ABSTRACT

COMPARING MOLECULAR PLASTICITY RESPONSES OF *ARABIDOPSIS THALIANA* RILS TO COLD STRATIFICATION

By Mohamed I. Yakub

Phenotypic plasticity is the ability of genetically identical organisms to produce differing phenotypes in response to environmental heterogeneity. While there are two central models for the genetic basis of phenotypic plasticity few studies have identified the genetic and molecular basis of plasticity. I used three recombinant inbred lines (RILs), of *Arabidopsis thaliana* generated by crossing two natural genotypes, and obtaining progeny after recombination, for a global gene expression study. Of the three RILs, one bolted (transition to reproductive tissue) later if the seeds experienced a cold-stratification period relative to the seeds that did not experience this cold-treatment (sensitive-positive), one bolted earlier if the seeds experienced the cold-stratification relative to the non-cold treated seeds (sensitive-negative), and one did not change when it bolted regardless of the cold stratification of seeds (homeostatic). I found different sets of genes differentially expressed in the three RILs suggesting that the response to the same regime of cold-stratification is via different pathways. The homeostatic genotype increased transcription of protein metabolism genes in response to cold stratification; whereas the plastic genotypes differentially expressed genes involved in stress response. This cold-stratification which is a common dormancy breaking cue for these plants may also be perceived as a stress, as the plants differentially expressed stress genes. Thus both gene expression and perception of environment may be involved in plastic responses to environmental heterogeneity.

COMPARING MOLECULAR PLASTICITY RESPONSES OF *ARABIDOPSIS THALIANA* RILS TO COLD STRATIFICATION

by

Mohamed I. Yakub

A Thesis Submitted
In Partial Fulfillment of the Requirements
For the Degree of

Master of Science - Biology

at

The University of Wisconsin Oshkosh
Oshkosh WI 54901-8621

October 2010

COMMITTEE APPROVAL

Advisor

1 Dec 2010 Date Approved

Member

11/30/10 Date Approved

Member

12/1/2010 Date Approved

PROVOST
AND VICE CHANCELLOR

12/17/10
Date Approved

FORMAT APPROVAL

11/19/2010
Date Approved

ACKNOWLEDGEMENTS

Many people have helped me throughout the course of my graduate career, and without them my work would be incomplete in some form. First and foremost, I would like to thank my advisor Dr. Lisa Dorn and committee members Dr. Bea Holton and Dr. Toivo Kallas, as well as Dr. Leonie Moyle (Indiana University) for everything I've learned in my path to be a better biologist. The 'Gene Team' was essential in all the work that went into this project: I would like to thank Matthew Rubin, Megan Nelson, Vai Lor, Jackie Mischka, George Weir IV, Kendra Cassanova, Lisa Degeronimo, and Ashley Winker for help with planting, lab-work, and discussing the results, along with other aspects of this project and the many many hours spent in lab and the greenhouse. I'm very grateful to my cohort of graduate students as well as the entire department of Biology and Microbiology, and Office of Science Outreach for making graduate school at UWO a positive, rewarding, and fabulous experience. I'm very grateful to Anne Murphy, Jeremiah Henning, and Shane Bierma for pushing me to complete this thesis and for being great friends. And thank you to everyone else who helped me, mentioned here or not.

In addition, this experience would not have been possible without the support of my family: Thank you Mom and Dad for encouraging me to follow my academic career, and thank you to my sibs Riswana Kadernani, Akbar Yakub, and Mohamed-Kassam Ismail for listening to me talk about my project countless times when you didn't want to listen, and for pushing me to succeed.

TABLE OF CONTENTS

Page

LIST OF TABLES

Following Page 50

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

1.0 Phenotypic Plasticity

Phenotypic plasticity is the ability of an organism to alter its phenotype in response to environmental heterogeneity (Bradshaw 1965; Scheiner 1993; Schlicting 1986). Phenotypic plasticity has been observed in many different species across kingdoms under varying environmental conditions: for example reptiles' sex determination is dependent on the incubation temperature of the egg (Crews et al. 1994; Shine 1999); the snail species *Physella virgata virgata* changes its growth rate and reproductive pattern in the presence of its crayfish predator (Crowl and Covich 1990); the colony form of the fungus *Aureobasidium pullulans* differs when incubated at different temperatures and different light cycles (Slepecky and Starmer 2009) plants change their internode length in response to shading (Gonzalez and Gianoli 2004; Sultan 2000). This response to the environment can be a change in the chemistry, physiology, development, morphology, or the behavior of the organism (Agrawal 2001).

Different populations may exhibit different plasticities for certain traits across environments, depending on genetic variation within and between populations. The direction as well as strength of plasticity in multiple different environments can be estimated by raising the same genotype, i.e. clones in different environments, and measuring the trait or phenotype in these environments (Dorn, Hammond-Pyle, and Schmitt 2000; Weinig 2000; Yakub, Nelson, and Dorn, *n.d.* see Appendix A).

To illustrate phenotypic plasticity, the mean value for the trait of interest within each environment for each genotype can be graphed as reaction norms (Scheiner 1993). For example, Figure 1-1 shows three reaction norm graphs illustrating plasticity that are plots of the mean phenotype of each of three genotypes with two alleles in different environments. The lines connecting the means of each genotype across environments, called reaction norms, show the direction and strength of plasticity for that genotype, not a continuum between the environments. Sloped reaction norms indicate plasticity while flat ones indicate homeostasis or no plasticity. Figure 1-1A shows three hypothetical genotypes exhibiting no plasticity for the phenotype in these two environments as shown by the flat reaction norm lines. The hypothetical genotypes in figure 1-1A have genetic variation for the phenotypes, because they differ in their mean phenotype value within each environment. Figure 1-1B shows three genotypes that have genetic variation for the phenotype of interest because they differ in their mean within environments, and exhibit plasticity because they differ in their mean across environments, as shown by sloping reaction norms. The hypothetical population of genotypes shown in figure 1-1B does not display genetic variation for plasticity because the slopes of the reaction norms for each genotype are the same, i.e. parallel. Alternatively, in figure 1-1C, the hypothetical population of genotypes has genetic variation for the phenotype of interest and exhibit plasticity, but these genotypes differ in their degree and direction of plasticity indicating that they have genetic variation for plasticity, because the reaction norm slopes differ from each other.

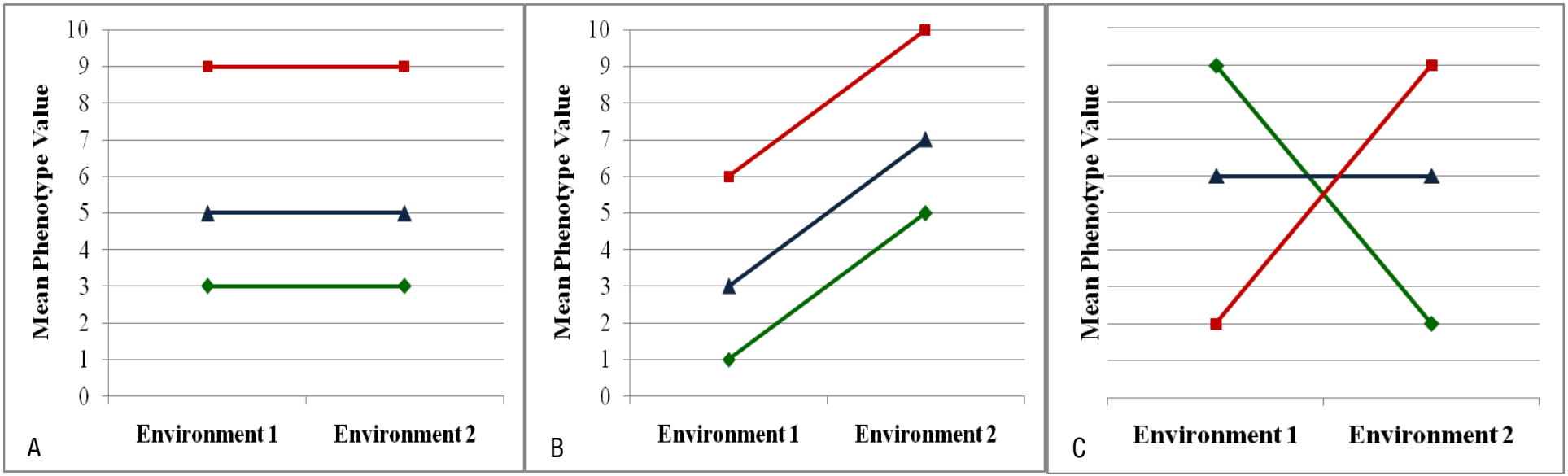

Figure 1-1: Reaction norm graphs illustrating plasticity (or lack thereof) and genetic variation. Each of these graphs represents a hypothetical population of three different genotypes, shown with three differing colors on the graphs. Populations exhibit plasticity if the reaction norm line between the two environments is sloped, and they exhibit homeostasis if the reaction norm line is straight. The line between the two environments does not represent a continuum between the two environments; rather, it's a visual illustration to show direction and strength of plasticity.

A: Genotypes do not exhibit plasticity, illustrated by the straight reaction norm lines are straight. This population has genetic variation for the phenotype, illustrated by the phenotype means differing within each environment.

B: Genotypes exhibit plasticity, seen because the reaction norm lines are sloped. Genotypes also have genetic variation for the phenotype, because the phenotype means are different within environments. Population has no genetic variation for plasticity, illustrated by parallel reaction norm lines.

C. Two of the three genotypes exhibit plasticity, illustrated by the sloped reaction norm lines, and one genotype shows homeostatic response across these environments. This population has genetic variation for the phenotype because the phenotype means are different within environments; this population also has genetic variation for plasticity, because the reaction norm lines are in differing directions and have different slopes.

Theory suggests that genetic variation in a population results in genotypes with plasticity allowing them to achieve optimum phenotypes across multiple environments, i.e. it is adaptive (Donohue *et al.* 2003; Dudley 2004; Poorter and Lambers 1986; Valladares and Pearcy 1998). Few studies have identified adaptive plasticity, possibly because few have tried. However, adaptive plasticity has been observed for a number of traits in response to shading (Dudley and Schmitt 1995 and 1996; Schmitt and Wulff 1993; Smith 1982) as well as in induced resistance against herbivores or pathogens (Agrawal 1998; Agrawal *et al.* 1999). If plasticity is adaptive, meaning plastic genotypes have highest fitness in all environments, then selection should favor plastic genotypes so that non-plastic genotypes would be culled from a population resulting in less genetic variation over time until only the optimum genotypes were present in any population. Without genetic variation for plasticity these genotypes would then not evolve further, but rather become stabilized. However, studies done using *Drosohila* in as early as 1966 showed that standing genetic variation at multiple loci does exist in populations (Hubby and Lewontin 1966; Lewontin and Hubby 1966). Recent studies have shown that while plasticity can be adaptive, some genotypes exhibit non-adaptive as well as maladaptive plasticities (Dorn, Hammond-Pyle, and Schmitt 2000; Weinig et al. 2006) showing that non-adaptive plastic genotypes do not get culled out in natural populations. Additionally some genotypes that exhibit plasticity may not have the highest fitness in multiple or either environments (Rubin and Dorn, *n.d.*; Yakub, Nelson, and Dorn, *n.d.*)

Plasticity can be considered a trait and may be subject to natural selection (West-Eberhard 1989; Williams 1966) if the direction and degree of plasticity is genetically

variable (Cook and Johnson 1968; Murfet 1977) and responds to selection (Bradshaw 1965; Cook and Johnson 1968; Scheiner and Lyman 1989). Variation among genotypes in their response to changing environment is termed genotype-environment interaction (Falconer 1981). If a population exhibits genetic variation for plasticity for a certain trait (such as the hypothetical population in figure 1-1C), specific genotypes that exhibit the trait with the highest fitness in multiple environments will be favored by natural selection, i.e. genotypes that are adaptive to those environments will likely be favored over those that aren't. Therefore, while the environment always acts as the selecting agent, the genotype of the organism as well as the environment contributes to plasticity (Scheiner 1993; West-Eberhard 1989)

1.1 Genetic Basis and Mechanisms of Phenotypic Plasticity.

While the exact genetic basis and mechanisms of phenotypic plasticity are not fully understood, there are several proposed mechanisms. Three of these models, the overdominance model, pleiotropy model, and epistasis model, are the leading models that explain the genetic basis of plasticity. The overdominance model suggests that plasticity is an accidental dysfunctional state; i.e. that individuals with reduced heterozygosity will lose the ability to maintain its phenotype (Gillepsie & Turelli 1989). In this model, the more homozygous a genotype, the more plastic it is; therefore, the heterozygous genotypes are homeostatic because they have the genetic variation to respond to multiple environments. Figure 1-2A shows a reaction norm graph with a hypothetical population of genotypes varying in their homozygosity. In this population the completely homozygous genotype, illustrated in green, is displaying a highly plastic phenotype and

the completely heterozygous genotype, illustrated in red, is displaying a homeostatic phenotype; the genotype with some plasticity illustrated in blue is due to some level of homozygosity.

While the overdominance model has not been disproved, it is not well supported either (Pigliucci 2001). This model was the focus of several empirical studies: Schlicting and Levin (1984) hypothesized that similarity in the plastic response depends on the degree of relatedness among species. They found no evidence of relationship between plasticity and heterozygosity among three *Phlox* species grown in six different environments. In a follow up study, they found inbreeding depression when they compared inbreeding *P. drumondii* for multiple generations but found no patterns of plasticity altered for any trait in any environment (Schicting and Levin 1986). Alternatively, Yampolsky and Scheiner (1994) identified a negative relationship between plasticity and heterozygosity for some traits when they quantified allozyme heterozygosity in *Daphnia magna*. Follow up studies have also had vague results, and with no strong evidence for this model, it has been pushed aside for other models that may explain plasticity further.

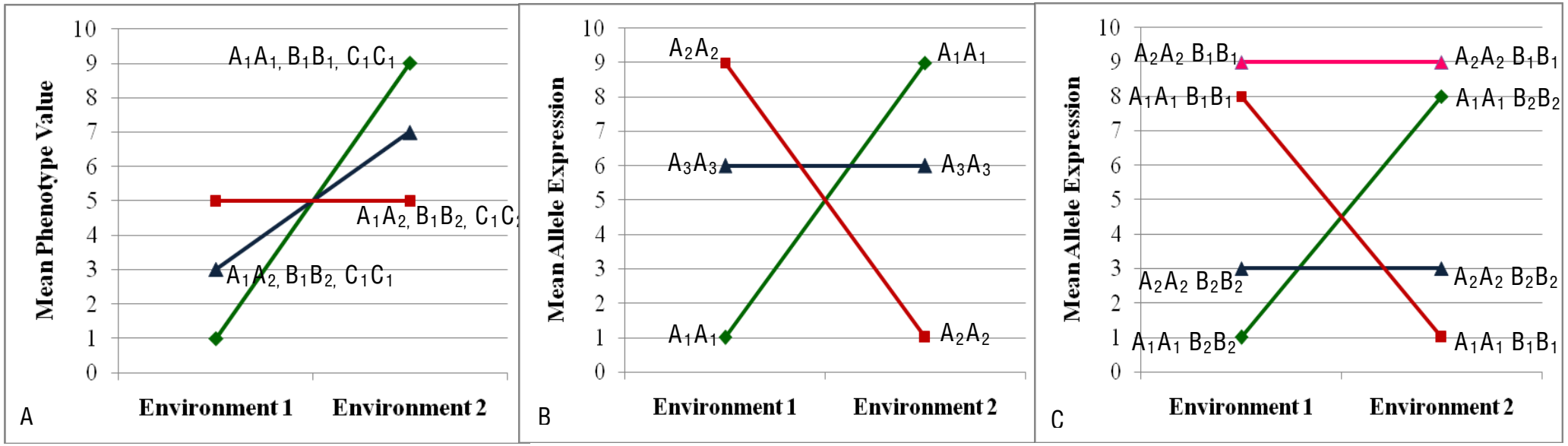

Figure 1-2: Reaction norm graphs illustrating the genetic basis of plasticity

A: Plasticity is due to high levels of homozygosity – i.e. the overdominance model. In this model, the highly homozygous genotype is plastic whereas the heterozygous genotype is homeostatic, as it has the genetic variation required for environmental buffering.

B: Plasticity is due to differential expression of the same alleles – i.e. the pleiotropy model. In this model, each genotype expresses relatively differing levels of the same allele in both environments, and this differential expression results in plasticity.

C: Plasticity is due to expression of different alleles – i.e. the epistasis model. In this model, each genotype expresses relatively different amounts of two genes which interact to exhibit plasticity; A1 allele is the plasticity gene, but amount and direction depends on the B gene with the B1 allele resulting in a sensitive positive phenotype and the B2 allele in a sensitive negative phenotype (sensitivity = mean expression in environment 1 – mean expression in environment 2)

The other two models, the pleiotropy and epistasis models focus on adaptive plasticity and how selection acts on it. The central dispute of plasticity is whether or not it is adaptive, and therefore a direct target of selection, i.e. can it be considered a trait, or is plasticity a by-product of selection acting on the traits in each environment and therefore indirectly plasticity. Further, while there is general agreement that adaptive plasticity can evolve under natural selection (Schlicting 1986; Stearns 1989), there is controversy over the mechanisms by which adaptive plasticity could evolve. The pleiotropy model suggests that plasticity depends on loci that are sensitive to the environment (Via 1993a and 1993b).

Pleiotropy, when one gene affects multiple traits may cause plasticity if the same sets of alleles are expressed at different levels in different environments (Schmalhausen 1949; Via 1993a and 1993b). In this model, when a new allele arises and/or mutations occur, they may be expressed differently across environments (Schmalhausen 1949); therefore creating genetic variation in phenotypic plasticity (Via and Lande 1985 and 1987). One way to visualize this model is to consider a trait expressed differently across environments due to expression of the same loci, but with individual alleles varying in their sensitivity (Via 1993a and Via 1993b). Figure 1-2B shows a reaction norm graph with a hypothetical population of genotypes, where plasticity is dependent on the same sets of alleles expressed in different levels depending on the environment. The genotype shown in red always expresses the A_2A_2 allele, but amount of expression is dependent on the environment, i.e. it is highly expressed in environment 1 but not so highly in environment 2, and this expression in turn controls the trait expression.

Via (1993a and 1993b) has argued in support of this model stating that plasticity is not the target of selection; rather it is a by-product of selection. This is because most ecologically important or life history traits have low additive genetic variance (Hegmann and Dingle 1982; Lynch and Sulzbach 1984) and are therefore under stabilizing selection, generally toward some optimum in a given environment. Adaptive phenotypic plasticity allows a genotype to produce a phenotype that's close to the optimum in each environment, and selection acts within each environment to adjust the trait value, i.e. selection acts only on the trait resulting in plasticity as a by-product of selection (Via 1993a and 1993b). Via (1993a) further reasons that if selection acts directly on plasticity, then in some environments, two genotypes that exhibit the same plasticity should also have the same fitness.

The epistasis model also suggests differential expression of genes, but rather than the same genes, this model suggests that two distinct sets of genes are differentially expressed, one set directly affects plasticity (plasticity loci) while the other affects the amount of plasticity (trait mean loci) (Schlicting 1986; Scheiner and Lyman 1991; Schlicting and Pigliucci 1993 and 1995). In this model, expression of trait mean loci is independent of the environment but the final phenotype depends on the environmentally dependant interaction of plasticity loci and trait mean loci (Schlichting and Pigliucci 1993 and 1995; Berrigan and Scheiner 2004). Figure 2C shows a reaction norm graph with a hypothetical population of genotypes, where plasticity is dependent on the interaction of two genes, A and B. When a genotype has the A_1 allele, it exhibits plasticity, but the direction of plasticity is dependent on which B allele that individual expresses; the B_2

allele is expressed highly in environment 2 relative to environment 1, and the interaction results in the final phenotype. Alternatively, when a genotype has the A_2 allele, it is homeostatic and does not exhibit plasticity, but the mean phenotypic value for that genotype also depends on which B allele it has.

Schlichting and Pigliucci (1993) define these plasticity genes as regulatory loci that control expression of multiple genes depending on the environment they encounter, and are independent of trait means; and this ultimately results in production of different phenotypes in different environments – i.e. plasticity. Basically these genes control the slope, not the height of a trait in a reaction norm and they may be expressed in one environment but not another. Schlicting and Picliucci (1993) further suggest that plasticity genes which would be regulatory genes that are independent of the trait means have been identified as those that are expressed during metamorphosis in amphibians (Semlitsch 1987; Newman 1988), seasonal polymorphisms of some insects (Moran 1992), as well as in plants during production of emergent versus submergent leaf forms (Cook and Johnson 1968). Schlicting and Pigliucci (1995) further restricted the definition of plasticity genes to those loci that exert environmentally dependent control over structural gene expression.

Schlicting and Pigliucci (1993) argued that in the previous model where sensitivity of individual alleles at certain loci vary directly in response to the environment would result in a gradual change in the phenotype, unlike plasticity which can be a drastic change. They reasoned that selection adjusts traits towards the new optimum via expression of these regulatory genes, because this would modify the system and therefore

plastic response of multiple traits. Via (1993a and 1993b) considered these to be modifier loci and suggested that they may cause intensity of allelic expression at trait loci to vary across environments, i.e. while the loci are directly affected by the environment, these modifier loci may increase or dampen the response, but would not be directly responsive to the environment for the plasticity observed.

These models are likely not mutually exclusive; rather, some plasticity genes may be environment-specific as well as trait-specific in their interaction with other genes, but may also be genotype specific (Rubin and Dorn, *n.d.*; Yakub and Dorn, *n.d.*). Therefore, the interaction of plasticity or modifier loci as well as mean loci is likely resulting in plasticity, and this plasticity is also dependent on the genotype.

2.0 *Arabidopsis thaliana*: a Useful Species for Studying Phenotypic Plasticity

Arabidopsis thaliana (Figure 1-3) is an annual plant from the mustard family (*Brassicaceae*) that is widely used in molecular ecology and genetics studies. Originally distributed through Europe and Asia, *A. thaliana* is now found throughout the world, with accessions (ecotypes) from natural populations available from The Arabidopsis Information Resource (2010). While *A. thaliana* is related to economically important crops such as: cabbage, broccoli, common radish, turnip, and horseradish, it is considered to be a weed without any economic value (Meyerowitz 1987).

A. thaliana is a small plant that is easy to grow requiring only moist soil and fluorescent lights for rapid growth (Meyerowitz and Pruitt 1985), and can also be grown in lab media such as nutrient agar. *A. thaliana's* generation time can be as little as 30

days from seed to seed because it primarily self-fertilizes. Additionally, new mutations can be made homozygous. Aside from selfing, it can also be cross-pollinated.

A. thaliana is an annual plant with simple developmental progress: after germination, the plant forms a rosette of leaves. An important life history characteristic in *A. thaliana* is bolting, the stage where the plant begins the irreversible process of flowering, i.e. reproduction. From this stage onwards the plant will no longer produce any more vegetative tissue; rather the developmental energy will be focused on reproduction. After floral production, *A. thaliana* produces fruits in the form of siliques, in which there are approximately 30 seeds per silique.

A. thaliana can display either a "winter annual" or "spring annual" life history (Figure 1-4). The seeds of winter annuals germinate in autumn and overwinter as rosettes. During the spring, the rosettes grow and then transition to flowering once they are relatively large, due to the long growth period. These plants then flower, set seed, and senesce before the summer drought arrives. Alternatively as spring annuals, the seeds overwinter delaying germinating until early spring. The germinants then flower when they're relatively small due to the short growing period, set seed, and die in that same season before the summer drought (Donohue 2002). Natural *A. thaliana* populations vary in this life history sequence (Evans and Ratcliffe 1972; Nordborg and Bergelson 1999; Ratcliffe 1965).

