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Four *TFL1*/*CEN*-Like Genes on Distinct Linkage Groups Show Different Expression Patterns to Regulate Vegetative and Reproductive Development in Apple (*Malus×domestica* Borkh.)

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Recent molecular analyses in several plant species revealed that *TERMINAL FLOWER1* (*TFL1*) and *CENTRORADIALIS* (*CEN*) homologs are involved in regulating the flowering time and/or maintaining the inflorescence meristem. In apple (*Malus×domestica* Borkh.), four *TFL1*/*CEN*-like genes, *MdTFL1*, *MdTFL1a*, *MdCENa* and *MdCENb*, were found and mapped by a similar position on putatively homoeologous linkage groups. Apple *TFL1*/*CEN*-like genes functioned equivalently to *TFL1* when expressed constitutively in transgenic *Arabidopsis* plants, suggesting that they have a potential to complement the *TFL1* function. Because *MdTFL1* and *MdTFL1a* were expressed in the vegetative tissues in both the adult and juvenile phases, they could function redundantly as a flowering repressor and a regulator of vegetative meristem identity. On the other hand, *MdCENa* was mainly expressed in fruit receptacles, cultured tissues and roots, suggesting that it is involved in the development of proliferating tissues but not in the control of the transition from the juvenile to the adult phase. In contrast, *MdCENb* was silenced in most organs probably due to gene duplication by the polyploid origin of apple. The expression patterns of *MdTFL1* and *MdCENa* in apple were also supported by the heterologous expression of β-glucuronidase fused with their promoter regions in transgenic *Arabidopsis*. Our results suggest that functional divergence of the roles in the regulation of vegetative meristem identity may have occurred among four *TFL1*/*CEN*-like genes during evolution in apple.

Keywords: Apple • *CENTRORADIALIS* (*CEN*) • Flowering time • Gene family • *Malus×domestica Borkh.* • *TERMINAL FLOWER1* (*TFL1*).

Abbreviations: *AP1*, *APETALA1*; *ATC*, *ARABIDOPSIS THALIANA CENTRORADIALIS*; *BFT*, *BROTHER OF FT AND TFL1*; CaMV, cauliflower mosaic virus; *CEN*, *CENTRORADIALIS*; DIG, digoxigenin; EST, expressed sequence tag; FBS, fruit-bearing shoot; *FLC*, *FLOWERING LOCUS C*; *FT*, *FLOWERING LOCUS T*; GUS, β-glucuronidase; I-PCR, inverse PCR; IS, inverted sequence; LD, long-day; *LFY*, *LEAFY*; LG, linkage group; *MFT*, *MOTHER OF FT AND TFL1*; ncRNA, non-coding RNA; RT–PCR, reverse transcription–PCR; *SOC1*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*; SS, succulent shoots; *TFL1*, *TERMINAL FLOWER1*; *TSF*, *TWIN SISTER OF FT*.

The nucleotide sequences reported in this paper have been submitted to the DDBJ under accession numbers AB366637 (*MdCENa* genomic DNA), AB366638 (*MdCENb* genomic DNA), AB366639 (*MdTFL1* genomic DNA), AB366640 (*MdTFL1a* genomic DNA), AB366641 (*MdCENa* mRNA), AB366642 (*MdCENb* mRNA), AB366643 (*MdTFL1a* mRNA), AB458503 (*MdAP1a* mRNA).

Introduction

Apple (*Malus×domestica* Borkh.), which belongs to the Rosaceae subfamily, Maloideae, is one of the most important fruit tree crops in the world from an economic standpoint. However, most fruit crops, such as apple, have a long generation

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time, which makes their breeding cycle slower (Sax 1957, Hackett 1985). Thus, understanding the phase transition of apple at a molecular level would provide an insight into the methods of acceleration of breeding in fruit trees. The shoot apex of higher plants passes through three more or less distinct phases during its post-embryonic development (Poethig 1990). The apple passes through a relatively long juvenile phase, during which the vegetative shoot elongates vigorously and does not bear floral buds for 4–8 years.

In woody plants, such as apple, attainment of the ability to flower indicates the end of the juvenile phase (**Fig. 1**). When actual flowering does not coincide with the end of the juvenile phase, the intervening period can be referred to as an adult vegetative phase or transition phase (Zimmerman 1972, Hanke et al. 2007). In the adult phase, reproductive parts of the apple tree produce fruit-bearing shoots (FBS) and enter the seasonal flower/fruit formation cycle (reproductive growth cycle) (**Fig. 1**). On the other hand, vegetative parts produce vegetative shoots, such as succulent shoots (SS) (**Supplementary Fig. S1A**), and enter the vegetative growth cycle (**Fig. 1**). After flowers bloom on FBS in the spring, a new budded shoot (bourse shoot) emerges from the axillary meristem on the bourse (**Supplementary Fig. S1B**). Approximately 95% of vegetative meristems on the bourse shoots become committed to floral development, which consequently convert into inflorescence meristems and then develop into floral meristems (Foster et al. 2003). The remaining 5% of vegetative meristems produce vegetative shoots. The morphology of SS, which emerge directly form the branch or trunk, is similar to that of juvenile shoots with vigorous growth, and SS do not bear floral buds for several years. Fruit ripens in the autumn and the apple tree enters the dormant period from late autumn. When the seeds from the ripened fruit germinate, they enter the ontogenetic growth cycle (**Fig. 1**).

In *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh.], the transition from the vegetative to the reproductive phase

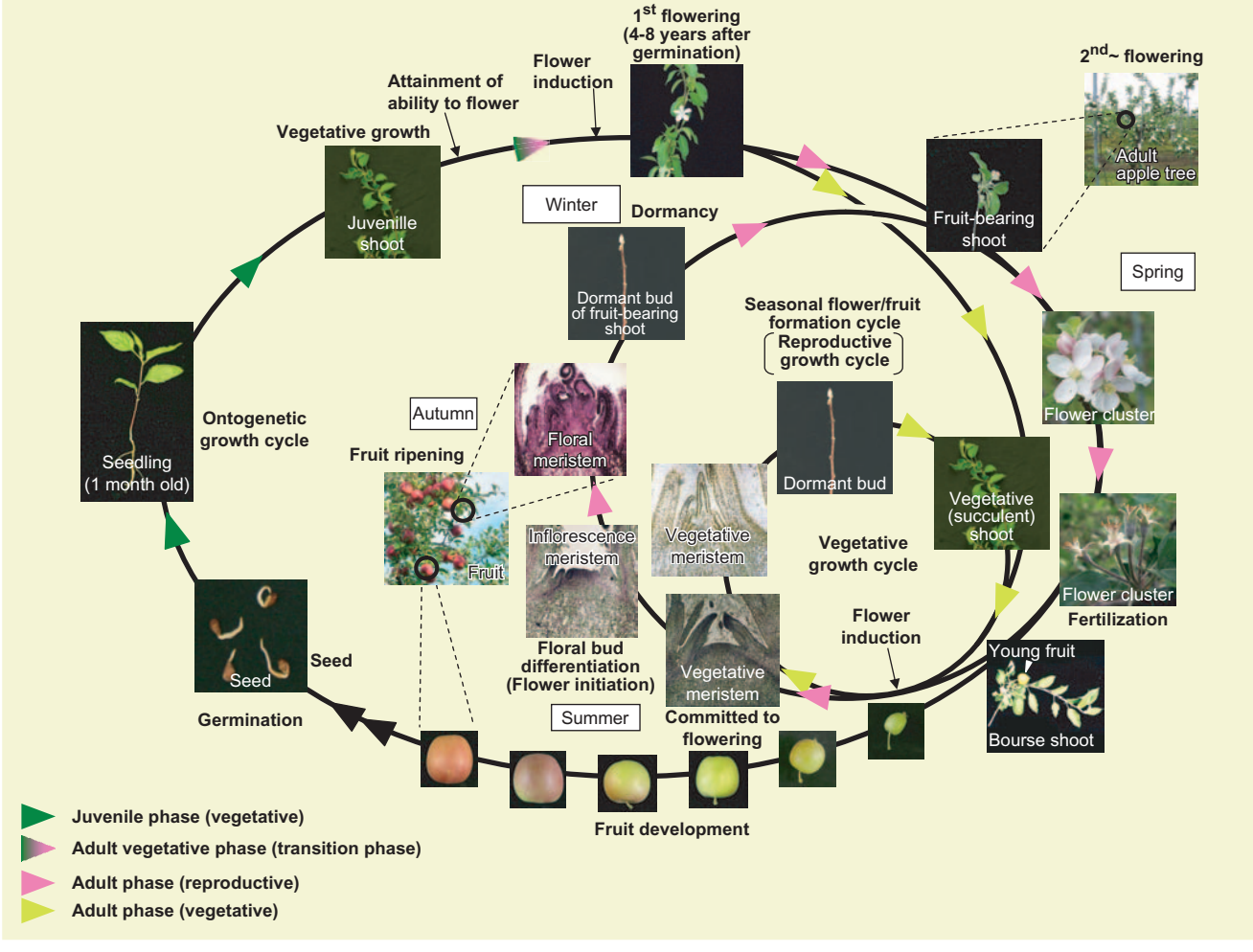


Fig. 1 Schematic representation of the apple growth cycle based on the studies of Hackett (1985), Zimmerman (1972), Poethig (1990), Kotoda et al. (2000), Foster et al. (2003) and Hanke et al. (2007).

is initiated by four independent pathways of signal transduction, i.e. the gibberellin-dependent, autonomous, vernalization-dependent and light-dependent pathways (for reviews, see Levy and Dean 1998, Mouradov et al. 2002, Simpson and Dean 2002, Boss et al. 2004, Komeda 2004). These signals are transmitted via integrator genes, such as *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) and *FLOWERING LOCUS C* (*FLC*), to the floral meristem identity genes *APETALA1* (*AP1*) and *LEAFY* (*LFY*) at the apical meristems. *TERMINAL FLOWER1* (*TFL1*) is a key gene involved in repressing flowering and maintaining the inflorescence meristem by preventing the expression of *AP1* and *LFY* (Bradley et al. 1997, Ratcliffe et al. 1998, Ratcliffe et al. 1999, Boss et al. 2004).

In apple, twin flowering genes, such as *MdAP1* (*MdMADS5*) (Yao et al. 1999, Kotoda et al. 2000, 2002) and *MdAP1a* (accession no. AB458503) of *AP1*-like genes, *MdTFL1* and *MdTFL1a* (*MdTFL1-2*) of *TFL1*-like genes (Esumi et al. 2005, Kotoda and Wada 2005, Kotoda et al. 2006, this study) and *MdLHP1a* and *MdLHP1b* of *LHP1*-like genes (Mimida et al. 2007), have been isolated and characterized. The abundance of *MdTFL1* and *AFL1* (a *LFY*-like gene of apple) transcripts in the apical buds of FBS varies considerably through the various developmental stages; *MdTFL1* is significantly down-regulated, whereas *AFL1* is up-regulated after floral bud differentiation (Kotoda and Wada 2005). These results suggest that *AFL1* and *MdTFL1* play antagonistic roles in regulating the phase transition and could potentially affect floral development throughout the growing season in apple. However, the expression patterns of homolog genes are not necessarily equivalent between apple and *Arabidopsis*. For example, *MdAP1* expression is prominent in sepals but is not detected in petals (Kotoda et al. 2000). The floral pathway genes in individual plant species are unique in their expression patterns and, possibly, in their protein–protein interaction networks. These differences are thought to be important in the variations of flowering time and in the distinctive morphological characteristics among plant species (Andersen et al. 2004). In particular, the *TFL1* homolog genes in the floral pathway are shown to have unique features in flowering time and plant architecture in each plant species.

