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Identification and characterization of *FT*/*TFL1* gene family in cucumber

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In *Arabidopsis*, *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*) are known to control growth habit of determinate or indeterminate type. In cucumber (*Cucumis sativus* L.), any *FT* or *TFL1* homolog is expected to be the candidate for the gene controlling the growth habit. Most cucumber cultivars show indeterminate type of growth habits. For more effective breeding, it is necessary to develop determinate type cultivars. In this study, we isolated one *FT* and five *TFL1* homologs in cucumber. On these homologs, the genomic organizations, phylogenetic relationships, expression patterns, and linkage to growth habit are elucidated herein. Through genetic analysis of F_2 individuals, two homologs did not co-segregate with the determinate-indeterminate phenotypes. On the other hand, different expression patterns of several homologs were detected between determinate and indeterminate cultivars.

Key Words: *Cucumis sativus* L., *FLOWERING LOCUS T*, *TERMINAL FLOWER 1*, *de*.

Introduction

In annual plant, the life cycle is separated into two different growth phases: vegetative and reproductive. The transition from the vegetative to the reproductive phase is tightly controlled by numerous physiological signals and genetic pathways, inducing flowering to coordinate with environmental conditions and the developmental stages of the plant. At the moment of this transition, the plant growth is switched from vegetative to reproductive phases, depending on the growth habit of indeterminate and determinate types. In indeterminate type, apical meristem grows indefinitely with generating floral meristems from auxiliary buds. On the other hand, in determinate type, apical meristem is eventually transformed into a floral meristem with terminating apical growth and leading subsequent vegetative growth only at lower axillary meristems.

In *Arabidopsis,* it is known that *FLOWERING LOCUS T* (*FT*) and *TERMINAL FLOWER 1* (*TFL1*) relate to the transition (Kobayashi *et al*. 1999). *FT* is expressed in vascular tissue of the leaf (Takada and Goto 2003, An *et al*. 2004) and FT protein moves to shoot apex (Corbesier *et al*. 2007). Then FT interacts with FLOWERING LOCUS D (FD), a bZIP transcription factor, and promotes the phase transition (Abe *et al*. 2005, Wigge *et al*. 2005). Thus, FT is suggested to be the candidate for "florigen" (Corbesier *et al*. 2007, Tamaki *et al*. 2007). On the other hand, *TFL1* is expressed in inner shoot apical meristem (SAM). TFL1 protein is also an agent of a mobile signal, which moves from inner to outer meristem cells (Conti and Bradley 2007). *TFL1* contributes to the maintenance of indeterminate shoot identity and the delay of flowering transition (Shannon and Meeks-Wagner 1991, Bradley *et al*. 1997, Ratcliffe *et al*. 1998). Although *FT* and *TFL1* play important roles on the antagonistic functions, their nucleotide sequences are highly homologous to each other. In the amino acid sequences of FT and TFL1, two crucial amino acid residues are suggested the keys for determining the functions (Tyr85 and Gln140 in FT; His88 and Asp144 in TFL1). These were revealed by the analysis of overexpression of chimeric protein in transgenic plants (Hanzawa *et al*. 2005) and the crystal structure analysis (Ahn *et al*. 2006). In *Arabidopsis*, the other homologs such as *MOTHER OF FT AND TFL1* (*MFT*), *BROTHER OF FT AND TFL1* (*BFT*), and *Arabidopsis thaliana CENTRORADIALIS* homologue (*ATC*) were cloned (Kobayashi *et al*. 1999). It is known that *MFT* has high homology with both *FT* and *TFL1*. *MFT* is suggested as a floral inducer and determiner of flowering time (Yoo *et al.* 2004). The function of *BFT* has not been identified yet. ATC protein is suggested to functionally substitute for TFL1 (Mimida *et al.* 2001). Up to now, functional orthologs of *FT* and *TFL1* in various plants except in *Cucumis* have been cloned (Bradley *et al.* 1996, Pnueli *et al.* 1998, Amaya *et al*. 1999, Jensen *et al*. 2001, Carmona *et al*. 2007, Igasaki *et al.* 2008, Komiya *et al.* 2008). Some genes were also demonstrated to affect indeterminate or determinate growth habit (Bradley *et al.* 1996, Pnueli *et al.* 1998, Amaya *et al*. 1999). *CENTRORADIALIS* (*CEN*) and *SELF-PRUNING* (*SP*) are functional homologs of *TFL1* in snapdragon and tomato, respectively (Bradley *et al*. 1996, Pnueli *et al*. 1998).

Most cucumber (*Cucumis sativus* L.) cultivars have indeterminate growth habits. While they supply several merits for cultivation such as long term of harvest, they also have several demerits such as management of vine, necessity of

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pinch, and difficulty in cultivation under field conditions due to weakness to wind. Meanwhile, a few determinate type cultivars, which have seven to ten nodes and possess short vine with main stem terminating in flower (Pierce and Whener 1990), are grown in the USA. The characteristics of them are early harvesting, available to mechanic harvesting, no need to pinch, and simplified management. Moreover, they withstand the wind blow, which makes their cultivation preferable under inferior field conditions. Although a few determinate cultivars are grown at present, it is necessary to choose determinate or indeterminate cultivars properly according to the environmental conditions. In cucumber, a single recessive gene, *de*, is known to control the growth habit (Denna 1971) and it is mapped on the molecular linkage map (Fazio *et al*. 2003). Although this allele is important for breeding, the DNA fragments corresponding to this gene has not been cloned yet.

In cucumber as well as *Arabidopsis*, snapdragon, and tomato, it is expected that any *FT* or *TFL1* homolog is the candidate for the gene controlling determinate growth habit. This is the first report on *FT*/*TFL1* homologs in cucumber, describing their genomic organizations, phylogenetic relationships, expression patterns, and linkage relationships with *de*.

