FINAL PROJECT REPORT

Project Title: Functional genomics of flowering in apple

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Other funding Sources

Agency Name: MSU Agricultural Experiment Station /Project GREEEN
Amount requested or awarded: 30,000

Total project funding: $96,319

Budget History:
Organization Name: Cornell University

Item	2007	2008	
Salaries	14,500	15,370	
Benefits	7,434	8,034	
Wages			
Benefits			
Equipment			
Supplies	3,000	1,000	
Travel			
Miscellaneous			
Total	24,934	24,404	

Footnotes: The salary and benefits were for a technician to work 50% time on transferring silencing constructs into apple. Supplies are for tissue culture, chemicals, enzymes, plastic ware, and potting supplies.

Budget 2:
Organization Name: Michigan State University

Item	2007	2008	
Salaries	12,854	13,240	
Benefits	885	912	
Wages			
Benefits			
Equipment			
Supplies	5,000	3,390	
Travel	600	600	
Miscellaneous	9,500		
Total	28,839	18,142	

The salary and benefits requested were for 1/2 grad student for first two years. Note that for the grad student, this amount includes tuition/fees ($4215) and stipend ($8640). Supplies were for molecular studies, and include enzymes, primers, and reagents. Expenses for travel were for one trip/year in years 1 and 2 from Michigan to New York.
Miscellaneous costs were for DNA sequencing of genes related to flowering.

Objectives:

1. Genomic census of *FT/TFL* gene family members in apple. We will identify all possible *FT/TFL* gene family members in apple, and determine their DNA sequence. As mentioned above, from previous studies we know that at least five exist in apple. This step is important, because additional genes may exist with even more important functions.

2. Gene expression atlas of the apple *FT/TFL* genes. We will extensively analyze the activity pattern of all *FT/TFL* genes identified through Objective 1, concentrating on flowering. This analysis will include expression in various parts and organs of the plant, changes in expression in response to phytohormones, effects of biotic and abiotic stresses on expression, and temporal control of expression during development (e.g. flower induction). The goal of this approach is to identify those members of the *FT/TFL* family that have the most important role in flowering.

3. Functional analysis. The best way to unambiguously determine the function of a gene is to examine the phenotypic consequences of loss of that gene's activity. In other words, how does flowering occur without that gene? In apple, genes can be repressed through a technique called RNA interference (RNAi), which PI Aldwinckle has several years' experience with other apple genes. We will examine the effect on flowering of suppression of the most important genes identified in Objective 1 and 2.

Summary of Significant Findings:

1. Genomic census of *FT/TFL* gene family members in apple.

- We used a novel molecular technique to isolate and sequence genomic DNAs corresponding to developmentally regulated genes from flower bud and leaf tissues (S Park, S Oh, S Mookerjee and S van Nocker, manuscript in preparation). This resulted in the acquisition of ~108,000 (flower bud) and ~226,000 (leaf) new apple genomic sequences representing ~28 million nucleotides of the apple genome and corresponding to nearly all apple genes previously identified by expressed sequence tag (EST) analysis (accession: http://vannocke.hrt.msu.edu/public/0822.fna; http://vannocke.hrt.msu.edu/public/1120.fna). Additional funding for this objective (one month postdoc salary equivalent) was leveraged through Michigan State University. This effort provided the genomic sequence for *FT/TFL* family members that was required to design gene-specific primers used in Objective 2 and construct the plant transformation vectors utilized in Objective 3. Based on this sequence we constructed a revised phylogenetic tree representing the apple *TFL/FT* gene family (Fig. 1).

