A Study of the Genetics of *Arabidopsis thaliana and Saccharomyces cerevisiae*

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In this research project the genetics of two different organisms were studied in the context of two different projects. Different strains of *Arabidopsis thaliana,* which had been bred to a homozygous knockout state using T-DNA and crossing, were studied for frequency of genomic insertion by means of Q-PCR, quantitative dual-target PCR, and gel electrophoresis. Control samples of known insert frequency (provided by Virginia Tech) were analyzed using these methods, and their data was analyzed to provide a standard against which to compare the unknown samples. Dozens of plants were analyzed for genomic insert frequency and the data obtained was inserted into a spreadsheet so that the data can be further analyzed and shared with the UNPAK consortium.

Additionally, the genetics of *Saccharomyces cerevisiae* yeast were studied to determine the function of the *brr6* gene. Different strains of the yeast, which had different point mutations in the *brr6* gene, were studied under fluorescence microscope to determine the gene's effect on survival, division, and morphology. It was determined that the mutations that were studied had some impact on morphology, and that some did impact survival. In addition, some *brr6-1* mutants were transformed with a fluorescence-marked tubulin gene and frozen down for later study.

INTRODUCTION

Arabidopsis thaliana

Arabidopsis thaliana is a quickly growing flowering plant that serves as a model organism for many plant-based genetic research programs[1]. A vast majority of the plant's genome has been annotated, and it has properties which make it an ideal model genetic organism, including fast growth, ease of care, prolific reproduction, and ease of genetic manipulation[1]. The role of the H-SC researchers is to genotype the homozygous mutant SALK lines of *A. thaliana.* These mutants have had their DNA altered with target DNA inserts (T-DNA), and then bred to a homozygous knockout state. T-DNA is a segment of DNA which is randomly inserted into the genome of the plant. Its purpose is to disrupt a gene in that plant, and hopefully produce a phenotypic change. These changes can then be studied to determine the function of the altered gene. The DNA of these plants is isolated, and then the levels of T-DNA are compared to a target gene, in this case, *PetC,* to determine whether each line has no inserts, one insert, or multiple inserts. It is necessary to know which lines have multiple inserts, because if those plants show phenotypic changes, it will be impossible to know which disrupted gene is responsible for the changes. The levels of DNA are compared and analyzed using real-time PCR and QD-PCR with gel electrophoresis.

Saccharomyces cerevisiae

Saccharomyces cerevisiae, known by the common names of baker's yeast and brewer's yeast, is a budding fungus that serves as a model organism for the study of genetics[2]. These experiments focused on the function of the *brr6* gene, which is important in the formation of nucleoporins. The goal of the research was to determine the role *brr6* plays in cell morphology, survival, and division. This was accomplished by studying *brr6* mutants with single point mutations. These mutants all involved the changing of a cysteine residue to another amino acid. Cysteine was chosen because it is believe to be important to brr6 localization and function. Table 1 shows all the mutants used, and how they were altered. Changing the cysteine to an arginine resulted is an opposite charge, whereas changing to a serine resulted in a charge neutralization.

Table 1: Mutations and Alterations

Mutant	Residue Changed	New AA
#3	None	None
#8	96	Arginine
#11	96	Serine
#14	115	Arginine
#17	115	Serine
#20	118	Arginine
#23	118	Serine
#26	124	Arginine
#29	124	Serine

METHODS

Arabidopsis thaliana

The plants samples which were studied were either harvested and dried at another college in the consortium and sent to our lab, or they were grown from seeds in our lab. In order to study the genome of the plants for T-DNA insert frequency, the plants needed to be processed to isolate DNA. First a plant tissue sample was placed into an Eppendorf tube, and ground using a plastic pestle. The sample was then covered with 250 µL of "Short" buffer. "Short" buffer was produced by combining 12.5 mL of 0.5 M EDTA, 25 mL of 0.2 M Tris Cl, 50 mL of 2 M LiCl, 2.5 g of SDS, and then diluting with DI water to a total volume of 250 mL. After addition of the buffer, the tissue was ground again. Following the second grind, 250 µL more "Short" buffer was added to the tube, and the sample was vortexed. The sample was then spun at 13,000 rpm for 10 minutes. Next, 400 µL of the supernatant was removed, added to 400 µL of isopropanol in a new tube, inverted to mix, and vortexed again at the same speed, for the same time. This spin settled the DNA from the sample on the wall of the tube. After spinning the supernatant was poured off. The DNA was then washed with 70% ethanol and spun again for 5 minutes. After washing the ethanol was poured off and the sample was allowed to dry. Finally the DNA was eluted in DI water and the sample was frozen at -20°C for future use.

After preparation the DNA samples were used for one of two experiments. In order to perform QD-PCR samples were prepared in thin walled PCR tubes. To each tube the following components were added: 10 µL Mean Green Master Mix, 4 µL DI water, 4 µL sample DNA, and 0.5 µL each of PetC upper primer, PetC lower primer, T-DNA upper primer, and T-DNA lower primer. The samples were then run in a PCR reaction for 27 cycles (the actual number of cycles varied from experiment to experiment, but 27 was found to be the ideal number of cycles after trial and error). After the PCR, gel electrophoresis was performed on the samples in order to separate the T-DNA from the reference gene (*PetC).* These gels were then photographed and the brightness of the bands was determined using ImageJ software and put into a Google Document to be shared with the other colleges in the consortium.