Materials and Methods

Plant materials

To identify and characterize *FT* and *TFL1* homologs in cucumber (*Cucumis sativus* L.), cv. Mogami, a Japanese cultivar, and cv. SpaceMaster, a cultivar in USA, were used as indeterminate and determinate type cucumber, respectively. Characteristics of cv. Mogami are a pickle type, tall stature, monoecious flower, standard leaf size, late flowering, and high yield. The genotype is *De*/*De*. Characteristics of cv. SpaceMaster are a slice type, dwarfness, monoecious flower, standard leaf size, early flowering, and high yield. The genotype of cv. SpaceMaster is *de*/*de*. In cv. SpaceMaster, apical meristems are transformed to floral bud and flowering clusters are formed when the plant reaches to the stage of ten true leaves (Wehner 2005). For linkage analysis, F_2 progenies were produced after self-pollination of an F_1 plant obtained from a cross between cv. Mogami and cv. SpaceMaster.

Cloning of the FT/TFL1 homologs

For cloning *FT* homologs in cucumber, primer pairs were designed from the sequence of *FT* of *Arabidopsis thaliana*; 5′-TTGGTGACTGATATCCCTGCT-3′ and 5′-TACACTG TTTGCCTGCCAAG-3′ as forward and reverse primers, respectively. *TFL1* homologs in cucumber were cloned by using degenerate primers designed from the sequences of *TFL1* of *A. thaliana*, *CEN* of snapdragon, and *SP* of tomato; 5′-CCTAGTGAYCCTTATMTRAGA-3′ and 5′-TATTCC AGGMACAACAGATG-3′ as forward primers and 5′-TCTTCTTGCAGCSGTTTCYCT-3′ as reverse primer. PCRs were carried out by using 20 ng of total genomic DNA of cv. Mogami, 2 pmol of each primer, 40 pmol of each dNTP, 1x EX Taq DNA polymerase Buffer (Takara), and 0.5 unit of EX Taq DNA polymerase (Takara) in a total volume of 10 µl. The PCRs were performed using 35 cycles of 30 s at 94°C, 30 s at 40°C, 1 min at 72°C. PCR products were loaded with 1 µl of blue dye and electrophoresed in 2.0% agarose gels. The gels were stained with ethidium bromide (EtBr) for making DNA fragments visible by UV irradiation. PCR products were also cloned into pGEM-T Easy Vector (Promega) and sequenced with 3100 Genetic Analyzer (Applied Biosystems).

For cloning of cDNA of *FT*/*TFL1* homologs, total RNAs were extracted from leaves and SAMs of cv. Mogami by LiCl-based method (Manickavelu *et al*. 2007). Reverse transcription and rapid amplification of cDNA ends (RACE) were conducted with the methods mentioned by Ushijima *et al.* (2003). The RACEs were performed with gene specific primers of each homolog (Table 1) and their products were sequenced.

To clarify the genomic structures of the *FT*/*TFL1* homologs, PCRs were conducted by using genomic DNA of cv. Mogami and each gene specific primer pairs (Table 2). For primer pairs of all homlogs, annealing temperatures were 55°C. The other conditions of PCR were the same as the PCR conditions mentioned above. The PCR products were sequenced.

To predict the copy number of the homologs, Southern hybridization was carried out by using *CsTFL1a* cDNAs as the probes.

Sequence comparison and phylogenetic analysis

Amino acid sequences of *FT* and *TFL1* homologs in other species were obtained by BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) and aligned with *FT*/*TFL1* homologs in cucumber by ClustalW software (http://clustalw.ddbj.nig.ac.jp/top-j.html/).

A phylogenetic tree based on their amino acid sequences was constructed by applying the neighbor joining (NJ) method (Saitou and Nei 1987) in Genetyx software (GENETYX corporation), when NJ tree was produced from the results of 10,000 bootstraps.

Expression pattern analyses by RT-PCR

To examine spatial expression patterns of homologs, total RNAs were isolated from the organs of SAM, root, leaf, tendril, stem, cotyledon, hypocotyl, flowering bud, petal, sepal, anther, ovary, and stigma of both cv. Mogami and cv. SpaceMaster. Total RNAs of each organ at several developmental stages were extracted and bulked. RT-PCRs were carried out by using cDNAs synthesized from 5 ng of total RNA and gene specific primer pairs of each homolog (Table 2). For primer pairs of all homologs and actin, annealing temperatures were 55°C. PCR cycle numbers for *CsTFL1a*, *CsTFL1cL1*, *CsTFL1Lc2*, *CsFT*, and *CsActin* were 35. Those for *CsTFL1b* and *CsTFL1d* were 50. The other conditions of RT-PCR were the same as the PCR conditions mentioned above.

Table 1. Primers for 3′ and 5′ RACEs of *FT*/*TFL1* homologs in cucumber

Homolog	3′ RACE	
	First-round PCR (5′-3′)	Second-round PCR (5′-3′)
CsTFL1a	ATGGCAATTAGATCAAAAGTAAG	CTTGAGGGAACACCTTCACTGGTA
CsTFL1b	AAATCTGAGTGCTTTTTGAGA	GGTAGGGAGGTAGTAAGCTA
CsTFL1Lc1	AAACGCACACTACTAGCTCTTA	TAGGAGATGTGGTTGATAATTTCGT
CsTFL1Lc2	AAACGCACACTACTAGCTCTTA	TAGGAGATGTGGTTGATAATTTCGT
CsTFL1d	CACCAGCCCTAAACTACCCT	ATGGCGATGGGGAAGATGCC
CsFT	ATGCCAAGAGATCGTGACCC	CCGATCTTCGAACCTTCTTC

Homolog	5′ RACE	
	First-round PCR (5′-3′)	Second-round PCR (5′-3′)
CsTFL1a	TCTCACAAGAAAATCTTCGAG	ATTCACTACTGACCGACGCTG
CsTFL1b	GTCGTTGTCGACCGCGAATGC	TTCACCGATTGTCTTCTCTTC
CsTFL1Lc1	TTGGCTCTTGAAGACGAGGAG	TCTCACAGTCTGTCTTCCTCG
CsTFL1Lc2	TTGGCTCTTGAAGACGAGGAG	TCTCACAGTCTGTCTTCCTCG
CsTFL1d	GTCTCTTGAAGGGTGTGGTG	CCTATGTTTGGACTTGGTTC
CsFT	TAGCACGAATCGATGTATCCCCACCGT	GGACTCTCATAGCATACTATCTCTTGCC

Table 2. Primers for analyses of genomic structures and expression patterns of *FT*/*TFL1* homologs in cucumber

