowing) within 1.5–2 months after grafting without vernalization treatment by grafting onto *R. sativus* rootstocks with high FT supply capacity. This is about half the time required to induce flowering by the conventional vernalization method. In the breeding area, the grafting method can be used firstly for shortening the time for establishing inbred lines, or time for backcrossing in the introduction of useful traits to existing cultivars, such as cytoplasmic male sterility or disease resistances. Another possible application is simultaneous flowering induction for crosses between cultivars that flower at different timing under natural conditions. In Egypt, an attempts has been reported to introduce a foreign cultivar of cabbage that is difficult to flower under natural conditions into local breeding by using the grafting method based on the result of Chapter 1 (EL-Eslamboly and Hamed, 2021). For commercial seed production, grafting method has the potential to be used for the development of the novel seed production techniques, such as off-season seed production when cabbage does not naturally flower, seed production of extremely late-bolting or non-flowering cabbage cultivars, or hybrid seed production between accessions with different natural flowering time. However, as I confirmed in Chapter 4, grafting method currently produces much smaller amount of seed per plant compared to the reported yield of cabbage seed production in the field (Al-Khatib et al., 1995), and is more labor-intensive. Thus, its applicability to commercial seed production would be limited for now. For example, grafting method could be possibly used for the seed production of the parental lines for hybrid seed production. On the other hand, for non-commercial use, it could be used for small-scale seed production to maintain genetic resources or for local seed self-sufficiency by farmers. For the broader application of grafting method for seed production, it will be needed to

improve the seed productivity of this method by developing rootstocks with larger plant size and higher FT supply capacity.

It will be of interest to investigate the flowering response of a wider variety of cabbage cultivars or other cruciferous crops grafted onto the *R. sativus* rootstocks as a future work. In several plant species, it has been shown that the floral initiation in SAM is determined by the balance between FT homologues and TFL1 homologues, which have antagonistic activity against FT (Lifschitz et al., 2014). Plants with strong activity of floral suppressors like TFL1 in SAM may be reluctant to be induced flower by grafting. By investigating flowering responses of broader range of crops grafted onto *R. sativus* rootstocks, it will be able not only to clarify the applicability of this technique, but also to gain new insights into the diverse mechanism of floral regulation in SAM in cruciferous crops. Environmental condition will be another point to be considered for the practical use of grafting method. In this study, grafting was conducted under warm and long-day conditions. However, it is possible that the grafting method is used in various environmental conditions when applied as a breeding and seed production technique. It will be important to clarify suitable rootstocks and control the environment to successfully induce flowering by grafting under such various environmental conditions. In the field of horticulture, various techniques have been developed to control flowering such as controlling temperature, photoperiod, and light quality, and the use of phytohormone (Proietti et al., 2022). It has also been shown that *FT* expression is also affected by these environmental factors and phytohormones (Kobayashi et al., 1999; Porri et al., 2012; Song et al., 2018; Susila et al., 2021). Thus, the FT supply capacity of the rootstock may be able to be controlled by those flowering control technologies. The expression level of *FT* in the leaves and the leaf area, which were found to be associated with the FT supply capacity of the rootstock in this study, will be good indicators in the process of optimizing the grafting method under various environments.

Through this study, I show the way to develop the “flowering induction rootstock”, which can stably induce flowering with high FT protein supply capacity and can be used for the practical breeding and seed production in cabbage. I believe the knowledge obtained in this study will also contribute to the development of the stable floral induction method by grafting in other crop species that have been considered to be difficult to induce flowering due to their environmental requirement for flowering.

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