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Research paper

Apple FLOWERING LOCUS T proteins interact with transcription factors implicated in cell growth and organ development

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Understanding the flowering process in apple (*Malus × domestica* Borkh.) is essential for developing methods to shorten the breeding period and regulate fruit yield. It is known that FLOWERING LOCUS T (FT) acts as a transmissible floral inducer in the *Arabidopsis* flowering network system. To clarify the molecular network of two apple FT orthologues, *MdFT1* and *MdFT2*, we performed a yeast two-hybrid screen to identify proteins that interact with *MdFT1*. We identified several transcription factors, including two members of the TCP (TEOSINTE BRANCHED1, CYCLOIDEA and PROLIFERATING CELL FACTORS) family, designated *MdTCP2* and *MdTCP4*, and an *Arabidopsis thaliana* VOZ1 (Vascular plant One Zinc finger protein1)-like protein, designated *MdVOZ1*. *MdTCP2* and *MdVOZ1* also interacted with *MdFT2* in yeast. The expression domain of *MdTCP2* and *MdVOZ1* partially overlapped with that of *MdFT1* and *MdFT2*, most strikingly in apple fruit tissue, further suggesting a potential interaction *in vivo*. Constitutive expression of *MdTCP2*, *MdTCP4* and *MdVOZ1* in *Arabidopsis* affected plant size, leaf morphology and the formation of leaf primordia on the adaxial side of cotyledons. On the other hand, chimeric *MdTCP2*, *MdTCP4* and *MdVOZ1* repressors that included the ethylene-responsive transcription factors (ERF)-associated amphiphilic repression (EAR) domain motif influenced reproduction and inflorescence architecture in transgenic *Arabidopsis*. These results suggest that *MdFT1* and/or *MdFT2* might be involved in the regulation of cellular proliferation and the formation of new tissues and that they might affect leaf and fruit development by interacting with TCP- and VOZ-family proteins.

DDBJ accession nos. AB531019 (*MdTCP2a* mRNA), AB531020 (*MdTCP2b* mRNA), AB531021 (*MdTCP4a* mRNA), AB531022 (*MdTCP4b* mRNA) and AB531023 (*MdVOZ1a* mRNA).

Keywords: apple (*Malus × domestica* Borkh.), FLOWERING LOCUS T, TCP family, Vascular plant One Zinc finger protein (VOZ), yeast two-hybrid.

Introduction

Apple (*Malus × domestica* Borkh.) is one of the most important commercial crops with over 60 million tonnes of production per year worldwide (FAOSTAT; Food and Agriculture Organization of the United Nations 2007 <http://faostat.fao.org/>). Commercial apple breeding programmes have been focused on a range of desired traits, such as texture, fruit size, fruit skin colour, disease

resistance and taste, but the relatively long juvenile period, which can last 4–8 years (Zimmerman 1972, Hackett 1985), has been and still is a hindrance to fast and efficient development of new cultivars. Therefore, shortening the juvenile period for earlier flowering is an important component of apple breeding. Elucidation of the molecular mechanism of the transition from the juvenile to adult phase would provide means to

accelerate breeding in fruit trees. In addition, elucidation of the transition process from the vegetative to the reproductive phase could provide an insight for understanding the alternate fruit bearing (biennial bearing) of apple and other perennial woody fruit trees, which is a significant problem for breeding and cultivation (Chan and Cain 1967).

In the *Arabidopsis* flowering network system, it is known that transition from the vegetative to the reproductive phase is initiated by four signalling pathways, namely gibberellin, autonomous, vernalization and light-dependent signalling pathways (Simpson and Dean 2002, Boss et al. 2004). The *FLOWERING LOCUS T (FT)* gene acts as the floral integrator of both the photoperiod and vernalization pathways. It is promoted by photoperiodic induction through the zinc finger protein, CONSTANS (CO), and is repressed by the vernalization integrator of *FLOWERING LOCUS C (FLC)* and *LIKE HETEROCHROMATIN PROTEIN1 (LHP1)/TERMINAL FLOWER2 (TFL2)*, a homologue of *HETEROCHROMATIN PROTEIN1 (HP1)* (Michaels and Amasino 1999, Kotake et al. 2003, Takada and Goto 2003). *FT* has been identified as a causative gene for a late-flowering mutant and encodes a protein similar to phosphatidylethanolamine-binding protein (PEBP) and Raf-1 kinase inhibitor protein (Kardailsky et al. 1999, Kobayashi et al. 1999). Studies in *Arabidopsis* demonstrated that the FT protein travels from the leaves to the shoot apex. It has been demonstrated that the FT protein acts as a transmissible floral inducer, such as a florigen (Corbesier et al. 2007, Jaeger and Wigge 2007, Lin et al. 2007, Mathieu et al. 2007, Notaguchi et al. 2008). *TERMINAL FLOWER1 (TFL1)*, another member of the PEBP-like gene family, acts as a floral repressor (Bradley et al. 1997). Analyses of the crystal structure of FT and TFL1 and transgenic plants with the chimeric fusion of *FT* and *TFL1* revealed that specific regions within their fourth exons function as a potential ligand-binding pocket, and that replacing these regions allows the opposite effects on flowering time (Ahn et al. 2006). These results suggest that there are fundamental differences in the interactive partners or protein-bound conformations between TFL1 and FT that selectively regulate their downstream signal transduction pathways in the active or inactive state (Ahn et al. 2006).

We have already identified and characterized two apple *FT*-like genes, *MdFT1* and *MdFT2*, which had 95% identity to each other at the nucleotide level (Kotoda et al. 2010). *MdFT1* was expressed highly in the apical buds of fruit-bearing shoots (FBS) during the transition period from vegetative to reproductive phase. On the other hand, *MdFT2* was highly abundant in the reproductive organs, such as young fruit (Kotoda et al. 2010). This differential expression suggests distinct roles, and the patterns of transcript accumulation imply that *MdFT1* possibly accelerates flowering and *MdFT2* functions in reproductive organ development. Therefore, two *FT*-like genes may have functionally diverged in apple. Moreover, the transgenic apple overexpressing *MdFT1* showed an extreme early-flowering phenotype

(Kotoda et al. 2010). Similar results were obtained in other tree species, such as Satsuma mandarin (*Citrus unshiu*) and poplar (*Populus deltoides*), showing that constitutive expression of *FT*-like genes in these plants induces precocious flowering (Endo et al. 2005, Böhlenius et al. 2006, Hsu et al. 2006). However, the true function of endogenous *FT* orthologues remains unclear in woody plants. Because the growth phase of most woody plants involves a relatively long juvenile period as compared with that of other perennial plants, knock-out trees lacking the expression of *FT*-like genes have not been reported.

The aim of this study was to identify proteins that bind to MdFT1 and MdFT2 using a yeast two-hybrid system in order to elucidate the molecular network of *FT* orthologues in apple. Further, we functionally characterized the transcription factors shown to interact with MdFT1 and MdFT2 proteins, two members of the apple TCP (TEOSINTE BRANCHED1, CYCLOIDEA and PROLIFERATING CELL FACTORS)-like family and one member of the apple VOZ (Vascular plant One Zinc finger protein)-like family.