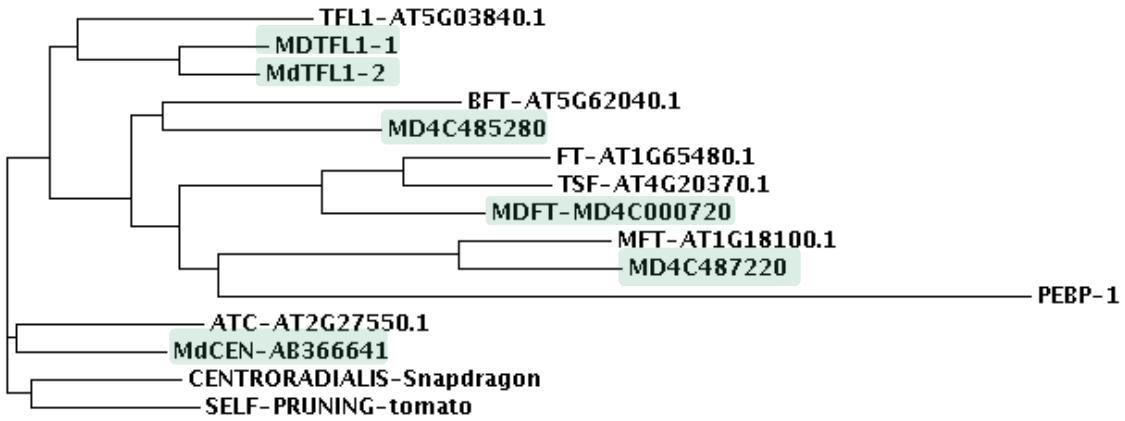

Fig 1. Phylogenetic tree representing the *TFL1*/*FT g*ene family from Arabidopsis, along with the homologs from apple, snapdragon, tomato, and human.

2. Gene expression atlas of the apple *FT/TFL* genes.

- *MdTFL1-1* and *MdTFL1-2* expression was detected only in the root, stem and apex of seedlings. *MdFT* expression was detected in the root, stem, apex and leaf of seedlings, and also in the flower bud and fruit. *MdBFT* was expressed in the root and stem of the seedling, and also in developing fruit. *MdMFT* was expressed most strongly in the seed (Fig. 2A). *MdCEN* had a strong level of expression in the root but was also detected in the vegetative tissues (stem, apex, and leaf) (Fig. 2B) (See Appendix A for sample information).

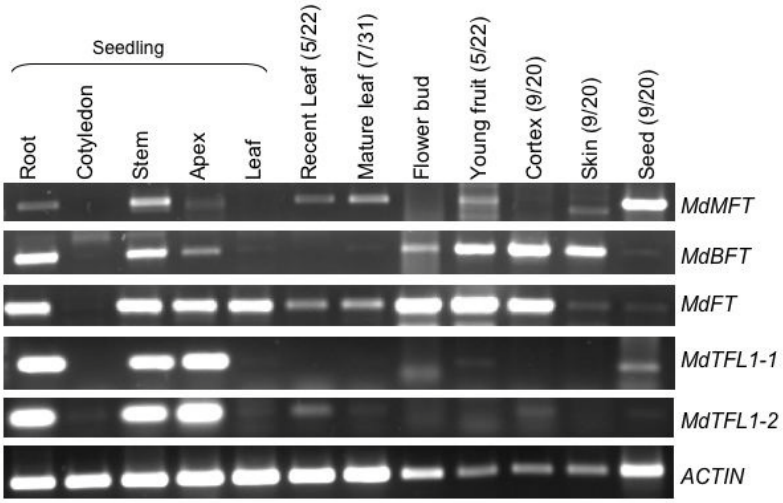

Fig 2A. Expression patterns for *MdTFL1-1*, *MdTFL1-2*, *MdFT*, *MdMFT*, and *MdBFT* in various parts of apple.

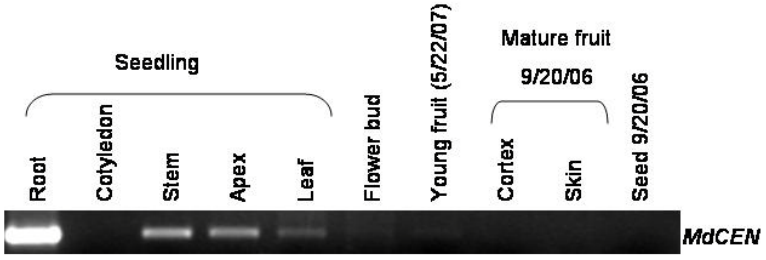

Fig 2B. Expression pattern of *MdCEN* in various apple tissues and parts.

