FINAL PROJECT REPORT

Project Title: Auxin and ethylene dynamics in the abscission zone

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Other funding Sources

Agency Name: Michigan Agricultural Experiment Station
Amount requested or awarded: Matching (Year 1 only).
Notes: This project is included in the PI's MAES 5-year Project. MAES pays partial faculty salary for the PI.

Total Project Funding: $56,236

WTFRC collaborative expenses $500

Budget History:

Item	2006	2007	2008
Salaries	8,676[1]	8,936	9,204
Benefits	819[2]	693	956
Wages	4,200	3,846	4,456
Benefits	0	0	
Equipment	0	0	
Supplies	1,800	1,600	4,200[3]
Travel	150	150	150
Miscellaneous	3,200[4]	3,200	
Total	18,845	18,425	18,966

Footnotes: [1]We have obtained matching funds from the Michigan State Agriculture Experiment Station. [2]Supported $^1/_2$ effort by a graduate student (stipend). [3]Costs include production and screening of microarrays. [4]Costs for DNA sequencing in Year 1 and Year 2.

OBJECTIVES

Interactions between two endogenous plant growth regulators, ethylene and auxins, play a crucial role in programming abscission of flowers and fruit. We proposed a model for the initiation of flower and fruit abscission, where loss of directional (polar) auxin flow through the abscission layers triggers enhanced ethylene signaling in abscission layer cells, culminating with activation of genes that promote cell separation. The objective of this project was to test this model through a methodical characterization of auxin and ethylene signaling components in the flower and fruit abscission layers, and to analyze the effects of cultural practices (including application of bloom and postbloom thinners) and environment on the interactions between auxin and ethylene signaling components.

Our specific objectives were:

1) Identification of auxin and ethylene signaling components active in the flower and fruit abscission layers. We sought to identify apple counterparts of known components of auxin and ethylene signaling (enzymes involved in biosynthesis, degradation, receptors, transporters, signaling intermediates, and regulatory proteins).

2) Design and construction of a microarray tool for gene expression profiling in apple

3) Studies of gene activity through microarray analysis. We sought to study these components in abscission-promoting circumstances, such as:

a) Flower abscission or retention associated with pollination/fruit set.
b) Fruitlet abscission associated with competition within a cluster.
c) Fruit abscission promoted by wounding.
d) Natural fruit abscission associated with maturity and ripening.
e) Flower abscission associated with bloom thinners.
f) Postbloom thinning by PGRs.
g) Fruit abscission promoted by reduced photosynthate
h) Fruit removal
i) Effects of PGRs on mature fruit retention or abscission

4) Construction of a map of regulatory pathways involving auxin and ethylene. The gene activity profiles reveal activity of each gene with respect to the individual abscission-promoting circumstances, and thus create a blueprint for the roles of auxin and ethylene in abscission. Hypothetically this can be used as a predictive tool for the design of more effecting thinning strategies.

SIGNIFICANT RESULTS (by objective)

1) Identification of auxin and ethylene signaling components active in the flower and fruit abscission layers.

- We analyzed the *expressed sequence tag (EST)*[1] information currently available in public sequence databanks
- We performed extensive literature searches to identify and catalog all suspected auxin-related and ethylene-related genes characterized in other plants.
- We used bioinformatics techniques to identify apple counterparts of known components of auxin and ethylene signaling, and identified a total of ~414 apple genes potentially involved in auxin signaling, and ~190 apple genes potentially involved in ethylene signaling.

[1] **EST (expressed sequence tag)** is a short DNA sequence of a randomly selected gene active in a given tissue. ESTs are a useful resource for gene discovery and for designing probes for DNA microarrays used to determine patterns of gene expression.

- We supplemented this gene set with 150 genes with roles in cell wall degradation, mostly pectinases, some of which are known to be activated during abscission in other plants.
- We compiled this information in a web-accessible database, Tree Fruit Technology (http://www.genomics.msu.edu/fruitdb). This work was published in the journal *Plant Physiology* (http://www.plantphysiol.org/cgi/content/full/141/3/811).

2) Design and construction of a microarray tool for gene expression profiling in apple

- We designed a microarray containing all DNA sequences (~2,200) of the gene sets that we identified. This microarray is now commercially available (CombiMatrix Corp.) and can be ordered by researchers. This tool is useful in studies of flowering, fruit ripening, color and aroma production, and other developmental processes important for production and storage.

