Mol Genet Genomics (2007) 278:295–305
DOI 10.1007/s00438-007-0250-0

ORIGINAL PAPER

Constitutive expression of two apple (*Malus* × *domestica* Borkh.) homolog genes of *LIKE HETEROCHROMATIN PROTEIN1* affects flowering time and whole-plant growth in transgenic *Arabidopsis*

Naozumi Mimida · Shin-ichiro Kidou · Nobuhiro Kotoda

Received: 18 February 2007 / Accepted: 15 May 2007 / Published online: 19 June 2007

Abstract Fruit trees, such as apple (*Malus* × *domestica* Borkh.), are woody perennial plants with a long juvenile phase. The biological analysis for the regulation of flowering time provides insights into the reduction of juvenile phase and the acceleration of breeding in fruit trees. In *Arabidopsis*, *LIKE HETEROCHROMATIN PROTEIN1* (*LHP1*) is involved in epigenetic silencing of the target genes such as flowering genes. We isolated and characterized twin apple *LHP1* homolog genes, *MdLHP1a* and *MdLHP1b*. These genes may have been generated as a result of ancient genome duplication. Although the putative MdLHP1 proteins showed lower similarity to any other known plant LHP1 homologs, a chromo domain, a chromo shadow domain, and the nuclear localization signal motifs were highly conserved among them. RT-PCR analysis showed that *MdLHP1a* and *MdLHP1b* were expressed constantly in developing shoot apices of apple trees throughout the growing season. Constitutive expression of *MdLHP1a* or *MdLHP1b* could compensate for the pleiotropic phenotype of *lhp1*/*tfl2* mutant, suggesting that apple *LHP1* homolog genes are involved in the regulation of flowering time and whole-plant growth. Based on these results, *LHP1* homolog genes might have rapidly evolved among plant species, but the protein functions were conserved, at least between *Arabidopsis* and apple.

Keywords Apple (*Malus* × *domestica* Borkh.) · Flowering time · *LIKE HETEROCHROMATIN PROTEIN1 (LHP1)* · *TERMINAL FLOWER2* (*TFL2*) · Ploidy · Hybridization

Abbreviations

FBS	Fruit-bearing shoot
LD	Long-day
SD	Short-day
SS	Succulent shoot
VS	Vegetative shoot

Introduction

The transition from juvenile to adult phase in plant species requires environmental cues, such as temperature, light intensity, and day length, and internal cues, such as phytohormones, age, and body size and thus the perennial fruit trees, such as apple (*Malus* × *domestica* Borkh.), have a long juvenile phase lasting about 4-8 years (Hackett 1985; Dennis Jr 2003). The long juvenile phase is the primary factor that limits the efficient breeding and the genetic and molecular analyses of fruit trees. Therefore, the studies of the molecular basis of flower differentiation would provide insights into the solution of such issues in the area of horticulture. Because apple is one of the most economically important fruit tree crops in the world, many researchers have studied this one genetically and physiologically from

Communicated by S. Hohmann.

Electronic supplementary material The online version of this article (doi:10.1007/s00438-007-0250-0) contains supplementary material, which is available to authorized users.

N. Mimida · N. Kotoda (✉)
Apple Breeding and Physiology Research Team,
National Institute of Fruit Tree Science,
92-24 Shimo-kuriyagawa, Morioka,
Iwate 020-0123, Japan
e-mail: koto@affrc.go.jp

S.-i. Kidou
Cryobiosystem Research Center,
Iwate University, Morioka, Iwate 020-8550, Japan

the view point of both industry and scientific interest. A decade ago, molecular studies on the morphogenesis in apple also started and several genes related to plant development have been isolated and characterized. Recent studies on the genetic control of flowering time in apple suggest that *MdTFL1*, a *TERMINAL FLOWER1*(*TFL1*)-like gene of apple, is a key regulatory gene involved in the maintenance of the juvenile and vegetative phase in apple (Kotoda et al. 2006). However, little is known about other genes regulating the flowering time of apple, and much less about the genetic framework of the life cycle of apple.

On the other hand, studies on *Arabidopsis thaliana* have led to the identification of many genes controlling the flowering time. Previous studies revealed that some mutants lacking the genes encoding Polycomb group (PcG) proteins or chromatin-remodeling factors show early flowering. *CURLY LEAF* (*CLF*) was first identified as a PcG gene in plant, which represses *AGAMOUS* (*AG*) expression (Goodrich et al. 1997). The *EMBRYONIC FLOWER2* (*EMF2*) encoding a PcG protein with a zinc finger motif is required for maintaining the vegetative development and regulating the development of the inflorescence and floral meristem (Yoshida et al. 2001). These PcG family proteins form large multimeric complexes and regulate developmental processes by preventing inappropriate expression of target genes (Chanvivattana et al. 2004). For example, CLF/SWINGER (SWN), EMF2, FERTILISATION-INDEPENDENT ENDOSPERM (FIE), and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) constitute a PcG complex, which predominantly maintains *AG* expression in a transcriptionally repressed state (Chanvivattana et al. 2004).

Arabidopsis LIKE HETEROCHROMATIN PROTEIN1 (*LHP1*) [*TERMINAL FLOWER2* (*TFL2*)] is a single-copy gene encoding a homolog protein of HETEROCHROMATIN PROTEIN 1 (HP1), which is composed of two characteristic domains: an N-terminal chromatin organization modifier (chromo) domain (CD) and a C-terminal chromo shadow domain (CSD) (Gaudin et al. 2001; Kotake et al. 2003). The HP1-family proteins are known to regulate chromatin function from mammals to yeast, and may act similarly as PcG proteins by silencing genes (Maison and Almouzni 2004). Animals have three isoform proteins, HP1α, HP1β, and HP1γ, whereas plants harbor a single-copy gene encoding a homologous protein more related to HP1γ according to its euchromatin localization (Minc et al. 2000; Zemach et al. 2006). The loss-of-function *lhp1/tfl2* allelic mutants showed the pleiotropic phenotypes, such as early flowering, terminal flower, small plant size, and curled leaf (Larsson et al. 1998; Gaudin et al. 2001; Kotake et al. 2003). In addition, the double mutants of *tfl1* and *tfl2* showed a more severe phenotype than that of a single mutant (Larsson et al. 1998). DNA microarray analysis in the *tfl2* mutant showed that the expression levels of euchromatin genes, such as *FLOWERING LOCUS T* (*FT*), which is a flowering integrator gene, and *APETALA3*, *PISTILLATA*, *AG*, and *SEPALLATA3*, which are floral homeotic genes, are up-regulated (Kotake et al. 2003; Takada and Goto 2003; Nakahigashi et al. 2005; Germann et al. 2006). Thus, the pleiotropic phenotypes of the *lhp1/tfl2* mutants are likely to be reflected by the fact that *LHP1* represses the expression of multiple genes.

