Expression of LhFT1, the Flowering Inducer of Asiatic Hybrid Lily, in the Bulb Scales

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Asiatic hybrid lily leaves emerge from their bulbs in spring, after cold exposure in winter, and the plant then blooms in early summer. We identified four FLOWERING LOCUS T (FT)-like genes, LhFT1, LhFT4, LhFT6, and LhFT8, from an Asiatic hybrid lily. Floral bud differentiation initiated within bulbs before the emergence of leaves. LhFT genes were mainly expressed in bulb scales, and hardly in leaves, in which the FT-like genes of many plants are expressed in response to environmental signals. LhFT1 was expressed in bulb scales after vernalization and was correlated to flower bud initiation in two cultivars with different flowering behaviors. LhFT8 was upregulated in bulb scales after cold exposure and three alternative splicing variants with a nonsense codon were simultaneously expressed. LhFT6 was upregulated in bulb scales after flower initiation, whereas LhFT4 was expressed constantly in all organs. LhFT1 overexpression complemented the late-flowering phenotype of Arabidopsis ft-10, whereas that of LhFT8 did so partly. LhFT4 and LhFT6 overexpression could not complement. Yeast two-hybrid and in vitro analyses showed that the LhFT1 protein interacted with the LhFD protein. LhFT6 and LhFT8 proteins also interacted with LhFD, as observed in AlphaScreen assay. Based on these results, we revealed that LhFT1 acts as a floral activator during floral bud initiation in Asiatic hybrid lilies. However, the biological functions of LhFT4, LhFT6, and LhFT8 remain unclear.

Keywords: alternative splicing, cold exposure, flower initiation, FLOWERING LOCUS T like genes, geophytes, Lilium sp., ornamental plants

INTRODUCTION

The genus Lilium consists of approximately 100 species that are distributed throughout the cold and temperate regions of the Northern Hemisphere and are classified into seven sections (van Tuyl et al., 2018). Lilies are important ornamental plants that include three main distinctive hybrid groups, i.e., Easter lilies, Asiatic hybrid lilies, and Oriental hybrid lilies (Dole and Wilkins, 2005). Asiatic hybrid lilies are derived from interspecific hybridization among L. dauricum, L. maculatum, L. lancifolium, etc., which are species that belong to the sections Sinomartagon and Dauriolirion (Marasek-Ciolakowska et al., 2018). Moreover, these plants are characterized by an upward-facing flower and...
little or no fragrance (Dole and Wilkins, 2005). The color of their flowers is often uniform or with a contrasting perianth segment tips and/or throat, and they exhibit a wide variety of flower colors in different shades, from white to red and yellow (Yamagishi, 2013). Commercial Asiatic hybrid lily cultivars are usually propagated by bulbs, rather than seeds (Beattie and White, 1993).

The typical life cycle of Asiatic hybrid lilies starts with bulb planting in the autumn (October–November), followed by the exposure of bulbs to the low temperatures of winter, which is necessary for flower initiation. Most Asiatic hybrid lilies are vernalized at 1°C–2°C for at least 6 weeks (Nau, 2011). The bulbs sprout in spring and flower in late spring to early summer (Okubo and Sochacki, 2012). Based on the timing of flower bud initiation, 85 Asiatic hybrid cultivars were classified into two types (Ohkawa et al., 1990). In the majority of cultivars (69%), flower bud differentiation starts and is completed after shoot emergence. Conversely, in the remaining cultivars (31%), flower bud initiation commences inside the bulb. With a few exceptions, the former type of Asiatic hybrid lily cultivars flower later than the latter (Ohkawa et al., 1990). Moreover, no relationship has been identified between shoot growth and flower bud initiation. In L. longiflorum, cold exposure is not an obligatory prerequisite for flowering, as an alternative flowering pathway can bypass vernalization in small bulbs (Lazare and Zaccal, 2016). Recently, the levels of glycerol in L. longiflorum bulbs was found to be associated with a delay in sprouting and flowering time and a reduction in abortion rate (Lazare et al., 2019).

The floral integrator FLOWERING LOCUS T (FT) is a key regulator of flowering time in Arabidopsis (Corbesier et al., 2007). The FT protein is induced under the flowering-inducive long-day photoperiod in leaves, and is then transported via the phloem to the shoot apical meristem (SAM), where it interacts with the bZIP transcription factor FD (Abe et al., 2005; Corbesier et al., 2007; Tamaki et al., 2007). This protein complex is assumed to comprise two FT monomers and two FD bZIP transcription factors, as well as a dimeric 14–3–3 protein, which acts to bridge the FT–FD interaction (Taoka et al., 2011). The florigen activation complex leads to the direct activation of the floral meristem identity genes, such as APETALA1 (API) and FRUITFULL (FUL) (Taoka et al., 2011). FT is a member of the phosphatidyl ethanolamine-binding protein (PEBP) gene family (Danilevskaya et al., 2008).

Arabidopsis carries another PEBP gene, TERMINAL FLOWER 1 (TFL1), which determines inflorescence development and suppresses flowering (Bradley et al., 1997). Several amino acids are important for the specific and unique function of FT and TFL1 in Arabidopsis (Hanzawa et al., 2005; Ahn et al., 2006). Floral activators such as FT contain a tyrosine (Y) at position 85, whereas floral repressors contain a histidine (H) at the analogous position 88 (Hanzawa et al., 2005). The amino acid residues at position 140 can also affect the function of the protein. In the FT activator, a glutamic acid (Q) is present at position 140, whereas an aspartate (D) is positioned at the same analogous position in the repressors (Ahn et al., 2006). In sugar beets (Beta vulgaris), BvFT2 is the functional FT ortholog, while BvFT1 is a flowering suppressor, despite being in the FT subfamily (Pin et al., 2010). Chrysanthemums (Chrysanthemum morifolium) are categorized as absolute short-day plants. In addition, CsFTL3 encodes a florigen that is induced under short photoperiod conditions in chrysanthemum plants, whereas CsAFT encodes an anti-florigen that acts systemically to inhibit flowering under long-day photoperiod conditions and plays a predominant role in the obligate photoperiodic response (Oda et al., 2012; Higuchi et al., 2013; Nakano et al., 2019).