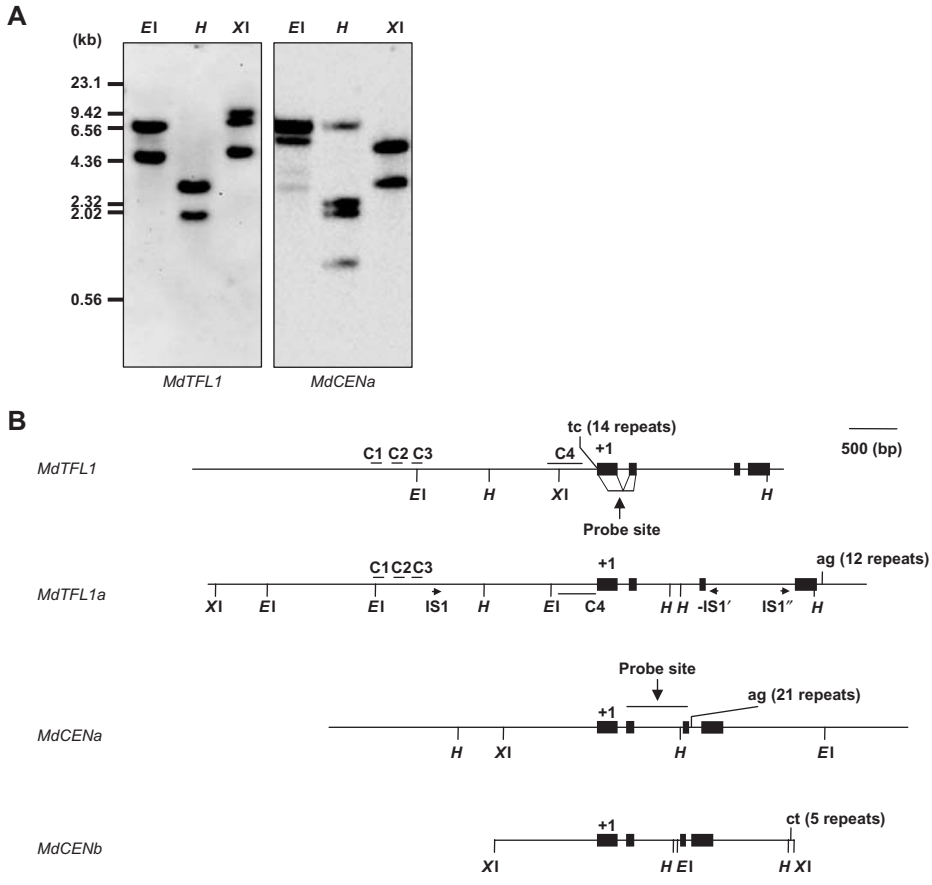
The *Arabidopsis* genome contains six genes in the *FT*/*TFL1* family, including the *MOTHER OF FT AND TFL1* (*MFT*), *FT*, *TWIN SISTER OF FT* (*TSF*), *BROTHER OF FT AND TFL1* (*BFT*), *ARABIDOPSIS THALIANA CENTRORADIALIS* (*ATC*) and *TFL1* (Bradley et al. 1997, Ohshima et al. 1997, Kardailsky et al. 1999, Kobayashi et al. 1999, Mimida et al. 2001, Yoo et al. 2004, Yamaguchi et al. 2005). The analyses of *TFL1*/*CENTROLADIALIS* (*CEN*; Bradley et al. 1996)-like genes in dicot plants suggested that the functional divergence among these groups occurred selectively in each plant species. For example, the *tfl1* mutant of *Arabidopsis* shows early flowering, and its apex of inflorescence is terminated by fused flowers (Bradley et al. 1997, Ohshima et al. 1997). On the other hand, the T-DNA-tagged mutant of the *ATC* of the *CEN* group (*CEN*-like genes) shows no novel phenotypes (Mimida et al. 2001, Yoo et al. 2005). In snapdragon (*Antirrhinum majus* L.), the *centroradialis* (*cen*) mutant forms a determinate inflorescence, but shows no early flowering (Bradley et al. 1996). In pea (*Pisum sativum* L.), two different mutants defective in the *PsTFL1a* and *PsTFL1c* gene of the *TFL1* group, *determinate* (*det*) and *late flowering* (*lf*), lead to terminal flower and early flowering, respectively (Foucher et al. 2003). This diversity of the function of the *TFL1*/*CEN*-like genes appears to be related to the indication by Ahn et al. (2006) that a particular region in the fourth exon of the *TFL1* gene has evolved more rapidly than that in the *FT* gene. Therefore, the analyses of *TFL1*/*CEN*-like genes will provide a novel explanation for the manner in which higher plants have come to gain diversity in morphology and variation in flowering time in a variety of species as well as provide useful information for the molecular breeding of crops.

We previously reported that apple transgenic plants expressing the antisense RNA of *MdTFL1* showed precocious flowering (Kotoda et al. 2003, Kotoda et al. 2006), revealing that *MdTFL1* controls the transition from the juvenile/vegetative to the reproductive phase in apple. However, it is suggested that several *TFL1*/*CEN*-like genes would also be present in the apple genome and might have different functions among them. Therefore, we isolated and characterized other *TFL1*/*CEN*-like genes from apple and elucidated the functional relationships among them.

Results

Four TFL1/CEN-like genes are present in the apple genome

We attempted to isolate apple *TFL1*/*CEN*-like genes by screening a genomic library and using a PCR strategy. As a result, four *TFL1*/*CEN*-like genes were obtained, and they were designated *MdTFL1*, *MdTFL1a*, *MdCENa* and *MdCENb*. *MdTFL1* and *MdTFL1a* corresponded to previously reported cDNAs (Esumi et al. 2005, Kotoda et al. 2005), whereas *MdCENa* and *MdCENb* were novel genes of the *FT*/*TFL1* family in apple. To assess the number of *TFL1*/*CEN*-like genes in the apple genome, genomic Southern hybridization was performed using the partial sequence of *MdTFL1* or *MdCENa* as a probe. Two to four major hybridizing bands were detected in each lane of DNA digested with *Eco*RI, *Hin*dIII or *Xba*I (**Fig. 2A**). The genomic regions to be hybridized with the probes for *MdTFL1* and *MdTFL1a* contained no sites of the restriction enzyme used for digestion. On the other hand, that for *MdCENa* had a *Hin*dIII site, and that for *MdCENb* had a *Hin*dIII site and an *Eco*RI site (**Fig. 2B**). In view of these considerations, there are at least two distinct *TFL1*-like genes



C

Consensus sequence

C 1 GCTWCTTTGAGYSATTGAGATGCCCTCTCWGCACKACTAGCAGCTTGGCAGSAGGCACTCAGAGAGCAGGTGGAGYTCCTTTATGCTCTTGKGMCTCMTC

C 2 ACAGTATATTTCCATTTCAAAATTTKYRTTACCCTAAMTGCMAAAGATRAGAKTTTCCYTKATTAGATTGTGAMTGTTCAGGAAKAATTCTAGGCAWTTTGYTGTTTTT

C 3 GRATTATATAATGATTTTCTGGCCTAWCTAAGGAAAAACATCCCCCTTWWTTTTTGAWMAAAAWAWTCAATAATTAGGTTTACAACCAATCAAGAACTAATTCCAAGGRKCC

R (A/G), Y (C/T), M (A/C), K (G/T), S (G/C), W (A/T)

Fig. 2 The apple genome has four *TFL1*- and *CEN*-like genes. (A) Genomic Southern blot analysis for *MdTFL1* (left) or *MdCENa* (right). The genomic DNA (15 µg) of the 'Fuji' apple was digested with *Eco*RI (*E*I), *Hin*dIII (*H*) or *Xba*I (*X*I) and subjected to hybridization. The molecular size markers are shown in kb on the left. (B) Schematic representation of the gene structures and restriction maps of four apple *TFL1*/*CEN*-like genes. The genomic sequences determined in this study are indicated by bars. The black boxes on the bars show exons. The relative positions of the initiation codon (ATG) are shown as +1. The positions of the highly conserved sequence between *MdTFL1* and *MdTFL1a* are shown by C1, C2, C3 and C4. The inverted repeat sequences in the *MdTFL1a* gene are indicated by IS1, -IS1′ and IS1″ (for sequences, see **Supplementary Fig. S2B**). The position and sequence direction of IS1, -IS1′ and IS1″ are indicated by arrows. The SSR shown by tc (14 repeats), ag (12 repeats), ag (21 repeats) and ct (5 repeats) was used as a marker for the genome mapping of *MdTFL1*, *MdTFL1a*, *MdCENa* and *MdCENb*, respectively. The probe regions used in the Southern blot are shown as bars indicated by vertical arrows on *MdTFL1* and *MdCENa*. The scale bar on the map represents approximately 500 bp. (C) The consensus sequence of C1, C2 or C3 (for the consensus sequence of C4, see **Supplementary Fig. S2A**).

(*MdTFL1* and *MdTFL1a*) and two *CEN*-like genes (*MdCENa* and *MdCENb*) in the apple genome.

The coding region of *MdTFL1* exhibited 93.6% identity to that of *MdTFL1a* at the nucleotide level, and these genes consisted of four exons, 198, 62, 41 and 218 bp, encoding a putative protein of 172 amino acids. However, the length of the second and third introns was considerably different between *MdTFL1* and *MdTFL1a* (**Fig. 2B**). To examine whether conserved sequences exist in the non-coding regions of *MdTFL1* and *MdTFL1a*, we searched the regions using MEME (the Multiple Em for Motif Elicitation), revealing that three conserved sequences were found in the putative promoter regions, which were represented as C1, C2 and C3 (100–112 bp in length, respectively). C1, C2 and C3 were

localized tandemly in the region between −2,075 and −1,436 in *MdTFL1* and between −2,075 and −1,436 in *MdTFL1a*, exhibiting 90.0, 88.1 and 90.2% identity, respectively, with the corresponding motifs of the two genes (**Fig. 2B, C**). In addition, there were conserved sequences in the 5′-flanking regions of *MdTFL1* and *MdTFL1a*, designated C4 (−450 to −72 of *MdTFL1* and −396 to −1 of *MdTFL1a*, respectively), which had 79% identity (**Supplementary Fig. S2A**). Furthermore, inverted sequences were found in *MdTFL1a*, represented as IS1, -IS1′ and IS1″ (**Fig. 2B**). The sequences of IS1, -IS1′ and IS1″ were present in the putative promoter region between −1,600 and −1,440, in the third intron between +1,187 and +1,339, and in the third intron between +1,858 and +2,013, respectively. They exhibited 66.9–70% identity at the nucleotide level among them (**Supplementary Fig. S2B**). The *MdTFL1* gene contained a simple sequence repeat (SSR) of tc (14 repeats) in the 5′-non-coding region and the *MdTFL1a* gene contained a SSR of ag (12 repeats) in the 3′-non-coding region (**Fig. 2B**). On the other hand, the coding region of *MdCENa* exhibited 95.6% identity to that of *MdCENb* at the nucleotide level. These genes consisted of four exons, 204, 62, 41 and 218 bp, encoding a putative protein of 174 amino acids. The *MdCENa* gene contained an SSR of ag (21 repeats) in the third intron, whereas a similar SSR was not found in the third intron of *MdCENb* (**Fig. 2B**).

Four TFL1/CEN-like genes are localized in a different linkage group

To assign the *TFL/CEN*-like genes in the apple linkage group (LG), genetic mapping was carried out using the SSRs present in each gene as DNA markers. The position of the target SSR sequence in each gene is shown in **Fig. 2B**. By analyzing two sets of seedlings from the cross between 'Ralls Janet' (Ra) and Mitsubakaido (*Malus sieboldii* Rehder) and between 'Delicious' (De) and Mitsubakaido as the mapping populations (Igarashi et al. 2008), we assigned *MdTFL1* to the north end of LG 12 of 'Ralls Janet' (Ra12) and to a region close to markers rD05A20-0.9 and E35M48-350 on LG 12 of 'Delicious' (De12), and *MdTFL1a* to the north end of LG 14 (De14) and to a region between markers E38M61-180 and E37M62-195d on LG 14 (Ra14), respectively (Fig 3A). On the other hand, *MdCENa* was assigned to the middle of LG 03 (Ra03 and De03), and *MdCENb* was assigned between E37M49-900 and CH02d08-L/S on LG 11 (Ra11) and around E32M59-180, rD11D66-0.85 and CH02d08-L/S on LG 11 (De11) (**Fig. 3B**).

