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Research paper

The *MdTFL1* gene of apple (*Malus* × *domestica* Borkh.) reduces vegetative growth and generation time

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TFL1 is known as a floral repressor in *Arabidopsis thaliana* (L.) Heynh. In apple there are two *TFL1* homologs, *MdTFL1-1* and *MdTFL1-2*. The *MdTFL1-1* gene was silenced in transgenic clones expressing a hairpin gene construct of a 323 bp fragment of *MdTFL1-1*. The hairpin gene construct was transferred to three different apple genotypes. Of 22 transgenic clones, 21 showed a significant reduction in *MdTFL1-1* mRNA expression. Precocious flowering was obtained for 20 clones, which flowered already during in vitro cultivation. Nineteen clones could successfully be transferred to the greenhouse where 18 of them flowered within a few weeks followed by the death or at least a strongly inhibited vegetative growth of the plant. Most of the transgenic flowers developed abnormally. Results obtained on greenhouse-grown plants of the transgenic clones and transgenic seedlings clearly demonstrated the major role of *MdTFL1* genes in maintaining the vegetative growth as prerequisite for a perennial lifecycle. It was shown that *MdTFL1* dsRNAi promotes a life history similar to annual plants. Preliminary results obtained from grafting experiments with non-transgenic scions grafted onto *MdTFL1* dsRNAi transgenic rootstocks indicated that the flower-inducing signal obtained after silencing of *MdTFL1* genes seems not to be graft-transmissible.

Keywords: early flowering, *Malus*, perennial lifecycle, silencing, TERMINAL FLOWER 1, transgenic.

Introduction

The cultivated apple *Malus* × *domestica* Borkh. is a perennial plant with a long-lasting juvenile (non-flowering) stage, which is characterized by vegetative growth and the inability of apple plants to respond to flower-inducing signals. This vegetative stage lasts under natural conditions 5–10 or even 12 years (Visser 1964, Fischer 1994). The juvenile stage ends with the vegetative transition. The plants attain the adult stage and get competent to respond to flower-inducing signals (Hanke et al. 2007). Once apple seedlings have reached the adult stage, they flower yearly or in a biennial rhythm. In contrast to annual plants, only a small portion of shoot meristems initiate flower buds terminally on fruiting spurs and terminally or laterally on long shoots. The other meristems remain vegetative (Bangerth 2009).

Genetic factors that control the vegetative transition from the juvenile to adult stage in apple are widely unknown. In previous years, several genes were identified in the model plant *Arabidopsis thaliana* (L.) Heynh that promote or prevent the stage transition. *Arabidopsis* is an annual, long-day plant that already responds to flower-inducing signals some days after seed germination. Although genes that promote flowering are increased in expression as the plant ages (Wu and Poethig 2006, Yamaguchi et al. 2009), it is difficult to discriminate clearly the transition from the juvenile to adult stage in *Arabidopsis*. In *Arabidopsis* flowering is promoted via several

interacting, flower-inducing pathways that respond to photoperiod, temperature, gibberellins and plant age (reviewed by Srikanth and Schmid 2011). Flower-inducing signals are mediated by these different pathways and converge at the regulation of the floral integrator gene *FLOWERING LOCUS T* (*FT*) that was identified as the long-distance, graft-transmissible, flower-inducing signal that is produced in leaves and induce flower development at the shoot meristems (Jaeger and Wigge 2007, Mathieu et al. 2007, Notaguchi et al. 2008). Besides *FT*, there are *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) and *LEAFY* (*LFY*) which act also as floral integrator genes. The *TERMINAL FLOWER 1* (*TFL1*) gene is a temporal and spatial repressor of flowering that acts antagonistically to *FT*. *TFL1* encodes a phosphatidylethanolamine-binding protein with homology to *FT*. The loss of *FT* function causes delay in flowering in *Arabidopsis*, whereas overexpression of *FT* results in precocious flowering (Kobayashi et al. 1999). Vice versa, overexpression of *TFL1* results in delayed flowering, whereas *Arabidopsis tfl1* mutant plants flower earlier than wild-type plants (Shannon and Meeks-Wagner 1991, Ratcliffe et al. 1999, Hanano and Goto 2011). *TFL1* is expressed in shoot apical and axillary meristems and inhibits the activity of *APETALA 1* (*AP1*) and *LFY* at the center of the shoot apex by delaying their up-regulation and by preventing the meristem from responding to *LFY* or *AP1*. In turn, the *FT*-promoted genes *LFY*, *AP1* and *CAULIFLOWER* (*CAL*) prevent *TFL1* transcription in floral meristems on the apex periphery, allowing the formation of indeterminate inflorescences (Ratcliffe et al. 1999, Parcy et al. 2002, Conti and Bradley 2007, Hanano and Goto 2011).

In apple, a homologous gene to *TFL1* (*MdTFL1*) was cloned by Kotoda and Wada (2005). Its expression was studied in different apple tissues and during vegetative and reproductive bud development. It was suggested that *MdTFL1* is involved in the maintenance of the vegetative stage in apple and that it functions analogously to *TFL1*. The function of *MdTFL1* was later clarified in apple using an antisense RNA approach. It was confirmed that *MdTFL1* functions like *TFL1* and that *MdTFL1* maintains the juvenile and vegetative stage in apple (Kotoda et al. 2003, 2006). Later on it was shown that there are two *MdTFL1* genes, *MdTFL1-1* (*MdTFL1*) and *MdTFL1-2* (*MdTFL1a*), in apple (Esumi et al. 2006, Hättasch et al. 2008, Mimida et al. 2009). To avoid any confusion evoked by the different style of gene nomenclature we will use *MdTFL1-1* and *MdTFL1-2* in accordance with Mimida et al. (2011*a*).

Transcript analyses in adult apple trees showed that *MdTFL1-1* and *MdTFL1-2* are expressed in vegetative shoot apices during flowering of floral buds that were initiated in the previous season and that the transcript levels of both genes increased again 2 weeks after flower induction. It was assumed that *MdTFL1-1* and *MdTFL1-2* inhibit precocious flower induction during blooming of the coexisting floral buds and that the activation of their expression after flower induction marks the onset of inflorescence development (Hättasch et al. 2008). Besides *MdTFL1-1* and *MdTFL1-2* two further members of the *TFL1*/*CEN*-like gene family (*MdCENa* and *MdCENb*) were identified in apple and mapped at a similar position on homologous chromosomes. Based on the transcription pattern, *MdCENa* and *MdCENb* seem not to be involved in the transition from the juvenile to adult stage. However, *MdTFL1-1* and *MdTFL1-2* were expressed in the vegetative tissues in both the adult and juvenile stages, and it was suggested that they could function redundantly as flowering repressors and regulators of vegetative meristem identity (Mimida et al. 2009). Based on these findings, it was of interest to suppress the *MdTFL1* genes *MdTFL1-1* and *MdTFL1-2* in apple in order to shorten the juvenile stage of young apple plants and to induce precocious flowering.

A chimeric gene construct encoding a double-stranded (ds) hairpin RNA that is based on 323 bp of the coding sequence of *MdTFL1-1* from the apple cultivar 'Pinova' was used for *Agrobacterium*-mediated leaf disk transformation of four different apple cultivars. The obtained transgenic plants were evaluated for the presence, integration and expression of the transgenic sequences at the molecular level. Transgenic plants grown in vitro, in the greenhouse on their own roots, or as transgenic seedlings of a cross between transgenic plants and the apple cultivar 'Weirouge' were evaluated for their phenotype. To study the graft transmissibility of *MdTFL1* silencing signals from rootstocks to scions, we used transgenic dsRNAi *MdTFL1* plants as rootstocks and grafted them with non-transgenic scions.