In this study, we report the isolation and characterization of two *LHP1* homolog genes in apple, *MdLHP1a* and *MdLHP1b*. These genes showed lower identity to other known *LHP1* homologs in plant species, but constitutive expression of *MdLHP1a* or *MdLHP1b* rescued the *Arabidopsis lhp1/tfl2* mutant. Consequently, *LHP1* homolog genes in the apple of woody perennial may also be involved in the regulation of the flowering pathway, and conserved functionally in plant species. In addition, these apple *LHP1* homolog genes may have been duplicated because the *Maloideae* subfamily possesses the complex genome derived from the origin of polyploidy.

Materials and methods

Plant materials

Different organ tissues and shoot apices of apple (*Malus* × *domestica* Borkh.) cultivars (cv.) 'Fuji' and 'Jonathan' were collected from the experimental field at the National Institute of Fruit Tree Science in Morioka, Japan. These cultivars were used for the seasonal and the tissue specific expression analyses because they are popular cultivars and the genetic background is not so different between them. The seedlings from 'Fuji' apple were used for the expression analysis of *MdLHP1a* and *MdLHP1b* in the juvenile phase. The *A. thaliana tfl2-2* loss-of-function mutant was obtained from the Arabidopsis Biological Resource Center at the Ohio State University and employed for the mutant rescue experiment.

cDNA library construction and cloning of *MdLHP1a* and *MdLHP1b*

Total RNA was isolated from flower buds of 'Fuji' apple collected on April 26, 2004, in Morioka, Japan, with the CTAB method as described by Kotoda et al. (2000). Poly $(A)^+$ RNA was isolated using a PolyA tract mRNA purification kit (Promega, Madison, WI, USA). The cDNA library was constructed using a ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA) and Packagene® Lambda DNA Packaging System (Promega). The average size of the inserted cDNAs was about 1.3 kbp. PCR amplification of *Arabidopsis LHP1* DNA fragments was carried out with a

pair of primers (LHP1F: 5′-TAG GAA ACG GAA GCG CAA ATA TGC AG-3′; LHP1R: 5′-CCA ACA CTT CCT GGA CAT TGT CTG AT-3′). The PCR-amplified fragment (gLHP1, 696 bp) was cloned into the pT7Blue vector (Novagen, Darmstadt, Germany). The gLHP1 labeled with [α-^{32}P]dCTP (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was used as a probe to screen 1.0×10^5 pfu of the apple cDNA library. A positive plaque was excised to the pBluescript SK(−) phagemid clone (*MdLHP1a1*), and this clone was then sequenced. Then, 5.0×10^5 pfu of the cDNA library was screened with a *MdLHP1a1* probe labeled with digoxigenin (DIG, Roche Diagnostics, Mannheim, Germany), which was amplified by PCR with a pair of primers designed for the 5′ region (MdLHP1F-*Nhe*I: 5′-GTA **GCT AGC** ACA A*AT G*AG AAC CAA GG-3′ with *Nhe*I site in bold and ATG in italic; MdLHP1-600R: 5′-GAC GAT GCC AAT TTC CAC AT-3′). The hybridization was performed in DIG Easy Hyb (Roche Diagnostics) at 42°C for 16 h followed by two rinses in 2× SSC containing 1% (w/v) SDS at room temperature for 5 min and two washes in 0.5× SSC containing 1% (w/v) SDS at 68°C for 20 min. Chemiluminescent signals were visualized using the LAS1000 image analyzer (Fuji Photo Film, Aichi, Japan). PCR amplification of *MdLHP1a* genomic DNA was carried out in a mixture of a pair of primers [MdLHP1F-*Nhe*I and MdLHP1a1R-*Xho*I: 5′-TGC **CTC GAG** A*CT A*AA ATG TAG GGT TGT AT-3′ with *Xho*I site in bold and CTA (stop codon) in italic], EX-Taq DNA polymerase (Takara Biomedical, Tokyo, Japan), and 250–300 ng of genomic DNA as a template. PCR was programmed for preheating at 96°C for 3 min followed by 30 cycles of 96°C for 20 s, 58°C for 30 s, and 72°C for 5 min. A 4.6-kb PCR-amplified product was cloned into the pT7Blue vector (Novagen).

Sequence analyses

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). Nucleotide and amino acid sequences were analyzed using CLUSTAL X multiple sequence alignment program Version 1.83 (Jeanmougin et al. 1998) and GeneDoc (Nicholas et al. 1997). The phylogenetic tree was displayed using the Njplot program (Perrière and Gouy 1996). A homology search was performed with BLAST2 of the GenomeNet (http://www.genome.ad.jp/).

Southern blot analysis

Southern blot analysis was carried out according to the method described by Sambrook et al. (1989). Fifteen micrograms of genomic DNA, isolated from the leaves of apple, was digested with *Eco*RI, *Pst*I, *Bam*HI, or *Eco*RV and then electrophoresed on a 0.8% agarose gel. The DNA bands were blotted onto a nylon membrane Hybond-N+ (GE Healthcare Bio-Sciences Corp.) and hybridized with the full-length cDNA of *MdLHP1a1* labeled with DIG by PCR as a probe. The hybridization signals were detected in the same manner as described in the section of cloning.

Expression analyses by RT-PCR and Northern hybridization

Total RNAs were extracted from flower buds (late April), fruit receptacles, and apical buds of fruit-bearing shoots (FBS) and succulent shoots (SS) (from June to October in 2004) of adult ‘Jonathan’ apple trees and stems, apical buds of vegetative shoots (VS), and leaves of 1-month-old seedlings and cotyledons and roots of 6-day-old seedlings. For RT-PCR analysis, first-strand cDNAs were synthesized from 2 μg of total RNAs in 30 μl of reaction mixture using the ReverTra Ace kit (Toyobo, Tokyo, Japan). The subsequent PCR reactions were performed with 0.5 μl of the first-strand cDNA reaction mixture as templates. A fragment of *MdLHP1a1/-a2* or *MdLHP1b1/-b2* was amplified with the following primers: sense primer (5′-CAG CAG CGT TCT ACT GAT CCT ATT TAC AAT-3′) and antisense primer (5′-GAT CCA GAT ACA AAT CTC ATA ATA TTT CCT AAT ACA G-3′) for *MdLHP1a1/-a2* and sense primer (5′-CAG AGC AAT CGC AGC ACA CTG GA-3′) and antisense primer (5′-CTC ATC CAA ACT CCG ACT AGT CCC-3′) for *MdLHP1b1/-b2*. As an internal control, a fragment of apple *Histone H3* was amplified with the following primers: sense primer (5′-TGA AGA AGC CCC ACA GAT A-3′) and antisense primer (5′-ACA CAA GAA ACT ATA AAC C-3′) as described by Kotoda et al. (2006). Thermal cycle programs were as follows: 96°C for 3 min followed by 40 cycles of 96°C for 20 s, 65°C for 30 s, and 72°C for 3 min for *MdLHP1a1/-a2* and *MdLHP1b1/-b2* and 96°C for 3 min followed by 32 cycles of 96°C for 20 s, 56°C for 30 s, and 72°C for 30 s for *Histone H3*. For Northern analysis, total RNA from wild-type and transgenic *Arabidopsis* plants was electrophoresed on a denaturing 1.2% agarose gel with formaldehyde and then blotted onto a Hybond N+ membrane (GE Healthcare Bio-Sciences Corp.). The blotted membrane was hybridized with a DIG-labeled *MdLHP1a1* probe. The hybridization was performed in DIG Easy Hyb at 50°C for 16 h. The wash and detection were performed in the same manner as that used for DNA.

