Week of 06-02-2013

Main goal of the week is to clone part plasmids for various GTs and promoters.

- GTs include UGT75B2, NP_567471, Q9LR44, UGT74E2, UGT74D1.
- Promoters include pBAD, pTrc, pTet, and a constitutive promoter provided by the Anderson Lab.

Primers arrived on 05-30-2013. BS66 and BS67 were diluted to 100micromolar by adding about 320 micro liters of miliQ water to both of them (miliQ water obtained by Ramya from I don't know where). Master Mix was created by Hojae Lee.
PCR tube was labelled GT-04. 49 microliters of master mix were added, and 0.5 microlitters of both primers. This particular PCR 1452bp and requires an extension time of about 25-30sec.

*Thermocycler was programmed to have an annealing temperature of 50C and an extension time of 1min. (Started at 10:50am).
*PCR finished at 12:45pm.

PCRs for GT1 and GT4 were found in righty B, on the infinite step at 4C

2 Gels were poured, and all 16 samples ran at 150V (20 microliters + 4 microliters of loading dye +GG). Images saved on the iGEM2013 dropbox (Names: 2013_06_02_0606 and 2013_06_02_0607).

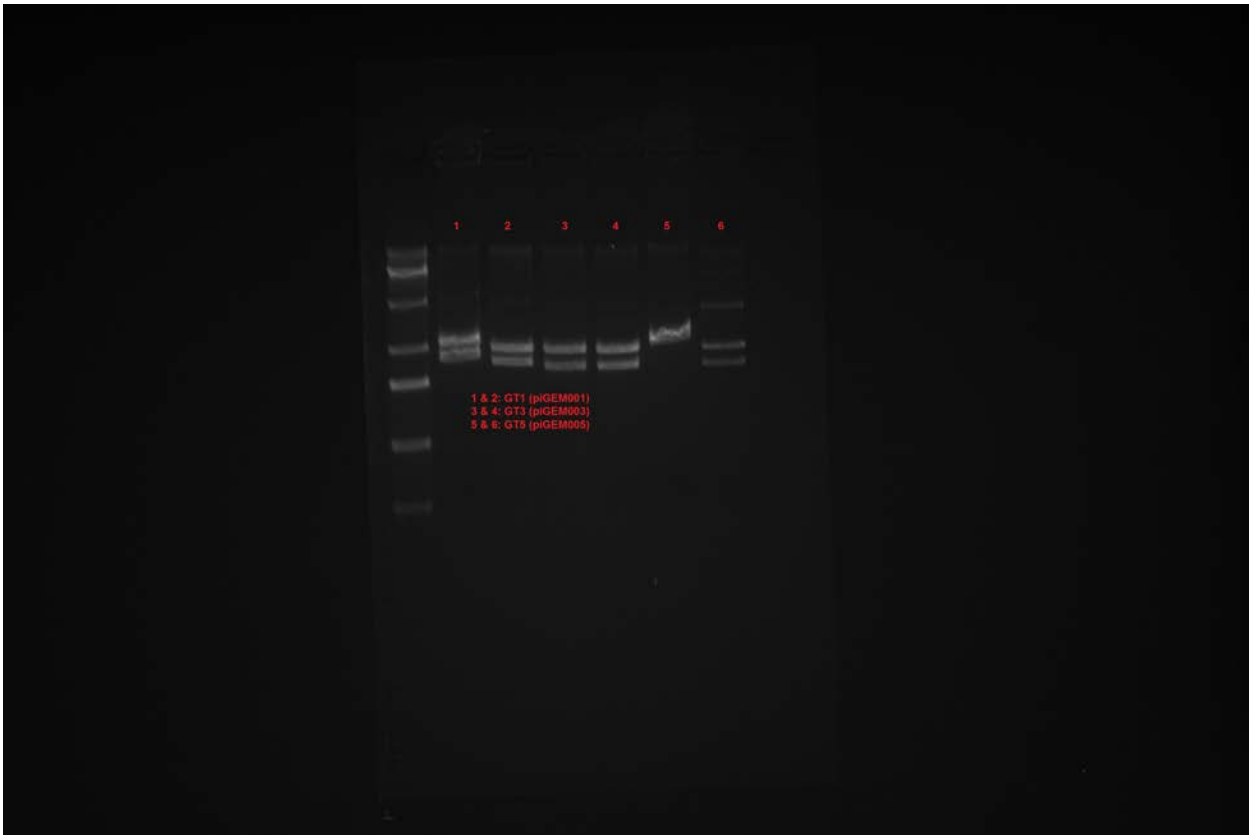
Conclusions from gels: No bands are decent enough for extraction. GT4 will be kept discarded for the time being.