Materials and methods

Vector design

A 323 bp fragment of the *MdTFL1-1* gene (accession no. DQ535888) was amplified from genomic DNA of the *Malus* × *domestica* Borkh. cultivar 'Pinova' using the primers attb1_tfl 5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG CCT CGG AGC CTC TGG TTG TT-3′ and attb2_tfl 5′-GGG ACC ACT TTG TAC AAG AAA GCT GGG TTC GGC ATC TCA TAA CTC ACC A-3′. The polymerase chain reaction (PCR) product was cloned into the vector pHELLSGATE12 (CSIRO) in a two-step procedure using pDONR™207 (Life Technologies GmbH, Darmstadt, Germany) as an intermediate vector. Polymerase chain reaction, as well as in vitro BP and LR clonase recombination reactions, was carried out according to the manufacturer's instructions (Invitrogen). The correct sequences and orientations of the introduced sense and antisense *MdTFL1-1* fragments were confirmed by sequencing. The construct pHELLSGATE12::*MdTFL1* was introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation. The structure of the T-DNA of pHELLSGATE12::*MdTFL1* is shown in Figure 1.

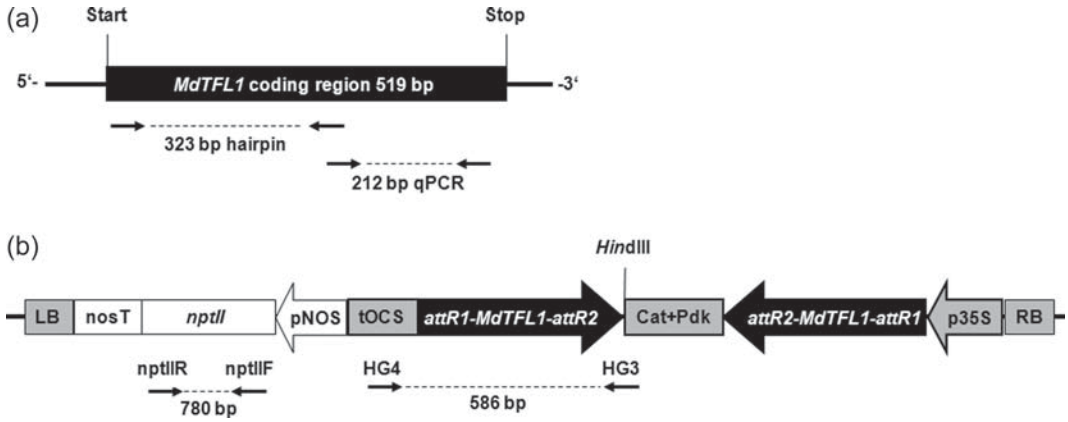

Figure 1. T-DNA of the binary plasmid vector pHELLSGATE12::*MdTFL1*. (a) Schematic presentation of the *MdTFL1-1* coding region including the regions used for cloning of the hairpin construct and for transcript analysis of transgenic plants, respectively. (b) Schematic presentation of the *MdTFL1-1* hairpin gene construct used for transformation. The construct based on the plasmid vector pHELLSGATE 12 (http://www.pi.csiro.au/rnai/vectors.htm). LB, left border sequence; nosT, terminator of the nopaline synthase gene; nptII, neomycin phosphotransferase II gene conferring resistance to kanamycin; pNOS, promoter of the nopaline synthase gene; tOCS, terminator of the octopine synthase gene; attR1-MdTFL1-attR2, 323 bp PCR fragment of the *MdTFL1-1* gene flanked by two attR recombination sites; Cat + Pdk, castor bean catalase-1 intron in reverse orientation fused with the pyruvate orthophosphate dikinase intron in sense orientation; RB, right border sequence.

Plant material and transformation

For plant transformation, proliferating axillary shoot cultures of the apple (*Malus* × *domestica*) cultivars 'Gala', 'Galaxy', 'Holsteiner Cox' and a seedling of the cultivar 'Pinova' (in the following referred to as 'PinS') were used. Plant transformation, regeneration, selection of transgenic plants, rooting and acclimatization were performed as described by Flachowsky et al. (2007). The plant transformation was carried out using the *A. tumefaciens* strain EHA105 containing the plasmid vector pHELLSGATE12::*MdTFL1*.

Polymerase chain reaction analysis

Genomic DNA was extracted from 0.1 g of leaf tissue using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany). The PCR mixture contained 50 ng of genomic plant DNA, 1× NH_4 buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM of each primer (nptIIF/nptIIR for *nptII* and HG3/HG4 for the *MdTFL1-1* hairpin) and 0.5 U of Taq DNA polymerase (Invitek, Berlin, Germany) in a total volume of 25 µl. All primer sequences are given in Table S1 available as Supplementary Data at *Tree Physiology* Online. The PCR reaction was performed by denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 58 °C (*nptII* and *MdTFL1-1* hairpin) and 1 min extension at 72 °C. After a final extension at 72 °C for 5 min the amplified fragments were separated on a 1% agarose gel.

Southern blot analysis

The detection of integrated T-DNA copies was performed by Southern hybridization. About 10 µg DNA was digested with 100 U of HindIII (MBI Fermentas, St Leon Roth, Germany) at 37 °C overnight. The restricted DNA was separated on a 0.8% agarose gel and transferred onto a nylon membrane (Roche Diagnostics, Mannheim, Germany). A digoxigenin-labeled probe amplified by PCR from the coding region of the *nptII* marker gene with the primers nptIIF/nptIIR was used for hybridization. Hybridization and detection were performed using the ECF Random Prime Labeling and Detection Kit (Amersham Biosciences, Freiburg, Germany) according to the manufacturer's manual.

Transcript analysis

Total RNA was extracted from frozen tissue using the Invisorb® Spin Plant RNA Mini Kit (Invitek). The DNase I treatment as well as the first strand cDNA synthesis was done as described by Flachowsky et al. (2007). Reverse transcriptase-PCR (RT-PCR) was performed in 25 µl standard volume using the primers nptIIF/nptIIR and HG3/HG4 as described before.

Quantitative real-time PCR (qRT-PCR) was performed using the iQ™ SYBR Green Supermix (Bio-Rad Laboratories GmbH, Munich, Germany) on an iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories). Gene-specific amplification was evaluated by melt curve analysis and agarose gel electrophoresis. Amplification and correlation efficiencies of each PCR assay were determined on diluted plasmid DNA containing the gene of interest. The PCR efficiency was used to transform the C_t values into raw data for relative quantification. The determination of PCR efficiency and the calculation of the mRNA transcript levels were done using Gene Expression Macro™ Version 1.1 (Bio-Rad Laboratories). The endogenous *MdTFL1-1* and *MdTFL1-2* gene transcription was quantified using the primers MdTFL1Fq/Rq for *MdTFL1-1* and

MdTFL1-2F/R for *MdTFL1-2*. All samples were normalized using the transcript level of RNA polymerase subunit II (RNAPOL II) as internal control. The scaling of the *MdTFL1-1* gene transcript level was performed in relation to the mRNA expression levels of the non-transgenic control genotypes 'PinS', 'Holsteiner Cox' and 'Galaxy', respectively, which were set to be 1.

Phenotyping of transgenic plants

In vitro grown shoots of transgenic clones and non-transformed control genotypes were evaluated on their morphological characteristics such as leaf size and morphology, length of the internodes and flower development. The morphological evaluation of transgenic and non-transgenic plants grown in the greenhouse on their own roots or grafted onto a transgenic plant used as rootstock was realized at the time when the plants set up flowers for the first time. Furthermore, we counted the sepals, petals, anthers and styluses per flower as well as the number of abnormal flower organs. Greenhouse plants were grown under the same conditions as described by Flachowsky et al. (2011). From April to December, the plants were grown under glasshouse conditions without additional light and temperatures comparable with the natural orchard conditions. From January to March an 'artificial winter' program was applied. The conditions of light and temperature were made similar to those in the orchard. The temperature was reduced from 3 °C (standard temperature during the winter months) to 0 °C from 1 h before to 9 h after sunrise by down-regulating the heater (only possible on days when the temperature is <0 °C). The heater was calibrated to a minimum temperature of 0 °C. In addition, the artificial circulation was reduced from 5 to 1 °C between 30 min before and 8.5 h after sunrise.