Figure 1-3: *Arabidopsis thaliana*, the model organism for molecular genetics and ecology is a useful species for studying phenotypic plasticity.

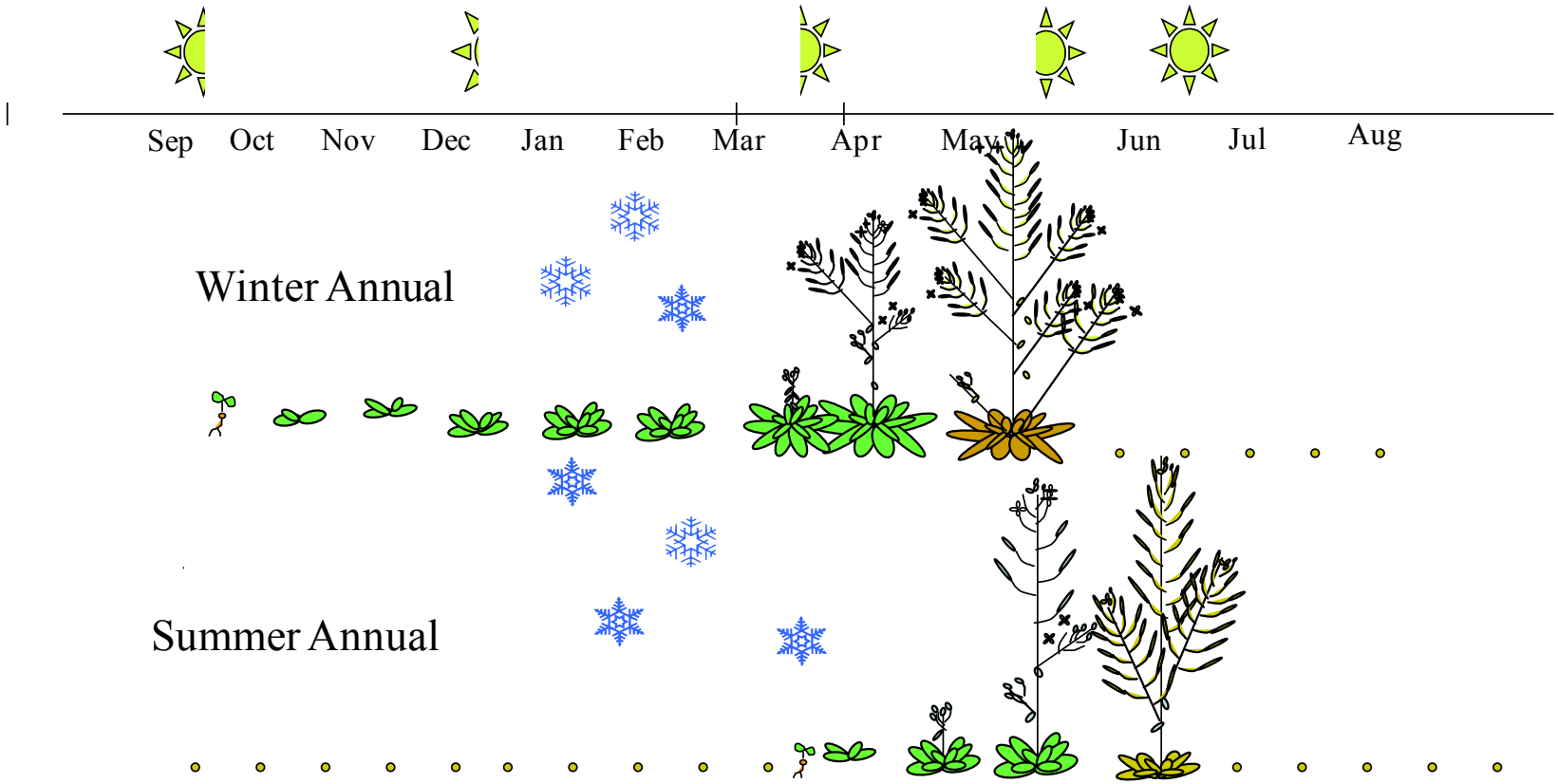

Figure 1-4: *Arabidopsis thaliana* life histories. Top: *A. thaliana* as a winter annual, overwintering as rosette (vegetative tissue) and flowering early in the spring with a large rosette; Below: *A. thaliana* as a summer annual, overwintering as seed, germinating in early spring, and then flowering in late spring with a smaller rosette.

2.1 Recombinant Inbred Lines.

Recombinant inbred lines (RILs) are genotypes generated by crossing over events from two parental genomes resulting in many progeny genotypes that are a mosaic of the parental genotypes (Fig 1- 5). RILs are created by crossing two inbred strains, and then repeatedly selfing the progeny to achieve homozygosity at every locus – i.e. the strains are fixed for different recombination events (Broman 2005; Burr *et al.* 1988).

Compared to F2 populations, RILs are advantageous for many studies because they constitute a permanent population in which recombination is fixed (Bailey 1971; Burr *et al.* 1988). RILs are primarily used in mapping studies because they can be replicated infinitely while still maintaining the genetic fidelity of each line (Lister and Dean 1993), and RILs have been used for mapping studies in many species including but not limited to mice (Bailey 1981; Taylor 1989), wheat (Blanco *et al.* 1998; Snape *et al.* 1985), pea (Ellis *et al.* 1992; Timmerman-Vaughan *et al.* 1996), maize (Burr *et al.* 1988; Burr and Burr 1991), as well as *Arabidopsis* (Alonso-Blanco *et al.* 1998; Lister and Dean 1993; Reiter *et al.* 1992). Since each genotype is essentially two shuffled genotypes, many replicates can be evaluated to reduce bias due to individual, environment, or measurement, and these replicates can be scored across multiple environments (Broman 2005; Pierce *et al.* 2004).

RILs are valuable in examining the interaction of genes and environment because during recombination, the control regions of genes may get detached from the genes they regulated. These control regions could then be recombined with other control regions and

genes. This causes variation of gene expression resulting in phenotypic variation, which can easily be measured.

2.2 Recombinant Inbred Lines and selection.

Genetic variation within any population is when there are multiple alleles at a locus present within the population or when the interaction of alleles at differing loci is different between individuals of the population. This genetic variation is important as it is the raw material for evolution changes over time from one generation to the next, with selection acting as one force shaping variation within populations (Conner and Hartl 2004). Selection alters genetic variation by changing allele frequencies in a population to a point of fixing one allele resulting in the loss of the others. Over time, selection weeds out certain alleles, and eventually certain genotypes, thereby reducing genetic variation in that population. One advantage of using recombinant inbred lines for selection studies is that these genotypes represent a population that has not undergone selection – i.e. in the lab/greenhouse setting these genotypes are not competing for resources and will not get culled-out or become extinct.

2.3 Cal x Tac RILs.

The set of recombinant inbred lines used for this experiment were generated from two natural *A. thaliana* ecotypes: Tac from Tacoma, Washington, USA and Cal from Calver, England. Cal seeds were acquired through the *Arabidopsis* Biological Stock Center at Ohio State University (CS1062), and Tac seeds were collected by T. Mitchell-Olds and L. A. Dorn in 1990. In order to minimize maternal effects, the stock of Tac seeds was maintained through single seed descent for two generations prior to crossing.

Tac and Cal differ in their location of origin, latitude, and longitude, as well as their germination requirements: while Tac requires a cold stratification of the seeds (over-winter of seeds) in order to germinate, Cal does not. Additionally, while the natural phenology of these ecotypes is not known, lab studies indicate that they differ in flowering and germination, and the recombinant inbred lines show variation in response to seed stratification (Donohue *et al.* 2005a and 2005b). Recombinant lines were created by crossing one genotype of each parent to each other; Tac was used as the maternal line.

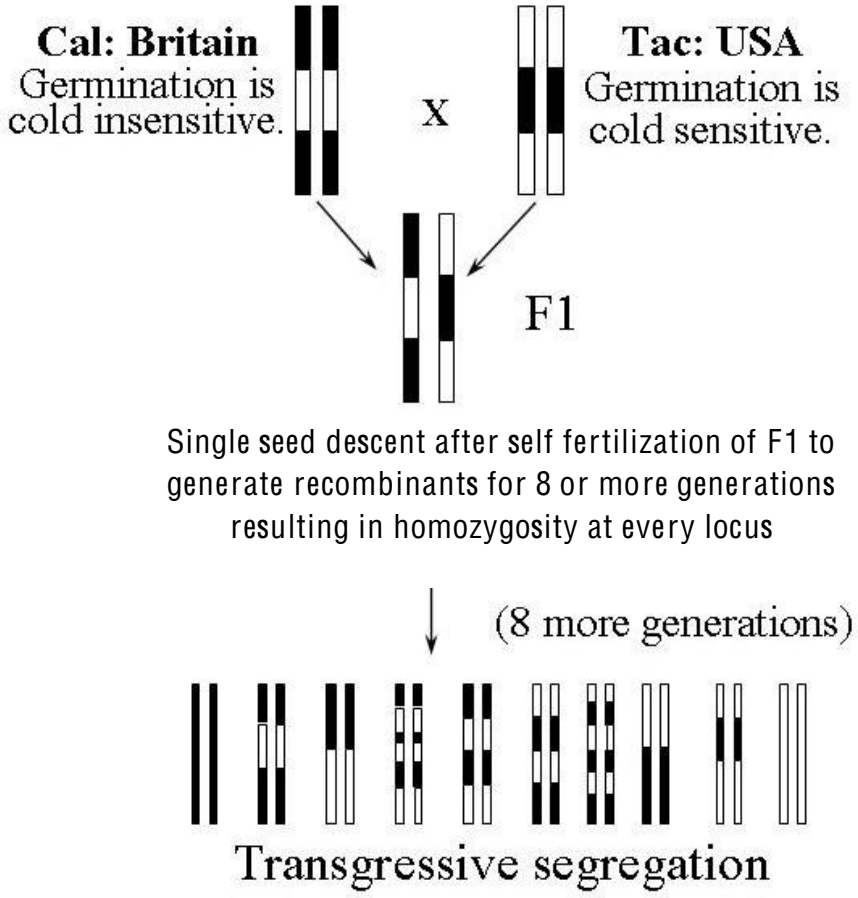

Figure 1-5: Generating recombinant inbred lines. Recombinant inbred lines (RILs) are generated by crossing two natural ecotypes to produce a heterozygous F1. The F1 is self fertilized to produce many F2 progeny with different recombination events which are all self fertilized via single seed descent for at least 8 generations to generate RILs which are homozygous at every locus.

3.0 Microarrays as Tools to Assess Global Gene Expression

DNA is the hereditary molecule present in every cell at every life stage of every living organism. But, proteins are the molecules which determine the phenotype (morphological, physiological, or biochemical) of the organism. As of June 2009, 371 eukaryotic genomes have been sequenced (National Center for Biotechnology Information 2009); however, understanding the function of these genes for these genomes, i.e. protein expression translated from DNA via RNA intermediates, is equally important.

One approach is to study the messenger RNAs (mRNAs) to identify genes being expressed in environments of interest. Historically, northern blot hybridization analysis was used to identify the presence of RNA. Northern blots detect the presence of specific mRNA molecules by denaturing the mRNAs, separating them according to size via electrophoresis, transferring to membrane to immobilize, and probing with labeled single-stranded DNA (Goldsby *et al.* 2003; Yang *et al.* 1993). Northern blot analysis can be used to determine the presence or absence of the relative amount of mRNA of a single gene at a time. Reverse-transcriptase polymerase-chain-reaction (RT-PCR) also identifies expression of mRNA using primers and polymerase to make many copies of the expressed mRNA. These copies can then be separated according to size via electrophoresis, and can then be viewed under ultraviolet light (Brooker 2005). While RT-PCR does not analyze the relative amount of RNA, this technology is sensitive enough to detect very small amounts of RNA. Several studies have used RT-PCR to identify gene expression of candidate genes: Lanciotti *et al.,* (1992) used RT-PCR to

detect and identify specific types of dengue viruses, whereas Datta *et al.,* (1994) used RT-PCR to identify mammary carcinoma cells in peripheral blood and bone marrow of patients with breast cancer.

Microarrays do not focus on one gene at a time. They examine multiple genes, up to entire genomes at the same time (Brooker 2005). They can be used to look at relative gene expression (Schena *et al.* 1995), identification of single nucleotide polymorphisms between organisms (Borevitz *et al.* 2003), as well as resequencing portions of the genome (Haccia 1999).

In most microarray studies, relative gene expression is compared between two samples. When doing a relative gene expression competitive study, for example, if comparing genes expressed in two environments, RNA is extracted from tissues exposed to the environments and reverse-transcribed to cDNA. The cDNA is then tagged with fluorescent dyes, often cyanine 3 which fluoresces green and cyanine 5 which fluoresces red (Stears, Martinsky, and Schena 2003). The tagged samples are then mixed and washed over the microarray slide to let complementary sequences bind, and the tag can then be used to visualize and calculate the relative expression of the particular biological molecule (Brown and Botstein 1999; Wisman and Ohlrogge 2000). Figure 6 illustrates microarray methodology. Of all the different microarray functions, the most well-known and commonly used are to profile mRNA levels (Shiu and Borevitz 2008). A variety of studies can be done, looking at different aspects of RNA expression: gene expression comparison from different tissues (Girke *et al.* 2000), gene expression in different strains/mutants (Gilad and Borevitz 2006), gene expression through some time period

(Girke *et al.* 2000), or commonly expressed genes across environments (Seki *et al.* 2001 and 2002).

Microarrays are advantageous relative to other expression methods because they compare multiple genes, even multiple genomes in parallel, the process is miniaturized and automated (Stears, Martinsky, and Schena 2003). Microarrays are flexible, universal, and can be manipulated, and the procedure is relatively fast (Brown and Botstein 1999). Additionally, an increasing number of labs work with many different organism microarrays, especially *Arabidopsis thaliana* microarrays, making the arrays readily available.

Since complex traits are the consequences of interactions between genes and the environment, understanding genes underlying phenotypic plasticity as well as understanding evolution of these genes is essential to identifying the mechanisms of plasticity and gene by environment interactions (Gibson 2002). Several studies have been done, to identify gene expression changes in the context of plasticity. Whitfield, Cziko, and Robinson (2003) examined gene expression profiles between nursing and foraging bees since this transition to foraging is environmentally dependent, whereas Aubin-Horth *et al.,* (2005) and Aubin-Horth, Letcher, and Hoffman (2005) compared plasticity and gene expression patterns between sneaker and anadromous male salmon. While typical anadromous salmon undergo marine migration to mature, some males, termed "sneakers," mature early, at a greatly reduced size in freshwater. Understanding gene expression patterns in relation to plasticity can be done via microarrays.

A typical microarray experiment compares samples from various sources; therefore, setting up the experiment accurately is important because there are many aspects to these experiments (Shiu and Borevitz 2008) including the number of biological and/or technical and dye bias (Churchill 2002; Yang and Speed 2002). The number of biological and technical replicates depends on the experimental hypothesis and varies between experiments (Churchill 2002). Studies have noted differences in the red and green intensities of the dyes; one way to account for this issue is via dye-swaps where each hybridization is done twice and the sample labeled green in one experiment is labeled red in the other and vice versa (Rosenzweig *et al.* 2004; Yang and Speed 2002). Factorial microarray designs take into account multiple factors and with enough replicates, use indirect comparisons between samples to reduce the number of microarrays without compromising data (Yang and Speed 2002).

Accurately designing and carrying out the microarray experiment generates large and complex data sets (Shiu and Borevitz 2008; Yang and Speed 2002), which after a background correction has to undergo several normalizations to eliminate questionable measurements and to adjust the relative measured intensities (Quackenbush 2002).

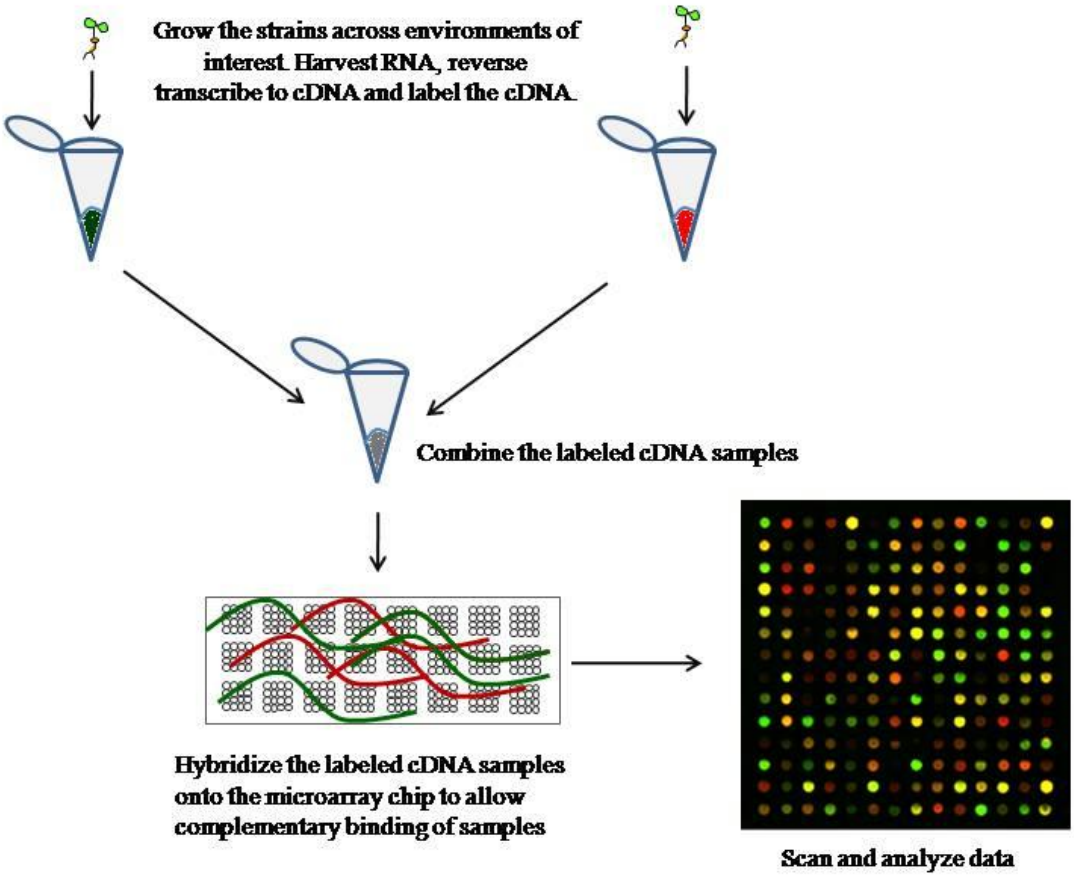

Figure 1-6: Summary of microarray procedure. RNA was extracted from each genotype within each environment. The RNA was reverse transcribed to cDNA, which was then labeled with the appropriate fluorescent dye (Cy 3 or Cy5). Two labeled cDNA samples were combined for each microarray, and the sample was then hybridized onto the microarray chip overnight to allow complementary binding of samples. The microarrays were washed, and then scanned and the data was then analyzed.

4.0 Previous Study

Recent studies looking at gene expression via microarrays have focused on understanding expression patterns that differ among cell types (Leonhardt *et al.* 2004), gene expression differences between mutants to understand gene function (Lloyd and Zakhleniuk 2004; Narusaka *et al.* 2003), as well as gene expression profiles in response to certain environmental stresses (Seki *et al.* 2001; Seki *et al.* 2002). These studies have shown that microarrays are an accurate and efficient method to observe gene expression patterns.

A study by Rubin and Dorn (*n.d.*) looked at gene expression patterns of three Cal x Tac recombinant inbred *A. thaliana* lines. Two of the three RILs exhibited extreme plasticity in opposing directions for days to bolting, in response to cold stratification of seeds, and the third genotype displayed no plasticity; it was homeostatic and early-bolting in both environments (Figure 1-7). The study focused on gene expression in cotyledon tissues, because these tissues would be the first plant tissue, post germination. They found that the homeostatic line significantly upregulated many genes relative to either of the plastic lines in response to the same cold treatment.

4.1 Present Study.

In the present study I look at gene expression patterns of less-extreme recombinant inbred *A. thaliana* cotyledons in response to cold treatment of the seeds, compared to cotyledons from seeds that were not cold treated. I want to compare gene expression patterns of the less-extreme RILs to the data from the extreme RILs, to identify overall gene expression patterns, as well as identify possible candidate genes.

From the recombinant inbred lines generated, I chose three lines that differed in their plasticities for bolting date in response to cold treatment of seeds. Since one of the parents of the RILs requires a cold treatment and the other does not, I were interested in identifying genotypes that would respond differently to the same environmental treatments of cold-treating the seeds compared to those that were not cold-treated. From the 105 RILs, I chose three less-extreme lines to compare to the prior study done by Rubin and Dorn (*n.d.*). I classified one genotype as less-extreme sensitive positive, in that it bolts earlier after a cold-treatment of seeds and the other as less-extreme sensitive negative which bolts later after the cold-treatment of seeds. I classified the third genotype as an insensitive-late bolting, because it does not change its bolting date in response to the cold; however, relative to the rest of the RILs, it bolts later (Figure 1-7).

The trait I am interested in is bolting date, as this is when the plant transitions from production of vegetative to reproductive tissue. Once *A. thaliana* plants bolt, they cannot produce any more vegetative tissue in the form of rosette leaves. However, I want to know which genes are differentially expressed in the early life stages, specifically during the cotyledon stage. Since the seeds are cold-treated, and the cotyledons are the embryonic tissue, genes differentially expressed at this stage will play a role in the plants continued development.

I hypothesized that gene expression patterns for the less extreme plastic RILs would be similar to the extreme plastic RIL gene expression, but to a lesser extent. For the homeostatic RILs, I hypothesized that a set of genes involved in environmental response would be differentially expressed.