Materials and methods

Plant materials

Tissue samples of apple (*Malus × domestica* Borkh.) cultivar 'Jonathan' were collected from the experimental field at the National Institute of Fruit Tree Science in Morioka, Japan. Seedlings obtained from the cross 'Fuji' and 'Orin' were used for expression analysis in the juvenile phase.

Yeast two-hybrid assays

Construction of a bait vector

To construct the DNA binding domain (BD) vector for screening proteins that interact with MdFT1, the full coding region of *MdFT1* was amplified by polymerase chain reaction (PCR) and the resulting product was cloned in-frame in the pBD-GAL4 Cam vector (designated MdFT1/BD).

Construction of a GAL4 AD vector library Total RNA was extracted from flowers, fruit and leaves of the apple cv. 'Jonathan' according to the cetyltrimethyl ammonium bromide method as described by Kotoda et al. (2000). Poly (A)⁺ RNA was isolated using a PolyAtract mRNA Purification Kit (Promega, Madison, WI, USA). The GAL4 AD vector library was constructed using a GAL4 Two-Hybrid Phagemid Vector Kit (Stratagene, La Jolla, CA, USA) and a Lambda DNA Packaging System (Promega).

Screening using the yeast two-hybrid system After converting the phage GAL4 AD vector library into a plasmid library, the yeast strain YRG-2 (Mata ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538 LYS2::UASGAL1-TATA GAL1-HIS3 URA3::UASGAL4 17mers(x3)-TATACYC1-lacZ) harbouring MdFT1/BD was co-transformed with the AD cDNA plasmid library (~5.5 × 10⁶ independent AD plasmids) by the

lithium method (Gietz and Woods 2002). The yeast transformants were incubated on selective agar medium with Minimal SD Base, -His/-Leu/-Trp DO supplement (Clontech Laboratories Inc., Mountain View, CA, USA) and 3 mM 3-amino-1,2,4-triazole (3-AT) containing 4% glucose as a carbon source at 30 °C for 4–5 days. The AD plasmids were extracted from the transformants grown on a triple dropout (-His/-Leu/-Trp) medium plate, and then re-transformed in *Escherichia coli* for sequence analysis.

A selection test for histidine (HIS3) reporter gene activity: to confirm positive candidates that interact with MdFT1 and also with MdFT2, we generated the bait vector construct of *MdFT2* and the yeast was transformed with a pair of bait (MdFT1/BD and MdFT2/BD) and prey (MdTCP2a/AD, MdTCP4a/AD and MdVOZ1a/AD) plasmids. The yeast transformants were tested for HIS3 reporter gene activity on selective agar medium with Minimal SD Base, -His/-Leu/-Trp DO supplement and 1 mM 3-AT containing 4% glucose at 30 °C for 3 days or 0 mM 3-AT containing 2% glucose at 20 °C for 7 days.

5-Bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) filter assay: the assay was performed for lacZ reporter gene expression by detecting β -galactosidase activity. A filter assay was performed according to protocols described in the GAL4 Two-Hybrid Phagemid Vector Kit instruction manual (Stratagene).

Screening an apple cDNA library

To isolate the full-length cDNAs of apple *TCP* and *VOZ1* homologues *MdTCP2*, *MdTCP4* and *MdVOZ1*, we screened an apple cDNA library that was constructed using a ZAP-cDNA Synthesis Kit (Stratagene) and a Packagene[®] Lambda DNA Packaging System (Promega). Approximately 5.0×10^5 plaques were screened with *MdTCP2*, *MdTCP4* or *MdVOZ1* fragments as probes obtained by the two-hybrid screening. Plasmids were rescued from the phage by *in vivo* excision with the ExAssist helper phage (Stratagene) for sequence analysis.

Hybridization analysis

Southern blot analysis was performed as described by Sambrook et al. (1989). Total DNA (15 μ g) isolated from the leaves of apple 'Fuji' was digested with EcoRI, HindIII or XbaI and then separated on a 0.8% agarose gel by electrophoresis. The DNA bands were blotted onto a Hybond-N+ nylon membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and hybridized with digoxigenin (DIG)-labelled full-length cDNA probes of *MdTCP2a*, *MdTCP4a* or *MdVOZ1a*. Hybridization was performed in DIG Easy Hyb (Roche) at 42 °C for 16 h followed by two rinses in 2 \times sodium saline citrate (SSC) containing 1% (w/v) sodium dodecyl sulphate (SDS) at room temperature for 10 min and two washes in 0.5 \times SSC containing 1% (w/v) SDS at 68 °C for 10 min. Chemiluminescent signals were visualized using the LAS1000 image analyser (Fuji Photo Film, Aichi, Japan).

Nucleotide and amino acid sequence analyses

Nucleotide sequences were 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). Sequence database searching was performed using BLAST2 of National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>), Genome Database for Rosaceae (GDR, <http://www.rosaceae.org/>) and Plant transcription factor database version 2.0 (Zhang et al. 2011, Center for Bioinformatics, Peking University, China, <http://plantfdb.cbi.pku.edu.cn/>). Amino acid sequences were analysed using the Clustal X multiple sequence alignment program ver. 1.83 (Jeanmougin et al. 1998) and the phylogenetic tree was displayed using the NJplot program (Perrière and Gouy 1996).

Expression analysis by reverse transcriptase (RT)–PCR and quantitative real-time RT–PCR

As described in our previous study (Mimida et al. 2009), total RNA samples were obtained from calyxes, petals, stamens, carpels, peduncles, fruit receptacles (FRs), seeds, fruit, mature leaves (MLs) and apical buds of FBS from June to the following April in the adult phase. Apical buds of vegetative shoots (VS), stems and roots were obtained from 2-week-old seedlings in the juvenile phase. First-strand cDNAs were synthesized from 3 μ g of total RNA in 60 μ l of reaction mixture using the ReverTra Ace Kit (Toyobo, Japan). Apple *Histone H3* was used as an internal control for gene expression (Mimida et al. 2009, Kotoda et al. 2010).

Subsequent PCR was performed with 1 μ l of the first-strand cDNA mixture and EX-Taq polymerase (Takara Biomedical, Otsu, Japan). Thermal cyclers programmes were as follows: 96 °C for 3 min followed by 32 cycles of 96 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s for *MdTCP2a* and *MdTCP2b*; 30 cycles of 96 °C for 20 s, 56 °C for 30 s and 72 °C for 30 s for *MdTCP4a/MdTCP4b* (the primer set of MdTCP4a/b F and MdTCP4a/b R amplifies both genes); and 32 cycles of 96 °C for 20 s, 56 °C for 30 s and 72 °C for 30 s for *MdVOZ1a*.

Quantitative real-time RT–PCR (qRT–PCR) analysis was carried out to validate the result of RT–PCR analysis following the manual for the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT–PCR reactions were performed with 0.15 μ g of the first-strand cDNA mixture as a template by using the power SYBR Green Master mix (Applied Biosystems). Thermal cyclers programmes were as follows: 50 °C for 2 min and 95 °C for 10 s followed by 40 cycles of 95 °C for 15 s and 60 °C for 15 s for each gene. Software analysis of absolute quantification was performed with sequence detection software version 1.3.1 (Applied Biosystems). The primer sets used in RT–PCR and qRT–PCR analysis are shown in Table 1.