Hybridization experiments on transgenic flowers

Crosses were made in an insect-protected glasshouse as described by Flachowsky et al. (2007). Pollen was collected from field-grown plants of different apple genotypes (cultivars and wild species) of the Dresden-Pillnitz *Malus* gene bank. Shoots of transgenic plants with flowers were selected and labeled. When a flower was fully open, a brush was used to spread pollen on the stigmas. Non-pollinated flowers were discarded.

Grafting experiments

For grafting experiments, micropropagated in vitro grown shoots were rooted and acclimatized to greenhouse conditions. Scions of the non-transformed control genotype 'PinS' were grafted onto transgenic rootstocks expressing the *MdTFL1-1* hairpin gene construct. The grafted scion was grown as the first-order shoot. Developing lateral shoots originating from the transgenic rootstock were grown as second- or third-order shoots.

Results

Development of a hairpin gene construct for silencing the MdTFL1 genes

Sequences of the members of the *MdTFL1/CEN*-like gene family of apple were aligned to find a region which is specific to the *MdTFL1* genes (*MdTFL1-1* and *MdTFL1-2*). The alignment consisted of published sequences of *MdTFL1-1*, *MdTFL1-2*, *MdCENa* and *MdCENb* (Table 1). A 323 bp sequence was selected and amplified from genomic DNA of 'Pinova' as described. The obtained fragment showed highest sequence identities with 99.7–100% to *MdTFL1-1* sequences of different apple genotypes. Identities of 94.4% (304/322 bp) were found between the amplified fragment and *MdTFL1-2* (accession no. EU672880). The fragment showed also identities of 75.6% (245/324 bp) to *MdCENa* (accession no. AB366637) and 75.3% (244/324 bp) to *MdCENb* (accession no. AB366638). A partial alignment is shown in Figure S1 available as Supplementary Data at *Tree Physiology* Online. The longest blocks of contiguous sequence identities were 14 bp between the vector sequence and *MdCENa* and *MdCENb*, respectively. Undesired gene silencing of both *CEN*-like genes of apple can therefore be excluded. Subsequently, the sequence of the *MdTFL1-1* fragment was blasted to the recently published genome sequence of the apple cultivar 'Golden Delicious'. Significant sequence identities of 322/323 bp and 300/316 bp to *MdTFL1-1* (MDP0000255437 and BAD06418.1) and *MdTFL1-2* (MDP0000126761 and BAD10967.1) were found, as expected. Furthermore, sequence identities to a number of other predicted gene sequences were detected. However, none of the matched genes contained boxes of identical gene sequences which were longer than 14 bp. Undesired gene silencing of other genes than *MdTFL1-1* and *MdTFL1-2* seems not to be expectable. Therefore, we decided to use the amplified fragment with highest homology to *MdTFL1-1* for cloning of the pHELLSGATE12::MdTFL1 plant transformation vector.

Generation of transgenic clones expressing a MdTFL1-1 hairpin gene construct

The hairpin gene construct containing the selected 323 bp sequence of the *MdTFL1-1* gene in sense and antisense orientation was transferred into wounded in vitro leaf explants of the apple cultivars 'Gala', 'Galaxy' and 'Holsteiner Cox' as well as into the apple genotype 'PinS'. A total of 4266 leaf explants were inoculated with the *A. tumefaciens* strain EHA105 containing the binary plasmid vector pHELLSGATE12::*MdTFL1*. After two rounds of selection a total of 22 putative transgenic shoots were obtained (see Table S2 available as Supplementary Data at *Tree Physiology* Online). These shoots were tested for the presence of transgenes by PCR and Southern hybridization analysis. The presence of the *nptII* selectable marker gene was

Table 1. Gene sequences used for alignment for vector construction

Gene	Accession number	Origin	LG/Chr.	Mapped in	Reference
MdTFL1-1					
MdTFL1	AB052994	*M. × domestica*			Kotoda and Wada 2005
MdTFL1-1	AB162040	'Fuji'			Esumi et al. 2006
MdTFL1-1	DQ535888	'Pinova'			Hättasch et al. 2008
MdTFL1	AB366639	'Fuji'	12	'Ralls Janet'	Mimida et al. 2009
			12	'Delicious'	Mimida et al. 2009
MdTFL1	MDP0000255437	'Golden D.'	12	'Golden D.'	Velasco et al. 2010
MdTFL1-2					
MdTFL1-2	AB162046	'Fuji'			Esumi et al. 2006
MdTFL1-2	EU672880	'Pinova'			Hättasch et al. 2008
MdTFL1a	AB366640	'Fuji'	14	'Ralls Janet'	Mimida et al. 2009
			14	'Delicious'	Mimida et al. 2009
MdTFL1a	MDP0000126761	'Golden D.'	4	'Golden D.'	Velasco et al. 2010
MdCENa					
MdCENa	AB366637	'Fuji'	3	'Ralls Janet'	Mimida et al. 2009
			3	'Delicious'	Mimida et al. 2009
MdCENa	MDP0000761080	'Golden D.'	10	'Golden D.'	Velasco et al. 2010
MdCENb					
MdCENb	AB366638	'Fuji'	11	'Ralls Janet'	Mimida et al. 2009
			11	'Delicious'	Mimida et al. 2009
MdCENb (?)	MDP0000127457	'Golden D.'	13	'Golden D.'	Velasco et al. 2010

LG, linkage group; Chr., chromosome; 'Golden D.', 'Golden Delicious'; (?), best hit for *MdCENb* homologs in the 'Golden Delicious' genome sequence, but the predicted coding sequence is much longer than the described *MdCENb* gene sequence.

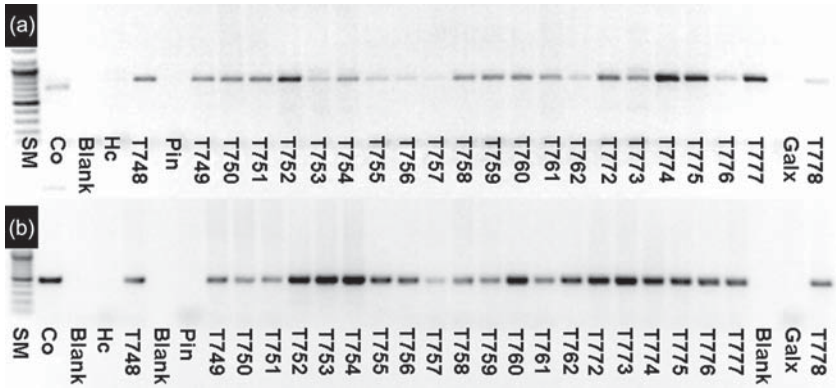

Figure 2. Molecular evaluation of transgenic apple lines. (a) The presence of the *nptII* marker gene was tested using the primers nptIIF/R. All putative transgenic plants showed PCR products of the expected size of 780 bp. (b) The presence of the *MdTFL1-1* hairpin gene construct was tested using the primers HG3/HG4. All putative transgenic plants showed PCR products of the expected size of 586 bp. SM, molecular size marker (100 bp DNA ladder, MBI Fermentas); Co, plasmid DNA of the transformation vector used as positive control; Blank, ddH_2O used as negative control; Hc, DNA of the non-transformed 'Holsteiner Cox'; Pin, DNA of the non-transformed 'PinS'; Galx, DNA of the non-transformed 'Galaxy'; T748-T778, transgenic apple clones.

tested using the primer pair nptIIF/IR. All plants showed the *nptII* gene-specific PCR product of 780 bp (Figure 2a). The presence of the *MdTFL1-1* hairpin gene construct was tested using the primers HG3 and HG4. The HG3 primer is specific to the 3′-end of the *Cat* intron sequence that is located between the sense and antisense fragment *MdTFL1-1*. The HG4 primer is specific to the *ocs* terminator downstream of the hairpin construct. Using the primers HG3 and HG4, a fragment of 586 bp containing a small part of the *Cat* intron, the 323bp *MdTFL1-1* sequence and a few bases of the *ocs* terminator was amplified for all shoots (Figure 2b). Subsequently, all shoots were tested for the integration and copy number of the transferred T-DNA by Southern hybridization. At least one hybridization signal was detectable for each plant. Thereby, 20 of 22 transgenic shoots showed only one signal indicating single copy integration. Two transgenic shoots showed multiple fragments, which indicates the integration of more than one T-DNA copy per shoot. Based on the results obtained by PCR and Southern hybridization all shoots were considered as *MdTFL1-1* dsRNAi transgenic. In total, 20 of 22 transgenic shoots originated from 'PinS'. No transgenic plant was obtained from the apple cultivar 'Gala' (see Table S2 available as Supplementary Data at *Tree*

Physiology Online). From 'Galaxy' and 'Holsteiner Cox' only one transgenic shoot per genotype was obtained, resulting in transformation efficiencies of 1.49% ('PinS'), 0% ('Gala'), 0.16% ('Galaxy') and 0.07% ('Holsteiner Cox'). Subsequently, all transgenic shoots were micropropagated and cultivated in vitro.