Relationship among the genes of the TFL1/CEN group in dicot plants

We carried out the alignment and phylogenetic tree analysis using the deduced amino acid sequences of the *TFL1/CEN*-like gene together with our isolated genes. As a result, MdTFL1 and MdTFL1a were more similar to TFL1 (84 and 83% similarity, respectively) than to CEN (82 and 81%, respectively). On the other hand, MdCENa and MdCENb were more similar to CEN (85 and 86%, respectively) than to TFL1 (83%). Alignment of the deduced amino acid sequences of the *TFL1/CEN*-like gene showed that the His88 and Asp139 in apple TFL1/CEN-like proteins, which are amino acids characteristic of TFL1/CEN-like proteins, were conserved among other plant species (**Fig. 4A**, see asterisks). The substitutions of valine (V) with phenylalanine (F) or cysteine (C) at position 73 in MdTFL1 or MdTFL1a, respectively, were characteristic of TFL1 group members in the Maloideae species of the Rosaceae family (**Fig. 4A**, see an arrow). The apple TFL1/CEN-like proteins were classified into two distinct groups, called 'TFL1' and 'CEN' on the phylogenetic tree (**Fig. 4B**). MdTFL1 and MdTFL1a belonged to the TFL1 group members in the Maloideae of the Rosaceae. MdCENa and MdCENb showed the highest similarity (91%) to VvTFL1A, a TFL1/CEN ortholog of grapevine (*Vitis vinifera* L.), among TFL1/CEN-like proteins in the phylogenic tree (**Fig. 4B**).

Four TFL1/CEN-like genes show different expression patterns in various tissues and apical buds throughout the growing season in apple

Expression analysis by reverse transcription–PCR (RT–PCR) was performed in various tissues for four apple *TFL1/CEN*-like genes. *MdTFL1* was expressed mainly in apical buds of FBS and cultured tissues (shoots) derived from adult trees and apical buds of vegetative shoots, stems and roots of 14-day-old seedlings in the juvenile phase, whereas *MdTFL1a* was expressed mainly in stamens, peduncles and apical buds of FBS and apical buds of vegetative shoots, stems and roots of seedlings (**Fig. 5A**). On the other hand, the transcripts of *MdCENa* were detected in petals, fruit receptacles, cultured tissues and roots whereas those of *MdCENb* could not be detected in any tissues at the 35 cycles of PCR (**Fig. 5A**) although RT–PCR Southern analysis revealed that the expression pattern of *MdCENb* was similar to that of *MdCENa* with a considerably lower expression level. To confirm the expression of *MdCEN* (*MdCENa* and *MdCENb*), an additional RT–PCR was performed using common PCR primers for *MdCENa* and *MdCENb*, reflecting the expression pattern of *MdCENa* (**Fig. 5A**). These expression patterns examined were confirmed by RT–PCR Southern analysis, where transcripts at the lower expression level could be detected.

To examine whether the expression levels of the *TFL1/CEN*-like genes change in the apical buds of SS and FBS throughout the growing season, RT–PCR analysis was carried out at different PCR cycles using total RNAs extracted from the apical buds of SS and FBS of the apple cultivar 'Jonathan' from June to October in 2004. It is noteworthy

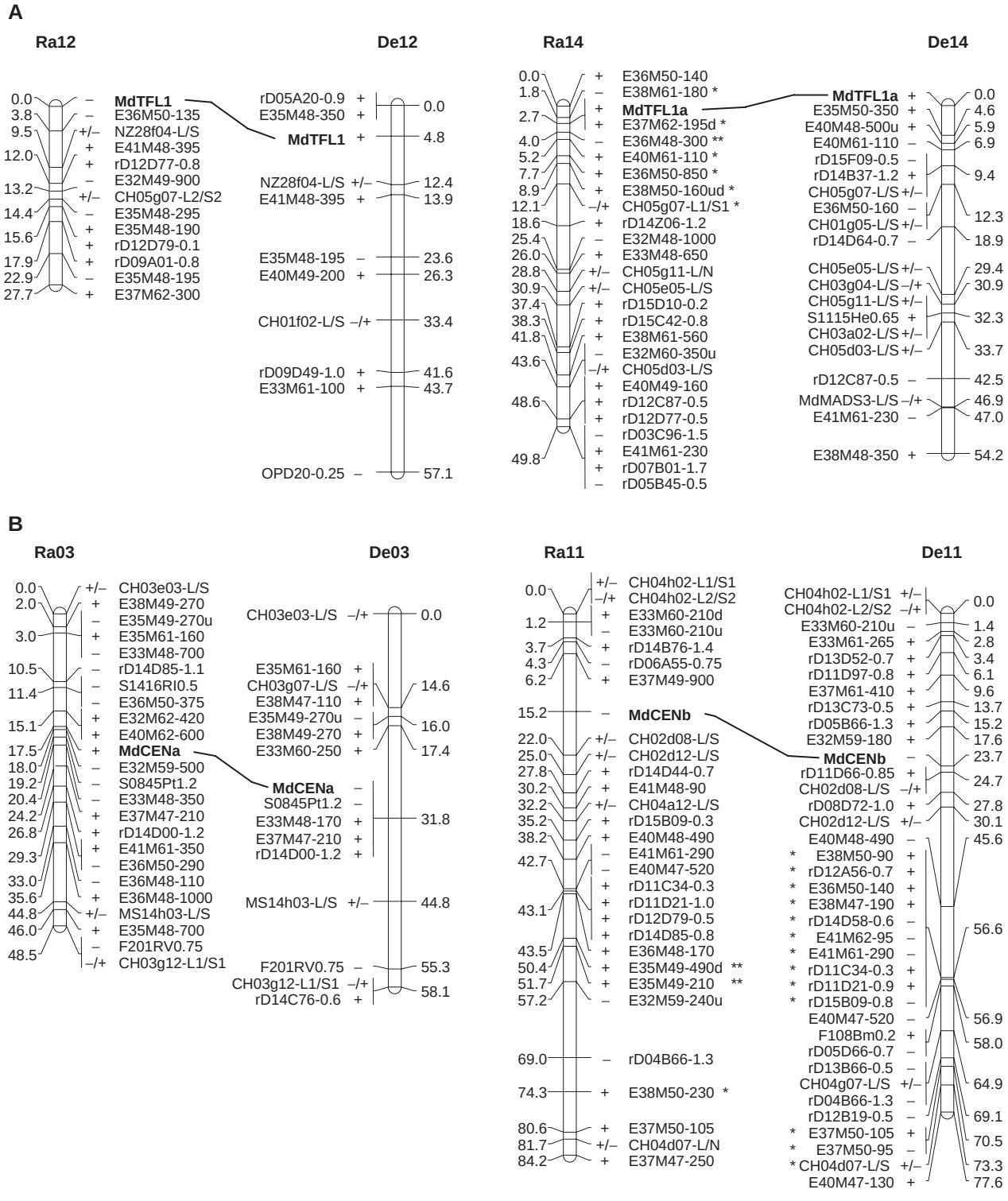


Fig. 3 Location of the *TFL1/CEN*-like genes on the linkage maps of the apple cultivars 'Ralls Janet' (Ra) and 'Delicious' (De). (A) Location of *MdTFL1* and *MdTFL1a* on linkage groups 12 (Ra12, De12) and 14 (Ra14, De14), respectively. (B) Location of *MdCENa* and *MdCENb* on linkage groups 3 (Ra03, De03) and 11 (Ra11, De11), respectively. The DNA markers on each linkage group are described in detail by Igarashi et al. (2008). Segregation ratios deviating significantly from the expected ratios ($P < 0.05$, 0.01) are indicated with one or two asterisks, respectively. The linkage phase information is provided as + or –, indicating on which of the homologous chromosomes the marker/allele is located.

