**Studies on the floral regulatory mechanism in a non-flowering cabbage mutant that spontaneously reacquires flowering ability**

（偶発的な開花復帰性をもつ非開花性キャベツ変異体の
開花制御機構に関する研究）

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## Abstract

‘nfc’ (non-flowering cabbage) was discovered as a non-flowering natural mutant among the open-pollinated cabbage line ‘T15’ (*Brassica oleracea* L. var. *capitata* L.) in 1978. ‘nfc’ has been propagated vegetatively by cutting for over 40 years. Cabbage is a plant-vernalization-type plant, which can sense low temperatures only after developing to a certain size, and also requires long-term low-temperature exposure for flowering. ‘nfc’ hardy flowers under floral inductive conditions where ‘T15’ and other cabbage cultivars flower at 100%. Meanwhile, another phenomenon in which lateral shoots generated near the base of the main stem of ‘nfc’ flowered in spring has been occasionally observed. Elucidation of the mechanism controlling the unique flowering characteristics of ‘nfc’ is expected to provide new insights into the physiology of plant flowering. The main objective of this study was to elucidate the non-flowering mechanisms and the causes of spontaneous flowering in ‘nfc’.

In Chapter 1, to confirm the reproducibility of the flowering characteristics of ‘nfc’, I cultivated ‘nfc’ and the parental line ‘T15’ in the open field for three years. Throughout the 3-year cultivation period, all ‘T15’ plants flowered, while the flowering rates of ‘nfc’ were 0, 32, and 4%, in the first, the second, and the third years, respectively. Consistent with previous observations, only the lateral shoots of ‘nfc’ flowered, while the terminal bud of ‘nfc’ continued to grow vegetatively. The flowering dates of the flowering ‘nfc’ plants were later than those of ‘T15’, and the average numbers of flowering shoots per plant of the flowering ‘nfc’ were lower than those of ‘T15’.

I hypothesized that ‘nfc’ is a chimeric plant composed of wild type and mutant cells with and without flowering ability, respectively. To test the hypothesis, I isolated

mesophyll protoplasts from ‘nfc’ and produced 12 protoplast lines consisting of several regenerated plants derived from the same protoplast, and investigated the flowering characteristics of them. The results showed that both flowering and non-flowering plants appeared in five protoplast lines, rejecting the hypothesis that spontaneous flowering of ‘nfc’ is due to chimerism. Furthermore, flowering of ‘nfc’ could not be explained by differences in plant size between flowering and non-flowering plants and differences in average temperature among cultivation years.

In Chapter 2, ‘nfc’ scions which were artificially induced to flower by grafting onto radishes (*Raphanus sativus* L.) were crossed with three *B. oleracea* cultivars (‘T15’, ‘W1’, and ‘Kairan’ in order of the closest relationship to ‘nfc’) to obtain $F_1$ plants, and $F_2$ populations were generated by self-pollinating each $F_1$ plant. Although the frequency of non-flowering plants differed, the flowering dates were widely distributed in a continuous manner in all $F_2$ populations. In each $F_2$ population, I performed whole genome sequencing of the parental lines, the earliest 10–20% flowering individuals (Early-bulk), and the latest 10–20% flowering individuals including non-flowering individuals (Late-bulk), and calculated the difference of ‘nfc’ type allele frequencies between Early-bulk and Late-bulk in each polymorphism (ΔSNP-index, Late-bulk－Early-bulk) (QTL-seq analysis).

As a result, a positive peak of ΔSNP-index exceeding a 99.9% confidence interval was detected around 51 Mb of chromosome 9 in ‘nfc’×‘W1’ $F_2$ and ‘kairan’×‘nfc’ $F_2$. On the other hand, no significant peak was detected in ‘nfc’×‘T15’ $F_2$. QTL analysis using ‘nfc’×‘W1’ $F_2$ confirmed that this genomic region is a QTL involved in the non-flowering trait. Furthermore, QTL analysis using ‘kairan’×‘nfc’ $F_2$ narrowed down the QTL region to the region corresponding to 50,177,696–51,474,818 bp (approximately 1.3 Mb interval) of chromosome 9 in the ‘TO1000’ reference genome. In ‘TO1000’, 241 genes were located in this region, including four homologs of flowering time-related genes in *Arabidopsis thaliana* L.. The coding sequences of the four genes were identical between ‘T15’ and ‘nfc’. RNA-seq analysis of leaves and shoot apices revealed that two of the four genes were differentially expressed (highly expressed in ‘nfc’). Therefore, I identified these two genes (LOC106318712 and LOC106318713) as candidate causal

genes responsible for the non-flowering trait of ‘nfc’. The two genes were both *BoFLC1*, homologs of *FLOWERING LOCUS C* (*FLC*), which encode MADS-box transcription factors and integrate the vernalization and autonomous flowering pathways, and were located tandemly 17.5 kb apart. I named LOC106318712 and LOC106318713 *BoFLC1a* and *BoFLC1b*, respectively. The amino acid sequence of *BoFLC1b* of ‘T15’/‘nfc’ was identical to that of previously reported *BoFLC1*, which has been demonstrated to function as a floral repressor by transformation to *Arabidopsis*. Three isoforms were expressed in *BoFLC1a*, one of which was likely to function as a floral repressor like *BoFLC1b* because it had 94% amino acid sequence homology and a similar predictive conformation with BoFLC1b (ColabFold: AlphaFold2 using MMseqs2 and HHsearch).

