

unPAK-ing Gene Function: Using knockout mutants to identify genes that alter life history traits in *Arabidopsis* under heat stress.

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Abstract

Despite having full genome sequences for many species, the functions of most genes in those genomes remain unknown. The genome sequence of the model plant *Arabidopsis thaliana* has been available for fifteen years, yet gene function is known for only about 10% of its 26,800 genes. Gene function is often investigated by generating a mutant that has the gene of interest 'knocked out', so the mutant lacks the gene product. The mutant is then grown under various conditions and phenotyped for traits such as life history or morphology. In some cases, gene function remains unclear because the effects of the knockout mutation are phenotypically visible only under stressful conditions. As part of unPAK (Undergraduate Phenotyping of *Arabidopsis* Knockouts), a collaborative project involving 11 institutions, we are investigating the phenotypic effects of 204 different knockouts in the Columbia genotype of *A. thaliana*. We are comparing the phenotypes of mutants to the unaltered genotype and 10 other *A. thaliana* genotypes representing natural populations. We exposed our plants to short-term extreme heat stress just before the plants transitioned from vegetative to reproductive growth then phenotyped plants from the germination stage through senescence. We have completed growth of the heat stress plants and are now repeating the experimental setup under non-stressful conditions for comparison, to identify genes whose role (absence) is revealed under heat stress. Our results advance the understanding of gene function and interesting mutant phenotypes in the research of plant functional genomics.

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Methods

Planting Design. We grew a total of 204 mutant genotypes and 11 phytometers (control genotypes). We grew the mutants in two independent sets of 122 mutant genotypes with 40 genotypes represented in both sets and 82 genotypes unique to a set. Each set also included the 11 phytometers as a control. Every genotype was represented by multiple plants - 6 replicates per genotype for mutants and 12 replicates per genotype for phytometers. Plants were grown in trays, 12 trays per set, with 72 pots per tray. Each tray contained one replicate of each phytometer and 61 mutants. Mutant genotypes were assigned randomly to each tray such that a single genotype was only represented once per tray.

Growth Conditions. Prior to being sown to soil, the seeds underwent cold-treatment to increase standardization of germination time. Seeds were placed onto moist filter paper in the dark at 4°C for one week. Seeds were planted into moist soil and moved into a controlled environment room (K109) for the rest of the experiment. Two seeds were planted in each pot. Two weeks after planting, each pot was thinned to contain a single seedling. Plants experienced 16h light and 8 h dark at 22°C (except for the heat stress, described below). Trays were watered as needed. Plants were harvested for additional phenotyping after senescence.

Heat Stress Treatment. During the 5th week of the experiment, just as plants reached the stage of bolting, accidental heat stress occurred due to a malfunction in the air circulation system. Without air circulation, temperature increased and decreased dynamically in accord with the activation and deactivation of the light sources. Plants were removed from the growth chamber upon discovery of the problem and were returned after repair, about 7 hours later. The heat stress lasted a total of 14.5 hours, with a max temp of 43°C, which is about 20°C higher than normal. Plants were closely monitored for stress responses in the week following the heat stress.

Phenotypes Recorded. Plant phenotypes were observed throughout the life cycle and the following traits were recorded:

- Germination: For two weeks post-planting, we recorded the date when cotyledons were visible.
- Bolting: When the plant's inflorescence (the part from which its stem and flowering parts grow) reached at least 5 mm, the diameter of the rosette (mm) and the date were recorded..
- Flowering: We recorded the date that open flowers were first observed.
- Number of branches - including basal, main stem, and branches of branches.
- Plant height (cm) from the rosette to the terminal bud of the main stem.
- Number of good fruits (fruits capable of forming normal seed).
- Length (mm) of 3 representative good fruit, chosen haphazardly
- Number of aborted fruits.

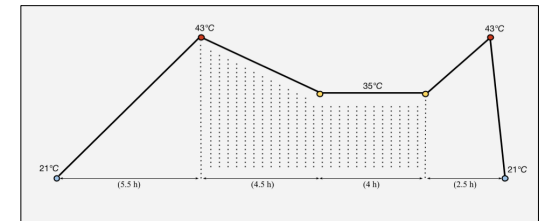


Figure 1. Diagram describing the change in temperature during the course of the heat stress. Lights were off during the dotted area of the graph.

Results & Discussion

Overall effects of knockout mutations compared to the phytometers.