Construction of transformation vector

To construct a vector for the constitutive expression of *MdLHP1a*, the coding region of *MdLHP1a1* was amplified

by PCR with a pair of primers MdLHP1F-*Nhe*I and MdLHP1a1R-*Xho*I. The PCR products were subsequently digested with *Nhe*I and *Xho*I and then cloned into the *Xba*I–*Sal*I sites of the plasmid vector pBI9526Ω (a derivative of pBI221) bearing the cauliflower mosaic virus (CaMV) 35S promoter fused with the Ω sequence (Gallie and Walbot 1992) and the nopaline synthase terminator (Tnos) (designated as 35SΩ:*MdLHP1a1*:Tnos/pBI9526Ω). After 35SΩ:*MdLHP1a1*:Tnos/pBI9526Ω was digested with *Eco*RI, the DNA fragment of 35SΩ:*MdLHP1a1*:Tnos was cloned into the respective sites of the pSMAK312Blue binary vector. The binary vector obtained was named 35SΩ:*MdLHP1a1*. To construct a vector for constitutive expression of *MdLHP1b*, the coding region of *MdLHP1b1* was amplified with a pair of primers MdLHP1F-*Nhe*I and MdLHP1b1R-*Kpn*I [5′-ATC **GGT ACC** *TTA* AAA TGT AGG ATT GTA TCG-3′ with *Kpn*I site in bold and TTA (stop codon) in italic]. It was then cloned into the *Xba*I–*Kpn*I sites of modified pSMAK193E (35SΩ/pSMAK193E) to be placed between the promoter 35SΩ and the terminator of the *Arabidopsis* rubisco gene (TrbcS). The resultant binary vector was named 35SΩ:*MdLHP1b1*. The pSMAK binary vector series, pSMAK312Blue and pSMAK193E, were made from the pSMAK251 vector. The characterization of pSMAK251, which contains genes encoding *β*-glucuronidase (GUS) and neomycin phosphotransferase II (NPTII) in its T-DNA region, will be published elsewhere (H. Ichikawa, in preparation).

Arabidopsis transformation

Wild-type (ecotype Columbia) and *tfl2-2 Arabidopsis* plants were transformed with 35SΩ:*MdLHP1a1* and 35SΩ:*MdLHP1b1* using an *Agrobacterium tumefaciens* strain, EHA101 (Hood et al. 1986), by the infiltration method as described by Bechtold et al. (1993). Bleach-sterilized seeds were place on MS plates (Murashige and Skoog 1962) containing carbenicillin (250 mg/l) and kanamycin (35 mg/l). *Arabidopsis* seeds of the T3 plant (primary transformants were defined as T1 plants) were sown on agar plates followed by stratification at 4°C for 3 days and grown in a growth chamber controlled at 22°C under long-day (LD) conditions (16 h light/8 h darkness) or short-day (SD) conditions (8 h light/16 h darkness).

Results

Isolation of four cDNAs of *LHP1* homolog genes from apple

To isolate the apple *LHP1* homolog genes, the PCR-amplified DNA fragment (696 bp) of *Arabidopsis LHP1* was used as a probe to screen a cDNA library prepared from flower buds of the apple cultivar (cv.) ‘Fuji’. One positive plaque was obtained after screening ∼100,000 plaques. The cDNA derived from the plaque was designated as *MdLHP1a1* (DDBJ accession number, AB290726) after *Malus* × *domestica LHP1*. Moreover, we screened about 500,000 plaques from the cDNA library with an *MdLHP1a1* probe. A total of seven positive plaques were obtained and then subjected to sequence analysis, revealing that they could be classified into four different cDNAs including *MdLHP1a1*, three of which were designated as *MdLHP1a2*, *MdLHP1b1*, and *MdLHP1b2* (DDBJ accession numbers, AB290727, AB290728, and AB290729, respectively). The features of four apple *LHP1*-like cDNAs are collectively shown in Table 1. The alignment in nucleotide sequence showed that *MdLHP1a1* was 99.1 and 90.4% identical to *MdLHP1a2* and *MdLHP1b1*, respectively, and that *MdLHP1b1* was 99.1% identical to *MdLHP1b2*. In comparison with the deduced amino acid sequences of the apple *LHP1*-like genes, four substitutions were found between MdLHP1a1 and MdLHP1a2 and between MdLHP1b1 and MdLHP1b2. On the other hand, 35 substitutions and five deletions/insertions were found between MdLHP1a1 and MdLHP1b1 (Fig. 1a). These results suggested that a pair of *MdLHP1a1* and *MdLHP1a2* and a pair of *MdLHP1b1* and *MdLHP1b2* would be allelic genes. The predicted amino acid sequences of MdLHP1a1 shared lower similarity to those of the HP1 homolog proteins from other plant and animal species [45.1% similarity to SlLHP1 (tomato), 41.6% to AtLHP1 (*Arabidopsis*), 34.4% to ZmLHP1 (maize), 34.2% to OsLHP1 (rice), 31.3% to DcLHP1 (carrot), 16.7% to DmHP1 (fruit fly), and 14.6% to HsHP1 (human)], whereas the CD (79.6, 98.0, 89.8, 87.8, 87.8, 67.3, and 63.3% similarities, respectively), CSD (64.8, 62.0, 66.2, 69.0, 71.0, 22.5, and 32.4% similarities, respectively), and three nuclear localization signal motifs among them were

Table 1 The features of four apple *LHP1*-like cDNAs

	cDNA in length (bp)	5′-UTR in length (bp)	3′-UTR in length (bp)	Amino acids in length	Predicted molecular mass (kDa)
MdLHP1a1	1,845	79	404	453	50.1
MdLHP1a2	1,804	75	367	453	50.2
MdLHP1b1	1,795	152	272	456	50.4
MdLHP1b2	1,793	147	275	456	50.4

