APPROPRIATE PLANT GENOTYPES FOR URBAN ECOLOGICAL RESTORATION: AN INVESTIGATION INTO URBAN STRESS RESPONSE

by

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ABSTRACT OF THE DISSERTATION

APPROPRIATE PLANT GENOTYPES FOR URBAN ECOLOGICAL RESTORATION: AN INVESTIGATION INTO URBAN STRESS RESPONSE

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Given the unprecedented increase in urbanization and its effect on natural ecosystems, the effort to restore human-impacted land is timely and essential. Flexible and stress-resistant plant genotypes may provide a practical solution for restoration of constantly changing and stressful environments, yet there has been little progress linking general stress tolerance with plants being used in urban restoration. This dissertation project uses a novel, experimental approach to test and determine the importance and effectiveness of phenotypically plastic and stress-resistant plant genotypes in the ecological restoration of urban and degraded land. Using a model system involving the annual cress, *Arabidopsis thaliana* and the heat-shock protein (HSP) induced stress response system; I began this dissertation by testing if the presence of an induced stress response (HSP17.6) was essential for overall success. I found that mutant plants lacking a working HSP17.6 response generally showed an inability to cope with various types of abiotic urban stress. This difference was generally more pronounced in high stress conditions, providing

evidence of adaptive plasticity. I then expanded the investigation by using six field-collected *Arabidopsis* genotypes and by adding a molecular analysis of the expression of both HSP17.6 and HSP101. I tested for natural variation in stress response, and then sought to use that information to predict success in various stressful conditions. While I found natural variation both in phenotype and the expression of HSP genes in stress, I saw little correlation between HSP expression and fitness, suggesting that predicting plant success via such molecular data may have limited utility. I did, however, identify "stress-resistant" genotypes, which consistently performed best across all stress treatments. Finally, I tested whether those stress-resistant genotypes continued to exhibit success in novel stress conditions. They did, which suggests that simple preliminary stress tests can provide a reasonable and quick method of genotype selection, especially for practitioners restoring urban and degraded land. I conclude that stress-resistant genotypes may be the best option when planting in heterogeneous soils with unknown stressor combinations in novel urban restoration sites.

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with the humble knowledge that I could not have done it without them and I am most grateful for their love, support and influence in my life.

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Portions of this dissertation will be submitted for review for publication.

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CHAPTER 1

INTRODUCTION

The Research Problem

Biodiversity, ecological processes, and ecosystem services are currently threatened by the unprecedented rate of global urbanization (McDonald et al., 2008; Goddard et al., 2009; Shochat et al., 2010). Urban development has been linked to increased biotic and abiotic contamination, elevated temperatures and habitat fragmentation (Zhao et al., 2006; Grimm et al., 2008). These urgent problems necessitate the development of effective methodologies to restore degraded urban and suburban habitats to functioning ecosystems.

The science of restoration ecology develops and tests methods and techniques for recovering ecosystems that have been degraded, damaged, or destroyed (Society for Ecological Restoration International, 2004). While its goals are admirable, this emerging discipline has frequently been criticized for its static and historical approach to restoring ecosystems (Choi, 2007). Urban areas are generally heterogeneous and dynamic and their restoration requires site-specific action, which addresses both the unique ecology and evolution of these sites. Restoration genetics, a synthesis of restoration ecology and population genetics, stresses the importance of genotype selection and evolutionary trajectories in restoring and recreating populations (Montalvo et al., 1997; Rice and Emery, 2003; Falk et al., 2006). Handel et al. (2004) hypothesized that restoration attempts hindered by factors such as extreme environmental site conditions could be enhanced with use of 'unusual' genotypes. Moreover, Jones and Monaco (2009) have

argued that some human-impacted areas have passed an “ecological threshold,” beyond which local genotypes may no longer be adaptive. For these situations, genetically manipulated plant populations may provide a practical alternative.

In heterogeneous and dynamic environments, genotypes that are more flexible (phenotypically plastic) in certain stress responses might be expected to perform better than genotypes specifically adapted to stable, unchanging habitats. Phenotypic plasticity is the ability of an individual genotype to alter its phenotype in response to changes in environmental conditions (Miner et al., 2005) and is specific to a particular trait (Scheiner, 1993). Induced plasticity, through an immediate adjustment of gene expression, produces an alternate phenotype that may enhance fitness. By studying plant responses to altered resources and stress, various investigators have demonstrated that plasticity, measured on both a morphological (Schmitt et al., 1999; Heschel et al., 2004) and molecular (Wang and Li, 2008) level can indeed be adaptive. This epigenetic phenomenon itself can be heritable, leading to trans-generational stress memory (Molinier et al., 2006; Chinnusamy and Zhu, 2009), which may be essential if populations are to cope with changing conditions.

Given its potential benefits, it is surprising that the consideration of plasticity is almost absent from the restoration ecology literature (but see Valladares and Gianoli, 2007). Genotypes that exhibit a higher level of stress plasticity could provide a cost-effective, long-term approach to improving urban environments. By using plants that have flexible responses to specific stressors, restoration practitioners could better assure population establishment and persistence.

Objective and Questions

This dissertation project uses a novel, experimental approach to test and determine the importance and effectiveness of phenotypically plastic and stress-resistant plant genotypes in ecological restoration of urban and degraded land. Specifically, this project aims to answer the following questions:

1) Can the success of a plant genotype be determined by the presence of an induced stress response and will the benefit of this response be more pronounced as stressful conditions increase?

2) Is there significant natural variation in stress response? Can that information predict plant performance and success in stressful environments?

3) Will stress-resistant plant genotypes, as defined by consistent performance across a broad array of previous stress treatments, successfully tolerate heterogeneous and unknown stressor combinations in a variety of new sites?

Study Species

Arabidopsis thaliana (*L.*) *Heynh.* (Brassicaceae) is an annual flowering plant native to western Eurasia and northern Africa (Hoffmann, 2002) and has become naturalized in the United States (Mitchell-Olds and Schmitt, 2006). This cosmopolitan cress can now be found in many disturbed sites around the world (Hoffmann, 2002). *Arabidopsis thaliana* was originally embraced for its small size, short generation time and high fecundity, but its small genome size, efficient transformation and successful

sequencing efforts have secured its popularity as the preferential model plant for genetic studies (Koornneef and Meinke, 2009).

I purchased all seeds through the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. Wildtype seeds are from the Columbia (*Col-1*) ecotype, and the HSP17.6 mutant seeds were created through a T-DNA insertion in *Col-0* (Alonso et al., 2003). I also selected six European ecotypes from "The 1001 Genomes Project for *Arabidopsis thaliana*" (Weigel and Mott, 2009) and purchased those seeds from ABRC (Table 1.1).

Study Sites

I used six sites throughout this dissertation work for soil chemistry and heterogeneity analysis and potting medium for growth chamber 'site soil' experiments (Fig. 1.1). The two least disturbed sites are post-agricultural fields in Central New Jersey: meadows within the Duke Farms Estate (DUK) in Hillsborough, Somerset County; and old fields on the Sauer property (SRG) in Sergeantsville, Hunterdon County. Although located within the New York metropolitan area, both locations have access restrictions and remain largely undisturbed. They represent early successional reference targets for urban restoration efforts.

The other four sites can be classified as urban, industrial or degraded, and represent potential restoration sites: an urban park currently going through a major renovation and restoration (DOP) in Brooklyn, New York; a closed, capped and covered landfill (FKL) located in Staten Island, New York; a former arsenal and superfund site situated in the EPA Region 2 Compound (EPA) in Edison, Middlesex County, New

Jersey; and an abandoned railroad site adjacent to a closed oil refinery (BAY), located in Bayonne, Hudson County, New Jersey.

Sixty soil samples, ten from each site, have been analyzed for physical and chemical properties. Information on soil composition and heterogeneity will be discussed in the following chapters.

Research Approach

I focus on the stress-induced heat-shock (HS) family of proteins in the annual cress, *Arabidopsis thaliana*. This model system allows for precise monitoring of morphological and fitness data, the availability of loss-of function mutants in genes of interest, and overall convenience in molecular analyses. Methodologies for the following three research chapters of this dissertation are summarized below:

1) Determining the importance of an induced stress response: I compared wildtype and mutant (loss-of-function in the small heat-shock protein gene, HSP17.6) *A. thaliana* plants exposed to controlled salt and heat/drought stress gradients and a variety of urban and reference site soils. I collected phenological, morphological and fitness data to determine whether plasticity in HSP17.6 is essential for overall success, particularly under increasingly stressful conditions.

2) Testing for natural variation in stress response: I exposed six globally collected *A. thaliana* genotypes to controlled heat, drought and heat/drought stress treatments and gathered morphological and fitness data. I quantified the expression of two induced stress genes, HSP17.6 and HSP101, and then combined

performance and fitness data with molecular genetic information to identify the most and least stress-resistant (plastic) genotypes.

3) Establishing whether stress-resistant genotypes are indeed more successful in restoration conditions: I compared the most and least stress-resistant genotypes in controlled salt and salt/heat stress treatments and urban and reference site soils. I collected germination, phenological, morphological and fitness data to determine whether broad stress-resistance (plasticity) is essential for success in spatially heterogeneous and disturbed sites.

TABLES AND FIGURES

Table 1.1. Ecotypes selected for this study

Ecotype ID	Stock Number	Habitat Type (information)	Collection Location
Ange-1	CS28020	City (parking lot, railway station)	Angers (FRANCE)
Gy-0	CS76139	Agricultural (rural roadside)	La Minière (FRANCE)
Si-0	CS28739	City (roadside)	Siegen (GERMANY)
Ak-1	CS28011	Agricultural (vineyard)	Achkarren (GERMANY)
St-0	CS76231	City	Stockholm (SWEDEN)
Ull-2-3	CS76293	Agricultural	Ullstorp (SWEDEN)

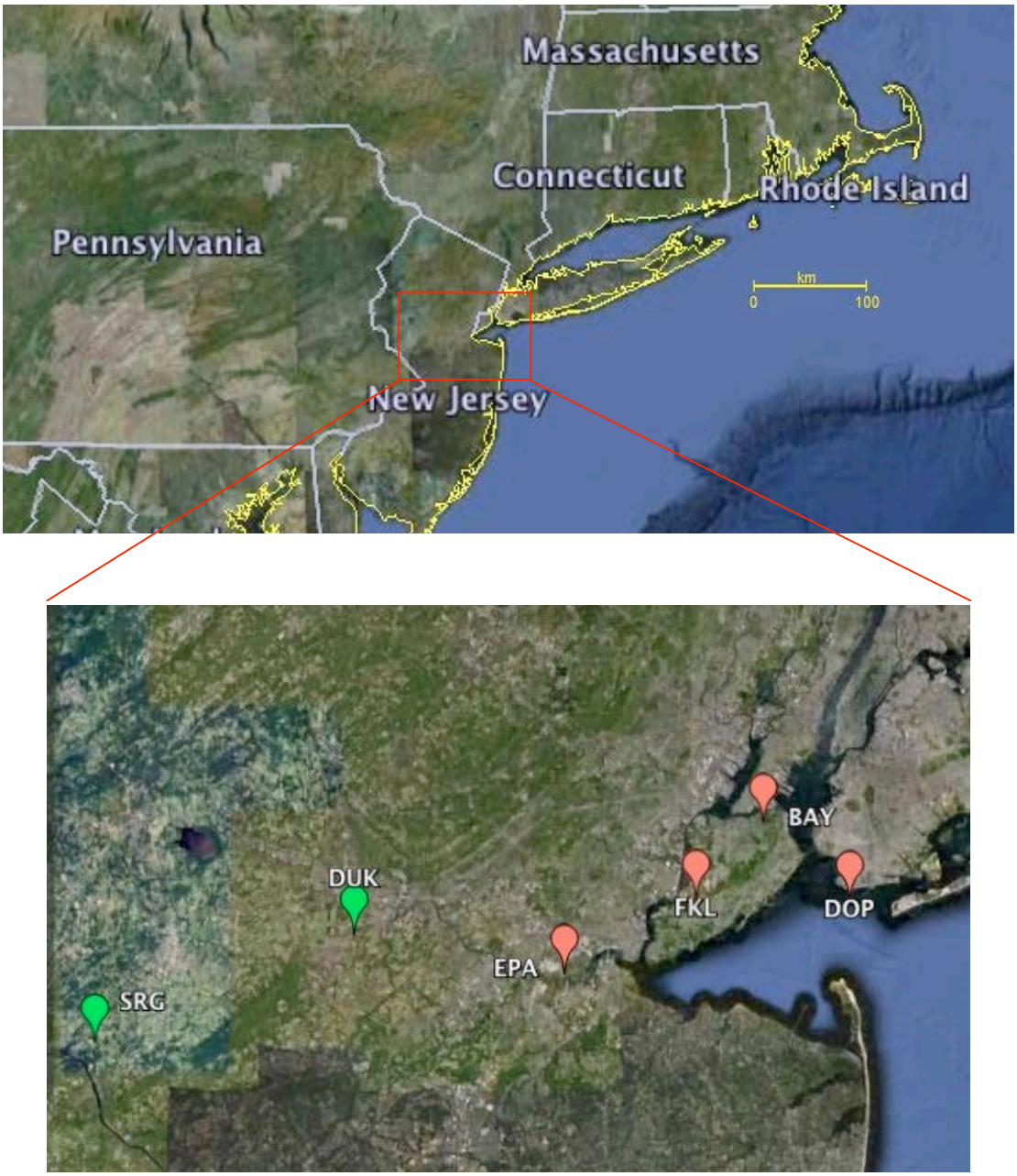

Figure 1.1. Map of the six study sites: green represents reference sites; red represents potential restoration sites (©2010 Google).

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CHAPTER II

STRESS-INDUCED HSP17.6 PLASTICITY IN *ARABIDOPSIS THALIANA* AND ITS SIGNIFICANCE IN URBAN ECOLOGICAL RESTORATION

ABSTRACT

Plants living in heterogeneous and constantly changing urban areas must be extremely flexible in their stress responses if they are to survive and flourish. Phenotypic plasticity is the ability of an individual genotype to modify its phenotype in response to changes in the environment. While this phenomenon has been extensively studied, very little attention has been paid to the practical use of plastic genotypes. This study examined the benefits of one induced stress response, heat shock protein (HSP) 17.6 induction in *Arabidopsis thaliana* plants exposed to typical urban stressors. I compared wild type and loss-of-function mutant plants across a salt stress gradient, a heat/drought stress gradient as well as on a variety of soils from potential restoration sites. I found that wild type individuals had longer lifespans, produced larger plants, and displayed increased fitness estimates (silique number, seed weight) than did the mutants. Mutant plants lacking a plastic HSP17.6 response generally showed an inability to cope with these various types of abiotic urban stress. This difference was generally more pronounced in high stress conditions, providing evidence that *A. thaliana* HSP17.6 induction is a type of adaptive plasticity. I argue that this type of study has important implications for the ecological restoration of degraded and urban areas.

INTRODUCTION

Context of the Problem

Plant performance should be considered within the context of the environment (Bradshaw, 1965). Individuals exposed to environmental variation throughout their entire lives must be able to tailor their responses to prevailing (but changing) conditions if they are to survive and flourish. Phenotypic plasticity is the ability of an individual genotype to alter its phenotype in response to changes in environmental conditions (Pigliucci, 2001; West-Eberhard, 2003; DeWitt and Scheiner, 2004; Sultan and Stearns, 2005). There have been many studies measuring the scope and breadth of plasticity (e.g., Quinn and Wetherington, 2002; Sultan, 2003), examining its fitness advantages and costs (e.g., Relya, 2002; Miner et al., 2005), and designing intricate models (e.g., Scheiner, 1993; Lande, 2009) for prediction and explanation of these plastic responses. Simultaneously, however, very little attention has been paid to the practical use of phenotypically plastic genotypes.