Tranformations/Plating of GT 1,2,3,5 and promoters===

GT1 (labelled M 1+2+3)
GT2 (labelled WT 4+5)
GT3 (labelled WT 6)
GT5 (labelled WT 8+9)
pBad-strong RBS (labelled PR1)
pBad-2A (labelled PR2)
pBad-2A-extended (labelled PR3)
pTrc_strongRBS (labelled PR 6+7)--> Re-GG by Roy
pTrc_2A (Labelled PB 1+2) --> Re-GG by Roy
pTet-2A (labelled PB3)
pCon-2A (Labelled pCON-Oligo Anneal) -- GG by me on 06-03.

* GT1,2,3,5,pBad-strong, pBad-2A, pBad-2A-Extended, and pTet-2A, were transformed into TG1 cells (chemically competent) at 1:00p. Placed for recovery in 100microliters of LB @37C for 1hr. Around 2:30, 15 microliters of recovered transformation were plated in LB/CAM plates and placed in 37C incubator.

*Results in this gel suggest that GT's parts plasmids are correct. We will proceed to sequence verify and assemble cassettes.



===Sequencing of parts plasmids===

The following parts plasmids were sent for sequencing with AW39 and AW38 pre-mixed with 1 microliter of 100micromolar oligo, 9 microliters of ddH2O, and 5 microliters of plasmid.
Labelling: piG001,003,005,006,007,012.

==06-05-2013==

===Plate Analysis===

Transformations containing GT assemblies only, showed about 10 colonies each (all white).

Transformations containing FMO or P450 only, showed many colonies, above 60 (all white).

Cotransformations of FMO and P450 with GTs initially showed no colonies, about 4 hours later colonies were visible.

*Colonies for the single transformations were picked, and cultures in 5ml of amp or kan media respectively.

*Colonies for the co-transformants were also picked, and grown in kan+amp media. We forgot to check for the type of promoter on each co-transformant; so expression of some GT's may not exist due to lack of inducer.

==06-06-2013==

===First growth of Cotransformations===

Overnight cultures were removed from 37 Shaker at 9:00am.

*piG19+ZNR459: Correspond to pbadGT3+p450 uninduced and shows a relatively "normal color" (white and cloudy) but with some small blue crystals which settle to the bottom of the tube.