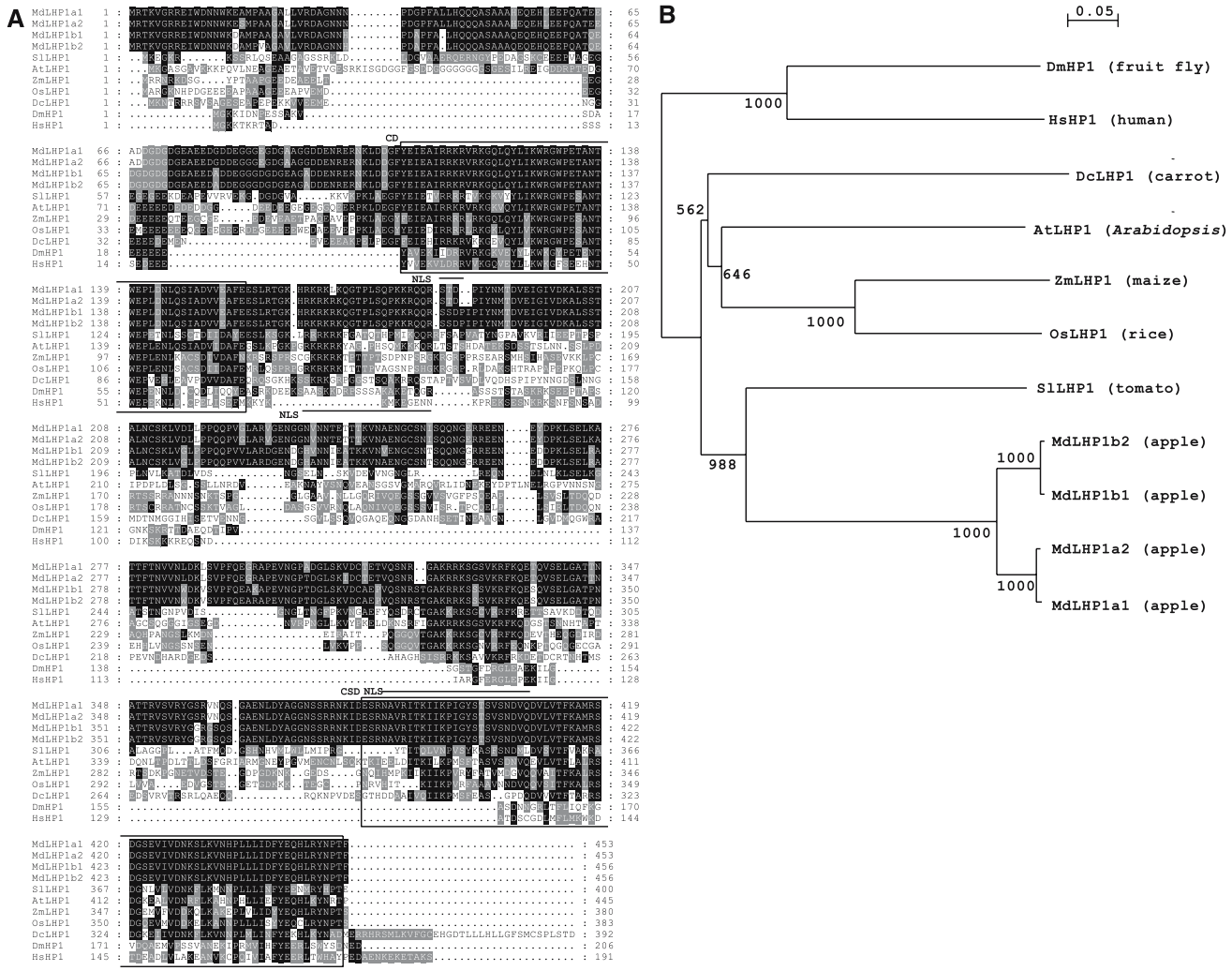

Fig. 1 Comparison of the predicted amino acid sequences of MdLHP1s and HP1 homologs from plant and animal species. **a** Schematic alignment of the predicted amino acid sequences of HP1-like proteins from apple (MdLHP1a1, MdLHP1a2, MdLHP1b2, and MdLHP1b2, in this paper), tomato (SlLHP1/LeHP1, AAL25116), *Arabidopsis* (AtLHP1/TFL2, BAB70689), maize (ZmLHP1, AAM93210), rice (OsLHP1, NP_920664), carrot (DcLHP1, BAA25905), fruit fly (DmHP1, P05205), and homo sapiens (HsHP1, NP_036249). Identical residues are outlined in *white* against black. Highly conserved regions of the chromo domain (*CD*), chromo shadow domain (*CSD*), and putative nuclear localization signal (*NLS*) are indicated by *boxes* for CD and CSD and *underlines* for NLS (Gaudin et al. 2001; Kotake et al. 2003; Zemach et al. 2006). **b** Phylogenetic tree of the HP1-like proteins. The branch lengths are proportional to the sequence divergence. *Numbers* along branches are bootstrap values (1,000 replicates). The *scale bar* represents 0.05 substitutions/site

well conserved, except those of DmHP1 and HsHP1 (Fig. 1a). The phylogenetic analysis indicated that apple LHP1 homologs constitute a small clade and are most similar to tomato SlLHP1 in the HP1 family (Fig. 1b).

Genomic organization

Southern hybridization was performed to assess the copy number of *LHP1* homolog genes in the apple genome (Fig. 2). The genomic DNA of the apple cv. 'Fuji' was digested with *Eco*RI, *Pst*I, *Bam*HI, or *Eco*RV, and the blotted membrane was then subjected to hybridization with full-length *MdLHP1a1* cDNA. Two to three major hybridizing bands were detected in each digested sample. In addition, PCR products (4.6 kb) of apple genomic DNA amplified with primers for *MdLHP1a1/-a2* were digested with each restriction enzyme used in Southern blot analysis to confirm the internal restriction enzyme sites in the probe region (S1). Furthermore, the 4.6-kb PCR products were cloned to characterize the gene structures of *MdLHP1a1/-a2*. Eight individual clones were selected randomly, and their sequences were analyzed. Among them, two clones had the same coding sequence as *MdLHP1a1*, which was designated *gMdLHP1a1* (DDBJ accession number, AB292418). The remaining six clones contained the same coding sequence as *MdLHP1a2*, designated *gMdLHP1a2* (DDBJ accession number, AB292419). The *gMdLHP1a1* sequence contained *Pst*I and *Eco*RV sites, whereas *gMdLHP1a2* has

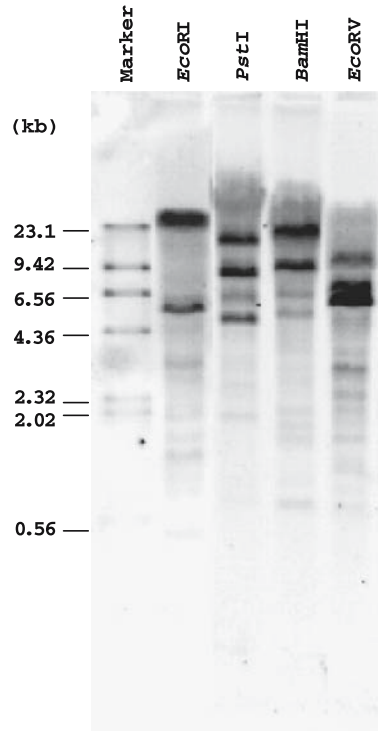

Fig. 2 Southern hybridization analysis of *LHP1* homolog genes in apple. Genomic DNA (10 μg) of 'Fuji' apple digested with *Eco*RI, *Pst*I, *Bam*HI, or *Eco*RV was electrophoresed in a 0.8% agarose gel, blotted onto nylon membranes, and hybridized with an *MdLHP1a1* probe. The molecular size marker is shown in kbp on the *left*

only an *Eco*RV site (Fig. 3). The band pattern of the PCR products digested with each restriction enzyme (S1) was consistent with the result of the sequence analysis of them. These findings, together with the results described in the section above, showed that 'Fuji' apple had two loci of *LHP1* homologs, *MdLHP1a* and *MdLHP1b*, each having two alleles, *MdLHP1a1/-a2* and *MdLHP1b1/-b2*, respectively. The *MdLHP1a1/-a2* (*MdLHP1a*) gene comprised six exons and five introns, such as the *LHP1* gene (Fig. 3). Moreover, the length of exons was well conserved between *LHP1* and *MdLHP1a*, although the fourth intron in *MdLHP1a* was about 28 times longer than that in *LHP1* (Fig. 3).