Transcription of transgenes and MdTFL1 genes

Total RNA of each clone was isolated from young leaves to investigate the transcription of the transgenes by RT-PCR analysis. The expression of the marker gene *nptII* was analyzed using the primers nptIIF/R. The expected 780 bp PCR product, indicating the transcription of the *nptII* gene, was amplified for each clone (Figure 3a). The expression of the *MdTFL1-1* hairpin gene construct was analyzed using the primers HG3/4. For each clone, a PCR product of the expected size of 586 bp was amplified (Figure 3a).

To determine the relative level of the endogenous *MdTFL1-1* and *MdTFL1-2* mRNA expression in transgenic in vitro shoots, we performed a qRT-PCR using the primers MdTFL1Fq/Rq for *MdTFL1-1* and MdTFL1-2F/R for *MdTFL1-2*. No transcripts of *MdTFL1-2* were detectable in transgenic and non-transgenic shoots. The *MdTFL1-1* mRNA transcript level of the transgenic clones was significantly reduced except for clone T748 that was derived from the cultivar 'Holsteiner Cox' (Figure 3a and b). The reduction of the *MdTFL1-1* transcript level of the other 21 transgenic clones ranged from 12 to 98%. The strongest reduction was found for clone T773. This clone expressed only 2% of the *MdTFL1-1* mRNA transcripts compared with the non-transformed control genotype 'PinS'. The experiment was repeated with similar results (data not shown).

Phenotyping of MdTFL1-1 dsRNAi in vitro plants

Transgenic apple clones were grown in vitro for 19–24 months. After 6 months first flowers were detected on shoots of clone T777. After 24 months 20 out of the 22 transgenic clones had started flowering in vitro, except for clones T748 from 'Holsteiner Cox' and clone T778 from 'Galaxy' that grew vegetatively all the time. All flowering in vitro plants developed solitary terminal flowers. Inflorescences consisting of several flowers were not found. After full bloom the growth of the reproductive shoot stopped and the terminal flower including the upper part of the main shoot was aborted. Subsequently, one of the lateral shoots proceeded as a new main shoot. New flowers were developed laterally in the leaf axils or terminally on the new main shoot. Transgenic plants that started flowering during in vitro cultivation flowered permanently for 2–4 months. Afterwards the plants stopped flower development and started again 10–12 months later.

The flowers of transgenic in vitro plants were normal in size when compared with flowers of 'Pinova' trees grown in the field, but they showed often an increased number of petals ranging from 6 to 20 petals per flower. An example is shown in Figure 4. The flowering plants produced also smaller leaves that appeared to be rounder than leaves of the non-transformed control plants grown in vitro.

Besides flower induction, there are further biological processes such as floral bud dormancy, which were completely overridden in the *MdTFL1* silenced apple clones. Flower development in apple takes normally 9–10 months from flower induction (middle of June in Europe) until full bloom (beginning of May in the following year). However, in vitro plants of the

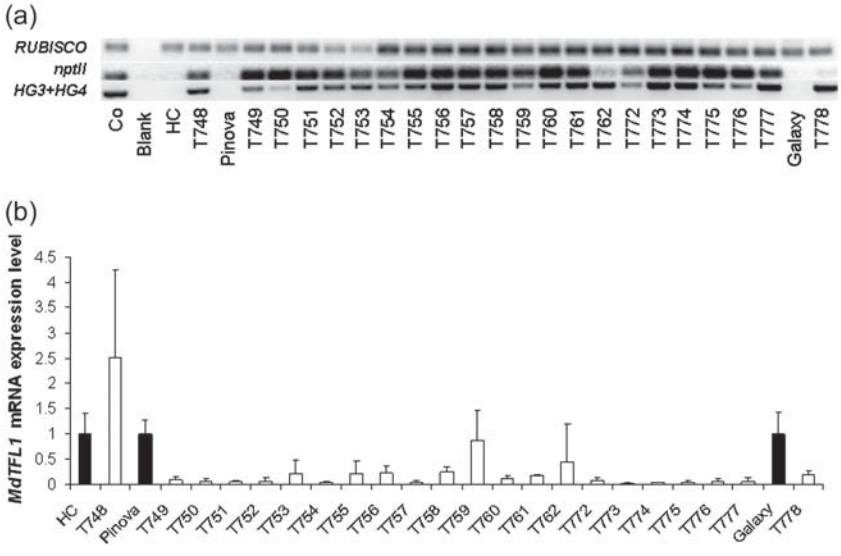

Figure 3. Evaluation of transgenic plants for the transcription of transgenes and *MdTFL1-1*. (a) Reverse transcriptase-PCR-based detection of *nptII* gene and *MdTFL1-1* hairpin gene transcripts. mRNA transcripts of *RUBISCO* were used as control. (b) Relative quantification of *MdTFL1-1* transcript levels by qRT-PCR.

Figure 4. In vitro grown shoots of the transgenic line T773 of the apple genotype 'PinS' containing the T-DNA of the binary plasmid vector pHELLSGATE12::*MdTFL1*. Transgenic shoots of several clones set up their first flowers immediately after transformation and regeneration. These plants flowered permanently for 2–4 months, stopped flowering and started again 10–12 months later. Developing flower buds are marked by yellow arrows. Black arrows show flowers with an increased number of petals.

MdTFL1-silenced apple clones required only 2–3 weeks from flower induction to full bloom without any chilling requirement. Thus, also developmental processes that take place after flower induction such as flower initiation, flower organ differentiation and blooming run significantly faster.

Phenotyping of greenhouse plants

Plants of 19 transgenic clones including also very small plants of clones T762 and T774 were successfully transferred to the greenhouse. No greenhouse plants could be established for clones T749, T756, T762 and T774. Plants of these clones were already flowering during the cultivation in 5 cm plastic pots. Plants of clones T749 and T756 died after full bloom and could not be evaluated in detail. Plants of clones T762 and T774 started to flower during cultivation in 5 cm plastic pots. Some plants died after flowering, whereas other plants of T762 and T774 survived for some weeks after full bloom, but their vegetative growth was inhibited.

No flowers developed on plants of clone T748 within 24 months of greenhouse cultivation, as observed for the in vitro grown plants of this clone. Most plants of the remaining 18 transgenic clones started flowering within a few weeks after transfer to the greenhouse. Their growth was reduced compared with the non-transformed control plants (Figure 5a–c). Plants of several lines developed a leaf rosette directly above the soil level (Figure 5b, e, f). Leaves of the rosette appeared to be rounder as leaves of the non-transformed control plants. Adjacent to the leaf rosette the transgenic plants developed a single main shoot with or without leaves along the shoot (Figure 5b, e). The growth of the main shoot stopped after a terminal flower had been developed. Other plants set up their first flowers directly above the leaf rosette (Figure 5c, d). Plants without leaves along the main shoot died directly after flowering, which is characteristic for annual plants, but not for perennial plants like apple. Plants that contained leaves along the main shoot aborted the terminal flower after full bloom, stopped their growth and developed new flowers in the leaf axils (Figure 5f). Subsequently, these plants died or they developed a lateral shoot below the aborted terminal flower. This shoot grew subsequently as the main (first-order) shoot and developed a new terminal flower within the next few weeks.

