FINAL PROJECT REPORT

Project Title: Developing genetic tools for regulation of flowering, thinning, and fruit drop

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Justification

The continuing infusion of technology into the U.S. apple industry poses new challenges for production and will require development of superior cultivars that combine excellent fruit quality and disease resistance with production characteristics optimized for the 21st century. The identification of major genes controlling traits important for high-tech production is the first step for the development of such cultivars. Production-related traits such as flowering and flower/fruit abscission have been largely ignored in breeding efforts, and the genetic basis for such traits has generally not been explored.

A considerable problem in apple production is overcropping. The natural tendency of commercial apple cultivars to bear excessively large crops can lead to small, poor quality fruit and exacerbate alternate bearing. This problem can be addressed by flower and/or fruit thinning, but results can be unpredictable and this operation adds to production costs. The ability to control overcropping by reducing flower number, rather than through thinning, has great potential. Apple flowers are borne in an inflorescence containing a terminal (king) flower, which usually develops into the highest-quality fruit, and several lateral flowers. Natural genetic alleles specifying minimal flower number could be used in breeding efforts for cultivars that produce large, top-quality fruit with minimal synthetic input.

Another production problem is pre-harvest drop, which typically reduces yield by 10-15% and can often be catastrophic. Some popular cultivars, such as McIntosh and its sports, are highly prone to this problem. Although drop can be controlled somewhat by application of AVG (ReTain) or NAA, it is desirable to eliminate these synthetic inputs. The potential for mechanical harvesting of apples will necessitate the development of methods to precisely control abscission and this will be facilitated by identification of the major genes controlling these traits.

The proposed one-year project seeks to evaluate natural diversity in flowering and fruit abscission in apple species and cultivars in order to understand the genetics of these traits. This collaborative project will be among the first steps in a long-term effort to identify major genes controlling these traits, understand how these genes are regulated, and develop molecular markers for use in breeding strategies. Our breeding goals are distinct from most traditional breeding efforts for two reasons. First, we are employing 'wide' genetic crosses, between wild species or cultivars exhibiting maximum trait variation, in order to represent the full range of trait variation in the progeny populations. Second, we are concentrating on traits that are suited to the increasingly high-tech production that will dominate in the 21st century.

Objectives

1 a) Estimate genetic diversity in flower number-per-inflorescence by evaluating this trait for each of the ~2000 cultivars/species currently flowering in the USDA National *Malus* germplasm collection.

b) Estimate genetic diversity in timing of fruit drop by generating quantitative data for this trait for selected cultivars/species in the USDA National *Malus* germplasm collection.

c) Select parents for genetic crosses that will allow analysis of the genetics of flower number, timing of fruit drop, and other traits critical for high-tech production.

2) Evaluate the hypothesis that two specific candidate genes previously identified through our work act as 'master regulators' of flower number and abscission zone formation in apple.

Methods (Objective 1)

The USDA National Plant Germplasm System maintains an apple (*Malus*) collection on a 50-acre farm located near Geneva, New York. This collection contains commercial cultivars, heirloom varieties, and wild *Malus* species. The entire collection is comprised of almost 4,000 distinct genotypes. Approximately one-half of the cultivars/species are maintained as mature trees and will be available for evaluation of flower number and fruit drop in '05. All work will be carried out in collaboration with P. Forsline, curator of the USDA-Geneva collections, and G. Fazio, USDA plant geneticist.

Objective 1a) A preliminary evaluation of flower number for a small subset (96) of USDA-Geneva *Malus* cultivars/species revealed a range from ~3 to ~10 flowers per inflorescence (http://www.ars-grin.gov/cgi-bin/npgs/html/desc.pl?115042). This revealed that significant natural genetic variation does indeed occur for this trait. Evaluation of the entire *Malus* collection will be done during the spring '05 flowering period. Dr. Forsline has committed to evaluating this trait as a high priority for the '05 season.