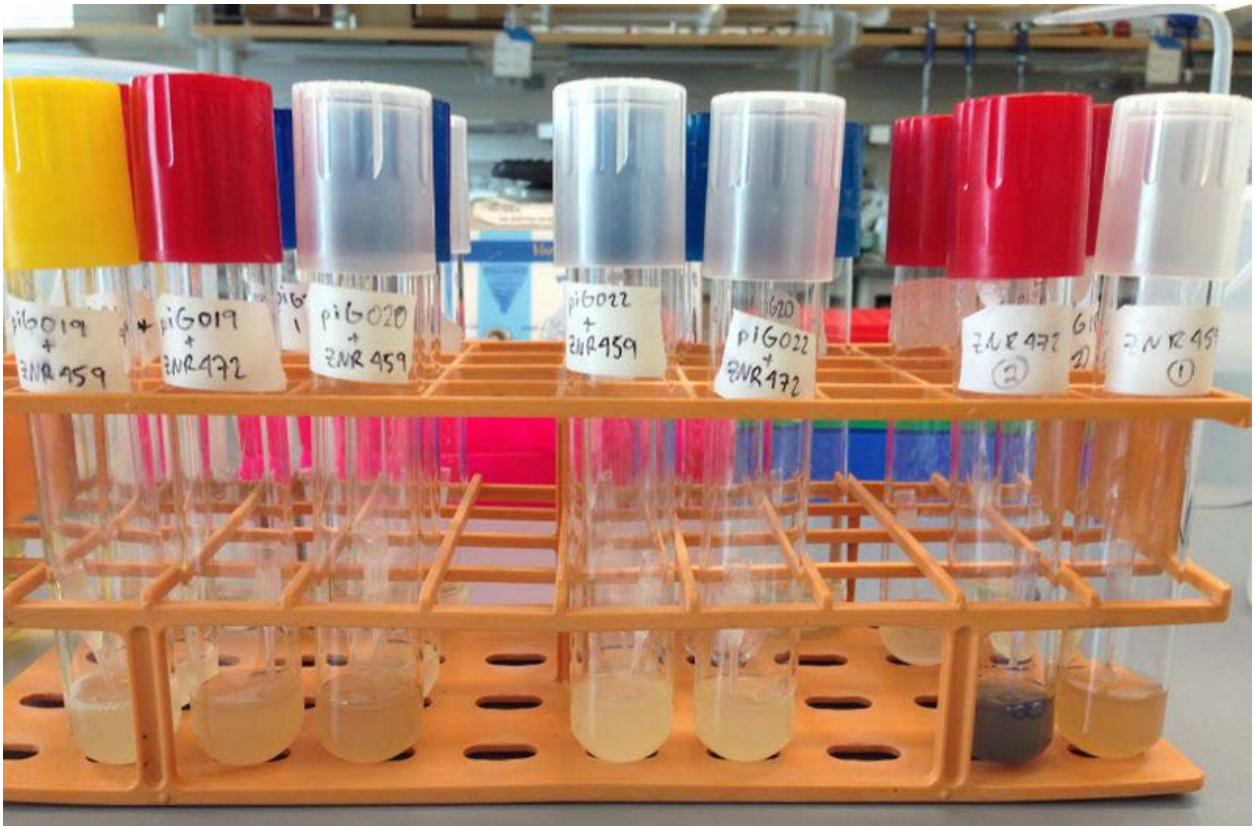
*piG19+ZNR472: Corresponds to pbadGT3+FMO uninduced and shows a darker/brownish color than with p450. No Crystals at bottom.

*piG20+ZNR459: Corresponds to pbadGT5+p450. Brownish-redish color. No crystals.

*piG22+ZNR472: Corresponds to pConGT3+FMO. shows normal white/cloudy color and no crystals.

*piG22+ZNR459: Corresponds to pConGT3+p450. Shows normal white/cloudy color and no crystals.

Refer to the following picture for more accurate representations of results.



G-Blocking Glu

Notes:

- Will probably have to design them as 3A-3B parts with MBP as part 3B. Prior literature have had to express them with MBP to improve solubility.
- Will need to find rice genomes (or extract them from berkeley bowl rice?)
  - Find Genome extraction protocol from plants.
- Maybe ask british teams to get us Woad Genome?
- Arboretum?

Need to discuss/plan using BW27783 rather than TG1 cells to perform the multi-gene cassettes.

Questions to ask Terry/John:
Risk Assessment. All parts of the project are up in the air. Should we be thinking of alternative projects?

06-11-2013

[edit]Microscope Usage

Turn On microscope (switch on the bottom-back-left of microscope). Turn On lamp (Box on top of the shelf - and let it warm until the bulb sign stops blinking). Turn on computer if need be and log in as Zach.

- 20X objective does not use oil.
- All others, a drop of oil can be applied by lightly squeezing bottle while being completely vertical over the objective.

We will be using condenser ph3. Change by using the closest 2 buttons on focus dials on the right of the microscope.
Microscope starts with a completely closed shutter. Open it all the way by clicking the closest black button on the left of the flat surface above the reflector (below the screen and to the left).
Dial below eye-pieces adjusts reflected light brightness.
Farther pair of buttons on the focus dial on the right of the microscope change the fluorescence settings. We will be using TR or texas red for m-Kate.
Finally, you can turn on or off reflected light and fluorescence light with the two buttons on the right of the microscope.
Also, the dial on the left of the microscope splits light between eye pieces and computer.
Software's name is AccioVision 4.7