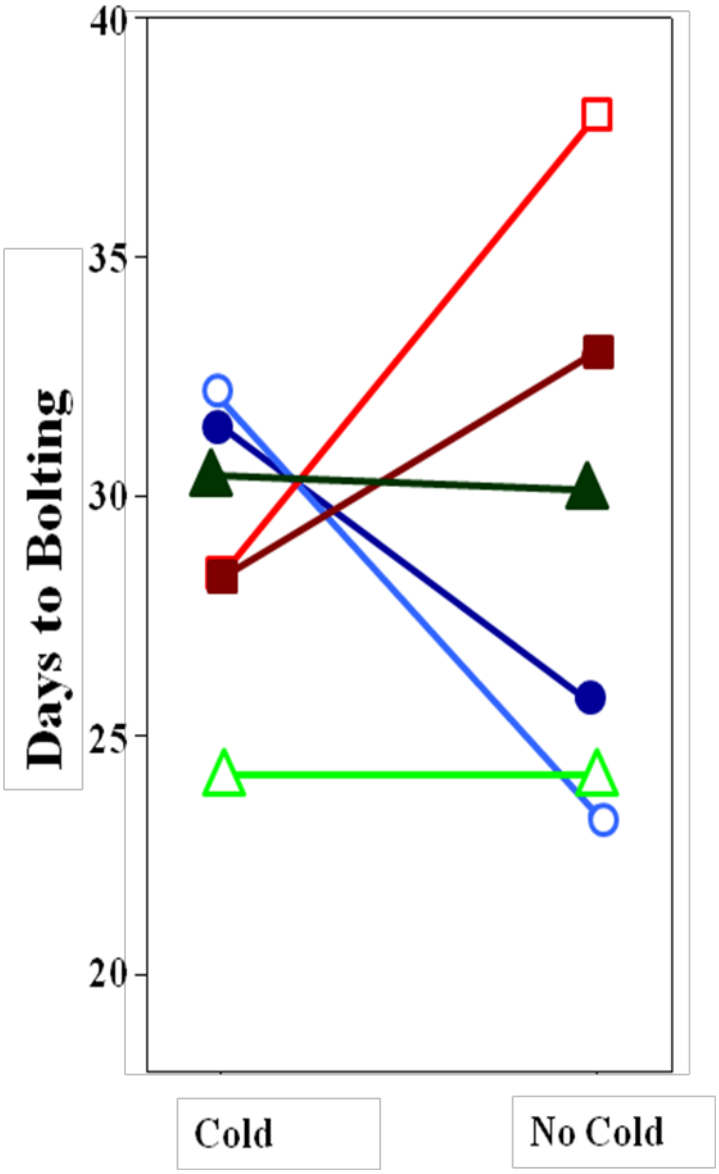

Figure 1-7: Reaction norm graph of the RILs chosen for the global gene expression studies. Open shapes represent genotypes chosen for previous study by Rubin and Dorn; shaded shapes represent genotypes for current study. Lines with shapes are as follows: open square (□) represents extreme sensitive negative RIL 30, shaded square (■) represents less-sensitive negative RIL 116, open circle (○) represents extreme sensitive positive RIL 81, shaded circle (●) represents less sensitive positive RIL 104, open triangle (△) represents early bolting homeostatic RIL 43, shaded triangle (▲) represents late bolting homeostatic RIL 4. Sensitivity is calculated as the mean trait value in the non-cold environment subtracted from the mean trait value in the cold treated environment.

4.2 Present Study Objectives.

The specific objectives of the present study were to:

1. determine overall gene expression patterns of the three selected recombinant inbred lines in response to cold treatment of the seeds compared to no-cold treatment of seeds.
2. compare the gene expression patterns of these two less-sensitive recombinant inbred lines to gene expression patterns from two other Cal/Tac recombinant inbred lines, which displayed extreme plasticity in either direction.
3. compare gene expression patterns of the insensitive-late bolting recombinant inbred line to gene expression patterns of another Cal/Tac recombinant inbred line, which was insensitive to the cold treatment of seeds, for days to bolting, but bolted relatively early.
4. identify possible candidate genes to further study expression in these and other selected recombinant inbred lines and parents.
5. identify candidate genes to study upstream gene regulatory sequences in these and other selected recombinant inbred lines as well as other natural *A. thaliana* ecotypes.

CHAPTER II

COMPARING MOLECULAR BASES AND OVERALL PATTERNS OF PLASTIC RESPONSES IN RESPONSE TO COLD STRATIFICATION OF ARABIDOPSIS THALIANA RECOMBINANT INBRED LINES

1.0 Introduction

Phenotypic plasticity is the ability of an organism to alter its phenotype in response to environmental heterogeneity (Bradshaw 1965; Scheiner 1993; Schlicting 1986). This change in phenotype of the same genotype when experiencing a different environment indicates the interaction of the genotype and environment during developmental processes. Studies have shown that plasticity can be adaptive, i.e. the genotype that changes its trait or phenotype achieves higher fitness relative to the genotype that does not change (Dudley and Schmitt 1995 and 1996; Dorn, Hammond-Pyle, and Schmitt 2000; Schmitt and Wulff 1993; Smith 1982). Other studies have identified plasticity that is non-adaptive (plasticity does not cause higher fitness) as well as mal-adaptive plasticity (plasticity results in reduced fitness) (Dorn, Hammond-Pyle, and Schmitt 2000; Poulton and Winn 2002; Weinig 2000). Costs of plasticity have also been observed (Dechaine et al. 2007; Dorn, Hammond-Pyle, and Schmitt 2000; Stinchcombe, Dorn, and Schmitt 2004; van Kleunen, Fischer, and Schmid 2000; Weijschede et al. 2006; Weinig et al. 2004). However, the genetic basis and mechanisms of phenotypic plasticity are still not fully understood. Two proposed models to explain the genetic bases of plasticity are the allelic sensitivity model also known as the pleiotropy model and gene regulation model also known as the epistasis model.

Pleiotropy, when one gene affects multiple traits may cause plasticity if the same sets of alleles are expressed at different levels in different environments (Schmalhausen 1949; Via 1993a and 1993b). In this allelic sensitivity model, when a new allele arises it may be expressed differently across environments (Schmalhausen 1949), therefore creating genetic variation in phenotypic plasticity (Via and Lande 1985 and 1987). One way to visualize this model is to consider a trait expressed differently across environments due to expression of the same loci, but with individual alleles varying in their sensitivity (Schlichting and Pigliucci 1993; Via 1993a).

The gene regulation or epistasis model also suggests differential expression of genes, but rather than the same genes, this model suggests that two distinct sets of genes are differentially expressed; one set directly affects plasticity (plasticity loci) while the other affects amount of plasticity (trait mean loci) (Scheiner and Lyman 1991; Schlicting 1986; Schlicting and Pigliucci 1993). In this model, expression of trait mean loci is independent of the environment but the final phenotype depends on the interaction locus and environmental interaction of plasticity loci and trait mean loci (Berrigan and Scheiner 2004; Schlichting and Pigliucci 1993 and 1995).

To identify candidate plastic genes, several studies have focused on quantitative trait loci (QTL) mapping of phenotypes of interest in mapping populations (Kliebenstein et al. 2002; Leips and Mackay 2000; Paterson et al. 1991; Ungerer et al. 2003). In a QTL study focused on understanding commercially important traits in tomatoes, Paterson et al. (1991) crossed the wild tomato species *Solanum cheesmanii* (formerly known as *Lycopersicon cheesmanii*) to the domestic tomato *Solanum lycopersicum* (formerly

known as *Lycopersicon esculentum*). They grew the F2 and F3 progeny at three different locations (two in California and one in Israel), and identified 4 QTL common among the three sites, but found more overlapping QTL between the California sites that either overlap with the Israel site; this suggests that there is genetic based environmental dependent response for life history traits. Leips and Mackay (2000) identified six QTL with significant epistatic interaction that affected lifespan in *Drosophila melanogaster*. Kliebenstein et al. (2002) identified one to five QTL for each of the 8 traits they measured in *Arabidopsis thaliana* recombinant inbred lines' response to methyl jasmonate treatment; and Ungerer et al. (2003) also identified several QTL for the inflorescence traits in *A. thaliana's* response to differing photoperiods. These QTL studies identify genomic regions that contribute to the phenotype, the magnitude of the response, as well as interactions of the QTL with the environment. These studies identify regions on the chromosome that may be involved in the plastic response; these QTL regions may consist of multiple genes and often a fine-scale mapping experiment is required as a follow-up to identify some candidate genes. Furthermore, some fine-scale mapping can become restricted due to lack of markers in certain genomic regions or lack of recombination in the region of interest. Additionally, fine-scale mapping to identify one or two genes is complex due to linkage, i.e. the lack of recombination in certain genomic regions.

An alternative method to identify candidate genes is via a global gene expression microarray study. In this study I used microarrays to look at gene expression patterns of two less-extreme recombinant inbred *A. thaliana* cotyledons in response to cold treatment

of the seeds, compared to cotyledons from seeds that were not cold treated, as well as the response of one homeostatic late flowering RIL. I wanted to compare gene expression patterns of the less-extreme RILs to the data from the extreme RILs (Rubin and Dorn *n.d.*), to identify overall gene expression patterns, as well as possible candidate genes that result in a phenotypically plastic response.

Seed stratification is a commonly experienced environmental cue for *A. thaliana* that is typically involved in breaking seed dormancy (Baskin and Baskin 1983; Munir *et al.* 2001) and influences flowering time (Nordborg and Bergelson 1999). Furthermore one of the parents of this set of RILs requires this cold stratification of seeds to break dormancy whereas the other does not require this cold stratification.

The plasticity trait interests us is bolting date, and the RILs I use display plasticity for bolting date in response to the cold stratification (see 'Study species and populations in materials and methods for details on the RILs). However, I want to know which genes are differentially expressed in the early life stages, specifically during the cotyledon stage. Since the seeds are cold-treated, and the cotyledons are the embryonic tissue, genes differentially expressed at this stage will play a role in the plant's continued development.

I address the following questions. Is there any overall gene expression pattern across the RILs in response to the same regime of cold stratification? Are there any genes differentially expressed across all the RILs? Are the same stress response genes differentially expressed across the RILs? How do the overall gene expression patterns of these three RILs compare to the previous study (Rubin and Dorn, *n.d.*)?

2.0 Materials and Methods

2.1 Study Species and Populations.

Arabidopsis thaliana (Brassicaceae) is a widely studied, naturalized annual with populations that exhibit winter annual life-histories germinating in the fall and overwintering as vegetative tissue or rosettes (Stinchcombe, Dorn, and Schmitt 2004), and others that exhibit a spring annual or rapid-cycling life history overwintering as seed and germinating in the spring (Munir et al. 2001; Nordborg and Bergelson 1999).

I used recombinant inbred lines (RILs) derived from two homozygous accessions of *A. thaliana*: Cal from Calver, England and Tac from Tacoma, Washington with Tac as the maternal parent. Cal seeds were acquired from the *Arabidopsis* Biological Resource Center at Ohio State University (stock CS1062) and Tac seeds were collected from Tacoma, Washington by Thomas Mitchell-Olds and Lisa A. Dorn (Donohue et al. 2005a and 2005b). One hundred and twenty recombinant lines were maintained for at least eight generations via single seed descent resulting in 99.6% homozygosity. See Donohue et al. (2005a) for more details on these genetic lines and their maintenance

2.2 Choosing Genotypes.

In summer 2005, two cohorts of 20 replicates of all one hundred and twenty RILs were planted to survey for life history traits and estimate phenotypic plasticity in response to a cold stratification treatment of seeds. A cold stratification treatment of 4°C for ten days in the dark was imposed on seeds from one cohort mimicking spring annual conditions, and no cold stratification was imposed on seeds from the other cohort, mimicking winter annual conditions; see Rubin and Dorn (*n.d.*) for information about this

study. Two RILs that displayed non-extreme plasticities in opposing directions to this cold stratification treatment of seeds, and one homeostatic RIL (RIL 4) that bolted at the midpoint of the season relative to the other RILS were chosen for a global gene expression microarray study (Figure 2-1). I refer to RIL 116 as sensitive negative and RIL 104 as sensitive positive, with sensitivity calculated as the mean days to bolting for the RIL without the cold stratification subtracted from the mean days to bolting for the RIL after the cold stratification treatment.

2.3 Experimental Design and Setup.

To achieve an unbiased data set based on dye-bias and replicates, I used 4 biological replicates per genotype per environment. Using the loop design (Figure 2-2), each genotype per environment was represented four times (twice labeled with cyanine 3 which fluoresces green and twice with cyanine 5 which fluoresces red), resulting in a total of twelve microarrays. *Arabidopsis* oligonucleotides microarrays, each containing 26,000 oligonucleotides, were obtained from the University of Arizona (http://www.ag.arizona.edu/microarray).

In summer 2008, two cohorts of 150 replicates of the three RILs chosen for the microarray study (4, 104, and 116) were planted in a random design. Similar to the previous method (Rubin and Dorn, *n.d.*), 10 seeds were placed on the surface of damp soilless mix (Sunshine SB300 Universal Soilless Mix; Mfg SunGro) in 45 - 2x2 pots. To impose the cold stratification treatment, the flats were placed in the dark in a 4°C cooler for fourteen days. On the thirteenth day, the second cohort of seeds was planted in pots as above, but these were immediately placed in the greenhouse at University of Wisconsin

Oshkosh. On the fourteenth day, the stratified flats were placed in the greenhouse, and all the pots were randomized in flats to avoid biased growth in the greenhouse. Prior to the emergence of true leaves (at approximately 4-5 days), 4 replicates of 20 cotyledon tissue samples per genotype were pooled together and flash frozen in liquid nitrogen for RNA extraction. I left one plant per pot, i.e. a total of 15 replicates per genotype for phenotypic data and fitness estimates.

Total RNA was extracted using RNeasy Plant Mini Kits (*Qiagen* Cat 74904), and I used at least 500ng of starting total RNA in a 20µl sample solution for the microarray procedure per sample. I used a modified version of the microarray target preparation and hybridization protocol provided by the University of Arizona (http://ag.arizona.edu/microarray/methods.html), briefly described below. The micorarrays were hydrated and the DNA probes were immobilized as per the protocol provided by the University of Arizona, prior to hybridization of labeled target onto the array.

I labeled the RNA with amino-allyl nucleotides, and reverse transcribed it to cDNA using random primers. Unincorporated amino-allyl nucleotides were removed using a *Qiaquick* PCR purification kit (*Qiagen* Cat 28104). cDNA was coupled to monoreactive cy-dyes (*Amersham Biosciences* Cat 23001 and 25001), and unincorporated cy-dye was removed using the *Qiaquick* PCR purification kit, following the kit procedure four times. Both labeled targets were mixed, and the solution was denatured and hybridized onto the microarrays for 14 hours at 55°C in a humid hybridization chamber, in the dark. Post hybridization, the microarray slides were washed

in SDS and SSC solutions for 5 minutes each, four times total, and spun dry in centrifuge tubes with kimwipes at the bottom.

Microarrays were stored in a dry, light-tight container at room temperature prior to scanning. Hybridized arrays were scanned using GenePix 4000b at the Gene Expression Center at University of Wisconsin Madison, and grids were overlayed on the microarray images using VersArray version 4.5 (BioRad 2002) program which also detected intensities of the spots.

Of the twelve arrays, two arrays had significantly high levels of background, such that the spots were not readily distinguishable from the background. In summer of 2009, I planted 60 replicates of the three RILs (4, 104, and 116) in 96-well flats in a random design imposing a cold stratification treatment on one set of seeds, and no cold stratification on the other, similar to the method done previously, described above. Similar to the previous method, 3 replicates of 15 to 20 cotyledon tissue samples per genotype were pooled together and flash frozen for RNA extraction. The same extraction, microarray, scanning, and gridding protocols and programs were used for these two microarrays. I left 15 replicates per genotype per treatment for phenotypic measurements and fitness estimates.

2.4 Statistical Analyses.

To determine which genes were significantly differentially expressed for each genotype in each treatment, I performed a mixed model analysis of variance (Proc Mixed-Model, SAS), in which dye and array were random variables, background was a continuous variable, and genotype was a fixed variable. The data were transformed and

normalized to remove any spots which had differential expression due to array, dye, and background. The residual values were averaged within arrays for sequences that had more than one spot, and across arrays to account for the replicate arrays. I refer to this as the general analysis.

As a comparison, I also did the same analysis after correcting for background. This was done by comparing the intensity of each spot to the background on that slide, and then including that value into the complete analysis across all slides. While it is more stringent, this method accounts for differing background hybridization on each slide. I refer to this as the background corrected analysis.

3.0 Results

3.1 Identification of Significant Genes and Comparison to Previous Study.

Statistical analysis of the probes identified different patterns of differential gene expression between the 3 RILs. Based on the two methods of analysis I did (general and background corrected), I detected differing numbers of genes significantly differentially expressed ($p<0.05$; counting both up and down regulated genes) in the three RILs. I focused on the results from the background corrected data; while this may be conservative in identifying candidate genes, this reduces the probability of type I error.

I detected 1045 genes significantly differentially expressed ($p<0.05$; counting both up and down regulated genes) in RIL 4 (the homeostatic genotype), 1345 genes significantly differentially expressed in RIL 104 (sensitive positive genotype; bolts late after cold treatment of seeds relative to no-cold treatment), and 1376 genes significantly

differentially expressed in RIL 116 (sensitive negative genotype; bolts early after cold treatment relative to no-cold treatment). Considering that some genes, while significant ($p<0.05$) were not very differentially expressed in the cotyledons from the cold treated seeds relative to the cotyledons from non-cold treated seeds, I focused on the genes that had a fold change of 1.2 or greater in either direction, reducing the number of genes to 134 in RIL 4, 248 in RIL 104, and 241 in RIL 116.

Comparing gene expression in plants that had been cold stratified as seeds, I found that the homeostatic RIL 4 had approximately even numbers of genes being up and down regulated (55 genes down regulated, 79 genes up regulated; fig 2-3A). In contrast while the sensitive positive RIL 104 had more genes down regulated after the cold treatment (183 genes down regulated, 65 genes up regulated; fig 2-3B), the sensitive negative RIL 116 had more genes up regulated after the same cold stratification (90 genes down regulated, 151 genes up regulated; fig 2-3C). I had expected overall patterns to be similar to the previous study focusing on the extreme plastic RILs (Rubin and Dorn, *n.d.*); however, these patterns are not.

Rubin and Dorn (*n.d.*) found that in order to be phenotypically homeostatic, the genotype had to differentially express many genes. The early bolting homeostatic RIL used in the Rubin and Dorn study (Fig 2-1) significantly ($p<0.05$) up regulated many genes after the cold treatment; alternatively the late bolting homeostatic RIL 4 did not seem to have such a general (high differential gene expression) effect associated with displaying a homeostatic phenotype. Data from the extremely plastic RILs (Fig 2-1) suggested that the extremely plastic sensitive negative (bolts early in the cold) was not

changing expression of enough genes to be phenotypically homeostatic, resulting in plasticity. I therefore hypothesized that in order to be less plastic, in the same direction, the less extreme sensitive negative RIL 116 would have to up regulate more genes in the no cold, i.e. it was more homeostatic relative to the extreme plastic genotype. I did not find the expected pattern; rather RIL 116 had more genes being significantly up regulated after the cold treatment compared to the no-cold treatment. Gene expression patterns of the extreme plastic sensitive positive (bolts later in the cold) suggested that it was phenotypically plastic because it failed to up regulate enough genes in response to the cold stratification, i.e. if it had up regulated more genes, it would have been phenotypically homeostatic. I hypothesized that the less plastic sensitive positive RIL 104 would up regulate more genes without the cold treatment; up regulating as it becomes more homeostatic relative to the extreme plastic genotype. For this genotype, my data did support the hypothesis as more genes were down regulated in response to the cold, i.e. up regulated without the cold treatment.

Comparing these two studies, these data suggest that there is not one single model for how the different genotypes respond to the cold in order to maintain a homeostatic phenotype, assuming that plasticity is due to the failure of expressing the right genes. I am also considering the cold environment to be an environmental cue rather than a stress and comparing this to the non cold treatment, which I am thinking of as a stress. These data don't suggest whether or not the cold treatment is a cue or a stress.

3.2 Functional Categories.

The functional categorization of the significantly differentially expressed genes with a fold change of greater than 1.2 shows that the cellular component, biological process, as well as molecular function of many genes is unknown (Fig 2-4 and Table 2-1). Studying gene expression patterns of these genes may provide further understanding of the functions and cellular location of these gene products. Overall patterns of cellular component of these genes are similar across all three genotypes (Fig 2-4B and Table 2-1B), suggesting that the genes involved in the plasticity response are spread throughout the cell. Overall patterns of molecular functions (Fig 2-4C and Table 2-C) as well as biological processes (Fig 2-4A and Table 2-A) of the differentially expressed genes are similar across all three genotypes.

One way to compare the functional categorization of genes expressed is to compare the ratios of expressed genes to the ratios of genes for each functional category in the genome, i.e. comparing the percentage of expressed genes in each functional category per genotype to the percentage of genes in that functional category in the entire genome. The pie charts in figure 2-4 (A-C) are visual representations of the functional categorization of expressed genes in each of the three genotypes as well as the functional categorization of the entire genome. The accompanying tables 2-1 (A-C) explain the color representation as well as number of genes and percentages for each genotype and whole genome. Further, this functional categorization only focuses on significant differential gene expression in response to cold stratification, regardless of up or down

regulation, i.e. categories with a higher ratio of genes expressed indicate higher total gene expression, not specific for one or the other environment.

For comparing the functional categorization, genes that account for less than 4% of the genome in any category, I considered significant deviation when any genotype differentially expressed approximately double the genes in that category; for genes that represent more than 4% of the genome in any category, I considered significant deviation when the genotype differentially expressed $\geq$1.5 times the ratio of genes. The categories with gene expression that deviated significantly from the whole genome ratio are marked with an asterisk in the corresponding table 2-1. For the biological processes, there was no category where all three RILs deviated significantly from the whole genome ratios (Fig 2-4A; Table 2-1A). RIL 4 and RIL 116, however, did significantly have more plasma membrane genes differentially expressed, and RIL 104 had more, though not quite significantly more, plasma membrane genes differentially expressed as well (Figure 2-4B; Table 2-1B). All three RILs also had relatively, but not significantly (i.e. not double the ratio) more genes involved in transporter activity differentially expressed (Fig 2-4C; Table 2-1C).

Functional category comparison showed that the homeostatic RIL 4 had more genes expressed that are involved in DNA/RNA metabolism, response to abiotic/biotic stimuli, as well as responses to stress (Fig 2-4A; Table 2-1A). These suggest that in order to remain phenotypically homeostatic, the genotype had to differentially express stress genes, and overall increased metabolism of DNA and RNA. Further, of the differential genes expressed by RIL 4, there was a higher expression of genes whose products end up

in the golgi apparatus functions and less in extracellular matrix (Fig 2-4B; Table 2-1B). Since the golgi apparatus primarily processes and packages macromolecules, these data also indicate that the phenotypically homeostatic genotype has to differentially express genes involved in stress and response pathways.

Functional category comparison of the plastic genotypes was very different relative to the homeostatic RIL. Both plastic genotypes had a significant increased ratio for signal transduction genes suggesting that the environmental condition may be acting as a stimulus, but the genotypes are not necessarily responding to this as a stress, or via stress or such response pathways. The sensitive positive RIL 104 (bolts early after the cold treatment) had more cell organization and biosynthesis genes differentially expressed (Fig 2-4A; Table 2-1B). RIL 104 also had a higher ratio of genes that encode proteins located in the cell wall and in the nucleus (Fig 2-4B; Table 2-1B), and more genes products function in DNA/RNA binding (Fig 2-4C; Table 2-1C). These suggest that this plastic genotype responds to the environmental treatments by modifying the cell walls, rather than by stress responses. Furthermore, RIL 104 also increased DNA/RNA binding. Alternatively, aside from the signal transduction genes, the sensitive negative RIL 116 (bolts later after the cold treatment) had significantly more transcription factor genes (Fig 2-4C; Table 2-1C), indicating that this genotype responds to the environment by modifying overall transcription; not focused on any pathways or cellular component modification. The functional categorization indicates that these three genotypes respond very differently at the molecular level, in response to the same cold and non cold treatments, in order to display their phenotypes.

3.3 Gene Expression Overlap.

Of the significantly differentially expressed genes, few genes overlapped across two of the three RILs, and there was no overlap of any gene across all three RILs. Since I was using RILs, I had expected some genes, likely environmental or stress response genes to be differentially expressed in all three genotypes. Some genes were significantly differentially expressed in two of the three genotypes: seven genes were differentially expressed in both RIL 4 and RIL 104; eight genes were differentially expressed in both RIL 116 and RIL 104; and only one gene was differentially expressed in both RIL 4 and RIL 104 (Table 2-2).