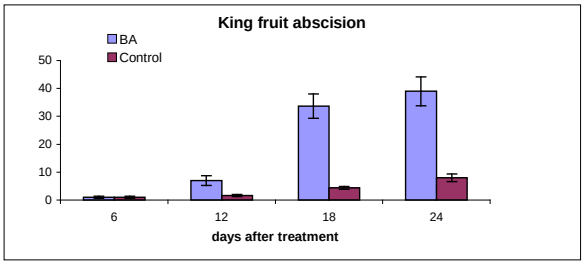
3) Studies of gene activity

Flower abscission associated with bloom thinners.

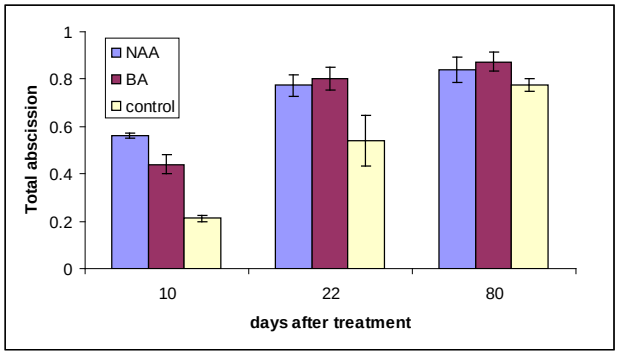
- We sampled flower and abscission zone tissues from bloom thinning trials on Gala in the Wenatchee area. This was in collaboration with the more extensive thinning trials done by WTFRC staff. Treatments were ReTain (200 ppm), MCP (Nate Reed, AgroFresh), Ethrel (3 pts/acre), CFO/Lime sulfur, Retain pretreatment/CFO/Lime sulfur, and MCP pretreatment/CFO/Lime sulfur. To dissect the molecular mechanisms of flower abscission promoted by chemical thinners, we dissected abscission zone tissues from flowers from trees treated with lime sulfur. To help evaluate the potential role of ethylene in promoting thinning in response to lime-sulfur, we also analyzed tissues from trees treated with lime sulfur that had also been pretreated with AVG, an inhibitor of ethylene biosynthesis, or MCP, a strong repressor of ethylene sensitivity. When evaluated 2d following application, flowers treated with lime-sulfur were found to generate markedly more ethylene than the control or plants treated with lime sulfur pretreated with AVG. Other treatments did not result in ethylene evolution. We conclude lime sulfur, which WTFRC data showed was by far the most effective, may work through ethylene signaling. Gene expression analysis is in progress.

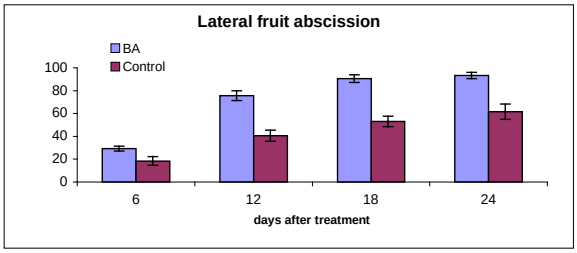
Postbloom thinning by PGRs

Research was conducted on Gala at MSU. Various concentrations of NAA (naphthaleneacetic acid)] and BA (6-Benzylaminopurine, benzyladenine) active ingredient were used in these experiments at 10-12 mm king fruit size. Each compound application and control included two replicates (three trees/replicate). Chemical compounds were dispersed in 0.1% surfactant Silwet-77 immediately before canopy application. All of the control trees were treated with 0.1% Silwet-77.



Abscission was calculated by fruit counts on





representative limbs. Total abscission was expressed at a percentage of total initial 10-12 mm fruit. Abscission zone samples were collected into liquid nitrogen in the above time points.

Both NAA and BA promoted fruit abscission early in the season, but ultimate fruit numbers were not significantly different than control. BA showed no marked preference for promoting drop of king fruit vs. lateral fruit. Still, documenting effects of chemical fruit thinning by NAA and BA early in the season when sampling was done allows interpretation of the gene expression data.