Homolog	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
CsTFL1a	TGTGAGAGTGTGCTTTATTTCAAATC	ATGCATCCATAGAACATAAGG
CsTFL1b	AAATCTGAGTGCTTTTTGAGA	CACTACTGTTGCCAGACGAC
CsTFL1Lc1	AAACGCACACTACTAGCTCTTA	CTAGAGGTAAATGGAGATGA
CsTFL1Lc2	TTGGCTCTTGAAGACGAGGAG	CATTTTCTTCTAGCGGCGGT
CsTFL1d	CACCAGCCCTAAACTACCCT	CGGCAACAGGAAGAGAAAGA
CsFT	GCCAACGGAAACATCTCTT	TTGCGTAAAAAGTGGGTTTA

To survey temporal expression patterns, total RNAs were isolated from leaves and SAMs of six growth stages of both cv. Mogami and cv. SpaceMaster, namely, Stage 1: early stage (possessing three true leaves), Stage 2: before male flowering, Stage 3: after male flowering, Stage 4: before female flowering, Stage 5: after female flowering, and Stage 6: late stage (holding mature fruits). It was observed that main stem of cv. SpaceMaster terminated but that of cv. Mogami did not terminate in Stage 2. *CsTFL1a*, *CsTFL1b*, *CsTFL1Lc1*, *CsTFL1Lc2*, and *CsTFL1d* were amplified from total RNA of SAMs. *CsFT* was amplified from total RNA of leaves. RT-PCRs were performed with the same conditions as analyses of spatial expression patterns.

To estimate in detail on temporal expression pattern of *CsTFL1b* at Stage 2 in SAMs, additional analysis were carried out by using total RNA of both cv. Mogami and cv. SpaceMaster. RT-PCR was performed with 40, 45, and 50 PCR cycles. Activity of *CsActin* was also examined with 20, 25, and 30 PCR cycles as positive control. Genomic DNAs of *CsTFL1b* were amplified with 35 PCR cycles by using gDNA of cv. Mogami and cv. SpaceMaster as template. Each PCR was performed with the same conditions mentioned above.

To reinforce these results, a repetition of each RT-PCR was conducted with total RNA from another plant individual.

Linkage analysis

Phenotypes of 68 F_2 individuals were assessed whether indeterminate or determinate type. To confirm the Mendelian ratio, statistical analysis was performed on the phenotypic data by chi-square test.

Sequence comparisons of gDNA of each *FT*/*TFL1* homolog between cv. Mogami and cv. SpaceMaster were conducted. The homologs in which polymorphisms were detected were examined on the linkage relationships to the phenotypes. PCRs were conducted by using gDNAs of F_2 individuals as the templates. For *CsTFL1a*, the amplifications were carried out with oligonucleotides of 5′-GTAATTGGA GATGTTGTTGATCCC-3′ and 5′-GAAAGAGAAGAAG GGAAGAATTC-3′ as forward and reverse primers, respectively. For *CsFT*, 5′-TTGGTGACTGATATTCCAGCT-3′ and 5′-TGTATTGAAGTTCTGACGCCAA-3′ were used for forward and reverse primers, respectively. The annealing temperatures were 55°C. PCR cycle number was 35. The other conditions of PCR were the same as the PCR conditions mentioned above. The PCR products were separated by 10% polyacrylamide gel electrophoresis (Yamanaka *et al*. 2001) and visualized by EtBr staining. The linkage analysis between the phenotype and *FT/TFL1* homologs was performed with the program MAPMAKER/EXP. ver. 3.0 using the Kosambi function (Lander *et al*.1987). The linkage criteria were an LOD score of >3.0.

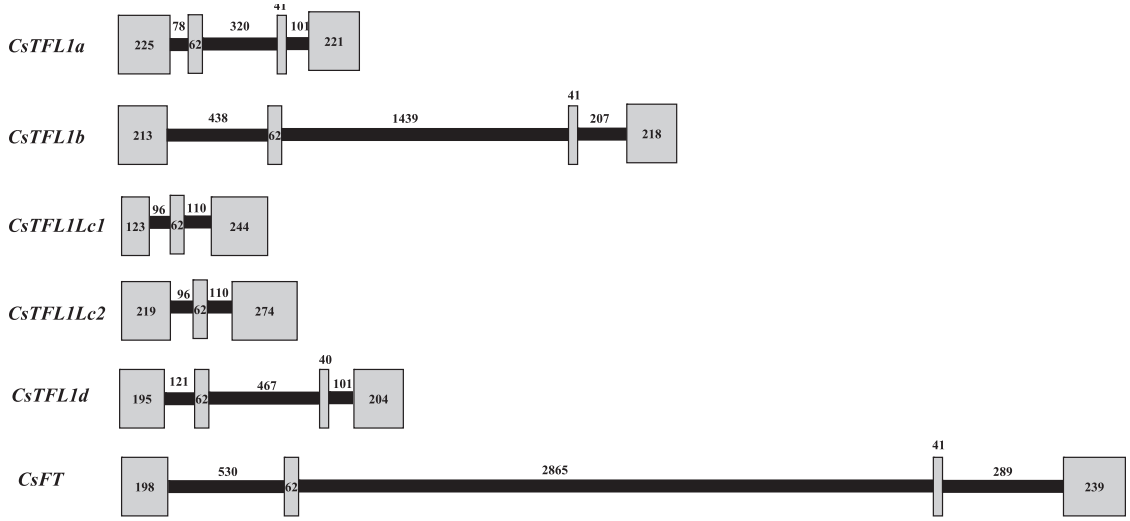

Fig. 1. Genomic organizations of *FT*/*TFL1* homologs in cucumber. The boxes and the lines indicate the exons and the introns, respectively. The numbers represent their lengths.