Objective 1b) Approximately 1500 USDA-Geneva *Malus* cultivars/species were previously evaluated by USDA staff for timing of fruit drop relative to fruit maturity. Of these, 41 (3%) were described as 'DROPS BEFORE MATURE' and 109 (7%) were described as 'PERSISTS WELL INTO WINTER' (http://www.ars-grin.gov/cgi-bin/npgs/html/desc.pl?115061). We will develop a meaningful method to more precisely quantify fruit drop/retention timing relative to the maturity of the fruit, and apply this method for the quantitative evaluation of those varieties identified as early- or late-dropping. This objective will be carried out from summer to early winter '05.

Objective 1c) The subsets of species/cultivars that exhibit maximal divergence in flower number or fruit drop/retention will be evaluated for suitability for use as parents in genetic crosses. These crosses will generate the segregating populations required for generating a linkage map. Although the focus of this work will be to identify and map major loci associated with flower number and fruit abscission, we will also want to identify and map major loci associated with other traits that contribute to optimal production efficiency. The reasoning behind this is that it will require a great deal of effort to create a genetic linkage map, but it will be relatively simple to assign trait loci to such a map. Since in nearly all cases the genetics of these other traits is also a 'black box', this approach will greatly expand the utility of our project. Many or most of the species/cultivars in these subsets will already have been evaluated for ~150 other critical traits (http://www.ars-grin.gov/cgi-bin/npgs/html/desclist.pl?115) related to disease, morphology, and phenology, and these evaluations will guide our selections. The chosen subset will be additionally evaluated for traits related to fruit flavor and post-harvest characteristics. This will be done in collaboration with R. Beaudry (MSU-Horticulture/Postharvest group).

Whereas most apple species and cultivars can be interbred, apple is occasionally subject to gametophytic self-incompatibility, and other less-understood phenomenon can also suppress compatibility between two species or cultivars. To help resolve these potential problems, where practical we will carry out test crosses between potential parents in Spring '05 (larger-scale crosses to generate the mapping populations will be done in Spring '06).

Methods (Objective 2)

In the first part of Objective 2 we will test the function of an apple gene that we hypothesize has a key role in determining flower number in the apple inflorescence. This gene, which we call *MALUS TERMINAL FLOWER (MTF)* is a counterpart (homolog) of the *TERMINAL FLOWER* gene from the reference research plant *Arabidopsis thaliana*. In Arabidopsis, this gene acts as a master regulator of inflorescence development; in Arabidopsis plants that have mutation in this gene, the normally indeterminate inflorescence terminates in a solitary flower. In common apple cultivars, the inflorescence produces 3-5 lateral flowers before terminating in a solitary flower, and we hypothesize that the *MTF* gene might be shut off in the inflorescence after these lateral flowers are formed. The potential ability to control activity of this gene in apple could lead to the ability to reduce the number of flowers, thereby influencing crop load, fruit quality, and alternate bearing at the level of flower initiation. We will test our hypothesis several ways. First, we will introduce the *MTF* gene into an Arabidopsis mutant that lacks the *TFL* gene and evaluate the potential of *MTF* to 'rescue' the defective inflorescence development. Second, we will evaluate activity of the *MTF* gene during inflorescence development in a common apple cultivar, and in apple species and cultivars that vary in the number of flowers, to determine if there is an association between gene activity and flower number.

In the second part of this objective we will similarly study the function of an apple gene that we call *MALUS JOINTLESS (MJT)*. This gene was discovered as a result of our current WTFRC-funded project to study fruit abscission. The *MJT* gene is an apple counterpart of the tomato gene *JOINTLESS*, a master regulator of formation of the tomato flower/fruit abscission zone. In tomato plants that have genetic mutations in *JOINTLESS*, the flower/fruit abscission zone is completely absent. Natural varieties of tomato that have these mutations have been widely utilized for the tomato processing industry, since ripe fruit do not fall to the ground and can be efficiently machine-harvested. We have found that a homolog of *JOINTLESS* exists in apple, and although its function is not known, we predict that this protein is a master regulator of abscission zone formation in the apple flower/fruit. The potential ability to regulate such a gene in apple would allow the grower to virtually eliminate pre-harvest drop. We will evaluate this hypothesis by introducing the apple *MJT* gene into tomato varieties that have mutation in the *JOINTLESS* gene and lack abscission zones. If the apple gene is a true regulator of abscission zone formation in apple, it should restore the formation of the abscission zone in the resulting tomato plants. In addition, we will begin a process to disrupt the activity of this gene in apple (this will be a ~3 year process, with minimal effort needed following the first year).