Some samples also went through a real-time PCR. These samples were prepared in a similar fashion to the QD-PCR, except each sample only got one set of primers (either T-DNA or PetC) and a different Master Mix was used. The samples were placed into a 96 well plate and run in a real-time PCR thermocycler. The computer then graphed out the reaction, and these graphs were compared and contrasted with the QD-PCR data. The purpose of the real-time PCR data is to serve as a comparison point for the visual data from the gel. That way the visual ratio obtained from the gels can be standardized with the actual levels of DNA in the samples.

Saccharomyces cerevisiae

The study of the *brr6* gene began with several mutants that were prepared previously by the Dartmouth Medical School Cole Lab. Each of the mutants studied had a single mutation of one amino acid in the brr6 protein. In addition, each mutant also expressed green fluorescence protein (GFP) in the nuclear membrane. To begin each of the mutants was separately removed from its stock yeast peptone dextrose (YPD) plate and grown for varying times in SC liquid media which lacked leucine and contained dextrose. These mutants would only show their effects when grown in media containing dextrose (+dex) and lacking leucine (-leu). If grown on media containing galactose (+gal), the mutations would not reveal themselves. This is because the yeast contains two copies of the *brr6* gene, but only one has been mutated. If grown on galactose, the normal copy of *brr6* would overpower the mutant copy. By growing the yeast on dextrose, the normal copy of the gene is turned off. After growing the yeast were immobilized on agar slides and sealed with a molten solution containing equal parts of paraffin, Vaseline, and lanolin. Some samples were also treated with a cell wall stain, which would appear blue under the fluorescence scope. The yeast were then observed under fluorescence microscope. They were compared with wild type yeast with GFP in the nuclear membrane to study any deformities.

In addition to the study of *brr6* gene, a transformation experiment was also performed. In that procedure a *brr6* mutant that was produced by Christian Lehman and Alan Fish was taken and transformed with a plasmid containing a fluorescently-labeled tubulin gene. This transformation was performed by adding 50 µL of yeast in media to tubes, then to each adding 5 µL salmon sperm DNA which had been denatured. Then 1 µL of the desired plasmid was added to each of three tubes, with a fourth left as a control. To each tube was added 300 µL of PEG/1xTE/1xLiAc solution. The cultures were then incubated without agitation for 30 minutes at 30°C, then for 20 minutes at 42°C. Next the samples were spun down briefly, and the supernatant removed. Finally the cells were resuspended in 100 µL DI water, and then plated on YP –leu +gal plates. After incubation the successful transformations were removed, grown up again in liquid media, and then mixed with 50% PEG and

frozen via submersion in liquid nitrogen for future study.

RESULTS

Arabidopsis thaliana

Among the plants that were tested this summer were six controls from VA Tech: *Columbia, Atpen1-1, Atpen1-2, Atpen1-3, B2/B3,* and *Jar1.* Each of these mutants had a known number of T-DNA inserts: *Col* with none, *B2/B3* with two, and the rest with one each. These plants were processed by the above described methods and run out on agarose gels. The following images were produced. The first image contains four experimental plants, which were not related to the VA Tech controls. Note that T-DNA is approximately 600 bp in size and *PetC* is 500 bp.

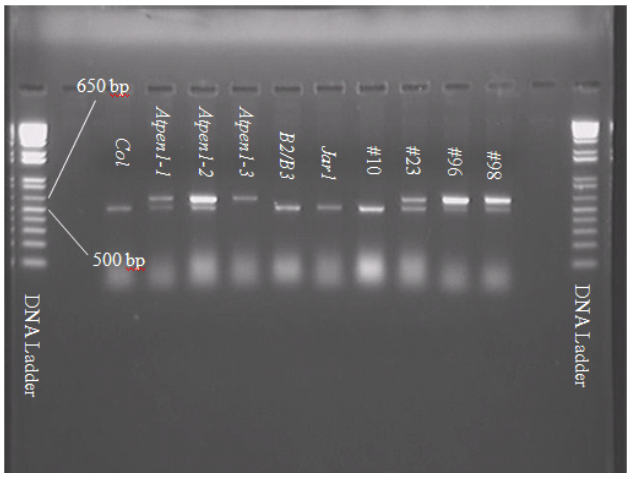


Fig 1. Sample 1

The second figure was produced using 27 cycles and its purpose was to redo the *B2/B3* control from above because the sample above lacked a T-DNA band, which means the above sample must have been damaged somehow, because it is known that *B2/B3* has two T-DNA inserts. For the redo four new *B2/B3* plants were selected and their leaves were processed as described in the "Methods" section (sample 2 was not used in the gel). Also in the gel are samples which had been previously processed through ImageJ software, with their theoretical brightness ratio in parentheses.

