FINAL PROJECT REPORT

Project Title: Breeding in the 21st century: technology platform for fast breeding

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Other funding sources

Agency Name: National Science Foundation
Funding amount: $8000 for undergraduate student support

Total Project Funding: $24,673

Budget History:

Item	**2012 –** Funding request for 10 months
Salaries	
Benefits	
Wages[a]	10,500
Benefits	4,473
Equipment	
Shipping	
Supplies[b]	3,000
Travel	1,000
Plot Fees[c]	700
Miscellaneous[d]	5,000
Total	**$ 24,673**

Footnotes:

a. Technical help – Time slip employee for plant handling, transgenic experiments, micropropagation and assisting in crosses, data collection etc.
b. Tissue culture supplies and reagents
c. Greenhouse/tissue culture facility use
d. Greenhouse materials/plant screening/transgenics and breeding/grafting program costs

OBJECTIVES

Note: This final report is for the project funded in May 2012 for 10 months which builds upon ongoing work in our programs since 2011 with support from the WTFRC.

The WSU Apple Breeding Program (WABP) has identified reduction of generation cycle as a priority in order to rapidly combine desirable traits in new varieties.

The primary objective of this project is to *reduce the generation cycle* in breeding so that time and resources can be saved in combining multiple traits faster to produce the varieties desired by the farmers and the consumers. The techniques being developed in this project are already being implemented in apple variety development worldwide; failure to invest in this technology will result in the loss of a competitive advantage within the WABP. Tree fruit breeding programs individually use a physiological, genetic or transgenic approach to reduce generation cycle.

The three approaches of reducing generation cycle that are being tested in our programs are:

1. Environmental and horticultural approach

For this approach, seedlings are grown under modified environmental conditions to accelerate growth and development with the goal of breaking juvenility in a shorter time. Seedlings for this aspect were selected from crosses that had already been made for the WABP with a reasonable level of diversity including desirable fruit quality traits and seasonality [e.g. Akane (early variety) and Fuji (late variety)] whilst not using Cripps Pink. These are baseline comparison seedlings which are not expected to be precocious.

2. Genetic approach

For this approach, varieties known to induce early flowering in their offspring (precocity) are used as parents. Parental overlap was intentionally maintained with individuals used in objective 1 for a direct comparison. Cripps Pink (Elite cultivar) and *M. zumi* (Wild donor) were used as the precocious parent.

We have made significant progress in using modified environmental conditions to accelerate growth of own-rooted WABP seedlings. Physiological manipulation of seedling growth and development in the greenhouse is primarily being carried out at WSU Pullman greenhouse. The horticultural practice of grafting seedlings onto precocious rootstocks is routinely used within the WABP.

3. Transgenic intermediate approach

This approach uses a quick flowering transgenic plant as an intermediate so that seedlings from any cross can flower within a year reducing the generation cycle to one year or less. This technique is particularly useful in enabling the breeder to incorporate diverse germplasm into the breeding program and rapidly go through several generations of selection (to remove the poor characteristics that frequently accompany novel traits from wild germplasm). The transgene can then be selected out of the final generation cross resulting in a non-transgenic selection with no regulatory issues attached.

Specifically, the objectives for the 10-month project were:

1. Maintain WABP seedlings (non-precocious and precocious) in the greenhouse and, monitor and regulate growth and development to record flowering timelines
2. Micropropagate and multiply WA 38 and WSU 48 for transformation experiments
3. Establish *M. zumi* in tissue culture for micropropagation
4. Perform transformation experiments with Royal Gala, WA38 and WSU 48

SIGNIFICANT FINDINGS

Significant findings for objective 1

- Apple seedlings follow a unique growth pattern under our modified environmental conditions different from what has been reported for growth in the orchard.
- Careful observations were made over the last year and a preliminary phenotype-guided growth model has been established that guides decisions for fertilization, pruning and administration of cold treatment to meet chilling requirement. This model is incomplete as most of the seedlings are currently in cold treatment or just emerging from that.
- A clear impact of genetic background under modified environmental conditions on the growth rate is visible based on number of nodes generated per month.