Phenotypic plasticity is considered to be adaptive when a genotype displays a broader tolerance to a changing environment and in turn, increases performance and fitness across environments (Ghalambor et al., 2007). By studying plant responses to altered levels of resources (Dorn et al., 2000; Schmitt et al., 2003; Heschel et al., 2004) and stress (Shao et al., 2007; Gimeno et al., 2008; Wang and Li, 2008), numerous investigators have demonstrated that plastic responses can indeed be adaptive. High levels of heterogeneity and disturbance as well as the presence of reliable cues can select for plasticity in plants (Bradshaw, 1965; Hoffman and Parsons, 1991; Sultan and

Spencer, 2002; Alpert and Simms, 2002; Dudley, 2004; Engelmann and Schlichting, 2005). While many recent empirical studies (Nussey et al., 2005; Pelletier et al., 2007; Molina-Montenegro et al., 2010) and meta-analyses (Hollander, 2008; Crispo et al., 2010) support these criteria, in urban settings, questions remain about the pace of change and unpredictability of variation.

When considering heterogeneous urban environments, plants that possess greater adaptive plasticity in certain traits might be selected for because the rapid change and fragmentation of these human-influenced lands might not allow for long-term evolution or migration (Sultan, 2004). In addition, plasticity can provide plant genotypes with immediate tolerance, 'buying some time' for longer term evolutionary responses (West-Eberhard, 2003; Badyaev, 2005; Richards et al., 2006). The success of organisms in novel habitats may even be attributed to the evolution of adaptive phenotypic plasticity (Agrawal, 2001).

Plants demonstrating quick and flexible stress responses may be the best option for restoring heterogeneous and constantly changing urban sites, and yet the consideration of phenotypically plastic genotypes is surprisingly absent from the restoration ecology literature. Except for a few theoretical mentions (Rice and Emery, 2003; Valladares and Gianoli, 2007; Funk et al., 2008), studies and reviews focusing on the population genetics of restoration primarily emphasize the importance of local adaptation (Hufford and Mazer, 2003; Gustafson et al., 2005) and genetic diversity (Falk et al., 2001; Broadhurst et al., 2008) in restored populations. While these are obviously important factors for population success, plants that show evidence of adaptive plasticity could provide a complementary approach to improving restoration, especially in urban

settings. By restoring sites using plants already known to have flexible responses to specific stressors, managers might select particular genotypes to better ensure population establishment and persistence.

Heat Shock Proteins

Concentrating here on adaptive plasticity, I examine the heat shock protein (HSP) system, a well-documented plastic response system (Pigliucci, 1996; Rizhsky et al., 2004; Timperio et al., 2008). HSPs primarily act as molecular chaperones, responsible for protein folding and assembly, as well as degradation of damaged or misfolded proteins (Hu et al., 2009). Many heat-shock proteins are involved in thermotolerance, but they may also act to buffer impact of harsh environmental conditions such as salt, drought, heavy metals and chemical toxicity (Mahajan and Tuteja, 2005; Timperio et al., 2008), all of which are common in the urban environment. While stress response is often a complex and multi-scale process, variation in a single HSP can be consequential for fitness (Feder and Hofmann, 1999).

Recent studies have monitored the expression of a small heat shock protein, HSP17.6 in *Arabidopsis thaliana* plants during exposure to single and multiple stressors, and found the elevation of this protein correlated with increased salt and heat/drought tolerance (Sun et al., 2001; Rizhsky et al., 2004; Kilian, et al., 2007). Furthermore, HSP17.6 belongs to the HSP 20 family, a group of small heat shock proteins that have been observed to possess high levels of gene expression in *A. thaliana* under general stress (Swindell et al., 2007).

HSP17.6 expression is an excellent model with which to investigate plastic response to urban stress. Urbanization has been linked to increased abiotic and biotic contamination, elevated temperatures and altered hydrology (Zhao et al., 2006; Grimm et al., 2008). In the following experiments, we imposed both salt and heat/drought stress treatments because of their ability to induce a response in HSP17.6 (Sun et al., 2001 and Rizhsky et al., 2004, respectively) while mimicking conditions of urban heat islands, altered hydrology, and road salting, all of which affect flora in the urban environment (Pickett et al., 2001; Paul and Meyer, 2001; Cunningham et al., 2008; Williams et al., 2008).

Objectives and Hypotheses

This study was designed to: (1) examine the adaptive benefits of heat shock protein (HSP) 17.6 induction for *Arabidopsis thaliana* exposed to various stressors, and (2) use those experiments to suggest how one might improve urban ecological restoration practice. The rationale here is that plants expressing higher levels of HSP17.6 expression will be able to quickly induce the necessary stress response and subsequently increase their survival and fitness over a variety of stress treatments. Under highly stressful conditions and on heterogeneous urban soils, wildtype strains should out-perform HSP loss-of-function mutant strains by larger margins than in less stressful treatments.

METHODS

Study Species

Arabidopsis thaliana (*L.*) *Heynh*. (Brassicaceae) is an annual flowering plant native to western Eurasia and northern Africa that is now found in many disturbed sites throughout the world (Hoffmann, 2002). This small mustard plant has gained popularity as a model in genetic studies because of its quick and prolific reproduction, small genome, and successful sequencing (Koornneef and Meinke, 2010). Later improvements in mutagenesis by transfer-DNA (T-DNA) transformation have increased its value as a genetic model (Feldmann and Marks, 1987; Krysan et al., 1999), since the abundance of available mutant strains can now be used to analyze the function and importance of virtually any gene of interest (Bolle et al., 2011)

Here I compare two genotypes: Columbia ecotype (*Col-1*), the wildtype (hereafter, WT) and an accepted standard for genetic studies (Meinke et al., 1998, www.1001genomes.org), and loss-of-function HSP17.6 mutant (hereafter, mutant). The mutant seeds were derived from the *Col-0* line and created through a T-DNA insertion (Alonso et al., 2003), which disabled the HSP17.6 gene. Potential pleiotropic effects from this insertion are discussed below. Seeds from these two genotypes represent single ancestral lines, and are available from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University.

Because transgenic mutants were used, I performed all experiments inside of controlled growth chambers at the New Jersey Agricultural Experimental Station, NJAES Greenhouses at Rutgers University, New Brunswick, NJ. While there was no field

component in this series of experiments, the use of non-plastic mutants provided an effective method to compare performance and fitness of plants that showed presence and absence of this plastic response.

Experimental Design

I grew the two *Arabidopsis* genotypes differing in HSP17.6 function in growth chambers (Model #GC15-31-CW-C3-X-HL-PW-CF, Environmental Growth Chambers, Chagrin Falls, OH) and compared their performance over two stress treatment gradients and in six site soils. Throughout all experiments, unless noted otherwise, germination protocol, growth chamber conditions and data collection methods were as follows.

Soil Collection Sites. Six different site soils, varying in disturbance and land-use, were used for the following growth chamber experiments and represented a range of conditions found in restoration sites around the New York/New Jersey metropolitan area. Two similar post-agricultural meadows, each with over 30 years since any major disturbance, represented restoration endpoint targets, or reference sites and were used as controls: (1) soil from meadows within the Duke Farms Estate (DUK) in Hillsborough, Somerset County, New Jersey (lat 40.55° N, long 74.62° W) and (2) soil from early successional fields on the Sauer property (SRG) in Sergeantsville, Hunterdon County, New Jersey (lat 40.44° N, long 74.98° W). The other four soils represented a sampling of potential restoration sites; each typifying a differing set of urban or industrial conditions: (3) an urban park currently going through a major renovation and restoration (DOP) in Brooklyn, New York (lat 40.58° N, long 73.99° W); (4) a closed, capped and covered

landfill (FKL) located in Staten Island, New York (lat 40.58° N, long 74.18° W); (5) a former arsenal and superfund site situated in the Environmental Protection Agency Region 2 Compound (EPA) in Edison, Middlesex County, New Jersey (lat 40.51° N, long 74.36° W); and (6) an abandoned railroad site adjacent to a closed oil refinery (BAY), located in Bayonne, Hudson County, New Jersey (lat 40.66° N, long 74.10° W).

For soil analysis, I randomly collected ten soil samples within a 10m x 10m plot from all six sites and sent them to the University of Massachusetts at Amherst Soil and Plant Tissue Testing Laboratory. Due to potential contamination issues, BAY soil was not accepted at UMass, and instead was analyzed at the USEPA compound in Edison, NJ. BAY soil samples had very high heavy metals (Fig. 2.3), but because of high hydrocarbon content, no other soil tests could be performed. Additional site information and a summary of soil properties can be found in Table 2.1 and Figures 2.1 to 2.3.

For the site soil experiment, I preserved the heterogeneity of the field sites by planting seeds among 24 individual soil samples (see design below), randomly collected from the 10m x 10m plot within each site. Potting medium (PMP High Organic Arabidopsis Medium; Lehle Seeds, Round Rock, TX) was used for the salt and heat/drought stress gradient experiments.

Germination Protocol. I soaked WT and mutant (HSP17.6) *Arabidopsis* seeds on filter paper, cold stratified them in the dark at 4°C for 2 days and then transferred them into soil under controlled growth chamber temperature and light conditions (see below) to stimulate and synchronize germination (Pigliucci and Schlichting, 1996). The majority of seeds germinated within the first 48 to 96 hours. I measured germination rate and did

not replace non-viable seeds. To prevent desiccation, I kept plastic domes over the flats, misted seeds and sub-irrigated daily until bolting (stalk emergence). I then removed the covers and watered plants every 2-3 days, or as needed, with distilled water. Throughout the experiment, I used an Arasystem (Betatech, Gent, Belgium), a series of plastic flats and tubes designed specifically for *Arabidopsis* growth and seed collection.

Controlled Growth Chamber Conditions. Conditions in the growth chamber consisted of a 14-hour day (~ 140 µE/m2/sec) with 25°C daytime temperature and 70% humidity, and with 23°C nighttime temperature and 60% humidity (Scholl, 1996; Weigel and Glazebrook, 2002). Flats were rotated every three or four days to minimize any effects of growth chamber position (Potvin et al., 1990).

Salt Stress Gradient Experiment. Thirty-four replicates of two genotypes were grown in three treatments, for a total of 204 plants across six flats of potting medium under control conditions (individual flats were randomized, blocked two per treatment). Flats remained in the control chamber for the entire experiment. At three weeks past germination, I continued the control watering regime (above), but replaced distilled water with a 50mM NaCl solution for two weeks for low salt stress in two flats and a 100 mM NaCl solution for two weeks for high salt stress in two flats (modified from Sun et al., 2001; Fig. 2.4). Salt stress required blocking by flat since watering was done by sub-irrigation. After the stress treatment was complete, I resumed using distilled water until harvesting.

Heat/Drought Stress Gradient Experiment. Thirty replicates of two genotypes were grown in three treatments, for a total of 180 plants, in potting medium, designed as above (Fig 2.5). Two flats were assigned to the control: plants remained in original growth chamber and watered every 2-3 days with distilled water; two flats of low heat/drought stress: plants moved to 32°C for 6 hours after 4 days with no water; and two flats of high heat/drought stress: plants moved to 38°C for 6 hours after 6 days no water (modified from Rizhsky et al., 2004). Experimental growth chambers maintained consistent light and humidity levels as the control chamber; measurements were taken each hour and did not fluctuate.

Site Soils Experiment. Twelve replicates of two genotypes were raised in six soil treatments, for a total of 144 plants (individual flats were randomized). Three flats of *A. thaliana* remained in one growth chamber under control conditions for the entire experiment; seeds were placed in six soil treatments: DUK, SRG, DOP, FKL, EPA, and BAY. Plants remained in the soil treatments from germination to harvest. While germination data is reported for all site soils, BAY soil is omitted from other analyses due to complete seedling mortality after germination. In some of the following analyses, when noted, I pooled DUK and SRG together as "reference site" soil and DOP, FKL and EPA as "degraded site" soil to determine overall differences of soil type.

Data Collection and Analysis

We collected the following data on individual plants: (1) germination date; (2) bolting date; (3) days to bolting (bolting date – germination date); (4) number of basal

leaves (counted at bolting date); (5) flowering date; (6) days to flowering (flowering date – germination date); (7) senescence date; (8) lifespan (senescence date – germination date); (9) final height (measured at senescence date); (10) number of lateral branches from the main stem (counted at senescence date); (11) number of siliques (fruits, counted at senescence date); and (12) total seed weight (of the whole plant, measured after harvest). All the following statistical analyses were performed using JMP version 8 for Macintosh, unless noted otherwise (SAS Institute Inc., Cary, NC, USA).

Germination. As germination data were binary, I performed contingency analysis on the stress gradient data and nominal logistic regression on the site soils data to compare proportions of germinated seeds. To determine whether these proportions were significantly different, I employed Fisher's exact test on stress gradient data. To address effects of soil, genotype and soil*genotype interaction for the site soils experiment; I performed a fixed effects likelihood ratio analysis. I then calculated odds ratios (e.g., Carlson and Holsinger, 2010) to reveal the strength of association between the values for germination of WT and mutant seeds. For each stress gradient experiment, I recorded germination rates before the stress was applied to the plants; I report these data individually since experiments were run at different times.

Phenological and Morphological Data. I analyzed differences in phenology (days to bolting, days to flowering, lifespan) and morphology (number of basal leaves, final height, number of lateral branches) between WT and mutant genotypes exposed to different stress treatments and soil types using two-way ANOVA and, when necessary,

generalized linear models. To determine which stress treatments affected differences in WT and mutant performance means, I employed Tukey-Kramer HSD post-hoc tests.

Fitness Data. I analyzed differences in fitness estimates (number of siliques, seed weight) between WT and mutant genotypes using two-way ANOVA and generalized linear models. Total seed weight produced per plant was used as a proxy for seed number because they were highly correlated (r^2=0.85, $p<0.0001$) and seed weight could be more accurately determined.

Plasticity Analysis. For selected data, I plotted genotypic reaction norms. These plots graphically depict plasticity by plotting the phenotype on the y-axis against various environmental conditions along the x-axis (Stearns, 1989). Steep slopes indicate high phenotypic plasticity and flatter reaction norms refer to low plasticity. Reaction norms are also very useful in revealing any significant genotype-environment interactions, indicated when genotypes' lines are non-parallel (Lewontin, 1974).

In some cases, to increase normality and decrease heteroscedacity, I log-transformed specified data sets. Two-way ANOVA was the preferred method of analysis, but was usually only performed if the one-way data were homoscedactic and residuals were normally distributed. If simple transformation was not able to ensure that, generalized linear models were employed. All generalized linear models were link-functioned to their proper distributions and tested for overdispersion. The only exception to this approach was for seed weight and overall fitness analysis for both stress gradient

experiments. Data were slightly heteroscedactic, but quite resistant to transformation. For these two datasets, I performed two-way ANOVA, as the F-test is robust to violated assumptions for large sample sizes (Underwood, 1997).

RESULTS

Stress Gradient Experiments

Germination. Germination rates of WT seed were significantly higher than those of mutant seed in both the salt stress gradient experiment and the heat/drought stress gradient experiment (Fig. 2.6). Odds ratios indicated that germination was over three times more likely to occur in WT rather than mutant seeds (G_{WT}/G_{mutant} = 3.12 and 3.32, respectively). These represent real differences between the strains yet do not reveal any information about stress treatment effects, since treatments were applied weeks after germination occurred.

Phenology. Time to bolting and time to flowering traits were not significantly affected by genotype or stress treatment, again most likely due to the timing of stress application. However, there were significant genotype, treatment and interaction effects for lifespan in salt stress and heat/drought stress (Table 2.2).

Salt Stress: Salt stress treatment profoundly affected lifespan, however, only the high salt treatment resulted in significantly shorter lifespan for both genotypes. A significant interaction effect indicated that the genotypes responded differently to the levels of salt stress. WT plants were able to perform consistently throughout the control and low salt treatments and only senesced early when exposed to the high salt treatment, while the mutant plants were negatively affected as soon as the low or high salt treatment was introduced (Fig 2.7).

Heat/Drought Stress: Heat/drought stress treatment also strongly affected lifespan, yet the only significant genotype difference was detected in the high heat/drought treatment. Again, a significant interaction effect indicated that the strains responded differently to the levels of heat/drought stress. WT plants performed consistently throughout all treatments, curiously showing a lack of response to this type of stress. Mutant plants had shortened lifespans when either low or high stress treatments were introduced (Fig. 2.7).