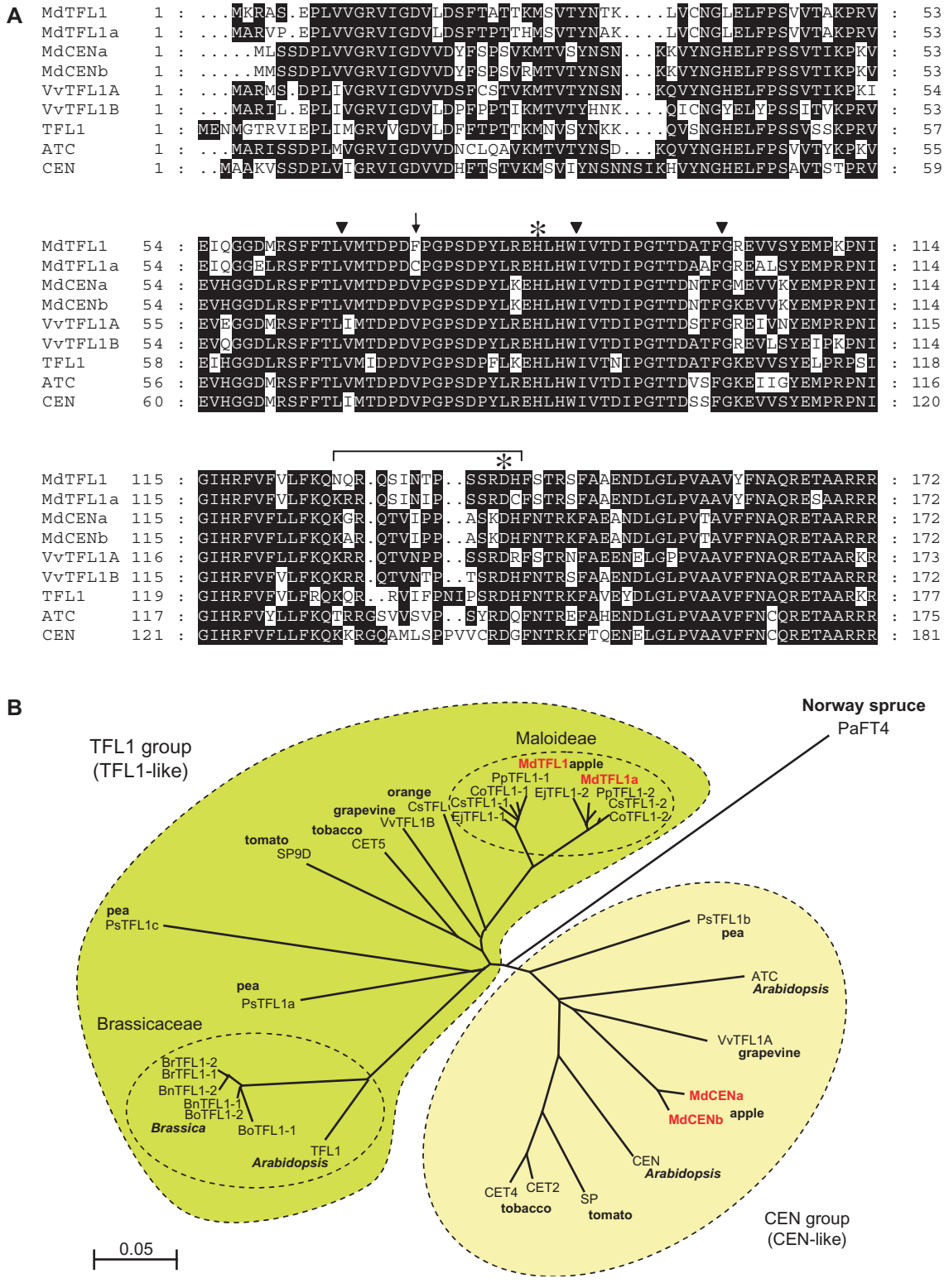

A
MdTFL1 1 : ...MKRAS.EPLVVGRVIGDVLDSFTATTKMSVTYNTK....LVCNGLELFPSVVTAKPRV : 53
MdTFL1a 1 : ...MARVP.EPLVVGRVIGDVLDSFTPTTHMSVTYNAK....LVCNGLELFPSVVTAKPRV : 53
MdCENa 1 : .....MLSSDPLVVGRVIGDVVDYFSPSVKMTVSYNSN...KKVYNGHELFPSSVTIKPKV : 53
MdCENb 1 : .....MMSSDPLVVGRVIGDVVDYFSPSVRMTVTYNSN...KKVYNGHELFPSSVTIKPKV : 53
VvTFL1A 1 : ...MARMS.DPLIVGRVIGDVVDSFCSTVKMTVTYNSN...KQVYNGHELFPSSVTIKPKI : 54
VvTFL1B 1 : ...MARIL.EPLIVGRVIGDVLDPFPPTIKMTVTYHNK....QICNGYELYPSSITVKPRV : 53
TFL1 1 : MENMGTRVIEPLIMGRVVGDVLDFFTPTTKMNVSYNKK....QVSNGHELFPSSVSSKPRV : 57
ATC 1 : ...MARISSDPLMVGRVIGDVVDNCLQAVKMTVTYNSD...KQVYNGHELFPSVVTYKPKV : 55
CEN 1 : ..MAAKVSSDPLVIGRVIGDVVDHFTSTVKMSVIYNSNNSIKHVYNGHELFPSAVTSTPRV : 59
MdTFL1 54 : EIQGGDMRSFFTLVMTDPDFPGPSDPYLREHLHWIVTDIPGTTDATFGREVVSYEMPKPNI : 114
MdTFL1a 54 : EIQGGELRSFFTLVMTDPDCPGPSDPYLREHLHWIVTDIPGTTDAAFGREALSYEMPRPNI : 114
MdCENa 54 : EVHGGDLRSFFTLVMTDPDVPGPSDPYLKEHLHWIVTDIPGTTDNTFGMEVVKYEMPRPNI : 114
MdCENb 54 : EVHGGDLRSFFTLVMTDPDVPGPSDPYLKEHLHWIVTDIPGTTDNTFGKEVVKYEMPRPNI : 114
VvTFL1A 55 : EVEGGDMRSFFTLIMTDPDVPGPSDPYLREHLHWIVTDIPGTTDSTFGREIVNYEMPRPNI : 115
VvTFL1B 54 : EVQGGDLRSFFTLVMTDPDVPGPSDPYLREHLHWIVTDIPGTTDATFGREVLSYEIPKPNI : 114
TFL1 58 : EIHGGDLRSFFTLVMIDPDVPGPSDPFLKEHLHWIVTNIPGTTDATFGKEVVSYELPRPSI : 118
ATC 56 : EVHGGDMRSFFTLVMTDPDVPGPSDPYLREHLHWIVTDIPGTTDVSFGKEIIGYEMPRPNI : 116
CEN 60 : EVHGGDMRSFFTLIMTDPDVPGPSDPYLREHLHWIVTDIPGTTDSSFGKEVVSYEMPRPNI : 120
MdTFL1 115 : GIHRFVFVLFKQNQR.QSINTP..SSRDHFSTRSFAAENDLGLPVAAVYFNAQRETAARRR : 172
MdTFL1a 115 : GIHRFVFVLFKQKRR.QSINIP..SSRDCFSTRSFAAENDLGLPVAAVYFNAQRESAARRR : 172
MdCENa 115 : GIHRFVFLLFKQKGR.QTVIPP..ASKDHFNTRKFAEANDLGLPVTAVFFNAQRETAARRR : 172
MdCENb 115 : GIHRFVFLLFKQKAR.QTVIPP..ASKDHFNTRKFAEANDLGLPVTAVFFNAQRETAARRR : 172
VvTFL1A 116 : GIHRFVFLLFKQKRR.QTVNPP..SSRDRFSTRNFAEENELGPPVAAVFFNAQRETAARKR : 173
VvTFL1B 115 : GIHRFVFVLFKQKRR.QTVNTP..TSRDHFNTRSFAAENDLGLPVAAVFFNAQRETAARRR : 172
TFL1 119 : GIHRFVFVLFRQKQR..RVIFPNIPSRDHFNTRKFAVEYDLGLPVAAVFFNAQRETAARKR : 177
ATC 117 : GIHRFVYLLFKQTRRGSVVSVP..SYRDQFNTREFAHENDLGLPVAAVFFNCQRETAARRR : 175
CEN 121 : GIHRFVFLLFKQKKRGQAMLSPPVVCRDGFNTRKFTQENELGLPVAAVFFNCQRETAARRR : 181
B
TFL1 group
(TFL1-like)
Maloideae
MdTFL1 apple
MdTFL1a
PpTFL1-1
CoTFL1-1
EjTFL1-2
PpTFL1-2
CsTFL1-2
CoTFL1-2
orange
CsTFL1
CsTFL1-1
EjTFL1-1
grapevine
VvTFL1B
tobacco
CET5
tomato
SP9D
pea
PsTFL1c
pea
PsTFL1a
Brassicaceae
BrTFL1-2
BrTFL1-1
BnTFL1-2
BnTFL1-1
BoTFL1-2
Brassica
BoTFL1-1
TFL1
Arabidopsis
Norway spruce
PaFT4
PsTFL1b
pea
ATC
Arabidopsis
VvTFL1A
grapevine
MdCENa
MdCENb
apple
CEN
Arabidopsis
CET4
CET2
tobacco
SP
tomato
CEN group
(CEN-like)
0.05

Fig. 4

that the flowering stage of apple was mid-May, and the timing of floral bud differentiation for the following spring was early July in Morioka, Japan (Kotoda et al. 2000). In the apical buds of SS, the expression of *MdTFL1* was maintained throughout the actively growing season and gradually decreased after mid-September (**Fig. 5B**). *MdTFL1a* was also expressed between June and October, with the transcriptional signals showing a broad monophasic peak (**Fig. 5B**). In contrast, both the transcripts of *MdCENa* and *MdCENb* were undetectable even by Southern blot after RT–PCR amplification (data not shown). In the apical buds of FBS, strong expression of *MdTFL1* was observed in mid-June at no more than 30 cycles of PCR, with the signals gradually decreasing from late June (**Fig. 5C**). The expression pattern of *MdTFL1a* showed three peaks in early June, late August and October (**Fig. 5C**), although the expression level of *MdTFL1a* seemed to be relatively lower than that of *MdTFL1*. On the other hand, *MdCENa* expression signals were considerably lower even at 35 cycles of PCR, although the expression pattern of *MdCENa* was somewhat similar to that of *MdTFL1a* except that the second peak of the expression of *MdCENa* was in late July (**Fig. 5C**). With regard to *MdCENb*, the expression could not be detected even by Southern blot after RT–PCR amplification (data not shown).

Spatial expression patterns of MdTFL1 and MdCENa with the β-glucuronidase (GUS) reporter gene in Arabidopsis

Because apple is a woody perennial plant, it is difficult to observe a spatial gene expression pattern in the whole plant throughout its life cycle. Therefore, to examine the spatial expression patterns of *MdTFL1* and *MdCENa*, we generated four types of transgenic *Arabidopsis* plants with the *GUS* reporter gene driven by the regulatory sequences of putative promoters (designated *pMdTFL1*:*GUS* and *pMdCENa*:*GUS*) or genomic fragments of those genes (designated *gMdTFL1*:*GUS* and *gMdCENa*:*GUS*) (**Fig. 6A**). Consequently, we obtained >30 independent transgenic lines for each *GUS* fusion transgene. Among them, representative transgenic lines, *pMdTFL1*:*GUS* (#3), *gMdTFL1*:*GUS* (#9), *pMdCENa*:*GUS* (#2) and (#3) and *gMdCENa*:*GUS* (#11), were selected, and the GUS staining patterns of these plants were observed throughout the developmental stages from seed to adult plant. In *pMdTFL1*:*GUS* (#3), no GUS expression was observed in the mature seed (**Fig. 6B**), 36-h-old germinating seed (**Fig. 6C**), and 52-h-old seedling (**Fig. 6D**). After 82 h, GUS expression was first detected weakly in the root–hypocotyl transition zone (**Fig. 6E**). In 5-day-old seedlings, strong GUS expression was observed in the hypocotyl and vascular tissue of the primary root but not in the elongation and meristematic zone (**Fig. 6F**). In a 16-day-old plant, GUS expression was clearly detected in the vegetative apical meristem, hypocotyl and roots developed in the early stage but rarely in the lateral roots (**Fig. 6G, I**). Upon progression of development, GUS expression was not detected in the whole inflorescence, including the reproductive organs (data not shown). In *gMdTFL1*:*GUS* lines, the spatial patterns of GUS expression were similar to those in *pMdTFL1*:*GUS* lines, although GUS was rarely expressed in the vegetative meristems (**Fig. 6H, J**).

On the other hand, in the *pMdCENa*:GUS lines, there were two types showing relatively different levels of GUS expression (higher or intermediate). In *pMdCENa*:*GUS* (#2), which showed a higher level of GUS expression as a whole, no GUS expression was observed in the mature seed (**Fig. 6K**). GUS staining was first detected weakly in cotyledon tips immediately after germination (**Fig. 6L**), and then weak GUS expression was detected in the root–hypocotyl transition zone 52 h after sowing (**Fig. 6M**). At 82 h after sowing, GUS expression appeared in the specialization zone of the primary root (**Fig. 6N**). In 5-day-old seedlings, strong GUS expression was observed in the primary root and the tips of expanded cotyledons (**Fig. 6O**). In a 16-day-old plant, GUS expression was also strongly detected in the tips of true leaves and both primary and lateral roots (**Fig. 6P**). The GUS expression patterns of *pMdCENa*:GUS (#3) which showed a intermediate level of GUS expression and *gMdCENa*:*GUS* (#11) were similar to that of *pMdCENa*:*GUS* (#2). However, the GUS

Fig. 4 Relationship among TFL1/CEN-like proteins found in dicot plants. (A) Comparison of the deduced protein sequence of *MdTFL1*, *MdTFL1a*, *MdCENa* and *MdCENb* (accession Nos. AB052994, AB366643, AB366641 and AB366642, respectively) with those of *TFL1* (*Arabidopsis*; Bradley et al. 1997, Ohshima et al. 1997), *CEN* (snapdragon, Bradley et al. 1996), and *VvTFL1A* (*VvTFL1*) and *VvTFL1B* (grapevine; Boss et al. 2006, Carmona et al. 2007). Sequence identities among TFL1- and CEN-like proteins are shown on a black background. The gaps indicated by dashes are attributed to the lack of amino acids. The asterisks indicate the amino acid positions related to antagonistic functions between TFL1 and FT (Hanzawa et al. 2005). The bracket represents the region of a potential ligand-binding pocket in TFL1/CEN-like proteins (Ahn et al. 2006). The triangles show the intron positions, which are conserved in TFL1/CEN-like proteins. The arrow indicates the position of the conserved amino acids in the members of the TFL1 group in the subfamily Maloideae of the Rosaceae. (B) Phylogenetic analysis of TFL1- and CEN-like proteins from apple, *Arabidopsis*, *Brassica* spp. (*Brassica napus* L., *Brassica oleracea* L. and *Brassica rapa* L.), Chinese quince (*Chaenomeles sinensis* Koehne.), grapevine, Japanese pear [*Pyrus pyrifolia* (Burm.) Nak.], loquat [*Eriobotrya japonica* (Thunb.) Lindl.], orange (*Citrus sinensis* L. Osbeck), pea, quince (*Cydonia oblonga* Mill.), tobacco and tomato (*Solanum lycopersicum* L.). The unit for the scale bar displays branch lengths (0.05 substitutions/site). The protein sequence data were obtained from the DNA database (DDBJ/EMBL/GenBank). For PsTFL1b, the sequence information of partial cDNA was used. The conifer Norway spruce (*Picea abies* L. Karst.) FT homolog, PaFT4, was used as an outgroup.

