# RNAi- SILENCING OF *MdTFL1* INDUCES EARLY FLOWERING IN APPLE (*Malus domestica* Borkh.)

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## ABSTRACT

The present study was carried out in the institute of Biological Production Systems, Fruit Science Section, Leibniz University Hannover, Germany during post doctoral scholarship of the first auther 2007 . 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.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. 'Elstar' and 'Gala' *via Agrobacterium tumefaciens*-mediated transformation. Regenerated shoots were proven for transgenity by PCR, 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. Some of the plants started to flower six month after the transformation under *in vitro* conditions. The plants were transferred to the greenhouse where they continued to flower.

**Keywords:** *Agrobacterium, Malus*, transformation, *in vitro* flowering

## 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 nontransformed 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*. The antisense approach did not silence *MdTFL1* completely (Kotoda *et al.* 2006). Therefore 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 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.

## 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 primer 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, pDONRTM207 (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 CaMV promoter, resulting in a transgene that produces self-complementary hpRNA in transgenic plants. The vector pHELLSGATE12 carries additionally on its T-DNA an nptII 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 cocultured with *Agrobacterium tumefaciens* strain EHA105 carrying the above mentioned vector, as described by Szankowski *et al.* (2003). The regeneration medium for 'Gala' consisted of MS salts and vitamins (Murashige and Skoog 1962), 3% sorbitol, 2.6 µM NAA, 22.1 µM TDZ and 0.3% gelrite. 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**

For the detection of transgenes in regenerated plants, DNA was extracted from leaves according to the method of Doyle and Doyle (1990) and tested by PCR and Southern blot analysis. Since the *MdTFL1* sequence is present in the *Malus* genome, the transgenity was proven by detection of the *nptII* marker gene. The presence of the *nptII* gene in pHELLSGATE::TFL1 transgenic plants was confirmed by using 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. The PCR reaction conditions were 95°C for 3 min, followed by 35 cycles at 94°C for 90 s, 60°C for 90 s, 72°C 150 s and with a final extension at 72°C for 10 min.

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 the 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 month 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.

## ACKNOWLEDGEMENTS

Alaa El-Din Saad Omar wishes to express his sincere gratitude to the Egyptian government for the financial support. Sascha Waidmann thanks Stiftung Gisela for financial support.

Figures

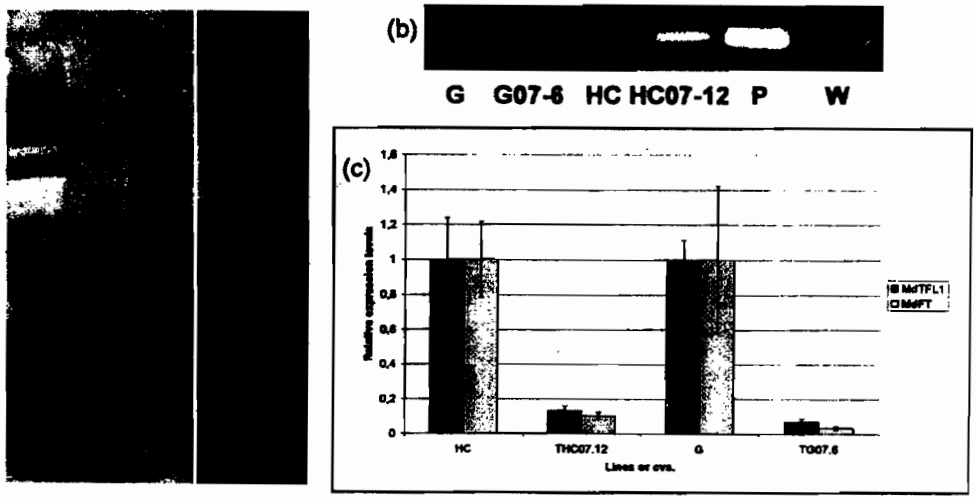


Figure 1: Molecular analyses of transgenic 'Holsteiner Cox' and 'Gala' plants. a) Southern blot analysis using an nptII specific probe. M molecular weight marker II Dig labelled (Roche), G untransformed 'Gala', HC untransformed 'Holsteiner Cox', G07-6 transgenic 'Gala' line, HC07-12 transgenic 'Holsteiner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

