RNAi-Silencing of *MdTFL1* Induces Early Flowering in Apple

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Abstract

We used an RNAi based approach to induce post-transcriptional gene silencing of the *MdTFL1* gene in apple in order to reduce the juvenile phase. The MdTFL1 protein is homologous to TFL1 of *A. thaliana* which suppresses the floral meristem identity genes *LFY* and *AP1* and maintains the inflorescence meristem. A binary vector was constructed which contains a constitutively expressed *nptII* gene and a chimeric gene construct encoding for a hairpin RNA homologous to the coding sequence of *MdTFL1*. The vector was used to transform the apple (*Malus domestica* Borkh.) cvs. 'Holsteiner Cox' and 'Gala' via *Agrobacterium tumefaciens*-mediated transformation. Regenerated shoots were proven for transgenity by Southern blot and RT-PCR. Quantitative real time PCR analysis showed that the expression of *MdTFL1* was markedly reduced in transgenic lines compared to non-transformed control plants. The regenerated transgenic plants started to flower six month after the transformation under in vitro conditions. The plants were recently transferred to the greenhouse where they continued to flower.

INTRODUCTION

Efficient breeding of fruit trees such as apple is limited by the long period of juvenility lasting several years. During recent years, many factors controlling the transition period from juvenile to adult stage were identified, mainly using the model plant *Arabidopsis thaliana*. Several genes such as *LEAFY* (*LFY*), *APETALA1* (*AP1*), *TERMINAL FLOWER 1* (*TFL1*), and *FLOWERING LOCUS T* (*FT*), which control flowering time, have been isolated from *Arabidopsis*. The MdTFL1 protein of apple is homologous to TFL1 of *A. thaliana* which suppresses the floral meristem identity genes *LFY* and *AP1* and maintains the inflorescence meristem. In order to clarify the function of *MdTFL1* in apple, the gene was down-regulated in transgenic plants by antisense suppression (Kotoda et al., 2003, 2006). Although *MdTFL1* was not completely suppressed, one of the transgenic plants flowered 8 months after the transfer to the greenhouse, whereas the non-transformed control plants have not flowered in nearly 6 years. No effects on the expression of *AFL1* (*LFY*) and *MdMADS5* (*AP1*) were detected. The authors conclude that both genes were regulated independently from *MdTFL1*. In order to suppress *MdTFL1* more efficiently we used an RNAi-based approach to induce early flowering by down-regulation of *MdTFL1* in apple. A chimeric gene construct encoding for a hairpin RNA (hpRNA) homologous to the coding sequence of *MdTFL1* was used for the transformation of *Malus domestica* cvs. 'Gala' and 'Holsteiner Cox'. Transgenic plants were evaluated for gene integration and expression during in vitro cultivation. Flower initiation and development were studied on in vitro shoots and/or glasshouse-grown plants.

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MATERIAL AND METHODS

Agrobacterium Strain and Binary Vectors

The Gateway technology (Invitrogen, Carlsbad, CA, USA) was used to construct a binary vector for gene silencing by RNA interference. A 317 bp PCR fragment of the *MdTFL1* from a 'Pinova' progeny was amplified 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 primers introduced recombination sites attB1 and attB2 attached at both ends. Cloning into pHELLSGATE12, kindly provided by CSIRO, containing attR1 and attR2 sites, was performed via an intermediate vector with attP sites, pDONR™207 (Invitrogen), in a two step process. PCR, in vitro BP and LR clonase recombination reactions were carried out according to the manufacturer's instructions (Invitrogen). Sequence and orientation of introduced *MdTFL1* fragments were confirmed by sequencing. The fragments are arranged as an inverted repeat downstream the 35S CaMV promoter, resulting in a transgene that produces self-complementary hpRNA in transgenic plants. The vector pHELLSGATE12 carries additionally on its T-DNA an *npt*II gene conferring resistance to kanamycin. The construct was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation.

Plant Transformation and Regeneration

In vitro shoots of *Malus domestica* Borkh. cvs. 'Holsteiner Cox' and 'Gala' were used for transformation. Young leaves were co-cultured with *Agrobacterium tumefaciens* strain EHA105 carrying the above mentioned vector, as described by Szankowski et al. (2003). The regeneration medium consisted of MS salts and vitamins (Murashige and Skoog, 1962), 3% sorbitol, 0.3% Gelrite, and 2.6 µM NAA, 22.1 µM TDZ or 1 µM IBA and 3 µM TDZ for 'Gala' and 'Holsteiner Cox' respectively. pH of all media was adjusted to 5.7. Explants were selected on medium supplemented with 50 mg/l kanamycin. Rooting of the regenerated shoots was induced on MS medium supplemented with 1.5 µM IBA for 'Holsteiner Cox' and 7.3 µM IBA for 'Gala'. The potted plants were acclimatized to greenhouse conditions.