Proposed schedule of accomplishments:

'Deliverables' upon one-year project termination:

- Flower number-per-inflorescence data for the USDA National *Malus* Germplasm Collection
- Quantitative data for the timing of fruit drop for ~150 cultivars previously designated as early- or late-dropping.
- Identification of appropriate parents for genetic crosses to be carried out in spring of '06.
- Completion of the functional analysis of the apple *MTF* and *MJT* genes
- Correlation of the apple *MTF* gene activity with morphology during inflorescence development and in cultivars exhibiting variation in flower number

The anticipated outcome of the long-term effort are genetic maps of apple that reveal the location of genes controlling traits important for profitability of the apple industry. Although other apple genetic maps already exist, most were created from crosses of domesticated cultivars that lack the full range of the traits expressed in apple species (referred to as 'narrow crosses'), and will not be useful to identify the major genes controlling many traits. This project is timely for two reasons: 1) many of the *Malus* cultivars/species in the USDA-Geneva collection, especially those wild genotypes collected from central Asia, are now coming into production and are available for evaluation, and 2) We intend to coordinate the completion of the genetic maps (5-6 years) with the predicted availability of new genomic information for apple, including a reference physical map. This new information will greatly facilitate the identification of the major genes. Because the apple *MTF* and *MJT* genes possibly play a major role in determining flower number and abscission, respectively, it is not unlikely that these genes will correspond to the major genetic loci that we will identify through mapping. We intend to develop this part of the project for funding through the USDA National Research Initiative Competitive Grants Program.

Literature Review

Most commercial apple varieties tend to bear fruit more heavily or exclusively in alternate years. This biennial bearing habit is disadvantageous not only because of very low production in the 'off' year, but also because overcropping in the 'on' year leads to poor fruit quality. Accordingly, the U.S. apple industry has consistently identified biennial bearing correction and flowering manipulation as a top research priority. In apple, flower initiation is inhibited by developing fruit, and most likely by gibberellins (GAs) produced in the seed (Review: Dennis and Neilsen, 1999). A potential control strategy for alternate bearing is the application of GAs at the time of flower initiation to those trees that are otherwise expected to bear heavily (Dennis and Edgerton, 1966; McArtney and Li, 1998). To refine this strategy and develop new methods to control biennial bearing, "..a better understanding especially of the early phases of the flower-formation process is badly needed" (Tromp, 2000). The signaling pathway leading to suppression of flowering by GA is completely unknown. Although GAs play an inhibitory role in flowering in many other species as well, molecular studies have addressed only their flowering promotive effects (eg in Arabidopsis).

Physiology, molecular biology and morphology of flowering in apple

Molecular studies in reference plants have shown that flowering results from transcriptional cascades involving multiple classes of gene. For example, in Arabidopsis the meristem identity genes *LEAFY (LFY)* and *APETALA1 (AP1)* become expressed early in the initiation process; *LFY* itself is transcriptionally activated subsequent to perception of inductive photoperiods (mediated through induction of *CO*, *FT*, and/or *SOC1*) and GAs (through induction of *SOC1* and/or *AtMYB33*). The *TERMINAL FLOWER (TFL)* gene is upregulated in concert with *LFY* and represses *LFY/AP1* expression in the center of the indeterminate inflorescence (Review: Jack, 2004). *LFY*- and *AP1*-related genes have been identified in apple using homology-based approaches (Sung et al., 1999; Kotoda et al., 2000; Wada et al., 2002). The *LFY*-related *AFL1* and *AP1*-related *MdMADS2* are upregulated in the shoot apex during floral initiation and are sufficient to trigger flowering when expressed heterologously in transgenic Arabidopsis or tobacco.