In addition to functioning as activators or repressors of flowering, members of the PEBP family are also involved in a variety of other processes (Wickland and Hanzawa, 2015), such as tuberization (Navarro et al., 2011), bulb formation (Lee et al., 2013), stomatal opening (Kinoshita et al., 2011), and photoperiodic control of seasonal growth in trees (Bohlenius et al., 2006; Hsu et al., 2006, 2011). In Arabidopsis, FT genes are involved in the regulation of H+–ATPase by blue light in stomatal guard cells, resulting in the regulation of stomatal opening by FT (Kinoshita et al., 2011). The tuberization of potatoes (Solanum tuberosum) under a short-day photoperiod is controlled by a homolog of FT, StS6P6A (Navarro et al., 2011). Interestingly, an StS6P6A-specific small RNA is induced by elevated temperatures and suppresses the tuberization of potatoes (Lehretz et al., 2019). Four FT-like genes (AcFT1, AcFT2, AcFT4, and AcFT6) have been identified in onions (Allium cepa) (Lee et al., 2013). AcFT2 is expressed during vegetative growth and likely regulates growth cessation and bud set, which promotes flowering. AcFT4 functions as an inhibitor of bulbing, whereas AcFT1 as a promoter of bulbing. Moreover, AcTFL1 is highly expressed during bulbing and inflorescence development in onions (Dalvi et al., 2019). The expression levels of AcTFL1 within the bulb are lowest in the outmost layers and highest in the innermost layers.

Some species of the genus Lilium have been studied regarding the genes that control flowering and vernalization. An RNA-seq analysis of molecules involved in the vernalization response in the Oriental hybrid lily “Sorbonne” identified two vernalization genes, SHORT VEGETATIVE PHASE (LoSVP) and VERNALIZATION 1 (LoVRN1), as well as the floral transition key gene SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (LoSOC1) (Liu et al., 2014; Li et al., 2016). Similarly, L. lancifolium (which is a breeding material for Asiatic hybrid lilies), the Asiatic hybrid lily “Tiny ghost,” and L. longiflorum “White Heaven” were also subjected to transcriptome profiling during the vernalization process by RNA-seq, which led to the identification of several cold signal transduction genes (Huang et al., 2014; Wang et al., 2014; Hamo et al., 2015; Villacorta-Martin et al., 2015). L. × formolongi, which is a lily hybrid between L. formosanum and L. longiflorum, flowers within 1 year of sowing. CO-LIKE (COL), FT, TREHALOSE-6-PHOSPHATE SYNTHASE (TPS), SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE (SPL) homologs may play significant roles in the flowering-induction and transition process of L. × formolongi (Li et al., 2017). Thus, most flowering studies of the genus Lilium were based on integrated expression analyses and the detailed function of FT-like genes remains unclear. Conversely, the LIFT gene from L. longiflorum is upregulated by cold exposure, and the overexpression in Arabidopsis and lily plants leads to an early-flowering phenotype (Leeggangers et al., 2018). Therefore, LIFT may be involved
in the vernalization response of lily and may be able to replace cold exposure.

Several studies of FT-like genes have been reported in various monocot horticulture plants, with the exception of lily. Tulips (Tulipa gesneriana) carry three FT-like genes, TgFT1, TgFT2, and TgFT3 (Leeggangers et al., 2017, 2018). TgFT2 is considered to act as a flowering inducer, as it is involved in floral induction in tulips, whereas TgFT3 is assumed to have a bulb-specific function.

In Chinese narcissuses (Narcissus tazetta), which are plants that exhibit summer dormancy, high temperatures are necessary for release from dormancy (Li et al., 2013; Noy-Porat et al., 2013). Under high temperature conditions (25°C–30°C) in the dark, NtFT expression occurred simultaneously with floral induction in the bulb meristems of Chinese narcissus plants, indicating that floral induction is affected by high temperature, but not by photoperiod or vernalization (Li et al., 2013; Noy-Porat et al., 2013).

In this study, we attempted to isolate and characterize FT-like genes in ornamental Asiatic hybrid lily.

**MATERIALS AND METHODS**

**Plant Materials**

The bulbs (circumference, 16 cm) of the Asiatic hybrid lily ‘Lollypop’ [original name, ‘Holebibi’ (Matthews, 2007)] were purchased from the Niigata Flower Bulb Growers Cooperative Association (Niigata, Japan) in September 2016 and 2017 (Figures 1A, B). The bulbs of L. leichtlinii ‘Hakugin’ which is one of the breeding materials of this Asiatic hybrid lily, were also harvested in August 2015 from a field of Hokkaido University (Sapporo, Japan) and were stored at 20°C until use. Cold treatment of bulbs was performed in peat moss in the dark at 4°C for 4 months. In early March, all bulbs were planted at a density of 12 bulbs per 16 L planter, which was filled with a mix of akadama and leaf mold (2:1) containing 2.5 g·L⁻¹ Magamp K (Hyponex Japan, Osaka, Japan). The plants were grown in a field of Shizuoka University (Shizuoka, Japan) under natural conditions.

During cold treatment and after planting, scales, SAMs, and leaves were sampled. The second and third outer scales and SAMs containing the basal plate were collected from bulbs, whereas SAMs with a height of approximately 1 cm were collected from sprouted plants. The mature leaves were also collected from sprouted plants.

**Observation of Floral Development**

Floral differentiation of SAM was observed in at least three independent samples for each point using a stereomicroscope. Floral developmental stage was defined as follows: (1) Vegetative (Figure 1C); (2) flower initiation (Figure 1D); (3) bract formation (Figure 1E); (4) outer perianth primordia (Figure 1F); (5) inner perianth primordia, (6) perianth development; (7) stamen differentiation; (8) pistil primordia (Figure 1G); (9) pistil differentiation; and (10) pistil development (Figure 1H; Fukai and Goi, 2001).