Expression patterns of *MdLHP1a* and *MdLHP1b* in various tissues and in apical buds throughout the growing season in apple

We performed RT-PCR analysis to clarify the expression patterns of *MdLHP1a* and *MdLHP1b* in apple. Total RNAs were prepared from various tissues, such as floral buds before bud break in late April; receptacles; apical buds of FBS in August; leaves of the apple cv. 'Fuji' in the adult phase; stems, apical buds of VS, and leaves of 1-month-old apple seedlings; and cotyledons and roots of 6-day-old apple seedlings in the juvenile phase. Transcripts of *MdLHP1a* were detected mainly in apical buds in both the adult and juvenile phases (Fig. 4a). On the other hand, transcripts of *MdLHP1b* were detected in all tissues used, with relatively higher expression in the apical buds of FBS in the adult phase (Fig. 4a). To investigate the change of the expression of *MdLHP1a* and *MdLHP1b* in the apical buds of FBS, which have flowers and fruit in the following year, and SS, which are called water sprouts and have no flowers or fruit, RT-PCR analyses for *MdLHP1a* and *MdLHP1b* were carried out using total RNAs extracted from the apical buds of FBS and SS shoots of the apple cv. 'Jonathan' from June to October 2004. 'Jonathan' apple was used in the seasonal expression analysis, because flower development of this cultivar had been studied well. It is noteworthy that the flowering stage of 'Jonathan' apple was early May, and the time of floral bud differentiation was early July in Morioka, Japan (Kotoda et al. 2000). *MdLHP1a* and *MdLHP1b* were expressed constantly in apical buds of both kinds of shoots throughout the growing season (Fig. 4b).

Rescue of the *Arabidopsis tfl2* (*lhp1*) mutant by constitutive expression of apple *MdLHP1a1* or *MdLHP1b1*

In order to analyze the functions of *MdLHP1a* and *MdLHP1b* using transgenic *Arabidopsis*, we generated two

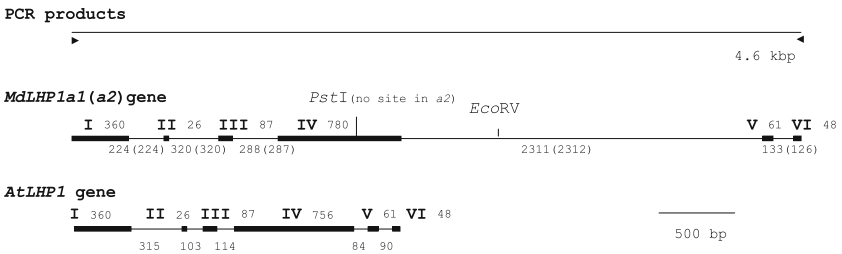

Fig. 3 Schematic representation of the apple *MdLHP1a* gene in comparison with the *Arabidopsis LHP1* gene. The PCR products covering the *MdLHP1a1* and *MdLHP1a2* alleles are shown as a *solid line*, and the *arrowheads* indicate the region for gene-specific primers. Introns are shown as *solid lines*, while exons are shown as *black boxes* and numbered with *Roman numerals* above the black boxes. The lengths (bp) of introns and exons are indicated below (introns) and above (exons) the map. The *scale bar* represents 500 bp

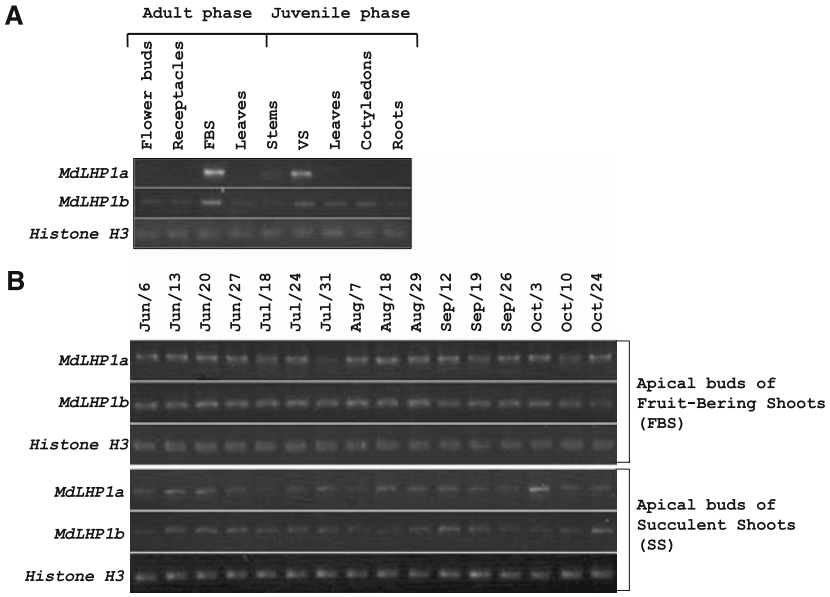

Fig. 4 Expression analysis of *MdLHP1a* and *MdLHP1b*. **a** RT-PCR products of *MdLHP1a* and *MdLHP1b* in various tissues. Total RNAs were isolated from flower buds, fruit receptacles, and apical buds of fruit-bearing shoots (*FBS*) on August 7, from mature leaves of 'Fuji' apple in the adult phase, and from stems, apical buds of vegetative shoots (*VS*), and leaves of 1-month-old seedlings and cotyledons and roots of 6-day-old seedlings in the juvenile phase. **b** RT-PCR products of *MdLHP1a* and *MdLHP1b* in apical buds of FBS or in apical buds of succulent shoots (*SS*) of 'Jonathan' apple from June to October in 2004. The date of sampling the apical buds of both shoots is indicated *above the lanes*. Apple *Histone H3* was used as an internal control of gene expression