Table 1. PCR primer sequences for gene expression analysis

Primer	Oligonucleotide sequence
MdTCP2a F	5'-GAA TCA GAG CCA GCA CCT TTC-3'
MdTCP2a R	5'-GTT GTT GTT TAT GCT CTG TTG GTA ACT-3'
MdTCP2b F	5'-GGG AAC CAA AGT CAG CAC C-3'
MdTCP2b R	5'-GGA AAT GTT GTG CGG GTA AGC-3'
MdTCP4a/b F	5'-CAG CAG CAGC CGT CGT TGT TCG G-3'
MdTCP4a/b R	5'-GAG ATG AGA TGA GAG CTT GAG CCC TTT TGA AGA G-3'
MdTCP4a real-time F	5'-AGA GAA TGG TGG CTT GGA ACA-3'
MdTCP4a real-time R	5'-GAT GAT CCA CCG CCG TTA TC-3'
MdVOZ1 F	5'-GCA CCT CTA TGA GTA CGA GAT C-3'
MdVOZ1 R	5'-GGA GCC CAT CAA ACT TCT ATC AG-3'

Vector construction

To construct transgenes of $35S\Omega:MdTCP2a$, $35S\Omega:MdTCP4a$ and $35S\Omega:MdVOZ1a$, the full-length cDNAs of *MdTCP2a*, *MdTCP4a* and *MdVOZ1a* were amplified by PCR with a pair of primers containing XbaI or KpnI. The PCR products were subsequently digested with XbaI and KpnI, and then cloned in the XbaI–KpnI sites of the modified pSMAK193E binary vector ($35S\Omega/pSMAK193E$) (Mimida et al. 2009) to place it between the cauliflower mosaic virus (CaMV) 35S promoter fused with the Ω sequence (Gallie and Walbot 1992) and rubisco terminator (TrbcS).

To generate $35S\Omega:MdTCP2a$ -SRDX, $35S\Omega:MdTCP4a$ -SRDX and $35S\Omega:MdVOZ1a$ -SRDX, the full-length cDNAs of *MdTCP2a*, *MdTCP4a* and *MdVOZ1a* were amplified by PCR with a pair of primers containing XbaI or SmaI. The PCR products were subsequently digested with XbaI and SmaI and then cloned in the XbaI–SmaI sites of the modified pSMAK193E binary vector ($35S\Omega$ -SRDX/pSMAK193E) to clone it in-frame with the 12 amino acid repression domain (SRDX) (Hiratsu et al. 2003).

Arabidopsis transformation

Arabidopsis (Columbia) was transformed using the *Agrobacterium tumefaciens* strain EHA101 by floral dipping (Clough and Bent 1998). Transformed *Arabidopsis* plants were selected on MS plates (Murashige and Skoog 1962) containing carbenicillin (250 mg^{-1} l) and kanamycin (35 mg^{-1} l). T1 or T2 plants (primary transformants were defined as T1 plants) were grown on MS plates containing kanamycin for 5 or 10 days, and then transferred to the soil and grown under long-day conditions (16 h light/8 h darkness) at $22\text{ }^{\circ}\text{C}$.

Results

Identification of proteins that interact with MdFT1 by the yeast two-hybrid system

A yeast two-hybrid system with the full-length *MdFT1* as a bait was used for screening $\sim 5.5 \times 10^6$ plasmids from the

cDNA AD library prepared from the flowers, fruit and leaves of 'Jonathan' apple. As a result, 26 clones were obtained and sequence analysis revealed that seven of the clones represented transcription factors, one represented the kinase/receptor class, 11 were classified as others and seven fell into the junk DNA category (Table 2). The transcription factors isolated in this study were homologues of the *Arabidopsis* TCP and VOZ family of genes. Three candidate clones of the TCP-family genes carried cDNA inserts of different sizes derived from a single gene, designated *MdTCP4a*, and one candidate carried an insert of another gene, designated *MdTCP2a*. Both *MdTCP2a* and *MdTCP4a* contained a TCP domain with a proposed basic helix–loop–helix motif (data not shown). All candidate clones of *AtVOZ1* homologues carried cDNA inserts with different sizes derived from a single gene, designated *MdVOZ1a*. It contains three conserved regions: acidic stretch region, zinc finger motif and basic region (data not shown).

To confirm that the candidate clones interact with MdFT1, the growth test for HIS3 reporter gene activity and an X-gal filter assay were performed using the yeast transformants harbouring a pair of bait and prey plasmids (*MdFT1*/BD and *MdTCP2a*/AD, *MdTCP4a*/AD or *MdVOZ1a*/AD). As a result, the activities of HIS3 and β -galactosidase were detected in yeast cells with all combinations analysed (Figure 1a and b). In addition, it was confirmed that MdFT2 also interacted with *MdTCP2a* and *MdVOZ1a* (Figure 1c).

Comparison of amino acid sequences and phylogenetic analysis of TCP2, TCP4 and VOZ1 homologues

To assess the number of *TCP2*, *TCP4* or *VOZ1* homologues in the apple genome, genomic Southern hybridization was performed using full-length cDNAs of *MdTCP2a*, *MdTCP4a* or *MdVOZ1a* as probe, respectively. One to four major hybridizing bands were detected in each lane of DNA digested with EcoRI, HindIII or XbaI, suggesting that at least two distinct loci for each gene exist in the apple genome (Figure 2). In an attempt to isolate apple *MdTCP2a*, *MdTCP4a* or *MdVOZ1a* homologues, $\sim 5.0 \times 10^5$ plaques were screened from a cDNA library with an *MdTCP2a*, *MdTCP4a* or *MdVOZ1a* probe, respectively.

We obtained a total of 22 positive plaques by screening a cDNA library with an *MdTCP2a* probe and then classified them into two different cDNAs, *MdTCP2a* and another gene that we designated *MdTCP2b*. The amino acid sequence alignment showed that *MdTCP2a* had 91.8 and 44.2% similarity to *MdTCP2b* and *TCP2*, respectively, and that *MdTCP2b* had 43.0% similarity to *TCP2*.