[edit]FMO+piG022

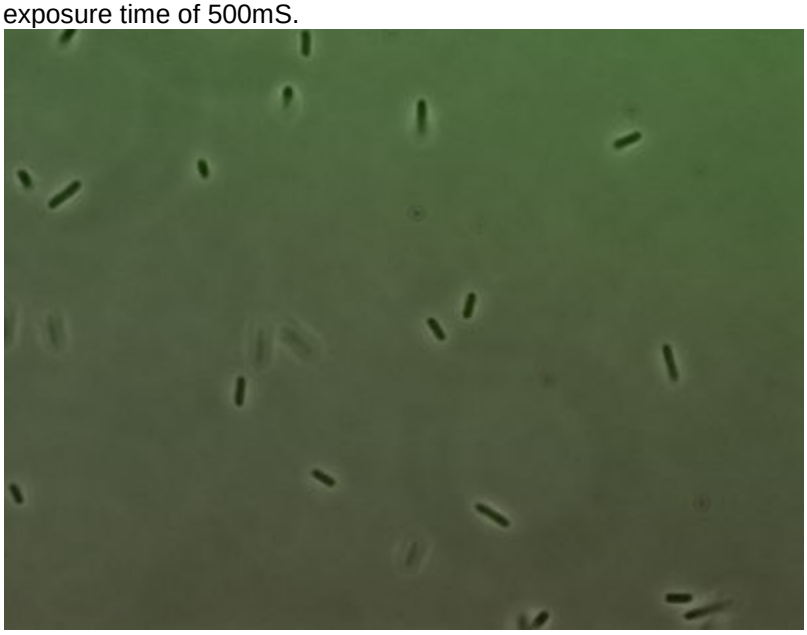
Co transformed FMO and p450 (ZNR 379 and 380) with piG22 as well as single transformations of all plasmids.
Plated co-transformations in Kan/amp plates, piG22 alone in Kan plate, and ZnR379/380 in amp plates.
This time, variable to be tested include High and Low tryptophan as before, induction with arabinose as before, repression with glucose as before, and "N" for no induction or repression.

Repression of p450 expression with arabinose and glucose

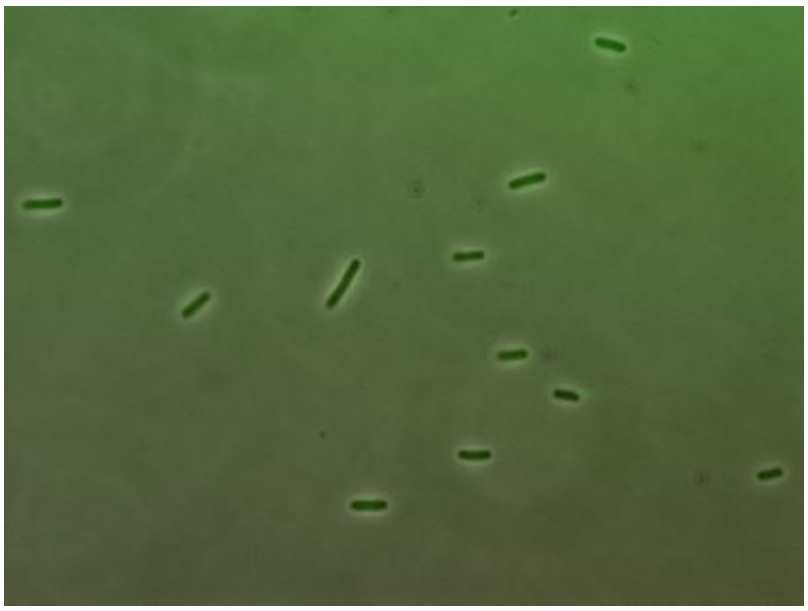
Yesterday's inoculation of p450 alone cultures did work. These tubes were left in the incubator and replicates were created to follow the experiment with the rest of the tubes.
The following picture was taken at 4:30pm which corresponds to about 18hrs of incubation.

- Picture depicts the repression of p450 expression with the addition of both arabinose and glucose.