Fruit removal

Work was done in three consecutive years on Gala and Golden Delicious. Fruit were removed from trees after June drop by cutting, leaving the pedicel stub attached to the branch. This was marked and analyzed for abscission. Abscission of the majority of the pedicels occurred five days later. We dissected abscission zone tissues from a separate set of pedicel stubs at 2h, 8h, 1d, 2d and 4d after fruit removal. Two controls were the adjacent segment of the pedicel stub not containing the abscission zone, and abscission zones from non-removed fruit pedicels. Experiments utilized three temporal replicates. These samples were used in gene expression profiling using the microarrays developed in Objective 2.

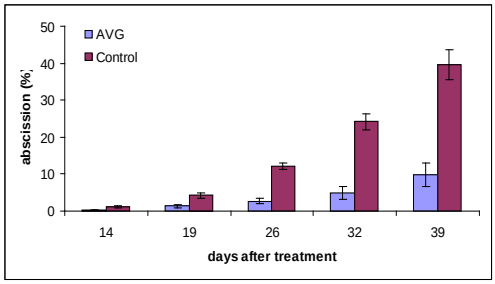
Results from this analysis are available at *http://vannocke.hrt.msu.edu/public/fruitremoval.xls*. This analysis identified many genes that were upregulated or downregulated in the pedicel abscission zone at various times after treatment. However, nearly all of the identified genes were also similarly upregulated or downregulated in the adjacent, non-abscission zone control tissues. Potentially, this suggests that abscission is initiated as a localized *response* to regional changes in gene expression. However, we also identified several genes that did appear to be abscission zone specific, including transcription factors, known components of auxin/ethylene signaling, and cell-wall modifying genes. We are concentrating on a subset of these that we suggest have a particularly important function based on their identities:

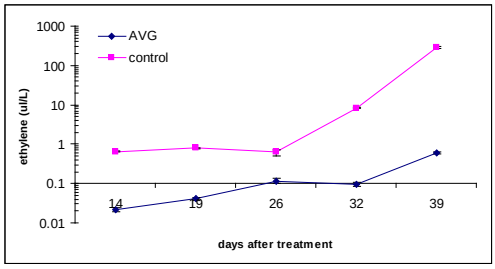
Gene i.d.	**Response**	**Identity**	**Presumed function**
MD4C492040	2h	MdHAE, a HAESA-like gene	Ethylene-independent abscission
MD4C054590	2h	MYB-class transcription factor	Gene regulation
MD4C506930	2h	HB-1 transcription factor	Gene regulation
MD4C464220	2h, 4h	WRKY transcription factor	Gene regulation
MD4C514850	4h	polygalacturonase	Cell wall degradation
MD4C515220	4h	ABA response element binding protein	ABA signaling

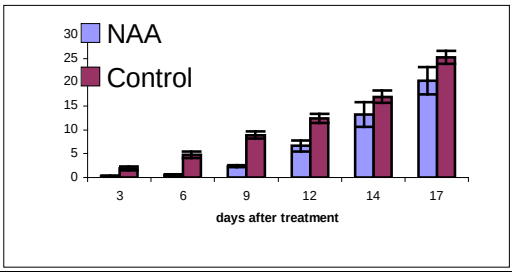
MD4C458550	24h	Pectate lyase	Cell wall degradation
MD4C503670	24h	PHD finger family protein	Gene regulation
MD4C496200	24h	Auxin-responsive SAUR protein	Auxin signaling
MD4C401830	24h	Auxin-responsive SAUR protein	Auxin signaling
MD4C501810	24h	MYB-class transcription factor	Gene regulation
MD4C191660	24h	ACC oxidase	Ethylene signaling
MD4C230030	48h	Auxin-response factor	Auxin signaling
MD4C2400230	48h	AUX1-like protein	Auxin signaling
MD4C268730	48h	Calmodulin-binding protein	Signal transduction
MD4C045340	48h	PIN1-like auxin transporter	Auxin signaling
MD4C188060	96h	MYB-class transcription factor	Gene regulation
MD4C509730	96h	SCL-class transcription factor	Gene regulation
MD4C214670	96h	Auxin-responsive protein	Auxin signaling
MD4C421570	96h	SPL-class transcription factor	Gene regulation

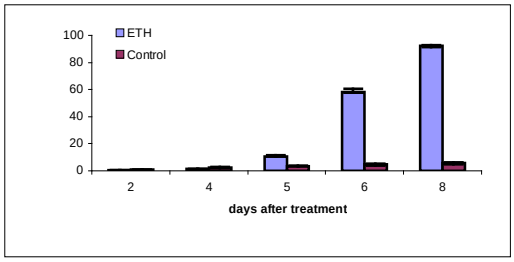
<u>Effects of PGRs on mature fruit retention or abscission</u>