Significant findings for objective 2 and 3

- Modified sanitizing and operating protocols were developed to counter the large pathogen load infesting the elite WSU selections and *M. zumi* accessions.

- Tissue culture protocols for maintaining WA 38, WSU 48 and *M. zumi* have been established. Each individual requires a unique combination of phytohormones and salts in tissue culture for robust growth and development.

Significant findings for objective 4

- Transformation protocols for Royal Gala as a standard for transgenic experiments have been established and several putative transgenic Royal Gala plants are ready to be tested for transgenic status.
- Micropropagation of WA 38 is underway for transformation experiments.

RESULTS & DISCUSSION

Detailed results and discussion follow this table that summarizes the progress and milestones achieved as an indicator of success in the funded project.

Time Frame	Objectives	Progress	Milestones
May 2012 – March 2013	1. Maintain WABP seedlings (non-precocious and precocious) in the greenhouse and, monitor and regulate growth and development to record flowering timelines	Careful phenotyping of seedling growth and development has provided decision making guidance for fertilization, irrigation, pruning and chilling treatment	A preliminary greenhouse growth model established
	2. Micropropagate and multiply WA 38 and WSU 48 for transformation experiments	Tissue culture protocols refined and established. Plant sanitization protocols established. More plant material will be established in tissue culture from fresh material in the Spring	Micropropagation procedures established.
	3. Establish *M. zumi* in tissue culture for micropropagation	Individuals from *M. zumi* have been established in suitable custom tissue culture media	Plantlets established in tissue culture.
	4. Perform transformation experiments with Royal Gala, WA 38 and WSU 48	Several transformation experiments performed with Royal Gala. WA 38 and WSU 48 micropropagation is ongoing for subsequent transformation experiments	Methods to generate transgenic Royal Gala plants established.

Objective 1: Maintain WABP seedlings (non-precocious and precocious) in the greenhouse and, monitor and regulate growth and development to record flowering timelines.

Status: A total of 510 seedlings; 85 WABP seedlings from crosses made in 2010 derived from non-precocious parents and 427 WABP seedlings derived from crosses made in 2011 derived from precocious parents are currently in the WSU Pullman facilities. The 2010 seedlings provided us a test set of how to grow and maintain apple seedlings in the greenhouse. We utilized the observations in successfully growing 427 seedlings representing 8 parental combinations with desirable traits (Figure 1A and B – please see at the end of the document).

Observations: Post-germination in March 2011, majority of the seedlings grew rapidly to a size of 18 to 24 inches within 8 weeks and by November 2011 many seedlings touched the greenhouse roof

indicating a height of 6 to 8 feet on average. It was interesting to note that some of the seedlings segregated for compact size. Some were super dwarfs (Fig 1C) while a few individuals were dwarf (Fig 1D). It is important to note that such seedlings would not have survived in a nursery operation but have been carefully maintained in the greenhouse and may possess unique fruit traits. While this is still speculation, the hallmark of any breeding exercise is the available genetic diversity and survival of maximum number of diverse seedlings bodes well for generating individuals with a unique combination of traits.

Growth and Development: Seedlings derived from each cross exhibit variable phenotypes in shoot growth with some seedlings being vigorous. This could be genetic or the impact of environmental conditions. Seedlings from each cross were randomly separated into Group A and Group B. In November 2012, Group A seedlings were placed in the cold to mimic natural dormancy conditions while Group B seedlings remained in the greenhouse. At the time of this report, Group A seedlings have completed dormancy and are now emerging from it (Fig 2A) and Group B seedlings were defoliated in early December for initiating dormancy. This process resulted in the production of unusual bud "clusters" on one tree (Fig 2B). It is not clear what these are but they appear abnormal and are similar to flower bud clusters in cherry. In summary, growth and development under modified environmental conditions are allowing survival of unusual seedlings with diverse growth and development behaviors.