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FIGURE 1 | Vegetative and reproductive development stages in the Asiatic hybrid lily ‘Lollypop.’ (A) Bulbs before chilling exposure. Bar, 2 cm. (B) Anthesis. Bar, 2 cm. (C–H) Definition of the floral development stages of the SAM. Bar, 500 μm. br, bract; op, outer perianth; ip, inner perianth; st, stamen; pi, pistil. (C) Vegetative SAM. (D) Flower initiation, rounded SAM. (E) Bract formation. (F) Formation of outer and inner perianthes primordia. (G) Formation of pistil primordia. (H) Pistil development. (I) Flower bud transition after planting. Flower developmental stages are defined in “Materials and Methods.” We observed three independent SAMs each week. (J) Alteration of plant height and leaf number after planting. The blue arrow indicates leaves emerged from the soil at three weeks after planting. The red arrow indicates “Lollypop” blooming at 75 days after planting.
Isolation of FT Orthologs From an Asiatic Hybrid Lily

The deposited transcriptome data (ERR578452 to ERR578471) of *L. longiflorum* were assembled using the trinity program of the DDBJ read annotation pipeline (Nagasaki et al., 2013); 300,375 contigs were obtained. *Lilium FT/TFL1* orthologs were identified in these contigs by the blast program using *FT/TFL1* orthologs from several plant species as queries. Several primers based on the nucleotide sequences of nine *FT/TFL1* candidate genes from *L. longiflorum* were designed to amplify *FT/TFL1* and flowering-related gene orthologs from an Asiatic hybrid lily, as shown in Supplementary Table 1.

Shoot apical meristems, scales, and leaves of *L. leichtlinii* ‘Hakugin’ were sampled at several developmental stages. Total RNA was isolated from each sample using the Fruit mate for RNA purification (Takara Bio, Shiga, Japan) and RNAiso Plus (Takara Bio). cDNAs were synthesized using a PrimeScript II 1st strand cDNA synthesis kit (Takara Bio). Using the primers that were designed as described above, we amplified putative *FT/TFL1* orthologs using the *L. leichtlinii* ‘Hakugin’ cDNA. The reaction mixture (25 μL) consisted of 1× *Ex Taq* buffer, 200 μM dNTPs, 0.2 μM each primer, 0.25 U of *Ex Taq* polymerase (Takara Bio) and 1 μL of template cDNA. The thermal cycler program was set as follows: 94°C for 2 min; followed by 35 cycles of 94°C for 20 s, 40°C–55°C for 40 s, and 72°C for 1 min; and a final step at 72°C for 10 min. The amplified fragments were subcloned into the pGEM-Teasy vector system (Promega, Madison, WI, United States). The sequences of all constructs were confirmed by DNA sequencing (Fasmac, Kanagawa, Japan).

Based on the partial sequences obtained for each *FT/TFL1* fragment, we used the rapid amplification of cDNA ends (RACE) technology to obtain full-length cDNA sequences with the SMARTer RACE 5’/3’ kit (Takara Bio). The open reading frame (ORF) sequences were amplified using *Ex Taq* polymerase, and the primer sets are listed in Supplementary Table 1. The thermal cycler program was set as follows: 94°C for 2 min; followed by 35 cycles of 94°C for 20 s, 60°C for 40 s, and 72°C for 2 min; and a final step at 72°C for 10 min. The amplified fragments were cloned and sequenced as described above. A phylogenetic tree of FT-like protein was constructed using ClustalW with neighboring-algorithm and visualized using MEGA ver. 7 software (Kumar et al., 2016).

Expression Analysis

Total RNA was isolated from the SAMs, scales, and leaves of each treated plant. cDNA was synthesized from total RNAs, as described above. For the RT-PCR analyses, a reaction mixture (50 μL) consisted of 1× *Ex Taq* buffer, 200 μM of dNTPs, 0.4 μM of each primer, 0.25 U of *Ex Taq* polymerase Hot Start version (Takara Bio), and 1 μL of template cDNA. The PCR cycling conditions were as follows: 2 min at 94°C; 26–34 cycles for 20 s at 95°C, 40 s at 55°C, and 1 min at 72°C; and final extension for 10 min at 72°C. The sequences of the primers are listed in Supplementary Table 2. The PCR products were separated on 1.5% agarose gels in TAE buffer and stained with ethidium bromide.

Reverse transcription-quantitative PCR (RT-qPCR) analyses used a Thermal Cycler Dice Real-Time System (TP850, Takara Bio), according to MIQE guidelines (Bustin et al., 2009). Briefly, the reaction mixture (10 μL) consisted of 1× KAPA SYBR Fast qPCR Master Mix (KAPA Biosystems, Wilmington, MA, United States), 0.2 μM of each primer, and 1 μL of template cDNA. Cycling conditions were as follows: 95°C for 20 s; followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. The sequences of the primers used in this study are listed in Supplementary Table 2. The specificity of amplification was checked by the addition of a dissociation analysis step after the cycle reactions. Data were analyzed by second derivative maximum methods using Thermal Cycler Dice Real-Time System II software version 5.00 (Takara Bio). Transcript levels were calculated relative to the actin-encoding *LhACT* gene (AB438963) used as a reference (Yamagishi et al., 2014; Suzuki et al., 2016). In a preliminary experiment, similar amplification rates of *LhACT* were detected among scale, SAM, and leaf samples of ‘Lollypop’ at different developmental stages (Supplementary Figure 1). The RT-qPCR analyses used six biological replicates.

Yeast Two-Hybrid Analysis Between *LhFT* Proteins and *LhFD*

To investigate whether the *LhFT* proteins interact with the *LhFD* protein, we employed a yeast two-hybrid analysis using the Matchmaker Two-Hybrid System 3 (Clontech, Takara Bio, Shiga, Japan) as described previously (Nakatsuka et al., 2019). The coding regions of *LhFT1*, *LhFT4*, *LhFT6*, *LhFT8*, and *LhFD* were cloned into either pGAD-T7 (GAL4 activation domain) or pGBK-T7 (GAL4 DNA-binding domain) vectors. All the constructs were transformed into *Saccharomyces cerevisiae* AH109 (Clontech). Transformed yeast cells were grown on SD selective medium without leucine (−Leu) and tryptophan (−Trp) at 30°C for 3 days. A survival test was performed for each transformed yeast culture using selective quadruple-dropout medium (−Leu, −Trp, histidine [−His], and adenine [−Ade]) supplemented with 15 mM of 3-amino-1,2,4-triazole (3-AT) at 30°C for 3 days.