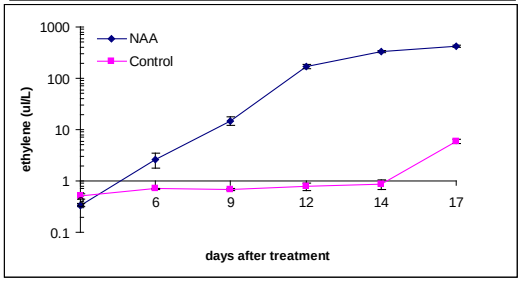
Research was conducted on 20-y-old Spur Macs at MSU. Three different chemical compounds, Retain [active ingredient: aminoethoxyvinylglycine (AVG))], Ethephon [active ingredient: ethephon (2-chloroethyl phosphoric acid)], and Fruitone-N [active ingredient: NAA (naphthaleneacetic acid)] were used in this experiment. Each compound application and control included two replicates (three trees/replicate). All of these three chemical compounds were applied to whole apple tree in this study. Chemical compounds were dispersed in 0.1% surfactant Silwet-77 immediately before canopy application. All of the control trees were sprayed with 0.1% surfactant Silwet-77 at the same time with treatments. Retain was applied one month prior to expected harvest date at 70mg/L active

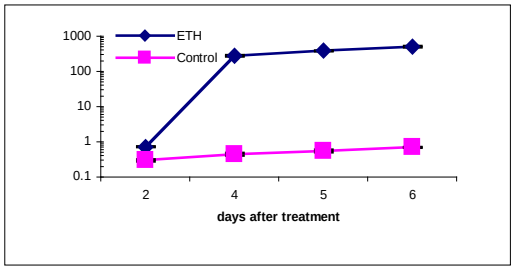












ingredient. Ethephon was applied two weeks before anticipated harvest data at 300 mg/L active ingredient. Fruitone-N was also applied 2 weeks before anticipated harvest date at 20mg/L active ingredient.

Fruit abscission and ethylene measurement were carried out starting from one day up to six weeks after treatment. Thirty fruits from three trees per treatment were collected for ethylene measurement at intervals of 5 to 7 days after retain application, or 2 to 3 days after Ethephon and Fruitone-N application. The number of abscised fruit was counted at each time point as above. Total abscission was expressed at a percentage of total initial fruit load. Abscission zone samples were collected into liquid nitrogen in the above time points.

AVG and NAA significantly influenced both ethylene evolution and fruit abscission. Ethephon promoted almost total fruit drop within 8 d of application in both years. These samples serve as important standards for the interpretation of microarray gene expression data, because they are associated with documented PGR effects.

RESULTS AND DISCUSSION

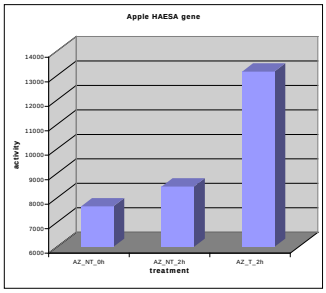
To recap, we developed a model for fruit abscission involving the interaction between two endogenous PGRs, auxin and ethylene. Specifically, at a very early stage in abscission we have found changes in the activity of a number of genes that participate in mediating auxin signal transduction, and this preceded observed changes in the activity of several genes that function in ethylene signaling. Taken together with a variety of studies of the effects of bloom and postbloom thinners, and with very recent findings in basic plant biology, our results allow us to propose a model for the initiation of flower and fruit abscission. In this model, loss of directional auxin transport through the abscission layers triggers enhanced ethylene signaling in abscission layer cells, culminating with activation of genes that promote cell separation.