Morphology. For all morphological traits measured, there were significant treatment and genotype effects, with the exception of number of lateral branches, which were not affected by genotype (Tables 2.3-2.5).

Salt Stress: Not surprisingly, salt stress significantly decreased the number of basal leaves, lateral branches and final height values of both strains (Fig. 2.8). While WT plants appeared to have a larger advantage over the mutants in the most stressful salt treatments for both number of basal leaves and final height, there were no significant interaction effects for either trait.

Heat/Drought Stress: Heat/drought stress also significantly decreased the values of all morphological traits measured. A significant interaction effect was detected for height, as the divergence between the WT and mutant significantly increased in the high heat/drought stress treatment, indicating a WT advantage (Fig. 2.8).

Genotypic Fitness. For all fitness traits measured, significant treatment and genotype effects were observed (Tables 2.6 and 2.7).

Salt Stress: Salt stress significantly decreased silique number and total seed weight of both strains (Fig. 2.9). A significant interaction effect was found for silique number due to the genotypes' differential response to the high salt treatment. Total seed weight did not indicate any WT advantage in the stressful treatments, and interestingly showed a convergence of the genotypes in the high salt treatment.

Heat/Drought Stress: Heat/drought stress also significantly decreased all fitness traits measured. While WT plants seemed to have a larger advantage over the mutants in the most stressful heat/drought treatments for silique number, there were no significant interaction effects for this trait. Curiously, both genotypes displayed almost parallel responses in total seed weight values as stress increased, as verified by no significant interaction effect (Fig 2.9).

Soil Testing Results

DUK and SRG, the two reference sites, had higher levels of organic matter and significantly more essential nutrients (e.g., P, K, Mg) than the degraded sites, FKL, DOP and EPA. In many cases, the degraded sites showed very high (and varying) levels of potentially dangerous contaminants and metals like salt, aluminum, lead, chromium, and cadmium. pH levels were also notably different; in the degraded sites pH averaged 7.3, while the reference sites averaged 6.2. In general, the degraded sites had much higher variances, indicating the 10 samples tested from those sites were highly heterogeneous. See Figures 2.1 to 2.3 for selected datasets.

While there was limited information about the quality of BAY soil, the available data suggested that this site was contaminated far beyond the other urban and degraded

sites. For example, BAY soil had over 35 times the amount of chromium, 250 times the amount of lead, 500 times the sodium and 1000 times the aluminum as the other sites. This may explain the complete seedling mortality observed in this soil.

Site Soils Experiment

Germination. I found an overall WT advantage that significantly strengthened as soils became more stressful. Over all soils combined, WT seeds had a significant germination advantage over mutant seeds. Odds ratios indicated that germination was over two times more likely to occur in WT rather than mutant seeds ($G_{WT}/G_{mutant} = 2.59$). As expected, percent germination decreased as soil stress increased (Fig. 2.10). Interestingly, when genotype and soil type were analyzed together, wildtype advantage decreased slightly as soil stress increased (Fig. 2.11), probably driven by high mortality in the BAY soil. These results still suggest that the HSP17.6 response does facilitate germination in the most stressful soils, which supports its adaptive role in heterogeneous and highly degraded soil.

Phenology and Morphology. There were no significant genotypic differences or interaction effects found in bolting or flowering time, lifespan, number of basal leaves, number of lateral branches, or final height of the plants across the five site soils. Not surprisingly, performance was solely influenced by soil treatment (data not shown); which presumably overwhelmed any genotype effect.

Genotypic Fitness. Silique number and total seed weight were affected by genotype and soil treatment (Tables 2.8 and 2.9), and again WT advantage significantly strengthened as soils became more stressful, as suggested by a significant interaction effect in seed weight, and various within treatment analyses (Table 2.9 and Fig. 2.12). Genotypes responded identically in both reference soils, and only in degraded soils did WT produce higher values for both fitness estimates, suggesting a potential adaptive role in HSP17.6 response for reproductive success in heterogeneous and highly degraded soils.

DISCUSSION

Stress tolerance, genotypic differences, and phenotypic plasticity

Overall, this study shows a clear adaptive benefit for *Arabidopsis thaliana* in possessing the HSP17.6 response. WT genotypes had longer lifespans, produced larger plants, and, most importantly, displayed increased fitness characteristics. Mutants lacking a plastic HSP17.6 response generally showed an inability to cope with various types of abiotic stress. This difference was commonly more pronounced in high stress conditions, providing compelling evidence of adaptive plasticity for *A. thaliana* HSP17.6 induction.

Phenology. In the stress gradient experiments, WT plants lived longer than mutant plants. There were no genotypic differences seen in the controls, yet low salt stress and high heat/drought stress displayed significant within treatment differences between WT and mutant, indicating WT advantage in these stress treatments (Fig.2.7). Some studies have looked at lifespan per se as an adaptive plastic response (Lin et al., 1998; Münch et al, 2008). The response of decreased lifespan as salt stress increased (Fig 2.7A) most likely represents an inevitable aspect of response (Sultan, 1995). In other words, there is no functional phenotypic response in adjusting lifespan to increase fitness. Presumably it is because the plant is given a sub-optimal set of conditions and is simply not able to survive for a lengthy time. However, the heat/drought results are more revealing (Fig 2.7B); in this case, WT showed an interesting lack of response to this type of stress. The differential genotype response, conceivably caused by the presence or

absence of HSP17.6, supports the hypothesis that this plastic induction is necessary for tolerating and surviving stressful conditions.

Morphology. WT plants grew taller than mutant plants; final height displayed WT advantage in the highest heat/drought treatment (Fig. 2.8 F), although a similar trend was seen in the salt treatment (Fig. 2.8 C). Apparently a working HSP17.6 response gave plants the ability to achieve full height in the face of high stress. Height is fundamental to the ecology of many plants, as it is highly correlated with lifespan, seed characteristics and the ability to compete for light (Moles et al., 2009). In a comparable study, Islam et al. (2007) looked at three rice genotypes exposed to six different salt treatments. While they found similar reductions in plant height as salinity stress increased, the genotype that showed the smallest height reduction actually showed the highest susceptibility to salt stress. The authors suggest this could be due to the individual genetic capacities of the genotypes, which must be considered by any analysis of complex stress responses.

Fitness. Silique number data were variable across the stress gradient experiments. In salt stress, silique number decreased as stress increased, and WT expressed a significantly higher margin of success in the most stressful treatment (Fig 2.9 A). This result was consistent with my hypothesis. In heat/drought stress, a very different pattern emerged. There was hardly an effect of stress on silique number and in fact, there was a slight increase in WT silique number in the highest stress treatment (Fig. 2.9 C). When plotting silique number across individual site soils, WT advantage was highest in two

degraded sites (DOP and EPA), supporting my hypothesis that the lack of this plastic response can be detrimental in highly stressful and heterogeneous soils (Fig. 2.12 A).

The inconsistent silique data are interesting because they indicate that this trait could be either susceptible or impervious to particular environmental conditions. The literature primarily supports silique numbers decreasing with increased stress. For example, Young et al. (2004) examined the effects of heat stress on *Brassica napus* reproduction and saw a significant (3- to 7-fold) decrease in siliques developing from flowers under heat stress. The data seen here are only troubling in the sense that numerous studies on plasticity and adaptation to stress in *Arabidopsis* use silique number as a proxy for fitness (e.g., Camara et al., 2000; Bossdorf et al., 2010). In this study, silique number changed depending on the genotype and environmental conditions, occasionally in a counterintuitive way. Care must be taken to standardize any experiment in which silique number serves as a substitute for fitness.

A more appropriate measure of fitness for a selfing plant like *Arabidopsis* might be seed weight (Simon et al., 2008); this metric is frequently used in experiments looking at stress response of this annual (e.g., Pagan et al., 2009; Jin et al., 2011). As previously mentioned, I found a high positive correlation between seed weight and seed number, and with variable silique response data, perhaps total seed weight produced is a more accurate way to approach reproductive output.

WT plants displayed higher seed weight values across stress treatments, however, again showed varied responses. In salt stress, genotypes showed a convergence between the two genotypes at the highest stress level (Fig. 2.9 B). This could be due to the severity of the salt stress and/or the subsequent reduction in sample size, as fewer individuals

survived. Conceivably, at very high stress, WT advantages can deteriorate, as a plant, no matter how plastic, can only tolerate so much. In heat/drought stress, there was no WT advantage seen at all, the parallel reaction norms simply represented the inherent differences between the genotypes; no interaction was seen (Fig 2.9 D). Across site soils, there was no difference observed between the genotypes in both reference sites, but WT did show significant genotypic advantages in two of the degraded sites, FKL and EPA (Fig. 2.12B). These seed weight results supported the hypothesis that HSP17.6 induction is important for maintaining reproductive success in degraded soils.

Seed weight can be very important in establishing advantages for a subsequent plant generations. Heavier seeds have been observed to germinate more often and more quickly (Tremayne and Richards, 2000), and may eventually produce more flowers (Stanton, 1984). Unfortunately, the inconsistency of the silique and seed data in the stress gradient experiments obscure any determination to whether total seed weight in these experiments is correlated with individual seed weights. In the salt experiment, WT plants had higher numbers of siliques than mutants, yet when looking at the seed weight data that difference disappears. Young et al. (2004) found decreased gametophyte function and seed production in *Brassica napus* exposed to heat stress. These responses could explain reduced number of seeds within an unchanging number of siliques.

Genotype and environment. While plasticity studies commonly use the genotype-environment interaction term as a measure of differential success between genotypes, I only found a few instances of genotype x environment (G X T) interaction throughout the entire set of experiments. This was somewhat surprising as it was expected that the WT

and mutant genotypes would react quite differently to the various stress treatments applied. This is consistent with Pigliucci and Schmitt (1999); they saw a similar pattern when comparing WT and mutant photomorphogenic *Arabidopsis* genotypes in response to different light qualities. While mutants had different levels of response, the overall shape of the reaction norms was very similar to that of the WT. More recently, Debat et al. (2009) noticed that *Drosophila* individuals containing various mutations in wing development followed nearly parallel reaction norms to the WT when exposed to various heat treatments, suggesting an overall stronger effect of temperature on the trait. They also found few significant G X E interactions.

While the effect of genotype was evident in this study, the stronger indicator of plant success was the environment. The soil stress treatments were by far the most responsible in driving the significant differences in plant performance. This is not surprising; field scientists, geneticists and plant breeders have often addressed the fact that plants are regularly more influenced by their environment rather than genotype (e.g., Araus et al., 2003; Islam et al., 2007; Collins et al., 2008; Johnson et al., 2008).

Use of mutants to determine adaptive plasticity. There has been a recent call for focusing instead on gene-environment interactions per se (Weinig and Schmitt, 2004; Eagles et al., 2008). For some time, researchers have been aware of the possible benefits of disabling certain genes to determine the evolutionary importance of specific traits (Pyke, 1994). The use of loss-of-function mutants to test the benefits of adaptive plastic responses has generally been successful (Cao et al., 2005; Fujii and Zhu, 2009). Schmitt

et al. (1995, 1999, 2003) have repeatedly shown convincing evidence of adaptive plasticity of phytochrome-mediated shade avoidance by comparing WT and mutants.

When working with mutants, potential pleiotropic and epistatic effects should not be ignored, as the causes behind the growth and fitness differences observed may be constrained by other genes unrelated to the trait of interest (Ackerly et al., 2000). It is understood that heat shock proteins have pleiotropic effects (Meyers, 1995). HSP17.6, for instance, is involved in stress response to salt (Sun et al., 2001), heat and drought (Rizhsky et al., 2004), and wounding (Swindell, 2007). An individual HSP can be involved in various biochemical pathways responsible for different modes of stress tolerance and various phenotypes. Pleiotropy and epistasis are important concepts to address in any study looking at the action of one gene, but findings from manipulations of single HSPs most often are consistent with outcomes of correlative studies (Feder and Hofmann, 1999). The differential genotypic responses in stress seen in this study provide strong evidence that the differences in growth and fitness documented here are in fact due to the lack of HSP17.6 plasticity, and not other aspects of disabling this HSP gene.

Application to Urban Restoration Ecology and Future Considerations

The obvious question surrounding this work on *Arabidopsis* and its heat shock response as a model system is whether and how the results are to inform the highly applied discipline of ecological restoration. Using this model system has provided me the opportunity to obtain loss-of-function mutants with which to test the adaptive nature of a plastic response. *Arabidopsis*, although cosmopolitan, is not frequently found in the New York metropolitan area (Steve Clemants, pers. comm.), and it will not be used in local

restoration practice. Also, *A. thaliana* is an annual, and while that has been helpful in estimating fitness, it will not help us to quantify the long-term success of alternative restoration practices, most of which will involve perennial planting stock. The lack of field experiments was inevitable; transgenic mutants prevented any plant material from being used outside. That being said, for basic questions to be answered, a tractable and manageable model system is a necessity. This is one of the first studies of the potential utility of phenotypic plasticity. My results indicate that heat shock proteins in plants provide adaptive plasticity in the face of urban (salt and heat/drought) conditions and that they can have an impact on plant growth and fitness. This study emphasizes the importance of understanding how urban and climatic stress affects reproductive development, and highlights the potential for selecting more tolerant genotypes for use in ecological restoration of urban and degraded land.

I am currently scaling up this investigation to six field-collected *Arabidopsis* genotypes and adding a molecular analysis of the expression of both HSP17.6 and HSP101, a larger heat-shock protein. Once the most and least stress-resistant strains are isolated, they will be planted in growth chambers with similar stress treatments and in two experimental gardens (DUK and EPA sites). These steps should move the analysis of alternative restoration plantings one step closer to the field, providing a platform for wider extrapolation to other species, genes, and restoration challenges.

Conclusions

Identifying adaptive plasticity for the HSP17.6 response across heterogeneous soils and stressful climatic conditions is the first step in establishing the principle that plastic responses can be utilized in urban restoration. The experiments performed here and those like them are incredibly timely and necessary to fully understand the predictable stresses caused by progressive urbanization.

TABLES AND FIGURES

Table 2.1. Study site soil and vegetation information

Site	Soil Type	Vegetation	Location	Surrounding Area
DUK	Loam	Post agricultural fields; grasses and herbs surrounded by mature forest	Hillsborough, NJ	Preserved lands (e.g., fields and forests); borders on commercial highway and county roads
SRG	Loam	Post agricultural fields; grasses, herbs and shrubs surrounded by mature forest	Sergeantsville, NJ	Fields and farms; borders on rural county roads and forests
FKL	Clay Loam	Grasses and herbs, few shrubs and trees	Staten Island, NY	Landfill, major highways, densely populated area
DOP	Sandy Loam	Grasses and herbs, few shrubs, young and mature trees	Brooklyn, NY	Urban park, city streets, borders on bay waters
	Sandy Loam	Grasses and herbs, few shrubs, young and mature trees	Edison, NJ	Compound borders on commercial highway, densely populated area
BAY	Sandy Loam	Grasses and herbs, few shrubs and young trees	Bayonne, NJ	Closed refinery, abandoned rail road, industrial

Table 2.2. Lifespan; Generalized linear model (log-linked for Poisson distribution) results for **(A)** salt and **(B)** heat/drought experiment: Whole model results and effect likelihood ratio analysis. Genotype and soil treatment significantly affected lifespan of *Arabidopsis thaliana*. There were significant interactions.

(A)

MODEL	-LogLikelihood	df	Chi-Square	P>Chi-Square
Difference	22.689	5	45.378	<0.0001*
Full	445.452			
Reduced	468.141			

STAT	Chi-Square	df	P>Chi-Square	Overdispersion
Pearson	166.262	143	0.0891	1.1627

SOURCE	df	L-R Chi-Square	P>Chi-Square
Genotype	1	8.526	0.0035*
Soil Treatment	2	26.549	<0.0001*
Soil*Genotype	2	7.882	0.0194*

(B)

MODEL	-LogLikelihood	df	Chi-Square	P>Chi-Square
Difference	27.293	5	54.585	<0.0001*
Full	653.136			
Reduced	680.429			

STAT	Chi-Square	df	P>Chi-Square	Overdispersion
Pearson	146.104	166	0.8647	0.8801

SOURCE	df	L-R Chi-Square	P>Chi-Square
Genotype	1	8.553	0.0035*
Soil Treatment	2	33.688	<0.0001*
Soil*Genotype	2	17.127	0.0002*

Table 2.3. Number of basal leaves; Generalized linear model (log-linked, Poisson) results for **(A)** salt and 2-way ANOVA results for **(B)** heat/drought experiments: Whole model results and effect likelihood ratio analysis. Only soil treatment significantly affected basal leaves produced by *Arabidopsis thaliana*.