chimeric constructs, 35SΩ:*MdLHP1a1* and 35SΩ:*MdLHP1b1* (Fig. 5a). The infiltration method (Bechtold et al. 1993) was employed to introduce those constructs into the *tfl2-2* (*lhp1*) null mutant (Kotake et al. 2003). Consequently, seventeen and seven independent transgenic lines were obtained for the constructs 35SΩ:*MdLHP1a1* and 35SΩ:*MdLHP1b1*, respectively (described as 35SΩ: *MdLHP1a1/tfl2-2* and 35SΩ:*MdLHP1b1/tfl2-2* for each transgenic plant, respectively). T3 transgenic plants were grown under LD conditions (16 h light/8 h darkness) for the examination of their phenotypes, such as plant size, inflorescence morphology, and flowering time (the number of rosette leaves produced at flowering). Five lines of 35SΩ:*MdLHP1a1/tfl2-2* and four of 35SΩ:*MdLHP1b1/tfl2-2* were selected to examine the phenotypes. In the representative transgenic lines of 35SΩ:*MdLHP1a1/tfl2-2* (#1) and 35SΩ:*MdLHP1b1/tfl2-2* (#2), the phenotypes, such as the small plant size and curled leaves that are characteristic of the *tfl2-2* mutant, were rescued (Fig. 5b). Inflorescence shoots, including secondary ones, kept producing flowers in the transgenic lines of 35SΩ:*MdLHP1a1/tfl2-2* and 35SΩ:*MdLHP1b1/tfl2-2* (Fig. 5e, f), showing that both *MdLHP1a1* and *MdLHP1b1* rescued the determinate inflorescences of the *tfl2-2* mutant. The flowering time of the transgenic plants was slightly earlier than that of the wild-type plants but later than that of the *tfl2-2* mutants (Table 2). 35SΩ:*MdLHP1a1/tfl2-2* (#1) plants flowered with 8.0 rosette leaves, and 35SΩ: *MdLHP1b1/tfl2-2* (#2) plants flowered with 8.8 rosette leaves. In contrast, wild-type plants flowered with 8.9 rosette leaves, and *tfl2* plants flowered with 5.3 rosette leaves. The cell size in leaves of the *lhp1* (*tfl2*) mutant is different from that of the wild type under LD conditions (Gaudin et al. 2001). The plant size of wild-type *Arabidopsis* plants increases more when they are grown under SD conditions (8 h light/16 h darkness) than under LD conditions. Because the *tfl2* mutant is photoperiod-insensitive, the cell size of the *tfl2* mutant was apparently smaller than that of the wild type under SD conditions (Fig. 5g, h). We observed the upper epidermis of a third rosette leaf in the 35SΩ:*MdLHP1a1/tfl2-2* (#1) plant under SD conditions, showing partial, if not complete, restoration in the cell size of the rosette leaves (Fig. 5i).

To examine whether transgenic *Arabidopsis* with 35SΩ:*MdLHP1a1* or 35SΩ:*MdLHP1b1* shows additional novel phenotypes, these constructs were also introduced into the wild-type *Arabidopsis* (Columbia). More than thirty kanamycin-resistant lines were obtained for each construct (described as 35SΩ:*MdLHP1a1*/wt or 35SΩ: *MdLHP1b1*/wt, respectively). Most of the T1 transgenic plants showed slightly delayed flowering (70–80% of all lines), but some of them showed pleiotropic phenotypes, such as the *tfl2-2* mutants with early flowering, terminal flowers, and curled leaves (10%) (data not shown). These phenotypes were inherited by subsequent generations. The phenotypes of five representative T3 transgenic lines of 35SΩ:*MdLHP1a1*/wt and four of 35SΩ:*MdLHP1b1*/