Results

Cloning of FT/TFL1 homologs

In this study, we identified six homologs of *FT*/*TFL1* in cucumber. One homolog is highly homologous to *FT* and five homologs are highly homologous to *TFL1*. They were named as *CsFT* (AB383152), *CsTFL1a* (AB383153), *CsTFL1b* (AB383154), *CsTFL1Lc1* (AB383155), *CsTFL1Lc2* (AB383156), and *CsTFL1d* (AB383157) after their homologies. Sequence data of these genes are deposited in the DNA Data Bank of Japan (DDBJ) data libraries. The genomic organizations were revealed by sequence comparison between gDNAs and cDNAs (Fig. 1). Four homologs, *CsTFL1a*, *CsTFL1b*, *CsTFL1d*, and *CsFT*, were found to consist of four exons and three introns. These results agree with *FT*/*TFL1* homologs in the other species (Bradley *et al*. 1996, Pnueli *et al*. 1998, Amaya *et al*. 1999, Jensen *et al.* 2001, Carmel-Goren *et al*. 2003, Carmona *et al.* 2007, Igasaki *et al*. 2008, Komiya *et al.* 2008). In contrast, two homologs, *CsTFL1Lc1* and *CsTFL1Lc2,* consist of three exons and two introns. They have similar sequences to each other with slight differences in the 1st and 4th exons, and 5′UTR, and 3′UTR.

In total, six and nine signals were detected by Southern blot analysis with gDNA digested with *Hin*dIII and *Msp*I, respectively (Fig. 2). Since each homolog is highly homologous to each other (Table 3), *CsTFL1a* cDNA probe must have caused cross hybridization to the other homologs. All six homologs in cucumber do not contain any *Hin*dIII restriction site. But *CsTFL1b*, *CsTFL1Lc1* and *CsTFL1Lc2* contain an *Msp*I site. From these results, it was suggested that the copy number of *FT*/*TFL1* homologs in cucumber was six. Thus, it is implied that all *FT/TFL1* homologs in cucumber have been cloned in the present study.

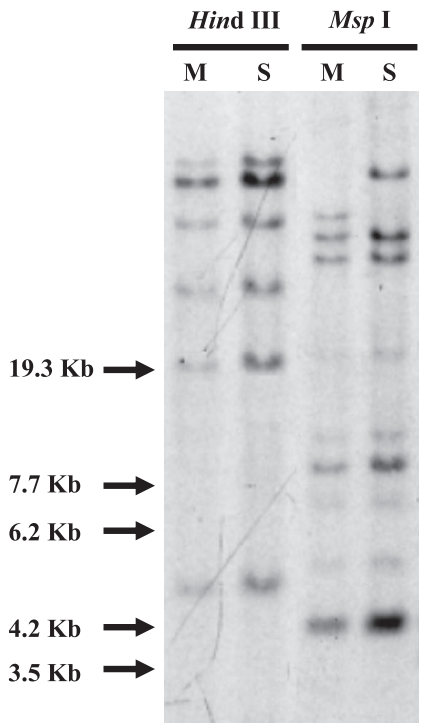

Fig. 2. Southern blot analyses of *FT*/*TFL1* homologs in cv. Mogami and cv. SpaceMaster. Genomic DNAs were respectively digested by *Hin*dIII and *Msp*I. *CsTFL1a* cDNA was used as probe. M and S indicate cv. Mogami and cv. SpaceMaster, respectively.

Table 3. Sequence similarities among *FT/TFL1* homologs (%)

	CsTFL1a	*CsTFL1b*	*CsTFL1Lc1*	*CsTFL1Lc2*	*CsTFL1d*	*CsFT*
CsTFL1a		82	81	82	81	73
CsTFL1b			76	80	78	70
CsTFL1Lc1				97	78	74
CsTFL1Lc2					80	78
CsTFL1d						74

Phylogenetic analysis

Amino acid sequences of FT/TFL1 homologs in cucumber were aligned. They showed high similarities to FT/TFL1 homologs in the other species (Fig. 3). Percentages of similarities of CsTFL1a, CsTFL1b, CsTFL1Lc1, CsTFL1Lc2, and CsTFL1d to TFL1 are 79%, 83%, 75%, 77%, and 76%, respectively. CsFT to FT is 84%.

All FT/TFL1 homologs in cucumber have crucial amino acid residues of Tyr85 and His88. CsTFL1a, CsTFL1b, and CsTFL1d have His88 of TFL1. CsTFL1Lc1, CsTFL1Lc2, and CsFT have Tyr85 of FT. Although CsTFL1Lc1 and CsTFL1Lc2 possess one crucial amino acid residue of FT (Tyr85), their amino acid sequence homologies to TFL1 (75% and 77%, respectively) are higher than those to FT (69% and 73%, respectively). On Gln140 of FT and Asp144 of TFL1, CsFT has Gln140. CsTFL1a and CsTFL1d have Asp144.

For estimating putative functions of each *FT*/*TFL1* homolog in cucumber, phylogenetic relationships of *FT*/*TFL1* homolog were analyzed among the other plant species (Fig. 4). Three major clades composed with FT, TFL1, and MFT groups were constructed. CsTFL1a, CsTFL1b, CsTFL1Lc1, CsTFL1Lc2, and CsTFL1d were clustered in TFL1 group. CsFT was clustered in FT group.

Expression pattern analysis

For elucidating spatial expression patterns of each homolog, RT-PCRs were performed by using 13 organs (Fig. 5). Spatial expression patterns of all homologs in cv. Mogami were similar to those in cv. SpaceMaster. As expected, *CsFT* and *CsTFL1* homologs were expressed in the leaves and SAMs, respectively. These are coincident with the expression patterns of their orthologs in *Arabidopsis*, snapdragon, tomato, and some other species (Bradley *et al*. 1996, Pnueli *et al*. 1998, Amaya *et al*. 1999, Jensen *et al.* 2001, Carmel-Goren *et al*. 2003, Carmona *et al.* 2007, Igasaki *et al*. 2008, Komiya *et al.* 2008). In addition, *CsFT* of both cv. Mogami and cv. SpaceMaster was strongly expressed in the leaves as well as petals and sepals and weakly expressed in SAMs, ovaries, and stigmas. In cv. Mogami, strong expressions of *CsTFL1a* were found in the SAMs, tendrils, stems, cotyledons, flowering buds, and ovaries. On the contrary, little expressions were observed in the roots, leaves, and hypocotyls. In cv. SpaceMater, little expressions of *CsTFL1a* were observed in SAMs, tendrils, stems, cotyledons, flowering buds, and ovaries. No signals of *CsTFL1b* were observed in the whole organs with 30 RT-PCR cycles. But in the case of increased cycles of RT-PCR at 50, bands were strongly detected in the SAMs, roots, leaves, anthers, ovaries, and stigmas and weakly detected in the tendrils, stems, flowering