Intuitively, we might predict that knocking out an entire gene would have a dramatic and visibly perceptible effect on the organism. However, we found that average trait values of individuals with a single knockout mutation are not significantly different than average trait values of the individuals from the non-mutated Columbia genotype (Table 1). This apparent robustness in the face of gene knockout suggests that most individual genes may not be critical to normal organismal function. For example, widespread gene redundancy may act as insurance against silencing mutations, while polygenic and epistatic phenotypes may be buffered as well.

In contrast to the lack of difference between mutants and the unaltered Columbia genotype, we found that mutant genotypes differed significantly from the other naturally occurring genotypes for several traits (Trait 1). Average values differed significantly for germination rate in Set 1, and rosette diameter and date to flower in both sets. For these traits in our experiment, single knockout mutations produced phenotypes that were not significantly different from Columbia but were markedly different from phenotypes produced by natural allelic variation. Interestingly, the mutants did not tend to differ from the non-mutants in a consistent way (e.g. always having a "worse" trait value). For example, knockouts flowered sooner than the natural genotypes in Set 2 but later than the natural genotypes in Set 1.

Linking phenotype with genotype for particular knockout genotypes.

In addition to comparing groups of genotypes as above, we also analyzed the variation among all genotypes with ANOVA, performing pairwise contrasts among all 133 genotypes in a Set for rosette diameter and days to flower, both of which had a significant Accession effect. After adjusting *P*-values for multiple comparisons (Holms correction), we examined the group of significant contrasts between the phytometer and mutant lines to determine whether the observed phenotypic difference corresponded to what was already known about gene function.

• Rosette Diameter

- In Set 1, the rosette of mutant *SALK_004769C* was significantly smaller than that of phytometer CS22647 ($P < 0.001$). The mutated gene for this accession was at the *AT1G03600* locus. This gene is known to be expressed during the visible-leaf stages, which corresponds to our observation of having a difference in rosette size.
- In Set 2, the rosette of mutant *SALK_051118C* was significantly larger than that of phytometer CS22618 ($P = 0.001$). The mutated gene for this accession was at the *AT1G71692* locus. This locus is associated with transcriptional regulation and developmental growth.

• Days to Flower

- In Set 1, we found that *SALK_032713C* flowered significantly later, between 3 and 5 days, than any of the phytometers ($P < 0.001$). *SALK_032713C* has a knock-out mutation in the *At1g54450* gene. This gene is associated with meristem initiation and growth and thus may influence the time to flowering.
- In Set 2, we found a significant difference in days to flower between *SALK_032048C* and the phytometer CS22618 ($P = 0.03$). The gene for this accession is *AT5G38310*, which is associated with the flowering stage as well as with petal differentiation and expansion.

Table 1. Sample sizes, means and standard deviations of phenotypes of each set for three groups of genotypes. "Columbia" is the non-altered genome of *A. thaliana* and "Natural" represents the 10 other phytometer genotypes as representative of natural variation.

GROUP ¹		SET 1			SET 2		
		N	MEAN	ST.DEV	N	MEAN	ST.DEV
Germination rate	Columbia	12	1.00 ^{a,b}	0.00	12	0.88	0.31
	Knockout	722	0.96 ^a	0.14	721	0.95	0.16
	Natural	120	0.89 ^b	0.23	120	0.91	0.21
Bolt rate	Columbia	12	1.00	0.00	11	1.00	0.00
	Knockout	720	0.99	0.09	717	0.99	0.11
	Natural	117	0.99	0.09	118	0.99	0.09
Rosette diameter	Columbia	12	20.45 ^{a,b}	5.73	11	19.40 ^a	5.51
	Knockout	712	19.25 ^a	5.74	708	22.92 ^a	6.00
	Natural	116	22.92 ^b	9.24	116	28.05 ^b	11.19
Flowering rate	Columbia	12	1.00	0.00	11	1.00	0.00
	Knockout	714	1.00	0.00	709	1.00	0.00
	Natural	116	0.96	0.20	117	0.99	0.09
Days to flower	Columbia	12	4.67 ^{a,b}	0.65	11	4.73 ^{a,b}	1.01
	Knockout	712	4.72 ^a	1.69	708	4.41 ^a	1.29
	Natural	111	4.49 ^b	1.15	115	5.10 ^b	3.43

¹For a given trait within a Set, group means that do not share a letter are significantly different with $P < 0.05$.