Analysis of Transgenic Plants

Integration of the transferred T-DNA was proven by detection of the *npt*II selection marker gene by Southern blot analysis. The effect of *MdTFL1* hpRNA on the mRNA level of *MdTFL1* was tested via real-time PCR.

DNA was extracted from leaves according to the method of Doyle and Doyle (1990) and used for Southern blot analysis. Twenty micrograms of genomic DNA were restricted with 20 units *Hin*dIII at 37°C over night followed by the addition of another 10 units of the enzyme. After 4h the DNA was fractionated in a 1% agarose gel and blotted on a positively charged nylon membrane (Roche, Mannheim, Germany). Membranes were hybridized with the digoxygenin-labelled PCR probe for *nptII*, incubated with alkaline phosphate conjugated antidigoxygenin followed by the chemiluminescent substrat CDP-Star and finally exposed to a chemiluminescent detection film (Roche, Mannheim, Germany). All steps were performed following the supplier's instructions (Roche). For *npt*II probe generation by PCR the primers 5'- CCA CAG TCG ATG AAT CCA GA-3' and 5'- AGC ACG TAC TCG GAT GGA AG -3', which amplify a 200 bp fragment, were used.

RNA isolation was performed using the *Plant RNA Reagent* (Invitrogen) according the manufacturer's instruction. cDNA synthesis and quantitative real time PCR was performed as described by Li et al. (2007).

RESULTS AND DISCUSSION

There are two ways to induce early flowering by transgenic approaches. One is the constitutive expression of genes that promote flowering, such as *LFY*, *AP1*, and *FT*, while

the other is the suppression of genes that delay flowering, such as *TFL1*.

We used an RNAi based approach to suppress *MdTFL1* expression in apple. For this purpose a fragment of the gene was cloned into a binary vector to produce double-stranded hairpin-like RNA molecules. After *Agrobacterium tumefaciens* mediated transformation, one putative 'Gala' line and one putative 'Holsteiner Cox' line regenerated on kanamycin selective medium. Southern blot and RT-PCR analyses confirmed the integration and expression of the *npt*II gene in the regenerated lines (Fig. 1a and b). Multiple copies were detected in both the transgenic 'Gala' line and the transgenic 'Holsteiner Cox' line (Fig. 1a). Quantitative Real time PCR analysis revealed that the expression of the *MdTFL1* gene as well as *MdFT* gene is suppressed in both lines (Fig. 1c). Six months after the initial transformation, the *MdTFL1* suppressed 'Holsteiner Cox' line flowered under in vitro conditions. Plants were rooted and transferred to the greenhouse, where they continued to flower. The transgenic greenhouse grown plants showed reduced growth compared to untransformed control plants (Fig. 2). Some of the flowers had an aberrant phenotype as some anthers were malformed. The plants of the transgenic 'Gala' neither flowered under in vitro conditions nor in the greenhouse until now.

This study confirms that *MdTFL1* is involved in the maintenance of juvenility in apple and suppression of the expression leads to early flowering.

CONCLUSIONS

RNAi-mediated posttranscriptional gene silencing of *MdTFL1* in apple resulted in early flowering in apple. The shortening of the juvenile period by using this technique can accelerate breeding processes.