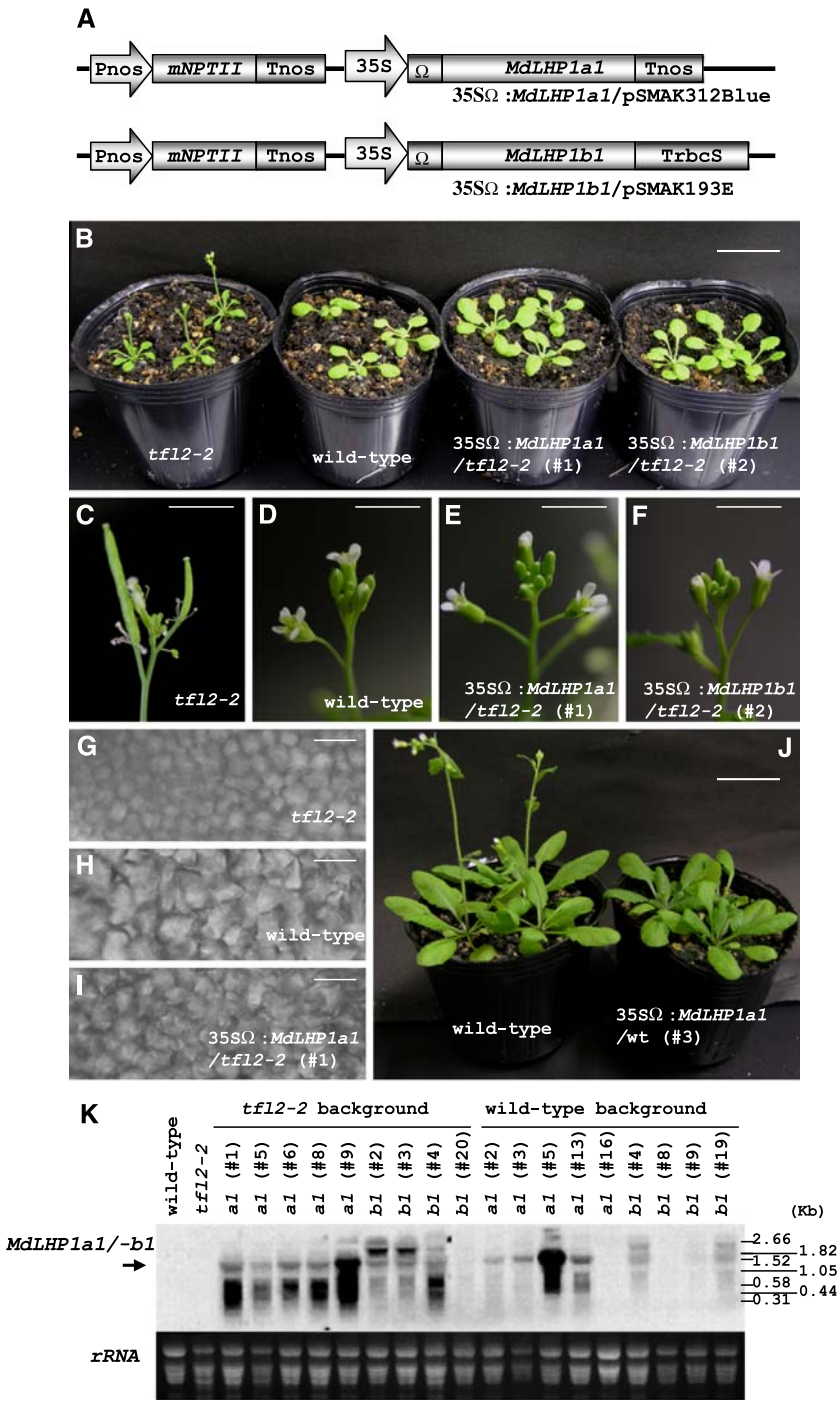

Fig. 5 Constitutive expression of *MdLHP1a1* and *MdLHP1b1* in the *Arabidopsis tfl2-2* mutant and wild-type plants: effects on flowering time and growth. **a** Schematic representation of the transformation vectors, 35SΩ:*MdLHP1a1* and 35SΩ:*MdLHP1b1* used in this study. **b** Seventeen-day-old plants after sowing. From left to right, *tfl2-2*, wild-type, 35SΩ:*MdLHP1a1/tfl2-2* (#1), and 35SΩ:*MdLHP1b1/tfl2-2* (#2). **c**–**f** Inflorescences of the plants shown in **b**, which were photographed 23 days after sowing. **g**–**i** Micrographs of the epidermal cells on a third rosette leaf from *tfl2-2* mutant plants (**g**), wild-type plants (**h**), and 35SΩ:*MdLHP1a1/tfl2-2* (#1) transgenic plants (**i**), which were grown on MS medium plates under short-day (*SD*) conditions (8 h light/16 h dark). **j** Twenty-three-day-old wild-type plants and 35SΩ:*MdLHP1a1*/wt (#3) transgenic plants. **k** Hybridization pattern with an *MdLHP1a1* probe for total RNA from transgenic and control *Arabidopsis* plants. A photograph of the ethidium bromide-stained gel before blotting is shown below. An *arrowhead* indicates the expected size of the full-length mRNA of *MdLHP1a1* and *MdLHP1b1*. The molecular size marker is shown in kbp on the *right*. *Scale bars* are 2 cm in **b** and **j**, 5 cm in **c**–**f**, and 20 μm in **g**–**i**. The plants shown in **b**, **c**–**f**, and **j** were grown in potted soil under long-day (*LD*) conditions (16 h light/8 h dark)

wt showing delayed flowering were examined under LD conditions. 35SΩ:*MdLHP1a1*/wt (#3) plants flowered with 10.4 rosette leaves, whereas wild-type plants flowered with 8.9 rosette leaves (Fig. 5j; Table 2). Delayed growth of transgenic plants with *MdLHP1a* or *MdLHP1b* constructs appeared to develop a few additional rosette leaves, although the appearance of the transgenic plants was similar to that of the wild-type plants. Thus, constitutive expression of *MdLHP1a1* or *MdLHP1b1* could affect flowering time in the wild-type *Arabidopsis* plants.

Northern hybridization analysis in transgenic *Arabidopsis* with *MdLHP1a1* or *MdLHP1b1*

To determine whether or not the expression level of the transgene is correlated with the degree of the phenotype in transgenic plants, Northern hybridization analysis was

Table 2 Flowering time of *tfl2-2* (*lhp1*) and wt plants transformed with 35SΩ:*MdLHP1a1* and 35SΩ:*MdLHP1b1*

Strain	Number of plants[a]	Number of rosette leaves[b]	Number of cauline leaves[b]
Wt (Col)	15	8.9 ± 0.2	2.7 ± 0.2
tfl2-2	13	5.9 ± 0.1	2.6 ± 0.1
35SΩ:*MdLHP1a1/tfl2-2*			
#1	14	8.0 ± 0.3	2.1 ± 0.1
#5	13	7.3 ± 0.2	2.4 ± 0.2
#6	17	9.3 ± 0.3	3.1 ± 0.1
#8	12	7.8 ± 0.2	2.4 ± 0.2
#9	10	7.8 ± 0.3	2.6 ± 0.2
35SΩ:*MdLHP1b1/tfl2-2*			
#2	12	8.8 ± 0.4	2.8 ± 0.1
#3	12	7.3 ± 0.2	2.6 ± 0.2
#4	13	7.2 ± 0.4	2.4 ± 0.2
#20	12	7.7 ± 0.3	2.8 ± 0.1
35SΩ:*MdLHP1a1/wt*			
#2	12	10.4 ± 0.6	2.9 ± 0.2
#3	12	10.4 ± 0.6	2.4 ± 0.2
#5	12	10.3 ± 0.4	2.7 ± 0.1
#16	12	9.2 ± 0.3	2.3 ± 0.1
35SΩ:*MdLHP1b1/wt*			
#4	7	9.7 ± 0.5	2.9 ± 0.1
#8	11	8.9 ± 0.4	2.4 ± 0.2
#9	12	7.8 ± 0.4	2.1 ± 0.1
#19	12	9.5 ± 0.3	2.7 ± 0.2

[a] Plants were grown on the potted soil at 22°C under the day length conditions at 16 h of light/8 h of darkness (long-day conditions)

[b] The number of rosette and cauline leaves were counted at flowering. Values are mean ± standard error (*SE*)

performed using a DIG-labeled *MdLHP1a1* probe. As a result, the expression of the transgenes was confirmed in all transgenic lines with the *tfl2-2* mutant background (Fig. 5k). In contrast, several lines showed little expression of the transgenes with the wild-type background (Fig. 5k). The degree of delayed flowering was not exactly due to the expression level of *MdLHP1a1* or *MdLHP1b1* in transgenic plants, although the delayed flowering in these transgenic plants appeared to depend on the expression of *MdLHP1a1* or *MdLHP1b1* (Fig. 5k). For example, lines 35SΩ:*MdLHP1a1/tfl2-2* #2 and #9 were considerably different in the expression level of *MdLHP1a1*, but there was little difference between them in the total numbers of rosette leaves produced at flowering (Fig. 5k; Table 2). A similar result was obtained in lines 35SΩ:*MdLHP1a1*/wt #2 and #5 (Fig. 5k; Table 2). In addition, smaller than expected sizes of the *MdLHP1a1*/*MdLHP1b1* transcripts were detected, predominantly in the transgenic plants with the *tfl2-2* background, as compared with those with the wild-type background (Fig. 5k).

Discussion

In this study, we isolated and characterized four cDNAs of apple *LHP1* homologs, *MdLHP1a1*, *MdLHP1a2*, *MdLHP1b1*, and *MdLHP1b2*. The genomic Southern hybridization and sequence analyses revealed that the apple cv. 'Fuji' had two loci of *LHP1* homologs, *MdLHP1a* and *MdLHP1b*, each having two alleles, *MdLHP1a1*/*-a2* and *MdFL2b1*/*-b2*, respectively, and that the genomic structure of *MdLHP1a* was similar to that of *Arabidopsis LHP1* (Fig. 3). A comparison of the amino acid sequences revealed that the plant LHP1 homologs exhibited lower similarity with each other, suggesting that a rapid divergence of these genes had occurred among plant species through evolution (Fig. 1a, b). In accordance with the dynamic modification of the chromatin structures in the evolutionary timescale, the LHP1 family regulating the chromatin structure might also have evolved rapidly and been widely distributed among plant species. However, CD, CSD, and nuclear localization signal motifs characteristic of the LHP1 family were highly conserved in apple and other plant LHP1 homologs (Fig. 1a). In animals, the CD of a human PcG is mainly involved in recognition of histone modifications and essential for protein-protein interaction within a multimeric protein complex; the CSD of HP1 protein is involved in self-association and interaction with the chromatin assembly factor 1 (CAF1) and the transcriptional intermediary factor 1β (TIF1β) proteins (Brasher et al. 2000; Eissenberg 2001; Cammas et al. 2004). In plants, the CD of SlLHP1 (LeHP1, tomato LHP1) is required for the interaction of SlLHP1 with K9-methylated Histone H3, which was revealed by Glutathione-S-transferase (GST) pull-down assays in vitro (Zemach et al. 2006). Furthermore, the CSD of both AtLHP1 and SlLHP1 is responsible for homo-dimerization (Gaudin et al. 2001; Zemach et al. 2006) and SlLHP1 CSD could participate in interaction with chromocenters in *Arabidopsis* cells (Zemach et al. 2006). In this study, we demonstrated that the constitutive expression of *MdLHP1a1* or *MdLHP1b1* could restore the flowering time and cell size of rosette leaves in the *tfl2-2* mutant (Fig. 5; Table 2). Observations of transgenic *tfl2-2* plants with *MdLHP1a1*/*-b1* have shown that these genes function equivalently to *Arabidopsis LHP1*. Based on these results, *LHP1* homolog genes in plants are essential for its growth, as they are in animals; CD and CSD, which are highly conserved in HP1 family, would especially be important for the biological function of HP1 homolog proteins.