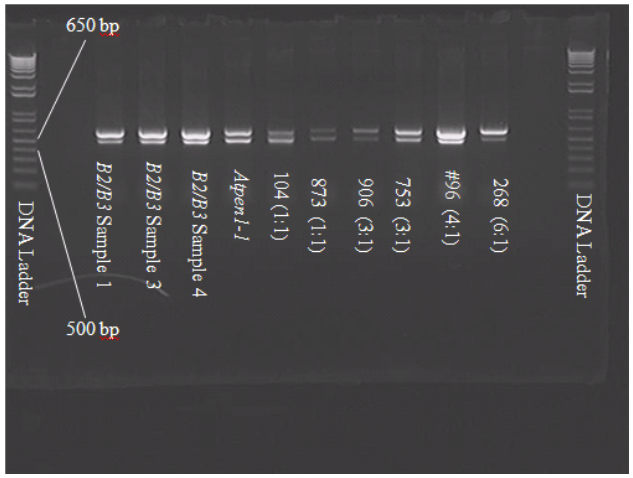


Fig 2. Sample 2

Additionally, *Col, Atpen1-1, B2/B3,* and *Jar1* were also put into a real time PCR reaction, to see actual DNA levels and compare them to the gel pictures. The purpose of the real-time experiments, both with these controls and the experimental plants, was to provide a certain and reliable comparison to the ratios of T-DNA to *PetC* in each sample. While this method is much more reliable than the gel method, it is much too expensive in terms of time, labor, and resources to use for every experimental plant. Therefore it is used to help perfect the gel method, which is a much more efficient way to study the experimental plants. Figures 3-6 (at end) show the real time graph.

These controls gave a clear picture as to what ratio of brightness should be observed in the gels. They also gave a clear image as to the relationship between what the real time data should look like compared to the T-DNA:*PetC* ratio. The data produced showed that twice the amount of T-DNA (meaning a double insert), appears as approximately a 1.2:1 ratio according to the ImageJ data. A single insert is an approximately a 0.95:1 ratio. While the data seems to suggest that 1.2 may be the minimum for declaring a sample as "multiple insert", the great variation in ImageJ data suggests that the method may be flawed. Further study is required of the gel pictures and ImageJ procedure to distinguish multiple inserts. The method and data, as they stand, are uncertain at best. The real time data, however, appears to be quite consistent. A single insert shows T-DNA appearing one cycle before the *PetC*. A double insert shows T-DNA and *PetC* appearing at the same time. The clarity and consistency of the data suggests that real-time PCR reactions may be much more reliable in determining the multiplicity of inserts in the experimental strains.

In addition to the above control, dozens of experimental plants with unknown numbers of inserts were studied, and the raw data produced was fed a Google Document Spreadsheet to be shared with the other researchers in the consortium. The data will be fully analyzed once a surefire system for classifying multiple inserts is established. The raw data from these plants is found in Table 2 (at end).

Saccharomyces cerevisiae

In addition to pictures, a mitotic survey was taken of several of the mutants. In these surveys the cells were counted and categorized to determine how division and survivability was affected (see Table 3). Table 4 only looked at the division of two other mutants, and whether or not the dividing daughter cells separated properly after division.

	Single & Normal	Dividing Normally	Morphology Altered	Nucleus Malformed	Abnormal Division	Failed Separation	Total	Notes
Nup-60 GFP 7/16/12								
Number	157	5	1	0	0	0	163	
Percentage	96.32%	3.07%	0.61%	0.00%	0.00%	0.00%	100.00%	
Nup60 GFP Brr6-1 7/16/12								
Number	21	4	2	41	5	72	145	
Percentage	14.48%	2.76%	1.38%	28.28%	3.45%	49.66%	100.00%	
Mutant #8 7/16/12								
Number	71	0	6	9	5	63	154	
Percentage	46.10%	0.00%	3.90%	5.84%	3.25%	40.91%	100.00%	
Mutant #14 2 hours 7/16/12								(Greater than 90% of cells were dead)
Number	8	0	0	0	0	0	8	
Percentage	100.00%	0.00%	0.00%	0.00%	0.00%	0.00%	100.00%	
Mutant #17 7/16/12								(Greater than 50% of cells were dead)
Number	11	2	0	4	1	2	20	
Percentage	55.00%	10.00%	0.00%	20.00%	5.00%	10.00%	100.00%	

Table 2

	Single	One Bud	Clumps/ Chains	Total
Nup-60 GFP 7/25/12				
Number	130	65	52	247
Percentage	52.63%	26.32%	21.05%	100.00%
Nup60 GFP Brr6-1 7/25/12				
Number	81	84	82	247
Percentage	32.79%	34.01%	33.20%	100.00%

Table 3

Analysis

Arabidopsis thaliana

The data produced from the gels varied greatly in quality and consistency. Some plant samples were poorly preserved and therefore did not produce viable results. Additionally the quality and brightness of the gel pictures was extraordinarily difficult to reproduce consistently. These problems, combined with the uncertain accuracy and reliability of the ImageJ software, make it very difficult to rely on the brightness ratios to determine multiplicity of T-DNA inserts. Although reconciling this data with the real time graphs does help in clearing up some inconsistencies, it is still difficult to classify some of the plants. Repetition of trials and further comparison of gel data and Q-PCR data may allow these plants to be more easily classified in the future.