Nine positive plaques were obtained with an *MdTCP4a* probe, and subsequent sequence analysis revealed that they could be classified as two different cDNAs, namely *MdTCP4a* and another gene that we designated *MdTCP4b*. The amino

Table 2. Summary of screening for MdFT1 interacting proteins using the yeast two-hybrid system

Clone (no.)	Number of AD clones obtained	Consensus gene set number (chromosome number)	<i>Arabidopsis</i> homologue (accession number)	Annotation
2, 21, 28	3	MDP0000729316 (chr 5, <i>MdVOZ1a</i>) [MDP0000879912(chr10)]	ath:AT1G28520	Transcription factor, <i>AtVOZ1</i> homologue.
4, 11, 34	3	MDP0000243495 (chr11, <i>MdTCP4a</i>) [MDP0000184743 (chr10, <i>MdTCP4b</i>)]	ath:AT3G15030	Transcription factor, <i>TCP4</i> homologue.
36	1	MDP0000763497 (chr 5, <i>MdTCP2a</i>) [MDP0000287069 (chr 5, <i>MdTCP2b</i>)]	ath:AT4G18390	Transcription factor, <i>TCP2</i> homologue.
5	1	MDP0000608985 (chr 4)	ath:AT1G04280	<i>REV3C</i> homologue.
9	1	MDP0000181717 (chr 9)	ath:AT3G04680	ATP/GTP-binding/pre-mRNA cleavage complex family protein.
10	1	MDP0000270976 (chr 1)	ath:AT2G46080	Zinc ion binding protein.
17	1	MDP0000135679 (chr 1)	ath:AT5G10780	Transmembrane protein.
18	1	MDP0000870498 (chr 2)	ath:AT5G66230	Unknown protein.
22	1	MDP0000340981 (chr13)	ath:AT1G28120	Ubiquitin thioesterase Otubain family protein.
26	1	MDP0000299813 (chr13)	ath:AT3G10700	ATP binding/galactokinase/ kinase/ phosphotransferase.
30, 38	2	MDP0000145618 (chr 8), MDP0000184474 (chr 5)	ath:AT1G71230	COP9 signalosome subunit protein homologue.
33	1	MDP0000896759 (chr13)	ath:AT2G02710	Two-component sensor molecule, <i>TWIN LOV PROTEIN 1 (TLP1)</i> homologue.
35	1	MDP0000924612 (chr 5)	ath:AT4G17190	<i>FARNESYL DIPHOSPHATE SYNTHASE 2 (FPS2)</i> homologue.
44	1	MDP0000608985 (chr 4)	ath:AT5G36230	Translation initiation factor containing basic leucine zipper and W2 domains 2.
Junk DNA	7	-	-	Frame-shift or non-coding DNA

Sequence database similarity searching was performed using the Genome Database for Rosaceae (GDR, <http://www.rosaceae.org/>). The consensus gene set number between brackets shows the homologue of the isolated gene.

acid sequence alignment showed that *MdTCP4a* had 88.7 and 56.1% similarity to *MdTCP4b* and *TCP4*, respectively, and that *MdTCP4b* had 55.6% similarity to *TCP4*.

Recently, the apple genome project has progressed (Velasco et al. 2010) and expression sequence tag (EST) data are also available (Newcomb et al. 2006, Zhang et al. 2011). Interrogation of the DNA database identified numerous TCP-family homologues in apple (Figure 3a). The TCP-family proteins were grouped as three distinct clades: TEOSINTE BRANCHED1 (TB1) and CYCLOIDEA (CYC) form the TB1/CYC clade, while CININNATA (CIN) and PROLIFERATING CELL FACTORS (PCF) form individual clades (Aguilar-Martinez et al. 2007). The four TCP-family homologues isolated from apple belonged to the CIN clade in the phylogenetic tree constructed using the amino acid sequences (Figure 3a).

Hybridization with an *MdVOZ1a* probe resulted in a total of eight positive plaques. However, all of them carried a cDNA insert derived from a single gene, *MdVOZ1a*. The alignment of amino acid sequence and phylogenetic analysis showed that *MdVOZ1a* belonged to the VOZ1 clade in the VOZ family, and had 76.4 and 47.1% similarity to *AtVOZ1* and *AtVOZ2*, respectively (Figure 3b). As a result of database searching, five homologues belonging to VOZ1 and VOZ2 clades were found as EST clones and in the apple genome (Figure 3b).

Expression patterns of *MdTCP2*, *MdTCP4* and *MdVOZ1*

Tissue-specific expression patterns of *MdTCP2a*, *MdTCP2b*, *MdTCP4a/MdTCP4b* (or *MdTCP4a*) and *MdVOZ1a* were investigated by RT-PCR and qRT-PCR analyses. The *MdTCP2a* transcript accumulated to relatively high levels in petals and fruit, and was also detected in calyxes, stamens, seeds, MLs and FBS in the adult phase, and in VS in the juvenile phase of seedlings (Figure 4a). The expression pattern of *MdTCP2b* was similar to that of *MdTCP2a* in the highly expressing tissues, but was not detected in juvenile VS. Reverse transcriptase-polymerase chain reaction analysis showed relatively high levels of *MdTCP4a/MdTCP4b* expression in petals and MLs, lower levels of expression in calyxes, stamens, seeds and FBS in the adult phase, and low levels in MLs and VS in the juvenile phase (Figure 4a). Quantitative real-time RT-PCR analysis confirmed high levels of *MdTCP4a* transcripts in petals (Figure 4a). *MdVOZ1a* transcript accumulation was observed in multiple tissues, and was detected strongly in fruit, calyxes and petals, but its expression was considerably lower in sexual organs, such as stamens and carpels, and MLs of the seedlings (Figure 4a).

Floral bud differentiation and development occur from summer through to spring in the apical buds of FBS (Kotoda et al. 2000, Mimida et al. 2009). We have previously analysed

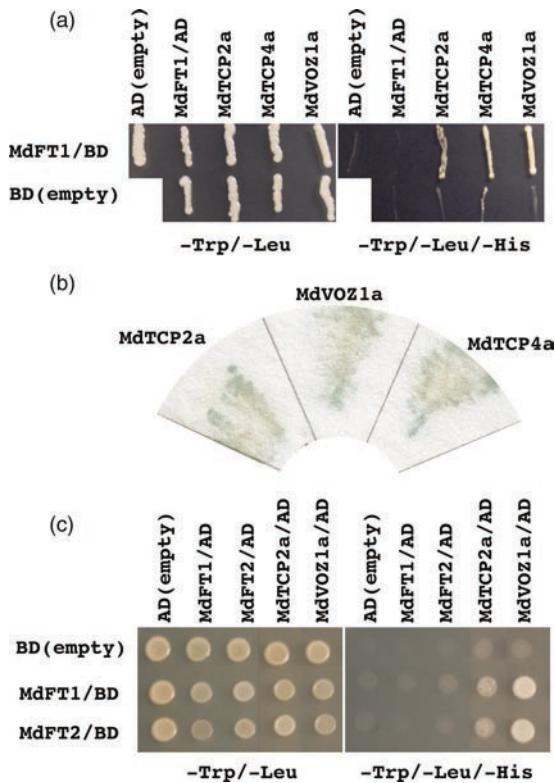


Figure 1. Interactions with MdFT1 and MdFT2 proteins detected in yeast. (a) Interaction of MdFT1 with MdTCP2a, MdTCP4a and MdVOZ1a. A selection test was performed for the HIS3 reporter gene of yeast transformants on the selective plates with Minimal SD Base and -His/-Leu/-Trp DO supplement containing 1 mM 3-AT and 4% glucose at 30 °C for 3 days. (b) X-gal filter lift assay for β -galactosidase activity using yeast transformant cells. (c) Interaction of MdFT1 and MdFT2 with MdTCP2a and MdVOZ1a. A selection test was performed for the HIS3 reporter gene of yeast transformants on the selective plates (-His/-Leu/-Trp) with 0 mM 3-AT and 2% glucose at 20 °C for 7 days. Wild-type cells were transformed with a pair of bait and prey plasmids: MdFT1/BD or MdFT2/BD and MdFT1/AD, MdTCP2a/AD, MdTCP4a/AD or MdVOZ1a/AD by the lithium method.