AlphaScreen-Based *in vitro* Protein–Protein Interaction Assay

The ORFs of *LhFTs*, *LhFD*, *AtFT*, and *AtFD* were modified with two-step PCR using gene-specific primer pairs with S1 or T1 linker sequences for the first step and primers attB1-S1 and attB2-T1 for the second step. The DNA fragments were cloned into pDONR221 vector using the gateway cloning system (Thermo Fisher Scientific). These expression vectors were generated using LR clonase recombination with pEU-E01-GW-AGIA (Yano et al., 2016) or pEU-E01-GW-bs (Iwasaki et al., 2016) vector for cell-free protein synthesis or transient expression vectors. All primer sequences are listed in Supplementary Table 3.

*In vitro* transcription and wheat cell-free protein synthesis were performed using the WEPRO1240 expression kit (Cell-Free Sciences, Matsuyama, Japan) according to the manufacturer’s instructions. *In vitro* biotin labeling of recombinant protein was performed as previously described (Sawasaki et al., 2008).
AlphaScreen-based in vitro protein–protein interaction assay was performed as previously described with slight modifications (Nemoto et al., 2017). The assay was performed using a reaction mixture of 15 µL containing AlphaScreen buffer [100 mM Tris–HCl (pH 8.0), 0.1% Tween20, 1 mg·mL⁻¹ BSA], 1 µL of C-terminal biotinylated LhFD, and 1 µL of C-terminal AGIA-tagged LhFTs in a 384-well Optiplate (PerkinElmer, Waltham, MA, United States). After incubation at 25°C for 1 h, 10 µL of detection mixture containing AlphaScreen buffer, 1 µg·mL⁻¹ anti-AGIA antibody (Yano et al., 2016), 0.1 µL of streptavidin-coated donor beads (PerkinElmer), and 0.1 µL of protein-A-coated acceptor beads (PerkinElmer) were added to each well of the 384-well Optiplate, followed by incubation at 25°C for 1 h. Luminescence was analyzed using the AlphaScreen detection program. All data represent the average of three independent experiments, and the background was controlled using a biotinylated dihydrofolate reductase (DHFR) from E. coli.

Complement Expression of LhFT Genes in the Arabidopsis ft-10 Mutant

The ORFs of LhFTs were located between the cauliflower mosaic virus (CaMV) 35S promoter and the Arabidopsis heat-shock protein terminator (HT) of a binary vector, pSHyg-35SproGUS-HT the hygromycin-resistance gene was modified from the pSMAB704 backbone vector (Igasaki et al., 2002). These constructs were then transformed into the Agrobacterium tumefaciens EHA101 strain. Arabidopsis ft-10 mutant was transformed using the floral dip method, as described (Clough and Bent, 1998). Positive transformants were selected on germination medium supplemented with 30 mg·L⁻¹ hygromycin, with T2 seeds being obtained after self-pollination. Transgenic plants were grown at 22°C under 16-h day florescence light. Homozygotic T2 lines of each transgenic plant were used to investigate flowering time.

The total RNA was isolated from the mature leaves of 3-week-old seedling in Col-1 and T2 line, as described above. The expression levels of transgene LhFTs and AtACT2 as internal standard were investigated by RT-PCR analysis, as described above.

Statistical Analysis

Data are presented as the mean ± SE. Statistical comparisons were carried out using Tukey–Kramer and Student’s t-tests.

RESULTS

Growth Behavior of the Asiatic Hybrid Lily ‘Lollypop’ and L. leichtlinii ‘Hakugin’

Before the investigation of the temporal and spatial expression of LhFT genes in pre-vernalization and during planting, we examined the floral development stages of the SAM of bulbs in each phase (Figure 1I). The SAM of bulbs that were pre-vernalized over 4 months was kept in the vegetative phase. One week after planting on soil, the SAM of bulbs exhibited either a swollen round apex or produced a bract. Two weeks after planting, SAMs displayed perianth differentiation. Therefore, the SAM of bulbs of ‘Lollypop’ was assumed to engage in flower bud initiation at ~2 weeks after planting. Three weeks after planting, leaves emerged from the soil, and flower buds were observed 8 weeks after planting (Figure 1I). The increase in the number of developed leaves stopped 82.3 ± 1.7 leaves at 10 weeks after planting, and flowers bloomed completely at 75 days after planting, resulting in a plant height of 45.3 ± 1.0 cm (Figure 1I).

Lilium lacifolium ‘Hakugin’ was observed to be swollen around the apex at 7 weeks after planting in the soil, and it bloomed at 16 weeks after planting. Both floral initiation and blooming of ‘Hakugin’ was 6 weeks later compared to those of Asiatic hybrid ‘Lollypop’.

Isolation of LhFT Orthologs

We obtained four FT-like genes, which were termed LhFT1 (accession number, LC544113), LhFT4 (LC544114), LhFT6 (LC544115), and LhFT8 (LC544117), from L. leichtlinii ‘Hakugin.’ A phylogenetic analysis showed that they were classified into two subgroups (Figure 2). LhFT1 and LhFT8 were classified into the FT-like IA subgroup. The deduced amino acid sequence of LhFT1, which belonged to the FT-like IA subgroup, exhibited 96.6% and 94.9% identity with that of LfT1 from L. × formolongi (Li et al., 2017) and TgFT2 from T. gesneriana (Leegganggters et al., 2018), respectively. The deduced amino acid sequence of LhFT8 exhibited 96.6% and 77.5% identity with that of LFT from L. longiflorum (Leegganggters et al., 2018) and PaFt1 of Phalaenopsis aphrodite (Jang et al., 2015). LhFT1 showed 72.2% identity with LhFT8 based on amino acid sequences (Supplementary Figure 2). Conversely, LhFT4 and LhFT6 were classified into the FT-like IB subgroup (Figure 2). LhFT4 exhibited 75.7% identity with LhFT6 based on amino acid sequences. The deduced amino acid sequence of LhFT6 showed 86.5% identity with that of TgFT3 from T. gesneriana (Leegganggters et al., 2018) and AcFt6 from A. cepa (Lee et al., 2013), respectively. The deduced amino acid sequence of LhFT4 correlated well with that of LfT13 (98.1% identity), as reported by Leegganggters et al. (2018). The LhFT4 mRNA had a length of 1,164 bp, but its maximum ORF was 339 bp, from the 447th to 815th nucleotides, and encoded 112 amino acid residues. Thus, LhFT4 protein was shorter than LfT13 protein (181 residues), which resulted from the shortening of its N terminus (Supplementary Figure 2). After isolation of LhFTs from L. leichtlinii ‘Hakugin,’ we confirmed nucleotide sequences of the corresponding genes in the Asiatic hybrid ‘Lollypop,’ which showed 7 to 10 nucleotide substitutions compared with those of ‘Hakugin.’ Therefore, we designed primers for expression analysis based on the conserved sequences of LhFTs in ‘Lollypop’ and ‘Hakugin.’