MdLHP1a and *MdLHP1b* were constitutively and abundantly expressed in apical buds of proliferating fruit-bearing and succulent (vegetative) shoots of apple trees throughout the growing season (Fig. 4). In transgenic *Arabidopsis* expressing *MdLHP1a1* or *MdLHP1b1* both in *tfl2-2* and

wild-type backgrounds, the amount of the transcripts of *MdLHP1a1* and *MdLHP1b1* might not be correlated with the extent of the phenotypes, such as flowering time (Fig. 5k; Table 2). In *Arabidopsis*, *LHP1* and *EMF2*, which maintain the gene activity states epigenetically, and are expressed predominantly in proliferating cells. In addition, overexpression of these genes does not affect the growth phases. These observations suggest that HP1-family and PcG genes are post-transcriptionally regulated (Gaudin et al. 2001; Yoshida et al. 2001; Hsieh et al. 2003; Kotake et al. 2003). It was consistent with these observations that the phenotype of transgenic plants with *MdLHP1a1/-b1* was not necessarily correlated with the extent of transcription level. In Northern blot analysis, the aberrant or incomplete transcripts of *MdLHP1a1/-b1* were found in transgenic *Arabidopsis* plants with *MdLHP1a1/-b1*, predominantly in the *tfl2-2* mutant background. On the other hand, few aberrant transcripts were found in transgenic plants with *MdLHP1a1/-b1* in the wild-type background, although the expression of *MdLHP1a1/-b1* in the wild-type background was no less than that in the *tfl2-2* background. The lower level of aberrant transcripts in the wild-type background may be due to a mechanism that represses the abnormal accumulations of the functional transcripts of *LHP1*-like genes. In some (10%) of the transgenic *Arabidopsis* with 35SΩ:*MdLHP1a1/-b1* in the wild-type background, down-regulation or silencing of endogenous *LHP1* might cause the expression of *tfl2-2* mutant-like phenotypes by a mechanism, such as co-suppression or RNA interference (RNAi).

In addition to *MdLHP1a* and *MdLHP1b*, several pairs of two duplicated genes regulating floral identity or flowering time have been identified in the apple genome; for example, pairs of *APETALA1* (*AP1*)-like genes from the apple cv. 'Granny Smith', 'Jonathan', 'Fuji', and 'Pinova' (Yao et al. 1999; Kotoda et al. 2000, 2002; our unpublished results), *LEAFY* (*LFY*)-like genes from 'Jonathan' and 'Fuji' (Wada et al. 2002; Esumi et al. 2005), and *TFL1*-like genes from 'Jonathan' and 'Fuji' (Esumi et al. 2005; Kotoda and Wada 2005; Kotoda et al. 2006; our unpublished results) have been isolated and characterized. The pair of paralogous genes related to apple flowering seems to have similar regulatory functions, considering the results from the analysis of transgenic *Arabidopsis* ectopically expressing those genes, although the expression levels and patterns in apple are not identical between the twin paralogs. The fact that there are pairs of two copy genes in the apple genome supports the hypothesis that the *Maloideae* subfamily (a basal haploid number, $n = 17$), which includes the genera *Malus* (apple) and *Pyrus* (pear), had evolved from the polyploid ancestor. It was hypothesized that *Maloideae* subfamily had been created by hybridization between ancestors of two other *Rosaceae* subfamilies, *Spiraeoideae* ($n = 9$) and *Amygdaloideae* ($n = 8$) (Sax 1933; Luby 2003) or that the origin of *Maloideae* ($n = 18$) had occurred from members of a lineage containing the ancestors of *Gillenia* ($n = 9$), and then its progeny had lost one chromosome ($n = 17$) (Evans and Campbell 2002). Because polyploidy (genome doubling) can easily produce functional divergence among homolog genes, this is one of the evolutional strategies in plants that enhances environmental adaptation (Adams and Wendel 2005). As an example of studies on such an evolutionary polyploid complex genome, the chromosomal rearrangement of amphidiploid *Brassica napus* (AACC) resynthesized by hybridization between allogenic *B. rapa* (AA) and *B. oleracea* (CC) affects the gene expression of *FLOWERING LOCUS C* (*FLC*) homologs, leading to a divergence in flowering time (Pires et al. 2004; Adams and Wendel 2005). In consideration for *Brassica* genome study, the polyploid complex genome may produce a variety of phenotypes in apple as well. Sung et al. (2006) reported that *Arabidopsis LHP1* also plays a crucial role in vernalization by regulating *FLC*. Interestingly, the vernalization requirement in some *Arabidopsis* ecotypes for its development resembles the chilling requirement in deciduous trees, such as the apple, which are adapted to survive at cooler temperatures in winter, although the apple is a perennial plant and its life cycle is different from that of annual plants, such as *Arabidopsis*.

Taken together, *LHP1* homolog genes of chromatin regulator might have rapidly evolved and be diverged widely among plant species following the dynamic modification of chromatin structures with chromosomal rearrangement during evolution, but the protein functions were conserved, at least between *Arabidopsis* and apple. Recently, we produced transgenic apples with an RNAi construct for *MdLHP1a/MdLHP1b*. These transgenic approaches may provide not only the insight into the practical aspects, such as the manipulation of flowering and the acceleration of breeding, but also a novel notion of the mechanism controlling flowering or dormancy via epigenetic system in fruit trees.

Acknowledgments We thank Dr. H. Ichikawa, National Institute of Agrobiological Sciences (Tsukuba, Japan), for kindly providing the binary vectors, pSMAK312Blue and pSMAK193E. We also thank Dr. E.E. Hood, Texas A&M University College Station, for kindly providing *Agrobacterium tumefaciens* EHA101. *A. thaliana tfl2-2* seeds were kindly provided by the Arabidopsis Biological Resource Center at Ohio State University. This work was supported by the "PROBRAIN (Program for Promotion of Basic Research Activities for Innovative Biosciences)" from the Bio-Oriented Technology Research Advancement Institution (BRAIN). This publication constitutes the contribution number 1445 of the National Institute of Fruit Tree Science.

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