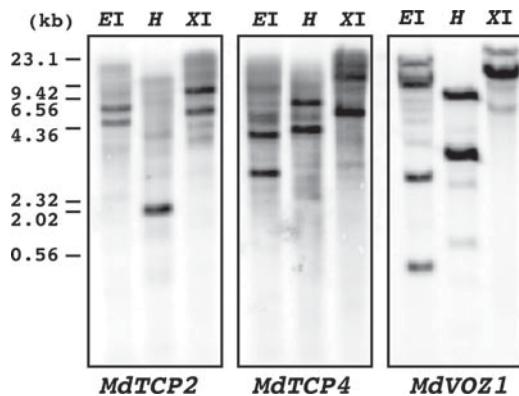


Figure 2. Genomic Southern blot probed with *MdTCP2a* (left), *MdTCP4a* (middle) and *MdVOZ1a* (right), respectively. Genomic DNA (15 μ g) of 'Fuji' apple was digested with EcoRI (EI), HindIII (H) or XbaI (XI) and subjected to hybridization analyses.

the seasonal expression patterns of genes related to flowering, such as *MdFT1*, *MdFT2*, *MdTFL1*, *MdCEN* and *MdAP1*, in apical buds (Mimida et al. 2009, Kotoda et al. 2010). Therefore, RT-PCR and qRT-PCR analyses were performed to establish seasonal expression patterns of *MdTCP2a*, *MdTCP2b*, *MdTCP4* and *MdVOZ1a* using total RNA isolated from the apical buds of FBS in each month from June to the following April. Similar levels of *MdTCP2a* transcript were detected across all seasons (Figure 4b). In contrast, *MdTCP2b* expression was significantly higher in the later stage of flower bud development. The expression levels of *MdTCP4a*/*MdTCP4b* or *MdTCP4a* were low from June to the following February but increased in March and April. The expression of *MdVOZ1a* decreased from June to September and increased from January to April (Figure 4b).

Analysis of apple TCP4, TCP2 or VOZ1 homologues in transgenic Arabidopsis

To establish the possible roles of *MdTCP2a*, *MdTCP4a* or *MdVOZ1a*, the gain-of-function and loss-of-function approach was used in transgenic *Arabidopsis* plants. Constitutive expression constructs, 35S Ω :*MdTCP2a*, 35S Ω :*MdTCP4a* and 35S Ω :*MdVOZ1a*, and chimera repressor constructs, 35S Ω :*MdTCP2a*-SRDX, 35S Ω :*MdTCP4a*-SRDX and 35S Ω :*MdVOZ1a*-SRDX (Figure 5a), were introduced into the *Arabidopsis* plants. Over 50 independent lines were obtained for each transgene. The T₁ or T₂ generation of these transgenic plants were grown in long-day conditions and their phenotypes were examined.

As compared with the wild-type plants (Figure 5b and h), most transgenic plants with 35S Ω :*MdTCP2a* and 35S Ω :*MdTCP4a* displayed a phenotype with elongated hypocotyls in green (Figure 5c, d, f, i, j and l). A few of them also had fused cotyledons similar to those of double mutants of *cup-shaped cotyledon1* (*cuc1*) and *cuc2* (Aida et al. 1997, Figure 5d and j). In addition, plants expressing *MdTCP4a* had narrow leaves and reduced plant size (Figure 5e, l and p). A few plants showed a phenotype with increased number of shoots (data not shown).

Transgenic plants with 35S Ω :*MdTCP2a*-SRDX or 35S Ω :*MdTCP4a*-SRDX showed similar phenotypes to each other. Newly emerging leaves on the adaxial side of cotyledons were observed in some transgenic plants (Figure 5e, g, k, m and o). Others had wavy leaves similar to those of the jaw mutant (Palatnik et al. 2003, Figure 5n) and were sterile.

The 35S Ω :*MdVOZ1a* construct induced sterile flowers with enhanced elongation of flower stalks, abnormal inflorescences and terminus by a flower (Figure 5q, r, s and t) at a frequency of <10%. In <10% of all transformants, 35S Ω :*MdVOZ1a*-SRDX induced sterile flowers and abnormal inflorescences with three to five flowers, resulting in plants with terminal flowers or dwarfed shape (Figure 5u, v, w, x and y).