(A)

MODEL	**-LogLikelihood**	**df**	**Chi-Square**	**P>Chi-Square**
Difference	58.602	5	117.2045	<0.0001*
Full	533.582			
Reduced	592.184			

STAT	**Chi-Square**	**df**	**P>Chi-Square**	**Overdispersion**
Pearson	53.450	131	1.000	0.4080

SOURCE	**df**	**L-R Chi-Square**	**P>Chi-Square**
Genotype	1	6.222	0.0126*
Soil Treatment	2	110.354	<0.0001*
Soil*Genotype	2	0.558	0.7564

(B)

R-Square	0.1206
Observations	171

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	5	102.580	20.52	4.5246	
Error	165	748.169	4.53	**Prob > F**	
Total	170	850.749		0.0007*	

SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	9.977	2.2003	0.1399
Soil Trt	2	2	91.260	10.0631	<0.0001*
Gen*Trt	2	2	1.259	0.1378	0.8714

Table 2.4. Number of lateral branches; Generalized linear model (log-linked, Poisson) results for **(A)** salt and 2-way ANOVA results (square root trans.) for **(B)** heat/drought experiments: Whole model results and effect likelihood ratio analysis. Genotype and soil treatment significantly affected number of branches.

(A)

MODEL	**-LogLikelihood**	**df**	**Chi-Square**	**P>Chi-Square**
Difference	58.602	5	117.2045	<0.0001*
Full	533.582			
Reduced	592.184			

STAT	**Chi-Square**	**df**	**P>Chi-Square**	**Overdispersion**
Pearson	53.450	131	1.000	0.4080

	SOURCE	**df**	**L-R Chi-Square**	**P>Chi-Square**
	Genotype	1	6.222	0.0126*
	Soil Treatment	2	110.354	<0.0001*
	Soil*Genotype	2	0.558	0.7564

(B)

R-Square	0.2451
Observations	171

SOURCE	**df**	**SS**	**MS**	**F-Ratio**
Model	5	8.474	1.70	10.7124
Error	165	26.105	0.16	**Prob > F**
Total	170	34.579		<0.0001*

SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	1.257	7.9492	0.0054*
Soil Trt	2	2	7.074	22.3553	<0.0001*
Gen*Trt	2	2	0.011	0.0345	0.9661

Table 2.5. Final height; 2-way ANOVA results for **(A)** salt and **(B)** heat/drought experiments: Whole model results and effect likelihood ratio analysis. Genotype and soil treatment significantly affected the final height of *Arabidopsis thaliana*. There was only a significant interaction effect found in heat/drought stress.

(A)

R-Square 0.6576

Observations 137

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	5	7629.714	1525.94	50.3174	
Error	131	3972.751	30.00	**Prob > F**	
Total	136	11602.465		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	168.133	5.5441	0.0200*
Soil Trt	2	2	7275.602	119.9551	<0.0001*
Gen*Trt	2	2	132.203	2.1797	0.1172

(B)

R-Square 0.6232

Observations 171

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	5	8512.668	1702.53	54.5694	
Error	165	5147.907	31.20	**Prob > F**	
Total	170	13660.575		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	376.7998	12.0771	0.0007*
Soil Trt	2	2	7771.4340	124.5445	<0.0001*
Gen*Trt	2	2	412.6692	6.6134	0.0017*

Table 2.6. Silique number; 2-way ANOVA results (both datasets log transformed) for **(A)** salt and **(B)** heat/drought experiments: Whole model results and effect likelihood ratio analysis. Genotype and soil treatment significantly affected the amount of siliques produced by *Arabidopsis thaliana*. There was only a significant interaction effect found in salt stress.

(A)

R-Square 0.7563

Observations 136

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	5	70.3889	14.0778	80.7078	
Error	130	22.6758	0.1744	**Prob > F**	
Total	135	93.0646		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	3.4896	20.0056	<0.0001*
Soil Trt	2	2	65.2012	186.8990	<0.0001*
Gen*Trt	2	2	1.6299	4.6722	0.0110*

(B)

R-Square 0.3210

Observations 171

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	5	12.1108	2.42	15.6041	
Error	165	25.6122	0.16	**Prob > F**	
Total	170	37.7230		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	4.0065	25.8106	<0.0001*
Soil Trt	2	2	7.1379	22.9921	<0.0001*
Gen*Trt	2	2	0.8514	2.7426	0.0673

Table 2.7. Total seed weight; 2-way ANOVA results (both datasets log transformed) for **(A)** salt and **(B)** heat/drought experiments: Whole model results and effect likelihood ratio analysis. Only genotype and soil treatment significantly affected the amount of siliques produced by *Arabidopsis thaliana*; there were no significant interaction effects found.

(A)

R-Square	0.5289
Observations	136

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	5	0.003542	0.000708	29.1916	
Error	130	0.003155	0.000024	**Prob > F**	
Total	135	0.006697		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	0.000464	19.1402	<0.0001*
Soil Trt	2	2	0.002823	58.1533	<0.0001*
Gen*Trt	2	2	0.000102	2.1090	0.1255

(B)

R-Square	0.5068
Observations	171

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	5	0.0144	0.002883	33.9156	
Error	165	0.0140	0.000085	**Prob > F**	
Total	170	0.2844		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	0.0064	75.7553	<0.0001*
Soil Trt	2	2	0.0076	44.4794	<0.0001*
Gen*Trt	2	2	0.0001	0.6170	0.5408

Table 2.8. Silique number; 2-way ANOVA results (log transformed) for site soil experiment: Whole model results and effect likelihood ratio analysis. Genotype and soil treatment significantly affected the amount of siliques produced by *Arabidopsis thaliana*. There was significant interaction effect found.

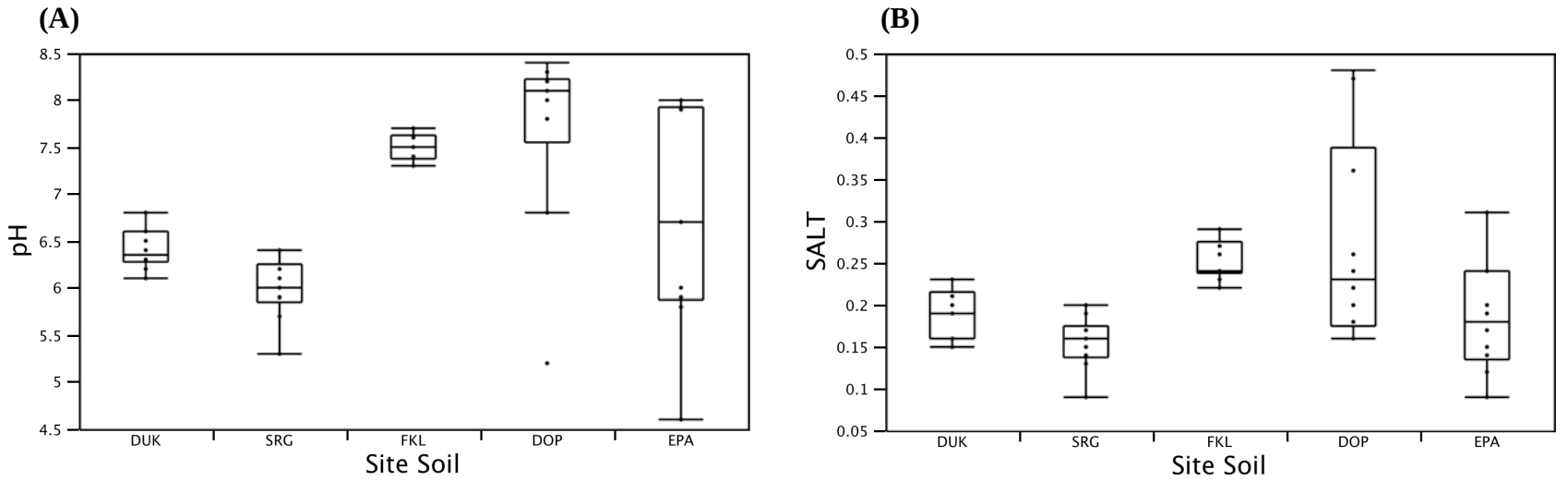
R-Square	0.6330
Observations	171

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	9	16.7087	1.8565	15.3339	
Error	80	9.6859	0.1211	**Prob > F**	
Total	89	26.3946		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	2.1245	17.5474	<0.0001*
Soil Trt	4	4	13.0336	26.9127	<0.0001*
Gen*Trt	4	4	1.8796	3.8811	0.0062*

Table 2.9. Total seed weight; 2-way ANOVA results (log transformed) for site soil experiment: Whole model results and effect likelihood ratio analysis. Genotype and soil treatment significantly affected the amount of siliques produced by *Arabidopsis thaliana*. There was no significant interaction effect.

R-Square	0.4855
Observations	91

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	9	9.6416	1.0713	8.4916	
Error	81	10.2189	0.1262	**Prob > F**	
Total	90	19.8605		<0.0001*	
SOURCE	**N**	**Df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	1	1	2.4626	19.5194	<0.0001*
Soil Trt	2	2	6.4988	12.8782	<0.0001*
Gen*Trt	2	2	1.2383	2.4539	0.0523



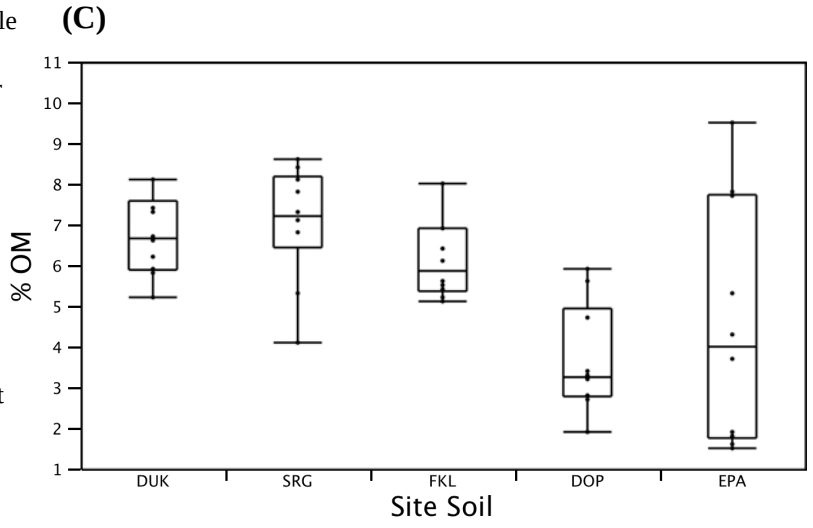


Figure 2.1. Quality and heterogeneity analysis of soil properties: **(A)** pH, **(B)** soluble salt and **(C)** percent organic matter. **(A)** Both reference sites had significantly lower pH than all degraded sites ($F_{4, 45}$ = 10.2741; p<0.0001). **(B)** DOP and FKL showed significantly higher levels of salt than SRG ($F_{4, 45}$ = 5.7377; p=0.0008). **(C)** Both reference sites had higher percent organic matter than DOP and EPA ($F_{4, 45}$ = 7.2266; p<0.0001). Heterogeneity was determined by Levene's test for unequal variances. EPA and DOP displayed higher than average heterogeneity in pH and salt ($F_{4, 45}$ = 7.6706; p<0.0001 for pH; $F_{4, 45}$ = 8.8514; p<0.0001 for salt); EPA displayed higher than average heterogeneity for % organic matter ($F_{4, 45}$ = 4.7171; p=0.0029).

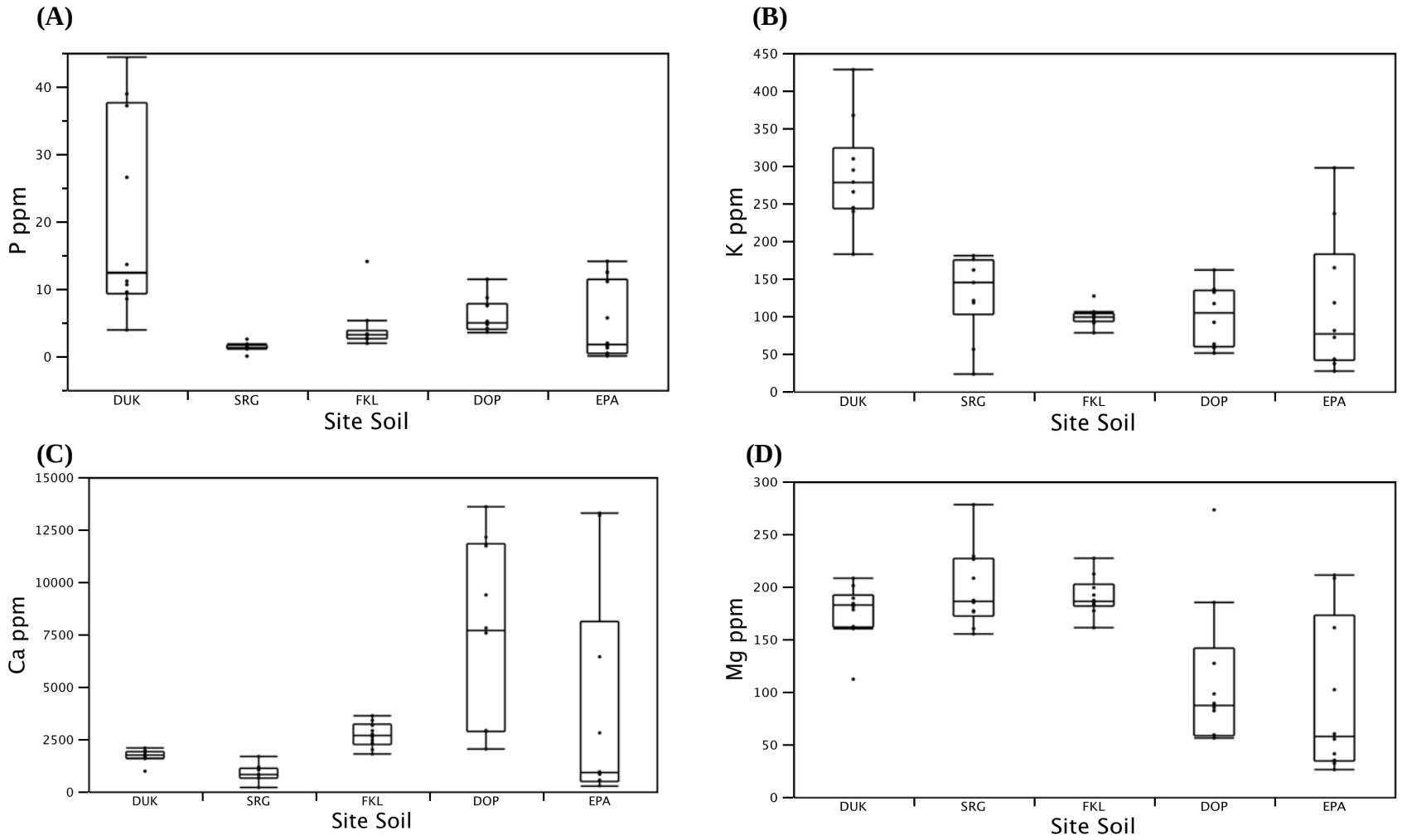


Figure 2.2. Continued on next page.