LhFT1, LhFT6, and LhFT8 proteins contain a tyrosine (Y, FT-type) at positions 87, 89, and 84, respectively, whereas LhFT4 protein contains a histidine (H, TFL1-type) residue at position 17 (Supplementary Figure 2). In segment B motif, LhFT1, LhFT4, and LhFT8 proteins contain a glutamine (Q, FT-type) at positions 139, 72, and 141, respectively, whereas the LhFT6 protein contains a proline (P) residue at position 136 (Supplementary Figure 2). Aspartic acid (D) at position 17 and
Kurokawa et al. Expression of Cymbidium goeringii CymFT (NP_193770.1); AcFT6 (AGZ20211.1), and AcFT3 (AGZ20208.1), substitutions per site. The FT assay measures the robustness of the tree. The scale indicates the average number of neighbor-joining method. Bootstrap values from 1,000 replicates were used to construct the phylogenetic tree. The tree was constructed using different monocot plants. The phylogenetic tree showed that LhFD was classified into eudicots (Supplementary Figure 3). The phylogenetic tree showed that LhFD was classified into eudicots and non-Poacea monocots subgroup (Supplementary Figure 3). The deduced amino acid sequence of LhFD had a length of 816 bp and encoded a sequence of 221 amino acids. The ortholog, FD, is similar to the characteristic features of the FT protein but was similar to TFL1-like proteins. Moreover, LhFD did not exhibit several characteristic features of the TgFT3 protein from tulips.

Spatial and Temporal Expression Profiles of FT-Like Genes in the Asiatic Hybrid Lily ‘Lollypop’ and L. leichlinii ‘Hakugin’

To investigate the expression profiles of LhFT genes in ‘Lollypop’ bulbs during the period from chilling exposure of bulbs to leaf emergence from the soil, we also performed a semiquantitative RT-PCR analysis using primer sets that amplified the ORF of TFL1-like genes in ‘Lollypop’ bulbs. The expression of LhFT1 in bulb scales increased gradually as chilling exposure progressed and was further increased after planting (Figure 3A). Therefore, it was thought that the activation of LhFT1 transcription occurred right after bulb planting. Similarly, the expression of LhFT6 was activated in the scales after planting (Figure 3A). Two LhFT6 mRNA variants were detected in the bulb scales (Figure 3A), whereas no amplified fragment of this gene was observed in SAMs throughout the chilling and planting periods (Figure 3B). The LhFT6 genome was composed of four exons and three introns, and its full length (from the initial codon to the stop codon) was 1,152 bp (Figure 4A). The amplified fragment of 700 bp...
Expression of FT-Like Genes in Bulb Scales

FIGURE 3 | Expression patterns of LhFT and flowering-related genes in ‘Lollypop’ bulbs during chilling and planting. (A) Semiquantitative RT-PCR analysis of LhFT, LhFD, LhMADS5, and LhACT genes using scales (A) and SAMs (B) of ‘Lollypop’ bulbs. Scale and SAM samples were collected from bulbs exposed to chilling for 0, 4, 8, 12, and 16 weeks, and from bulbs grown for 1, 2, and 3 weeks after chilling for 16 weeks. The gene names and number of cycles are indicated to the left of the panel. The amplified fragment lengths are indicated to the right of the panels. The 516-bp fragment of LhFT6 corresponds to the normal transcript, whereas the 700-bp fragment corresponds to the alternative transcript. The 540-bp fragment of LhFT6 corresponds to normal transcript, whereas the 630-bp, 725-bp, and 820-bp fragments correspond to alternative transcripts.

FIGURE 4 | Structure of the genome and mRNA variants of LhFT6 (A) and LhFT8 (B). The boxes and lines indicate exons and intron, respectively. Filled and empty boxes indicate coding and non-coding regions, respectively. The numbers within the boxes correspond to the number of exons, whereas the numbers outside the boxes indicate the length (in base pairs). We were not able to obtain the complete sequences of the second intron of LhFT8 because it was too long to allow amplification and sequence analysis. Black and green arrows indicate primer position in RT-PCR and RT-qPCR analyses.
planting and remained constant thereafter. *LhFT4* was expressed constantly in both scales and SAMs during chilling and planting (Figures 3A,B). Although the expression of *LhFD* was detected in both scales and SAMs, it was stronger in SAMs than it was in scales. The expression of *LhMADS5*, which is a floral identity gene, was detected exclusively in SAMs at planting and 1 week after planting, which implied that floral bud differentiation started in SAMs (Figures 1, 3B).

We also investigated the expression levels of *LhFT3* and *LhMADS5* in bulb scales of *L. leichtlinii* 'Hakugin' (Supplementary Figure 4). The expression of *LhFT1* was detected 1 week after planting, which then increased markedly at 4 weeks after planting. The expression profiles of *LhFT6* were similar to those of *LhFT1*. *LhFT8* was detected in alternative splicing variants, and its expression decreased within two weeks of planting. No expression of *LhFT4* was detected in bulb scales. Increased expression of *LhFT1* and *LhFT6* at 4–6 weeks after planting was correlated with flower initiation, which occurred 7 weeks after planting.