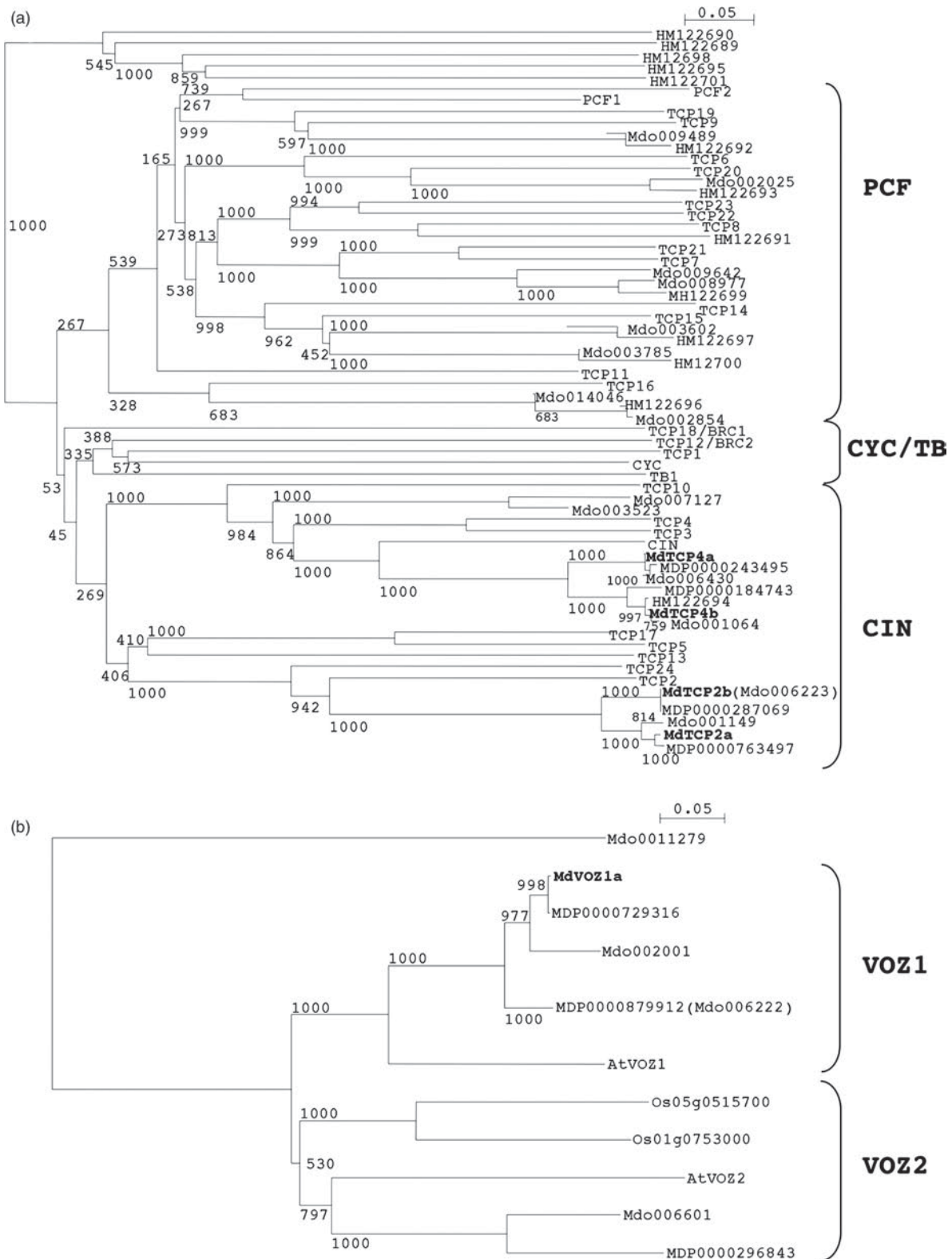


Figure 3. Relationship of the predicted TCP- or VOZ-family proteins between apple and other plant species. (a) Phylogenetic tree of TCP-family proteins of *Arabidopsis* and apple. (b) Phylogenetic tree of VOZ-family proteins of *Arabidopsis*, rice and apple. These trees were constructed using *Arabidopsis* TCP-family proteins described by Aguilar-Martinez et al. (2007), *Arabidopsis* VOZ-family proteins described by Mitsuda et al. (2004), Os01g0753000, Os05g0515700 and other amino acid sequences obtained from Plant transcription factor database version 2.0 (Mdo), Genome Database for Rosaceae (HDP) and the New Zealand Institute for Plant & Food Research Ltd (HM) in addition to MdtTCP2a, MdtTCP2b, MdtTCP4a, MdtTCP4b and MdtVOZ1a shown in bold in this paper. Numbers along branches are bootstrap values (1000 replicates). The unit for the scale bars displays branch lengths (0.05 substitutions/site).

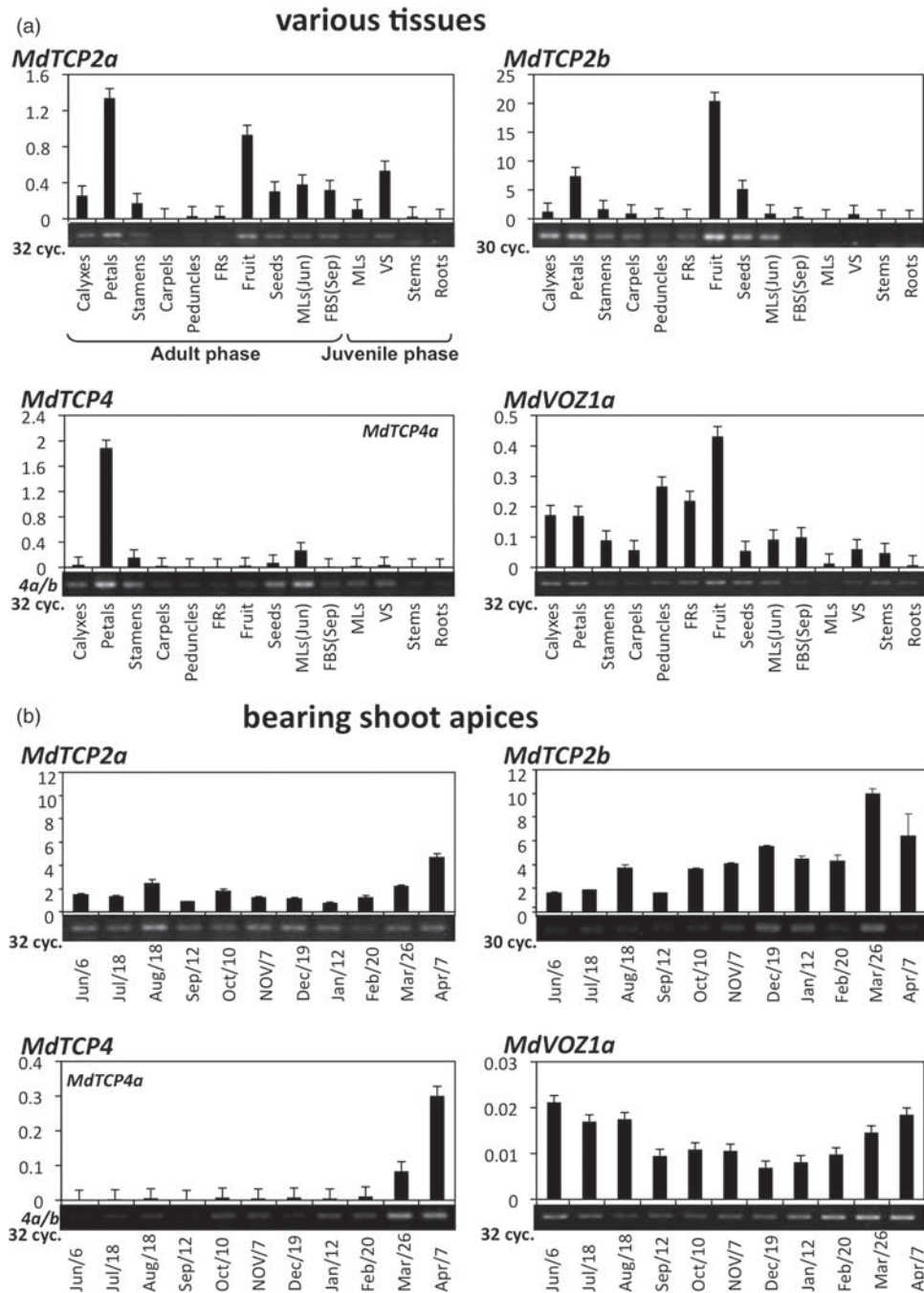


Figure 4. Expression analyses of *MdTCP2*, *MdTCP4* and *MdVOZ1* by RT-PCR and qRT-PCR. (a) Expression pattern of *MdTCP2a*, *MdTCP2b*, *MdTCP4a*/*MdTCP4b* (RT-PCR), *MdTCP4a* (qRT-PCR) and *MdVOZ1a* in various tissues. Samples from left to right are as follows: calyxes, petals, stamens, carpels, peduncles, fruit receptacles (FRs), fruit, seeds, mature leaves (MLs) and apical buds of fruit-bearing shoots (FBS) on September 12 [FBS (Sep)] in the adult phase; MLs, apical buds of vegetative shoots (VS), stems and roots of 2-week-old seedlings in the juvenile phase. (b) Expression pattern of *MdTCP2a*, *MdTCP2b*, *MdTCP4a*/*MdTCP4b* (RT-PCR), *MdTCP4a* (qRT-PCR) and *MdVOZ1a* genes in apical buds of FBS from June to April. Values are means \pm standard deviation of the results from three replicates. The units on the vertical axis show 1.0×10^{-9} g of gene transcripts/ 1.5×10^{-4} g of total RNA. It is noteworthy that in Morioka, Japan, the timing of the flowering of the floral buds of 'Jonathan' is early May, the timing of floral differentiation and fruit ripening is the beginning of July and mid-October, respectively, and the dormancy period starts from November. The number of PCR cycles is indicated at the left of each panel.