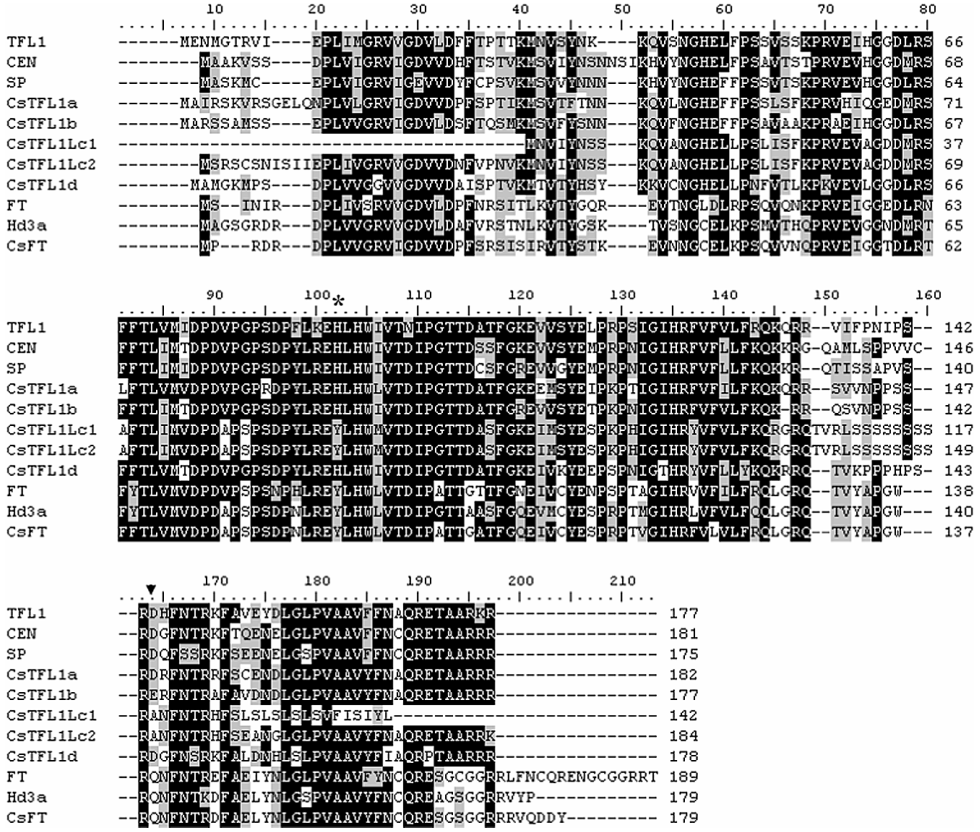

Fig. 3. Alignment of amino acid sequences of *FT*/*TFL1* homologs in cucumber, *TFL1* (T44654) and *FT* (AB027504) of *Arabidopsis*, *SP* of tomato (U84140), *CEN* of snapdragon (S81193), and *Hd3a* of rice (AB052942). The black and gray areas indicate identical and similar amino acid, respectively. The asterisk and the arrowhead indicate the positions of crucial amino acid residues of Tyr85 of FT/His88 of TFL1 and Gln140 of FT/Asp144 of TFL1, respectively.