While the data from this project may not be as conclusive as would be hoped, it does show some significant trends. It revealed that a multiple insert will most likely not appear as a 2:1 insert, but can be as low as 1.2:1. Some examples that were clearly multiple inserts, such as SALK_111268 have ratios ranging from 1.3 to 2.0 without background brightness removed, or from 1 to 3.6 with the background brightness removed. It does seem conclusive that anything above 1.5 can be considered a definite multiple insert. This conclusion comes from comparing the gel data to the real-time data of SALK_111268 and B2/B3.

It may be wise to learn more about the ImageJ software and figure out its limitations. It may be that nothing is wrong with the software: it may just be natural variations in the samples causing the disparity in the data. Trying out similar software may

be worthwhile. Another problem is whether it is better to remove the background brightness or leave it. Data with the background left in appears to be more consistent and to have fewer errors. These are just some points that may be studied to make the data more consistent and reliable.

Saccharomyces cerevisiae

The *brr6* mutants displayed varying levels of deformity. Yeast #3 contained no mutation, and served as a control. Mutant #8 showed deformity rates similar to the control, however it had a much higher rate of failed separation. Mutant #11 showed greater levels of morphology error, but the levels were not as high as *brr6-1,* which is a version which is known to be heavily deformed. Mutants #14, #17, #20, #23, and #26 showed very high rates of deformity and very low rates of survival. Even when grown on dextrose for as short a time as two hours, between 50% and 95% of these cells would die. Mutant #29 showed a similar level of deformity as Mutant #11. Overall these particular mutations to *brr6* either produced minor morphological and mitotic errors or resulted in the death of the mutants. None of the mutants produced consistent error on a reasonable level without dying off.

Mutant	Effects
#3	No effect. This *brr6* gene is the same as wild type, with the addition of the GFP. No defects were detected.
#8	Slightly higher rates of morphology and nuclear envelop formation error were discovered. There was a very high rate of failed separation of daughter cells (>40%).
#11	Some instances of failed separation were observed. A great majority of the nuclei were malformed.
#14	These cells were essentially unable to survive on dextrose. Greater than 90% of the cells were dead, and the few survivors were severely deformed.
#17	These cells were essentially unable to survive on dextrose. Greater than 50% of the cells were dead, and the survivors were severely deformed.
#20	These cells showed few defects, but also suffered from very low survival rates.
#23	These cells had a very poor survival rate and often failed to separate.
#26	These cells had a very poor survival rate and also suffered from incomplete division.
#29	These cells displayed some instances of failed separation and some morphological and nuclear malformation.

Table 4: Some of the specific problems that appeared with each mutation

It seems that alterations made to residue positions 115 and 118 have a high probability of causing cell death and high rates of deformity. For future study these cultures should be repeated and they should all be carefully counted to find exact rates of deformity. Time constraints only allowed a few of these mutants to be studied in that fashion.

Conclusion

Arabidopsis thaliana

The data produced during this research will serve to further the UNPAK program and will eventually lead to a fully annotated library of the *A. thaliana* genome. This will lead to a better understanding of plant genetics and may lead to great advancements in plant genetics and could result in the production of genetically superior crops. Cataloging these gene knockouts and their effects will greatly advance this project and is an important part of its fruition.