### Quantitative Expression Profiles of FT-Like Genes in the Asiatic Hybrid Lily ‘Lollypop’

Using RT-qPCR, we investigated the expression profiles of *LhFT* and flowering-related genes using bulb scales, leaves, and SAMs of ‘Lollypop’ (Figure 5). The primer set used for *LhFT6* and *LhFT8* genes in the RT-qPCR analysis did not detect alternative variants (Figure 4). The expression of *LhFT1* was detected mainly in bulb scales, and not in SAMs. The expression levels of *LhFT1* were increased by 13.7-fold in bulb scales at 26 days compared with day 0 after planting, whereas the expression levels of *LhFT1* were weak in just-emerged leaves (Figure 5). The strong expression of *LhFT1* in bulb scales continued until 34 days after planting, after which the mother bulbs disappeared gradually. *LhFT4* was detected at weak but constant levels in bulb scales and leaves during plant development. In the SAM of non-vernalized bulbs and in differentiated floral meristem, the expression levels of *LhFT4* were about 2-fold those detected in other phases. The expression of *LhFT6* was detected exclusively in bulb scales, and was 59.8-fold higher at 34 days compared with day 0 after planting (Figure 5). This peak in *LhFT6* expression was observed 1 week later than that of *LhFT1*. The highest expression level of *LhFT6* was detected in bulb scales right after chilling exposure for 4 months, and was 75.6-fold higher than that of non-vernalyzed bulbs (Figure 5). Furthermore, the expression levels of *LhFT8* in bulb scales were reduced after planting. In leaves and SAMs, *LhFT8* transcripts were hardly detected throughout the investigation period. *LhFD* exhibited its highest expression levels in SAMs of non-vernialized bulbs. Subsequently, the expression levels of *LhFD* were reduced by 50% after chilling exposure and planting. Modest expression peaks of *LhFD* in bulb scales and leaves were detected right after chilling exposure and at 34 days after planting, respectively. The expression of *LhMADS5*, which is an *AP1* ortholog, was increased in SAMs after planting, and peaked at 34 days after planting. The expression levels of *LhMADS5* were 24.9-fold higher at 34 days compared with day 0 after planting. The expression of *LhMADS5* was also detected in leaves and was increased up to 40 days after planting.

Taken together, these results showed that enhanced expression of *LhFT1* and *LhFT6* was detected in bulb scales 1 week after planting and correlated well with the timing of floral initiation in SAMs. Conversely, *LhFD* was expressed constantly in SAMs throughout the chilling and planting periods, but was also expressed weakly in scales. Finally, *LhFT8* was expressed during chilling exposure.

### Overexpression in the Arabidopsis ft-10 Mutant

To evaluate the flowering-induction activity of *LhFT* genes, we produced transgenic plants using the late-flowering *Arabidopsis ft-10* mutant. We selected three representative *T*$_2$ lines for each construct and investigated their gene expression patterns and phenotypes. In all selected transgenic *T*$_2$ lines, the expression of transgenes was detected (Figure 6). Any alternative splicing variants were not detected in *LhFT6*- and *LhFT8*-overexpressing plants. *LhFT1*-expressing plants nos. 2-1, 3-2, and 4-2 developed 8.1, 27.0, and 8.4 rosette leaves, respectively, during bolting, which was significantly lower compared with the 40.9 rosette leaves observed in *GFP*-expressing *ft-10* plants as a vector control (Figure 6A). The overexpression complemented the *ft-10* mutant, resulting in similar early-flowering time compared with the wild-type Col-1 plants (10.4 rosette leaves). Similarly, *LhFT8*-expressing plants nos. 2-7, 3-1, and 5-1 developed 15.8, 17.6, and 14.1 rosette leaves, respectively (Figure 6B), which was significantly lower than that observed in the *GFP*-expressing plant but higher than that in Col-1 and *LhFT1*-expressing plants nos. 2-4 and 4-2 (Figures 6A,B). Therefore, the overexpression also partially complemented the *ft-10* mutant phenotype. Conversely, *LhFT4* and *LhFT6* overexpression did not complement the *ft-10* mutant (Figures 6C,D). Taken together, these results suggest that *LhFT1* and *LhFT8* are potential inducers of the floral transition in Asiatic hybrid lilies.

### Protein–Protein Interactions Between LhFT Proteins and LhFD

To confirm the presence of protein–protein interactions between *LhFT* proteins and *LhFD*, we employed the GAL4 yeast two-hybrid system (Figure 7A). Yeast harboring AD:*LhFT1* and BK:*LhFD* grew on quadruple-dropout medium, which was indicative of protein–protein interactions (Figure 7A). However, yeast cells harboring the reversed vector combination, BK:*LhFT1* and AD:*LhFD*, did not survive on quadruple-dropout medium. In vitro protein–protein interaction assay, AlphaScreen, also showed that *LhFT1* protein interacted with *LhFD* (Figure 7B). This signal intensity emitted by *LhFT1–LhFD* is very similar to that by *AtFT–AtFD* (Figure 7C). In addition, *LhFT6–LhFD* and *LhFT8–LhFD* interactions were also detected with weaker signals than that of *LhFT1–LhFD* interaction, implying their weak but positive interactions with *LhFD* (Figure 7B). Taken together, these results suggest that...
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FIGURE 5 | Temporal expression patterns of LhFT and flowering-related genes in different organs of ‘Lollypop.’ The quantitative gene expression analysis of LhFT1, LhFT4, LhFT6, LhFT8, LhFD, and LhMADS5 in SAM, scales, leaf samples of Asiatic hybrid ‘Lollypop’ was performed. Bulb scales samples were collected from bulbs under different development phase, including non-vernalization (NV) and vernalization (= 0 day after planting), 26, and 34 days after planting. Leaf samples were collected from 26, 34, 40, and 47 days after planting. SAM samples were collected from non-vernalized bulbs and vernalized bulb (0 day) and from shoots at 26, 34, 40, and 47 days after planting. Blue and orange fill boxes indicated chilling exposure period for 4 months and floral initiation period, respectively. The expression levels of LhFT and flowering-related genes were investigated by RT-qPCR and normalized using that of LhACT. Values are expressed as the mean ± SE (n = 6). Temporal expression pattern per each organ is shown in Supplementary Figure 5.

the LhFT1, LhFT6, and LhFT8 proteins form heterodimers with the LhFD protein.

DISCUSSION

To identify the molecular mechanisms underlying floral initiation in Asiatic hybrid lilies, we identified four FT-like genes, LhFT1, LhFT4, LhFT6, and LhFT8, in the Asiatic hybrid lily ‘Lollypop’ and characterized their functions.