The first 131 flowers that were developed on transgenic plants grown in the greenhouse were evaluated for different morphological traits. Most of these flowers were abnormally formed. Several examples are shown in Figure 6. Only 17 flowers (13%) showed a normal phenotype with 5 sepals, 5 petals, 13–20 anthers and 5 styluses. About 64% of the flowers showed an increased number of petals, 46% developed

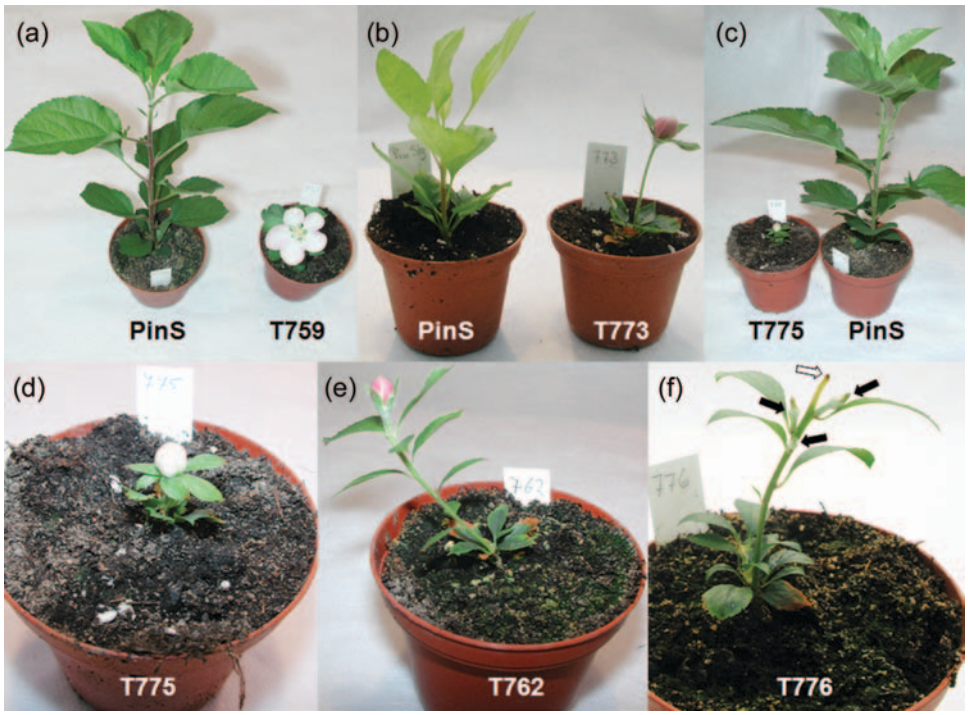

Figure 5. Evaluation of transgenic greenhouse plants grown on their own roots. (a–c) Transgenic plants of three different clones with terminal flowers compared with non-transformed control plants of the apple genotype 'PinS' at the same plant age. The transgenic plants showed a reduced growth. (b–f) Transgenic plants developed a leaf rosette directly above the soil level. (b) Transgenic plant of clone T773 consisting of a leaf rosette, a single main shoot without lateral leaves along the shoot and a terminal flower. Such plants died mostly directly after blooming. (c + d) Transgenic plant of clone T775 with a terminal flower directly above the leaf rosette. (e) Transgenic plant of clone T762 consisting of a leaf rosette, a single main shoot with lateral leaves along the shoot and a terminal flower. Such plants developed a side-shoot or lateral flowers in the leaf axils after blooming of the terminal flower. The side-shoot grew then as main shoot and developed a new terminal flower. (f) Transgenic plant of clone T776 after blooming of the terminal flower. The terminal flower has been discarded (white arrow) and new flowers have been developed in the leaf axils (black arrows).

individual anthers that looked like petals, 24% developed no styluses and 20% had sepals that were acuminate and elongated. Other abnormalities like fused sepals and petals (8%), sepals which looked like leaves (10%), stunted (15%) or enlarged (11%) sepals were also frequently found (Table 2, Figure 6). These floral abnormalities indicate that *MdTFL1* genes are also involved (directly or indirectly) in floral organ development.

Pollination experiments

To investigate the fertility of transgenic plants, flowers of greenhouse-grown shoots of T752, T755, T759, T775, T776 and T777 were pollinated with pollen of the apple cultivar 'Weirouge' that is grown in the experimental field. Three to five days after fertilization, the receptacle of pollinated flowers started to swell, and fruits developed 8 weeks after pollination. The growth of the main shoot bearing the pollinated flower stopped immediately after pollination. Several plants developed a single lateral shoot below the pollinated flower (fruit), which grew subsequently as the main (first-order) shoot. This shoot developed a new flower terminally or in leaf axils within the next few months. Sometimes we found developing fruits and new developed flowers in parallel on the same plant (Figure 7a–c). Other plants stopped their growth directly after pollination. These plants developed no lateral shoots (Figure 7d, e) and it seemed that all resources were used for the developing fruit. Several plants aborted their leaves, but the fruits of these plants developed normally (Figure 7d). In total, 10 fruits were harvested after 5–6 months of evaluation. Most fruits showed a normal fruit size of 5–6 cm in diameter and a usual number of 5–7 seeds (Figure 7f). This result demonstrates that flowers of transgenic apple plants are fertile.

Evaluation of transgenic seedlings

A total of 41 seeds were planted in the greenhouse; 32 of these seeds germinated. Only 25 seedlings developed well whereas the others died during early stages of plant development. All 25 seedlings were tested by PCR for the presence of the *nptII* gene and the *MdTFL1-1* hairpin gene construct. Both transgenes could be detected in 16 out of the 25 seedlings. The transcription of the transgenes was tested by RT-PCR. Both transgenes were transcribed in all transgenic seedlings as expected. An example of this investigation is shown in Figure S2 available as Supplementary Data at *Tree Physiology* Online. All

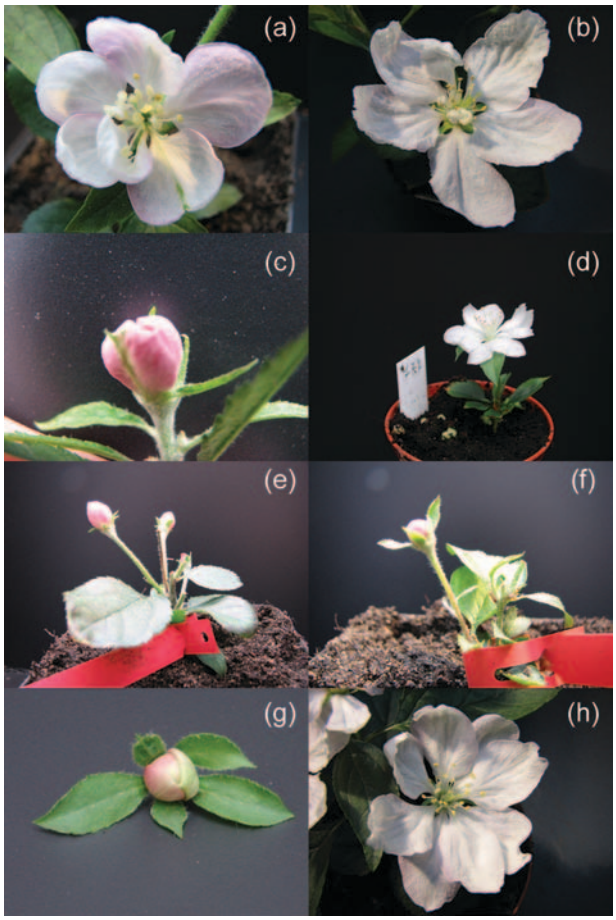

Figure 6. Frequent abnormalities found on flowers of transgenic apple plants. (a) No styluses have been developed. (a, b) Individual anthers grew like petals. (c) Sepals were acuminate and elongated. (d, h) The number of petals was increased. (e) Sepals were stunted. (f, g) Sepals were changed to leaves. (g) The trichome development on petals was increased.