Overlap patterns of gene expression show that six of the seven genes had opposing differential expression in RIL4 and RIL 104, i.e. up regulated in one genotype and down regulated in the other (Table 2-2). This opposing pattern suggests that these two genotypes likely use different molecular responses to the cold stratification, corresponding to the different phenotypic responses. The F-box family protein (Phloem protein 2-11) gene and protein kinase family protein (F1312.1) gene are known to be involved in protein metabolism, and were up regulated in the homeostatic RIL 4 and down regulated in the sensitive positive RIL 104. The F box-family protein is involved in N-myristoylation (Boisson, Giglione, and Meinnel 2003), which is plays a role in signal transduction in response to environmental stress (Podell and Gribskov 2004). Additionally, mediator-related RNA polymerase transcriptional regulation (MHC9.3) gene, which encodes a transcription regulator is also up regulated in RIL 4 and down regulated in RIL 104. MHC9.3 has been linked with control of cell proliferation as well

as regulation of flowering time (Backstrom et al. 2007). The BIN4 gene which encodes a protein that forms part of the topoisomerase VI complex that was down regulated in RIL 4 and up regulated in RIL 104 seems to be a housekeeping gene at the cotyledon stage, as it is involved in endoreduplication (Breuer et al. 2007; Kirik et al. 2007), a process that duplicates the genome without mitosis. These overlap patterns suggest that in order to remain homeostatic in response to the cold stratification, RIL 4 has to up regulate protein metabolism compared to the plastic RIL 104.

Overlap comparisons between the two plastic genotypes RIL 104 and RIL 116 show the same overall gene expression pattern for seven of the eight genes (Table 2-2), i.e. differentially expressed in the same direction. This pattern suggests that while there may be different genes/mechanisms for the opposing plasticity responses, these common genes respond the same way to the same cold treatment. Two genes that are down regulated in both RIL 104 and RIL 116 are known to be involved in cell death: BAP1 inhibits programmed cell death, i.e. it's a defense response (Yang, Li, and Hua 2006) and BAG regulates apoptotic like processes, also a defense response (Doukhanina et al. 2006). Down regulating defense response genes indicates that abiotic stress response pathways are different from defense responses. Protein Phosphatase 2C (PP2C) is a negative modulator of protein kinase pathways which are involved in stress responses (Xue *et al.* 2008); down regulation of this gene indicates that the protein kinase pathways are upregulated to respond to this cold stress using a similar molecular pathway between 104 and 116. There was only one gene of unknown function overlap between RIL 4 and RIL 116, which was up-regulated in both lines (Table 2-2).

3.4 Stress Response Genes.

Of the genes significantly differentially expressed, all three genotypes differentially expressed several genes that are known to be involved in stress response as well as those involved in response to biotic or abiotic stimuli (Table 2-3) suggesting that the cold stratification, while a common cue for these plants, may also be a stressor. All three genotypes up regulated multiple genes that encode proteins involved in response to cold; however different genes were up regulated suggesting that there may be multiple pathways for plants to respond to the same cold stratification stress/cue. RIL 116, however down regulated Inducer of CBF Expression 1 (ICE 1), a gene which encodes a transcription factor that is a master regulator for a pathway in response to cold (Chinnusamy, Zhu, and Zhu 2006). This suggests that there are other cold responding pathways the plant can use, or by down regulating this master switch gene, the genotype then bolts earlier after a cold stratification.

All three genotypes down regulated genes involved in oxidative stress response, and also differentially expressed at least one gene involved in response to ultraviolet response (Table 2-3). This suggests that some of these gene products may not be targeted for single responses; rather these UV-response genes may be similar to heat-shock proteins, i.e. they respond to multiple types of stress or are involved in different stress response pathways. This also further indicates that there is molecular-crosstalk between pathways in response to abiotic factors.

Focusing on these genes that encode proteins involved in response to some form of stress, the sensitive positive RIL 104 (bolts later after the cold stratification of seeds)

down regulated more of these genes, whereas the sensitive negative RIL 116 (bolts earlier after the cold stratification of seeds) upregulated more of these stress response genes. This suggests that the different timing to bolting may be due to different pathways being used. Alternatively this could also mean that rather than the cold treatment always being the stressor, no-cold may be a stressor and the cold stratification a normal cue (similar to the parent, Tac). This suggests that for RIL 104, a cold stratification and then bolting later is the norm; whereas the no cold resulting in bolting earlier is the response.

4.0 Discussion

Phenotypic plasticity, the ability of genetically identical organisms to produce different phenotypes in different environments is one way that organisms respond to environmental heterogeneity (Bradshaw 1965). The genetic basis of phenotypic plasticity has been an ongoing debate with two leading proponent models: the allelic sensitivity model suggesting that the same sets of genes are expressed at differing levels in different environments (Schmalhausen 1949; Via 1993a and 1993b) and the gene regulation model suggesting two sets of genes are differentially expressed with one set controlling the trait and the other set controlling plasticity of the trait (Scheiner and Lyman 1991; Schlicting 1986; Schlicting and Pigliucci 1993 and 1995).

To test the idea of plasticity dependant on differential gene expression, I did a global gene expression study of two plastic and one homeostatic recombinant inbred lines response to cold stratification of seeds. I found that the three RILs differentially expressed different sets of genes to respond to the same regime of cold stratification, and

overall patterns differed between the three RILs as well. While there were some genes that were significantly differentially expressed across multiple RILs ($p<0.05$; fold change >1.2), there were no genes that were significantly differentially expressed across all three RILs. This suggests that these genotypes are responding to the cold stratification via different pathways resulting in the phenotypic plasticity or homeostasis.

Comparing the overall gene expression patterns of these RILs to the extreme plastic RILs, I found no immediate similar patterns. I had expected to find similar overall expressions, as well as the same response pathways being used to respond to the cold stratification in the extreme and less-extreme plastic lines. However, I observed different gene expression patterns as well different genes being differentially expressed. Rubin and Dorn (*n.d.*) observed that in order to be phenotypically homeostatic, the genotype was molecularly non-homeostatic, i.e. higher amount of gene expression was necessary in order to flower at the same time across the two environments. In this study I found that there was no such high gene expression effect to being homeostatic; this is likely because the homeostatic genotype used in this study is a late bolting genotype, i.e. regardless of environment it bolts after a certain period of time, likely using the autonomous flowering pathway (Simpson 2004). Alternatively the plastic responses seem to be due to lack of the right gene expression in the right environment, or due to over/under expression of certain genes.

These combined gene expression pattern data suggest that a modified overdominance model would explain plasticity, but rather than the genotypes being homozygous for plasticity and heterozygous for homeostasis, the response would be

based on which regulatory regions of genes are from which genotype. Using recombinant inbred lines, regulatory regions for genes get shuffled such that while the gene may be from one parent, some regulatory regions may be from the other parent. These gene expression data, phenotypic data for these genotypes (Rubin and Dorn, *n.d.*) and molecular marker data for these genotypes (Huang et al. 2010) could be used to describe a model for gene expression mechanisms of plasticity.

Some general stress response genes were differentially expressed suggesting that one of these two common environmental cues (either cold or no-cold) may also be a form of stress for these plants. Furthermore, some UV response genes were also differentially expressed suggesting that the stress responses pathways for different stresses may be the same, or may have significant cross-talk – i.e. they may have conserved-pathways. These results are consistent with physiological studies showing that plants respond to different types of stress with the same "stress-response" pathways (Huang et al. 2008; Kreps et al. 2002). Furthermore, Swindell, Huebner, and Weber (2007) using transcriptional response profiles of ten *A. thaliana* biotic and abiotic stress responses showed that genes with the most differential expression for temperature stress were variable in expression among the *A. thaliana* populations. Thus the plants may use the same pathways or components of the same pathways for different responses.

Cold acclimation involves adjustment of the metabolism and growth via induction, up regulation, as well as down regulation of many genes in plants (Kreps et al. 2002). While this study is not looking at cold acclimation, the response would involve differential gene expression. In response to the cold stratification, oxidative stress

response genes were down regulated in all three genotypes. While reactive oxygen species are toxic byproducts of aerobic metabolism, they are also key regulators of metabolic and defense pathways and can be used to kill pathogens. In response to general stress factors that disrupt cellular homeostasis, such as drought, dessication, salt, UV radiation and more, reactive oxygen species are generally up regulated. This suggests that either the cold is not a stress, which in turn would mean that the no cold environment is the stressor. Alternatively, the cold stratification at the seed level is likely not disrupting cellular homeostasis at the cotyledon stage resulting in down regulation of this group of genes.

Many of the genes differentially expressed in all three genotypes have unknown characteristics and functions; therefore, some are likely involved in response to stresses such as cold stratification. Additionally, the differential expression of the varying groups of genes indicates that while there may be crosstalk between stress response pathways (as suggested by the range of stress response genes), not all pathways are used for response to cold stratification.

This study was focusing only on gene expression to look at basic gene expression patterns. Studies have shown that quantified gene expression is not directly correlated with protein expression; rather there are post transcriptional and/or post translational modifications (Harford and Morris 1997), as well as RNA and protein stability and half lives (Varshavsky 1996) that play a role in the overall trait or phenotype expression. Gygi et al. (1999) found that protein and mRNA levels varied by as much as 20 fold in yeast. Alternatively, Futcher et al. (1999) found high correlations between mRNA and protein

expression after data normalization, also in yeast. This indicates that while gene expression patterns may not completely correlate with protein expressions, gene expression patterns could provide basic understanding and ideas of proteins to focus further studies.

5.0 Conclusion

Our results show that different sets of genes are needed for the plastic response suggesting that both the allelic-sensitivity model as well as the gene regulation model may be interacting during the plant development to display plasticity. Further, a modified thought of the overdominance model may also be incorporated in a new model necessary to understand such gene expression pattern data. While I have not identified specific candidate genes in this study, several genes that were differentially expressed in response to the cold stratification may be involved in the plant plastic response of bolting date to the cold treatment. Expression of these genes should be tested in these RILs as well as the parents (Cal and Tac) via quantitative reverse transcription polymerase chain reaction (qRT-PCR). Additionally, combining the gene expression data from this study and the Rubin and Dorn (*n.d.*) study with the molecular marker QTL study of these RILs (Huang et al. 2010) may provide further understanding of the genes involved in the plastic response.

Furthermore, some of these arrays need to be hybridized again to get data without the background interference of dyes, and fitness data of the RILs are needed to understand if these plastic responses are adaptive.

6.0 Acknowledgements

I would like to thank M. Rubin, V. Lor, J. Mischka, G. Weir, K. Cassanova, L. Degeronimo, C. Fisher for all the help in planting, data and tissue collection, and fruit counts. I would also like to thank the University of Arizona for providing the microarrays at a reduced cost and the University of Wisconsin Madison Biotechnology Center for access to their microarray scanner and discussions of microarray data analysis.

This work was supported by National Science Foundation-Research Experience for Undergraduates grant to L.A. Dorn. Any opinions, findings, conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

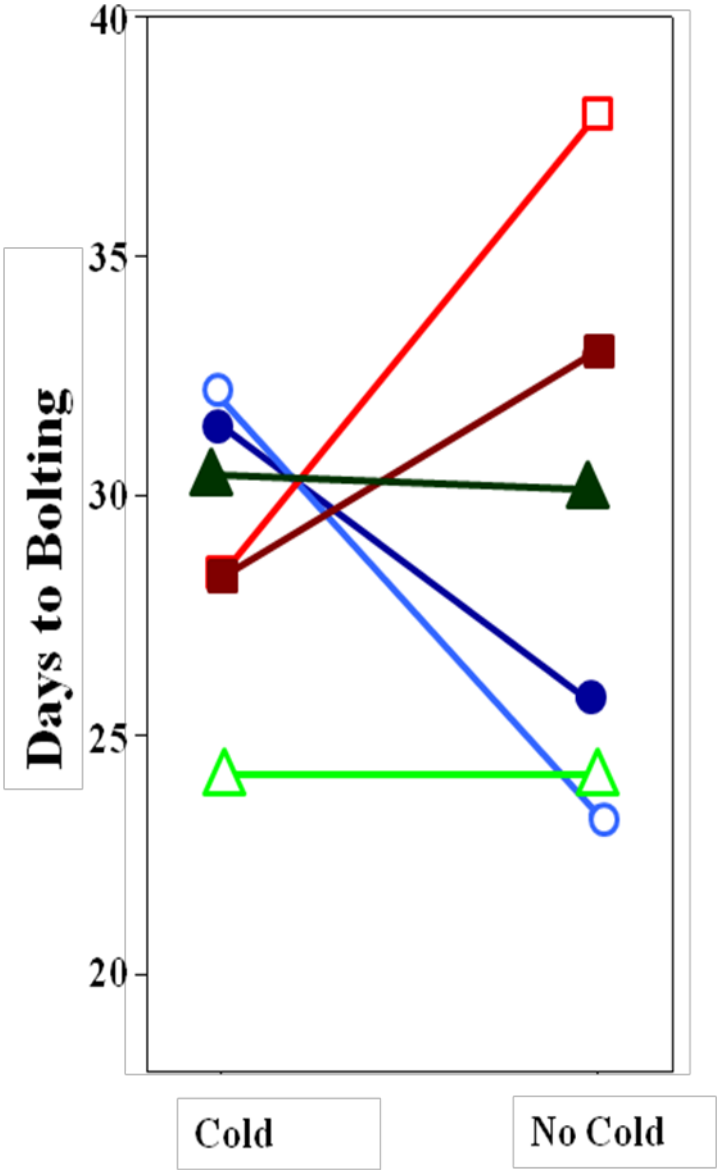

Figure 2-1: Reaction norm graph of the RILs chosen for the microarray studies. Open shapes represent genotypes chosen for previous study by Rubin and Dorn; shaded shapes represent genotypes for current study. Lines with shapes are as follows: open square (□) represents extreme sensitive negative RIL 30, shaded square (■) represents less-sensitive negative RIL 116, open circle (○) represents extreme sensitive positive RIL 81, shaded circle (●) represents less sensitive positive RIL 104, open triangle (△) represents early bolting homeostatic RIL 43, shaded triangle (▲) represents late bolting homeostatic RIL 4. Sensitivity is calculated as the mean trait value in the non-cold environment subtracted from the mean trait value in the cold treated environment.

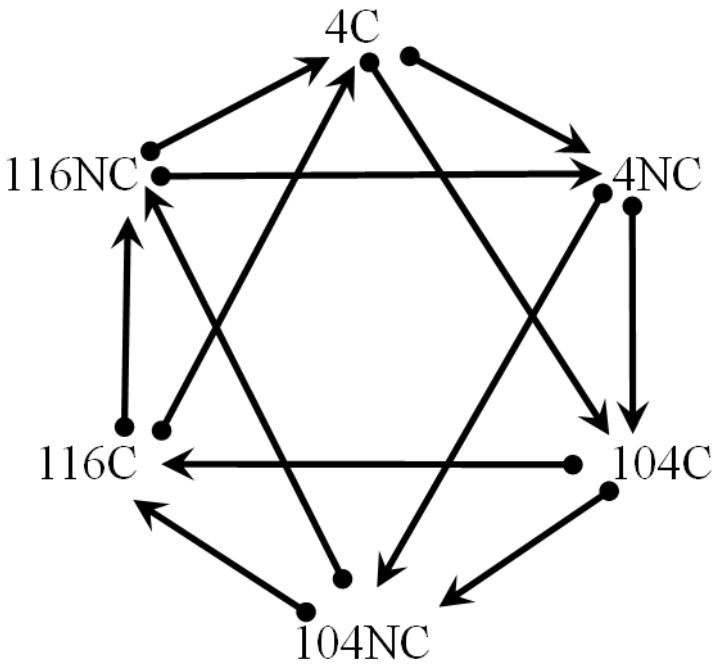

Figure 2-2: Factorial (loop) design for the microarray study where each genotype is represented four times (twice with red dye and twice with green) to achieve an orthogonal experimental design. The arrow indicates that the dotted end represents those genotypes that would be labeled with the red dye and the pointed end represents those genotypes that would be labeled with the green dye.

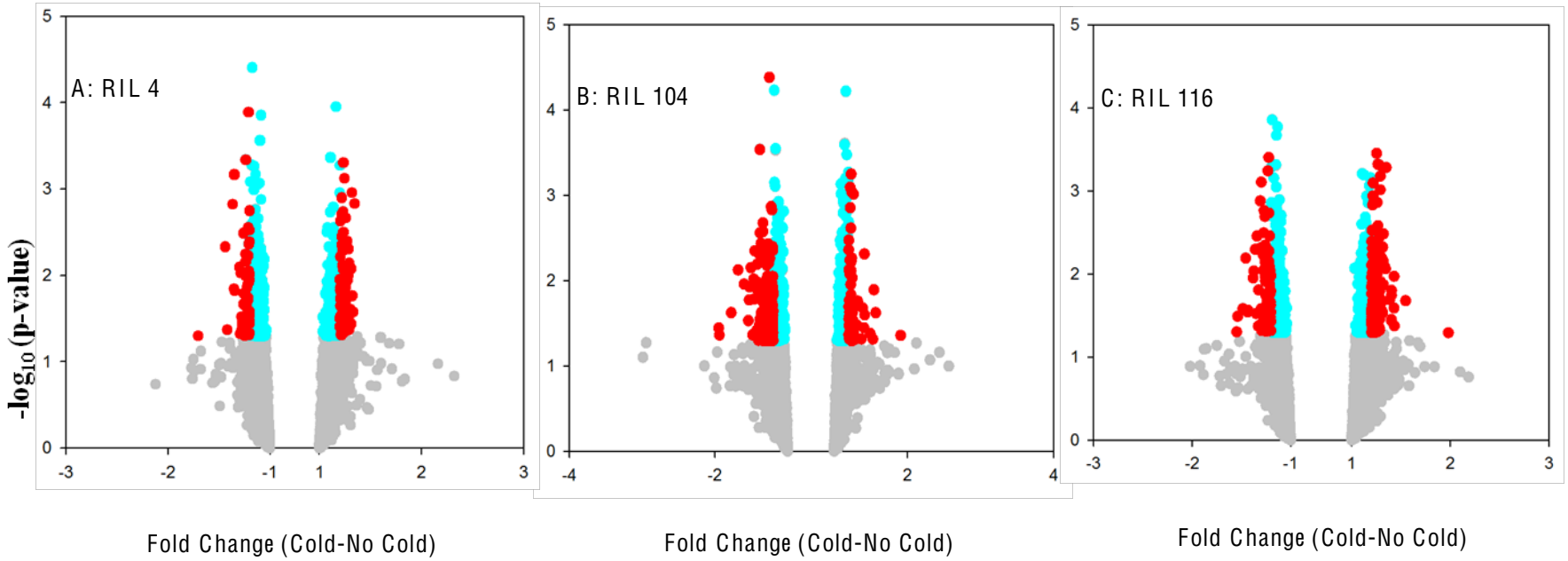

Figure 2-3: Volcano plots illustrating overall patterns of differential gene expression in the three RILs, in response to cold stratification. The y-axis is negative log value of the p-value and the x-axis is the $\log_2$ value of the fold change (cold-no cold) – i.e., positive indicates up regulation of a gene in the plants that experienced the cold stratification and negative indicates down regulation of a gene in the cold-stratified plants. Each gray dot indicates a gene, blue dots indicate genes that are significantly differentially expressed with $p<0.05$, and red dots indicate the significantly differentially expressed genes ($p<0.05$) that have a fold change of greater than 1.2 in either direction.

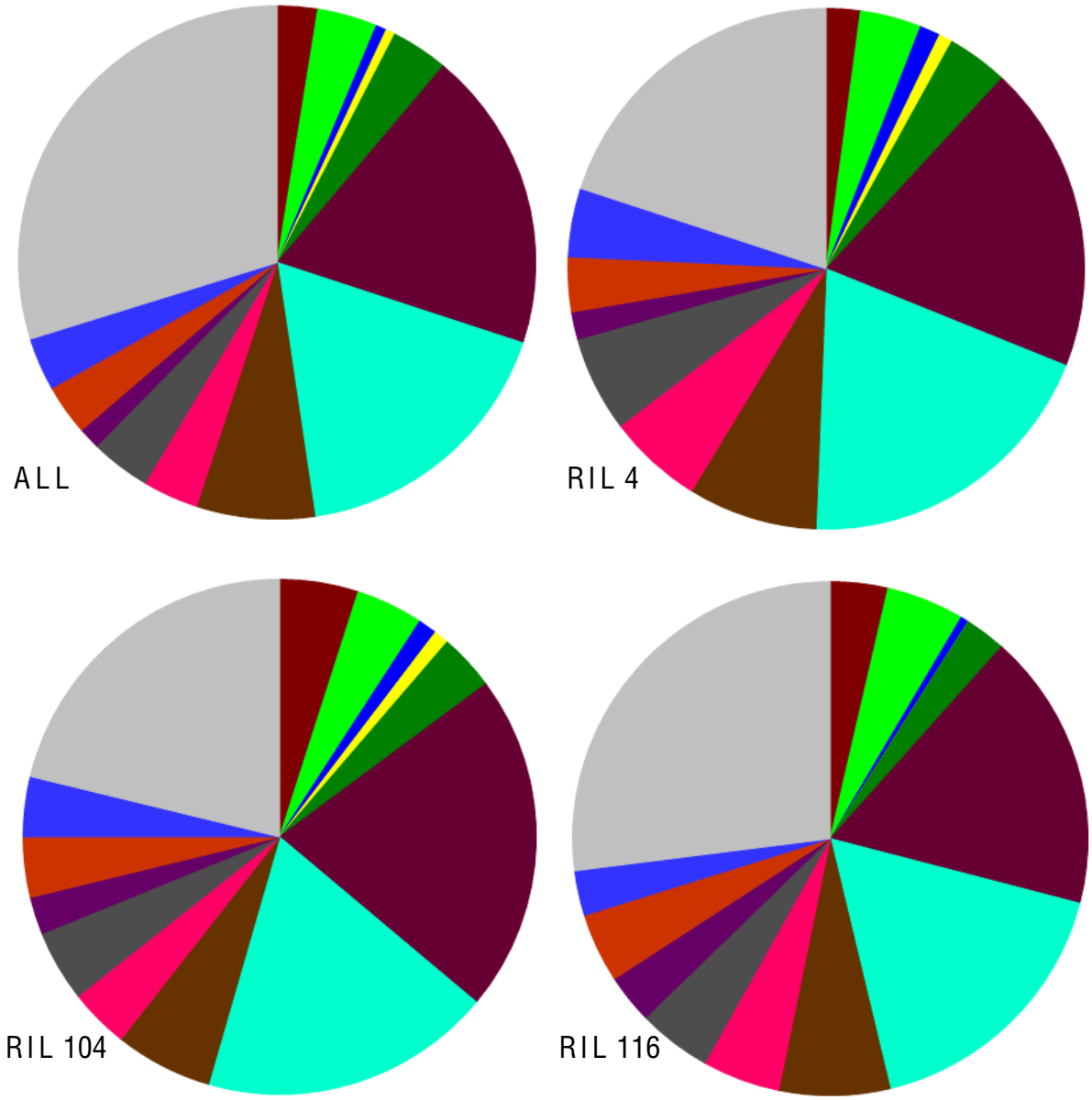

Figure 2-4A: Functional Categorization of *Biological Processes* of significantly differentially expressed genes ($p<0.05$) with fold change of greater than 1.2 in one environment relative to the other compared to the functional categorization of the whole genome.