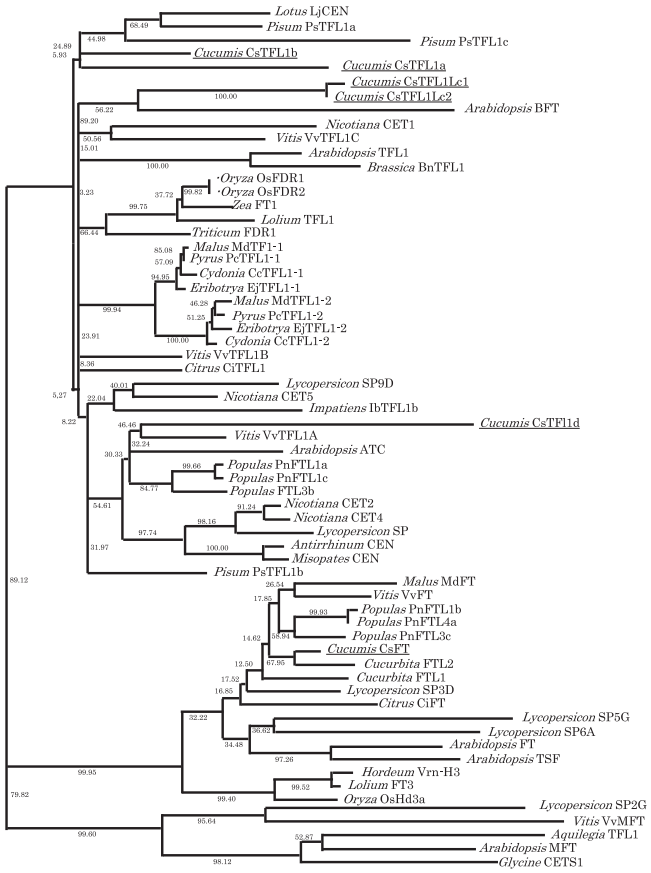

Fig. 4. Phylogenetic tree consisted by *FT*/*TFL1* homologs in cucumber and the other species. Accession numbers are as follows; *Antirrhinum CEN* (S81193); *Arabidopsis FT* (AF152096), *TSF* (AF152907), *TFL1* (U77674), *MFT* (AF147721), *ATC* (AB024714), and *BFT* (NM_125597); *Aquilegia TFL1* (Q2V822); *Brassica BnTFL1* (O82152); *Citrus CiFT* (AB027456) and *CiTFL1* (AY344245); *Cydonia CoTFL1-1* (AB162043) and *CoTFL1-2* (AB162049); *Cucubita FTL1* (A6YE09) and *FTL2* (A6YE10); *Eriobotrya EjTFL1-1* (AB162045) and *EjTFL1-2* (AB162051); *Hordeum Vrn-H3* (3302428); *Glycine CETS1* (A4ZFE0); *Lolium TFL1* (2714296A) and *FT3* (3221354C); *Impatiens IbTFL1* (Q2WCW5); *Lotus LjCEN* (Q6TDS5); *Lycopersicon SP* (U84140), *SP6A* (AY186737), *SP3D* (AY186735), *SP9D* (AY186738), *SP2G* (AY186734), and *SP5G* (AY186736); *Misopates CEN* (Q2WBN1); *Malus MdFT* (AB161112), *MdTFL1-1* (AB052994) and *MdTFL1*-2 (AB162046); *Nicotiana CET1* (AF145259), *CET2* (AF145260), *CET4* (AF145261), and *CET5* (AF145262); *Oryza OsFDR1* (Q53Q71), *OSFDR2* (Q9XGS5), and *OsHd3a* (AB052942); *Pisum PsTFL1a* (AY340579), *PsTFL1b* (AY340580), and *PsTFL1c* (AY343326); *Populus PnFTL1a* (AB181183), *PnFTL4a* (AB161108), *PnFT1b* (AB161109), *PnFTL3b* (AB181240), *PnTFL1c* (AB104629), and *PnFT3c* (AB110009); *Pyrus PcTFL1-1* (AB162042) and *PcTFL1-2* (AB162048); *Triticum FDR1*(Q70JR8); *Vitis VvTFL1A* (A4GG71), *VvTFL1B* (A4GG72), *VvTFL1C* (A4GG73), *VvFT* (A1BQ40), and *VvMET* (ABI99469); *Zea FT1* (Q005X8). The Under lines indicate *FT*/*TFL1* homologs in cucumber. Bootstrap values are shown on each branch.

buds, and petals. *CsTFL1Lc1* and *CsTFL1Lc2* were expressed in the all organs. Expression of *CsTFL1d* could be found in all organs at 50 RT-PCR cycles. These results of *CsTFL1b*, *CsTFL1Lc1*, *CsTFL1Lc2*, and *CsTFL1d* were coincident between cv. Mogami and cv. SpaceMaster.

For analyzing temporal expression patterns, RT-PCRs were conducted by using six growth stages of leaves and SAMs for *CsFT* and *CsTFL1* homologs, respectively (Fig. 6). At Stage 2, main stem of cv. SpaceMaster terminated but that of cv. Mogami did not terminate. Although the expression levels of *CsFT* increased from the Stages 2 to 5 in cv. Mogami, it was expressed in all stages in cv. SpaceMaster. *CsTFL1* homologs except *CsTFL1Lc1* and *CsTFL1d* are upregulated at flowering stage. Their expression patterns are coincident with that of *TFL1* (Bradley *et al.* 1997, Ratcliffe *et al*. 1999). *CsTFL1Lc1* was expressed in all stages of both cv. Mogami and cv. SpaceMaster. *CsTFL1d* was expressed more strongly in cv. SpaceMaster than in cv. Mogami at all stages. *CsTFL1a* increased from the Stage 3 to 6. The expression pattern of *CsTFL1a* was similar between cv. Mogami and cv. SpaceMaster. In contrast, the expression patterns of *CsTFL1Lc2* showed difference between the two cultivars. It was expressed earlier stages in cv. Mogami than cv. SpaceMaster. On *CsTFL1b*, the highest expression was observed in the Stage 2 of both cv. Mogami and cv. SpaceMaster (Fig. 6). Moreover, it was revealed that the expression of *CsTFL1b* in cv. Mogami was much higher than that in cv. SpaceMaster by detailed analysis at Stage 2 (Fig. 7). Weak signal was observed in cv. SpaceMaster at 50 RT-PCR cycles. On the other hand, weak signal was observed in cv. Mogami at 40 RT-PCR cycles and strong signal was detected with 50 RT-PCR cycles.

Although spatial expression patterns of *CsFT*, *CsTFL1b*, *CsTFL1Lc2*, and *CsTFL1d* were similar between two cultivars (Fig. 5), their temporal expression patterns were different between cv. Mogami and cv. SpaceMaster (Fig. 6 and Fig. 7) for the reason that bulks from total RNAs of several growth stages were used for RT-PCR of spatial expression analyses.

Linkage analysis

The 68 F_2 individuals showed segregation on growth habit in which 51 and 17 represented indeterminate and determinate types, respectively. This corresponded to expected numbers following Mendel's law as 51 : 17 = indeterminate: determinate ($\chi^2 = 0$, $p \geq 0.9$).

Sequences of all homologs except *CsTFL1a* showed no difference in coding region. However, one base pair insertion was found in the 1st exon of *CsTFL1a* in cv. SpaceMaster. The polymorphisms were detected with 10% polyacrylamide gel electrophoresis (Fig. 8). The genotypes segregated according to expected values as 17 : 34 : 17 = cv. Mogami type: heterozygous type: cv. SpaceMaster type ($\chi^2 = 0$, $p \geq$ 0.9). In addition, *CsFT* showed polymorphisms of SSR in the 1st intron between cv. Mogami and cv. SpaceMaster. The genotypes also segregate according to expected values calculated as 17 : 34 : 17 = cv. Mogami type: heterozygous type: cv. SpaceMaster type ($\chi^2 = 0$, $p \geq 0.9$). However, there are no linkage relationships between the phenotypes and the genotypes of both of *CsTFL1a* and *CsFT*.