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Figures

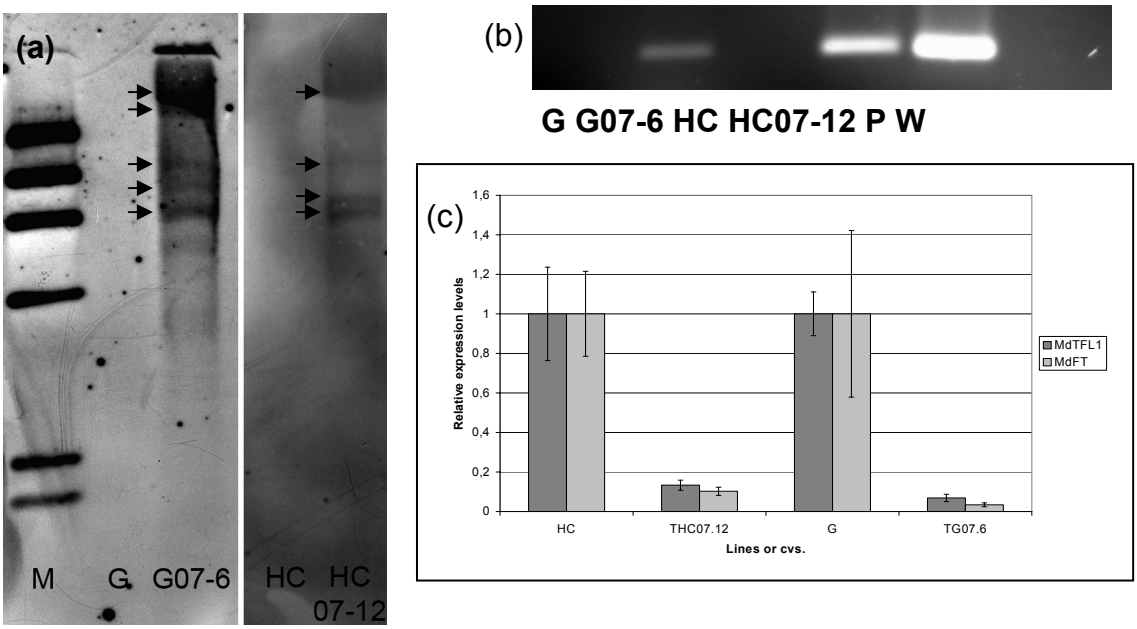

Fig. 1. Molecular analyses of transgenic 'Holsteiner Cox' and 'Gala' plants. a) Southern blot analysis using an *nptII* specific probe. b) RT-PCR products of *nptII* mRNA (cDNA). c) Expression levels of *MdTFL1* and *MdFT* determined by quantitative real-time PCR. The values are expressed in comparison to the transcript levels in non-transformed 'Gala' and 'Holsteiner Cox'. Bars represent standard deviation. *M* molecular weight marker II Dig labelled (Roche), *G* untransformed 'Gala', *HC* untransformed 'Holsteiner Cox', *G07-6* transgenic 'Gala' line, *HC07-12* transgenic 'Holsteiner Cox' line, *P* plasmid, *w* blank.

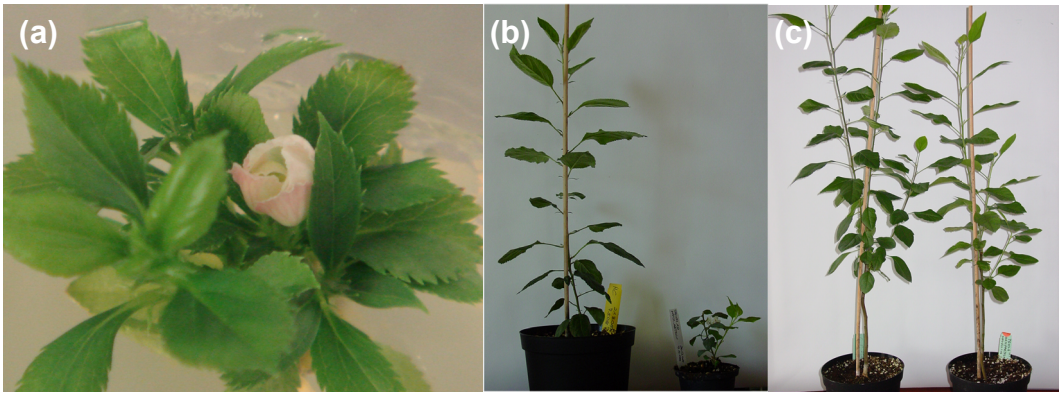

Fig. 2. Development of flowers of shoots of transgenic, *MdTFL1* silenced 'Holsteiner Cox' plants and phenotypical comparison of glasshouse plants of transgenic and non-transformed plants of 'Gala' and 'Holsteiner Cox'. a) Flowers of in vitro shoots of transgenic 'Holsteiner Cox' were obtained 6 month after the initial transformation. b) The growth of the transgenic, flowering 'HC' line was reduced in comparison to the untransformed control, while c) the growth of the transgenic 'Gala' line (not flowering so far) appeared to be normal.