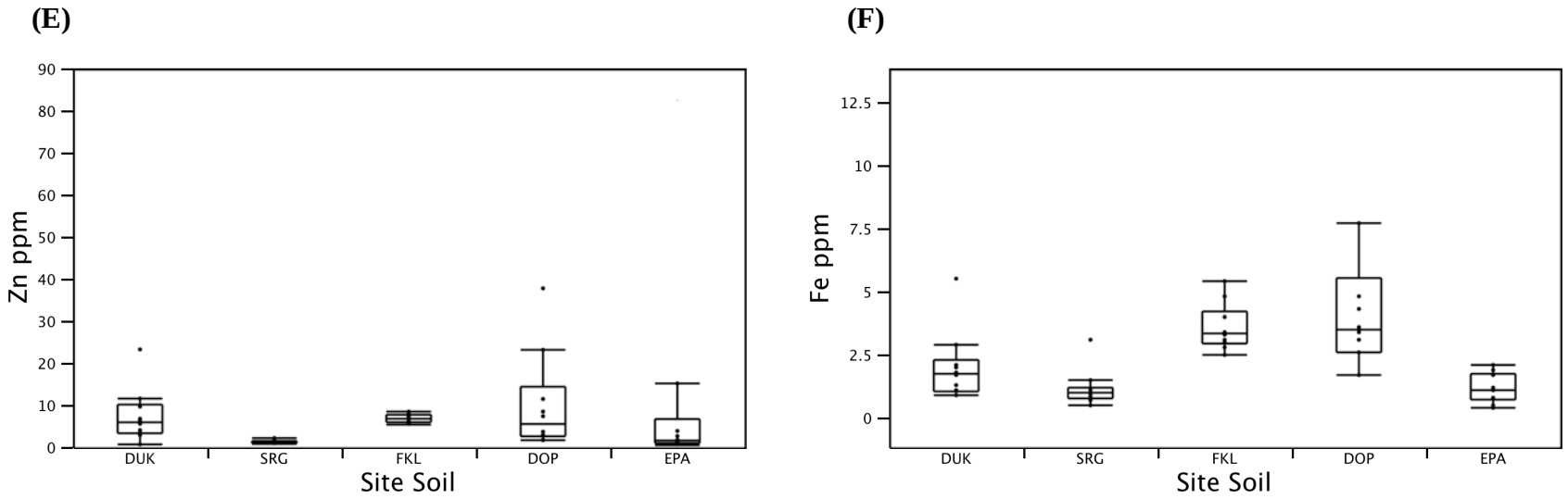


Figure 2.2. Quality and heterogeneity analysis of soil nutrients: **(A)** phosphorus, **(B)** potassium, **(C)** calcium, **(D)** magnesium, **(E)** zinc and **(F)** iron. **(A, B)** DUK had significantly higher P and K levels than all other sites ($F_{4, 45}$ = 10.3530; p<0.0001 for P; $F_{4, 45}$ = 18.3427; p<0.0001 for K). **(C)** DOP soils contained significantly more Ca than DUK, SRG and FKL ($F_{4, 45}$ = 6.6258; p=0.0003). **(D)** Both reference sites and FKL showed significantly higher Mg levels than DOP and EPA ($F_{4, 45}$ = 9.2192; p<0.0001). **(E)** Zinc levels did not differ across sites. **(F)** DOP had significantly higher levels of Fe than both reference sites and EPA ($F_{4, 45}$ = 7.7903; p<0.0001). Heterogeneity was determined by Levene's test for unequal variances. While DUK showed higher than average heterogeneity for **(A)** phosphorus levels ($F_{4, 45}$ = 26.5174; p<0.0001), EPA and/or DOP displayed higher than average heterogeneity for **(B-F)** all other nutrients ($F_{4, 45}$ = 4.4298; p=0.0042 for K; $F_{4, 45}$ = 16.6689; p<0.0001 for Ca; $F_{4, 45}$ = 5.3318; p=0.0013 for Mg, $F_{4, 45}$ = 3.9462; p=0.0079 for Zn; $F_{4, 45}$ = 7.7903; p<0.0001 for Fe).

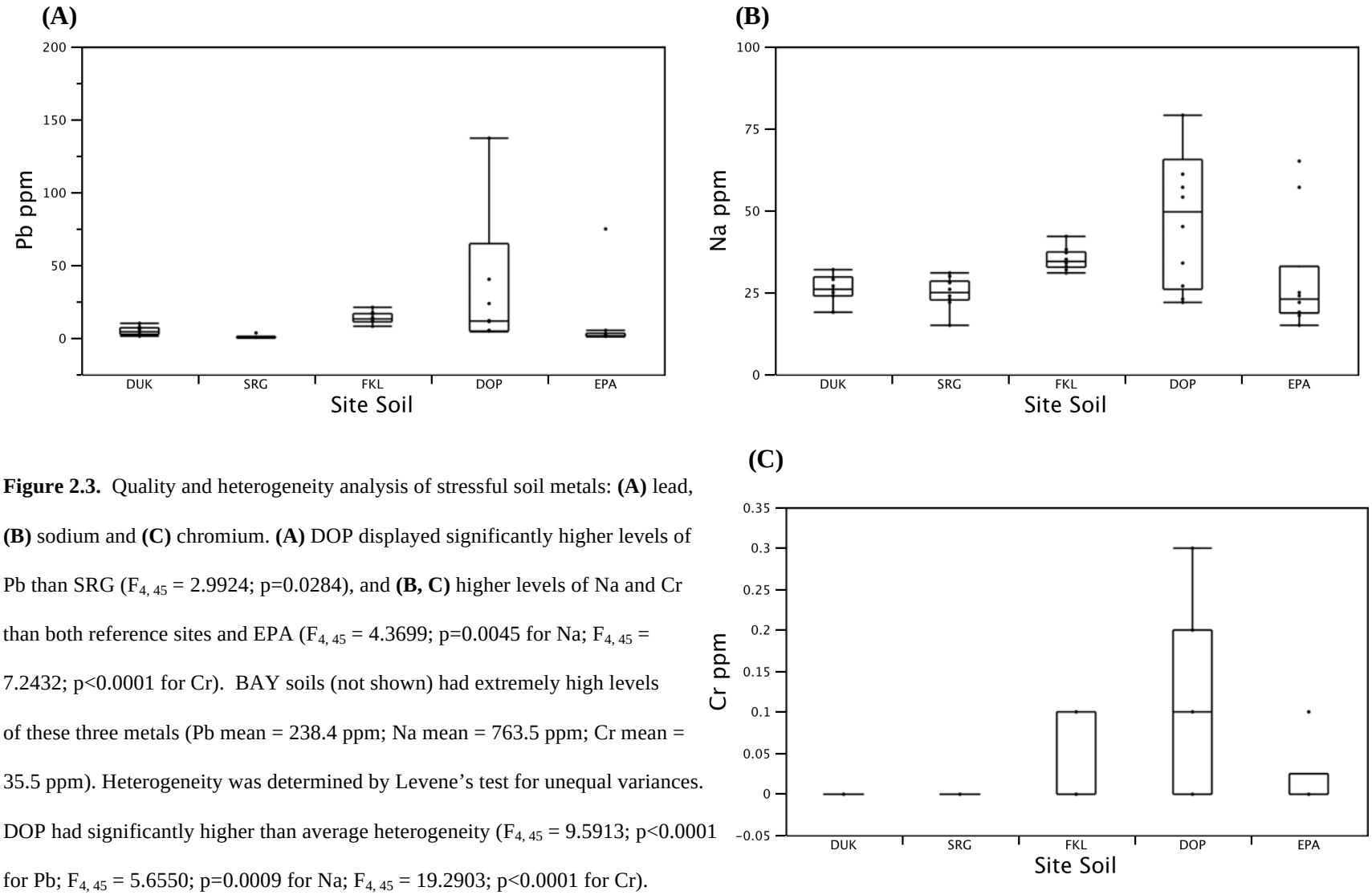


Figure 2.3. Quality and heterogeneity analysis of stressful soil metals: **(A)** lead, **(B)** sodium and **(C)** chromium. **(A)** DOP displayed significantly higher levels of Pb than SRG ($F_{4, 45}$ = 2.9924; p=0.0284), and **(B, C)** higher levels of Na and Cr than both reference sites and EPA ($F_{4, 45}$ = 4.3699; p=0.0045 for Na; $F_{4, 45}$ = 7.2432; p<0.0001 for Cr). BAY soils (not shown) had extremely high levels of these three metals (Pb mean = 238.4 ppm; Na mean = 763.5 ppm; Cr mean = 35.5 ppm). Heterogeneity was determined by Levene's test for unequal variances. DOP had significantly higher than average heterogeneity ($F_{4, 45}$ = 9.5913; p<0.0001 for Pb; $F_{4, 45}$ = 5.6550; p=0.0009 for Na; $F_{4, 45}$ = 19.2903; p<0.0001 for Cr).

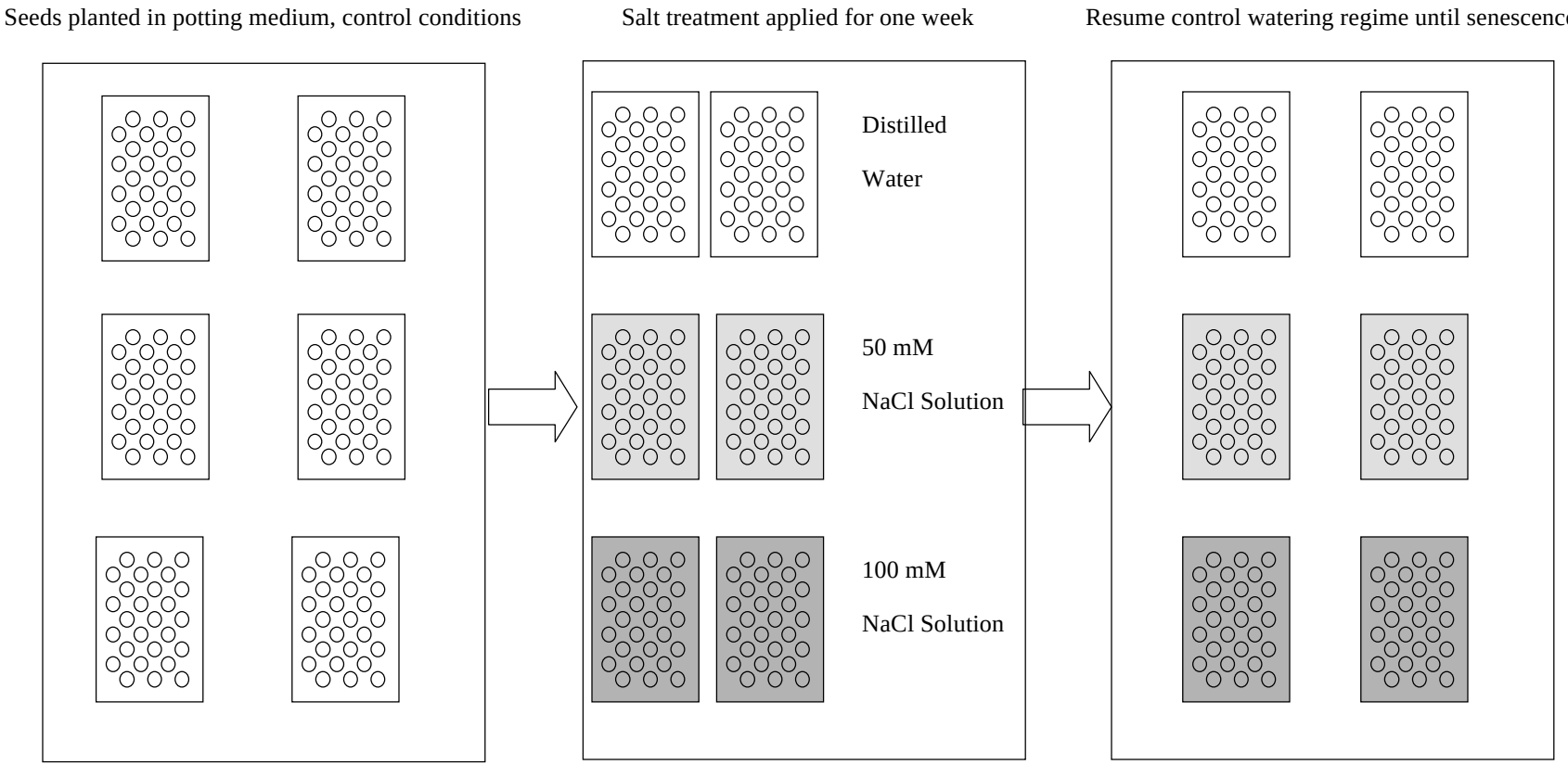


Figure 2.4. Experimental design of salt stress experiment. 34 replicates x 2 genotypes x 3 treatments (204 plants across six flats of potting medium under control conditions; individual flats were randomized, blocked two per treatment). Plants remained in one chamber; boxes in the illustration represent time periods. At three weeks, four flats were subjected to salt stress as indicated above for one week. After which, plants were watered regularly until senescence.

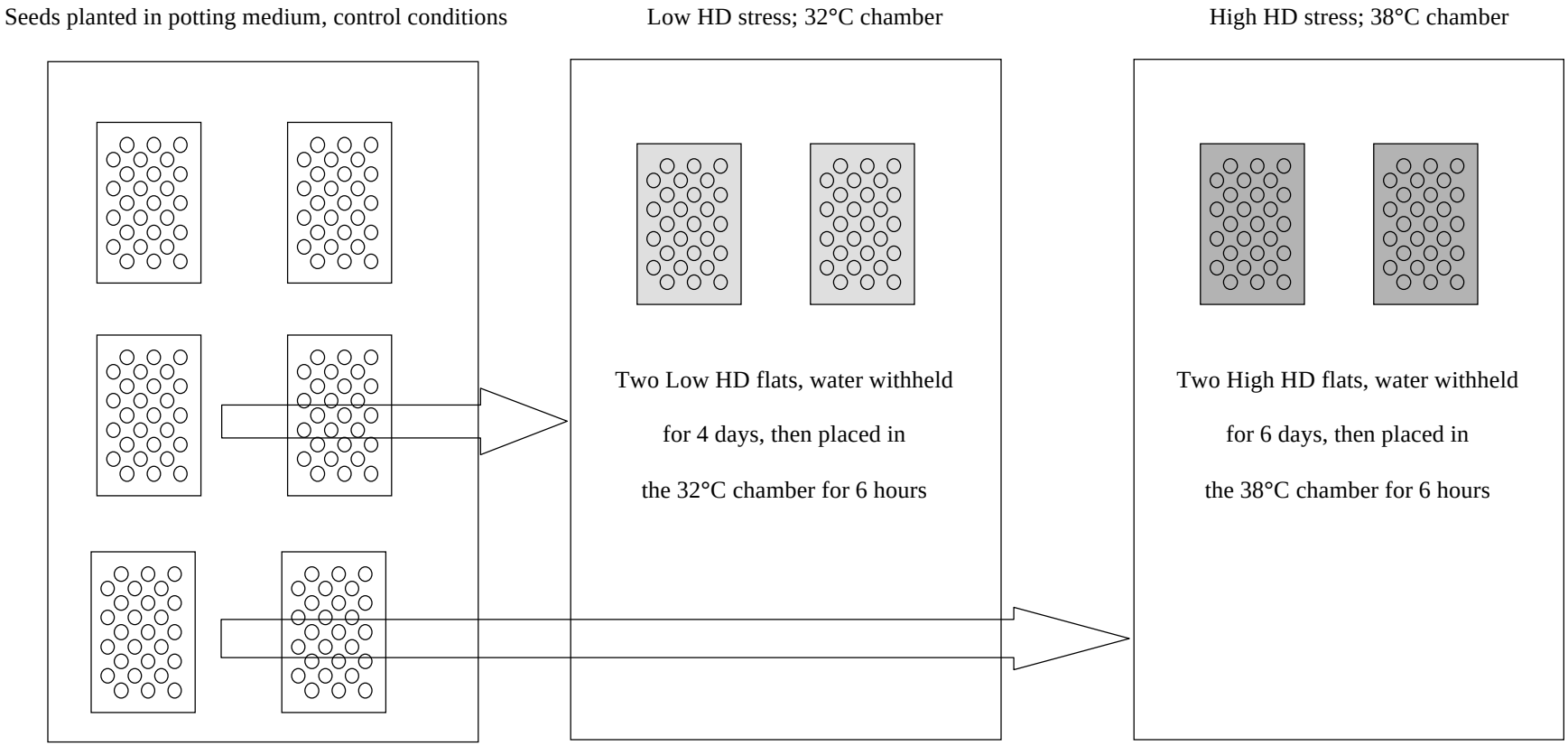


Figure 2.5. Experimental design of heat/drought stress experiment. 30 replicates x 2 genotypes x 3 treatments (180 plants, across six flats of potting medium under control conditions; individual flats randomized, blocked two per treatment). Three weeks after germination, C: two flats remained in original growth chamber, watered every 2-3 days with distilled water; two low HD flats: plants moved to 32°C for 6 hours after 4 days no water; and two high HD flats: plants moved to 38°C for 6 hours after 6 days no water. After the treatments above, plants returned to the control chamber and were watered regularly until senescence.