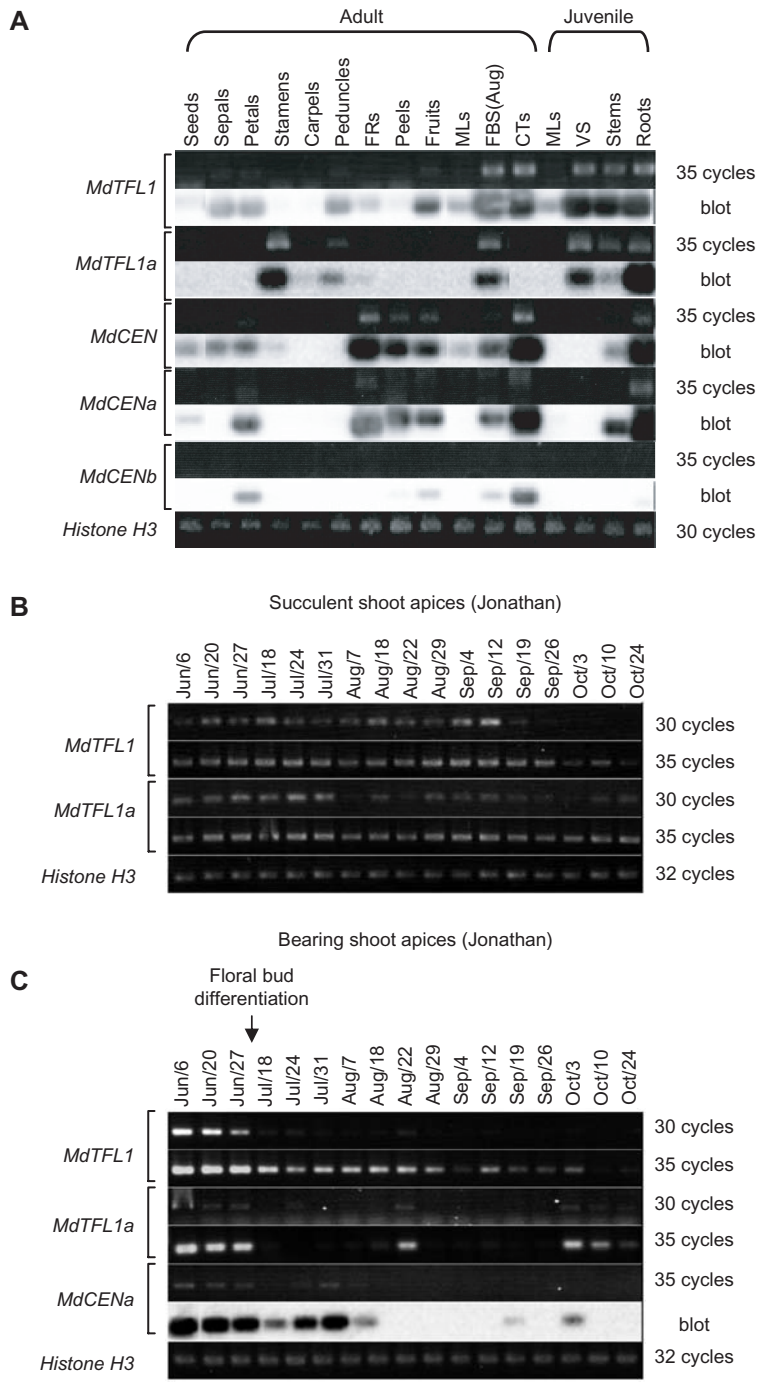


Fig. 5 Expression analyses of apple *TFL1*/*CEN*-like genes. (A) Expression patterns of four apple *TFL1*/*CEN*-like genes in various tissues of 'Fuji' apple and seedlings ('Fuji' × 'Orin'). The samples from left to right are as follows: seeds, sepals, petals, stamens, carpels, peduncles, fruit receptacles (FRs), fruit peels, fruits, mature leaves (MLs) and apical buds of FBS on August 7 [FBS(Aug)] in the adult phase; cultured tissues (CTs); and MLs, apical buds of vegetative shoots (VS), stems and roots of 2-week-old seedlings in the juvenile phase. For the CTs, shoots in the culture box 1 month after subculture were collected and used for the experiment. (B) RT–PCR products of *MdTFL1* or *MdTFL1a* in apical buds of SS of 'Jonathan' apple from June to October in 2004. (C) RT–PCR products of *MdTFL1*, *MdTFL1a* or *MdCENa* in apical buds of FBS of 'Jonathan' apple from June to October in 2004. The number of PCR cycles is indicated at the right of each panel. RT–PCR Southern blot (indicated as 'blot' at the right of A and C) was carried out to confirm the results of RT–PCR analysis. Vertical arrows indicate the timing of floral bud differentiation of 'Jonathan' apple in (C). Apple *Histone H3* was used as an internal control of gene expression.

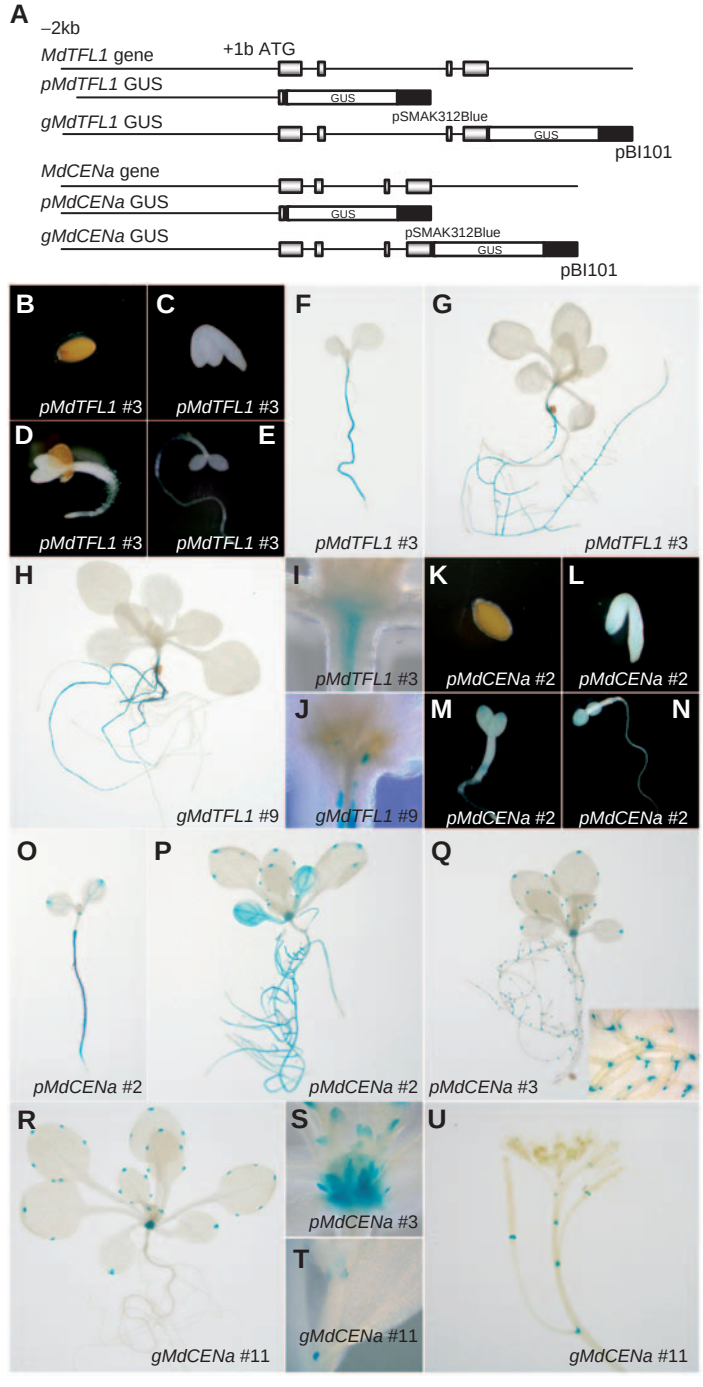


Fig. 6 Histochemical analysis of transgenic *Arabidopsis* plants carrying the *β-glucuronidase* (*GUS*) gene fused with *MdTFL1* or *MdCENa*. (A) Schematic representation of vector constructs for *pMdTFL1:GUS*, *gMdTFL1:GUS*, *pMdCENa:GUS* and *gMdCENa:GUS*; (B) mature seed of pMdTFL1 (#3); (C) 36-h-old germinating seed of *pMdTFL1* (#3); (D) 52-h-old seedling of *pMdTFL1* (#3); (E) 82-h-old seedling of *pMdTFL1* (#3); (F) 5-day-old seedling of *pMdTFL1* (#3); (G) 16-day-old seedling of *pMdTFL1* (#3); (H) 16-day-old seedling of *gMdTFL1* (#9); (I) vegetative meristem (VM) in a 16-day-old seedling of *pMdTFL1* (#3); (J) VM in a 16-day-old seedling of *gMdTFL1* (#9); (K) mature seed of *pMdCENa* (#2); (L) 36-h-old germinating seed of *pMdCENa* (#2); (M) 52-h-old seedling of *pMdCENa* (#2); (N) 82-h-old seedling of *pMdCENa* (#2); (O) 5-day-old seedling of *pMdCENa* (#2); (P) 16-day-old seedling of *pMdCENa* (#2); (Q) 16-day-old seedling of *pMdCENa* (#3); closed-up roots of (Q) are shown at the bottom on the right; (R) 18-day-old seedling of *gMdCENa* (#11); (S) VM in a 16-day-old seedling of *pMdCENa* (#3); (T) cauline inflorescence of *gMdCENa* (#11); (U) inflorescence of *gMdCENa* (#11). Transgenic plants in B–U were stained in a staining solution [1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), 0.1 M sodium phosphate buffer at pH 7.0, 5% methanol, and 0.01% Silwet L-77 (Nippon Unicar)] at 37°C for 24 h.