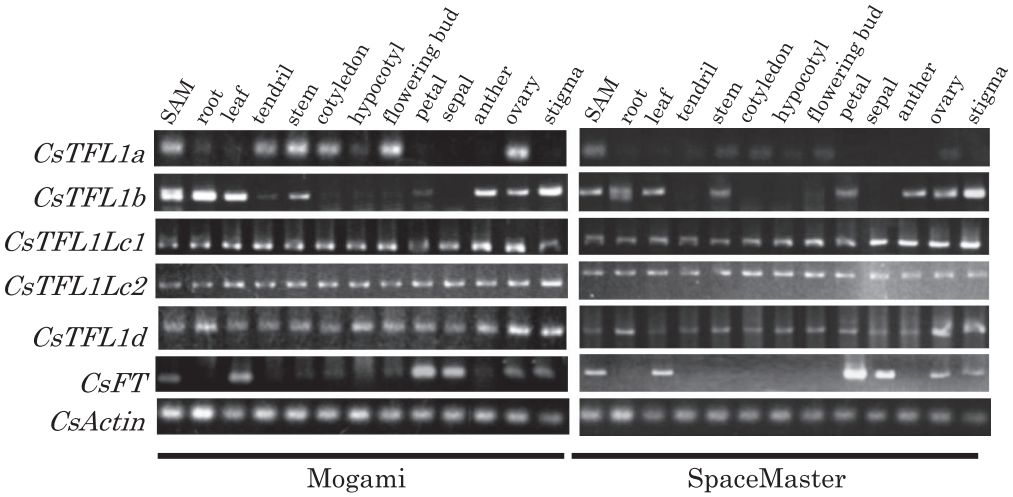

Fig. 5. Spatial expression patterns of *FT*/*TFL1* homologs in cv. Mogami and cv. SpaceMaster by using 13 organs; SAM, root, leaf, tendril, stem, cotyledon, hypocotyl, flowering bud, petal, sepal, anther, ovary, and stigma. The cycle numbers of RT-PCRs for *CsTFL1a*, *CsTFL1Lc1*, *CsTFL1Lc2*, *CsFT*, and *CsActin* were 35, and those for *CsTFL1b* and *CsTFL1d* were 50.

Polymorphisms for the other *FT*/*TFL1* homologs were not detected in both the exons and the introns between cv. Mogami and cv. SpaceMaster.

Discussion

In the present study, one *FT* and five *TFL1* homologs in cucumber were identified. Only one *FT* homolog was also found in grape (Carmona *et al*. 2007), while duplication and divergence of this homolog has been often reported in the other species such as poplar (Igasaki *et al*. 2008), rice (Komiya *et al*. 2008), and *Arabidopsis* (Kobayashi *et al*. 1999). In our study, it was suggested that *CsFT* could be *FT* ortholog in cucumber from the two results. First, amino acid sequence of CsFT show high conservation of FT (Fig. 3 and Fig. 4), and have crucial amino acid residues of Tyr85 and Gln140 of FT (Fig. 3). Second, spatial and temporal expression patterns of *CsFT* were similar to those of *FT* (Fig. 5 and Fig. 6). From linkage analysis between the genotypes of *CsFT* and the phenotypes, *CsFT* was suggested not to be *de*.

Although genomic structures of *CsFT*, *CsTFL1a*, *CsTFL1b*, and *CsTFL1d* are same as those of *FT/TFL1* homologs in the other species, *CsTFL1Lc1* and *CsTFL1Lc2* have unique structures that have never been reported in the other species (Fig. 1). Amino acid sequences of CsTFL1Lc1 and CsTFL1Lc2 showed higher homology with that of TFL1 than FT (Fig. 3). Nevertheless, these genes possess Tyr85 of crucial amino acid residue of FT. Thus, we did not name them as *CsTFL1* but *CsTFL1L* (*like*). Although CsTFL1Lc1 and CsTFL1Lc2 have no crucial amino acid residues of TFL1, these genes may contribute determinate growth habit like *ATC*. ATC also has no crucial amino acid residue of TFL1 and contribute determinate growth habit (Mimida *et al.* 2001). *CsTFL1a* and *CsTFL1d* are assumed to control determinate growth habit in cucumber in the same manner of TFL1 in *Arabidopsis* because these homologs have two

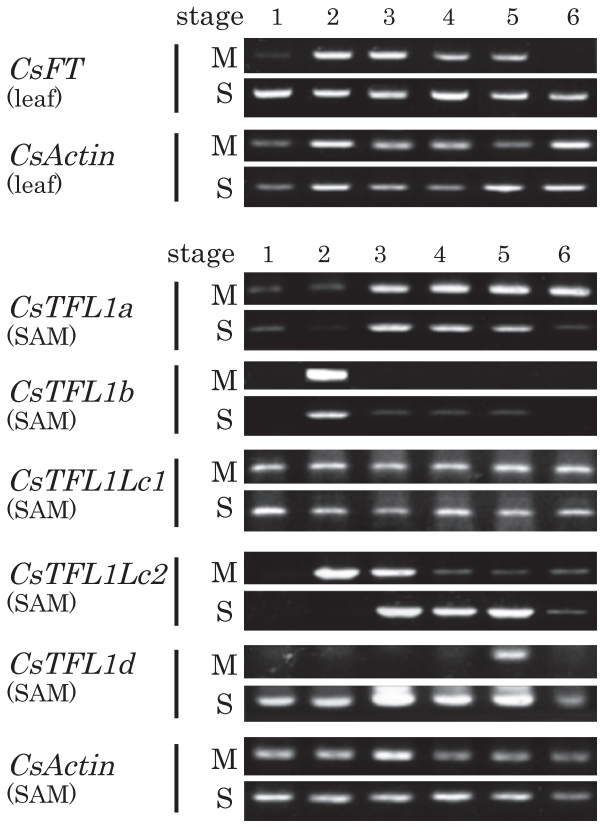

Fig. 6. Temporal expression patterns of *FT*/*TFL1* homologs in cv. Mogami (M) and cv. SpaceMaster (S). Expression of *CsFT* was examined in leaf. That of *CsTFL1a*, *CsTFL1b*, *CsTFL1Lc1*, *CsTFL1Lc2*, and *CsTFL1d* were examined in SAM. Activity of *CsActin* was surveyed in both leaf and SAM as positive control. Six developmental stages of the leaf and SAM were used for analysis; Stage1: early stage (possessing three true leaves), Stage 2: before male flowering, Stage 3: after male flowering, Stage 4: before female flowering, Stage 5: after female flowering, and Stage 6: late stage (holding mature fruits). The cycle numbers of RT-PCRs for *CsTFL1a*, *CsTFL1Lc1*, *CsTFL1Lc2*, *CsFT*, and *CsActin* were 35, and those for *CsTFL1b* and *CsTFL1d* were 50.