- We then analyzed expression of *FT/TFL1* gene family members in various parts and organs of the mature flower (Fig 3). Although *MdMFT* expression was not detected in immature flowers (Fig. 2A) it was found in the style at the time of anthesis. *MdBFT* expression was mainly localized to the flower pedicel. *MdFT* was detected in all parts of the flower analyzed (Fig. 3).

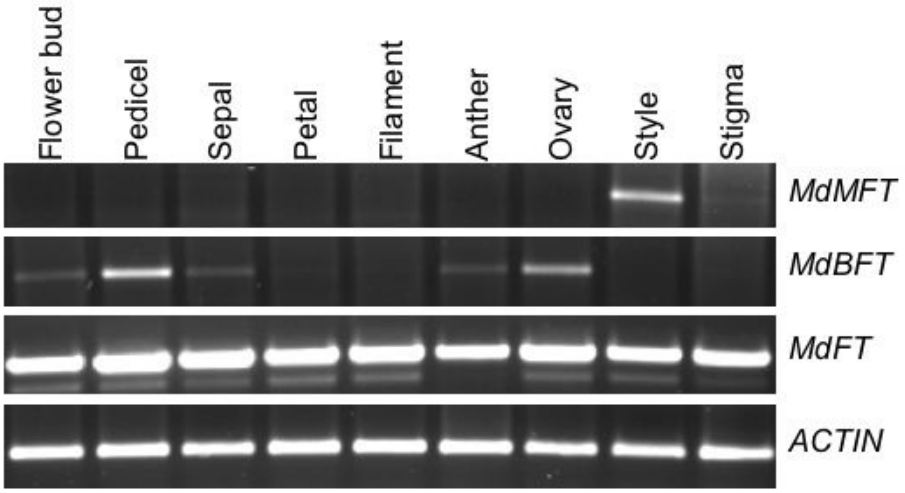

Fig 3. Expression pattern of *MdMFT*, *MdBFT*, and *MdFT* in different parts of the mature flower.

- We also analyzed expression of those genes expressed in the fruit (*MdFT* and *MdBFT*; Fig. 2A) at various stages of fruit development and in various parts of the fruit (Fig 4). *MdBFT* was expressed in all parts of the fruit and in all developmental stages tested, with relatively higher expression in the cortex. *MdFT* was detected in both the fruit skin and cortex in the early stages and only in the cortex in the later stages of development.

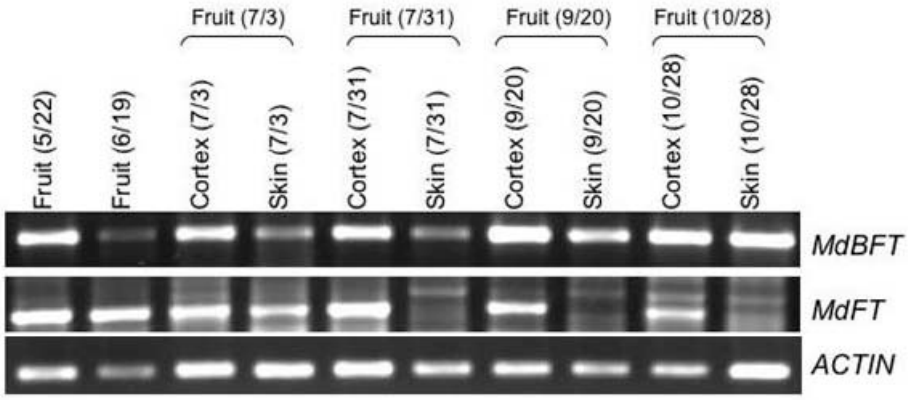

Fig 4. Expression of *MdBFT* and *MdFT* in apple fruit tissue at different stages of development.

- Because *MdMFT* and *MdTFL1-1* were detected in seed tissues (Fig 2A), we analyzed expression of these genes at different stages of seed development (Fig 5). *MdMFT* expression was strongest in seeds in green fruit, but was detected at very early stage and in ripe fruit. *MdTFL1-1* was expressed almost exclusively at an early developmental stage (Fig. 5).