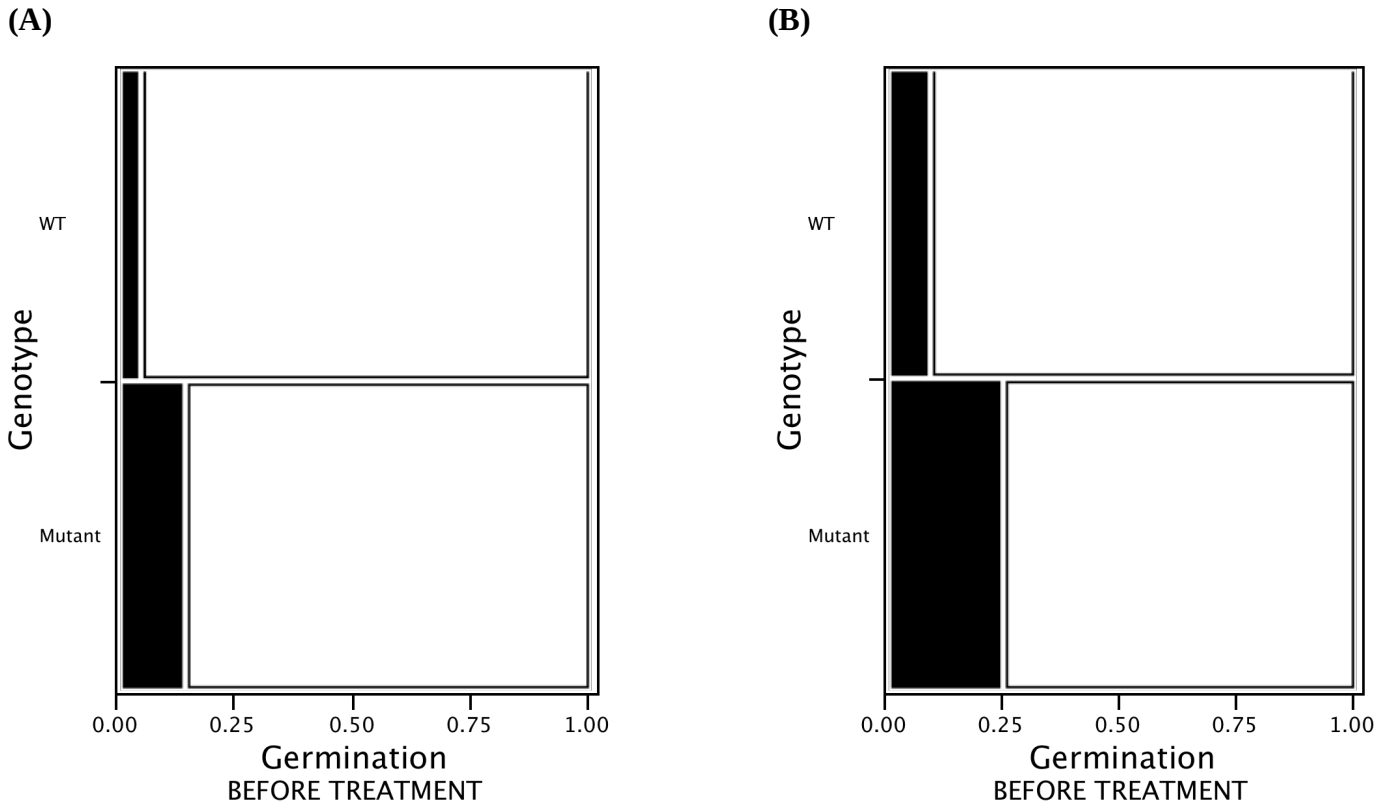


Figure 2.6. Contingency plots of germination rates in both **(A)** salt and **(B)** heat/drought experiments (before stress was applied). White represents germination; **(A)** N=203 of which 95% WT seed and 86% mutant seed germinated (Fisher's Exact Test, p=0.0319); **(B)** N=180 of which 91% WT seed and 76% mutant seed germinated (Fisher's Exact Test, p=0.0085).

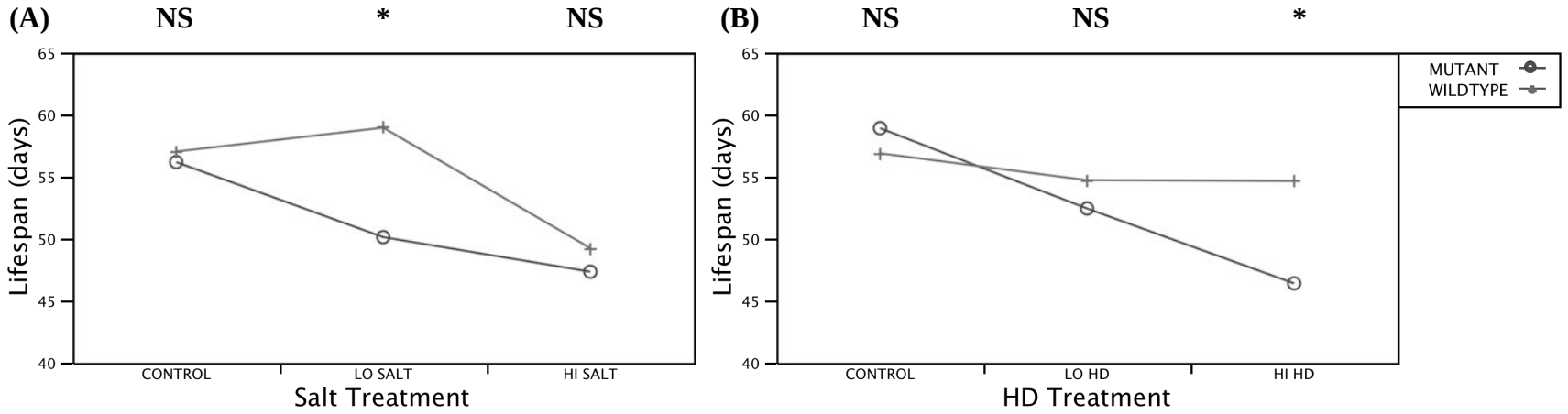


Figure 2.7. Lifespan reaction norms of WT and mutant *Arabidopsis thaliana* in response to (A) salt and (B) heat/drought stress. **(A)** There was no difference in control, yet the low salt treatment displayed a significant within treatment genotypic difference (t_{52} = 4.318; $p<0.0001$). Interestingly, the two genotypes converged in the high salt treatment, perhaps due to the severity of the treatment. **(B)** The lifespan plot for heat/drought stress showed no significant genotypic difference in control and low stress treatments, but did in the high stress treatment (t_{53} = 4.695; $p<0.0001$), indicating WT advantage for this trait in high heat/drought stress. Two-way analyses are presented in Table 2.2. Values reported are least-squares means.

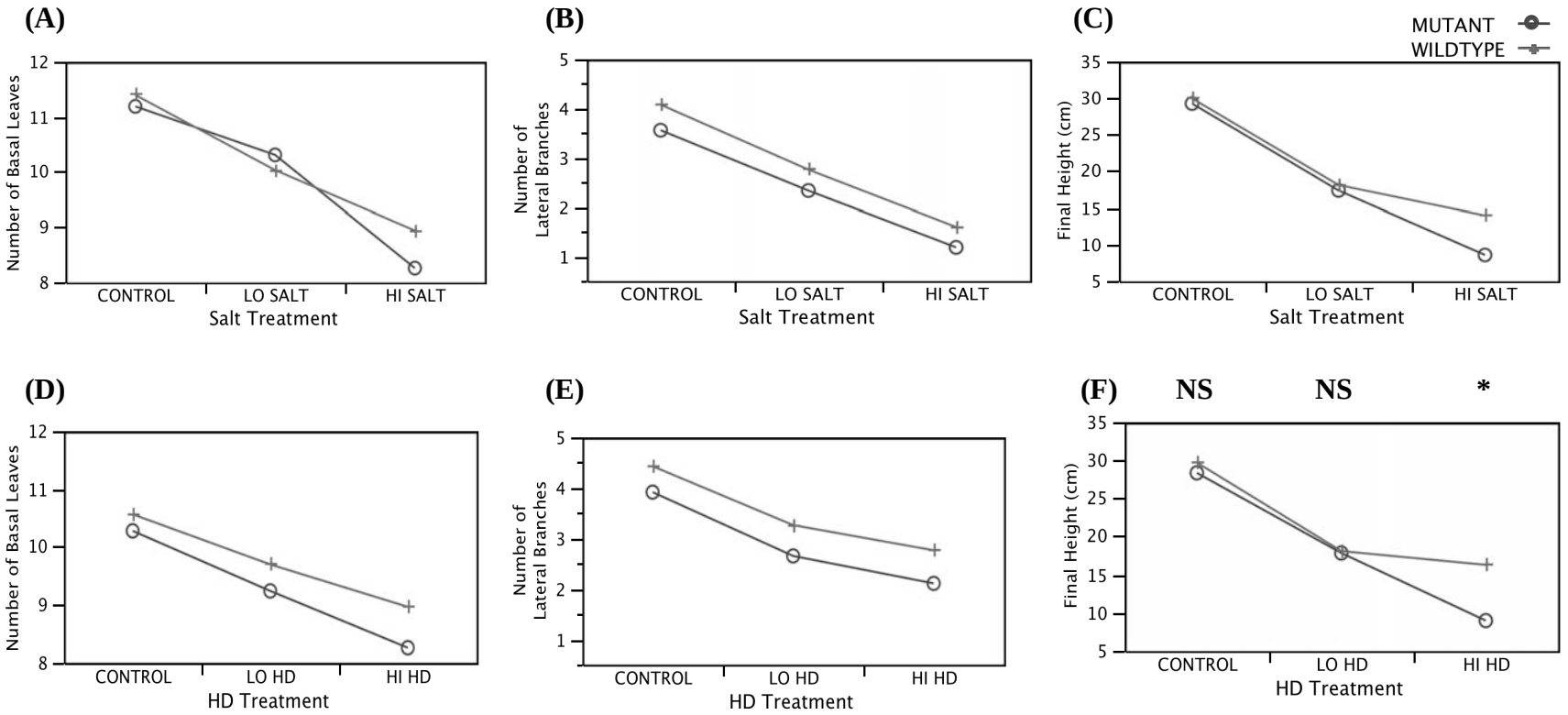


Figure 2.8. Morphological reaction norms of WT and mutant *Arabidopsis thaliana* in response to **(A-C)** salt and **(D-F)** heat/drought stress. While it seems as though WT shows an advantage in high stress for basal leaves and height in both stressors, only **(F)** height in heat/drought stress treatment showed a significant genotypic difference here ($t_{47} = 5.589$; $p<0.0001$), verifying WT advantage for this trait in high heat drought stress. Two-way analyses are presented in Table 2.3, 2.4 and 2.5. Values plotted are least-squares means.

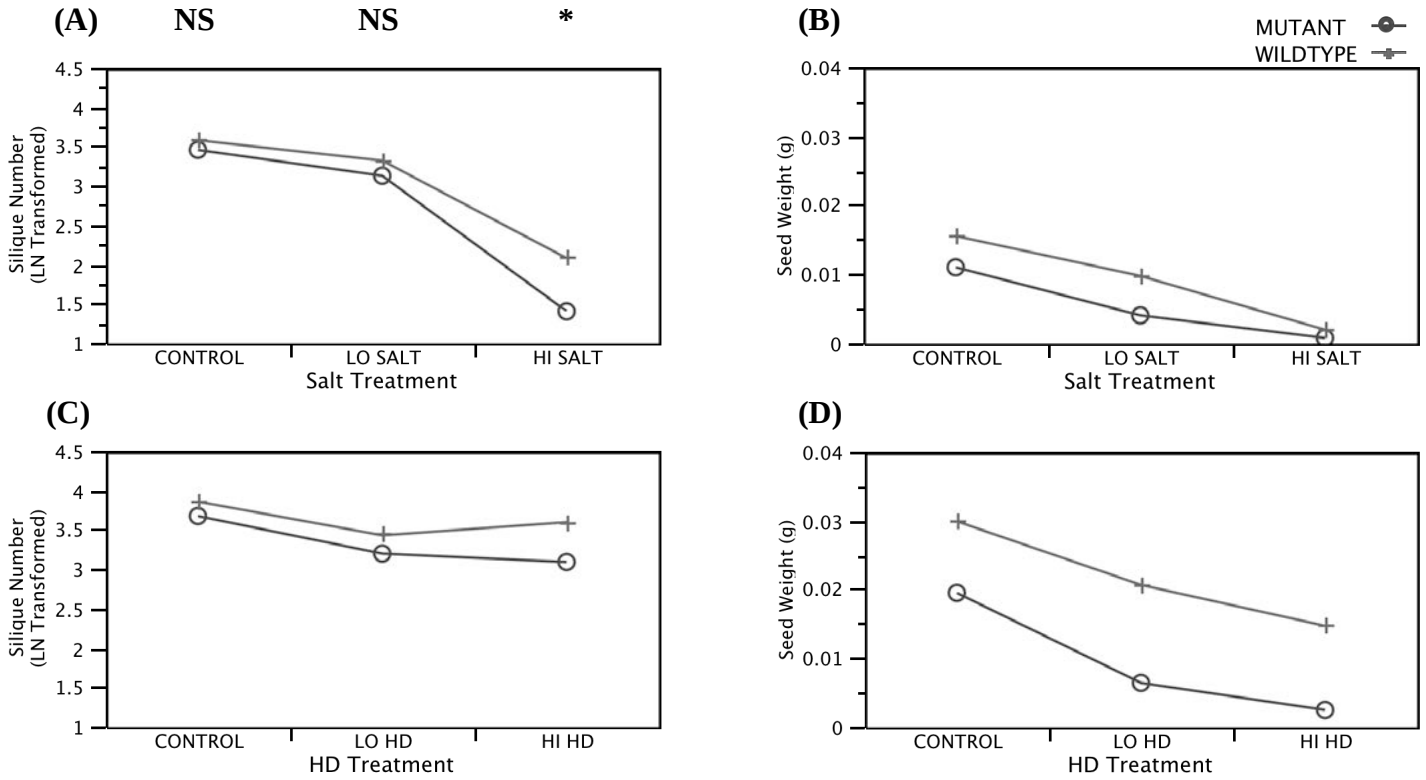


Figure 2.9. Fitness reaction norms of WT and mutant *Arabidopsis thaliana* in response to **(A-B)** salt and **(C-D)** heat/drought stress gradient treatments. While it appears as though WT shows an advantage in high stress for silique number, only **(A)** silique number in salt stress treatment showed a significant genotypic difference here (t_{21} = 4.1882; p=0.0004), verifying WT advantage for this trait in high heat drought stress. Two-way analyses are presented in Table 2.6 and 2.7. Values plotted are least-squares means.