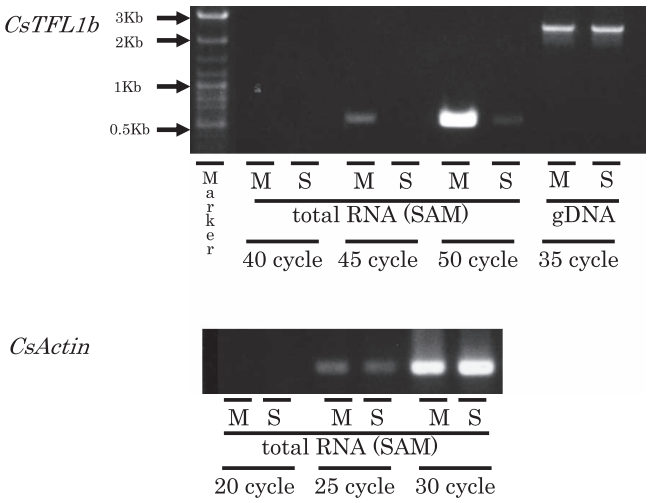

Fig. 7. Temporal expression patterns of *CsTFL1b* at Stage 2 in SAM in cv. Mogami (M) and cv. SpaceMaster (S). Expression patterns of *CsTFL1b* were shown in upper figure. The numbers of RT-PCR cycles were 40, 45, and 50. Genomic DNAs of *CsTFL1b* were shown at last two lanes in upper figure. Activity of *CsActin* was also examined at Stage 2 in SAM with 20, 25, and 30 PCR cycles as positive control, displayed in lower figure.

crucial amino acid residues of TFL1 (His88 and Asp144). CsTFL1b have also one of the two residues of TFL1 (His88). From these results, *CsTFL1a*, *CsTFL1b*, and *CsTFL1d* are expected to have similar function of *TFL1*. Nevertheless, sequences in coding regions of *CsTFL1b* and *CsTFL1d* showed no difference between cv. Mogami and cv. SpaceMaster. *CsTFL1a* possessed 1bp insertion in cv. SpaceMaster, which leads to frameshift and forms premature stop codon. But genotypes of this gene did not show cosegregation to the phenotypes (Fig. 8). Thus, *CsTFL1a* may affect growth habit but may not be *de*.

In this study, phylogenetic analysis generated three major clades composed of FT, TFL1, and MFT group (Fig. 4). This result agree with the report of Carmona *et al.* (2007) in grapevine whereas Igasaki *et al.* (2008) described four clades composed of FT, TFL1, MFT, and BFT groups in poplar. CsFT was clustered in FT group. CsFT is just near to FT2 having same functions of FT in *Cucurbita moschata* (Lin *et al.* 2007). CsTFL1a, CsTFL1b, CsTFL1Lc1, CsTFL1Lc2, and CsTFL1d were clustered in TFL1 group. CsTFL1a and CsTFL1b are the neighbors of PsTFL1a demonstrating to have similar functions to *TFL1* in *Arabidopsis*. Although CsTFL1Lc1 and CsTFL1Lc2 have one crucial amino acid residue of FT, they were clustered in TFL1 group and were near to BFT which function has not been identified yet. Probably, the functions of *CsTFL1Lc1* and *CsTFL1Lc2* are equal to that of *TFL1* with taking into account the results of phylogenetic analysis. CsTFL1d was in the small clade of ATC and was near to VvTFL1A having similar function of TFL1 in *Arabidopsis* (Carmona *et al.* 2007).

Although all *TFL1* homologs showed similar spatial expression patterns between cv. Mogami and cv. SpaceMaster, there were some *TFL1* homologs observed different temporal expression pattern in SAM between two cultivars. The homologs showing difference in temporal expression patterns in SAM between cv. Mogami and cv. SpaceMaster such as *CsTFL1b* and *CsTFL1Lc2* (Fig. 6) are considered to be the candidate for controlling determinate growth habit. Temporal expression patterns of *CsTFL1a* and *CsTFL1Lc1* in SAM coincided with each other between cv. Mogami and cv. SpaceMaster. Moreover, *CsTFL1Lc1* was expressed in all stages. These expression patterns of *CsTFL1a* and *CsTFL1Lc1* are different from the expression patterns of *TFL1*, *CEN*, and *SP*. Interestingly, although *CsTFL1b*, *CsTFL1Lc2*, and *CsTFL1d* are expressed in the same organs of cv. Mogami and cv. SpaceMaster (Fig. 5), these homologs showed different temporal expression patterns in SAM between the two cultivars (Fig. 6). *CsTFL1b* showed weaker expression in cv. SpaceMaster than in cv. Mogami at Stage 2 (Fig. 6 and Fig. 7). In addition, temporal expression pattern of *CsTFL1Lc2* was disparate between cv. Mogami and cv. SpaceMaster at Stage 2, 4, and 5 (Fig. 6). These two homologs show different expression patterns at Stage 2. The lack or weakness of expression at the stage just before flowering in SAM of cv. SpaceMaster, at which main stems are terminated, may be related to the characteristic of determinate growth habit. *CsTFL1d* was expressed strongly in cv. SpaceMaster, determinate cultivar, than in cv. Mogami, indeterminate cultivar, at all stages. The expression patterns in SAM of *CsTFL1d* are different from expression patterns of *TFL1*, *CEN*, and *SP*.

The interrelationship between the genotypes of *CsTFL1b* or *CsTFL1Lc2* and the phenotypes are important to understand the relationship between the *TFL1* homolog and *de*. However, sequence comparisons of both *CsTFL1b* and *CsTFL1Lc2* between cv. Mogami and cv. SpaceMaster were difficult because there were no differences in the sequences not only in the exons but also in the introns. Identification of differences of flanking sequences between *de*/*de* and *De*/*De* and utilization of the difference in linkage analysis will clarify whether *CsTFL1b* or *CsTFL1Lc2* would be involved in determinate growth habit.

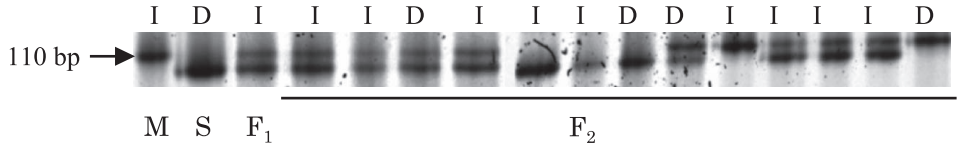

Fig. 8. Genotype analysis of *CsTFL1a*. I and D indicate indeterminate type and determinate type of phenotype, respectively. M and S indicate cv. Mogami and cv. SpaceMaster, respectively.

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