Discussion

Over the past decade, studies of the flowering process in *Arabidopsis* have provided us with the important findings of *FT* (reviewed in Turck et al. 2008). An existing model proposes

that the formation of a protein-protein complex between *FT* and *FLOWERING LOCUS D* (*FD*) is implicated in the activation of the *APETALA1* (*AP1*) gene promoter (Abe et al. 2005, Wigge et al. 2005). Taking into consideration that *FT* acts as a *trans-*

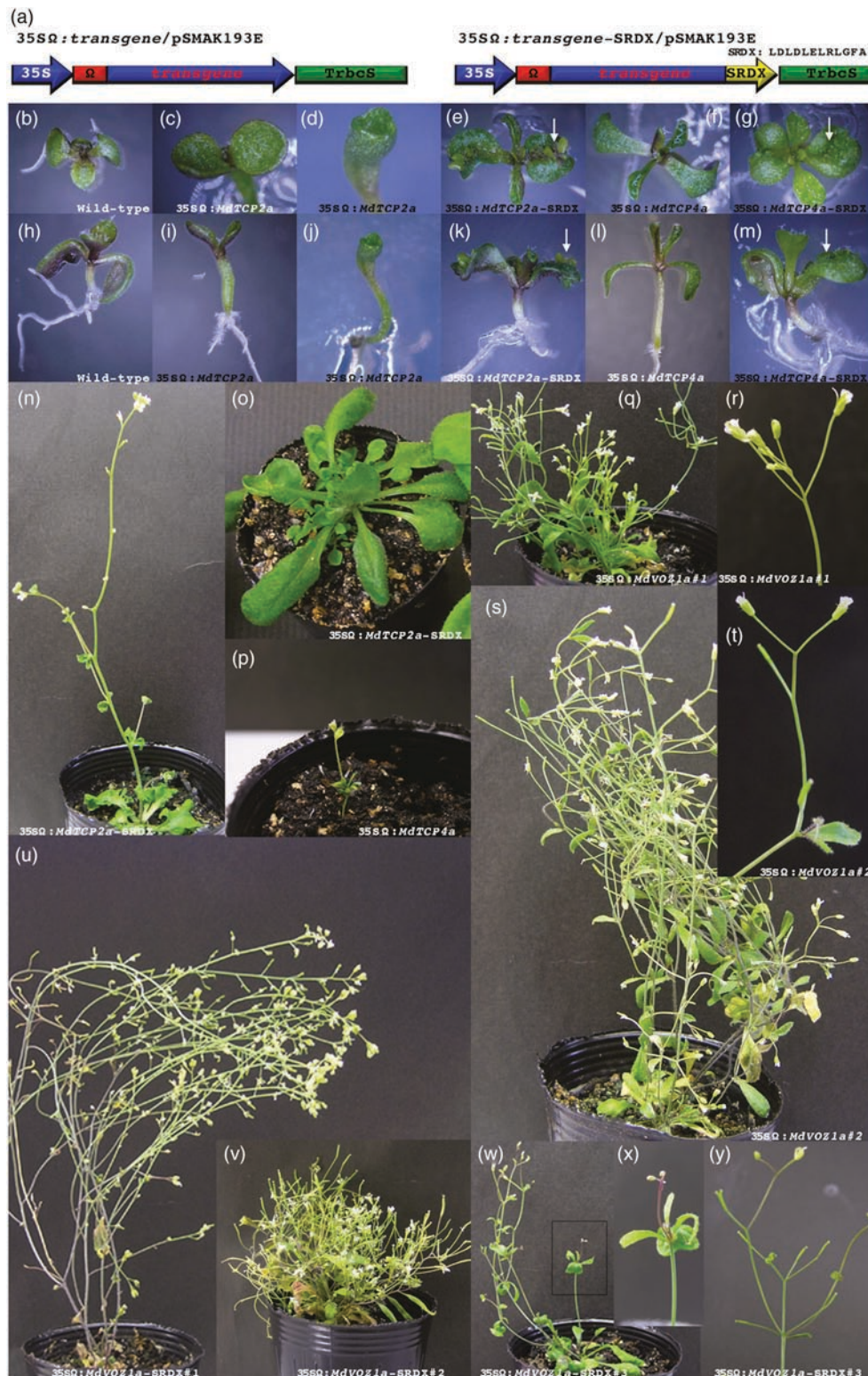


Figure 5. *Arabidopsis* transgenic plants expressing apple *TCP2*, *TCP4* and *VOZ1* homologues. (a) Schematic representation of the vector construction of transgenes, *MdTCP2a*, *MdTCP4a* and *MdVOZ1a*. (b–m) Typical lines of the wild-type (b, h), 35S::*MdTCP2a* (c, d, i, j), 35S::*MdTCP4a* (f, l), 35S::*MdTCP2a*-SRDX (e, k) and 35S::*MdTCP4a*-SRDX (g, m) plants. (n) 35S::*MdTCP2a*-SRDX plant (no. 1). (o) Rosette leaves of a 35S::*MdTCP2a*-SRDX plant (no. 2). (p) 35S::*MdTCP4a* plant (no. 1). (q) 35S::*MdVOZ1a* plant (no. 1). (r) Inflorescence of a 35S::*MdVOZ1a* plant (no. 1). (s) 35S::*MdVOZ1a* plant (no. 2). (t) Inflorescence of a 35S::*MdVOZ1a* plant (no. 2). (u) 35S::*MdVOZ1a*-SRDX plant (no. 1). (v) 35S::*MdVOZ1a*-SRDX plant (no. 2). (w) 35S::*MdVOZ1a*-SRDX plant (no. 3). (x) Close-up of the inflorescence in (w). (y) Developed inflorescence in the 35S::*MdVOZ1a*-SRDX plant (no. 3) in (w). Arrowheads in (e), (g), (k) and (m) indicate leaves formed on the adaxial side of the cotyledons. These plants were grown under long-day conditions (16 h light/8 h dark). Scale bars are 1 cm in (o) and (p).

element of gene promoters, it could interact with several transcription factors and control multiple genes. In other words, it could be inferred that FT has other functions in addition to flowering control (Lifschitz et al. 2006, Shalit et al. 2009). Recently, it was demonstrated that *MdFT2*, one of two apple FT orthologues, was highly expressed in developing fruit (Kotoda et al. 2010). Thus, *MdFT2* might be involved in reproductive organ development as well as in flowering.