These observations suggest that some mechanisms of floral initiation have been evolutionarily conserved in apple. However virtually nothing else is known about the molecular biology of floral initiation in apple, including the host of genes that are expected to regulate *LFY* and *AP1*, such as *TFL*. In Arabidopsis plants with mutations in *TFL*, the normally indeterminate inflorescence terminates in a single flower, and lateral shoots develop as solitary flowers (Shannon and Meeks-Wagner 1991; Alvarez et al. 1992). Thus, a presumed function of *TFL1* is to keep the inflorescence meristem in an indeterminate state. *TFL* encodes a member of a small protein family exhibiting limited homology to mammalian Raf kinase inhibitor protein (RKIP). RKIP is a membrane-

associated protein that regulates Raf-1 kinase, which is intimately involved in signal transduction cascades controlling cell proliferation and differentiation in mammals. The amino-terminus of RKIP is cleaved off to form a small peptide hormone, leading to the speculation that *TFL* may in a similar manner be the progenitor of a small signaling peptide involved in flowering (Bradley et al. 1997). In contrast to animals, where numerous peptide hormones are known and utilized extensively in medicine, almost nothing is known about peptide hormones in plants. The potential ability to regulate flower formation in apple through application of peptide hormones is an exciting possibility, since such hormones would act very specifically, in contrast to most growth regulators such as GAs or auxins which have a very general effect on growth and development.

In contrast to the molecular mechanism, the morphology of floral initiation and early development is well described. In typical apple varieties, 4-6 flowers are formed on a determinate inflorescence, which can be formed terminally or laterally (within axillary buds) on the current season's growth. Flowers initiate during the growing season, overwinter in a partially developed state, and complete development early in the subsequent growing season. New vegetative growth in the second season takes place from one or more axillary meristems proximal to the inflorescence (Fulford, 1966). Foster et al. (2003) distinguished eight stages of inflorescence initiation and development in the cv. Royal Gala. The initial morphological transition was a broadening of the apex (Stage 0 to Stage 1). Subsequently, apices became dome-shaped (Stage 2) followed by the appearance of lateral (Stage 3) and terminal (Stage 4) floral meristems. This study provides the morphological framework for a meaningful study of the underlying molecular biology of flowering.

Abscission zone development and activation

The abscission process is generally considered to consist of three more-or-less defined stages: induction, differentiation, and separation. The inductive and differentiative stages have been studied the least, especially in fruit abscission. The flower/fruit abscission zone is genetically determined very early in development, at the time when floral organs are initiated from the floral primordia (Smyth *et al*., 1990). Thus, in apple, this typically occurs during the previous season. At least in the tomato flower, the specification of the abscission zone is determined by the activity of a single, regulatory gene called *JOINTLESS* (Mao *et al*., 2000). Depending on the species, the abscission zone may be fully developed, or only partially developed at bloom time (Bonghi *et al*., 2000). In apple, the abscission zone continues to develop after bloom (Pandita and Jindal, 1991). The fully-developed abscission zone consists of layers of morphologically distinct cells ('abscission layers') arranged transversely to the axis of the pedicel. The vascular tissues, which pass through these layers, do not participate in abscission and thus can limit fruit drop at very late stages of abscission.

CITATIONS

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Wada M, Cao Q, Kotoda N, Soejima J and Masuda T. 2002. Apple has two orthologues of FLORICAULA/LEAFY involved in flowering. Plant Molec Biol 49, 567-577.

BUDGET
Developing genetic tools for regulation of flowering, thinning, and fruit drop
Steven van Nocker
Proposed project duration: One year, 2005
Current year request: $19,033

Item	Year 1 (2005)
Salaries[1]	15,317
Benefits[2]	1,216
Wages	0
Benefits	0
Equipment	0
Supplies[3]	1,600
Travel[4]	900
Miscellaneous	
Total[5]	19,033

[1]We request one-half stipend support for one PhD graduate student ($8,288, not inclusive of tuition and fees), and summer salary for S. van Nocker ($7,029; summer salary is no longer provided by MSU).

[2]Health benefits for graduate student ($727) and S. van Nocker ($538)

[3]S. van Nocker has funding from the USDA and NSF for projects using similar technologies that provides much of the infrastructure needed for the proposed project.

[4]To allow travel between East Lansing, MI and Geneva, NY in the spring, summer and fall.

[5]Total project cost is $28,800. We have requested partial matching support from the Michigan apple industry.