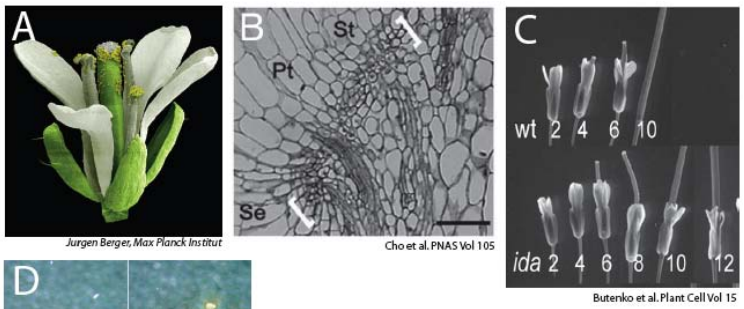
We found that among the first genes to be activated in the abscission zone upon fruit removal are a subset of genes associated with carbohydrate modification. The presumed role of these genes in abscission is to degrade the cell wall, allowing for cell separation. This was initially confusing, because in the system used for this study, separation of abscission layer cells takes place much later (3-4 d after activation), and previous studies demonstrated that a large variety of cell-wall-modifying genes became active only late in abscission. However, a possible scenario is that the early-induced carbohydrate-modifying genes participate in generating a signaling molecule that acts as an initiator of abscission. Hypothetically, degradation of the cell wall contributes to an extracellular pool of small oligosaccharides, some of which are well-known signaling intermediates in other pathways such as defense response. Analogous to the defense pathway(s), this could result in initiation of ethylene production and coordinated advance of the abscission process. Though highly speculative, this idea is supported by two recent findings: 1) Our observation that an *ACO* gene is induced at a later stage of abscission, suggesting activation of ethylene signaling, and 2) Recent findings from Michael McManus' lab (Ann Bot, Oct 2007) showing that abscission depends on a mobile signal, generated in the stele of the pedicel, that works with ethylene to promote abscission. In fact, vasculature is considered to be a main route of polar auxin transport; disruption of auxin flow resulting from a variety of cultural manipulations or environmental trauma could somehow act as a trigger to generate this signal.

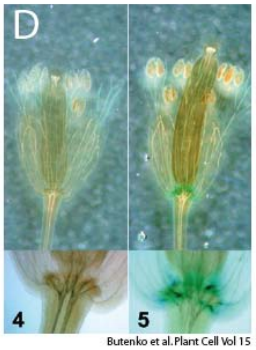
In contrast to expectations, we identified numerous abscission-associated genes that, based on identity, are presumed to act as positive regulators of auxin signaling. Our model predicts that initiation of abscission disruption of auxin flower results in decreased auxin in the abscission zone, but the activation of such genes indicates exactly the opposite! One possible explanation is that auxin

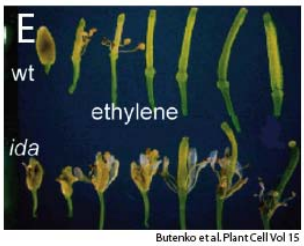
transport is indeed disrupted as expected, and this results in the accumulation of auxin in the disrupted region simply because it can't be exported. We have yet to analyze several experiments that should shed light on this apparent paradox.

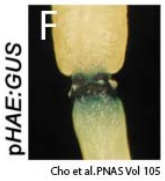
Ethylene-independent mechanisms of fruit abscission. We identified a gene that we call *MdHAE* as an abscission early-response gene. This gene was activated within 2 hours of fruit removal in the pedicel abscission zone (below, left). *MdHAE* is the apple counterpart of an abscission-control gene called *HAESA* identified in the reference plant Arabidopsis.



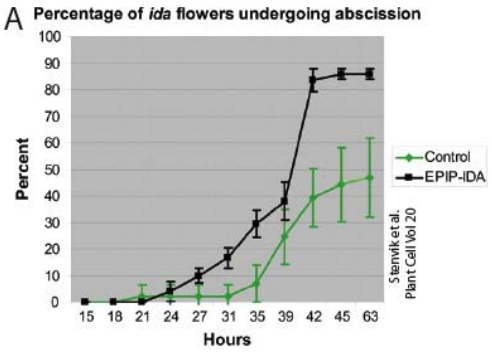




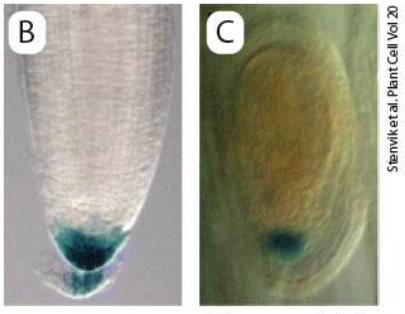




Arabidopsis does not shed fruit but does abscise flower petals. Flower petal shedding closely follows pollination, and takes place through activation of an abscission zone found at the base of the petal (panel B, at right). By looking for mutations that delay petal loss, researchers have identified several genes required for promoting abscission. One of these is called *IDA* (C). This gene becomes activated in the abscission zones just after pollination (D) and produces a small protein, which undergoes proteolysis to generate a small peptide. This peptide is thought to act as the molecular signal to activate other genes required for cell-cell separation. Interestingly, *IDA* seems to act independently of ethylene signaling (E), and so defines a previously unrecognized 'pathway' of abscission. Like apple *MdHAE*, *HAESA* is also activated in the abscission zone (F). *HAESA is though to represent the receptor for the IDA signaling molecule.*