In this study, we performed a large-scale yeast two-hybrid screen to identify proteins that interact with *MdFT1*. We have obtained several candidates that interact with *MdFT1*. They include the homologue of two-component sensor molecule *TWIN LOV PROTEIN 1 (TLP1, AT2G02710)*. *TLP1* is a photoreceptor protein for blue light and contains the light, oxygen or voltage (LOV) domain (Crosson et al. 2003). Apple *TLP1*-like genes might play a role in controlling flowering in response to photoperiod and light quality via interaction with *MdFT1* and/or *MdFT2*. However, the molecular mechanisms of flowering in the light-dependent pathway remain largely unknown in apple. The protein in the kinase/receptor class showed no sequence homology to SPAK [SELF PRUNING (SP)-associated kinase], which interacts with SP, a member of the PEBP-like protein family in tomato (Pnueli et al. 2001). Other candidate genes include several transcription factors (Table 1). Among them, *TCP* and *VOZ1* homologues were identified that interacted strongly with both *MdFT1* and *MdFT2* in yeast (Figure 1). Transgenic *Arabidopsis* plants expressing either *MdFT1* or *MdFT2* showed an early-flowering phenotype (Kotoda et al. 2010, Li et al. 2010). Taking these facts into consideration, it was suggested that *MdFT1* and *MdFT2* could function equivalently at the protein level. *TCP*-family genes play crucial roles in cell growth and proliferation (Cubas et al. 1999), whereas *AtVOZ* is possibly involved in the regulation of vacuolar pyrophosphatase (*V-PPase*) gene expression during pollen development (Mitsuda et al. 2004). These reports suggest that *MdTCP2a*, *MdTCP4a* and *MdVOZ1a* might also play important roles in regulating cell division and differentiation in apple.

The *Arabidopsis* *TCP* family consists of 24 paralogue genes (Koyama et al. 2007). Interestingly, the apple genome contains most homologues of *Arabidopsis* *TCP* genes (Figure 3a). *CIN* class genes, which might be functionally redundant, are involved in regulating *CUC* gene expression to promote meristem formation and to determine the correct position of organ primordia and boundaries (Koyama et al. 2007). Five of the *CIN* class genes are regulated at the transcriptional level by miRNAs (Palatnik et al. 2003). The phenotype of *Arabidopsis* plants transformed with the 35S Ω :*MdTCP2a* and 35S Ω :*MdTCP4a* constructs was similar to that of plants carrying 35S:miRNA-resistant *TCP2 (mTCP2)*, 35S:*mTCP4* and 35S:*mTCP3*. The phenotype of *Arabidopsis* with 35S Ω :*MdTCP2a*-SRDX and 35S Ω :*MdTCP4a*-SRDX was also similar to that of

35S Ω :*TCP3*-SRDX (Palatnik et al. 2003, Koyama et al. 2007, Figure 5). In addition, *MdFT1/MdFT2* and *MdTCP2a/MdTCP2b* exhibited overlapping expression profiles in reproductive organs and particularly *MdFT2* and *MdTCP2a/MdTCP2b* were highly expressed in the developing fruit of apple (Kotoda et al. 2010, Figure 4). Moreover, the *Arabidopsis* *CIN* class genes are expressed in immature siliques at a high level (Koyama et al. 2007). Our findings suggest that the formation of protein–protein complex by *MdFT1* or *MdFT2* and *CIN* class proteins, such as *MdTCP2a* and *MdTCP2b*, might play a role in cell growth and proliferation and affect leaf development, meristem formation and fruit development. Krieger et al. (2010) reported that heterozygosity for loss-of-function alleles of *SINGLE FLOWER TRUSS (SFT)*, a tomato FT orthologue, increases fruit yield by up to 60%, suggesting the involvement of FT orthologues in the fruit development of dicotyledonous crops. On the other hand, *MdTCP4a* was expressed specifically in petals and shoot apices in April during a period of flower development after dormancy (Figure 4). Therefore, *MdTCP4a* may affect floral organ development by interaction with *MdFT2*, the expression of which increases rapidly in apical buds of FBS after dormancy (Kotoda et al. 2010).

MdFT1 and *MdFT2* interacted with *MdVOZ1a* more strongly than with *TCP*-family proteins (Figure 1a and c). *MdVOZ1a* was expressed in calyxes, petals and fruit at a relatively high level (Figure 4a). Because a DNA database search revealed that there were at least four *VOZ1* homologues including *MdVOZ1a*, these homologues should also be investigated in future work. Both 35S Ω :*MdVOZ1a* and 35S Ω :*MdVOZ1a*-SRDX transgenes induced a sterile phenotype in *Arabidopsis*, suggesting that *MdVOZ1a* is also involved in the development of reproductive organs (Figure 5). *AtVOZ1*, a zinc-finger transcription factor, was identified as a *V-PPase* promoter-binding protein by a yeast one-hybrid system (Mitsuda et al. 2004). *V-PPase* acts as a vacuolar proton pump and is expressed in the young fruit of Japanese pear (Shiratake et al. 1997). *AtVOZ1* is expressed in vascular bundles of various tissues, stamens, and the junction of silique and pedicel, which is the position likely corresponding to apple fruit (Mitsuda et al. 2004). Expression of FT homologues has also been detected in the fruit of apple and citrus (Nishikawa et al. 2007, Kotoda et al. 2010). These results suggest that the protein complex of *MdFT1*–*MdVOZ1a* and/or *MdFT2*–*MdVOZ1a* might play an important role in the vacuole during fruit development, although *MdFT2* fused with the green fluorescent protein is reported to be localized in the cytoplasmic membrane (Li et al. 2010).

In grasses, vegetative meristem is not maintained under the constitutive expression of FT, because FT induces precocious flowering and represses vegetative growth. In a perennial woody plant like Norway spruce, however, FT orthologues have additional functions. They play a role in regulating seasonal growth and bud outgrowth in the juvenile phase (Gyllenstrand

et al. 2007). Assuming that the apple *FT* orthologues have additional functions to flowering control, they might be associated with events in the seasonal growth cycle. In potato, the *FT* orthologue is involved in the seasonal growth control of tuberization (Rodríguez-Falcón et al. 2006). The cells in organs such as apple fruit and potato tuber that contribute to tuberosity, contain large vacuoles. *MdFT1* and/or *MdFT2* might also be associated with fruit development by interacting with homologues of the TCP and VOZ family.

Flower bud formation in the following season is affected by crop load in the current season in apple. For example, fruit bearing without thinning induces alternate (biennial) bearing (Chan and Cain 1967). Phytohormones such as auxins and gibberellins produced during fruit development, are considered to be translocated to shoot apices and affect the transition from vegetative to reproductive growth (Chan and Cain 1967, Buban and Faust 1982). However, it remains unclear whether phytohormones are required for transcriptional activity of *MdFT1* and *MdFT2* in apple. In this paper, we suggest that *MdFT1* and *MdFT2* may have multiple functions, especially in flowering and fruiting, by interacting with proteins of the TCP and VOZ family of transcription factors in apple. To understand the flowering mechanism, and the relationships between flower bud formation and fruit development in perennial plants with alternate bearing habit, it will be necessary to further investigate the interaction of *MdFT1*/*MdFT2* and transcription factors including the TCP and VOZ family and their responses to phytohormones.

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