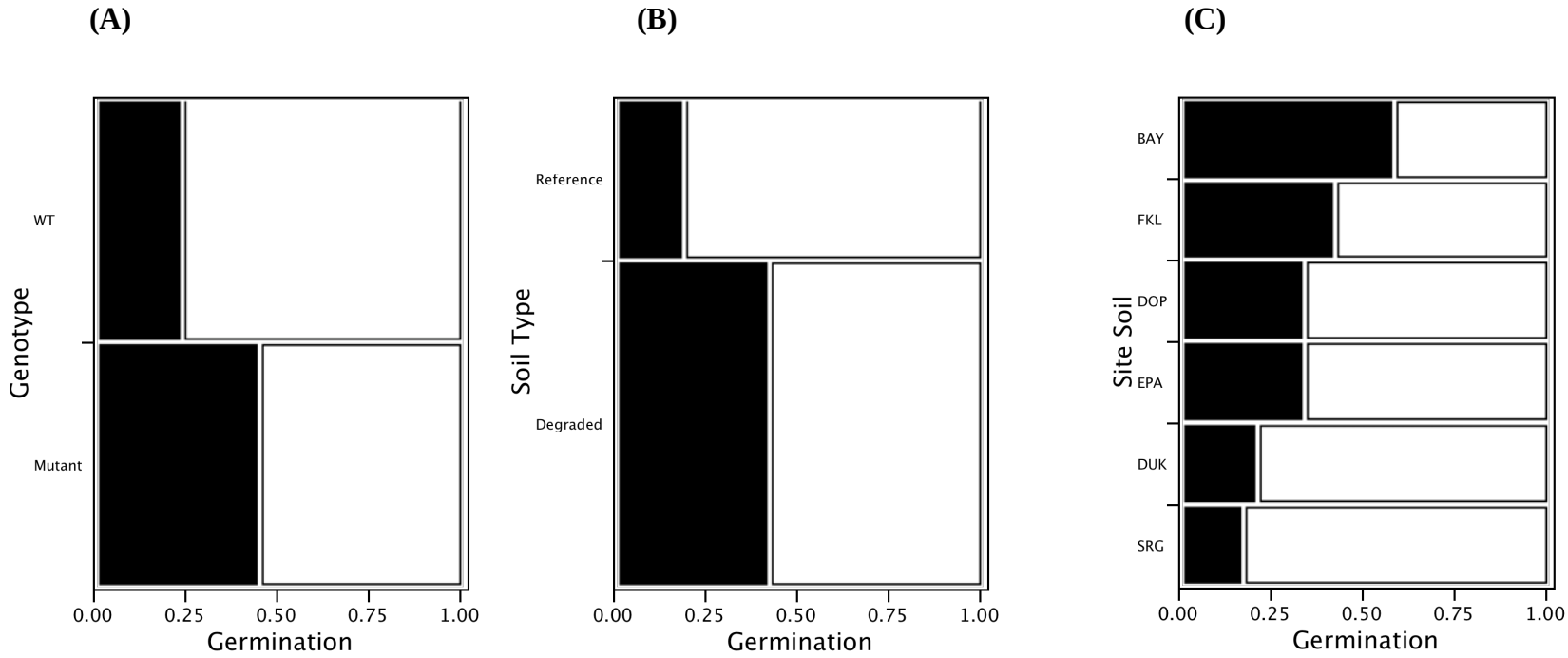


Figure 2.10. Contingency plots of germination rates of WT and mutant seed **(A)** over all soil, **(B)** pooled soil type and **(C)** across soil sites. N=144 of which germination occurred in **(A)** 76% of WT and 56% of mutant seed (p=0.0134, Fisher's Exact Test); **(B)** 81% of the time in reference soil and 58% of the time in degraded soil (p=0.0086, Fisher's Exact Test); and **(C)** ranged from 83% in SRG soils to 42% in BAY soils (p=0.0343, Pearson's test).

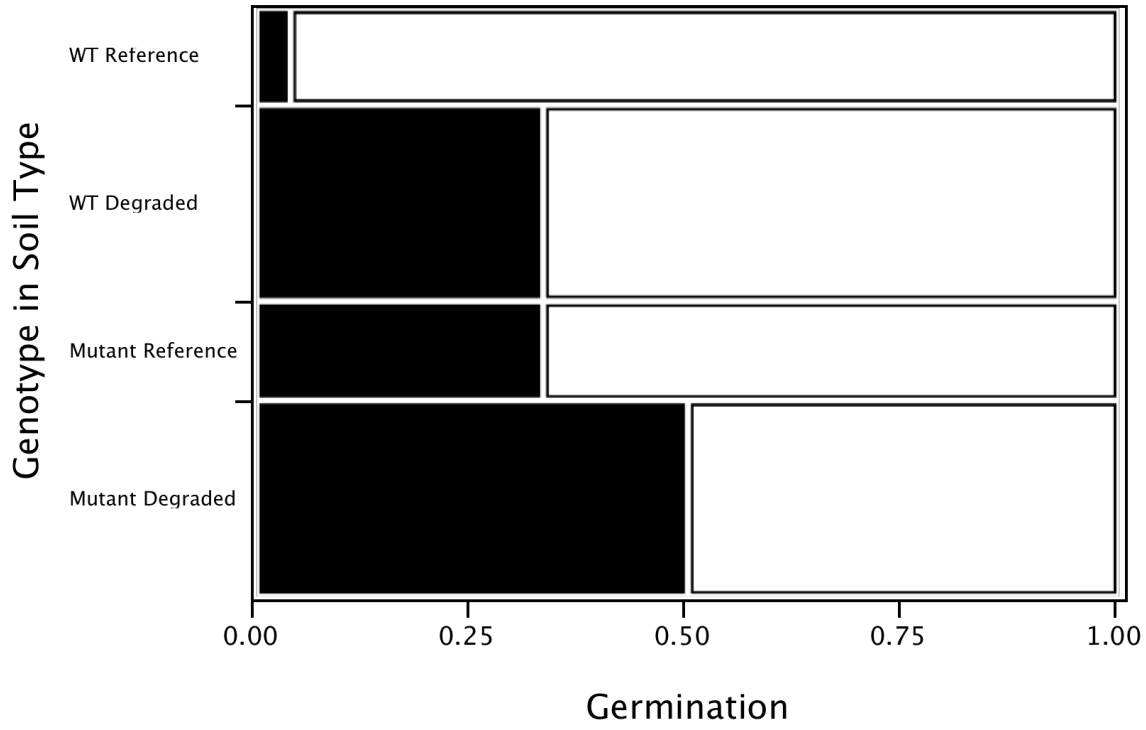


Figure 2.11. Contingency plots of germination rates of seed strain across soil type. N=144 of which germination occurred in 96% of wildtype seed in reference soil, 67% of wildtype seed in degraded soil, 67% of mutant seed in reference soil and 50% of mutant seed in degraded soil (p=0.0018, Pearson's test).

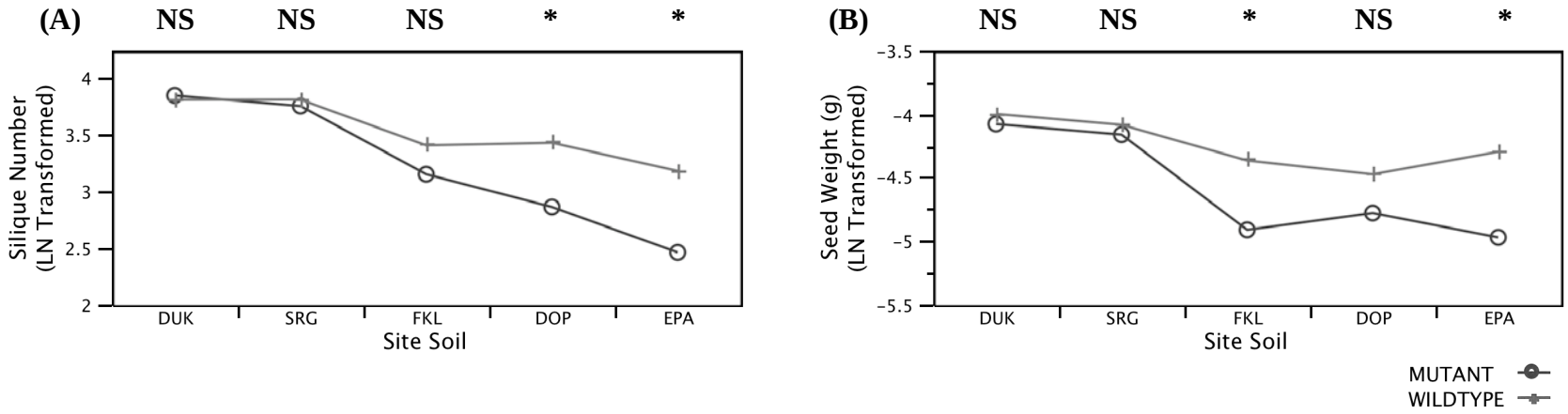


Figure 2.12. Fitness reaction norms for **(A)** silique number and **(B)** total seed weight of WT and mutant *Arabidopsis thaliana* in response to various site soil treatments. WT showed an advantage in high stressful soils for **(A)** silique number; the difference between genotypes was significant in DOP (t_{14} = 13.513; p=0.0035) and EPA (t_8 = 2.329; p=0.0490) soils. There was also a significant WT advantage in total seed weight; the difference between genotypes was significant in FKL (t_{14} = 3.957; p=0.0014) and EPA (t_{14} = 3.872; p=0.0017) soils. Two-way analyses are presented in Table 2.8 and 2.9. Values plotted are least-squares means.

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CHAPTER III

NATURAL VARIATION IN STRESS RESPONSE AND HSP INDUCTION IN *ARABIDOPSIS THALIANA*

ABSTRACT

This study investigates natural variation found among populations of *Arabidopsis thaliana* under heat and drought stress, measured at phenotypic and molecular levels. I exposed six Arabidopsis genotypes (an urban and rural pair from France, Germany and Sweden) to three stress treatments (heat, drought, heat/drought) in experimental growth chambers, analyzed their phenotypic performance and measured gene induction for both HSP17.6 and HSP101 with Real Time Quantitative PCR. I observed inherent natural variation among the six genotypes for five of the six phenotypic parameters tested, the sole exception being total seed weight. There was no difference in baseline HSP101 levels, although HSP17.6 levels differed among genotypes. Over the three stress treatments in the growth chamber, R-Sweden and R-France were most successful and urban genotypes were generally less fit. There was little correlation between HSP expression and fitness, suggesting that predicting plant success via such molecular data may have limited utility. While this work sheds valuable light on the molecular basis of heat and drought response for the HSP system, plant phenotypic performance itself may be a better predictor of how genotypes will tolerate novel stressors.

INTRODUCTION

Context of the Problem

Increasing urban development can be expected to increase local temperatures, via the urban heat island phenomenon (Grimm et al., 2008), and decrease soil moisture through altered hydrology (Paul and Meyer, 2001). It is anticipated that future global climate change will exacerbate the situation (Wilby, 2008; Risbey, 2010). Urban plants will have very few options to avoid such stressors, so acclimation and adaptation to warmer and drier conditions will depend on the available phenotypic variation (Aitken et al., 2008) and its underlying genetic diversity (Williams et al., 2008). This study tested the natural variation found among populations of *Arabidopsis thaliana* under heat and drought responses at both phenotypic and molecular levels.

Heat Shock Proteins and Stress Response

The production of heat shock proteins (HSPs) is a well-understood and frequently characterized example of thermotolerance (e.g., Lindquist and Craig, 1988; Vierling, 1991; Feder and Hofmann, 1999). While HSPs have been identified as prime candidates for the study of selection in plants experiencing frequent episodes of stress-inducing heat (Tonsor et al., 2008), many HSPs also act to buffer the impacts of drought, salt, heavy metals and toxic chemicals (Mahajan and Tuteja, 2005; Timperio et al., 2008), all of which are common stressors in urban environments.

There are five major classes of HSPs, categorized by their molecular weight: Hsp100, Hsp90, Hsp70, Hsp60 and Hsp20 (sometimes simply referred to as small, or

sHsp) (Wang, 2004). The majority acts as molecular chaperones, though each class of HSPs has different roles in maintaining homeostasis in face of various stressors (Fig. 3.1). Either by constitutive or stress-induced production, HSPs are responsible for protein folding, assembly, and degradation of damaged or misfolded proteins (Hu et al., 2009). HSPs can also stabilize proteins and membranes and help refold denatured proteins during stress events (Wang et al., 2004).

I focus on a pair of very different HSPs in this study representing different roles in abiotic stress response: HSP17.6 (Hsp20 class), responsible for protein stabilization and aggregation prevention; and HSP101 (Hsp100 class), involved in resolubilizing aggregated proteins and sending them off to be refolded by other HSPs or degraded by proteases (Wang, 2004). This dual characterization addresses two main components of HSP regulation simultaneously (Fig. 3.1).

HSP17.6 (AT-HSP17.6A). This cytosolic class I HSP can be induced by heat, drought, or a combination of the two in *A. thaliana* (Rizhsky et al., 2004). Swindell et al. (2007) found that HSP17.6, as well as other HSP17 proteins in *A. thaliana*, displayed large expression responses to an array of different stressors, in all parts of the plant, and across most developmental stages. When a plant is exposed to stress, HSP17.6 is expressed immediately (Nishizawa et al., 2006) and is thought to be responsible for preventing dangerous protein aggregation by binding to denatured proteins and creating stable HSP-substrate complexes. Damaged proteins are then released and repaired with cooperation from other HSP classes, such as the Hsp100 family (Haslbeck et al., 2005).

HSP101 (ATHSP101). HSP101 is also a cytosolic heat shock protein, and the most-studied HSP in plants (Tonsor et al., 2008). It is primarily induced by heat (e.g.

Queitsch et al., 2000), although Rizhsky et al. (2004) found that a combination of heat and drought increased transcription levels of this protein in *A. thaliana*. Campbell et al., (2001) also found that drought stress induced higher levels of HSP101 in wheat plants, suggesting that these proteins may be also involved in drought response. HSP101 transcripts and gene products are instantly increased upon plant exposure to heat stress (Hong and Vierling, 2001); they disaggregate proteins, assist with refolding, or send irreparably damaged proteins to proteases for degradation (Wang et al., 2004).

HSP induction is an excellent model with which to test plant responses to urban stress, as urbanization has been linked to elevated temperatures and altered hydrology (Zhao et al., 2006; Grimm et al., 2008). In the following experiments, we imposed both heat, drought and heat/drought stress treatments because of their ability to induce a response in HSP17.6 and HSP101 (Rizhsky et al., 2004, Tonsor et al., 2008, respectively) while mimicking conditions of urban heat islands and altered hydrology, both of which affect flora in the urban environment (Pickett et al., 2001; Paul and Meyer, 2001; Williams et al., 2008).

Objectives and Hypotheses

In the preceding chapter, I examined the benefits of one heat shock protein (HSP17.6) in *Arabidopsis thaliana* plants exposed to heat and drought and found a clear advantage in possessing the stress-inducible HSP17.6 response. Mutant plants lacking a working HSP17.6 gene generally showed an inability to cope with various types of abiotic urban stress. This difference was generally more pronounced in high stress conditions, providing evidence that *A. thaliana* HSP17.6 induction was adaptive. The

work presented in this chapter expands this investigation by using six field-collected *Arabidopsis* genotypes and by adding a molecular analysis of the expression of both HSP17.6 and HSP101.

This study was designed to: (1) determine if natural variation exists in phenotype and HSP (17.6 and 101) induction among six *Arabidopsis thaliana* genotypes exposed to various stressors, and (2) use that information to predict plant performance and success in stressful environments. The rationale here is that plants expressing higher levels of HSP17.6 and/or HSP101 expression will be able to quickly induce the necessary stress response and subsequently increase their survival and fitness over a variety of stress treatments. Plants expressing higher induction levels of HSPs in particular treatments will exhibit increased fitness in those treatments. Conversely, those that display lower levels of induction will exhibit decreased fitness.

METHODS

Study Species

Many plant HSP studies have been focused on *Arabidopsis thaliana* (L.) *Heynh.* (Brassicaceae). This cosmopolitan, highly-selfing annual, native to western Eurasia and northern Africa, can be now found worldwide in many disturbed habitats (Hoffmann, 2002). *Arabidopsis thaliana* is widely used as an experimental model for higher plants (Swarbreck et al., 2008) and is particularly favored for its quick and prolific reproduction, small genome and successful sequencing (Koornneef and Meinke, 2010).