Model of Plant Development: Careful observations of the ~500 seedlings over the last year have resulted in the establishment of a preliminary model that can be used to identify phenotypes and plantlets cycled through dormancy conditions to reduce generation times. The model identifies three developmental stages which are easily recognizable. First stage represents vigorous growth characterized by actively growing shoot tips. In the second stage, the shoot tips become less aggressive and start producing bract-like structures. Finally, when the plant is ready for dormancy the shoot tip appears like a rosette. In the final stages the leaves come off easily from the stem. At this point, the tree should be moved into the cold with slow reduction of temperature and light duration. Figure 3A represents this concept graphically; it shows one typical growth cycle. A plant goes through this cycle once when grown outside in an orchard. One could accommodate 2 or 3 such cycles in greenhouse/cold room conditions (Fig 3B). We intend to utilize this model to move seedlings through growth cycles and reduce generation time. It is critical to note that each individual seedling will behave differently requiring detailed and everyday attention in such experiments. While labor-intensive, it is a worthy approach as it can save several years in variety development and could be particularly useful to fully exploit seedlings coming through DNA-assisted selection. Once the investment has been made in the seedling using the portfolio of key markers we expect to have in the near future, we would predict the number of successful (un-culled) seedlings will be reasonably few; at this point, seedling death through transplanting to the nursery would be a greater loss so it is likely the WABP will need to use greenhouse facilities to rear these valuable seedlings. Being able to speed up this growth and maturation process using the model developed will increase the efficiency of the application of DNA-assisted selection and should ultimately reduce costs.

2. Micropropagate and multiply WA 38 and WSU48 for transformation experiments

In spring 2011 there was limited amount of plant material available. Despite that, several explants were established in tissue culture. Fungal and bacterial infection decimated the collection with a few surviving samples. This required the establishment of rigorous sanitization protocols. In addition to the use of bleach we used a sonication device to ensure that air bubbles created due to surface irregularities are dislodged. This resulted in substantial reduction of post-sanitization infection.

For micropropagation of WA 38 and WSU 48, five different apple micropropagation media for Royal Gala and Geneva rootstocks available in the lab were tested. While the experiment requires more

time, observations made thus far have resulted in the identification of suitable media formulation for WA 38 and WSU 48 growth and micropropagation.

Fresh plant material will be used in spring 2013 to initiate more new cultures.

3. Establish *M. zumi* in tissue culture for micropropagation
As was the case with WA 38 and WSU 48, a limited amount of plant material was available for *M. zumi*. However a few individuals have now been established in tissue culture; the repeated sanitization due to the high bacterial and fungal load on the initial plant material has resulted in reduced growth and vigor. Fresh plant material in spring 2013 will be used to establish larger numbers of plantlets in tissue culture.

4. Perform transformation experiments with Royal Gala, WA 38 and WSU 48

There are several groups that have successfully reported on genetic transformation of apples however techniques used are extremely variety/genotype-specific and none of the transformation protocols reported are for individuals that are relevant to the WABP. Royal Gala has been a workhorse for our transformation work. Our first goal was to test our methods and replicate what others have accomplished.

So far, three genetic transformation experiments in Royal Gala have been performed with considerable success. Transformation was performed using protocols provided by Jay Norelli at the USDA-ARS station in West Virginia. At present there are over 60 preliminary transgenic plants (Figure 4) that are undergoing rooting in antibiotic selection medium. In the next few weeks, their transgene integration status will be confirmed via molecular analysis.

While it is encouraging to see the level of success with Royal Gala, transformation of WA 38 and WSU 48 may require protocol modifications given the fact that different composition of growth media is required for the WSU genotypes for growth and micropropagation compared to Royal Gala.