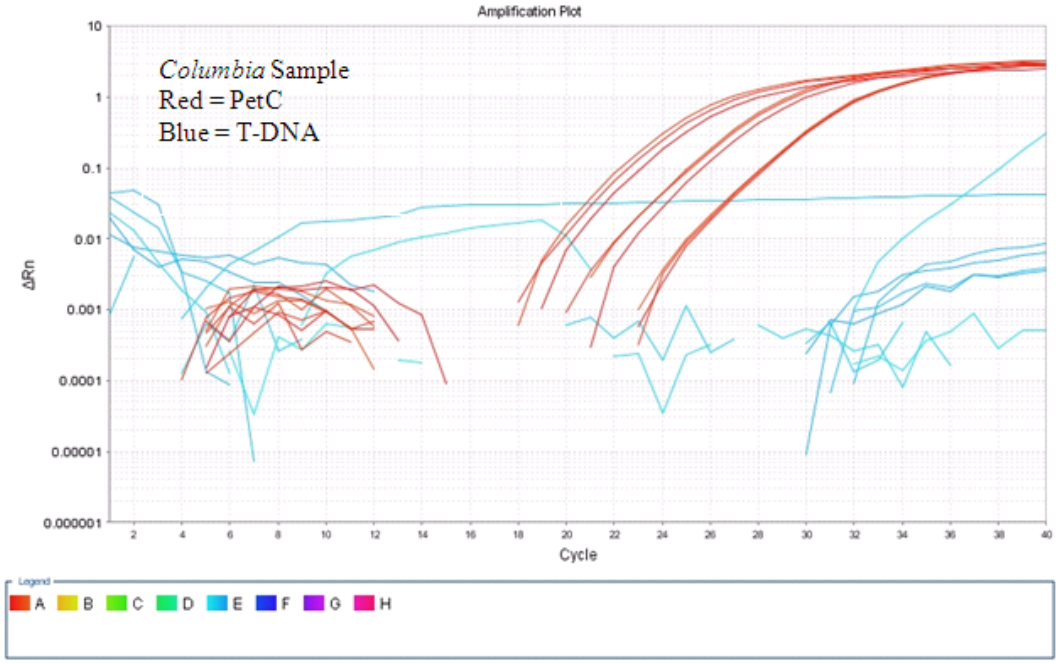
Saccharomyces cerevisiae

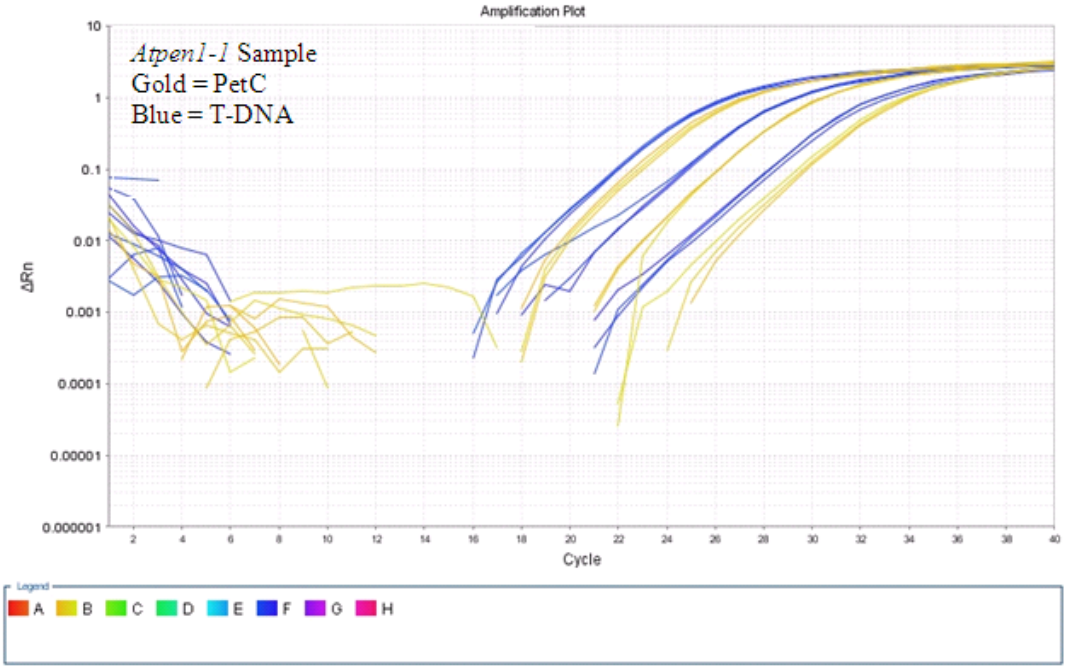
The study of the *brr6* mutants is important for understanding the mechanism by which yeast divide and reproduce. While it is known that *brr6* is involved in the production of nucleoporins, its exact role in that process is unknown. While these mutants were not wholly successful, they were a step in the right direction. An ideal mutant would be one that produces an obvious morphological or mitotic change without compromising the life of the cell. This would allow the study of the brr6 protein and learn more about its function. However this experiment did demonstrate that whatever the exact role of *brr6* is, it is clear that it is critical to cell survival and reproduction, and that alteration of the gene can easily lead to the death of the mutant.