Table 2-1A: Functional Categorization of *Biological Processes* of significantly differentially expressed genes ($p<0.05$) with fold change of greater than 1.2 in one environment relative to the other compared to the functional categorization of the whole genome. Genes: ALL indicates the number of genes in the genome involved in the particular biological process; %: ALL indicates ratio of whole genome involved in each particular biological process; Genes Exp indicates the number of genes expressed in that functional category for each RIL compared; % Exp indicates the ratio of expressed genes involved in that functional category for each RIL compared; * indicates significant deviation of that ratio from whole genome ratio

	Functional Category	Genes: ALL	%: ALL	Genes Exp: RIL 4	% Exp: RIL 4	Genes Exp: RIL 104	% Exp: RIL 104	Genes Exp: RIL 116	% Exp: RIL 116
	Cell organization and biogenesis	1346	2.52	5	2.13	21	4.95*	14	3.60
	Developmental processes	2006	3.75	9	3.83	18	4.25	19	4.88
	DNA or RNA metabolism	353	0.66	3	1.28*	5	1.18	2	0.51
	Electron transport or energy pathways	316	0.59	2	0.85	4	0.94	0	0.00
	Other biological processes	1913	3.58	9	3.83	15	3.54	10	2.57
	Other cellular processes	10140	18.97	45	19.15	90	21.23	68	17.48
	Other metabolic processes	9410	17.60	46	19.57	78	18.40	67	17.22
	Protein metabolism	3947	7.38	19	8.09	26	6.13	27	6.94
	Response to abiotic or biotic stimulus	1864	3.49	14	5.96*	16	3.77	19	4.88
	Response to stress	2012	3.76	14	5.96*	19	4.48	18	4.63
	Signal transduction	724	1.35	4	1.70	10	2.36	12	3.08*
	Transcription	1709	3.20	8	3.40	16	3.77	17	4.37
	Transport	1778	3.33	10	4.26	16	3.77	11	2.83
	Unknown biological processes	15940	29.82	47	20.00	90	21.23	105	26.99
	Total	53458	100.00	235	100.00	424	100.00	389	100.00

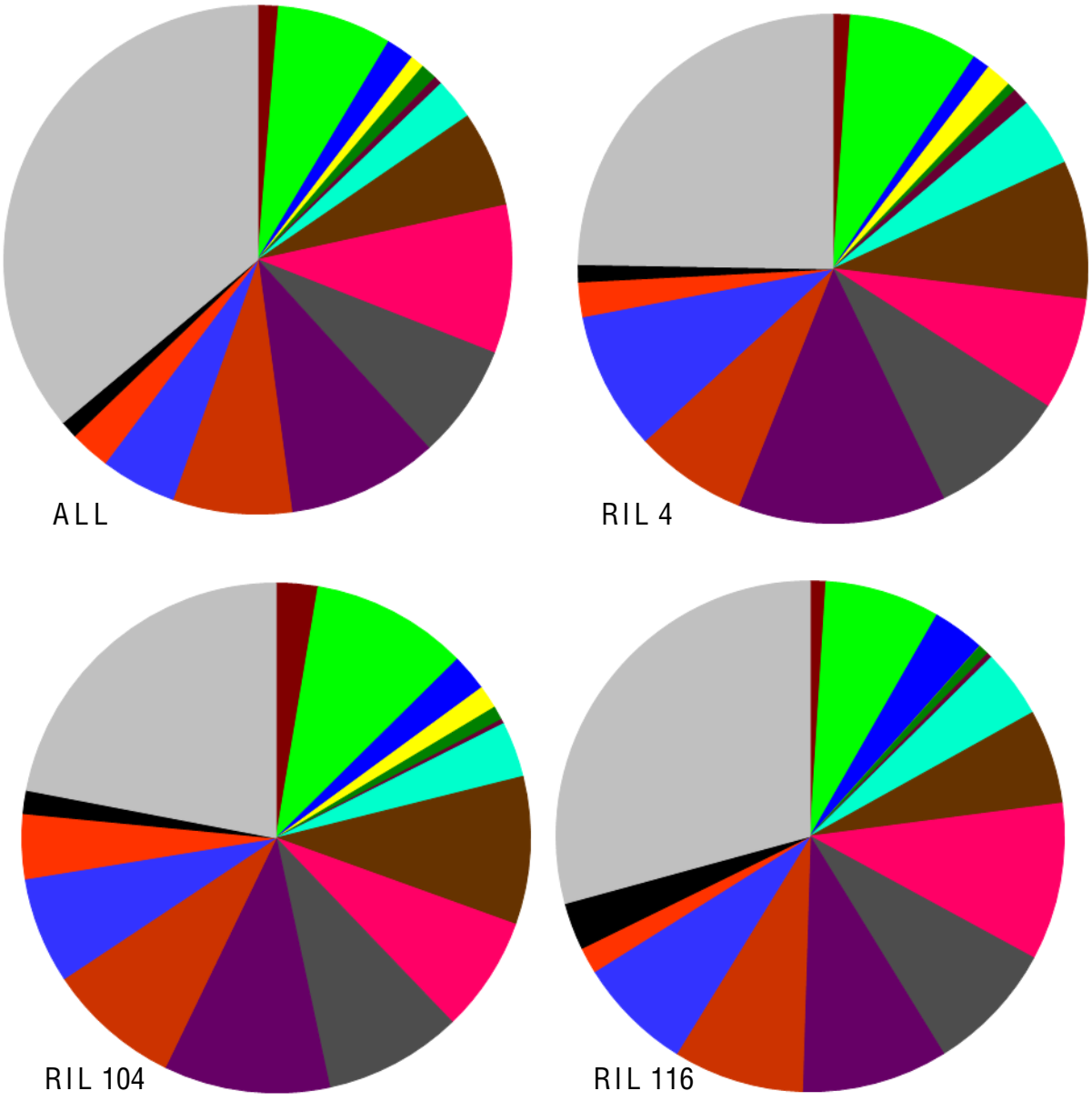

Figure 2-4B: Functional Categorization of *Cellular Component* of significantly differentially expressed genes ($p<0.05$) with fold change of greater than 1.2 in one environment relative to the other compared to the functional categorization of the whole genome.

Table 2-1B: Functional Categorization of *Cellular Component* of significantly differentially expressed genes ($p<0.05$) with fold change of greater than 1.2 in one environment relative to the other compared to the functional categorization of the whole genome. Genes: ALL indicates the number of genes in the genome localized in the particular cellular component; %: ALL indicates ratio of whole genome localized in each particular cellular component; Genes Exp indicates the number of genes expressed in that functional category for each RIL compared; % Exp indicates the ratio of expressed genes involved in that functional category for each RIL compared; * indicates significant deviation of that ratio from whole genome ratio

	Functional Category	Genes: ALL	%: ALL	Genes Exp: RIL 4	% Exp: RIL 4	Genes Exp: RIL 104	% Exp: RIL 104	Genes Exp: RIL 116	% Exp: RIL 116
	Cell wall	556	1.30	2	1.10	9	2.64*	3	1.00
	Chloroplast	3119	7.29	15	8.24	34	9.97	22	7.31
	Cytosol	758	1.77	2	1.10	8	2.35	10	3.32
	Endoplasmic reticulum	377	0.88	3	1.65	5	1.47	0	0.00
	Extracellular	441	1.03	1	0.55*	3	0.88	2	0.66
	Golgi apparatus	229	0.54	2	1.10	1	0.29	1	0.33
	Mitochondria	1122	2.62	8	4.40	12	3.52	13	4.32
	Nucleus	2609	6.10	16	8.79	32	9.38*	18	5.98
	Other cellular components	4034	9.43	13	7.14	25	7.33	30	9.97
	Other cytoplasmic components	3114	7.28	16	8.79	30	8.80	25	8.31
	Other intracellular components	4115	9.62	24	13.19	36	10.56	28	9.30
	Other membranes	3238	7.57	13	7.14	29	8.50	25	8.31
	Plasma membrane	2063	4.82	16	8.79*	23	6.74	22	7.31*
	Plastid	1094	2.56	4	2.20	14	4.11	5	1.66
	Ribosome	476	1.11	2	1.10	5	1.47	9	2.99
	Unknown cellular components	15425	36.06	45	24.73	75	21.99	88	29.24
	Total	42770	100.00	182	100.00	341	100.00	301	100.00

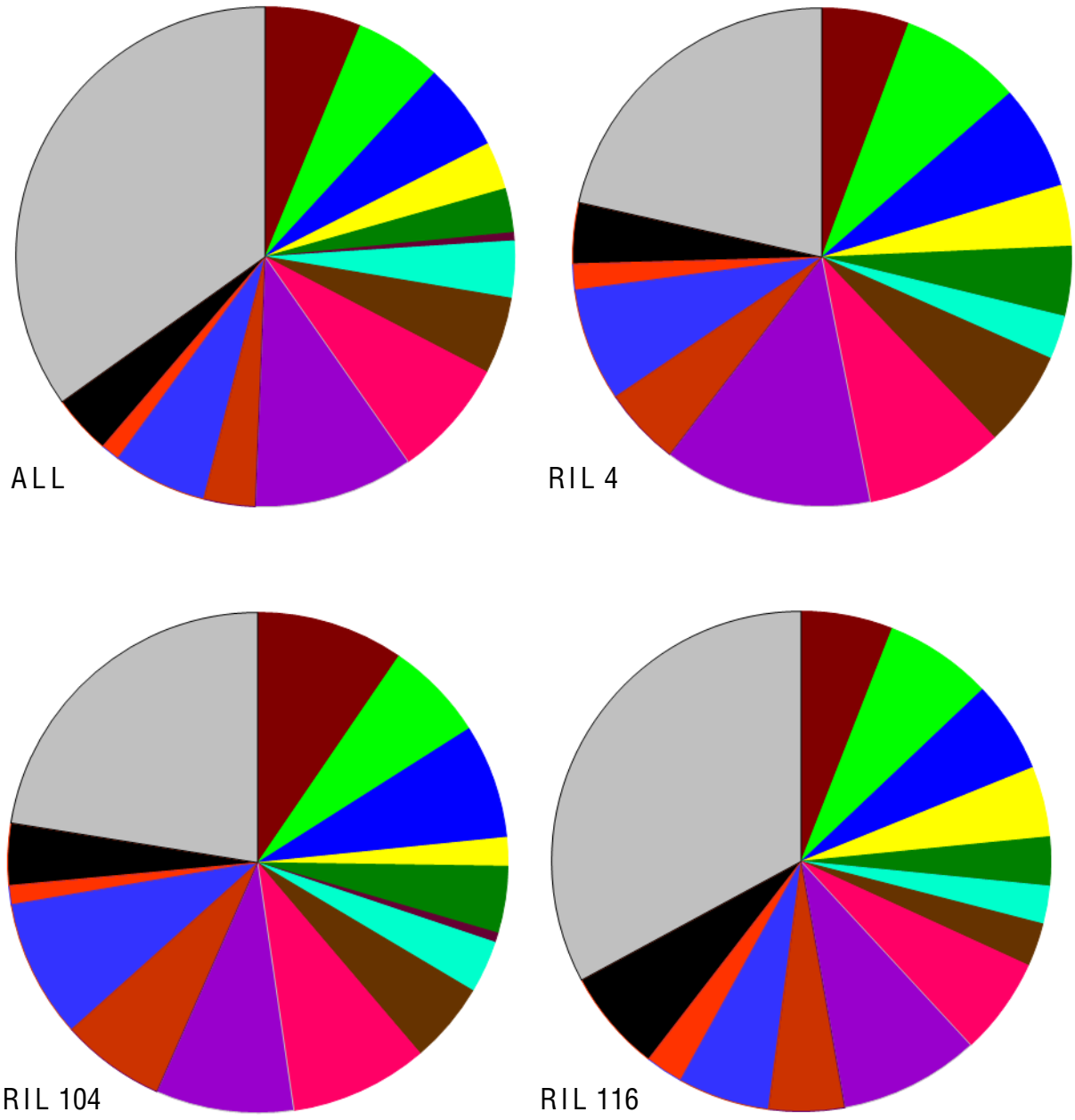

Figure 2-4C: Functional Categorization of *Molecular Functions* of significantly differentially expressed genes ($p<0.05$) with fold change of greater than 1.2 in one environment relative to the other compared to the functional categorization of the whole genome.

Table 2-1C: Functional Categorization of *Molecular Function* of significantly differentially expressed genes ($p<0.05$) with fold change of greater than 1.2 in one environment relative to the other compared to the functional categorization of the whole genome. Genes: ALL indicates the number of genes in the genome involved in the particular molecular function; %: ALL indicates ratio of whole genome involved in each particular molecular function; Genes Exp indicates the number of genes expressed in that functional category for each RIL compared; % Exp indicates the ratio of expressed genes involved in that functional category for each RIL compared; * indicates significant deviation of that ratio from whole genome ratio.

	Functional Category	Genes: ALL	%: ALL	Genes Exp: RIL 4	% Exp: RIL 4	Genes Exp: RIL 104	% Exp: RIL 104	Genes Exp: RIL 116	% Exp: RIL 116
	DNA or RNA binding	2714	6.22	10	5.65	31	9.54*	17	5.94
	Transferase activity	2457	5.63	14	7.91	21	6.46	20	6.99
	Hydrolase activity	2474	5.67	12	6.78	24	7.38	17	5.94
	Kinase activity	1339	3.07	7	3.95	6	1.85*	13	4.55
	Transporter activity	1242	2.85	8	4.52	14	4.31	9	3.15
	Receptor binding or activity	233	0.53	0	0.00	2	0.62	0	0.00
	Nucleic acid binding	1599	3.67	5	2.82	11	3.38	7	2.45
	Nucleotide binding	2166	4.97	11	6.21	17	5.23	8	2.80
	Other enzyme activity	3345	7.67	16	9.04	29	8.92	18	6.29
	Other binding	4516	10.35	24	13.56	29	8.92	26	9.09
	Other molecular functions	1457	3.34	9	5.08*	22	6.77*	14	4.90
	Protein binding	2660	6.10	13	7.34	29	8.92	17	5.94
	Structural molecule activity	538	1.23	3	1.69	4	1.23	7	2.45
	Transcription factor activity	1679	3.85	7	3.95	13	4.00	19	6.64*
	Unknown molecular functions	15198	34.84	38	21.47	73	22.46	94	32.87
	Total	43617	100.00	177	100.00	325	100.00	286	100.00

Table 2-2: List of significantly differentially expressed genes ($p<0.05$; fold>1.2) overlap in the RILs in response to cold stratification of seeds.

Gene	Name							Description		Biological Process		Cellular Component		Molecular Function
AT5G24630	BIN4	4	↓		104	↑		Forms part of the topoisomerase VI complex		Cell organization and biogenesis		Nucleus		DNA or RNA binding
AT5G50880	Unknown	4	↓		104	↑		hypothetical protein		Unknown		Unknown		Unknown
AT1G63090	Phloem Protein 2-11	4	↑		104	↓		F-box family protein		Protein metabolism		Unknown		Other binding
AT3G46930	F1312.1	4	↑		104	↓		protein kinase family protein		Protein metabolism		Unknown		Kinase activity
AT3G21350	MHC9.3	4	↑		104	↓		mediator-related RNA polymerase transcriptional regulation		Transcription		nucleus		Transcription factor activity
AT5G21930	ATHMA8	4	↑		104	↑		mediates copper transport to chloroplast thylakoid lumen		Transport		Chloroplast		Transferase activity
AT3G01750	F28J7.8	4	↑		104	↓		ankyrin repeat family protein contains ankyrin repeats		Unknown		Unknown		Other binding
AT4G28085	N/A	4	↑		116	↑		expressed protein		unknown		Other membranes		unknown
AT2G45040	T14P1.15	116	↓		104	↓		matrix metalloproteinase		Protein metabolism		Other membranes		Hydrolase activity
AT3G24954		116	↓		104	↓		leucine-rich repeat family protein		Unknown		Unknown		Unknown
AT5G02510	T22P11.100	116	↓		104	↓		hypothetical protein		Unknown		Unknown		Unknown
AT1G12060	ATBAG5	116	↓		104	↓		regulate apoptotic-like processes		Other metabolic processes		Unknown		Other binding
AT3G24730	K7P8.2	116	↓		104	↓		mitosis DIM1 family protein		Developmental processes		Nucleus		Other enzyme activity
AT2G45760	BAP2	116	↓		104	↓		Similar to BONZAI1-binding protein BAP1		Unknown		Unknown		Unknown
AT1G76420	ANAC031	116	↑		104	↓		no apical meristem (NAM) family protein N-term		Developmental processes		Unknown		Transcription factor activity
AT3G16560	MDC8.3	116	↓		104	↓		protein phosphatase 2C		Protein metabolism		Other cellular components		Other enzyme activity

Table 2-3: List of significantly differentially expressed genes ($p<0.05$; fold>1.2) that encode products involved in cold, heat, UV or other form of stress in the three RILs, and their regulation (up or down in cold treated relative to non-cold treated) in response to cold stratification of seeds

48 RIL	ID	Fold	Name	Biological Process	Response
4	AT1G62820	↑	F23N19.25	response to cold	Cold
4	AT5G16910	↑	CELLULOSE-SYNTHASE LIKE D2	response to cold	Cold
4	AT3G22840	↑	EARLY LIGHT-INDUCABLE PROTEIN	response to cold	Cold
4	AT2G13350	↑	F14O4.8	Unknown	Cold
4	AT2G43480	↑	T1O24.22	response to oxidative stress	Stress
4	AT1G06180	↑	MYB DOMAIN PROTEIN 13	response to salt stress, gibberellin stimulus, jasmonic acid stimulus, salicylic acid stimulus	Stress
4	AT1G53670	↓	METHIONINE SULFOXIDE REDUCTASE B 1	response to oxidative stress	Stress
4	AT3G60750	↓	T4C21.160	response to salt stress and cadmium ion	Stress
4	AT4G17340	↓	TONOPLAST INTRINSIC PROTEIN 2;2	transport, reponse to salt stress	Stress
4	AT1G21520	↓	F24J8.16	response to oxidative stress	Stress
4	AT1G03190	↓	ULTRAVIOLET HYPERSENSITIVE 6	DNA repair, response to UV, response to heat	UV
104	AT2G21060	↑	COLD SHOCK DOMAIN PROTEIN 4	regulation of transcription	Cold
104	AT1G45145	↑	THIOREDOXIN H-TYPE 5	response to oxidative stress, microbial phytotoxin, defense response to fungus	Stress

(table continues)

RIL	ID	Fold	Name	Biological Process	Response
104	AT3G45890	↑	ROOT UVB SENSITIVE 1	biological process unknown, response to UV-B, developmental process	UV
104	AT4G37930	↓	SERINE HYDROXYMETHYLTRANSFERASE 1	response to cold, plant-type hypersensitive response	Cold
104	AT1G56410	↓	HEAT SHOCK PROTEIN 70T-1	response to heat, protein folding	Heat
104	AT3G28910	↓	MYB DOMAIN PROTEIN 30	plant-type hypersensitive response, response to bacterium, salt stress, plant growth regulators	Stress
104	AT3G03250	↓	UDP-GLUCOSE PYROPHOSPHORYLASE 1	cellular response to phosphate starvation, response to salt stress	Stress
104	AT3G48360	↓	BTB AND TAZ DOMAIN PROTEIN 2	response to carbohydrate stimulus, auxin stimulus, nitrate, regulation of response to stress	Stress
104	AT3G28200	↓	T19D11.4	response to oxidative stress	Stress
104	AT4G35000	↓	ASCORBATE PEROXIDASE 3	response to oxidative stress	Stress
116	AT2G38905	↑	AT2G38905	response to cold, hyperosmotic salinity response	Cold
116	AT1G48000	↑	MYB DOMAIN PROTEIN 112	response to salt stress, abscisic acid stimulus, salicylic acid stimulus, cadmium ion	Stress
116	AT3G25780	↑	ALLENE OXIDE CYCLASE 3	response to fungus, salt stress	Stress
116	AT3G55730	↑	MYB DOMAIN PROTEIN 109	response to salt stress, plant growth regulators, regulation of transcription	Stress
116	AT5G41150	↑	ULTRAVIOLET HYPERSENSITIVE 1	nucleotide-excision repair, response to oxidative stress, radiation	UV
116	AT3G26744	↓	INDUCER OF CBF EXPRESSION 1	response to cold, freezing	Cold
116	AT5G07460	↓	PEPTIDEMETHIONINE SULFOXIDE REDUCTASE 2	response to oxidative stress	Stress

APPENDIX

Phenotypic Plasticity of Arabidopsis thaliana to the Evolutionarily Novel Environment UV-C is Moderated by Seed Stratification

M. I. Yakub, M. Nelson, and L. A. Dorn

Manuscript as submitted to Journal of Evolutionary Biology

ABSTRACT

PHENOTYPIC PLASTICITY OF ARABIDOPSIS THALIANA TO THE EVOLUTIONARILY NOVEL ENVIRONMENT

The evolution of adaptive phenotypic plasticity may be constrained by the frequency of environments and potential costs of plasticity to rare environments. Human activities have led to changes in the earth's atmosphere and climate that may expose extant plant species to the rarest of environmental factors, those never previously experienced in their evolutionary history (i.e. evolutionarily novel environments). If plants perceive these novel environments as stress then existing biochemical pathways may allow adaptive responses with no additional costs, relative to other stresses. We evaluated 29 *Arabidopsis thaliana* genotypes for the adaptive value of plasticity to an evolutionarily novel environment (UV-C) and a more familiar environmental cue (seed stratification). We found that a small number of genotypes were capable of adaptively plastic responses to UV-C when seeds were not stratified. However, seed stratification reduced plasticity to UV-C among these genotypes and altered the selective regime, thus buffering these genotypes from the effects of UV-C.

Introduction

Phenotypic plasticity, the ability of a plant to produce a new phenotype in a new environment may be an adaptive response of relatively immobile plants to environmental heterogeneity (Bradshaw 1965; Hedrick 1986). Plants may face a unique challenge if changes in Earth's atmosphere caused by human activity also causes the emergence of environmental factors that plants have not experienced in their evolutionary history (i.e. evolutionarily novel environments). Even if current terrestrial plant species inherited genes for plasticity to such environments, the subsequent lack of exposure to such environments may have caused the loss of this ability because of drift. The frequency of exposure to different environments has long been proposed as a potential constraint on the evolution of adaptive phenotypic plasticity (Gomulkiewicz & Kirkpatrick 1992). Furthermore, adaptively plastic responses may also be constrained by costs of maintaining biochemical pathways for dealing with rarely experienced environments (van Tienderen 1991; DeWitt et al. 1998). Several recent studies of multiple plant populations observed that some populations display non-adaptive and maladaptive plasticity, as well as maladaptive or non-adaptive homeostasis in environments they rarely experience while others display adaptive plasticity (Dorn et al 2000; Weinig 2000; Poulton & Winn 2002). Costs of plasticity have also been observed although rarely (Dorn et al 2000; van Kleunen et al. 2000; Stinchcombe 2004; Weinig et al. 2004; Weijschede et al. 2006; Deschaine et al. 2007). Evolutionarily novel environments provide an opportunity to examine whether

the rarity of an environment is what drives maintenance costs and non-adaptive responses to that environment.

To test if environment frequency and costly alternative biochemical pathways represent strong constraints on phenotypic plasticity, we examined the plastic responses of *Arabidopsis thaliana* to an extreme example of an evolutionarily novel environment, i.e. exposure to the more energetic ultraviolet (UV) radiation (< 290nm) of UV-C. While UV wavelengths are a normal component of light emitted by the sun, the ozone layer around the earth determines the relative amount of each type of UV that reaches the earth. Some UV-A (400nm – 320nm), most UV-B (320nm – 290nm), and all UV-C wavelengths are filtered out by the ozone layer. Therefore, terrestrial plants and their ancestral species have only ever experienced current UV levels and have evolved appropriate defenses for these conditions. For example, Weinig et al (2004) found *Impatiens capensis* displayed adaptive plasticity of the concentration of phenolics, a common plant response to UV light, when plants were exposed to ambient UV light vs. a blocked UV light treatment. However, they did not study plasticity to increased UV radiation or the responses to the more energetic UV-C, both environmental factors that may emerge if the worldwide depletion of the ozone layer continues unabated. Stricter controls on the release of chlorofluorocarbons into the atmosphere have begun to slow ozone depletion (Rowland 2006). Therefore, we do not know whether UV-C radiation will ever reach Earth, but UV-C is a good, easily controlled representative of an environment never previously experienced in a plant's evolutionary history (i.e. evolutionarily novel environment).