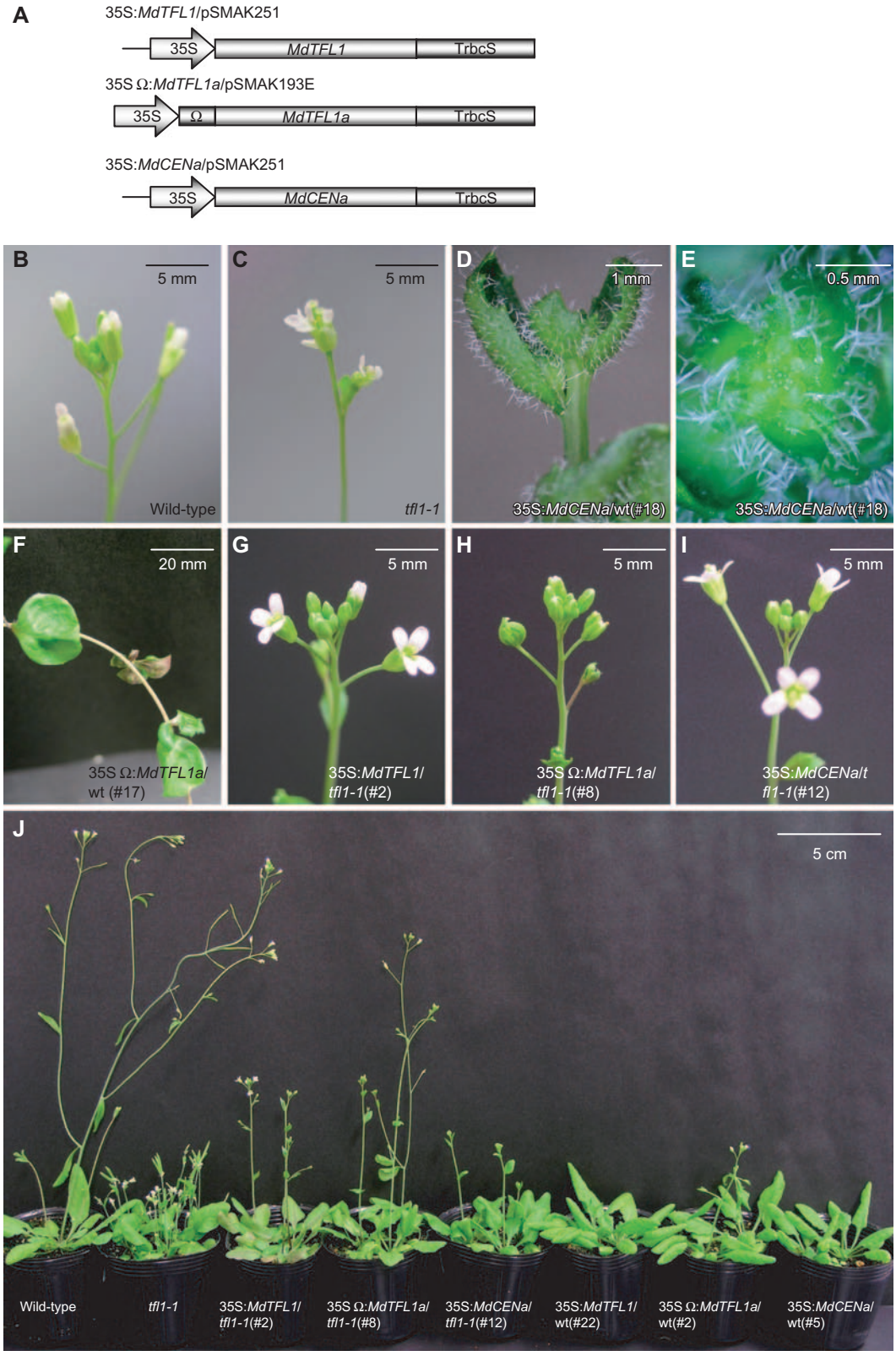


Fig. 7 Plant architecture of transgenic *Arabidopsis* with apple *TFL1/CEN*-like genes. (A) Schematic representation of the transformation vectors, 35S:*MdTFL1*, 35SΩ:*MdTFL1a* and 35S:*MdCENa*, used in this study. (B) Inflorescence of the wild type. (C) Inflorescence of *tfl1-1*. (D) Inflorescences of 35S:*MdCENa*/wt (#18). (E) Close-up view of the apical meristem of (D). (F) Cauline leaves of 35SΩ:*MdTFL1a*/wt (#17). (G) Inflorescence of 35S:*MdTFL1*/*tfl1-1* (#2). (H) Inflorescence of 35SΩ:*MdTFL1a*/*tfl1-1* (#8). (I) Inflorescence of 35S:*MdCENa*/*tfl1-1* (#12). (J) Appearance of control wild-type and transgenic *Arabidopsis* plants. From left to right, wt, *tfl1-1*, 35S:*MdTFL1*/*tfl1-1* (#2), 35SΩ:*MdTFL1a*/*tfl1-1* (#8), 35S:*MdCENa*/*tfl1-1* (#12), 35S:*MdTFL1*/wt (#22), 35SΩ:*MdTFL1a*/wt (#2) and 35S:*MdCENa*/wt (#5). Photographs of B, C, G–J at 35 d, D and E at 50 d, and F at 90 d after sowing. These plants were grown at 22°C under LD conditions (16 h light/8 h dark).

expression of *pMdCENa*:*GUS* (#3) was detected specifically in the bases and tips of the secondary roots (**Fig. 6Q**) and the GUS expression in the roots of *gMdCENa*:*GUS* (#11) was too weak to be detected (**Fig. 6R**). In aerial tissues, the GUS expression of *pMdCENa*:*GUS* and *gMdCENa*:*GUS* was detected strongly in the leaf tips, developing leaf primordia, stipules, axillary meristems on the base of rosette and cauline leaves, the junctions of siliques and pedicels (silique receptacles) and the base of flower stalks (**Fig. 6R–U**).

Constitutive expression of the apple TFL1/CEN-like genes affect flowering time and plant morphology in Arabidopsis

To determine the effects of apple *TFL1/CEN*-like genes on flowering time and inflorescence morphology, we generated transgenic *Arabidopsis* plants with *MdCENa* under the control of the cauliflower mosaic virus (CaMV) 35S promoter (35S) or with *MdTFL1a* under the control of the CaMV 35S promoter fused with the Ω sequence (35SΩ) (**Fig. 7A**). Consequently, we obtained >20 independent transgenic lines for 35S:*MdCENa* and 35SΩ:*MdTFL1a* (identified as 35S:*MdCENa*/wt and 35SΩ:*MdTFL1a*/wt, respectively). Transformants with 35S:*MdTFL1* (35S:*MdTFL1*/wt), as described in our previous paper, were used as a control for comparison (Kotoda and Wada 2005). Five lines of 35S:*MdCENa*/wt and 35SΩ:*MdTFL1a*/wt in the T_2 or T_3 generation were grown under long-day (LD) conditions (16 h light/8 h dark), and their phenotypes were examined. The morphology and flowering time of the inflorescence were affected in the 35S:*MdCENa*/wt and 35SΩ:*MdTFL1a*/wt lines. 35S:*MdCENa*/wt (#5) plants produced 18.1 ± 1.5 rosette leaves, and 35SΩ:*MdTFL1a*/wt (#2) plants produced 9.3 ± 0.2 rosette leaves. In contrast, the wild-type plants produced 6.8 ± 0.2 rosette leaves (**Table 1**). In 35S:*MdCENa*/wt lines with a strong phenotype, the inflorescences continued to develop after bolting. The coflorescences were generated from the main axis by the conversion of floral organs into inflorescences (**Fig. 7D**). The inflorescences of 35SΩ:*MdTFL1a*/wt with a strong phenotype were similar to those of 35S:*MdCENa*/wt and 35S:*MdTFL1*/wt, whereas the cauline leaves of 35Ω:*MdTFL1a*/wt became more rounded than those of 35S:*MdCENa*/wt or 35S:*MdTFL1*/wt (**Fig. 7F**). The three transgenic lines of 35SΩ:*MdTFL1a*/wt never flowered for >9 months (data not shown).

To investigate whether apple *TFL1* homologs function equivalently to *Arabidopsis TFL1*, we introduced the 35S:*MdTFL1* (Kotoda and Wada 2005), 35SΩ:*MdTFL1a* or 35S:*MdCENa* transgene into *Arabidopsis tfl1-1* mutant plants that show early flowering and determinate terminal flowering under LD conditions (**Fig. 7C, J** and **Table 2**). Consequently, >20 kanamycin-resistant lines with 35S:*MdTFL1*,

Table 1 The flowering time of transgenic plants (wild-type background) with 35S:*MdTFL1*, 35SΩ:*MdTFL1a* and 35S:*MdCENa*

Strain	No. of plants	No. of rosette leaves[a, b]	No. of cauline leaves[a, b]
Wt (Col)	20	6.8 ± 0.2	2.2 ± 0.1
35S:*MdTFL1*/wt (T_3)			
#21	9	10.9 ± 0.6	3.0 ± 0.3
#22	9	13.0 ± 0.6	3.3 ± 0.2
#28	10	15.8 ± 1.3	6.7 ± 0.3
35SΩ:*MdTFL1a*/wt (T_2)			
#2	10	9.3 ± 0.2	2.5 ± 0.2
#4	7	9.4 ± 0.4	2.6 ± 0.2
#13	10	9.5 ± 0.3	2.9 ± 0.2
#15	10	9.4 ± 0.2	2.7 ± 0.2
#17	15	8.3 ± 0.1	2.2 ± 0.2
35S:*MdCENa*/wt (T_2)			
#1	10	10.4 ± 0.2	2.6 ± 0.2
#2	10	9.5 ± 0.3	2.8 ± 0.2
#3	6	9.0 ± 0.6	2.7 ± 0.2
#5	10	18.1 ± 1.5	5.8 ± 0.6
#8	8	8.5 ± 0.4	2.4 ± 0.2
#10	5	8.6 ± 0.4	2.2 ± 0.2

Plants were grown on the potted soil at 22°C under the day length conditions of 16 h of light/8 h of darkness (long-day conditions).
[a]The numbers of rosette and cauline leaves were counted as an indicator of flowering time.
[b]Values are the mean ± SE.

Table 2 The flowering time of transgenic plants (*tfl1-1* background) with 35S:*MdTFL1*, 35SΩ:*MdTFL1a*, and 35S:*MdCENa*

Strain	No. of plants	No. of rosette leaves[a, b]	No. of cauline leaves[a, b]
Wt (Col)	20	6.8 ± 0.2	2.2 ± 0.1
tfl1-1	20	6.2 ± 0.2	0.9 ± 0.1
35S:*MdTFL1*/*tfl1-1* (T_3)			
#1	15	12.5 ± 0.4	4.7 ± 0.4
#2	15	12.6 ± 0.3	3.6 ± 0.2
#4	14	12.6 ± 0.3	5.4 ± 0.2
#5	15	12.8 ± 0.6	4.7 ± 0.3
35SΩ:*MdTFL1a*/*tfl1-1* (T_2)			
#4	15	9.7 ± 0.3	2.6 ± 0.1
#7	15	7.5 ± 0.3	2.0 ± 0.1
#8	20	11.2 ± 0.3	4.3 ± 0.3
35S:*MdCENa*/*tfl1-1* (T_2)			
#10	15	10.3 ± 0.3	1.7 ± 0.2
#12	15	11.7 ± 0.4	3.7 ± 0.1
#13	15	12.3 ± 0.6	3.7 ± 0.4
#15	14	14.7 ± 0.6	6.1 ± 0.4

Plants were grown on the potted soil at 22°C under the day length conditions of 16 h of light/8 h of darkness (long-day conditions).
[a]The number of rosette and cauline leaves were counted as an indicator of flowering time.
[b]Values are mean ± SE.

35SΩ:*MdTFL1a* or 35S:*MdCENa* were obtained (identified as 35S:*MdTFL1*/*tfl1-1*, 35SΩ:*MdTFL1a*/*tfl1-1* and 35S:*MdCENa*/*tfl1-1*, respectively). Among them, four lines of 35S:*MdTFL1*/*tfl1-1*, three lines of 35SΩ:*MdTFL1a*/*tfl1-1* and four lines of 35S:*MdCENa*/*tfl1-1* in the T_2 or T_3 generation were selected and grown under LD conditions. In these transgenic lines, all inflorescences, including coflorescences, were basically maintained (**Fig. 7G–I**), and the flowering time was more delayed than that of *tfl1* mutant and wild-type plants (**Fig. 7J** and **Table 2**). 35S:*MdTFL1*/*tfl1-1* (#5) plants produced 12.8 ± 0.6 rosette leaves, 35SΩ:*MdTFL1a*/*tfl1-1* (#8) plants produced 11.2 ± 0.3 rosette leaves and 35S:*MdCENa*/*tfl1-1* (#12) plants produced 11.7 ± 0.4 rosette leaves. In contrast, wild-type and *tfl1-1* plants produced 6.8 ± 0.2 and 6.2 ± 0.2 rosette leaves, respectively (**Table 2**).