References

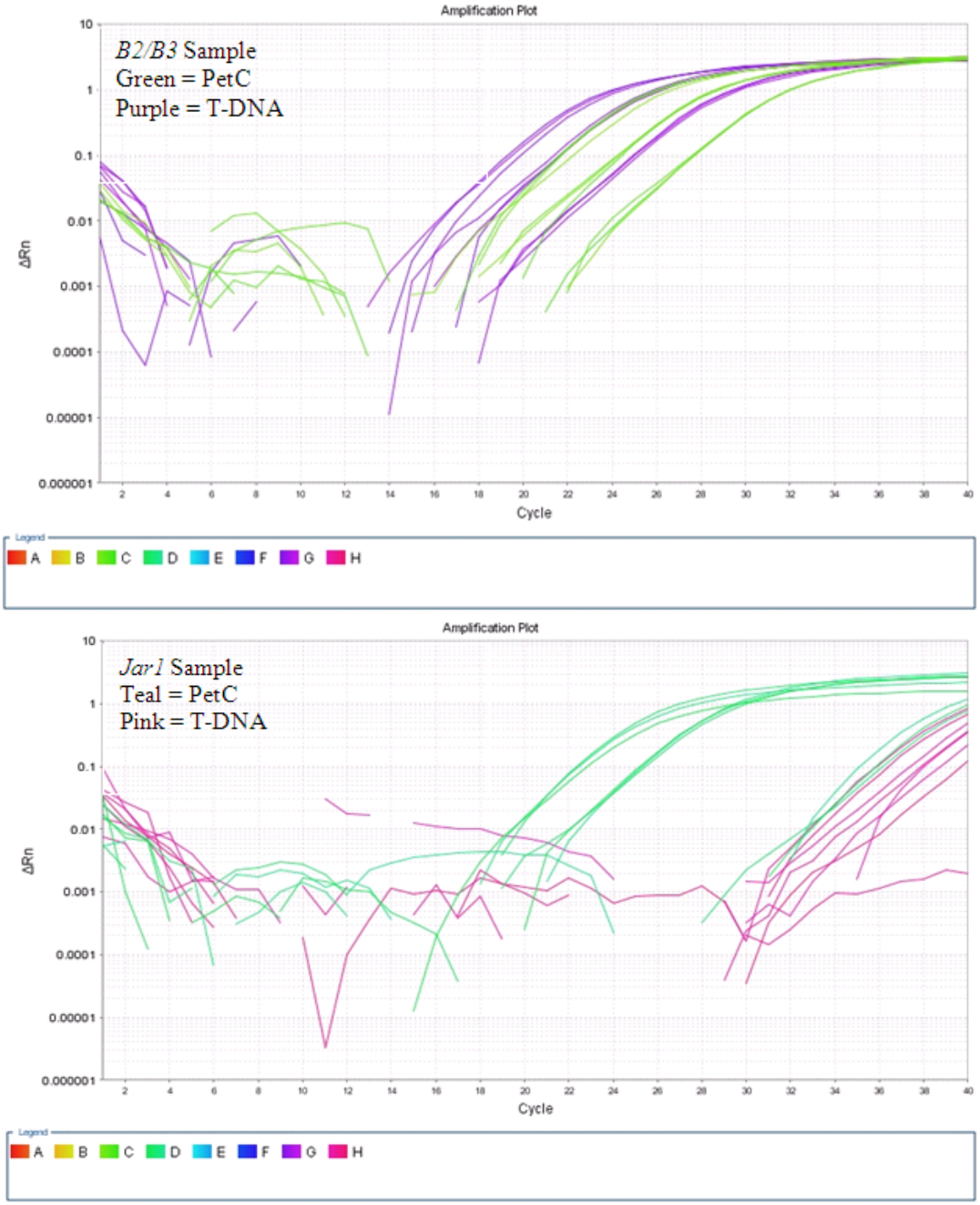
1. National Institutes of Health (NIH). *Arabidopsis*. Web. 16 August 2012. < http://www.nih.gov/science/models/arabidopsis/index.html>
2. *"Saccharomyces* spp." *Doctor Fungus.* Web. 21 August 2012. <http://www.doctorfungus.org/thefung//Saccharomyces.php>

Supplemental Tables and Figures





Figs. 3-6


Amplification Plot
B2/B3 Sample
Green = PetC
Purple = T-DNA
ΔRn
Cycle
Legend
A B C D E F G H
Amplification Plot
Jar1 Sample
Teal = PetC
Pink = T-DNA
ΔRn
Cycle
Legend
A B C D E F G H

	Top Band	Bottom Band	Background	SALK #	Tag Number		Top-Background	Bottom-Background	Ratio Top:Bottom (minus background)	Direct Ratio Top:Bottom
7-3-12.jpg	224.136	132.682	94.133	75753			130.003	38.549	3.372409142	1.689
	231.136	157.5	96.65	75796			134.486	60.85	2.210123254	1.468
	232.364	117.955	96.517	75879			135.847	21.438	6.336738502	1.97
	131.5	98.273	97.3	75797			34.2	0.973	35.14902364	1.338
	185.455	102.364	98.15	75841			87.305	4.214	20.71784528	1.812
	127.864	139.364	100.467	75873			27.397	38.897	0.704347379	0.917
	102.318	105.909	100.15	111091			2.168	5.759	0.376454246	0.966
	116.909	114.636	101.6	111104			15.309	13.036	1.174363302	1.02
	230.864	151.955	104	111105			126.864	47.955	2.645480138	1.519
	185.182	105.05	102.017	111140			83.165	3.033	27.42004616	1.763
7-5-12.jpg	56.947	76.974	56.439	111173			0.508	20.535	0.024738252	0.74
	57.211	57.789	54.193	111176			3.018	3.596	0.839265851	0.99
	137	82.211	63.228	111184			73.772	18.983	3.886213981	1.666
	74.421	84.921	65.614	111188			8.807	19.307	0.456155798	0.876
	253.684	105.737	75.228	111268			178.456	30.509	5.849290373	2.399
	72.132	71.632	71.354	111270			0.778	0.278	2.798561151	1.007
	83.868	74.737	71.417	111271			12.451	3.32	3.750301205	1.122
	61.789	66.289	67.733	111281			-5.944	-1.444	4.116343449	0.932
	86.789	70.947	63.567	110906			23.222	7.38	3.146612466	1.223
	65.895	63.026	62.683	110928			3.212	0.343	9.364431487	1.046
7-6-12.jpg	86.938	126.875	85.773	Columbia			1.165	41.102	0.028344112	0.685
	139.75	110.25	87.227	At-pen 1-1			52.523	23.023	2.281327368	1.268
	230.75	158.312	92	At-pen 1-2			138.75	66.312	2.092381469	1.458
	131.75	89.375	89.364	At-pen 1-3			42.386	0.011	3853.272727	1.474
	89.938	167.5	89.955	B2/B3			-0.017	77.545	-0.000219228	0.537
	90.812	134.875	89.924	Jar1			0.888	44.951	0.019754844	0.673
	94.5	212.25	91.955	Pot #10			2.545	120.295	0.021156324	0.445
	165.812	144.938	91	Pot #23			74.812	53.938	1.386999889	1.144
	230.062	122.562	89.978	Pot #96			140.084	32.584	4.299165234	1.877
	230.438	129.562	89.156	Pot #98			141.282	40.406	3.496559917	1.779