An extreme evolutionary response to environmental heterogeneity would be if plants evolved multiple biochemical pathways, each uniquely suited for sensing and responding to a single environmental factor. Thus, rarely used pathways would be subject to drift or might incur maintenance costs on an organism. In a more probable scenario, plants may evolve specialized pathways for responding to predictable environmental "cues" such as light sensing pathways of phytochromes and cryptochromes, but less specialized pathways to unpredictable "stressors" such as drought, heat or cold shock. Physiological genetics studies suggest that plants respond to multiple forms of stress with the same stress response genes (Kreps et al. 2002; Wang et al. 2004; Huang et al. 2008), while other studies have shown that some stress proteins have other functions besides responding to stress (Carranco et al. 1997; Su & Li 2008). Taken together these studies suggest that the evolution of phenotypic plasticity to rare environments need not result in costs of plasticity or require selection to maintain a functioning pathway if these environments are perceived by plants as a "stress" factor rather than a cue.

Changes instigated by novel environmental conditions may present unique challenges to the potential of plants to respond adaptively, because such environmental conditions will be superimposed on top of already existing or familiar environmental variation. Moreover, plants seldom experience a single environmental factor in isolation. For example, Murren et al. (2006) studied the effects of different combinations of soil mineral content and water availability naturally found among habitats of *Mimulus gutattus* in California on phenotypic variation. Each of these environmental factors affected phenotypes differently in isolation than in combination. Thus, familiar and novel

environments may interact to generate a new plastic response and/or change the selective regime to alter the adaptive value of that plasticity

Familiar environmental cues may also change when global atmospheric conditions change. For example, To test for the influence of existing environmental variation on plasticity to evolutionarily novel environments, we tested the influence of the familiar environmental cue, seed stratification, on the response to UV-C. ratification, cold temperatures experienced by overwintering seeds, depends on seasonal temperature differences that vary by geographic location. Seasonal temperature differences are important determinants of annual plant life histories (Munir et al 2001; Donohue et al. 2005). For example, *A. thaliana*, the mouse-eared cress, is an annual plant in the Brassicaceae family with a broad distribution throughout the northern hemisphere. In southern latitudes, it is primarily a winter annual plant, germinating its seeds in the autumn immediately following the spring flowering season. The cold temperatures experienced by overwintering rosettes, or vernalization, induces flowering with maximal vegetative tissue the following spring. Southern populations often require vernalization to flower, using it as a means of avoiding flowering in less than optimal conditions (Stinchcombe et al. 2004). In contrast, populations in cooler, more northern latitudes may reserve a cohort of seeds that display a spring annual or rapid-cycling life history pattern, delaying germination until the following spring (Nordborg & Bergelson 1999; Munir et al. 2000). Seed stratification is used to break seed dormancy and as a cue to accelerate flowering in the spring, as spring annuals must complete their growth and reproduction before the summer drought.

In this study, we used exposure to UV-C wavelengths as an evolutionarily novel environmental stress for the plant species *Arabidopsis thaliana* (Brassicaceae), and seed stratification as the evolutionarily familiar environmental cue.

we asked the following questions:

1. Is there genetic variation among *A. thaliana* genotypes for phenotypic plasticity to the evolutionarily novel environmental variable UV-C?

2. Is plasticity to UV-C mediated by the familiar environmental cue, seed stratification, and plasticity to cold mediated by UV-C?

3. Is the fitness value of UV-C response influenced by the familiar environmental cue of seed stratification?

Materials & Methods

Genotypes.

We examined the phenotypic plasticity of *A. thaliana* plants from 48 naturally occurring European populations distributed along a latitudinal gradient ranging from 32.6°N to 60.4°N. However, several plants from southern latitudes failed to flower without a vernalization treatment reducing our sample to 29 genotypes (in order of lowest to highest latitudes: Co-1, Di-1, Di-G, Bs-1, Mrk-0, Fe-1, Mz-0, Gy-0, Rak-2, Sav-0, Jm-0, Jl-3, Je54, Nw-0, Ga-0, Gie-0, Po-0, Aa-0, No-0, Dr-0, Kr-0, El-0, Eil-0, Nok-0, Cal-0, Bch-1, Lu-1, Ms-0, RLD-1).

These genotypes, which have been part of several recent flowering time studies (Stinchcombe et al. 2004), were chosen because this latitudinal gradient also represents a

seasonal temperature gradient, with northern latitudes more likely to have populations that experience seed stratification compared to southern latitudes.

Evolutionarily Familiar Environmental Cue: Cold Stratification Seed Treatments.

In May 2006 twelve replicate seeds of each genotype were placed on the surface of damp soilless mix (Sunshine SB300 Universal Soilless Mix; Mfg. SunGro) in random cells, one seed per cell in six 96 cell flats (i.e. 2 replicates per flat). To impose a stratification treatment on the seeds, these flats were then placed in the dark in a 4°C cooler for ten days. On the ninth day an additional twelve replicate seeds of each genotype were planted in six more flats as above, but these were immediately placed in the greenhouse at University of Wisconsin Oshkosh. On the tenth day, the stratification flats were placed in the greenhouse. We began the experiment with 576 plants and reduced our analysis to 348 plants after removing the genotypes lacking sufficient replication because of the vernalization requirement.

The Evolutionarily Novel Environment: UV-C treatment.

Just prior to the emergence of the inflorescence of the earliest bolting plants, we exposed half of the cold treated and half of the non-cold treated plants to thirty minutes of UV-C irradiance in a hood with a lamp emitting 100 μW/cm2 9 (NuAire Inc., Plymouth, Minnesota). To determine length of UV-C exposure that would result in a response, in a previous study, we exposed *A.* thaliana plants to UV-C for different time lengths ranging from 5 minutes to 6 hours. We found that a one hour exposure or more resulted in death whereas plants exposed for 30 minutes showed visible signs of stress, but survived

(Yakub and Dorn, *pers. obs.*) These flats were then placed back in the greenhouse to complete their life cycle.

Traits.

We surveyed plants on a daily basis and recorded the following dates: Germination Date, Bolting date, Flowering date and Senescence Date. From these dates, we calculated reproductive timing (the Number of Days to Bolting and Number of Days to Flowering) and the Flowering Interval, a measure of floral development time, from the difference between Flowering Date and Bolting Date and Lifespan as the difference between Senescence Date and Germination Date. We estimated reproductive size by measuring the Rosette Diameter at bolting and fitness from the number of fruits at senescence.

Data Analysis.

To test for among genotype genetic variation of plasticity to the novel UV-C environment we performed a 3-way Mixed Model Analysis of Variance (SAS Proc GLM) testing for the fixed effects of UV-C and cold stratification, and the random effects of genotype and all interactions on life history traits. Both the UV-C and cold stratification treatments were imposed at the flat level, therefore we also included the random effect of flat with treatment effects nested within flat. Genotype was considered a random effect despite pre-selection based on the latitude of their collection sites because we consider them representative of any genotypes of this species with an historical distribution along a latitudinal gradient.

Plasticity to UV - Genotypic Selection Analysis.

We estimated the strength, mode and direction of selection within UV-C treatments when seeds were stratified and when they were not stratified. Within each of the four treatment combinations, we estimated the genotypic selection differentials by simple linear regression of within environment genotype means of each trait (standardized to the within environment mean) against relative fitness calculated within that environment (Rausher 1992). To determine the mode of selection within environments, i.e. stabilizing or disruptive, we estimated the quadratic regression coefficient (γ) for each trait in a separate analysis. We tested for significant differences between the estimates of linear regression coefficients across environments by ANCOVA. The significance of non-linear regression coefficients was estimated in a second ANCOVA that included the quadratic terms.

Results

Genetic Variation for Plasticity to UV and Cold Stratification.

The 3-way ANOVA revealed there is genetic variation among these *A. thaliana* genotypes for plasticity of reproductive timing traits in response to UV-C (Table 1). However, the expression of that variation depended on the seed treatments (Cold vs. NoCold) as suggested by the significant 3-way interactions for both Days to Bolting and Flowering in the three-way ANOVA (Table 1).

Rosette Diameter, our measure of plant size, displayed plasticity to the UV-C treatment, indicated by the significant main effect for UV-C in the 3-way ANOVA (Table

1). In general, the UV treatment resulted in a significantly smaller rosette diameter, as we expected, given that UV-C causes leaf damage (Table 2). However, we did not detect genetic variation for plasticity of this trait to UV-C or stratification as we found no significant interactions of genotype with any treatment (Table 1). The lack of genetic variation suggests that mechanisms for recovering from leaf damage are highly conserved and not variable (although other analyses suggest otherwise, see below).

The Influence of Cold Stratification on Plasticity to UV.

To determine how the seed treatments influenced the expression of genetic variation for plasticity of reproductive timing traits to UV we performed a two-way ANOVA within each seed stratification treatment (Cold and No Cold). We found a significant UV treatment-by genotype interaction for both Days to Bolting and Days to Flowering, but only when seeds were not cold-stratified (Table 3a). Following the cold stratification treatments, a small number of genotypes changed the direction of their response to the UV treatment but most genotypes displayed less plasticity to UV-C following cold stratification (Figure 1). These results suggest that mechanisms for responding to cold stratification induced a more homeostatic (non-plastic) response that buffered those genotypes from the fitness effects of UV exposure.

The Influence of UV treatment on Plasticity to Cold Stratification.

Since the UV-C treatment was imposed after the seed treatments, an alternative interpretation of the significant three-way interactions (Table 1) for Days to Bolting and Flowering is that UV-C exposure influenced the expression of plastic or homeostatic responses already induced by the seed treatments. Therefore, we also performed two-way

ANOVAs within each UV treatment testing for effects of Cold Stratification, Genotype and Stratification by-Genotype interactions. In this analysis, we found a significant cold stratification-by genotype interaction for Days to Flowering when plants were not stressed via the UV-C exposure but not when plants were UV-C stressed (Table 3b). Thus, the expression of genetic variation was observed only when plants were not exposed to UV-C as many genotypes reduced their plastic response to cold if also exposed to UV-C homogenizing the plastic responses among genotypes (Figure 2a, b). However, one genotype, Lu-1 displayed the opposite pattern showing flowering time plasticity to cold when UV treated but not without the UV-C treatment (Figure 2a, b) suggesting some rare variation in the response mechanism. Thus, the evolutionarily novel stressor influenced the sensitivity of several genotypes to their familiar environmental cues primarily by reducing plasticity but at least one genotype displayed greater sensitivity to the familiar environmental cue after exposure to UV-C.

In contrast, the 2-way ANOVAs for Rosette Diameter showed the opposite pattern of UV-C influence with the expression of genetic variation for plasticity of Rosette Diameter to cold stratification observed only when plants were exposed to UV-C. (Table 2). The two-way ANOVAs performed within UV environments showed a significant two-way interaction of cold stratification and genotype for Rosette Diameter with exposure to UV-C but we detected no significant interaction when plants were not exposed to UV-C (Table 3b). Additionally, unlike the reproductive timing traits, the influence of the novel environment on plasticity of Rosette Diameter to cold stratification was much more variable. Many genotypes did not display plasticity of Rosette Diameter

to cold stratification when plants were not exposed to UV-C, but a few were extremely plastic (e.g. Mrk-0, Lu-1) (Figure 2c). After exposure to UV-C the plastic responses of several genotypes to stratification changed dramatically such that previously unresponsive genotypes displayed plasticity (e. g. Ms-0 , Co-1) to cold stratification while at least one previously plastic genotype (e. g.Mrk-0) displayed homeostasis (Figure 2d). The results for both reproductive timing and size traits suggest that the genetic mechanisms for responding to a familiar environmental cue can be influenced by an unpredictable or novel environment but that response is reflected differently for different traits.

Genotypic Selection analysis, Plasticity to UV & Stratification.

To test the effect of exposure to UV-C on selection in the different cold stratification treatments and vice versa we performed genotypic selection analysis within each of the four treatment combinations. We found that the strength, direction and mode of selection changed when plants were exposed to the evolutionarily novel UV-C environment but the effect of UV-C on selection depended on the influence of the familiar environmental cue, cold stratification.

When seeds were not stratified, the selection regimes for reproductive timing traits and size at reproduction differed between plants exposed to UV-C and those not exposed (Table 4). For plants not exposed to UV-C, we found significantly positive linear selection differentials for both Days to Bolting and Days to Flowering, and a significantly negative selection differential for Rosette Diameter suggesting that later flowering plants of small size had higher fitness in this combination of environments (Table 4). When

plants were exposed to UV-C, the selection differentials for reproductive timing traits were not linear. We found significantly negative quadratic terms for reproductive timing traits suggesting stabilizing selection favored plants flowering near the mean flowering time over all genotypes (Figure 3). Although the ANCOVA (not shown) failed to show that the slopes of the *linear* regression coefficients were significantly different between UV-C treatments, the slopes of the *non-linear* regression coefficients differed significantly between the UV-C treatments. Thus, the mode of selection changed more dramatically with the UV-C treatment then the strength of selection. Furthermore, plasticity to UV-C could be adaptive for genotypes that flowered relatively late in the No-UV environment and responded to UV-C by flowering earlier and nearer to the mean.

In contrast, when seeds were stratified, we found only weakly significant negative selection differentials for lifespan that did not differ across the UV and No UV treatments (Table 4). Therefore, in this experiment, the evolutionarily familiar environmental cue of cold stratification moderated the effects of the novel UV-C treatment on selection, i.e. the cold stratification was much more important in shaping phenotypic response.

When plants were not exposed to UV-C, selection favored late flowering genotypes in the No Cold treatment. However, there was no selective advantage among genotypes for any trait in the Cold stratification treatment. We detected significantly positive selection differentials for both Days to Bolting and Flowering in the No Cold treatment but no significant regression coefficients in the Cold stratification treatment (Table 4). Thus, any plasticity toward late flowering in the No Cold environments could be adaptive, as could homeostasis for a genotype flowering late in both environments,

suggesting that existing mechanisms can be used to produce adaptive phenotypic plasticity without the aid of stratification.

Discussion

Phenotypic plasticity in plants has long been proposed as an adaptive response to environmental heterogeneity that may shelter a plants genetic diversity from the consequences of environmental change (Bradshaw 1965; Hedrick 1986). Theory suggests that adaptive plasticity may depend in part, on the frequency of alternative environments (Golmuckiewicz & Kilpatrick 1992), and may be constrained by costs of maintaining the ability to be plastic to rarely experienced environments (vanTienderen 1991). These provisions might have important consequences if climate change and alterations of the earth's atmosphere dramatically alter the distribution of existing environmental heterogeneity and/or introduces environmental variants that are beyond the experience of any extant plant species. Recent studies have shown that both plants and animals can display adaptive responses to changes in climatic zones caused by human activity (Charmentier et al. 2008; Kelly & Goulden 2008), but we know nothing about how organisms will respond to environmental factors that have never existed in their evolutionary history (i.e. evolutionarily novel environments). In this study, we have shown that interactions between novel stressors and familiar cues allowed some genotypes of the model plant species, *A. thaliana* to avoid deleterious effects of an evolutionarily novel stress on the flowering time, an important life history trait in annual plants. Additionally, some but not all of the genotypes we tested were able to produce an

adaptive response to the evolutionarily novel environment without the influence of the familiar environmental cue, cold stratification. These results suggest that pathways used to respond to stress are sufficiently flexible to be effective against some novel environments and that they can be genetically variable.

To test the idea that lack of experience limits the ability of plants to respond adaptively to evolutionarily novel environments, we exposed plants to UV-C light, an extreme evolutionarily novel environment. We found that reproductive timing traits could be adaptively plastic to UV-C suggesting that existing mechanisms can be used to respond adaptively to an evolutionarily novel environment but few genotypes had this ability. These results are consistent with physiological studies showing that plants respond to different types of stress with the same "stress response" pathways (Kreps et al. 2002; Huang et al. 2008). Thus, the UV-C environment may not be "perceived" by plants as novel but just as stressful. Additionally, Swindell et al. (2007) using whole genome expression studies showed that genes with the most differential expression for temperature stress (high or low temperature stress) were variable in expression among 10 *A. thaliana* populations. Thus, existing mechanisms may be capable of inducing an adaptive response to evolutionarily novel environment and our results as well as other genomic studies show that these existing mechanisms vary among populations.

While we found that few genotypes produced an adaptive response to UV-C, we also tested if the familiar environmental cue, cold stratification, could significantly influence the potential for an adaptive response to the novel environment. This test is important because the emergence of evolutionarily novel environments due to alterations

of atmospheric conditions will likely be geographically widespread and thus superimposed on top of already existing environmental variation. Thus, for plants exposed to evolutionarily novel environments, the mechanisms induced by the existing environmental stresses may either amplify or dampen plasticity to the novel environment. More importantly, the combined environments may alter selection pressures on critical life history traits.

Seed stratification is a commonly experienced or evolutionarily familiar environmental cue for *A. thaliana* that is typically involved in breaking seed dormancy (Baskin & Baskin 1982; Munir et al. 2001) and influences flowering time (Nordborg & Bergelson 1999). We found that cold stratification of seeds reduced the plasticity of flowering time to UV-C in genotypes that had exhibited strong plasticity to UV-C when seeds were not stratified (Figure 1a and b). This reduced plasticity eliminated selection pressures on this trait that were otherwise observed when plants were not cold stratified (Table 4). These results suggest that mechanisms for responding to cold stratification induced a more homeostatic response that allowed these genotypes to escape the deleterious effects of UV-C exposure.

One way that plants can respond to stress is by accelerating growth and development rather than attempting to wait for conditions to improve. Stratification of seeds has been shown to accelerate growth and development to induce earlier flowering (Nordborg & Bergelson 1998) while UV stress has been shown to inhibit growth (Landry et. al 1995). Although identifying the biochemical mechanisms plants use to respond to stratification and UV is beyond the scope of this study, other studies showed that

stratification induced the expression of genes involved in metabolic processes that might help ameliorate the damage caused by UV. For example, Fait et al (2006) studied the production of metabolites in *Arabidopsis* seeds following 3 days of cold seed stratification (aka seed vernalization) and through the first 24 hours of germination. They compiled both gene expression and gas chromatography studies and found that enzymes involved in major metabolic processes such as the citric acid cycle (TCA) increased with stratification while those involved in the degradation of amino acids decreased as expected for a rapidly growing seedling. While they did not study the fate of these metabolites in plants whose seeds were not stratified or beyond the germination stage, it follows that variation among populations in their sensitivity to stratification may also reflect variation in metabolic processes that could be used to reverse or reduce the effect of stresses, including UV-C stress.

The genotypes in our study responded to stratification in ways that altered the effects of the UV-C environment, which selection analysis showed buffered those genotypes from the fitness effects of UV-C. This dampening of selective pressures by seed stratification is an example of how no single genotype necessarily gains or loses in frequency when a novel stressor arises, as long as all populations/genotypes experience a compensating familiar environmental cue, like seed stratification. However, in our example, the seasonal temperature differences that provide the stratification environment are geographically distributed along with the genetic variation of this selfing species (i.e. each population acts as a repository of a portion of the species genetic variation). Therefore, the effect of emerging novel environments on the persistence or local

extinction of many *A. thaliana* populations depends on the potential for changes in the frequency of more familiar environmental cues imposed by climate change. Moreover, even if the frequency of stratification environments does not change, a species like *A. thaliana,* with its genetic variation partitioned among populations distributed over a wide range of environments, will lose that portion of its genetic variation that does not experience a familiar environment capable of buffering the evolutionarily novel environment such as seed stratification.

While we have shown that a familiar environmental cue could influence sensitivity to an evolutionarily novel environment the reverse may also be true. We showed that UV-C influenced plasticity to stratification by affecting the phenotype of plants whose seeds were not previously stratified. While this is still fundamentally an illustration of the power of familiar environments to modulate the effects of novel environments, it can potentially identify the ways that stratification works to buffer the effects of UV-C. There are a number of explanations for how plants might alter plasticity to stratification through the influence of UV-C. For example, exposure to UV-C could generate somatic mutations to DNA that then results in alteration of the mRNA transcribed and thus the functionality of the proteins encoded by this DNA. UV-C may also directly damage proteins in plants that were not stratified if protection mechanisms, such as sumoylation (Kurepa et al. 2002) are induced by stratification. Alternatively, plants may interpret UV-C exposure as a stress that requires greater up-regulation of protein protection mechanisms than the normal levels induced by the familiar stratification cue.

In our analysis, several genotypes exhibited adaptive homeostasis or adaptive plasticity of flowering time to cold stratification when exposed to UV-C but not without exposure to UV-C. In the UV treatment, we detected only stabilizing selection in the No Cold environment for this trait and no selection in the Cold; therefore, any plastic or homeostatic response toward the mean flowering time in the UV, No Cold environments would be adaptive. When exposed to UV-C several genotypes flowered near the mean flowering time in both environments, thus displaying adaptive homeostasis of flowering time across stratification treatments (Figure 2b). However, only Ms-0, displayed adaptive plasticity across stratification treatments when exposed to UV-C. This is evidence that several genotypes may have interpreted the UV-C environment as a stress that required up-regulation of stress responses and/or protein protection mechanisms. While this experiment can only speculate on the responses of stress genes, molecular techniques are rapidly evolving to allow us to quantify the response of "stress' pathways in populations/genotypes exposed to familiar and unfamiliar environments. Thus, we can directly test our hypotheses about the ability of plants to respond to unfamiliar environments and the costliness of those responses.

We have shown that plant responses to evolutionarily novel environments such as exposure to UV-C can be adaptive suggesting that existing biochemical pathways can be used to respond to evolutionarily novel environments. However, the genetic variants capable of adaptive responses were rare in the sample of genotypes we tested. A larger experiment may find more genotypes with this ability. Alternatively, far more genotypes were affected by the interaction of the familiar environmental cue, cold stratification with

the novel UV-C environment by both reducing sensitivity to UV-C and altering the selective regime to buffer deleterious effects of UV-C.

Acknowledgements

This work was supported by a University of Wisconsin Oshkosh Student/Faculty Collaborative Grant to MIY and LAD. Beatrice Holton commented on an earlier draft. M. Rubin, J. Johnson, and V. Lor helped collect data and count fruits. LAD was supported by an NSF-REU DBI 0753525.

Thanks to two anonymous reviewers and L. Moyle (Indiana University) for comments.