Interestingly, researchers also found that a synthetic peptide corresponding to the active peptide of IDA (EPIP-IDA) could accelerate petal abscission when applied to the flower (Right, panel A). Moreover, they found that *IDA* is one of several related genes in Arabidopsis, that these additional genes, such as *IDL1* or *IDL2*, become activated in other events associated with cell-cell separation, such as in the root cap or in the abscission zone of the seed (B, C), and that *IDA*-like genes are found in other plants as well.



IDL1 gene activity in the root cap

IDL2 gene activity in the seed abscission zone

This data revealed a previously unknown class of gene with regulatory roles in abscission. I suggest that the homologous *MdHAE* gene in apple has a role in promoting flower and fruit abscission, and that derived synthetic peptides from apple counterparts of IDA-like proteins might be useful for thinning.

Executive Summary - Auxin and ethylene dynamics in the abscission zone

The goal of this three-year project is to analyze patterns of gene expression in the flower- and fruit-pedicel abscission zone under various abscission-promoting circumstances, in order to understand the dynamics of auxin and ethylene signaling that trigger abscission, and potentially to gain some information that could be used to design better thinning strategies.

We approached this by designing numerous field experiments that are known to influence flower or fruit abscission. We carried out these experiments in each of the years of the project, and in those cases where the treatments had a substantial effect on abscission, we saved the dissected abscission zone tissues for analysis. Our analysis tool was a DNA microarray, which we designed and constructed in collaboration with a company named Combimatrix. This is now a commercial product that is available from that company.

Among our most exciting findings to date:

- We see rapid induction of an apple gene related to *HAESA*, a gene from Arabidopsis that works in an ethylene-independent pathway of abscission involving a little-studied, abscission-promoting peptide. This leads to the possibility that apple thinning could be promoted by synthetic bioactive peptides!
- A subset of cell-wall modifying genes induced very early in the abscission processes. We hypothesize that these could promote the synthesis of small oligosaccharides that act as a signaling molecules to coordinate abscission.
- Induction of ACO oxidase in the abscission zone. We hypothesize that this gene is the basis for ethylene production that turns on cell-wall-modifying genes.
- Induction of genes thought to be auxin-responsive, suggesting a rise in auxin levels or auxin sensitivity. This is confusing and counter to our expectations that auxin signaling decreases in the abscission zone upon induction.

We encountered three blocks during the course of the project. The first was related to the field work. Some of our treatments, especially the post-bloom thinning trials carried out at MSU, did not affect abscission in the anticipated manner. Thus, many experiments carried out in the first year had to be repeated in the second, and ultimately the third year. Second, we found that the public genomic data for apple that we needed to construct the microarray was disorganized, filled with artifacts and frequently misannotated. Cleaning this up required almost a year of effort on my part. Third, we had some quality control problems with the microarrays. Some of our replicate analyses did not yield high-quality data, and could not be used, forcing us to confirm much of the results by laborious low-throughput techniques.

Genomic technologies have progressed quickly in the past three years, and microarray-based approaches are now mostly obsolete. Current high-throughput sequencing technologies are far more sensitive and comprehensive than microarrays in identifying the type of changes in gene activity patterns that we are interested in, and offer an exciting opportunity to expand the approach. Funds supported half-time effort by a graduate student, Lingxia Sun. The work will be further developed into one of the three research chapters of Lingxia's PhD thesis, and she plans to graduate at the end of the spring '09 semester. Consequently, we have to submit the work for publication before that time to get the reviewer feedback required for the thesis defense. So, we are continuing to work on the project and expect the unfinished aspects of the project to be tied up within the next five months. The work has already resulted in a publication in *Plant Physiology*, one of the two most highly cited plant research journals, and will be the basis for application to the USDA AFRI for major funding to extend the work.