7-10-12top32cycles.jpg	230.812	232.25	71.438	B2/B3 Sample 1			159.374	160.812	0.991057881	0.994
	227.812	231.75	72.062	B2/B3 Sample 2			155.75	159.688	0.975339412	0.983
	229.625	231.125	75	B2/B3 Sample 3			154.625	156.125	0.990392314	0.994
	230.625	231.438	74.5	B2/B3 Sample 4			156.125	156.938	0.994819611	0.996
	141.625	211.625	72.375	111104			69.25	139.25	0.497307002	0.669
	73.625	230.25	70	111104			3.625	160.25	0.022620905	0.32
	176.062	207.312	70.812	110906			105.25	136.5	0.771062271	0.849
	214.438	217.125	70.625	110906			143.813	146.5	0.981658703	0.988
	230.812	230.938	70	111268			160.812	160.938	0.999217009	0.999
	230.688	191.938	70.938	111268			159.75	121	1.320247934	1.202
7-10-12bottom16cycles.jpg	87.528	94.583	89	B2/B3 Sample 1			-1.472	5.583	-0.263657532	0.925
	89.722	91.722	90.778	B2/B3 Sample 2			-1.056	0.944	-1.118644068	0.978
	92.028	101.306	93.611	B2/B3 Sample 3			-1.583	7.695	-0.205717999	0.908
	92.972	109.194	95.444	B2/B3 Sample 4			-2.472	13.75	-0.179781818	0.851
	93.667	93.944	94.639	111104			-0.972	-0.695	1.398561151	0.997
	93.306	94.611	95.028	111104			-1.722	-0.417	4.129496403	0.986
	93.5	94.806	97.444	110906			-3.944	-2.638	1.495072024	0.986
	93	93.667	95.333	110906			-2.333	-1.666	1.400360144	0.993
	92.222	95	93.806	111268			-1.584	1.194	-1.326633166	0.971
	92.306	93.833	92.722	111268			-0.416	1.111	-0.374437444	0.984
7-11-12top32cycles.jpg	223.917	190.667	65	B2/B3 Sample 1			158.917	125.667	1.264588158	1.174
	228.833	206.167	65.667	B2/B3 Sample 3			163.166	140.5	1.161323843	1.11
	231.917	190.75	68	B2/B3 Sample 4			163.917	122.75	1.335372709	1.216
	214.25	224.083	67.5	At-pen 1-1			146.75	156.583	0.937202634	0.956
	100.917	129.667	66	111104			34.917	63.667	0.548431684	0.778
	137.833	193.5	64.667	75873			73.166	128.833	0.5679135	0.712
	129.167	125.333	64.333	110906			64.834	61	1.062852459	1.031
	122.667	89.667	66	75753			56.667	23.667	2.394346558	1.368