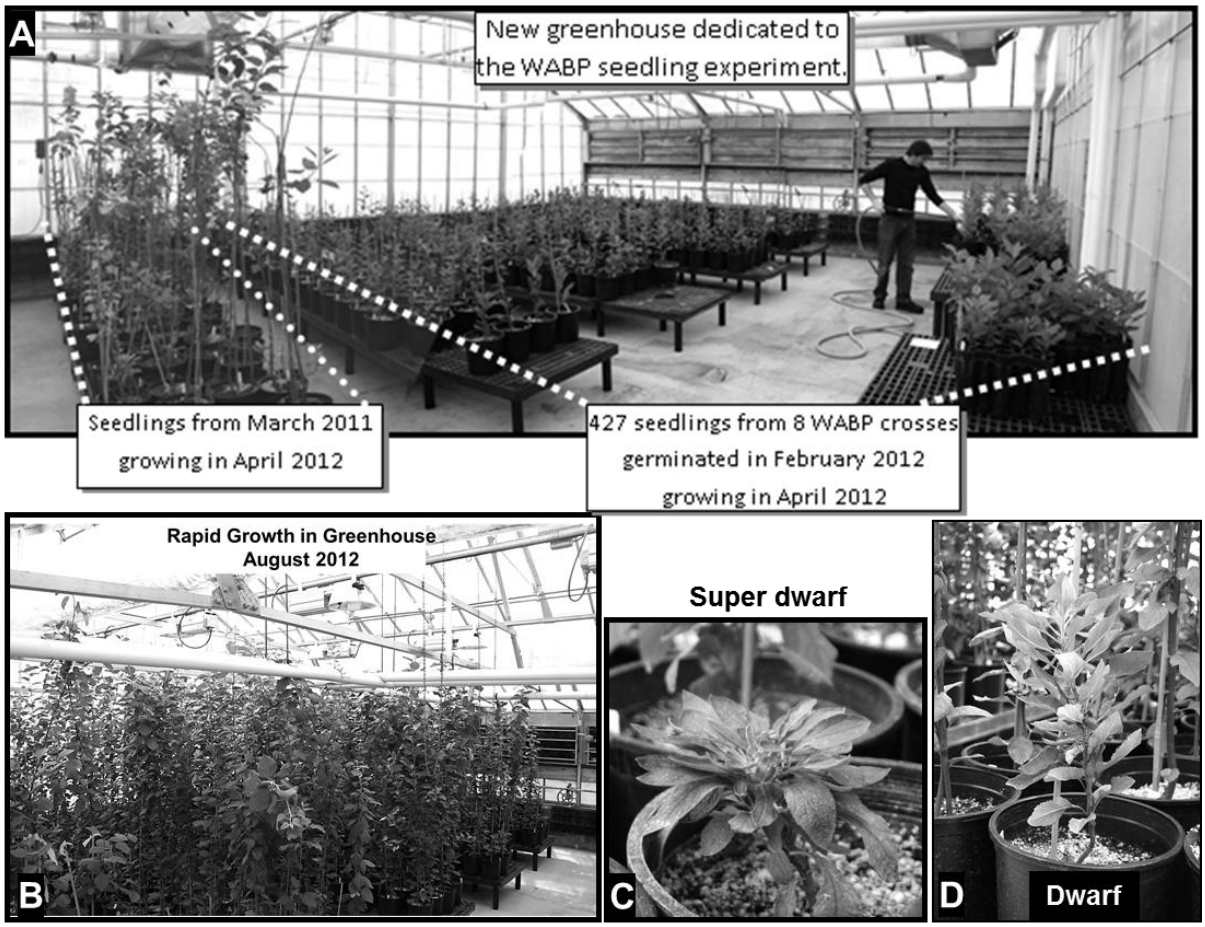

Figure 1: WABP seedling growth in WSU Greenhouse. A. 510 seedlings in the greenhouse. Notice the robust seedling growth as recorded in April 2012. B. The seedling stand is 6-8 ft tall in August 2012. C. A super dwarf seedling in the same population in September 2012. D. A dwarf seedling in the same population in September 2012.