Based on the deduced amino acid sequences of the LhFT genes, LhFT1 and LhFT8 were classified into the FT-like IA subgroup (Figure 2). LhFT1 belonged to same clade as LFT from L. longiflorum (Leeggangers et al., 2018), whereas LhFT8 belonged to another clade containing LfFT1 from L. × formolongi (Li et al., 2017) and LiFTL2 from the Asiatic hybrid lily ‘Connecticut King’ (Leeggangers et al., 2018; Figure 2). A complementation experiment using the Arabidopsis ft-10 mutant indicated that both LhFT1 and LhFT8 are potential inducers of the floral transition in Asiatic hybrid lilies (Figures 6A,B). The floral-inducer activity of LhFT1 was stronger than that of LhFT8. The yeast two-hybrid and AlphaScreen analyses showed that LhFD protein interacted strongly with the LhFT1 protein; however, the interaction between LhFD and LhFT6/8 proteins was not detected (Figures 7A,B). The yeast two-hybrid assay can indirectly detect yeast survival and cannot control the expression levels of a transgene fused with the relative large GAL4 protein. We believe that AlphaScreen is more sensitive than the yeast two-hybrid assay because it detects the direct interaction between native proteins with short tag. Therefore, AlphaScreen would be useful to evaluate FT–FD interactions in plants.

The expression levels of LhFT1 increased gradually in bulb scales during chill exposure, followed by a sharp increase at 1 week after planting (Figure 3A). This expression profile of LhFT1 correlated well with that of a floral identity gene (LhMADS5) and with the initiation of floral differentiation (Figures 1, 3). L. × formolongi is a lily that blooms within 1 year after sowing and expresses LfFT in its leaves (Li et al., 2017). TgFT2 from T. gesneriana is expressed in the stem and leaves during rapid shoot elongation and in flowers in the blooming period (Leeggangers et al., 2018). However, previous studies did not investigate the expression of FT-like genes in bulb scales of these lilies. Perhaps LhFT1 ortholog might be upregulated in the bulb scales of other species belonging to the family Liliaceae.
in response to environmental changes. In many plant species, 
FT-like genes are expressed in leaves under flowering-inducing 
conditions, followed by transportation via the phloem to the 
SAM (Abe et al., 2005; Corbesier et al., 2007; Tamaki et al., 
2007). However, the four LhFT genes identified here in an 
Asiatic hybrid lily were expressed mainly in bulb scales, in which 
the expression levels were higher than those detected in leaves 
(Figure 5). L. leichtlinii ‘Hakugin’ also led to the detection of a 
strong expression of LhFT1 in their bulb scales (Supplementary 
Figure 4). In ‘Hakugin,’ which underwent flower initiation 
6 weeks later than ‘Lollypop,’ the expression of LhFT1 also 
induced at 4 weeks after planting (Supplementary Figure 4). The 
later flowering of ‘Hakugin’ than ‘Lollypop’ correlated well with 
the delayed expression initiation of LhFT1 in its scales (Figure 3A 
and Supplementary Figure 4). These results strongly suggest 
that LhFT1 is a floral inducer in Asiatic hybrid lilies. Scales are 
leaf-like organs that make up the bulb, in addition to the true 
photosynthetic leaves and inflorescence base (Rees, 1972). The 
investigation of the levels of NFT1 in Chinese narcissuses revealed 
its expression in apices of bulbs and leaves, but not in scales of 
bulbs (Li et al., 2013). Thus, this study provided new knowledge, 
in that the expression of FT-like transcripts was induced in bulb 
scales of an Asiatic hybrid lily.

The deduced amino acid sequence of LhFT8 was highly 
similar to that of LIFT from L. longiflorum (Leeggangers 
et al., 2018) and orchid FT transcripts (Hou and Yang, 2009;
Since cold exposure for 9 weeks upregulates LIFT in the meristem of bulbs of *L. longiflorum*, LIFT has been proposed to be involved in the vernalization response of lilies. The overexpression of *LIFT* in wild-type *Arabidopsis* induces a mild early-flowering phenotype, and *LIFT*-overexpressing transgenic lilies exhibited flowering under non-inductive condition (Leeggangers et al., 2018). An *LhFT8*-overexpressing *Arabidopsis ft-10* mutant also exhibited a mild early-flowering phenotype (Figure 6B).