16 transgenic seedlings developed solitary, terminal flowers within a few weeks. Afterwards they stopped growing vegetatively. These seedlings were evaluated on vegetative growth for two consecutive years. Surprisingly, the transgenic seedlings remained very small in comparison with non-transgenic seedlings (Figure 8, Table 3). They developed only a few leaves and a greater number of flowers. The shoot growth was almost completely inhibited.

Grafting experiments

In order to test whether the silencing effect of *MdTFL1-1* dsRNAi is graft-transmissible, transgenic plants of T755, T759, T760 and T776 were used as rootstocks and grafted with scions of the non-transformed 'PinS'. The non-transgenic scions were top-grafted and grown as first-order shoots. Lateral shoots which developed from the transgenic rootstocks were grown as second-order shoots. All four grafted plants were grown in the greenhouse under long-day conditions with 16 h light and 8 h darkness. Within 14 months after grafting, all shoots (non-transgenic first-order shoots and transgenic second-order shoots) were evaluated on different morphological traits such as growth habit, leaf morphology and flower development. The growth of the transgenic second-order shoots was reduced as seen for the shoot growth of the transgenic, non-grafted control plants. The leaves of transgenic shoots were also smaller and rounder and all transgenic shoots flowered within 2–3 months after grafting. No flowers developed on the non-transgenic top-grafted scions. The non-transgenic scions showed a normal habit and normal leaf shape as wild-type plants (Figure 9). No differences were found between non-transgenic scions grafted onto *MdTFL1*-silenced plants and plants of the non-transgenic control plants grown on their own roots. Our preliminary results indicate that the effect originating from RNAi-based silencing of *MdTFL1* in transgenic rootstocks is not graft-transmissible in apple.

Discussion

The *TERMINAL FLOWER 1* (*TFL1*) gene is known to represses flowering in *A. thaliana*. This gene extends the vegetative growth stage and maintains the indeterminate nature of the inflorescence (Shannon and Meeks-Wagner 1991, Alvarez et al. 1992, Bradley et al. 1997, Boss et al. 2004). The *TFL1* gene prevents the expression of *LEAFY* (*LFY*) and *APETALA1* (*AP1*)/*CAULIFLOWER* (*CAL*), key genes which are known to be involved in flower induction (Bradley et al. 1997, Ratcliffe et al. 1998, Boss et al. 2004). Mutants of *A. thaliana* and *Pisum sativum* L. which are defective in *TFL1* or its homologous genes are significantly earlier in flowering (Bradley et al. 1997, Ohshima et al. 1997, Foucher et al. 2003). In apple, the presence of a *TFL1*-like mRNA was firstly described by Kotoda et al. (2003). Based on results obtained by Southern hybridization, Kotoda et al. (2003) assumed the presence of at least two *TFL1*-like genes in the apple genome. The assumption was confirmed by Esumi et al. (2006) and Hättasch et al. (2008). Esumi et al. (2006) isolated two different *MdTFL1*-like mRNA sequences from apple indicating the presence of two different genes. Genomic sequences of these two genes were subsequently isolated by Hättasch et al. (2008). Based on the nucleic acid sequence identity the genes were designated as *MdTFL1-1* and *MdTFL1-2* (respectively, *MdTFL1* and *MdTFL1a*). Based on genetic mapping it was shown that *MdTFL1-1* is located on chromosome 12 (LG12) and *MdTFL1-2* on chromosome 14 (LG14) of the apple genome (Mimida et al. 2009, Guitton et al. 2012). The mRNA expression pattern of *MdTFL1* was studied by Kotoda and Wada (2005). In apical meristems the *MdTFL1* mRNA was expressed strongly in early July, about 2 weeks before floral bud differentiation. A gradual decrease was detectable from early to late July. In contrast, an increase in the expression of the apple *FLO/LFY* ortholog *AFL1* was detected when the expression level of the *MdTFL1* genes was

Table 2. Frequent abnormalities of flowers of *MdTFL1* dsRNAi transgenic apple plants grown in the greenhouse

T-clone	Petals per flower	Sepals per flower	Anthers per flower	Styluses per flower	Flowers with individual abnormal flower organs									*n*
					A	B	C	D	E	F	G	H	I	
'PinS'														
T751	4.8 ± 0.4	11.6 ± 6.0	21.6 ± 4.4	2.0 ± 2.3	3						1	2	5	5
T752	5.0 ± 0.0	8.3 ± 4.9	19.0 ± 2.6	3.0 ± 2.6	2		2	1				1	2	3
T753	5.1 ± 0.7	7.0 ± 1.9	18.0 ± 4.2	3.3 ± 2.5	5	1		2	6	1	1	3	10	12
T754	5.5 ± 0.7	13.0 ± 4.2	23.0 ± 1.4	0	2	1						2	2	2
T755	5.0 ± 0.0	8.3 ± 2.1	18.7 ± 1.5	1.7 ± 2.9	1			1			1	2	3	3
T757	4.5 ± 0.7	8.0 ± 1.4	15 ± 4.2	0		1						2	2	2
T758	5.8 ± 2.0	5.7 ± 1.2	20.4 ± 4.8	3.7 ± 2.0	4	1	2	1	1	3	2		4	9
T759	5.0 ± 0.3	5.9 ± 1.3	18.9 ± 1.4	4.3 ± 1.3	10		10		1		2	1	9	21
T760	1	17	21	1									1	1
T761	5.0 ± 0.0	6.0 ± 0.9	16.7 ± 3.0	4.0 ± 2.0	2			2	4			1	4	6
T762	5.1 ± 0.7	7.4 ± 2.1	18.7 ± 5.0	5.6 ± 1.3	3	1	2				1		5	7
T772	4.0 ± 2.2	5.4 ± 0.9	16.4 ± 7.8	5.8 ± 0.8	3		3				1		1	5
T773	5.3 ± 0.6	8.7 ± 4.0	16.7 ± 3.8	2.3 ± 2.1	2				2	1	1	1	2	3
T774	0	11	9	0								1	1	1
T775	5.4 ± 0.7	6.5 ± 2.6	21.6 ± 5.0	4.5 ± 2.5	4	1		1	3		1	2	8	13
T776	5.4 ± 0.9	6.9 ± 2.1	22.9 ± 3.7	4.2 ± 2.7	4	2	5			1		2	9	13
T777	5.1 ± 0.3	6.6 ± 2.6	19.6 ± 4.5	3.0 ± 3.0	8	2	2	1	1			5	6	11
'Galaxy'														
T778	4.6 ± 0.8	6.9 ± 1.7	14.1 ± 3.4	1.6 ± 1.8	7	1		4	2	2	4	6	10	14
Total					60	11	26	13	20	8	15	31	84	131

Frequent abnormalities found on transgenic flowers: A, individual anthers grow like petals; B, sepals and petals were conjoint; C, sepals were acuminate and long; D, sepals were changed to leaves; E, sepals were stunted; F, trichome development on petals and/or the basal part of styluses; G, enlarged sepals; H, no styluses developed; I, increased number of petals; *n*, number of plants per T-clone; 748 (HC), 749 (P) and 750 (P), 756 (P) no flowers; other abnormalities: styluses were conjoint, anthers and styluses were conjoint, anthers malformed and/or conjoint.