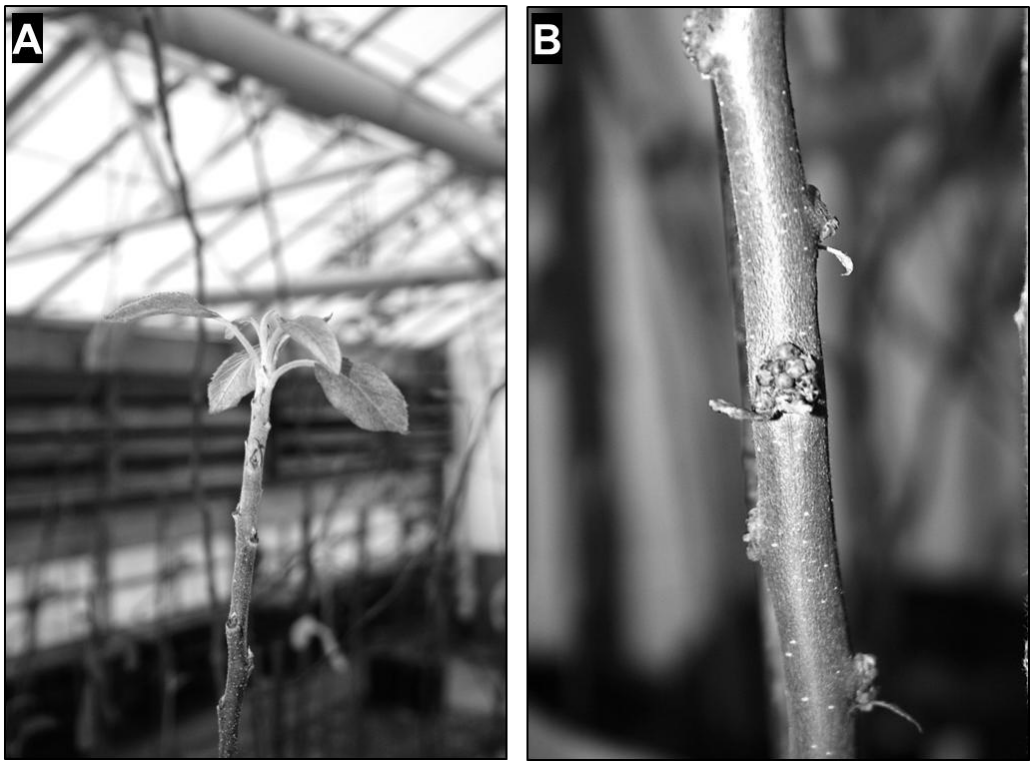

Figure 2: A. Resumption of growth in Group A seedlings after being moved out of dormancy. B. Appearance of unique cluster buds in one of the individuals.