Interestingly, *LhFT8* expressed its four alternative mRNA variants in the bulbs of Asiatic hybrid lilies during chilling exposure (Figures 3, 4). The expression of one functional and three alternative *LhFT8* variants was induced in both the scales and SAM of bulbs during the initial 8 weeks of chilling (Figure 3). The functional *LhFT8* mRNA detected in bulb scales and SAMs disappeared 3 weeks and 1 week after planting, respectively. The expression of the functional *LhFT8* gene was downregulated after the induction of floral meristems, unlike that observed for *LhFT1*. *LIFT* was considered to be involved in creating meristem competence to flowering cues in *L. longiflorum* (Leeggangers et al., 2018). Therefore, *LhFT8* is likely to be closely associated with the vernalization response of these plants, rather than act as a floral inducer. In perennial species, *FT*-like genes regulate growth cessation and dormancy (Bohlenius et al., 2006; Hsu et al., 2006, 2011; Ream et al., 2012). In perennial poplar, *PtFT1* expression in winter initiates the transition of vegetative meristems to the reproductive phase, whereas *PtFT2* controls vegetative growth by inducing growth cessation, bud set, and dormancy in the growing season (Hsu et al., 2006, 2011). In biennial sugar beets, the *FT* duplication products *BvFT1* and *BvFT2* have divergent functions (Pin et al., 2010). *BvFT2* is a flowering inducer, whereas *BvFT1*, resulting in part from a three-amino-acid change in segment B of *BvFT2*, is a flowering repressor, despite being in the FT-like IA subgroup (Pin et al., 2010, 2012). *BvFT1* is expressed at the juvenile stage, whereas *BvFT2* is expressed during the reproductive stage (Pin et al., 2012). *BvFT1* is a flowering inducer, whereas *BvFT1*, resulting in part from a three-amino-acid change in segment B of *BvFT2*, is a flowering repressor, despite being in the FT-like IA subgroup (Pin et al., 2010, 2012). A functional *LhFT8* gene was downregulated after the induction of floral meristems, unlike that observed for *LhFT1*. *LIFT* was considered to be involved in creating meristem competence to flowering cues in *L. longiflorum* (Leeggangers et al., 2018). Therefore, *LhFT8* is likely to be closely associated with the vernalization response of these plants, rather than act as a floral inducer. In perennial species, *FT*-like genes regulate growth cessation and dormancy (Bohlenius et al., 2006; Hsu et al., 2006, 2011; Ream et al., 2012). In perennial poplar, *PtFT1* expression in winter initiates the transition of vegetative meristems to the reproductive phase, whereas *PtFT2* controls vegetative growth by inducing growth cessation, bud set, and dormancy in the growing season (Hsu et al., 2006, 2011). In biennial sugar beets, the *FT* duplication products *BvFT1* and *BvFT2* have divergent functions (Pin et al., 2010). *BvFT2* is a flowering inducer, whereas *BvFT1*, resulting in part from a three-amino-acid change in segment B of *BvFT2*, is a flowering repressor, despite being in the FT-like IA subgroup (Pin et al., 2010, 2012). *BvFT1* is expressed at the juvenile stage, whereas *BvFT2* is expressed during the reproductive stage (Pin et al., 2012). *BvFT1* is expressed at the juvenile stage, whereas *BvFT2* is expressed during the reproductive stage (Pin et al., 2012).
Glu (E) at position 132 (Supplementary Figure 2). Therefore, this single residue alteration of segment B between LhFT8 and LhFT1 might be responsible for their functional differentiation. Alternative splicing occurs in >61% of intron-containing genes in Arabidopsis, 60% in Drosophila melanogaster, and more than 95% in humans (Capovilla et al., 2015). However, the biological significance of most alternative splicing event in plants remains largely unknown. In Brachypodium distachyon, which is a model plant for major crop cereals, BdFT2 undergoes age-dependent alternative splicing, resulting in two splicing variants, BdFT2α and BdFT2β (Qin et al., 2017). We were not able to clearly demonstrate the molecular mechanism underlying the LhFT8 alternative splicing. Alternative variants of LFT6 and LhFT8 seem to lack segment B in their C-terminal ends (Supplementary Figure 2). Segment B is an important domain for binding to 14-3-3 proteins (Taoka et al., 2011). Because AlphaScreen showed that LhFT6 and LhFT8 also interacted with LhFD (Figure 7), C-terminal deficiency in LhFT6 and LhFT8 proteins might function as either the negative auto-regulators or antagonists of LhFT1 protein.

Upregulation of LhFT6 in bulb scales after planting was detected prior to the timing of floral initiation (Figures 3A, 5). The overexpression of LhFT6 did not complement the delay in flowering in Arabidopsis ft-10 (Figure 6D). The LhFT6 protein showed weaker interaction with LhFD protein than LhFT1 (Figure 7B). LhFT6 was classified into the FT-like 1B subgroup (Figure 2), which includes AcFT6 from A. cepa (Lee et al., 2013) and TgFT3 from T. gesneriana (Leeggangers et al., 2018). TgFT3 expression was initiated earlier and increased in the stem and leaves during rapid shoot elongation. TgFT3 overexpression weakly repressed floral transition in Arabidopsis, and TgFT3 might act as negative regulator of flowering in tulips (Leeggangers et al., 2018). Further studies are required to identify the function of LhFT6.

LhFT4 exhibited a length of 1,164 bp and encoded 112 amino acids, i.e., it was shorter than other FT/TFL proteins (Supplementary Figure 2). The deduced amino acid sequences of LhFT4 were categorized into the FT-like 1B clade (Figure 2), but contained a histidine (H) residue that is known to be important for the specific and unique TFL1 function in Arabidopsis (Supplementary Figure 2). The LhFT4 protein showed 98.1% identity with that encoded by LiFTL3 from the Lilium spp. ‘Connecticut King’ (Leeggangers et al., 2018). However, the LiFTL3 mRNA encoded sequences of 181 amino acids, suggesting the existence of an LhFT4 allele encoding longer amino acid sequences in other cultivars of Asiatic hybrid lilies. The overexpression of LhFT4 did not affect the flowering time in either Arabidopsis ft-10 plants (Figure 6C). In addition, LhFT4 expression was detected at constitutive levels in all samples (Figures 3, 5). Therefore, we assumed that LhFT4 does not function in flowering signaling in Asiatic hybrid lilies.

Asiatic hybrid lilies create meristem competence to flowering cues by exposing bulbs to low temperatures, and then the bulbs sprout in spring and flower in late spring to early summer (Okubo and Sochacki, 2012). In the most cultivars, including ‘Lollypop,’ flower bud differentiation starts and is completed after shoot emergence (Ohkawa et al., 1990). LhFT8 was associated with the vernalization response in lily bulbs and was speculated to control the expression levels of LhFT8 mRNA by occurring as splicing variants. When the bulb is released from cold exposure, LhFT1 expression is induced in bulb scales, and floral transition occurs in SAM after shoot emergence. This study provides the first evidence that the expression of LhFT1 in bulb scales is regulated by alterations of temperature in Asiatic hybrid lilies and contributes to flowering initiation. Furthermore, the generation of LhFT8 splicing variants in lily bulbs during cold exposure might be involved in winter memory (Bouche et al., 2017). Further studies are required to reveal the function of LhFT8 alternative variants and their generation mechanism. Our findings can help reveal the molecular mechanism of flowering and vernalization in geophytes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ddbj.nig.ac.jp/, LC544113; https://www.ddbj.nig.ac.jp/, LC544114; https://www.ddbj.nig.ac.jp/, LC544115; https://www.ddbj.nig.ac.jp/, LC544116; https://www.ddbj.nig.ac.jp/, LC544117; https://www.ddbj.nig.ac.jp/, LC544118; https://www.ddbj.nig.ac.jp/, LC544119; https://www.ddbj.nig.ac.jp/, LC544120; https://www.ddbj.nig.ac.jp/, LC544121.

AUTHOR CONTRIBUTIONS

KK, TN, and MY conceived the experiments. KK and JK performed the gene expression analysis. KK and TN performed the yeast two-hybrid analysis and complement experiments of ft-10. KN, AN, and TS performed the AlphaScreen analysis. TN, KN, and MY wrote the manuscript. All authors approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.570915/full#supplementary-material
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