In *Arabidopsis*, the expression of *FLC* is repressed by vernalization gradually, enabling the activation of flowering-related genes such as *FLOWERING LOCUS T* (*FT*). Therefore, the relative expression levels in the upper leaves sampled from plants cultivated in the open field over time from October to April were quantified. The expression levels of *BoFLC1a* and *BoFLC1b* in ‘T15’ and ‘W1’ gradually decreased from October to January, while those in ‘nfc’ were higher at the early stage and did not decrease after exposure to low temperatures. In particular, the expression levels of *BoFLC1a* and *BoFLC1b* in ‘nfc’ in mid-April were about 10- and 20-fold higher than those in ‘T15’, respectively. The expression level of *BoFT* in ‘T15’ and ‘W1’ increased in April, while that in ‘nfc’ hardly increased. These results suggested that the high expression levels of both *BoFLC1a* and *BoFLC1b* contributed to the non-flowering trait of ‘nfc’. The fact that tandemly arranged *BoFLC1a* and *BoFLC1b* genes showed similar expression patterns and that ‘nfc’ is a mutant appeared from ‘T15’ suggests that a *cis*-regulatory mutation in the vicinity of these genes may have contributed to their upregulation. However, comparative analysis of a genomic sequence from 100 kb upstream of the transcription start site (TSS) of *BoFLC1a* to 100 kb downstream of the termination codon of *BoFLC1b* using short reads revealed no polymorphism with an absolute value of ΔSNP-index (SNP-index_nfc − SNP-index_T15) greater than 0.75.

Based on the results of Chapter 1, I considered the possibility that the flowering characteristics of ‘nfc’ is controlled by epialleles (alleles that are epigenetically regulated

without any DNA sequence changes and have metastable heritability and reversibility). In Chapter 3, to test this hypothesis, I prepared the first to third generations of the self-pollinated progeny of the 'nfc' plants that spontaneously flowered by themselves under natural floral inductive conditions (nfcV1, nfcV2, nfcV3), the first to fourth generations of the self-pollinated progeny of protoplast-regenerated 'nfc' plants (nfcP1, nfcP2, nfcP3, nfcP4), and the self-pollinated progeny of 'nfc' scion whose flowering was artificially induced by grafting (nfcG1). Then, I investigated whether the flowering characteristics of these plants were compatible with the property of epialleles. nfcG1 generally maintained the non-flowering trait, while 90% of nfcV1–3 and nfcP1–4 flowered. This suggested that plants that flowered in response to low temperatures reacquired flowering ability, which was metastably inherited by the progeny. Meanwhile, the remaining 10% reverted to non-flowering, indicating reversibility from flowering to non-flowering. There was a wide variation in flowering characteristics (the flowering date and the number of flowering shoots) among individuals of nfcV1–3 and nfcP1–4 and a positive correlation between the parent and the median of the progeny lines. These results suggest that the flowering characteristics of 'nfc' is controlled by epialleles. Furthermore, non-flowering plants appeared in 'T15' which had been propagated by cutting, suggesting that flowering/non-flowering in 'T15'/'nfc' was considered to be due to differences in epigenetic state. Expression analysis of *BoFLC1a* and *BoFLC1b* in 'T15', 'nfc', nfcG1, nfcV1, and nfcP4 plants showed that the expression levels of the two genes were different in each line and significantly correlated with flowering characteristics. Therefore, *BoFLC1a* and *BoFLC1b* were considered to be epialleles controlling flowering characteristics in 'nfc'.

Because flowering is an essential process for crossing and seed production in seed plants, the non-flowering trait is rare and difficult to analyze. In this study, I succeeded in identifying the QTL responsible for the non-flowering trait of 'nfc' by mapping analysis of $F_2$ population generated by crossing 'nfc' scions which was artificially induced to flower by grafting on a radish rootstock with other cultivars. Furthermore, the expression analysis indicated that the non-flowering trait of 'nfc' may be due to the constitutively high expression of *BoFLC1a* and *BoFLC1b*. I also showed

that the spontaneous flowering of ‘nfc’ is not due to tissue chimerism but the reversibility of epialleles. The expression levels of *BoFLC1a* and *BoFLC1b* were correlated with flowering characteristics, suggesting that both genes have become epialleles by epigenetic mutations. The establishment of epialleles and the stability and heritability of the epigenetic state are still unknown. Identification of the epigenetic mutation in ‘nfc’ and elucidation of its establishment mechanism will contribute to the diversification of flowering characteristics of Brassicaceae crops, as well as to a better understanding of the property of epialleles.