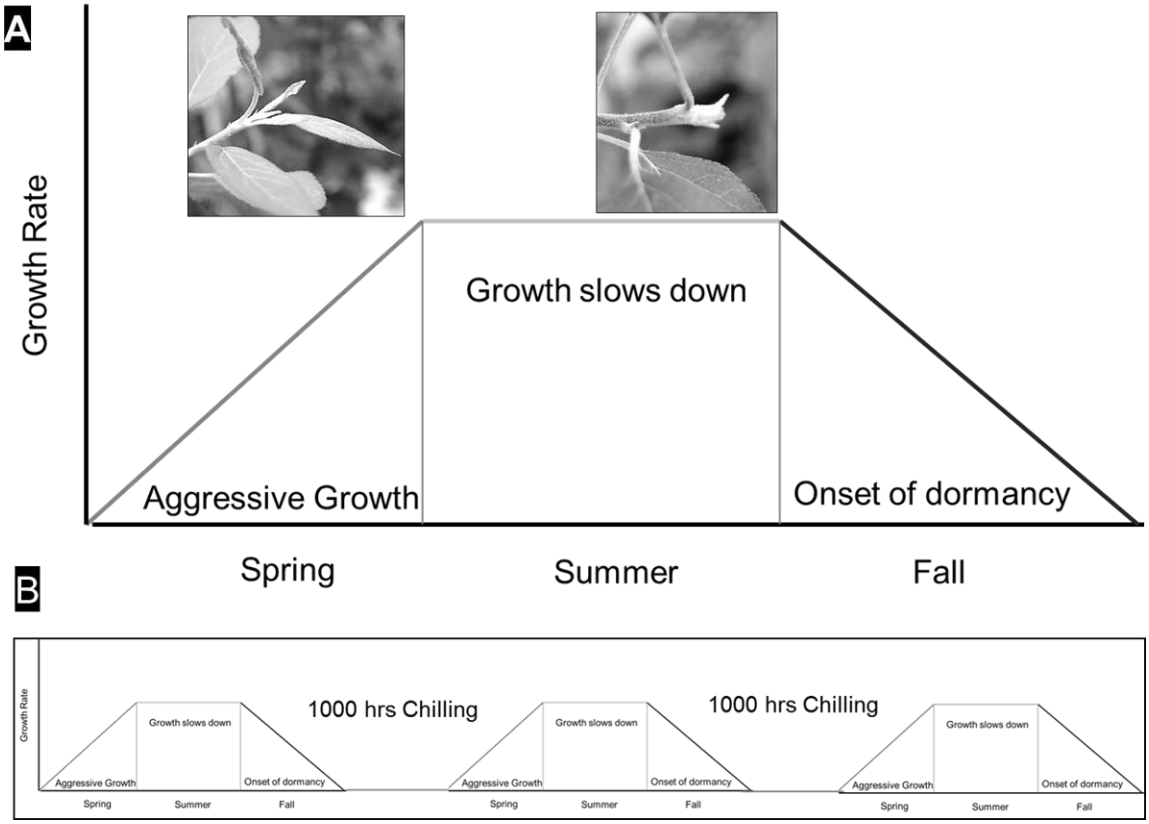

Figure 3: Model of plant growth. A. Representative graph of growth rates in the spring, summer and fall. Images of shoot tips taken at the time of aggressive growth and slowing down of growth are shown. This growth cycle is completed in one year in the orchard B. It is proposed that three such growth cycles could be completed in one year in the greenhouse.