	230.583	206.25	68.583	Pot #96			162	137.667	1.176752599	1.118
	205.167	159.083	65	111268			140.167	94.083	1.489822816	1.29
7-11-12bottom27cycles.jpg	224.188	154.156	67.188	B2/B3 Sample 1			157	86.968	1.805261705	1.454
	231.656	191.125	68	B2/B3 Sample 3			163.656	123.125	1.329185787	1.212
	231.438	210.062	68.281	B2/B3 Sample 4			163.157	141.781	1.150767733	1.102
	195.406	205.375	68	At-pen 1-1			127.406	137.375	0.927432211	0.951
	115.281	145.969	66.375	111104			48.906	79.594	0.6144433	0.79
	82.312	93.438	65.281	75873			17.031	28.157	0.604858472	0.881
	107.656	79.5	65.438	110906			42.218	14.062	3.002275636	1.354
	203.469	164.625	67.031	75753			136.438	97.594	1.398016271	1.236
	231.344	224.125	69	Pot #96			162.344	155.125	1.046536664	1.032
	215.156	108.75	67.031	111268			148.125	41.719	3.550540521	1.978
7-12-12_27cycles.jpg	114.071	124.714	71.429	110904			42.642	53.285	0.800262738	0.915
	106	83.714	70.286	110861			35.714	13.428	2.659666369	1.266
	200.5	85.286	71	110830			129.5	14.286	9.064818704	2.351
	118.286	108.5	71	110809			47.286	37.5	1.26096	1.09
	171	89.857	70.214	110714			100.786	19.643	5.130886321	1.903
	75.429	74.071	70.214	110713			5.215	3.857	1.352087114	1.018
	122.143	91.286	69.929	110696			52.214	21.357	2.444819029	1.338
	195.214	113.071	72	110691			123.214	41.071	3.000024348	1.726
	218.929	86.571	71.571	110684			147.358	15	9.823866667	2.529
	70.929	72.786	69.571	110610			1.358	3.215	0.422395023	0.974
7-16-12_27cycles.jpg	38.175	29	28.775	110590			9.4	0.225	41.77777778	1.316
	39.025	88.625	33.775	110587			5.25	54.85	0.095715588	0.44
	37.875	39.425	36.725	110563			1.15	2.7	0.425925926	0.961
	65.675	77.025	38.25	110551			27.425	38.775	0.707285622	0.853
	167.95	69.025	49.225	110534			118.725	19.8	5.996212121	2.433
	55.525	57.35	40.525	110520			15	16.825	0.891530461	0.968
	46	40.65	40.3	110440			5.7	0.35	16.28571429	1.132
	254.525	69.325	36.35	110355			218.175	32.975	6.616376042	3.671
7-18-12top.jpg	58.143	56.964	57.107		178		1.036	-0.143	-7.244755245	1.021

	65.107	63.964	63.25		179		1.857	0.714	2.600840336	1.018
	75.107	74.786	68.929		180		6.178	5.857	1.054806215	1.004
	253.821	253.821	72.536		181		181.285	181.285	1	1
	69.75	69.429	68.5		191		1.25	0.929	1.345532831	1.005
	234.929	96.179	67.607		192		167.322	28.572	5.856152877	2.443
	68.071	68.071	65.643		193		2.428	2.428	1	1
	95.75	70.536	66.429		196		29.321	4.107	7.139274441	1.357
	68.571	69.821	68.357		197		0.214	1.464	0.146174863	0.982
	72.321	73.25	64.393		209		7.928	8.857	0.895111211	0.987
7-18-12bottom.jpg	231	125.3	74		211		157	51.3	3.060428885	1.844
	231.233	171.633	76.467		212		154.766	95.166	1.626274089	1.347
	176.633	184.2	75.6		213		101.033	108.6	0.930322284	0.959
	229.833	185.967	76.467		214		153.366	109.5	1.400602744	1.236
	158.933	94.5	75		215		83.933	19.5	4.304256441	1.682
	198.833	89.1	74.133		216		124.7	14.967	8.331662992	2.232
	213.033	105.567	74.333		217		138.7	31.234	4.440673625	2.018
	96.8	93.467	73.533		218		23.267	19.934	1.167201766	1.036
	231.133	105.7	74.333		219		156.8	31.367	4.998884178	2.187
	226.3	100.533	75		226		151.3	25.533	5.925664826	2.251
7-23-12.jpg	119.038	99.346	68.423		221		50.615	30.923	1.636807554	1.198
	115.731	100.885	72.192		222		43.539	28.693	1.517408427	1.147
	232.923	135.038	71.808		224		161.115	63.23	2.548078444	1.725
	158.231	225.5	73.5		225		84.731	152	0.557440789	0.702
	94.462	118.038	71.231		226		23.231	46.807	0.496314654	0.8
	253.962	120.692	72.769		227		181.193	47.923	3.780919392	2.104
	113.577	146.577	70.038		228		43.539	76.539	0.568847254	0.775
	112.846	84.385	69.115		229		43.731	15.27	2.863850688	1.337
	252.346	108.769	70.846		230		181.5	37.923	4.786013765	2.32
	253.692	128.846	71.808		231		181.884	57.038	3.188821487	1.969
7-27-12.jpg	31	31.267	32.4		232		-1.4	-1.133	1.235657546	0.991
	33.2	34.2	36.4		233		-3.2	-2.2	1.454545455	0.971
	36	35.733	36.533		234		-0.533	-0.8	0.66625	1.007
	41	42.6	49.933		236		-8.933	-7.333	1.218191736	0.962
	37.467	38.6	39.2		237		-1.733	-0.6	2.888333333	0.971
	252.26	253.86	46.467		238		205.8	207.4	0.9922854	0.994

	7	**7**							**39**	
	253.667	**254.4**	**44.267**		**240**		**209.4**	**210.133**	**0.996511733**	**0.997**
	56.867	**41.067**	**38.467**		**241**		**18.4**	**2.6**	**7.076923077**	**1.385**
	36.6	**36.267**	**36.4**		**242**		**0.2**	**-0.133**	**-1.503759398**	**1.009**
	34.867	**35.133**	**37.867**		**243**		**-3**	**-2.734**	**1.097293343**	**0.992**

Table 2