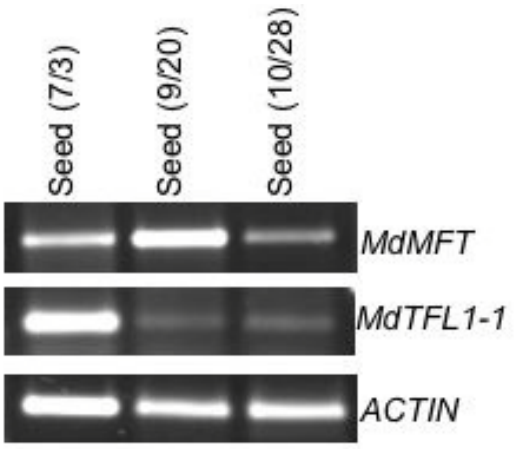

Fig 5. Expression of *MdMFT* and *MdTFL1-1* in different stages of seed development.

- Finally, we analyzed the expression of the two apple *TFL1* homologs (*MdTFL1-1* and *MdTFL1-2*) in flowering-committed shoot apices during floral initiation and development (Fig. 6). Interestingly, *MdTFL1-1* was expressed at increasingly levels during the period of floral initiation, with strongest expression at weeks 12 to 14 AFB concomitant with strong expression of the flowering initiator gene *AFL1* (the apple homology of *LFY)*. After this point, it was silenced, with expression reestablished at week 20 and beyond. *MdTFL1-2* was expressed constitutively throughout the season (Fig. 6).

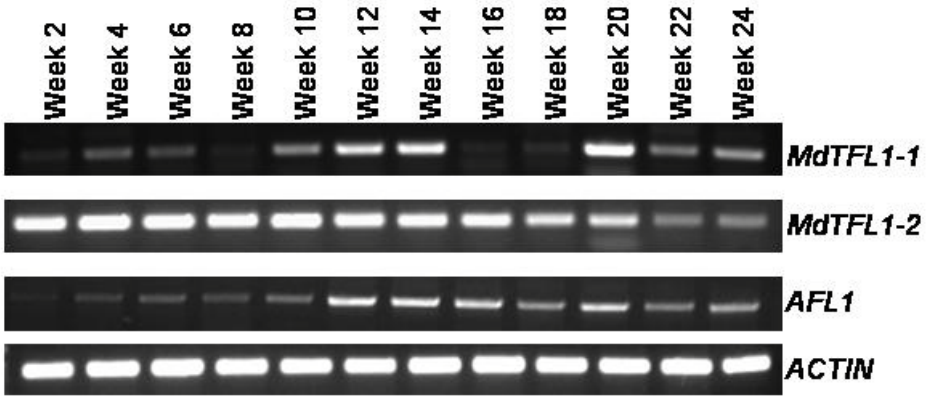

Fig 6. Expression patterns of *MdTFL1-1*, *MdTFL1-2*, and *AFL1* in 'Gala' buds collected from week 2-24 after full bloom.

3. Functional analysis

- **Artificial miRNA (amiRNA) technology was used to design amiRNAs targeting *MdTFL1-1*, *MdTFL1-2*, or both *MdTFL1-1* and *1-2*, while minimizing the risk of suppressing off-targets. This means that these genes can be silenced individually and their distict roles dissected. Initial transformation experiments with the amiRNA silencing constructs for TFL1-2 were done in Aldwinckle's lab, and transformed plants are now being selected.** Transformation experiments with 6 amiRNA silencing constructs for *MdTFL1-1*, *MdTFL1-2*, or both *MdTFL1-1* and *1-2*, were done in Aldwinckle's lab, and multiple transformed plants of Gala variety were produced (Table 2).

Table 2. Transformed lines of Gala apple obtained with 6 amiRNA silencing constructs for *MdTFL1* genes

Silencing Construct	# transformed lines of Gala
TFLa::2	35
TFLa::10	22
TFLa::21	10
TFLa::51	25
TFLa::55	10

The transformed lines have been confirmed as transformed by PCR. They have been propagated in tissue culture and observed for morphological differences from non-transformed Gala. This far no differences have been observed. Selected lines will be further propagated and grown as own-rooted or grafted plants to determine whether flowering time is affected by silencing the two genes.