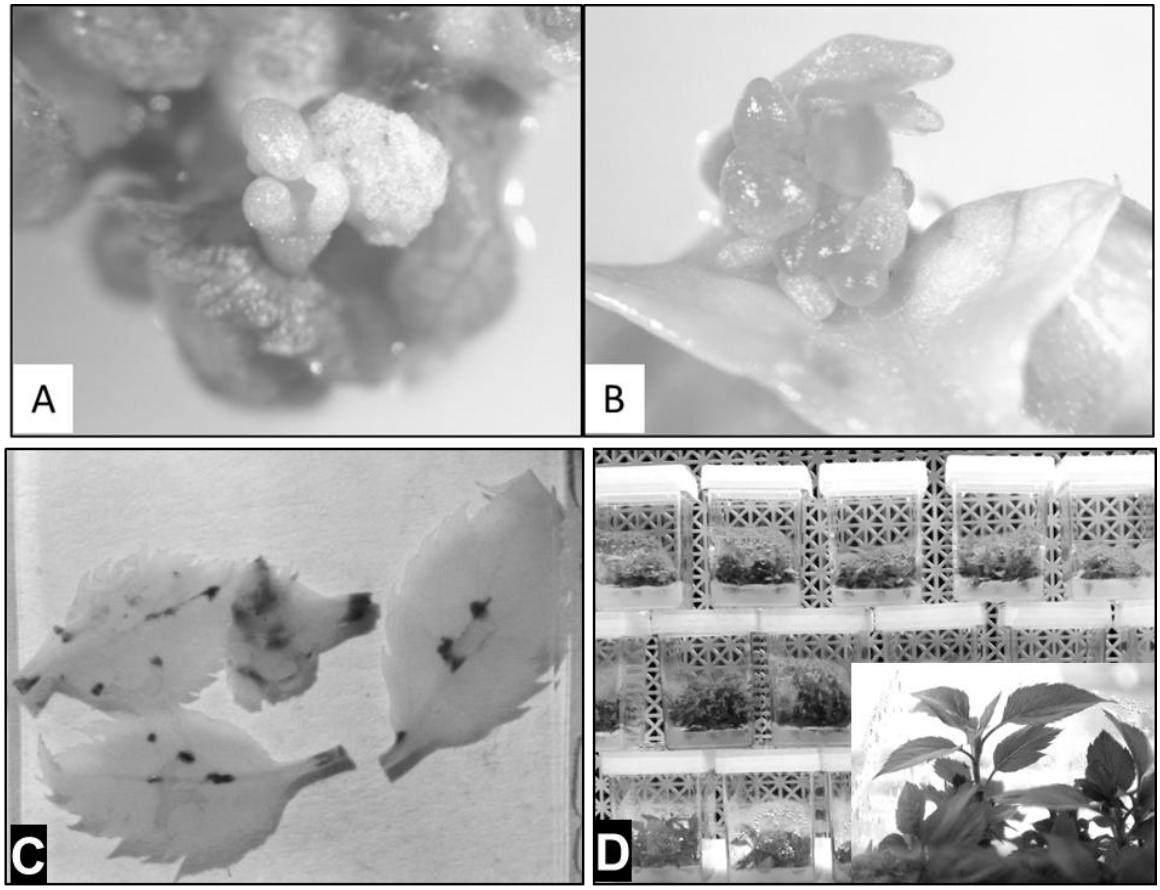

Figure 4: Transgenic experiments in Royal Gala. A: A putative transgenic shoot emerging from apple leaf tissue on antibiotic selection. B: Another transgenic event on another leaf explant. C. Dark spots indicate expression of transgenic protein in parts of the plant where the cells express the transgene. D. Representative transgenic plantlets thriving on very high concentration of antibiotic that allows only those plants to survive that harbor the transgene. Close up of one such plant is shown in the inset.

EXECUTIVE SUMMARY

The aim of this project was to progress earlier work initiated to *reduce the generation cycle* in breeding so that time and resources can be saved in combining multiple traits faster to produce the varieties desired by the growers and the consumers. This reduction in generation time has been identified as a priority in the WSU apple breeding program (WABP) in order to allow the rapid combination of desirable traits in new varieties.

Significant progress has been made in our ability to establish the required plant material in tissue culture (a key first step for the transformation process required for the fast generation cycling). Transformation experiments with our model variety Royal Gala also appear to be successful. Progress in understanding how to manipulate apple seedlings to rapidly go through juvenility in the greenhouse is underway; this model is however incomplete as most of the seedlings are currently in cold treatment or just emerging.

Summary of findings

Apple seedlings follow a unique growth pattern under our modified environmental conditions different from what has been reported for growth in the orchard. Careful observations were made over the last year and a preliminary phenotype-guided growth model has been established that guides decisions for fertilization, pruning and administration of cold treatment to meet chilling requirement. A clear impact of genetic background under modified environmental conditions on the growth rate is visible based on number of nodes generated per month.

Modified sanitizing and operating protocols were developed to counter the large pathogen load infesting the plant material required for initiating tissue culture. Tissue culture protocols for maintaining our target accessions have been established. Each individual requires a unique combination of phytohormones and salts in tissue culture for robust growth and development. Transformation protocols for Royal Gala as a standard for transgenic experiments have been established and several putative transgenic Royal Gala plants are ready to be tested for transgenic status. Micropropagation of WA 38 is underway for transformation experiments.

Future directions

We propose to continue this work focusing on the physiological manipulation of seedling growth and development as this will be a key tool in moving forward elite seedlings following the extensive DNA-assisted selection that we envisage will become a routine part of the WABP as more markers become available. A regeneration protocol is the next step required in establishing WA 38 as a quick-flowering transgenic that will serve as the intermediate parent in future rapid generation cycling crossing within the WABP.