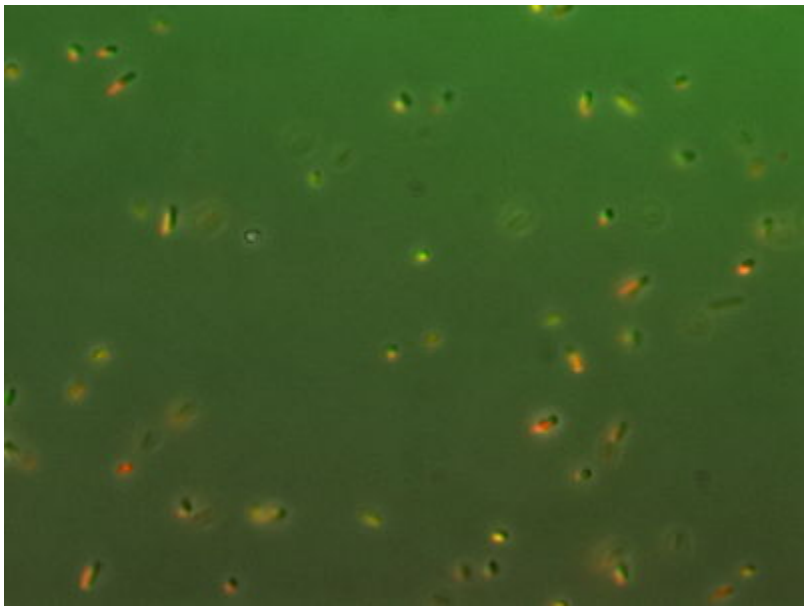
To confirm the repression, pictures were taken with microscope under Texas Red settings, with an exposure time of 500mS.



- p450 with high tryptophan and arabinose added.



- p450 with high tryptophan and glucose added.



- p450 with high tryptophan and nothing added.

In addition we ran the samples on tcan and saw no RFP on those with arabinose or glucose, but significant RFP with sample that had nothing added. Results are here.

piG22 Co-transformations

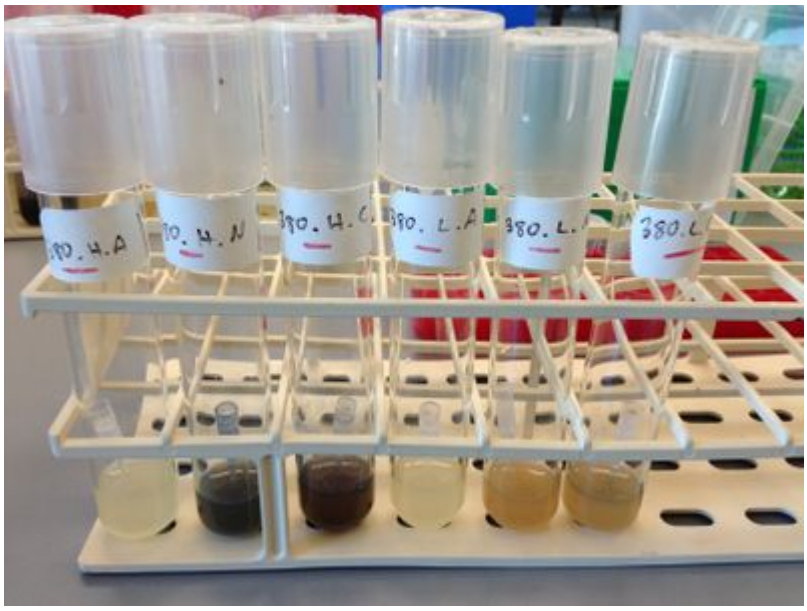
5ml culutures for 22/F/P cotransformations with no arabinose or glucose were removed from 30C at 9:20am.

- 500ul of each were transferred to 1.5ml tubes and spun at 1400xg for 1min. Supernatant was discarded and pellets were observed under green light. RFP was easily observed in F or P controls. All other tubes were debatable.
- Pellets were re-suspended on 250ul of PBS.

[edit]TECAN data of piG22

A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
22HN	22LN	22PHN	22PLN	22FHN	22FLN	FLN	FHN	PHN	PLN

- Row B had 1:2 dilutions of all samples. Dilutions were made with 100ul of PBS and 100ul of sample.



- Picture of samples was taken at 10:45am prior to tecan.

Making more PGGs

PGG006, 062, 078, 083 were inoculated from the glycerol stocks onto a 24well block.
A1 and B1 --> PGG006
A2 and B2 --> PGG062
A3 and B3 --> PGG078
A4 and B4 --> PGG083

- Cultures were incubated at 37C and then miniprepped. All minipreps were stored in the GG box in our -20.

[edit]Retried TECAN

Removed the cultures again and re-tried TECAN since the color had changed significantly to "more blue".

E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
22FLN	PLN	FHN	FLN	22FHN	22HN	22LN	22PLN	PHN	22PHN

- Row F had 1:2 dilutions of all samples.