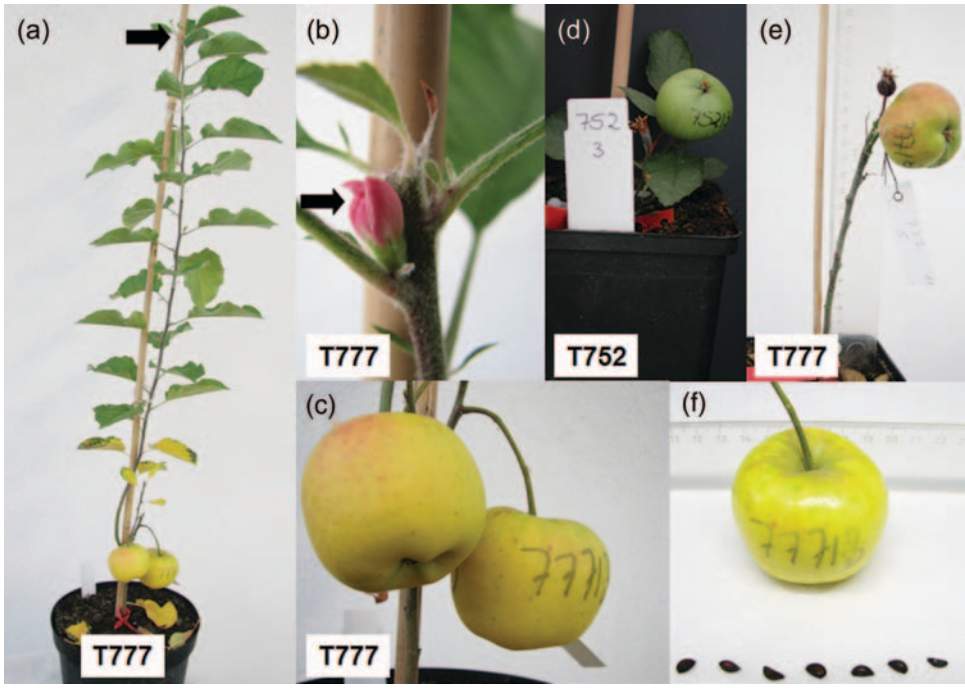

Figure 7. Pollination experiments with transgenic greenhouse plants. (a) Transgenic plant of clone T777 with two apple fruits and a flower developed in the leaf axils next to the shoot apex of the new main shoot. (b, c) enlarged sections of (a). After pollination, the growth of the main shoot stopped and a side-shoot developed as the new first-order shoot that formed a new axillary flower (black arrow figures a + b). (d, e) Small transgenic apple plants with fruits. (f) Transgenic apple fruit with fertile seeds.

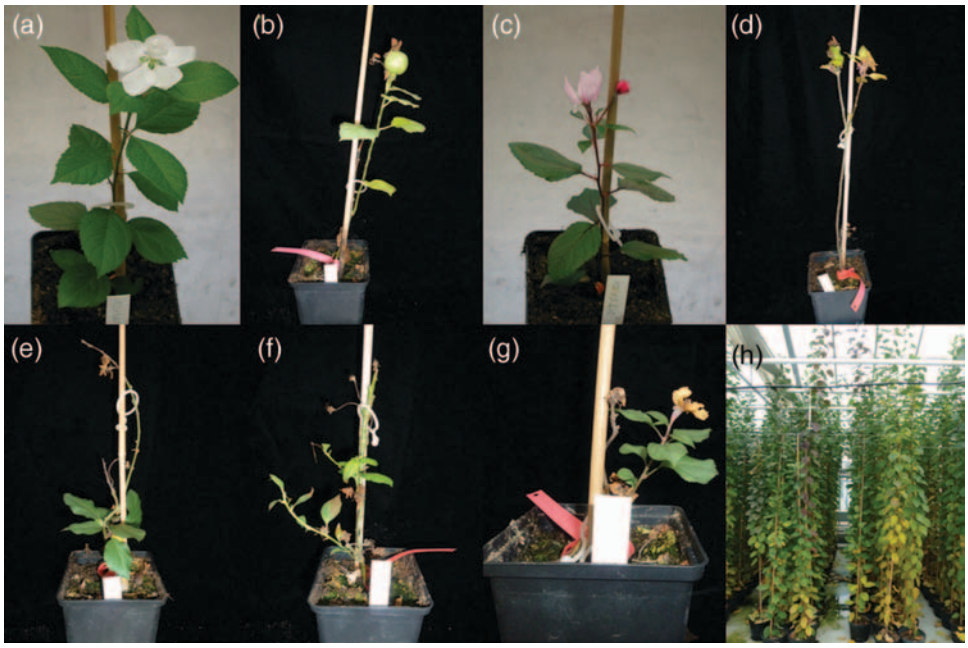

Figure 8. Transgenic seedlings grown in the greenhouse. (a) Transgenic seedling 10136/3 4 months after seed planting. (b) Transgenic seedling 10136/3 after 2 years of greenhouse cultivation. (c) Transgenic seedling 10131/1 4 months after seed planting. (d) Transgenic seedling 10131/1 after 2 years of greenhouse cultivation. (e–g) Transgenic seedlings 10135/4, 10136/4 and 10132/4 after 2 years of greenhouse cultivation. (h) Non-transgenic apple seedlings after 10 months of greenhouse cultivation.

Table 3. Evaluation of transgenic seedlings after 2 years of greenhouse cultivation

Name of seedling	Shoot length in cm	Shoot diameter in mm
10131/1	14	3
10131/2	10	2
10131/3	37	5
10131/4	60	5
10132/4	30	4
10133/1	69	5
10133/3	91	5
10135/1	49	5
10135/3	38	6
10135/4	34	5
10136/2	88	8
10136/3	22	4
10136/4	28	3
Non-transgenic[1]	223.8 ± 24.2	11.1 ± 0.9

[1]Mean of the 10 seedlings 14 months after seed planting.

relatively low (Kotoda and Wada 2005). Very similar results were obtained by Hättasch et al. (2008) and Mimida et al. (2009). Hättasch et al. (2008) studied the expression of *MdTFL1-1* and *MdTFL1-2* in terminal buds in lateral positions on current-year shoots, which preferentially become generative and develop flowers for the next season. They found relatively low mRNA levels of both genes between late May and early to mid June just before floral induction takes place in Germany. Mimida et al. (2009) studied the mRNA expression of both *MdTFL1* genes in succulent shoot apices and in bearing shoot apices as well. In succulent shoot apices both genes were expressed throughout the growing season. In bearing shoot apices a decrease in *MdTFL1-1* and *MdTFL1-2* expression was detected in late June immediately before floral transition takes place in Japan. Using in situ hybridization Mimida et al. (2011c) were able to confirm the decrease of *MdTFL1* gene expression in floral buds. Possibly, the low expression of both *MdTFL1* genes at this time allows the accumulation of transcripts of floral promoter genes, resulting in flower initiation (Hättasch et al. 2008). Based on these results and on results obtained from different flower-inducing genes such as *MdSOC1*, *MdMADS2*, *AFL1* and *AFL2* (Hättasch et al. 2008), it is assumed that the decrease in MdTFL1 expression followed by an increase in expression of different floral promoter genes marks the onset of flower induction. All results obtained by Kotoda et al. (2003, 2006), Kotoda and Wada (2005), Hättasch et al. (2008) and Mimida et al. (2009, 2011c) argue for the assumption that the *MdTFL1* genes may act as floral repressors in apple. The final proof was adduced by Kotoda et al. (2006) who showed that the overexpression of an antisense mRNA of *MdTFL1* (AB052994) resulted in early flowering. First solitary flowers were detected on transgenic scions of the apple cultivar 'Orin' 8 months after grafting.

In the present study, the *MdTFL1* genes of apple were silenced by an RNAi-based approach. A hairpin gene construct containing a 323 bp fragment of *MdTFL1-1* with a high level of sequence identities to other *MdTFL1-1* and *MdTFL1-2* sequences was established. In silico studies suggested that

unspecific gene silencing of non-target genes is not to be expected. The hairpin gene construct was transferred to the genome of three different apple genotypes. Twenty-two putative transgenic clones were selected as the total output of 10 separate transformation experiments. All 22 clones tested positive by PCR and Southern blot analysis. The transformation efficiency ranged between 0% ('Gala') and 1.49% ('PinS'). The mean transformation efficiency (0.52%) of our experiments is comparable with efficiencies described for other transformation experiments on apple (Bulley et al. 2007). Using molecular techniques we were able to show that the *MdTFL1-1* hairpin gene construct was integrated and transcribed in each transgenic clone. The expression of the endogenous *MdTFL1-1* gene was repressed as expected. No expression was detectable for *MdTFL1-2* in in vitro grown shoots. Thus, plants with very low level of *MdTFL1* expression compared with wild-type plants were generated.