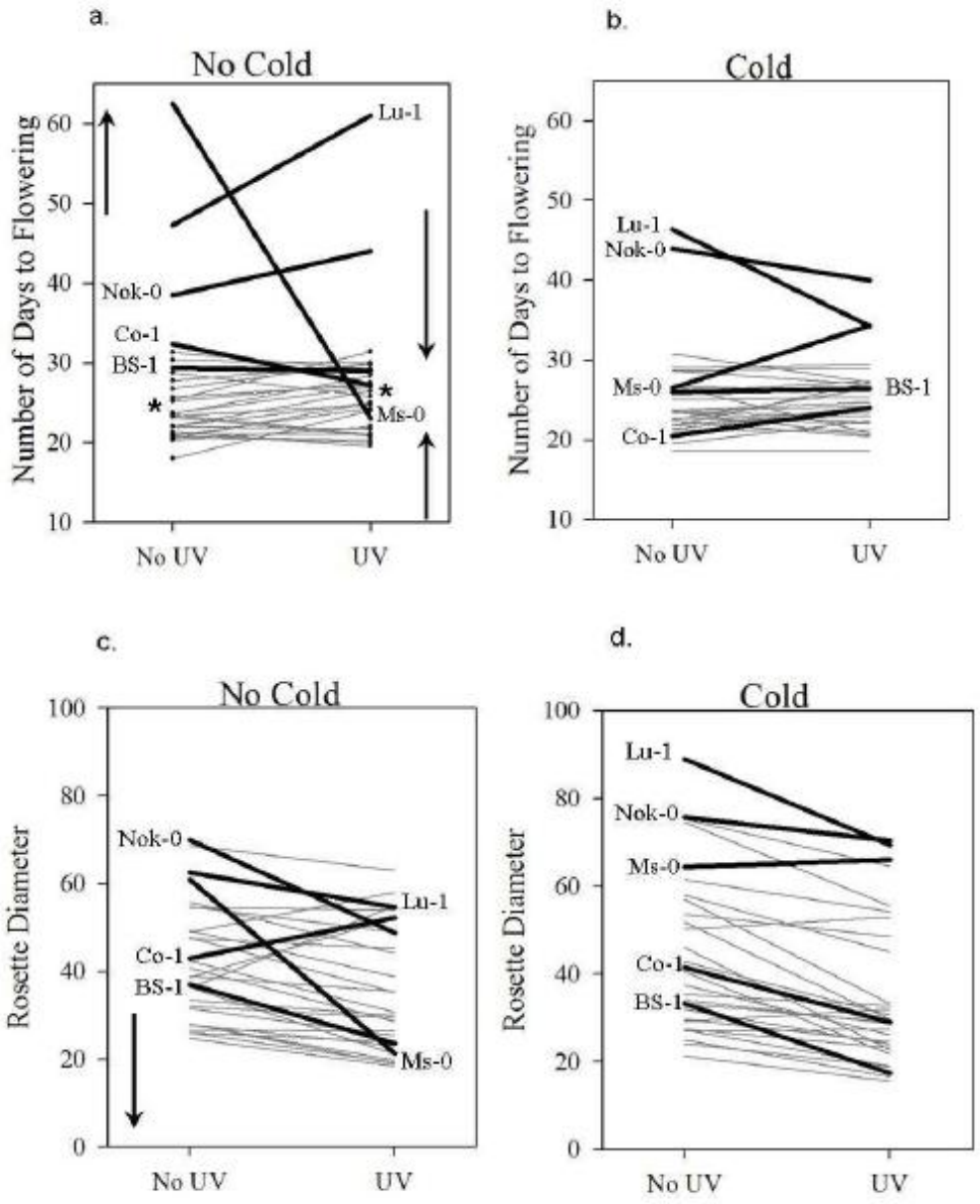

Figure 1. a. Reaction norm graph of the genotype means for Number of Days to Flowering in the No UV-C and UV-C treatments when seeds were not stratified. b. Reaction norm graph of the genotype means for Number of Days to Flowering in the No UV-C and UV-C treatments when seeds were cold stratified. c. Reaction norm graph of the genotype means for Rosette Diameter in the No UV-C and UV-C treatments when seeds were not stratified. d. Reaction norm graph of the genotype means for Rosette Diameter in the No UV-C and UV-C treatments when seeds were cold stratified. The reaction norms of genotypes with unusual or extreme responses are in bold and labeled.

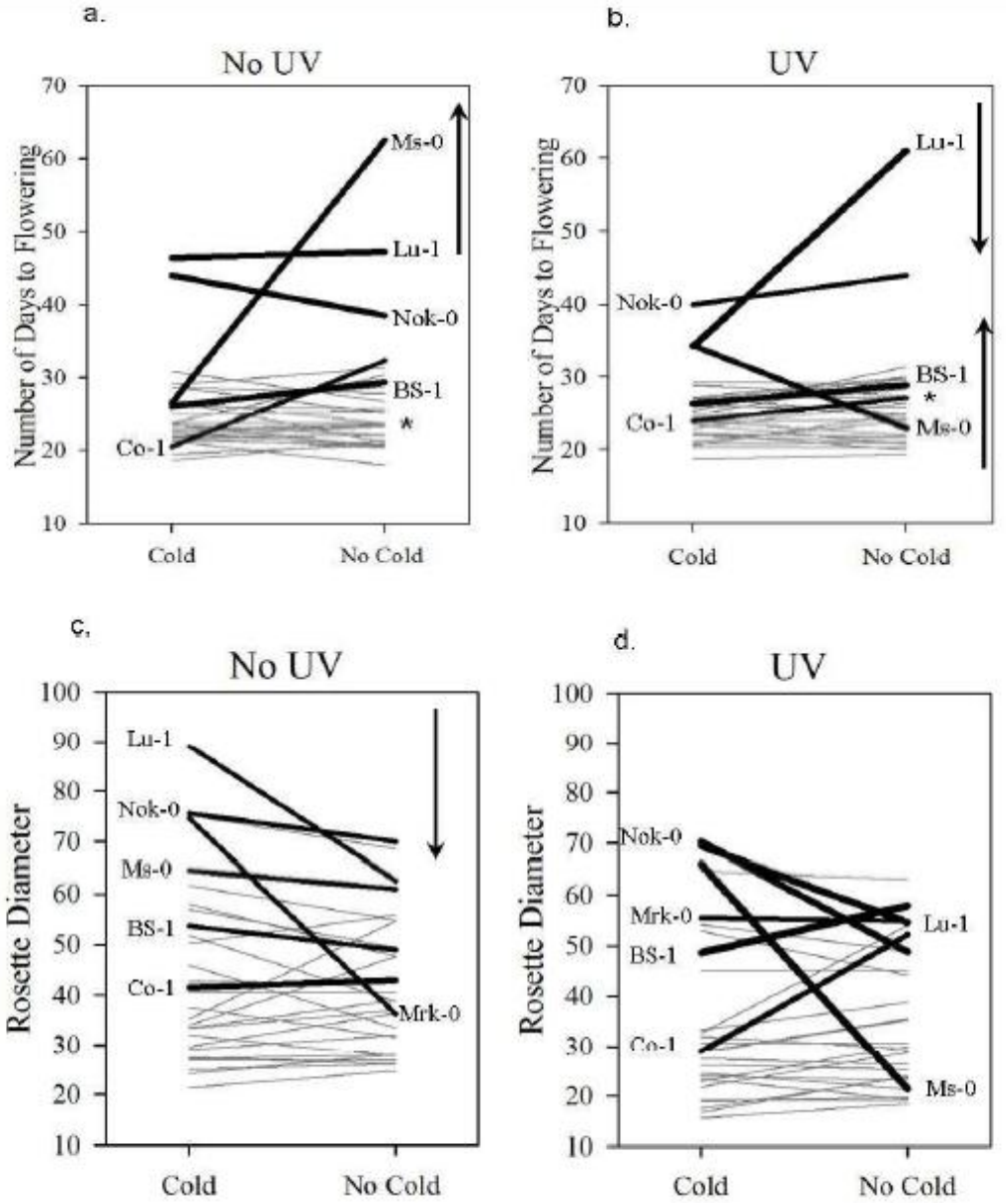

Figure 2. a. Reaction norm graph of the genotype means for Number of Days to Flowering in the Cold and No Cold treatments without the UV-C treatment. b. Reaction norm graph of the genotype means for Number of Days to Flowering in the Cold and No Cold treatments with the UV-C treatment. c. Reaction norm graph of the genotype means for Rosette Diameter in the Cold and No Cold treatments without the UV-C treatment. d. Reaction norm graph of the genotype means for Rosette Diameter in the Cold and No Cold treatments with the UV-C treatment. The reaction norms of genotypes with unusual or extreme responses are in bold and labeled.

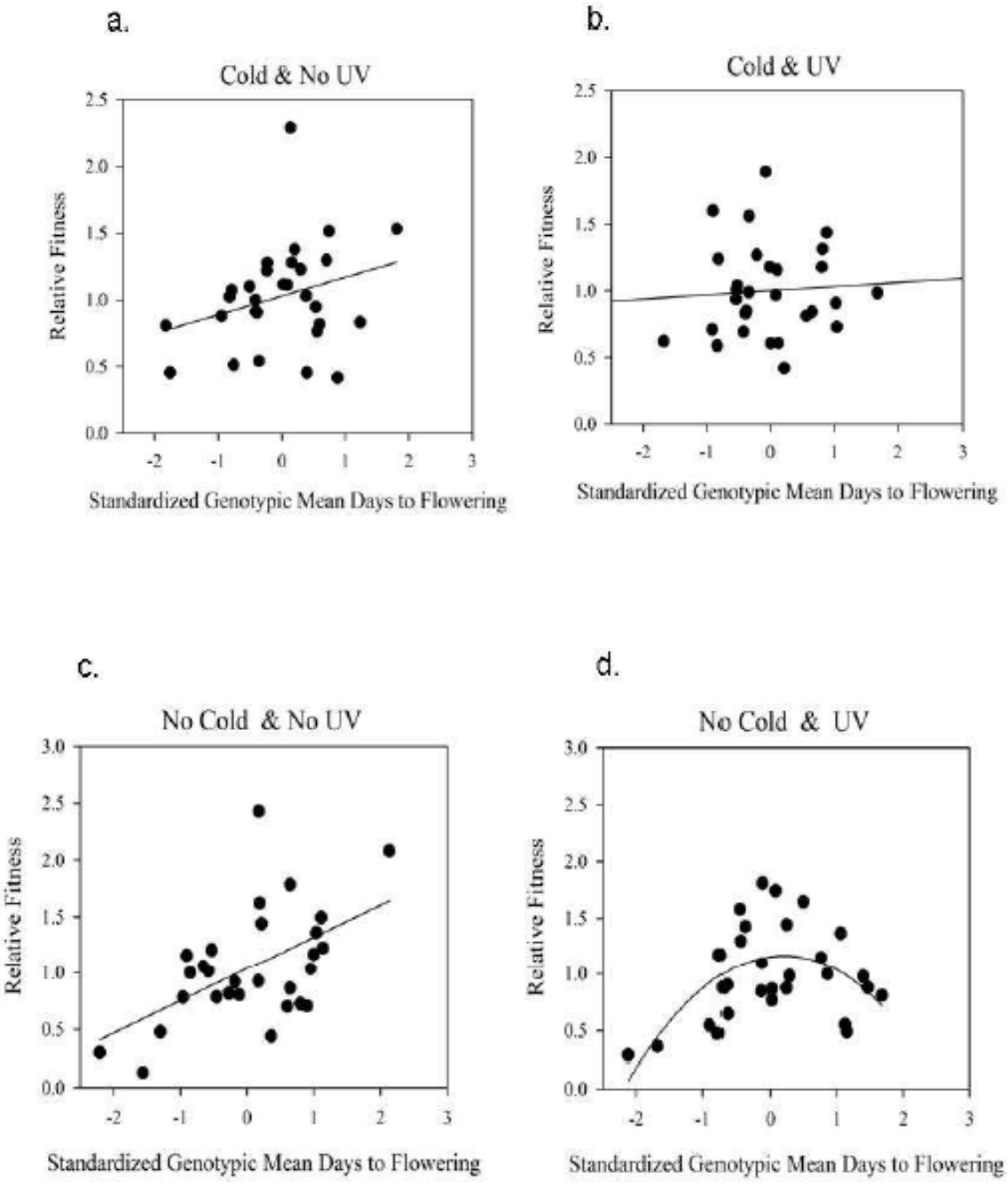

Figure 3. Scatterplot of the standardized genotypic means for Number of Days to Flowering (x-axis) against genotypic mean relative fitness (y-axis) in a. the Cold and No UV-C treatment, b. Cold and UV-C treatments, c. No Cold and No UV-C treatments, d. No Cold and UV-C treatments.

Table 1: Type III F-tests from a 3-way mixed model ANOVA. Bold terms are significant after a Bonferonni correction for multiple tests.

	Cold	UVC	Pop	Cold*UVC	Cold*Pop	UVC*Pop	Cold*UVC*Pop
df	1	1	28	1	28	28	28
Days to Bolting	0.84	0.11	33.86	0.11	0.65	0.66	**1.91****
Days to Flowering	0.48	0.49	**23.67***	0.18	0.67	0.74	**2.11****
Flowering Interval	1.7	**5.4***	**2.59****	0.00	**2.4***	**2.55****	0.54
Rosette Diameter	0.00	**30.07****	**17.07*****	1.46	1.34	0.69	1.29
Number of Fruits	1.56	0.80	1.00	0.87	1.01	1.03	0.91
Lifespan	0.17	0.66	-7.91	0.65	0.35	0.53	1.12

* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001

Table 2. Table of least squares means of untransformed data within environments and their standard errors. Significant t-tests for the Post-Hoc contrasts of NoUVC vs. UVC within environments are presented in bold.

	Cold		NoCold	
	NoUVC	UVC	NoUVC	UVC
Days to Bolting	21.76 ± se	22.09	22.98	23.40
Days to Flowering	25.64 ± se	25.49	26.64	26.89
Flowering Interval	3.87 ± se	4.05	3.66	3.92
Rosette Diameter	43.84 ± se	35.51	43.03	37.31
Number of Fruits	108.11 ± se	97.26	95.51	94.32
Lifespan	51.11	49.56	44.42	51.68

* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001

Table3. Type III F-test of the two way Mixed Model ANOVAs testing for the effect of UVC, population and UVC-by-population interactions within the stratification (Cold) and non-stratified (No Cold) treatments.

	Cold			No Cold		
	UVC	Pop	UVC*Pop	UVC	Pop	UVC*Pop
df	1	28	28	1	28	28
Days to Bolting	0.01	**9.13******	1.22	0.76	**14.40******	**2.22*****
Days to Flowering	0.02	**9.44******	1.44	1.54	**13.75******	**2.35*****
Flowering Interval	1.85	**3.22******	2.17	**19.84****	**3.90******	0.99
Rosette Diameter	**24.14****	**14.89******	0.84	7.42*	**10.36******	1.43
Number of Fruits	0.42	1.42	1.40	0.77	0.83	0.82
Lifespan	0.01	0.01	0.75	1.00	0.76	1.03

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

Table 4. Genotypic selection analysis estimating linear selection differentials and the quadratic term (γ) estimating non-linear selection within each of the four environments.

	Regression	Cold		NoCold	
Trait	Coefficients	NoUVC	UVC	NoUVC	UVC
Days to Bolting	Linear SD	0.12	0.02	0.27**	0.09
	Quadratic γ	-0.08	-0.04	-0.02	-0.51***
Days to Flowering	Linear SD	0.14	0.03	0.28**	0.09
	Quadratic γ	-0.05	-0.06	-0.02	-0.20***
Flowering Interval	Linear SD	0.00	-0.01	-0.22	-0.07
	Quadratic γ	-0.24	-0.07	-0.08	-0.18*
Rosette Diameter	Linear SD	-0.10	-0.05	-0.30**	-0.10
	Quadratic γ	-0.06	0.08	-0.22	-0.13
Lifespan	Linear SD	-0.41*	-0.36*	-0.10	-0.15
	Quadratic γ	0.17	0.49	0.09	-0.22

* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001

LITERATURE CITED:

Baskin, J.M. and Baskin, C.C. 1983. Seasonal changes in the germination responses of buried seeds of *Arabidopsis thaliana* and ecological interpretation. *Bot. Gaz.* 144: 540-543.

Bradshaw, A.D. 1965. Evolutionary significance of phenotypic plasticity in plants. *Adv. Genet.* 13: 115-155.

Carranco, R., Almoguera, C., and Jordano, J. 1997. A Plant Small Heat Shock Protein Gene Expressed during Zygotic Embryogenesis but Noninducible by Heat Stress *J. Biol. Chem.* 272: 27470-27475.

Charmantier, A., McCleery, R.H., Cole, L.R., Perrins, C., Kruuk, L.E.B., and Sheldon, B.C. 2008. Adaptive Phenotypic Plasticity in Response to Climate Change in a Wild Bird Population. *Science.* 320: 800-803.

Dechaine, J.M., Johnston, J.A., Brock, M.T., Weinig, C. 2007. Constraints on the evolution of adaptive plasticity: costs of plasticity to density are expressed in segregating progenies. *New Phytol.* 176: 874-882.

DeWitt, T.J., Sih, A., and Wilson, D.S. 1998. Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* 13: 77-81.

Donohue, K., Dorn, L.A., Griffith, C., Kim, E.S., Aguilera, A., Polisetty, C.R., and Schmitt, J. 2005. Niche construction through germination cueing: life-history responses to timing of germination in *Arabidopsis thaliana. Evolution.* 59: 4771-785.

Dorn, L.A., Pyle, E.H., and Schmitt, J. 2000. Plasticity to light cues and resources in *Arabidopsis thaliana*: Testing for adaptive value and costs. *Evolution.* 54: 1982-1994.

Fait, A., Angelovici, R., Less, H., Ohad, I., Urbanczyk-Wochniak, E., Fernie, A.R., and Galili, G. 2006. *Arabidopsis* Seed Development and Germination Is Associated with Temporally Distinct Metabolic Switches. *Plant Physiol.* 142: 839–854.

Gomulkiewicz, R. and Kirkpatrick, M. 1992. Quantitative Genetics and the Evolution of Reaction Norms. *Evolution.* 46: 390-411.

Hedrick, P.W. 1986. Genetic Polymorphisms in heterogeneous environments: A decade later. *Annu Rev Ecol Syst.* 17: 535-566.

Huang, D.Q., Wu, W.R., Abrams, S.R., and Cutler, A.J. 2008. The relationship of drought-related gene expression in *Arabidopsis thaliana* to hormonal and environmental factors. *J. Exp. Bot.* 59: 2991-3007.

Kelly, A.E. and Goulden, M.L. 2008. Rapid shifts in plant distribution with recent climate change. *Proc. Natl. Acad. Sci. U.S.A.* 105: 11823-11826.

Kreps, J.A, Wu, Y., Chang, H-S., Zhu, T., Wang, X., and Harper, J.F. 2002. Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiol.* 130: 2129-2141.

Kurepa, J., Walker, J.M., Smalle, J., Gosink, M.M., Davis, S.J., Durham, T.L., Sung, D-Y., and Vierstra, R.D. 2002. The SUMO protein modification system in *Arabidopsis*: accumulation of SUMO1 and 2 conjugates is increased by stress. *J. Biol. Chem.* 278: 6862-6872.

Munir, J., Dorn, L.A., Donohue, K., and Schmitt, J. 2001. The effect of maternal photoperiod on seasonal dormancy in *Arabidopsis thaliana* (Brassicaceae) *Am. J. Bot.* 88: 1240-1249.

Murren, C.J., Douglass, L., Gibson, A., and Dudash, M.R. 2006. Individual and combined effects of Ca/Mg ratio and water on trait expression in *Mimulus guttatus. Ecology* 87: 2591-2602.

Poulton, J. and Winn A.A. 2002. Costs of canalization and plasticity in response to neighbors in *Brassica rapa. Plant Species Biology.* 17: 109–118.

Rausher, M.D. 1992. The measurement of selection on quantitative traits – Biases due to environmental covariances between traits and fitness. *Evolution.* 46*:* 616-626.

Rowland, F.S. 2006. Stratospheric ozone depletion. *Phil. Trans. Roy. Soc. B-Biological Sciences.* 361: 769-790.

Stinchcombe, J.R., Dorn, L.A. and Schmitt, J. 2004. Flowering time plasticity in *Arabidopsis thaliana:* a reanalysis of Westerman & Lawrence (1970). *J. Evol. Biol.* 17: 197 – 207.

Su, P-H and Li, H-M. 2008. *Arabidopsis* Stromal 70-kD Heat Shock Proteins Are Essential for Plant Development and Important for Thermotolerance of Germinating Seeds. *Plant Physiol.* 146: 1231-1241.

Swindell, W.R., Huebner, M., and Weber, A.P. 2007. Plastic and adaptive gene expression patterns associated with temperature stress in *Arabidopsis thaliana. Heredity.* 99: 143-150

van Kleunen, M. and Fischer, M. 2005. Constraints on the evolution of adaptive phenotypic plasticity in plants. *New Phytol.* 166: 49–60.

van Kleunen, M., Fischer, M., and Schmid, B. 2000. Costs of plasticity in foraging characteristics of a clonal plant *Ranunculus reptans*. *Evolution.* 54: 1947–1955.

Van Tienderen, P.H. 1991. Evolution of generalists and specialists in spatially heterogeneous environments. *Evolution.* 45: 1317-1331.

Wang, W., Vinocur, B., Shoseyov, O., and Altman, A. 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends Plant Science.* 9: 244-252.

Weijschede, J., Martinkova, J., de Kroon, H., and Huber, H. 2006. Shade avoidance in *Trifolium repens:* costs and benefits of plasticity in petiole length and leaf size. *New Phytol.* 172: 655-666

Weinig, C. 2000. Differing selection in alternative competitive environments: shade-avoidance responses and germination timing. *Evolution* 54:124–136

Weinig, C., Graveur, K.A., Kane, N.C., and Schmitt, J. 2004. Testing adaptive plasticity to UV: Costs and benefits of stem elongation and light-induced phenolics. *Evolution.* 58: 2645-2656.

REFERENCES

Agrawal, A. A. 1998. Induced responses to herbivory and increased plant performance. *Science* 279: 1201-1202.

---. 2001. Phenotypic plasticity in the interactions and evolution of species. *Science* 294: 321-326.

Agrawal, A. A., J. K. Conner, M. T. J. Johnson, and R. Wallsgrove 1999. Ecological genetics of an induced plant defense against herbivores: additive genetic variance and costs of phenotypic plasticity. *Evolution* 56: 2206-2213.

Alonso-Blanco, C., A. J. M. Peeters, M. Koornneef, C. Lister, C. Dean, N. van den Bosch, J. Pot, and M. T. R. Kuiper 1998. Development of an AFLP based linkage map of *Ler*, Col and CVI *Arabidopsis thaliana* ecotypes and construction of a *Ler*/Cvi recombinant inbred line population. *Plant Journal* 14: 259-271.

Aubin-Horth N, C. R. Landry, B. H. Letcher, and H. A. Hoffman 2005. Alternative life histories shape brain gene expression profiles in males of the same population. *Proceedings of Biological Sciences: The Royal Society* 272: 1655-1662.

Aubin-Horth, N., B. H. Letcher, and H. A. Hoffman 2005. Interaction of rearing environment and reproductive tactic on gene expression profiles in Atlantic salmon. *Journal of Heredity* 96: 261-278.

Bäckström, S., N. Elfving, R. Nilsson, G. Wingsle and S. Björklund 2007. Purification of a plant mediator from *Arabidopsis thaliana* identifies PFT1 as the med25 subunit. *Molecular Cell* 26: 717-729.

Bailey, D. W. 1971. Recombinant inbred strains. *Transplantation* 11: 325-327.

---. 1981. Recombinant inbred strains and bilineal congenic strains. In *The Mouse in Biomedical Research,* 1 (H. L. Foster, J. D. Small, and J. G. Fox, eds). New York Academic Press, pp. 223-239.

Baskin, J.M. and C.C. Baskin 1983. Seasonal changes in the germination responses of buried seeds of *Arabidopsis thaliana* and ecological interpretation. *Botany Gazette* 144:9 540-543.

Berrigan, D., and S. Scheiner 2004. Modeling the evolution of phenotypic plasticity. In *Phenotypic Plasticity: Functional and Conceptual Approaches.* (T. J. DeWitt and S. M. Scheiner, eds). Oxford University Press, New York: pp. 82-97.