[edit]06-14-2013

[edit]Meeting Notes

Prof. Anderson questioned the necessity of bio-synthesizing indican instead of just feeding it. He also reminded us of some alternative reporter systems such as B-gal, X-gal, S-gal.

- Perhaps have to cultures that when combined they go from white to blue

Prof. Dueber suggested beginning the search for more things that can be glycosylated.
Conclusions:

- Production of toxics
  - Do a growth experiment to find if indigo is toxic.
  - Continue trying to produce indican.
  - Find a second toxic pathway.
- Rapid Sensor Read-Out.
  - Kinas
  - Modulated Enzyme (B-Glu, B-gal, etc).
- Co-culured
  - Export
  - Import

Need to: Design fluorescence vs indigo experiment. Design primers to get promoters. Design primers to mutate FMO, P450, and GT. Design primers for TnaB cloning. Disgn experiment for growth curves in presence of various things. Extraction of indican and proof of GT activity. Finish p15A stuff.

[edit]06-15-2013

No work performed.

[edit]06-16-2013

No work performed.

[edit]Week of 06-17-2013

Designed constructs with mutant FMO and P450 (piG121, and piG122 respectively). Planned growth curve experiment.

Will utilize MOPS (Prepared on 06-21) and express FMO, P450, and mutant FMO, mutant P450.

MOPS with different salts: NaBr, NaCl

MOPS with different food sources: Glycerol and Pyruvate (to avoid repression of our promoters).

Checked if mutant versions of FMO and P450 worked on 6-22

Week of 06-23

Designed multigenes combining all GTs including ItGT.

Prepared LB from scratch:

10g Triptone
5g Yeast Extract
10g NaCl (Also did NaBr)

Ran growth experiment at various levels of induction (0%, 30%, 60%, 100%). Induced immediately after innoculations and grew at 30C for 15Hrs. With time points every hour.

Confirmed that our constitutive promoter from Anderson lab is repressed in the presence of a variety of sugars. Will have to re-clone everything with actual constitutive promoters.

Different Arabinose concentrations to induce pBad.

0% - No arabinose added

50% - 125ul of 0.5M stock solution into 5ml of culture.

100% - 500ul of 0.5M stock solution into 5ml of culture.

Week of 06-30

Designed piG 271 through 276, cloned and test digested.

Started considering in-vitro testing of GTs.

Took a trip to the botanical gardens and obtained Isatis tinctoria (woad).

Week of 07-01

Tested B-Glucosidases with Indican. Showed B-Circulans B-Glucosidases

Week 07-08

Attempted RNA extraction for the first time on various using triazol reagents. And reverse transcribing with superscript 3 from life technology.

Week 07-15

Planed and cloned multigene assemblies with various GT's along with FMO.

Re-extracted RNA using the RNeasy kit. Used an RNA chip and nanodrop to quantify the RNA extraction. Concentrations ranged from 700ng/ul to 1900ng/ul.

Detailed explicitly RNA extraction protocol in 07-19-13.

Planned out degenerate primers

Week 07-21

Used Gene Racer kit to generate full length cDNA from Indigofera suffruticosa and Polygonum tinctoria. Ran first sent of PCRs with degenerate primers on cDNA libraries.

Week 07-28

Planning for a potential blue/white screen. Cloning FMO with random GTs and expressing them in library form to count background white colony formation.
0% white colonies observed. A lot of variability on the intensity of the blue.

Week 08-04

Re-ran PCRs as hotstarts and used Topo-Cloning system to get transcripts into Sequencing Vector. Sequencing results for first round of Topo-Clones returned no GTs. A lot of random plant transcripts were found via homology.

Week 08-11

Obtained NTGT2 and cloned as part of our homologus set of proteins.
Trip to advanced biofuels process unit.

Week 08-18

Contemplated potential strain engineering approaches and started working with OleD. Re-made MOPS for indigo titer experiments. Second round of TOPO Cloning and new primers have been designed. Found our first GT.

Week 08-25

Continued cDNA library screening. Found new Glucosyl transferase by using specific primers for suffruticosa on tinctoria.

Week 09-01

Found a new GT from Polygonum Tinctoria. Continued cloning strategies to obtain full length of all GTs and cloning OleD ASP for in vitro testing.
Began work on presentation materials.

Week 09-08

Continued cloning work of all GTs for in vitro testing. In vivo tests of plant GTs showed no indican production.