Recent results from other labs have shown limited response by Gala to silencing of TFL1, but earlier resonse in other varieties. Therefore we have now done transformation experiments with the amiRNA silencing constructs on M.26 apple rootstock, which is also readily transformed (Table 3).

Table 3. Putatively transformed lines of M.26 apple rootstock obtained with 3 amiRNA silencing constructs for *MdTFL1* genes

Silencing Construct	# regenerated lines of M.26
TFLa::2	49
TFLa::10	20
TFLa::21	regenerants now appearing

Since we get very few escapes among the regenerants on the highy selective regeneration medium, most of the regenerated lines are very probably transformed with the silencing constructs. The regenerated shoots are small (2-10 mm) at this time, and too young to exhibit possible morphological differences. They will be grown and propagated in tissue culture and subsequently grown as plants for observation of any alteration in flowering onset. Experiments will also be done with the other 3 amiRNA silencing constructs on M.26.

Discussion:

Genomic census of *FT/TFL* gene family members in apple.

We used a novel gene identification technique that appears to be very efficient in identifying gene regions within stretches of genomic DNA. This effort characterized the majority of transcribed gene space in the apple genome, and more than tripled the amount of publicly available sequence data for apple. Based on this we probably have identified all of the *FT/TFL* related genes in this apple. Our phylogenetic analysis provides clues for function (based on relationship with genes of known function) and suggest that *TFL1-1* and *TFL1-2* are the only authentic homologs of *TFL1*. Apple apparently has only one homolog of the *FT* gene, which is duplicated in Arabidopsis.

Gene expression atlas of the apple *FT/TFL* genes..

Results of Objective 2 are exciting for a number of reasons:

1. This is the first comprehensive analysis of this family of genes in apple. Based on widespread expression in non-reproductive tissues, the activity of these genes is probably not limited to flowering regulation, but probably extends to other developmental events.

2. The *MdBFT* gene likely has a function in the developing fruit, and should be further analyzed as a potential determinant of fruit quality.
3. Interestingly, both *MdMFT* and *MdTFL1-1* are strongly expressed in developing seeds. Potentially, this expression is related to the role of the developing seed in repressing floral initiation, which is already known to involve the phytohormon GA.
4. *MdFT*, which by homology with the *FT* gene of Arabidopsis is expected to promote flowering, is strongly expressed in seeding tissues and in the fruit. It is well known that flowering is repressed in seedlings and young plants through a phenomenon known as juvenility; if *MdFT* has a role as expected in promoting flowering in apple, its expression in seedlings suggests juvenility must involve a control point 'downstream' from *MdFT* activity.
5. We detected two seasonal peaks of expression of *MdTFL1-1*, first during the period of floral initiation, and second during the period of inflorescence development (Fig. 6). This pattern was reproducible in at least two biological replicates. *MdTFL1-2* expression, in contrast, was ubiquitous. We interpret this data as showing a specialized role for *MdTFL1-1* in flowering. Based on its known activity to antagonize *LFY* activity and repress inflorescence determinacy in Arabidopsis, we suggest that the early peak of expression (weeks 12-14) limits *AFL1* activity to the initiation of lateral flowers, and that its subsequent downregulation derepresses *AFL1* activity in the center of the inflorescence apex, allowing for terminal flower formation.

Based on these results, we now hypothesize that *MdTFL1-1* and *MdTFL1-2* have become functionally specialized in apple, with *MdTFL1-1* devoted to regulating inflorescence architecture, and *MdTFL1-2* devoted to maintenance of juvenility. This suggests that juvenility and inflorescence architecture can be regulated independently in apple – a tremendous opportunity for improving production.