Discussion

Because the sequences in the coding regions of *MdTFL1* and *MdTFL1a* were too similar to clarify the functional difference between the two genes by the knockdown strategy, we inferred the function from their expression patterns. As a result, relatively high levels of accumulation of both *MdTFL1* and *MdTFL1a* transcripts were observed in tissues of apple seedlings in the juvenile phase (**Fig. 5A**). In addition, both *MdTFL1* and *MdTFL1a* were expressed throughout the growing season in the apical buds of SS, which do not produce flower buds (**Fig. 5B**). These results suggested that not only *MdTFL1* but also *MdTFL1a* participates in the maintenance of the juvenile phase and vegetative shoot growth throughout the growing season, although the expression of *MdTFL1a* was maintained longer in SS. This is also supported by the phenotype of transgenic *Arabidopsis* expressing *MdTFL1a*, which showed prolonged vegetative (V) and first-inflorescence (I_1) phases (V phase, rosette leaves are produced; I_1 phase, shoots with a subtending cauline leaf are produced; phases defined by Ratcliffe et al. 1998) similar to that of transgenic *Arabidopsis* with *MdTFL1* (**Fig. 7J** and **Tables 1, 2**). In apical buds of FBS, *MdTFL1* and *MdTFL1a* were both expressed in June, which is the period of vegetative growth, and the expression of these genes decreased dramatically after late June, which is the transition period from vegetative growth to reproductive growth in the adult phase, suggesting that these genes regulate the fate of the inflorescence meristems coordinately at the appropriate time (**Fig. 5C**). These results are consistent with the fact that the shoot apical meristems stop producing any novel leaf primordia or axillary meristems at the onset of floral transition in early summer (Foster et al. 2003). Since flower buds of apple are mixed, containing both vegetative and reproductive parts, the two peaks of the accumulation of *MdTFL1a* transcripts in late summer and autumn might be associated with the growth of vegetative or floral primordia inside the floral buds after the onset of floral transition (**Fig. 5C**). Regarding *MdTFL1*, the expression patterns of *MdTFL1* were further supported by those of the *GUS* reporter gene in transgenic *Arabidopsis* lines with *pMdTFL1*:*GUS* and *gMdTFL1*:*GUS* (**Fig. 6F–J**). Heterologous GUS expression in the vegetative apical meristem of transgenic *Arabidopsis* (**Fig. 6I**) was consistent with the result of RT–PCR (**Fig. 5A**). In future studies, GUS expression analysis using transgenic apple with *pMdTFL1*:*GUS* or *gMdTFL1*:*GUS* will be required. The result of DNA blot and RT–PCR analysis and the phenotype of 35S:*MdTFL1*/wt obtained in this study on *MdTFL1*, which was used as a comparison with the other apple *TFL1*/*CEN*-like genes, was consistent with that of a previous study (Kotoda and Wada 2005).

On the basis of comparisons of *MdTFL1* with *MdTFL1a*, four conserved sequences were found in the putative promoter regions (C1, C2 and C3) and flanking regions close to the initiation codon (C4) (**Fig. 2B, C**). These common motifs may reflect the similar expression profiles of *MdTFL1* and *MdTFL1a* in the vegetative tissues. Interestingly, the third intron in the *MdTFL1a* gene was 10-fold longer than that of *MdTFL1* and contained two inverted sequences (-IS1′ and IS″, **Fig. 2B**). Moreover, a sequence similar to -IS1′ and IS1″ was also found in the putative *MdTFL1a* promoter region (IS1, **Fig. 2B**). The IS1 sequence was found to be well aligned with several expressed sequence tag (EST) clones in the DNA database derived from various tissues of apple, suggesting that active IS1-like sequences are present widely in the apple genome (data not shown). Recent studies revealed that small non-coding RNAs (ncRNAs) play a major role in regulating gene expression and that ncRNA genes coding functional RNA molecules are often found in the introns (Brown et al. 2003). It is possible that inverted sequences, such as the IS1 element in the *MdTFL1a* gene, become target sequences for the transcriptional regulator or are related to the intronic ncRNA and play a role in regulating gene expression.

The exon–intron organization, coding regions and expression patterns of *MdCENa* and *MdCENb* in the *CEN* group were highly similar. In addition, *MdCENa* and *MdCENb* were located at a similar position on LG 3 and LG 11 of apple linkage groups, respectively (**Fig. 3B**). However, the upstream putative promoter sequences of these genes were considerably different, and the expression level of *MdCENb* was much lower than that of *MdCENa* (**Fig. 5A**). Based on these results, the function of *MdCENb* may have been lost during evolution, probably due to gene duplication by the polyploid origin of the Maloideae (Sax 1933). On the other hand, *MdCENa* was expressed in reproductive organ tissues such as fruit receptacles, as well as in vegetative tissues, such as roots and cultured tissues, unlike *MdTFL1* and *MdTFL1a*. In addition, the expression of *MdCENa* could not be detected in the apices of SS and vegetative shoots of seedlings (**Fig. 5A, B**).

These results suggest that *MdCENa* would function in a different manner from *MdTFL1* and *MdTFL1a*, although *MdCENa* seemed to have the potential to complement the function of *TFL1*-like genes and maintain shoot growth from the analysis of the transgenic *Arabidopsis* expressing *MdCENa* in the wild-type and *tfl1* mutant background (Fig. 7 and **Tables 1, 2**). Transgenic tobacco plants constitutively expressing *CEN* from *Antirrhinum* showed a prolonged vegetative phase, but those expressing *TFL1* from *Arabidopsis* did not show an altered phenotype (Amaya et al. 1999). Additionally, Ahn et al. (2006) demonstrated that a chimeric fusion protein of TFL1 replaced with a 17 amino acid segment (called segment B) in the fourth exon of *CEN* could function like CEN in tobacco, suggesting that segment B is essential for the expression of CEN function. It is interesting that VvTFL1 (VvTFL1A) from grapevine, showing high similarity to MdCENa and MdCENb (**Fig. 4**), delayed flowering in tobacco, suggesting a CEN function for VvTFL1 (Boss et al. 2006). *MdCENa* also may have a *CEN* function, since apple *CEN* and *TFL1* group genes shared only six identical residues in this segment B (**Fig. 4A**). The GUS expression in the transgenic *Arabidopsis* lines of *pMdCENa*:*GUS* and *gMdCENa*:*GUS* also supported the results of RT–PCR analysis in that it was detected in the silique receptacles, roots and axillary meristems but not in the apical meristems (Fig. 6O–U). As a whole, the activity of the 2.15 kb promoter and 3.45 kb genomic regions of *MdCENa* seemed to reflect the actual gene expression in apple. The GUS expression patterns in the lines with *pMdCENa*:*GUS* and *gMdCENa*:*GUS* are reminiscent of that in transgenic *Arabidopsis* with *GUS* under the control of the *LpTFL1* (a perennial ryegrass *TFL1* homolog) promoter (*pLpTFL1*:*GUS*) (Jensen et al. 2001). *LpTFL1* controls floral transition in perennial ryegrass, and GUS expression of transgenic *Arabidopsis* with *pLpTFL1*:*GUS* is restricted to the vegetative axillary meristems in *Arabidopsis* (Jensen et al. 2001). Thus, *MdCENa* may be involved in the development of the proliferating tissues or organs including fruit receptacles, which develop into edible parts, but not in the control of the transition from the juvenile to the adult phase, as demonstrated by the fact that it was completely silenced in the apical buds of vegetative shoots of juvenile seedlings; however, it might influence the regulation of flowering in apple indirectly. The expression pattern of GUS in transgenic *Arabidopsis* with *pMdCENa*:*GUS* or *gMdCENa*:*GUS* is different from that of *ATC* in *Arabidopsis*, where *ATC* transcripts are detected in the tissues surrounding vascular bundles in the hypocotyl of 14-day-old plants (Mimida et al. 2001), suggesting that the function of *MdCENa* is different from that of *ATC*. The function of *ATC* in *Arabidopsis*, however, remains to be investigated.

The number of exons/introns and the sequence of coding regions in *TFL1/CEN*-like genes were basically conserved between apple and *Arabidopsis*, and the phenotypes of *Arabidopsis* plants constitutively expressing apple *TFL1/CEN*-like genes (Fig. 7 and **Tables 1, 2**) closely resembled those exhibited by *Arabidopsis* expressing *TFL1/CEN* group members from other plant species (Mimida et al. 2001, Jensen et al. 2001, Pillitteri et al. 2004, Ordidge et al. 2005, Guo et al. 2006, Boss et al. 2006, Carmona et al. 2007, Igasaki et al. 2008). However, apple *TFL1/CEN*-like genes showed expression patterns characteristic of each gene. These results suggest that each *TFL1/CEN*-like gene of apple has developed the role to specialize by changing its expression pattern in the process of evolution. It is widely known that nearly all higher eukaryotes, including flowering plants, have genomes that exhibit extensive gene redundancy due to genome doubling or polyploidy (Wendel 2000, Adams and Wendel 2005). In polyploid plants, there is a certain level of duplication within the genome and the expression of the duplicated genes (homoeologs) is retained or lost on an evolutionary timescale. The origin of the Maloideae subfamily of the Rosaceae, which includes apple, has also been studied for a long time (Sax 1933, Chevreau et al. 1985, Morgan et al. 1994, Evans and Campbell 2002, Luby 2003). Maliepaard et al. (1998) first identified the duplication of LG5 and LG10 in its entirety by examining the positions of EST-RFLP (restriction fragment length polymorphism) markers on an apple linkage map of the cross 'Prima' × 'Fiesta', which are domestic apple cultivars. In addition, LGs 3 and 11 and LGs 12 and 14 seem to be partly homoeologous to each other, respectively (Maliepaard et al. 1998, Gardiner et al. 2007). The homoeology of those linkage groups was also supported by our results showing that *MdTFL1* and *MdTFL1a* were mapped on LGs 12 and 14, respectively (**Fig. 3A**), and *MdCENa* and *MdCENb*, on LGs 3 and 11, respectively (**Fig. 3B**). Adams et al. (2003) show that a high percentage of genes exhibit silencing or biased expression that is developmentally regulated and that, in a few case, alternative homoeologs have been reciprocally silenced in different organs, suggesting subfunctionalization. Accordingly, *TFL1*-like genes might also have been subfunctionalized by altering the expression in some organs or tissues, whereas one of the *CEN*-like genes might have been silenced in most organs during evolution.

In this study, we collectively described four *TFL1/CEN*-like genes of apple for the first time and elucidated the function of each gene from the viewpoint of their different expression pattern, transformation of a heterologous plant and the hypothesis of a polyploidy origin of apple. However, molecular studies on the mechanism of flowering of apple have been made complicated by the fact that, in most cases, there are pairs of homologous genes in the apple genome (Mimida et al 2007). Our results suggest that *MdTFL1a* functions mainly in vegetative tissues, redundantly with *MdTFL1*, whereas *MdCENa* may function in the proliferating tissues whether they are vegetative or reproductive. Further studies will be required to clarify the function of *TFL1/CEN*-like genes

by analyzing transgenic apples, for example with an RNAi (RNA interference) construct, and by investigating their spatial and temporal expression patterns by *in situ* hybridization in order to develop the technology to control the flowering of apple in the juvenile and adult phase.

Materials and Methods

Plant materials

The tissue samples of the apple (*Malus*×*domestica* Borkh.) cvs. 'Fuji' (age: 20 years) and 'Jonathan' (age: 19–20 years) were collected from the experimental field at the National Institute of Fruit Tree Science in Morioka, Japan. The seedlings from a cross between the apple cvs. 'Fuji' and 'Orin' were used for the expression analysis of apple *TFL1*/*CEN*-like genes in the juvenile phase. The *A. thaliana tfl1-1* mutant (Columbia background) was obtained from the *Arabidopsis* Biological Resource Center at Ohio State University and employed for mutant rescue experiments.

Isolation of the genomic clones

For construction of the genomic library, the genomic DNA from leaves of 'Fuji' apple was extracted according to the CTAB (cetyltrimethylammonium bromide) method as described by Kotoda et al. (2000). The λ phage genomic library was constructed using a Lambda FIXII/*Xho*I Partial Fill-in Vector Kit (Stratagene, La Jolla, CA, USA) and a Lambda DNA Packaging System (Promega, Madison, WI, USA). The average size of the inserted genomic DNAs was about 10,000 bp. To obtain a *CEN*-like genomic sequence from apple, PCR amplification of DNA fragments was performed with a pair of degenerate primers 3F and 4R. The PCR-amplified fragments were cloned into the pT7Blue T-Vector (Novagen, Darmstadt, Germany), and these clones were then sequenced (designated gMdCENa34 and gMdCENb34). The gMdCENa34 or the first to second exon-coding region of *MdTFL1* cDNA labeled with a PCR digoxigenin (DIG) mixture (Roche Diagnostics, Mannheim, Germany) was used as a probe to screen 2.0×10^5 p.f.u. of the apple genomic library to obtain *TFL1*/*CEN*-like genes. The hybridization signals were detected in the same manner as described in the section on hybridization analysis. The λ clone DNAs from positive plaques were digested with the restriction enzymes *Not*I or *Xba*I and ligated into the corresponding site of pBluescriptII SK (+). These clones were then sequenced. For inverse PCR (I-PCR) of *MdCENb*, 15 µg of genomic DNA was digested with *Xba*I and self-ligated with T4 DNA ligase (TAKARA BIO INC., Otsu, Japan). I-PCR was carried out with primers MdCENb480R, MdCENbT8F and MdCENb3'UTRF. The amplified PCR fragments were cloned into the pT7Blue T-Vector, and these clones were then sequenced. Primer sequences and positions are shown in **Supplementary Table S1 and Supplementary Fig. S3A**, respectively.