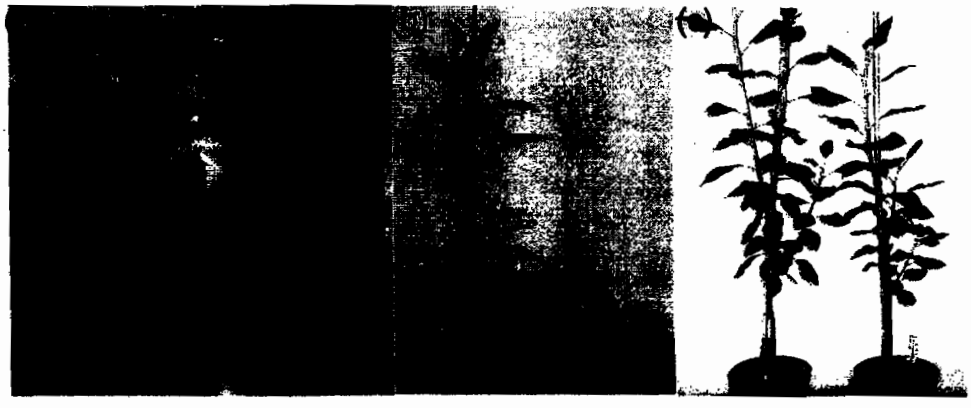

**Figure 2:** Development of flowers of shoots of transgenic, *MdTFL1* silenced 'Holsteiner Cox' plants and phenotypical comparison of glasshouse plants of transgenic and non-transformed plants of 'Gala' and 'Holsteiner Cox'. a) Flowers of in vitro shoots of transgenic 'Holsteiner Cox' were obtained 6 month after the initial transformation. b and c) The growth of the transgenic, flowering 'HC' line was reduced in comparison to the untransformed control, while the growth of the transgenic 'Gala' line (not flowering so far) appeared to be normal. *HC* untransformed 'Holsteiner Cox', *G07-6* transgenic 'Gala' line, *HC07-12* transgenic 'Holsteiner Cox' line.

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## الملخص العربي

# إنتاج نباتات تفاح مبكرة التزهير عن طريق استخدام تقنية RNAi

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أجريت هذه الدراسه بمعهد نظم إلانتاج البيولوجية – قسم علوم الفاكهة– جامعة هانوفر– المانيا – خلال المهمه العلميه للباحث الاول عام ٢٠٠٧. تعتبر كفاءة عمليات التربية في أشجار الفاكهة (مثل التفاح) محدودة لارتباطها بطول فترة الطفولة والتي تحتاج إلي سنوات عديدة ، لكن خلال السنوات الأخيرة تم تحديد عديد من العوامل التي تتحكم في الفترة الانتقالية من مرحلة الطفولة إلي مرحلة البلوغ من خلال استخدام نباتات *Arabidopsis thaliana* (كنبات مستخدم في تجارب النقل الجيني او الهندسة الوراثية) حيث تم عزل مجموعه من الجينات من هذا النبات والتي تتحكم في عملية التزهير مثل *LEAFY* (*LFY*), *APETALA1* (*AP1*), *TERMINAL FLOWER 1* (*TFL1*), *FLOWERING LOCUS T* (*FT*), وقد استخدم في هذه الدراسه تقنية RNAi(RNA interference) بهدف استحداث الصمت الجيني ما بعد النسخ لجين *MdTFL1*في التفاح لتقصير فترة الطفولة. ولهذا الهدف تم تركيب الناقل المزدوج (Binary vector) الذي يحتوي علي جين مقاوم للكناميسين (جين كاشف) وذلك بالإضافة إلي جزي RNA والمماثل للتتابع المشفر في جين ( *MdTFL1* ) في التفاح ( *Malus domestica* Borkh ) صنفي الالستر وجالا(Elstar & Gala) عن طريق النقل الجيني باستخدام الاجروباكتريم (*Agrobacterium tumefaciens*). وقدا ثبت التحليل الوراثي باستخدام تكنيكات PCR ، Southern blot للنباتات الناتجة أنها تحتوي علي جين *MdTFL1* – كما اظهر التحليل الكمي (RT-PCR) ان التعبير الجيني للجين*MdTFL1* قد انخفض بصورة واضحة في هذه النباتات الناتجة ( محولة أو مهندسة وراثيا) مقارنة بنباتات الكنترول ( غير المحولة أو غير المهندسة وراثيا) . وقد بدأت بعض النباتات الناتجة في التزهير بعد ٦ شهور من النقل الجيني تحت الظروف المعملية ، ونقلت باقي النباتات الي الصوب لاستكمال نموها ووصولها الي مرحلة الازهار.