3. Functional analysis.
Confirmation of the role of the *MdTFL1-1* and *MdTFL1-2* genes depends on determining what occurs when they are silenced (turned off). The powerful amiRNA technique should allow us to do this. We have obtained multiple lines of Gala with each of 6 silencing constructs. However thus far none of these lines have shown the phenotype (visible effect) of silencing either or both of the genes, although we will continue to observe them as they develop for any effects. Recent data from another lab in Germany produced similar inconclusive results with Gala. Therefore we are now exploring the effects of silencing the *MdTFL1-1* and *MdTFL1-2* genes in other varieties. We have started with the M.26 rootstock which is the best apple variety to transform. Although it is a rootstock, the effect of the *MdTFL1-1* and *MdTFL1-2* genes on flowering of M.26 should be applicable to fruiting varieties. We have just recovered multiple lines with three of the silencing constructs. These are still too young to show any early flowering effect, but we expect to be able to assess them meaningfully in the next two months. Experiments with the other silencing constructs are also planned. Results will be confirmed in a fruit variety, such as Fuji, which we can now also transform quite well.

Appendix A. **Material used for gene expression atlas:** Gala vegetative, floral, and fruit samples collected during ~~the~~ years 2006-07.

Tissue		**Collection date**
Seedling	Root	4 week seedling
	Stem	4 week seedling
	Leaf	4 week seedling
	Cotyledon	4 week seedling
	Apex	4 week seedling
Leaf	Recently expanded leaf	5/22/07
	Leaf 4	5/22/07
	Leaf 4	7/31/07
Apex	Apex	5/22/07
Flower	Bud and parts	04/04/08
Fruit	Fruit	05/22/07
	Cortex	07/03/07
	Cortex	07/31/07
	Cortex	9/20/06
	Skin	07/31/07
	Skin	09/20/06
	Seed	07/03/07

Full bloom dates:
2006: 05/07/06
2007: 05/07/07

Harvest dates:
2006: 09/05/06
2007: 09/04/07- 09/14/07

EXECUTIVE SUMMARY

Functional genomics of flowering in apple

Herb Aldwinckle and Steve Van Nocker

The overall goal of this project was to improve our understanding of the genetic regulation of flowering in apple, so that eventually better varieties without shortcomings like juvenility, biennial bearing and over-cropping can be produced by marker assisted breeding. The research may also yield information that could result in corrective treatments for existing varieties.

We approached the problem first by identifying all possible members of *FT/TFL* gene family, which is known to regulate flowering in other plants, in apple, and determining their DNA sequence. We confirmed that the apple genome contains five genes in this family, with a single gene related to the flowering time regulator *FT* and two duplicate genes related to the juvenility/inflorescence architecture gene *TFL1*. This effort added substantially to the public apple sequence database.

Second, we analyzed the activity pattern of all the identified *FT/TFL* genes. This analysis included expression of the genes in various parts and organs of the plant, and control of expression during development (e.g. flower initiation). The goal of this approach was to identify those members of the *FT/TFL* family that might play the most important role in flowering. We showed that *FT* is expressed in a surprisingly broad pattern, suggesting roles in addition to flowering. Two other members of the family are expressed strongly in fruit and seeds, suggesting unanticipated functions in these plant parts. Our findings on the duplicated apple *MdTFL1* genes suggests that one gene may be responsible for inflorescence architecture, while the other may be involved in repressing juvenility.

Finally we concentrated our functional analysis on these latter two genes. In order to show if these genes are those that are really critical to flowering, we knocked out (or silenced) them, to determine if the pattern of flowering was in fact altered. This work is in progress. We have silenced both genes individually and in combination in Gala apple, but have not yet seen effects on flowering behavior in the silenced plants in tissue culture. We have also silenced the genes in M.26 rootstock, but these experiments are still in their early stages. We will also silence the genes in another fruiting variety, probably Fuji, in case there are strong differences between varieties in the behavior of this family of genes.

It is still too early to say exactly which genes are most critical to onset of flowering in apple. The evidence thus far indicates strongly that one of the *MdTFL1* genes is likely to one of the most important genes involved. The transformation experiments in progress should provide additional important evidence.

Once the genes most critically involved in flowering onset are confirmed, we can proceed to develop markers for the use of those genes in marker-assisted apple breeding. New improved varieties will be of great value to the Washington apple industry, resulting in decreased production costs and higher quality fruit.