Blanco, A., M. P. Bellomo, A. Cenci, C. DeGiovanni, R. D'Ovidio, E. Iacono, B. Laddomada, M. A. Pagnotta, E. Porceddu, A. Sciancalepore, R. Simeone, and O. A. Tanzarella 1998. A genetic linkage map of durum wheat. *Theoretical and Applied Genetics* 97: 721-728.

Boisson, B., C. Giglione, and T. Meinnel 2003. Unexpected Protein Families Including Cell Defense Components Feature in the N-Myristoylome of a Higher Eukaryote. *Journal of Biological Chemistry* 278: 43418-43429

Borevitz, J. O., D. Liang, D. Plouffe, H-S. Chang, T. Zhu, D. Weigel, C. C. Berry, E. Winzeler, and J. Chory 2003. Large-scale identification of single-feature polymorphisms in complex genomes. *Genome Research* 13: 513-523.

Bradshaw, A. D. 1965. Evolutionary significance of phenotypic plasticity in plants. *Advances in Genetics* 13: 115-155.

Breuer, C., N. J. Stacey, C. E. West, Y. Zhao, J. Chory, H. Tsukaya, Y. Azumi, A. Maxwell, K. Roberts and K. Sugimoto-Shirasu 2007. BIN4, a novel component of the plant DNA topoisomerase VI complex, is required for endoreduplication in *Arabidopsis*. *The Plant Cell* 19:3655-3668.

Broman, K. W. 2005. The genomes of recombinanant inbred lines. *Genetics*: 169: 1133-1146.

Brooker, R. J. 2005. *Genetics: Analysis and Principles*. 2nd ed. New York: McGraw Hill.

Brown, P. O., and D. Botstein 1999. Exploring the new world of the genome with DNA microarrays. *Nature Genetics* 21: 33-37.

Burr, B., and F. A. Burr 1991. Recombinant inbreds for molecular mapping in maize; theoretical and practical considerations. *Trends in Genetics* 118: 55-60.

Burr, B., F. A. Burr, K. H. Thompson, M. C. Albertson, and C. W. Stuber 1988. Gene mapping with recombinant inbreds in maize. *Genetics* 118: 519-526.

Chinnusamy, V., J. Zhu, and J. K. Zhu 2006. Gene regulation during cold acclimation in plants. *Physiologia Plantarum* 126: 52-61.

Churchill, G. A. 2002. Fundamentals of experimental design for cDNA microarrays. *Nature Genetics* 32: 490-495.

Conner, J. K. and D. L. Hartl 2004. *A primer of Ecological Genetics*. Sinauer Associates Inc. Sunderland, Massachusetts, U.S.A.

Cook, S. A., and M. P. Johnson 1968. Adaptation to heterogeneous environments I. Variation in heterophylly in *Ranunculus dlammula* L. *Evolution* 22: 496-516.

Crews, D., J.M. Bergeron, J.J. Bull, D. Flores, A. Tousignant, J.K. Skipper, and T. Wibbels 1994. Temperature-dependent sex determination in reptiles: proximate mechanisms, ultimate outcomes, and practical applications. *Developmental Genetics* 15: 297-312.

Crowl, T.A., and A.P. Covich 1990. Predator-induced life-history shifts in a freshwater snail. *Science* 247: 949-951.

Datta, Y. H., P. T. Adams, W. R. Drobyski, S. P. Ethier, V. H. Terry, and M. S. Roth 1994. Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. *Journal of Clinical Oncology* 12: 475-482.

Dechaine, J.M., J.A. Johnston, M.T. Brock, and C. Weinig 2007. Constraints on the evolution of adaptive plasticity: costs of plasticity to density are expressed in segregating progenies. *New Phytologist* 176: 874-882.

Donohue, K. 2002. Germination timing influences natural selection on life-history characters in *Arabidopsis thaliana*. *Ecology* 83 (4): 1006-1016.

Donohue, K., L. Dorn, C. Griffith, E. Kim, A. Aguilera, C. R. Polisetty, and J. Schmitt 2005a. Environmental and genetic influences on the germination of *Arabidopsis thaliana* in the field. *Evolution* 59: 740-757.

Donohue, K., E. Hammond-Pyle, D. Messiqua, M. S. Heschel, and J. Schmitt 2003. Adaptive divergence in plasticity in natural populations of *Impatiens capensis* and its consequences for performance in novel habitats. *Evolution* 55: 692-702.

---. 2005b. The evolutionary ecology of seed germination of *Arabidopsis thaliana*: variable natural selection on germination timing. *Evolution* 59: 758-770.

Dorn, L. A., E. Hammond-Pyle, and J. Schmitt 2000. Plasticity to light cues and resources in *Arabidopsis thaliana*: testing for adaptive value and costs. *Evolution* 54: 1982-1994.

Doukhanina, E. V., S. Chen, E. van der Zalm, A. Godzik, J. Reed and M. B. Dickman 2006. Identification and functional characterization of the BAG protein family in *Arabidopsis thaliana*. *Journal of Biological Chemistry* 281: 18793-18801.

Dudley, S. A. 2004. The functional ecology of phenotypic plasticity in plants. In: DeWitt T.J., and S. M. Scheiner, eds. *Phenotypic Plasticity. Functional and conceptual approaches*. Oxford, UK: Oxford University Press, 151-172.

Dudley, S. A., and J. Schmitt 1995. Genetic differentiation in morphological responses to simulated foliage shade between populations of *Impatiens capensis* from open and woodland sites. *Functional Ecology* 9: 655-666.

---. 1996. Testing the adaptive plasticity hypothesis: density-dependent selection on manipulated stem length in *Impatiens capensis. The American Naturalist* 147: 445-465.

Ellis, T. H. N., L. Turner, R. P. Hellens, D. Lee, C. L. Harker, C. Enard, C. Demoney, and D. R. Davies 1992. Linkage maps in pea. *Genetics* 130: 649-663.

Evans, J., and D. Ratcliffe 1972. Variation in "after-ripening" of seeds in *Arabidopsis thaliana* and its ecological significance. *Arabidopsis Information Service* 9: 3-5.

Falconer, D. S. 1981. *Introduction to Quantitative Genetics*. 2nd ed. Longman, N.Y.

Futcher, B., G. I. Latter, P. Monardo, C. S. McLaughlin, and J. I. Garrels 1999. A sampling of the yeast proteome. *Molecular and Cellular Biology* 19: 7357-7368.

Galbraith, D. 2010. University of Arizona. http://ag.arizona.edu/microarray/

Gibson, G. 2002. Microarrays in ecology and evolution: a preview. *Molecular Ecology* 11: 17-24.

Gilad, Y., and J. Borevitz 2006. Using DNA microarrays to study natural variation. *Current Opinions in Genetics and Development* 16: 553-558.

Gillespie, J. H. and M. Turelli 1989. Genotype-environment interaction and the maintenance of polygenic variation. *Genetics* 121: 129-138.

Girke, T., J. Todd, S. Ruuska, J. White, C. Benning, and J. Ohlrogge 2000. Microarray analysis of developing *Arabidopsis* seeds. *Plant Physiology* 124: 1570-1581.

Goldsby, R. A., T. J. Kindt, B. A. Osborne, and J. Kuby 2003. *Immunology*. 5th ed.: New York: W. H. Freeman and Company.

Gonzalez, A. V., and E. Gianoli 2004. Morphological plasticity in response to shading in three Convolvulus species of different ecological breadth. *Acta Oecologica* 26: 185-190

Gygi, S. P., Y. Rochon, B. R. Franza, and R. Aebersold 1999. Correlation between protein and mRNA abundance in yeast. *Molecular and Cellular Biology* 19: 1720-1730.

Haccia, J. 1999. Resequencing and mutational analysis using oligonucleotides microarrays. *Nature Genetics* 21: 42-47.

Harford, J. B., and D. R. Morris 1997. Post-transcriptional gene regulation. Wiley-Liss, Inc., New York, N.Y.

Hegmann, J. and H. Dingle 1982. Phenotypic and genotypic covariance structure in milkweed bug life history traits. Dingle, H. and Hegmann, J. (eds.). *Evolution and Genetics of Life Histories.* Springer-Verlag, New York.

Huang, D.Q., W.R. Wu, S.R. Abrams, and A.J. Cutler 2008. The relationship of drought-related gene expression in *Arabidopsis thaliana* to hormonal and environmental factors. *Journal of Experimental Botany* 59: 2991-3007.

Huang, X, J. Schmitt, L. A. Dorn, C. Griffith, S. Effgen, S. Takao, M. Koornneef, and K. Donohue 2010. The earliest stages of adaptation in an experimental plant population: strong selection on QTLs for seed dormancy. *Molecular Ecology* 19 1335-1351.

Hubby, J.L. and R.C. Lewontin 1966. A molecular approach to the study of genic heterozygosity in natural populations I. The number of alleles at different loci in *Drosophila pseudoobscura*. *Genetics* 54: 577-594.

Kirik, V., A. Schrader, J. F. Uhrig and M. Hulskamp 2007. MIDGET unravels functions of the *Arabidopsis* topoisomerase VI complex in DNA endoreduplication, chromatin condensation, and transcriptional silencing. *The Plant Cell* 19:3100-3110.

Kliebenstein, D., D. Pedersen, B. Barker, and T. Mitchell-Olds 2002. Comparative Analysis of Quantitative Trait Loci Controlling Glucosinolates, Myrosinase and Insect Resistance in *Arabidopsis thaliana*. *Genetics* 161: 325-332.

Kreps, J.A., Y. Wu, H-S Chang, T. Zhu, X. Wang, and J.F. Harper 2002. Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiology* 130: 2129-2141.

Lanciotti, R. S., C. H. Calisher, D. J. Gubler, G. Chang, and A. V. Vorndam 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *Journal of Clinical Microbiology* 30: 545-551.

Leips, J., and T. F. C. Mackay 2000. Quantitative trait loci for life span in *Drosophila melanogaster*: interactions with genetic background and larval density. *Genetics* 155: 1773-1788.

Leonhardt, N., J. Kwak, M. Robert, G, Leonhardt, and J. Schroeder 2004. Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive

abscisic acid ypersensitive protein phosphatase 2c mutant. *Plant Cell* 16: 596–615.

Lewontin, R.C., and J.L. Hubby 1966. A molecular approach to the study of genic heterozygosity in natural populations II. Amount of variation and degree of heterozygosity in natural populations of *Drosophila pseudoobscura*. *Genetics* 54: 595-609.

Lister, C., and C. Dean 1993. Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana*. *Plant Journal* 4: 745-750.

Lloyd, J. C., and O. V. Zakhleniuk 2004. Responses of primary and secondary metabolism to sugar accumulation revealed by microarray expression analysis of the *Arabidopsis* mutant, *pho3*. *Journal of Experimental Botany* 55: 1221-1230.

Lynch, C. and D. Sulzbach 1984. Quantitative genetic analysis of temperature regulation in *Mus musculus*. II. Diallel analysis of individual traits. *Evolution* 38: 527-540.

Meyerowitz, E. 1987. *Arabidopsis thaliana*. *Annual Reviews in Genetics* 21: 93-111.

Meyerowitz, E. M., and R. E. Pruitt 1985. *Arabidopsis thaliana* and plant molecular genetics. *Science* 229: 1214-1218.

Moran, N. A. 1992. The evolutionary maintenance of alternative phenotypes. *American Naturalist* 139: 971-989.

Munir, J., L.A. Dorn, K. Donohue, and J. Schmitt 2001. The effect of maternal photoperiod on seasonal dormancy in *Arabidopsis thaliana* (Brassicaceae). *American Journal of Botany* 88: 1240-1249.

Murfet, I. C. 1977. Environmental interaction and the genetics of flowering. *Annual Review of Plant Physiology* 28: 253–278.

Narusaka, Y., M. Narusaka, M. Seki, J. Ishida, M. Nakashima, A. Enju, A. Kamiya, T. Sakurai, M. Satoh, M. Kobayashi, Y. Tosa, P. Park, and K. Shinozaki 2003. The cDNA microarray analysis using an *Arabidopsis* pad3 mutant reveals the expression profiles and classification of genes induced by *Alternaria brassicicola* attack. *Plant Cell Physiology* 44:377-387.

National Center for Biotechnology Information 2009. "Genome Project Statistic." Accessed June 26 2009. http://www.ncbi.nlm.nih.gov/genomes/static/gpstat.html

Newman, R. A. 1988. Adaptive plasticity in development of *Scaphiopus couchii* tadpoles in desert ponds. *Evolution* 42: 774-783.

Nordborg, M., and J. Bergelson 1999. The effect of seed and rosette cold treatment on germination and flowering time in some *Arabidopsis thaliana* (Brassicaceae) ecotypes. *American Journal of Botany* 86: 470-475.

Paterson, A. H. S. Damon, J. D. Hewitt, D. Zamir, H. D. Rabinowitch, S. E. Lincoln, E. S. Lander, and S. D. Tanksley 1991. Mendelian factors underlying quantitative traits in tomato: comparison across species, generations, and environments. *Genetics* 127: 191-197.

Pierce, J. L., L. Lu, J. Gu, L. M. Silver, and R. W. Williams 2004. A new set of BxD recombinant inbred lines from advanced intercross populations in mice. *BMC Genetics* 5: 7-23.

Pigliucci, M. 2001. *Phenotypic Plasticity: Beyond Nature and Nurture*. John Hopkins University Press, Baltimore, USA.

Podell, S., and M. Grobskov 2004. Predicting N-terminal myristoylation sites in plant proteins. *BMC Genomics* 5:37-52.

Poorter, H. and H. Lambers 1986. Growth and competitive ability of a highly plastic and marginally plastic genotype of *Plantago major* in a fluctuating environment. *Physiological Plantarum* 67: 217-222.

Poulton, J. and A. A. Winn 2002. Costs of canalization and plasticity in response to neighbors in *Brassica rapa*. *Plant Species Biology* 17: 109-118.

Quackenbush, J. 2002. Microarray data normalization and transformation. *Nature Genetics* 32: 496-501.

Ratcliffe, D. 1965. Germination characteristics and their inter- and intra-population variability in *Arabidopsis*. *Arabidopsis Information Service* 13.

Reiter, R. S., J. G. K. Williams, K. A. Feldman, J. A. Rafalski, S. V. Tingey, and P. A. Scolnik 1992. Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and random amplified polymporphic DNAs. *Proceedings of the National Academy of Sciences USA* 89: 1477-1481.

Rosenzweig, B. A., P. S. Pine, O. E. Domon, S. M. Morris, J. J. Chen, and F. D. Sistare 2004. Dye-bias correction in dual-labeled cDNA microarray gene expression measurements. *Environmental Health Perspectives*. 112: 480-487.

Rubin, M. J., and L. A. Dorn. Quantitative trait loci and global gene expression patterns offer insight into phenotypic plasticity in bolting time in an annual plant species. *Forthcoming*

Scheiner, S. M. 1993. Genetics and evolution of phenotypic plasticity. *Annual Review of Ecology and Systematics* 24: 35-68.

Scheiner, S.M. and R.F. Lyman 1989 Genetics of phenotypic plasticity. I. Heritability. *Journal of Evolutionary Biology* 2: 95-107.

---. 1991. The genetics of phenotypic plasticity. II. Response to selection. *Journal of Evolutionary Biology* 4: 23-50.

Schena, M., D. Shalon, R. W. Davis, and P. O. Brown 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270: 467-470.

Schlicting, C. D. 1986. The evolution of phenotypic plasticity in plants. *Annual Review of Ecology and Systematics* 17: 667-693.

Schlicting, C. D. and D. A. Levin 1984. Phenotypic plasticity in the annual *Phlox*: tests of some hypotheses. *American Journal of Botany* 71: 252-260.

---. 1986. Phenotypic plasticity: an evolving plant character. *Biological Journal of the Linnean Society* 29: 37-47.

Schlicting, C. D., and M. Pigliucci 1993. Control of phenotypic plasticity via regulatory genes. *American Naturalist* 142: 366-370.

---. 1995. Gene regulation, quantitative genetics, and the evolution of reaction norms. *Evolutionary Ecology* 9:154-168

Schmalhausen, I. I. 1949. *Factors of Evolution: The Theory of Stabilizing Selection.* Blakiston, Philadelphia, PA.

Schmitt, J., and R. D. Wulff 1993. Light spectral quality, phytochrome and plant competition. *Trends in Ecology and Evolution* 8: 47-51.

Seki, M., M. Narusaka, H. Abe, M. Kasuga, K. Yamaguchi-Shinozaki, P. Carninci, Y. Hayashizaki, and K. Shinozaki 2001. Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell* 13: 61-72.

Seki, M., M. Narusaka, J. Ishids, T. Nanjo, M. Fujita, Y. Oono, A. Kamiya, M. Nakajima, A. Enju, T. Sakurai, M. Satou, K. Akiyama, T. Taji, K. Yamaguchi-Shinozaki, P. Carninci, J. Kawai, Y. Hayashizaki, and K. Shinozaki 2002. Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold, and high-salinity stresses using a full-length cDNA microarray. *Plant Journal* 31: 279-292.

Semlitsch, R. D. 1987. Paedomorphosis in *Ambystoma talpoideum*: effects of density, food, and pond drying. *Ecology* 69: 994-1002.

Shine, R. 1999. Why is sex determines by nest temperature in many reptiles? Trends in *Ecology and Evolution* 14 (5): 186-189.

Shiu, S. H. and J. O. Borevitz 2008. The next generation of microarray research: applications in evolutionary and ecological genomics. *Heredity*: 100: 141-149.

Simpson, G. G. 2004. The autonomous pathway: epigenetic and post-transcriptional gene regulation in the control of *Arabidopsis* flowering time. *Current opinion in plant biology* 7: 1-5.

Slepecky, R.A., and W.T. Starmer 2009. Phenotypic plasticity in fungi: a review with observations on *Auerobasidium pullulans*. *Mycologia* 101 (6): 823-832.

Smith, H. 1982. Light quality, photoreception, and plant strategy. *Annual Review of Plant Physiology* 33: 481-518.

Snape, J. W., R. B. Flavell, M. O'Dell, W. G. Hughes, and P. P. Payne 1985. Intrachromosomal mapping of the nuclear organizer region relative to three marker loci on chromosome 1B of wheat (*Triticum aestivum*). Theoretical and *Applied Genetics* 69: 263-270.

Stearns, S. C. 1989. The evolutionary significance of phenotypic plasticity. *BioScience* 39: 436-445.

Stears, R. L., T. Martinsky, and M. Schena 2003. Trends in microarray analysis. *Nature medicine* 9: 140-145

Stinchcombe, J.R., L.A. Dorn, and J. Schmitt 2004. Flowering time plasticity in *Arabidopsis thaliana:* a reanalysis of Westerman & Lawrence (1970). *Journal of Evolutionary Biology* 17: 197 – 207.

Sultan, S. E. 2000. Phenotypic plasticity for plant development, function and life history. Trends in Plant *Science* 5: 537-542.

Swindell, W.R., M. Huebner, and A.P. Weber 2007. Plastic and adaptive gene expression patterns associated with temperature stress in *Arabidopsis thaliana. Heredity* 99: 143-150

Taylor, B. A. 1989. Recombinant inbred strains. In *Genetic Variants and Strains of the Laboratory Mouse*, 2 (M. F. Lyon and A. G. Searle, ed). Oxford University Press, pp. 773-796.

The *Arabidopsis* Information Resource. Accessed 2009. http://www.arabidopsis.org

Timmerman-Vaughan, G. M., J. A. McCallum, T. J. Frew, N. F. Weeden, and A. C. Russell 1996. Linkage mapping of quantitative trait loci controlling seed weight in pea (*Pisum sativum* L.). *Theoretical and Applied Genetics* 93: 431-439.

Ungerer, M. C., S. S. Halldorsdottir, M. D. Purugganan, and T. F. C. Mackay 2003. Genotype-Environment Interactions at Quantitative Trait Loci Affecting Inflorescence Development in *Arabidopsis thaliana. Genetics* 165: 353-365.

Valladares F. and R. W. Pearcy 1998. The functional ecology of shoot architecture in sun and shade plants of *Heteromeles arbutifola*. M. Roem., a Californian chapparal shrub. *Oecologia* 114: 1-10.

van Kleunen, M., M. Fischer and B. Schmid 2000. Costs of plasticity in foraging characteristics of a clonal plant *Ranunculus reptans. Evolution.* 54: 1947–1955.

Varshavsky, A. 1996. The N-end rule: functions, mysteries, uses. Proceedings of the *National Academy of the Sciences USA* 93:12142-12149.

Via, S., and R. Lande 1985. Genotype-environment interaction and the evolution of phenotypic plasticity. *Evolution* 39: 505-522.

---. 1987. Evolution of genetic variability in a spatially heterogeneous environment: effects of genotype-environment interaction. *Genetical Research* 49: 147-156.

Via, S. 1993a. Adaptive phenotypic plasticity: Target or by-product of selection in a variable environment? *American Naturalist* 142: 352-365.

---. 1993b. Regulatory genes and reaction norms. *American Naturalist* 142: 374-378.

Weijschede, J., J. Martinkova, H. de Kroon, and H. Huber 2006. Shade avoidance in *Trifolium repens:* costs and benefits of plasticity in petiole length and leaf size. *New Phytologist* 172: 655-666.

Weinig, C. 2000. Plasticity versus canalization: population differences in the timing of shade-avoidance responses. *Evolution* 54: 441-451.

Weinig, C., K.A. Graveur, N.C. Kane, and J. Schmitt 2004. Testing adaptive plasticity to UV: Costs and benefits of stem elongation and light-induced phenolics. *Evolution* 58: 2645-2656.

Weinig, C., Z.M. German, L.M. Demink, and J.A. Johnston 2006. Local and global costs of adaptive plasticity. *American Naturalist* 126: 826-836

West-Eberhard, M. J. 1989. Phenotypic plasticity and the origins of diversity. *Annual Review of Ecology and Systematics* 20: 249-278.

Whitfield, C. W., A. M. Cziko, and G. E. Robinson 2003. Gene expression profiles in the beain predict behavior in individual honey bees. *Science* 302: 296-299.

Williams, G. C. 1966. *Adaptation and Natural Selection*. Priceton, Princeton University Press.

Wisman, E., and J. Ohlrogge 2000. *Arabidopsis* microarray service facilities. *Plant Physiology* 124: 1468-1471.

Xue, T. T., D. Wang, S. Z. Zhang, J. Ehlting, F. Ni, S. Jakab, C. C. Zheng, and Y. Zhong 2008. Genome-wide and expression analysis of protein phosphatase 2C in rice and *Arabidopsis*. *BMC Genomics* 9:550-571.

Yakub, M., M. Nelson, and L. A. Dorn. Phenotypic plasticity of *Arabidopsis thaliana* to the evolutionarily novel environment UV-C is moderated by seed stratification. *Journal of Evolutionary Biology* (*forthcoming*).

Yampolsky, L. Y., and S. M. Scheiner 1994. Developmental noise, phenotypic plasticity, and allozyme heterozygosity in *Daphnia*. *Evolution* 48: 1715-1722.

Yang, H., J. McLeese, M. Weisbart, J. L. Dionne, I. Lemaire, and R. A. Aubin 1993. Simplified high throughput protocol for Northern hybridization. *Nucleic Acids Research* 21: 3337-3338.

Yang, H., Y. Li, and J. Hua 2006. The C2 domain protein BAP1 negatively regulates defense responses in *Arabidopsis*. *The Plant Journal* 48: 238-248.

Yang, Y. H., and T. Speed 2002. Design issues for cDNA microarray experiments. *Nature Review Genetics* 3: 579-588.