Cloning of cDNAs

To obtain the cDNAs of *MdTFL1a* and *MdCENa*, the first-strand cDNAs were synthesized from 1 µg of total RNAs from apical buds of FBS in 20 µl of a reaction mixture using the ReverTra Ace kit (Toyobo, Tokyo, Japan). A PCR fragment of *MdTFL1a* was amplified with the primers, MdTFL1aRTF1-*Xba*I and MdTFL1aRTR1, followed by a nested PCR with a pair of primers, MdTFL1aRTF1-*Xba*I and MdTFL1aRTR2, for *MdTFL1a*. A PCR fragment of *MdCENa* was amplified with the primers, MdCENaF1 and MdCENaR1, followed by a nested PCR with MdCENaF2-*Xba*I and MdCENaR2-*Sac*I for *MdCENa*. The amplified PCR products were cloned into the pT7Blue T-Vector. Primer sequences and positions are shown in **Supplementary Table S1 and Supplementary Fig. S3A**, respectively.

Mapping of apple TFL/CEN-like genes

SSR markers for the four genes were developed, and SSR marker detection was performed using a post-PCR fluorescence labeling method (Kukita et al. 2002) for an F_1 mapping population with 83 seedlings from the cross between 'Ralls Janet' and Mitsubakaido (*Malus sieboldii* Rehder) and another one with 72 seedlings from the cross between 'Delicious' and Mitsubakaido (Igarashi et al. 2008). The PCR primers used are as follows: MdTFL1P-SSR2-U and MdTFL1P-SSR2-L for *MdTFL1*; MdTFL1A-SSR1-U and MdTFL1A-SSR2-L for *MdTFL1a*; MdCENa-SSR3-U and MdCENa-SSR2-L for *MdCENa*; and MdCENb-SSR-U and MdCENb-SSR-L for *MdCENb* (for primer sequences, see **Supplementary Table S1**). For fluorescence labeling, some nucleotides were added to the 5′ end of the forward primers to obtain the sequence ATT. A 2 µl aliquot of the PCR product was added to 4 µl of the post-PCR labeling reaction buffer [15mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 0.1 µM R110-ddUTP, 0.006 U μl^{-1} Klenow fragment and 0.0288 U μl^{-1} Thermo Sequenase DNA Polymerase. The mixed solution was incubated at 37°C for 5 min, followed by 57°C for 15 min. The reaction was stopped by the addition of 6 µl of 20 mM EDTA. The appropriate amounts of labeling products were purified by ethanol precipitation before loading onto an ABI3100 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). The resulting electropherogram was analyzed using GeneScan Analysis Software and Genotyper Software (Applied Biosystem). The linkage map was constructed using JoinMap ver. 3.0 (Van Ooijen and Voorrips 2001).

Sequence analysis

The nucleotide sequence was determined using a DTCS Quick Start Kit for Dye Terminator Cycle Sequencing (Beckman Coulter, Fullerton, CA, USA) and an automated DNA sequencer CEQ 8000 (Beckman Coulter). Searching for the conserved sequence regions in the genomic *MdTFL1* and *MdTFL1a* was performed with the MEME system ver. 3.0

(http://meme.sdsc.edu/meme/website/intro.html). Amino acid sequences were analyzed using the CLUSTAL X multiple sequence alignment program ver. 1.83 (Jeanmougin et al. 1998) and GeneDoc (Nicholas et al. 1997). The phylogenetic tree was displayed using the Njplot-unrooted (Perrière and Gouy 1996). A homology search was performed with BLAST 2 through the GenomeNet (Bioinformatics Center, Kyoto University, http://www.genome.jp/).

Nucleic acid extraction and hybridization analysis

Southern hybridization was carried out according to the method described by Sambrook et al. (1989). The genomic DNA (15 µg) was isolated from mature leaves of 'Fuji', digested with *Eco*RI, *Hin*dIII or *Xba*I, electrophoresed on a 0.8% agarose gel and then blotted onto Hybond-N+ nylon membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The blotted membranes were hybridized with the DIG-labeled first to second exon-coding region of *MdTFL1* cDNA or gMdCENa34. The hybridization was performed in DIG Easy Hyb (Roche) at 42°C for 16 h followed by two rinses in 2× SSC containing 1% (w/v) SDS at room temperature for 10 min and two washes in 0.5× SSC containing 1% (w/v) SDS at 68°C for 10 min. Chemiluminescent signals were visualized using the LAS1000 image analyzer (Fuji Photo Film, Tokyo, Japan).

Expression analysis by RT–PCR

For RT–PCR analysis, total RNAs were extracted from sepals, petals, stamens, carpels, peduncles, fruit receptacles, fruit peels, whole young fruits, mature leaves and apical buds of FBS in 'Fuji' apple trees, and apical buds of FBS and SS (from June to October in 2004) in 'Jonathan' apple trees and leaves, apices of vegetative shoots, stems, and roots of 14-day-old seedling and in vitro-cultured tissues (shoots) of 'Fuji' apple. The first-strand cDNAs were synthesized from 3 µg of total RNAs in 60 µl of a reaction mixture using a ReverTra Ace kit (Toyobo). The subsequent PCRs were performed with 1 µl of the first-strand cDNA mix as templates. A DNA fragment of each transcript of *TFL1/CEN*-like genes was amplified with the following primers: MdTFL(286→307) and MdTFL1 (575→598) for *MdTFL1*; MdTFL(286→307) and MdTFL1a (585←608) for *MdTFL1a*; MdCEN-F and MdCEN-R for *MdCEN* (*MdCENa*/*MdCENb*); MdCEN-F and MdCENa-R for *MdCENa*; and MdCENb-F and MdCENb-R for *MdCENb* (for primer sequences and positions, see **Supplementary Table S1 and Supplementary Fig. S3B**, respectively). As an internal control, a fragment of apple *Histone H3* was amplified with a pair of primers: 5′-TGA AGA AGC CCC ACA GAT A-3′ and 5′-ACA CAA GAA ACT ATA AAC C-3′ as described by Kotoda et al. (2006). The thermal cycle programs were as follows: 96°C for 3 min, followed by 30–35 cycles of 9°C for 20 s, 60°C for 30 s, and 72°C for 30 s for *MdTFL1*, *MdTFL1a*, *MdCEN*, *MdCENa* and *MdCENb*, and 96°C for 3 min, followed by 30–32 cycles of at 96°C for 20 s, 56°C for 30 s, and 72°C for 30 s for *Histone H3*. The electrophoresed and blotted RT–PCR products were hybridized with the DIG-labeled full-length cDNA of *MdTFL1* or *MdCENa*.

Construction of the transformation vector for the constitutive expression of MdTFL1a and MdCENa

To construct a vector for the constitutive expression of *MdTFL1a*, the coding region of *MdTFL1a* was amplified by PCR with a pair of primers, MdTFL1aRTF1-*Xba*I and MdTFL1aR-*Kpn*I. An amplified PCR product was subsequently digested with *Xba*I and *Kpn*I and then cloned into the *Xba*I–*Kpn*I sites of the modified pSMAK193E binary vector (35SΩ/pSMAK193E) to be placed between the CaMV 35S promoter fused with the Ω sequence (Gallie and Walbot, 1992) and the terminator of rubisco (TrbcS). For *MdCENa*, the coding region of *MdCENa* was amplified by PCR with a pair of primers, MdCENaF2-*Xba*I and MdCENaR2-*Sac*I. An amplified PCR product was subsequently digested with *Xba*I and *Sac*I and then cloned into the *Xba*I–*Sac*I sites of pSMAK251 to be placed between the promoter 35S and the nopaline synthase terminator (Tnos). The transformation vector of *MdTFL1* for constitutive expression was used as described previously (Kotoda et al. 2005). Primer sets used in vector construction are listed in **Supplementary Table S1**.

Construction of the transformation vector for the MdTFL1 and MdCENa promoters with the GUS reporter gene

For the *pMdTFL1*:*GUS* chimeric gene, the genomic *MdTFL1* sequence from −2,129 to +9 was amplified by PCR with primers (−2kMdTFL1BEIF-*Bam*HI and MdTFL19bR-*Eco*RV). The PCR products were digested with *Bam*HI and *Eco*RV and then cloned in-frame into the *Bam*HI–*Sma*I sites of the pBI221 vector to be placed upstream of *GUS* (designated *pMdTFL1*:*GUS*:Tnos/pBI221). After being digested with *Eco*RI, the DNA fragment containing *MdTFL1* (−1,800 to +9):*GUS*:Tnos was cloned into the *Eco*RI site of the pSMAK312Blue binary vector. For the *gMdTFL1*:*GUS* chimeric gene, the genomic *MdTFL1* sequence from −2,129 to +1,832 (before the stop codon) was amplified by PCR with primers (−2kMdTFL1BEIF-*Bam*HI and +1.8kMdTFL1R) designed on the basis of the sequences for the cDNA and λ phage clone of *MdTFL1*. An amplified PCR product was cloned into the *Sma*I site of pBI101 (Clontech Laboratories Inc., Mountainview, CA, USA) in-frame by blunt end ligation. For the *pMdCENa*:*GUS* chimeric gene, the genomic *MdCENa* sequence from −2,129 to +20 was amplified by PCR with primers (−2kMdCENaF-*Eco*RI and +20MdCENaR-*Eco*RV). An amplified PCR product was digested with *Eco*RI and *Eco*RV and then cloned into the *Eco*RI–*Eco*RV sites of the pBI221 vector to be placed upstream of *GUS* in-frame (designated as *pMdCENa*:*GUS*:Tnos/pBI221). After being digested

with *Eco*RI, the cassette of *pMdCENa*:*GUS*:Tnos was cloned into the *Eco*RI site of the pSMAK312Blue binary vector. For the *gMdCENa*:*GUS* chimeric gene, the genomic *MdCENa* sequence from −2,129 to +1,349 (before the stop codon) was amplified by PCR with primers (−2kMdCENaF-*Eco*RI and +1.3kMdCENaR). An amplified PCR product was cloned into the *Sma*I site of pBI101 (Clontech) in-frame by blunt end ligation. Primer sets used in vector construction are listed in **Supplementary Table S1**.

Arabidopsis transformation

Arabidopsis was transformed with the constructs used in this study via the *Agrobacterium tumefaciens* strains, EHA101 (for the constructs based on the pSMAK series) or GV3126 (for the constructs based on the pBI101), by the infiltration method as described by Bechtold et al. (1993). To select the transgenic plants, bleach-sterilized seeds from infected plants were plated on an MS plate (Murashige and Skoog 1962) containing carbenicillin (250 mg l^{-1}) and kanamycin (35 mg l^{-1}). The obtained kanamycin-resistant seeds for the T_1, T_2 or T_3 generation (primary transformants were defined as T_1 plants) were sown on agar plates followed by stratification at 4°C for 3 d, and grown in a growth chamber controlled at 22°C under LD conditions (16 h light/8 h dark).

Histochemical analysis in transgenic Arabidopsis with the GUS gene

To detect GUS activity, GUS staining was performed according to the method described by Jefferson (1987). Whole plants of transgenic *Arabidopsis* with the *GUS* gene were stained in a staining solution [1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc), a 0.1 M sodium phosphate buffer at pH 7.0, 5% methanol, and 0.01% Silwet L-77 (Nippon Unicar, Tokyo, Japan)] at 37°C for 24 h. The stained plants were bleached with 70% ethanol.

Supplementary data

Supplementary data are available at PCP online.

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