Silencing of the *TFL1*-like genes of apple resulted in precocious flowering and reduced vigor. Compared with Kotoda et al. (2006) the effects found in the present study were much stronger. Most transgenic clones started flowering during in vitro cultivation already. Whether it results from the apple genotypes used for transformation or from the RNAi strategy used instead of the antisense technology cannot be stated at the moment. Interestingly, a similar strong acceleration of flowering was recently described after virus-induced gene silencing of *MdTFL1* in apple seedlings (Sasaki et al. 2011). Apple seedlings infected with an *Apple latent spherical virus* (ALSV) vector carrying the partial sequence of *MdTFL1* flowered within 1.5–2 months after inoculation.

However, the fact that similar results were obtained in all three studies on different genetically unrelated apple genotypes argues for a general function of *MdTFL1* genes in apple. The *MdTFL1* genes seem to play a role in (i) floral induction, (ii) floral organ development and (iii) tree architecture. Furthermore, the results obtained on greenhouse-grown plants of transgenic clones and seedlings support the hypothesis that the *MdTFL1* genes are (iv) key regulators of vegetative growth and therefore of particular importance for perenniality in trees. Plants of transgenic clones died directly after flower/fruit development or stopped at least their vegetative growth. Plants that survived the first growing season failed to develop a usual number of foliar leaves in the next season. Nearly all meristems developed flowers (Figure 9a, c, d). Transgenic seedlings stopped their growth after 3–4 months at a mean plant height of ~44 cm. No additional growth took place during the second and third growing season. The plants remained small, sometimes completely without leaves, and died gradually. The vegetative growth of *MdTFL1* dsRNAi apple plants was nearly completely blocked at the cost of plant lifetime. The results obtained in the present study give evidence for the hypothesis of Mimida et al. (2011*b*) who assumed that *MdTFL1* may have only one (main) function

Figure 9. Grafting experiments. (a) *MdTFL1-1* dsRNAi transgenic plants grown on their own roots in the first year of evaluation in the greenhouse. (b) Grafted plant consisting of a *MdTFL1-1* dsRNAi transgenic rootstock and a non-transgenic scion of 'PinS' in the first year of evaluation in the greenhouse. (c) Grafted plant of (b) compared with three *MdTFL1-1* dsRNAi transgenic plants in the second year of evaluation in the greenhouse. (d) Enlarged picture of two transgenic plants in (c). Transgenic plants developed only a few leaves in spring of the second season, a few flowers, and then stopped the vegetative growth.

in apple: maintaining vegetative growth in both the juvenile and adult plant. The expression pattern obtained after in situ hybridization suggested that *MdTFL1* genes seem to control vegetative growth for stem elongation (Mimida et al. 2011*c*). The expression of both *MdTFL1-1* and *MdTFL1-2* is regulated by phytohormones (Mimida et al. 2011*b*). Cytokinin seems to induce the expression of *MdTFL1* genes to maintain juvenility at the base of the plant, whereas the transition of the apical shoot meristem from the vegetative to the generative stage seems to be determined by cytokinin and auxin (Mimida et al. 2011*c*). The cytokinin, which is produced in the apical region of roots, seems to maintain the juvenile stage at the base of the apple plant (Mimida et al. 2011*b*). Auxin is produced in the apical region of young shoots and seems to control the juvenility by regulation of *MdTFL1* expression at the top of the plant (Mimida et al. 2011*b*). Gibberellic acids (GAs) produced in seeds of developing fruits inhibit floral induction in nearby shoot apical meristems (for review see Bangerth 2009). Whether they are

responsible for repression of *MdTFL1* gene expression has not been studied so far. However, an increase in *MdTFL1* gene expression in trees subjected to heavy crop treatment compared with trees subjected to flower thinning, which argues for a role of GAs in regulation of *MdTFL1* expression, has been demonstrated very recently (Kittikorn et al. 2011).

Further support of the hypothesis that the *MdTFL1* genes are mainly responsible for maintaining vegetative growth in apple came very recently from pear *Pyrus communis* L. (Freiman et al. 2011), potato *Solanum tuberosum* L. (Guo et al. 2010), *Arabis alpina* L. (Wang et al. 2011) and *Populus* spp. (Mohamed et al. 2010). Transgenic pear plants with silenced activities of *PcTFL1-1* and *PcTFL1-2* developed solitary flowers from apical and lateral buds, and showed reduced vegetative growth and vigor too (Freiman et al. 2011). Transgenic potato plants overexpressing the *StTFL1* gene produced significantly more tubers, which is also an indication for increased vegetative growth (Guo et al. 2010). In the perennial plant species *A. alpina* it was shown that *AaTFL1*, the *TFL1* homologous gene, prevents flower development of juvenile plants by blocking the expression of the *LFY* homologous gene *AaLFY* in juvenile shoot apical and axillary meristems during vernalization. Secondly, *AaTFL1* blocks flowering of young axillary meristems of flowering plants in order to maintain vegetative meristems in continued growth (Wang et al. 2011). These are crucial conditions for the life cycle of perennials with a distinct juvenile stage and repeated flower development. Furthermore, reduction of *AaTFL1* in *A. alpina* plants resulted in slightly earlier flowering and reduced the age at which *A. alpina* plants responded to flower induction by vernalization (Wang et al. 2011). Age-related changes and accelerated flower development were also found in transgenic poplar trees with overexpressed and silenced activity of the *PopCEN1* gene (Mohamed et al. 2010). Poplar contains only three members (*PoptrCEN1*, *PoptrCEN2* and *PoptrBFT*) of the *TFL1*/*CEN*-like gene family (Mohamed et al. 2010) whereas in apple four different members (*MdTFL1-1*, *MdTFL1-2*, *MdCENa* and *MdCENb*) exist (Mimida et al. 2009). The *PoptrCEN1*/*PoptrCEN2* gene function has a major role in age/size-related maturation and seasonal shoot development. It regulates first onset of flowering and the identity of axillary buds (Mohamed et al. 2010). In contrast to apple where down-regulation of *MdTFL1* resulted in the production of terminal flowers, transgenic poplar trees with reduced activity of *PoptrCEN1*/*PoptrCEN2* developed inflorescences from axillary meristems. The terminal vegetative meristem identity was not altered (Mohamed et al. 2010). Results obtained in poplar and apple suggest a similar, but not identical function of different members of the *TFL1*/*CEN*-like gene family in different tree species.

Graft-transmitted down-regulation of *MdTFL1* gene expression in apple appears tempting, because it would be very useful for breeding. Grafting of non-transformed seedlings from traditional crosses onto a silencing transmitter rootstock to break the juvenile stage would help to reduce the time that is needed for each breeding cycle. Fruits grown on grafted seedlings would be free of any foreign DNA. On this account we tested whether gene silencing of *MdTFL1* is systemically transported in apple and used plants of the transgenic clones as rootstocks. The transgenic rootstocks were grafted with non-transformed juvenile scions of the apple genotype 'PinS'. No flowers were obtained on non-transformed scions within 14 months of evaluation. Based on our preliminary results we assume that the silencing effect of *MdTFL1* is not systemically transported.

Conclusions

The results obtained in the present study clearly demonstrate that the *TFL1* genes of apple play a major role in maintaining the vegetative growth. Silencing of *MdTFL1* gene expression inhibits vegetative growth, accelerates flower development and reduces tree lifetime. Furthermore, it seems that the flower-inducing effect of *MdTFL1* silencing is not graft-transmissible from transgenic rootstocks to non-transgenic scions.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* online.

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Conflict of interest

None declared.

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