The "1001 Genomes Project" was initiated in 2008 to catalogue whole-genome sequence variation in 1001 accessions (strains) in *A. thaliana* (Weigel and Mott, 2009). These globally collected accessions exhibit high phenotypic variation in numerous traits, including stress response (http://www.1001genomes.org, 2011). From that extensive database, maintained by the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University, I selected six accessions, collected from natural populations across Europe. The six accessions represent one urban and one rural population from France, Germany and Sweden (hereafter: U-France and R-France, U-Germany and R-Germany, U-Sweden and R-Sweden). I selected the urban and rural counterparts to determine if there were any inherent differences between provenance and performance. The urban and rural strains for each country were roughly matched for geography, elevation and climate (Table 3.1).

The 1001 Genomes Project maintains that each available accession represents a single inbred line, so I refer to the six accessions as genotypes for the purposes of this

study. Elevated homozygosity (for a naturally selfing plant) can be useful when trying to compare natural genotypes, since genetic variation that exists in nature is typically subtler (and more likely adaptive) than that found in laboratory based loss-of-function mutants (Tonsor et al., 2005). To understand the consequences of this natural variation in HSP response, it is important to understand the mechanisms by which HSPs reduce stress on individuals (Feder and Hoffman, 1999). Here, I have tested phenotypic performance as well as HSP gene expression of *A. thaliana* exposed to various stressors attempting to provide an informative and holistic view of stress response, HSP function, and plant success.

Experimental Design

I exposed six Arabidopsis genotypes to three stress treatments (heat, drought, heat/drought) in experimental growth chambers, analyzed their phenotypic performance and measured the induction of gene expression for both HSP17.6 and HSP101. Throughout all experiments, unless noted otherwise, germination protocol, growth chamber conditions and data collection methods were as follows. An illustration of the experimental design, flats and treatments can be found in Figure 3.2.

Germination and growth protocol. I soaked *A. thaliana* seeds of the six genotypes on filter paper, cold stratified them in the dark at 4°C for two days and then transferred them into potting soil (PMP High Organic Arabidopsis Medium; Lehle Seeds, Round Rock, TX) in six flats under controlled growth chamber temperature and light conditions (see below) to synchronize and stimulate germination (Pigliucci and Schlichting, 1996).

The majority of seeds germinated within the first 48 to 96 hours. I recorded germination date and replaced non-viable seeds immediately. To prevent desiccation, I covered the flats with plastic domes, misted seeds and sub-irrigated daily until bolting (emergence of the stalk). I then removed the covers and watered plants every 2-3 days, or as needed, with distilled water. For the duration of the experiment, I used an Arasystem (Betatech, Gent, Belgium), which is a series of flats and plastic tubes designed specifically for *Arabidopsis* growth and seed collection.

Controlled Growth Chamber Conditions. Conditions in the growth chamber (Model #GC15-31-CW-C3-X-HL-PW-CF, Environmental Growth Chambers, Chagrin Falls, OH), consisted of a 14-hour day (~ 140 μE/m2/sec) with 25°C daytime temperature and 70% humidity, and with 23°C nighttime temperature and 60% humidity (Scholl, 1996; Weigel and Glazebrook, 2002). Flats were rotated every three or four days to minimize any effects of growth chamber position (Potvin et al., 1990).

Baseline Assessment. To document the inherent differences between genotypes, I germinated eight replicates of the six genotypes under control conditions (see above), for a total of 48 plants across two flats of potting medium (individual flats were randomized). At four weeks past germination, I randomly collected half of the replicates for preliminary molecular tests and qPCR validation. The other half remained in the growth chamber for performance analysis. Since the baseline plant material was used for necessary validation tests, I quantified baseline levels of HSP expression using the control group from the following experimental run (noted in RESULTS).

Experimental Stress Treatments. Thirty-two replicates of six genotypes were grown under control conditions, for a total of 192 plants across four flats of potting medium (individual flats were randomized, blocked two per initial control and drought treatment). Approximately three to four weeks after germination, before plants bolted, I applied three treatments: Drought stress (D), Heat stress (H) and Heat/Drought stress (HD) (Fig. 3.2).

Drought stress: At three weeks past germination, I withheld water in two of the four flats for seven days (Rizhsky et al., 2004) after which I watered plants as needed until harvest. Drought stress required blocking by flat since watering was done by sub-irrigation. Plants from the watered flats then either remained in the control chamber to become control (C) plants or were transferred to the hot chamber to become heat (H) stressed plants. Plants from the drought stressed flats either remained in the control chamber to become drought (D) stress plants or were transferred to the hot chamber to become heat/drought (HD) stressed plants.

Heat and Heat/Drought Stress: At four weeks past germination, I transferred 4 random replicates from each genotype from each flat (as explained above; 96 plants) to 38°C for 6 hours (Rizhsky et al., 2004). At the end of the heat-shock period, I immediately collected four plants per genotype per heat treatment (H and HD) for molecular analysis and returned the remaining heat-shocked plants to the control chamber. They remained there until senescence for performance data collection. Within one hour of collecting the H and HD samples, I had also collected C and D plants for molecular analysis. This helped to standardize the biological replicates for each stress experiment by ensuring similar induction rates by which to compare all treatments.

Performance Data Collection

I collected the following data on individual plants: (1) germination date; (2) bolting date; (3) days to bolting (bolting date – germination date); (4) flowering date; (5) days to flowering (flowering date – germination date); (6) senescence date; (7) lifespan (senescence date – germination date); (8) final height (measured at senescence date); (9) number of lateral branches (branches off the main stem), (10) number of siliques (counted at senescence date); and (11) total seed weight produced (by the whole plant, measured after harvest).

Performance Data Analysis

All statistical analyses were performed using JMP version 8 for Macintosh (SAS Institute Inc., Cary, NC, USA). To increase normality and decrease heteroscedacity, I log-transformed specified raw data sets.

I analyzed differences in phenology (days to flowering, lifespan), morphology (final height, number of lateral branches) and fitness estimates (number of siliques, total seed weight) between the six genotypes exposed to different stress treatments using one- and two-way ANOVA. One-way ANOVA was used in the baseline experiment to compare phenotypic trait values across six genotypes within one (control) condition; these results were presented as box-plots. Two-way ANOVA, illustrated by least squares means plots, were performed to simultaneously compare differential genotype performance over various stress conditions and to uncover any interactions between genotype and treatment. To determine if certain genotypes exhibited significantly higher fitness estimates than others, Tukey HSD post-hoc tests were employed.

Days to bolting and days to flowering were highly correlated (r^2=0.96, p<0.0001), so only flowering time was analyzed. Total seed weight produced per plant was used as a proxy for seed number because they were highly correlated (r^2=0.85, p<0.0001) and seed weight could be more accurately determined.

Molecular Methods

To measure the subtle variation in HSP17.6 and HSP101 expression among the six genotypes accurately, I used Real Time Quantitative Polymerase Chain Reaction (hereafter, qPCR). This technology, which has now become a standard and robust method for quantifying gene expression (VanGuilder et al., 2008), allows target mRNA, once reverse transcribed, to be amplified, detected and quantified in real time (Gibson et al., 1996). "Real time" refers to the ability to detect fluorescence that increases as a cDNA (complementary DNA, created from mRNA) molecule of interest is amplified in a thermocycler and measured after each cycle of PCR. The specific florescent technology used in this study was TaqMan (Applied Biosystems, Foster City, CA), a very reliable and specific chemistry that relies on probe hydrolysis (VanGuilder et al., 2008; Fig. 3.3).

To quantify change in gene expression accurately, one must normalize the transcript levels of target genes to those of an endogenous control or housekeeping gene that does not change when exposed to a particular stress (see the $2^{-\Delta\Delta Ct}$ Method described below). For this work, I selected the protein actin 2 (ACT2), a cytoskeletal protein frequently used in expression studies because it is not induced by heat (De Schutter et al., 2007), drought (Kant et al., 2007), or related abiotic stressors (Liu et al., 2010; Wan et al., 2010).

Molecular Data Collection

Harvesting and Preserving Plant Material. To preserve mRNA transcripts, I harvested four biological plant replicates from each of the six genotypes and placed them in labeled Eppendorf tubes in liquid nitrogen immediately after the stress treatment was applied; in cases involving heat stress (H and HD), this was done within the hot chamber. I then transferred the Eppendorf tubes to a -80º C Freezer (Model #ULT1786-5-AUA, Revco Scientific, Asheville, NC) where they remained until extraction. I used three biological replicates of the six genotypes (72 samples; 18 per treatment) for analysis; the extras remained on reserve.

RNA Extraction and Assessment. I extracted total RNA from each sample, removing any potentially contaminating genomic DNA with an optional DNase treatment, using the RNeasy Plant Mini Kit and protocol (Qiagen, Valencia, CA). After extraction, I performed a quality check on concentration, purity and integrity of all 72 samples (following Udvardi et al., 2008). Concentration of RNA was determined by measuring absorbance at 260 nm (A260) with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). To confirm purity, I took the ratio of A260/A280 and found all samples had a value greater than 1.8 (Udvardi et al., 2008). I then performed gel electrophoresis on 1.2% agarose gel strained with ethidium bromide to confirm the integrity of the RNA. Each sample showed satisfactory quality and was used in subsequent analyses. The water-eluted RNA was stored at -80º C until use.

Reverse Transcription. I performed reverse transcription on each of the 72 RNA samples with a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA; hereafter ABI) using protocol and reagents from the High Capacity cDNA Reverse Transcription Kit with RNAse Inhibitor (ABI). Thermal cycler conditions were 25º C for 10 minutes, 37º C for 120 minutes, 85º C for 5 minutes and then 4º C until removed. The cDNA was stored at -80º C until used for the experimental runs.

Real Time Quantitative PCR. I performed all preliminary validation tests, experimental design, and methods as outlined in Udvardi et al. (2008). MicroAmp Fast Optical 96-Well Reaction Plates (ABI) were prepared using TaqMan Gene Expression Master Mix reagents and protocol (ABI). In each 20 µl reaction, I combined sample cDNA with the master mix and a specific TaqMan Gene Expression Assay (ABI) for HSP17.6, HSP101, or ACT2. A total of 648 experimental (4 treatments x 6 genotypes x 3 biological replicates x 3 genes x 3 technical replicates) and 24 NTC (no template control, 3 per plate) wells were run on a StepOnePlus Real-Time PCR System (ABI). Thermal cycler conditions were 50º C for 2 minutes, 95º C for 10 minutes, then 40 cycles of 95º C for 15 seconds and 60º C for 1 minute, and then 4º C until removed and discarded. All data were collected and saved using StepOne Software v 2.2.1 (ABI).

Molecular Data Analysis

All statistical analyses were performed again using JMP version 8 for Macintosh. In qPCR, DNA quantification relies on plotting the fluorescence (Y axis) against the number of PCR cycles (X axis). A threshold fluorescence level is automatically set just

above background levels. The number of cycles at which a sample's fluorescence crosses that threshold is defined as the cycle threshold, or C_T (Fig. 3.4). To quantify the relative change in gene expression of HSP17.6 and HSP101, I used the $2^{-\Delta\Delta C}{}_T$ Method described by Livak and Schmittgen (2001). This equation calculates the amount of target gene transcripts, normalized against an endogenous control gene and relative to a control treatment (Applied Biosystems User Guide, P/N #4371095, 2008):

C_T = cycle number at which fluorescence exceeds threshold

$\Delta C_T = C_{T\ \text{GENE OF INTEREST}} - C_{T\ \text{ENDOGENOUS CONTROL GENE}}$

$\Delta\Delta C_T = \Delta C_{T\ \text{TREATMENT}} - \Delta C_{T\ \text{CONTROL}}$

This double standardization permits an accurate measure of the relative induction of the target genes (HSP 17.6 and 101), in each stress treatment. One-way ANOVA was used for the baseline analysis to compare HSP17.6 and HSP101 expression levels of the six genotypes within one (control) condition and were presented with box-plots. Two-way ANOVA were performed to visualize the differential genotypic inductions for each gene; least squares means plots illustrate the change in expression from baseline to stress-induced levels. To determine if certain genotypes exhibited significantly higher expression levels than others, Tukey HSD post-hoc tests were employed.

To precisely compare genotypes, $2^{-\Delta\Delta C}{}_T$ data were transformed due to high variance among biological replicates. The procedure, outlined by Willems et al. (2008) involved log transformation, mean centering and autoscaling (Table 3.2, Fig. 3.5). There was significant variation across the three biological replicates used, although it was clear that the genes of interest were being differentially induced across genotype. To standardize the replicates without changing their relationship, data were first log

transformed, however, that alone could not correct experimental differences between the biological replicates in each stress. I mean-centered each replicate experiment by subtracting the experimental average (average of 1.1C, 1.1D, 1.1H, 1.1HD) by the log transformed fold increase (0 values for control treatments). Mean centering provided a correction for the difference in background or control levels between biological replicates. I autoscaled the data (equalizing the standard deviation across all treatments in each biological replicate) by dividing the mean-centered values by the standard deviation for the same replicate. Autoscaling required one last correction to make the fold changes reflect the initial observation, and that was to multiply the autoscaled value by the average experimental standard deviations for each replicate (Willems et al., 2008). I performed one-way and two-way ANOVA on the transformed fold change values to compare induction rates of the six genotypes.

Determination of "most successful" and "least successful" genotypes. Fitness results (silique number and seed weight) were the primary determinants of success; however, I examined these jointly with the induction rates of the HSP genes. Plants that performed best in control treatments were not necessarily the best at tolerating stress. For this reason, the slopes of the least squares mean plots (from the two-way ANOVA of differential genotypic performance) were also considered when determining overall success of genotypes.

RESULTS

Baseline Assessment

Phenology. Genotypes displayed significant differences in flowering time; R-France (28 days) and R-Germany (29 days) flowered significantly earlier than the others, while U-Sweden (38 days) flowered the latest (Fig. 3.6). Although both early flowering genotypes were rural, R-Sweden (37 days) did not have a similar pattern; it flowered more like the urban strains. This very well may be due to its cold climate provenance, remaining longer in basal rosette form may be advantageous in the early spring. Genotypes R-Germany and R-France exhibited significantly shorter lifespans (both 57 days) than the rest, while U-Germany lived the longest (60 days; Fig. 3.6). While long lifespan can be an important factor in overall success, three days difference is probably not very noteworthy.

Morphology. Genotypes differed in final height; U-Germany (39 cm) and R-France (36 cm) grew significantly taller than U-France (30 cm) and U-Sweden (28 cm; Fig. 3.7). As height can be correlated with lifespan, seed characteristics and the ability to compete for light (Moles et al., 2009), ten centimeters may give an individual plant an advantage. Similarly, R-France (8 branches) genotypes produced significantly more lateral branches than U-France (6 branches) and U-Sweden (5 branches; Fig. 3.7). There were no evident urban/rural differences.

Fitness. Genotypes R-France and U-Germany produced significantly more siliques than U-Sweden. Interestingly, this did not translate into any seed weight differences, there was no significant difference found in total seed weight among the six genotypes (Fig. 3.8).

HSP Expression. Using the control data from the experimental run, I quantified constitutive HSP expression to determine inherent differences among genotypes. HSP17.6 displayed variation; R-France and U-Germany genotypes expressed significantly higher levels of the gene than R-Sweden, although presumably, the very low value of R-Sweden drove that effect. While there was slight variation among genotypes in HSP101, ANOVA showed no significant differences (Fig. 3.9).

Success. In the absence of stress, R-France and U-Germany were clearly the most successful genotypes. R-France flowered earliest, grew tall and produced the most branches, which most likely led to its high number of siliques. U-Germany lived the longest, grew tallest and also had high numbers of siliques. Interestingly, of all the six genotypes, these two genotypes had the highest constitutive levels of HSP17.6. U-Sweden and to a lesser extent, U-France, were the least successful in non-stress conditions, as they were shorter and produced fewer branches and siliques.

Experimental Stress Treatments: Drought

Phenotype. There was very little phenotypic response to drought stress alone. Genotypes generally responded with minor decreases in phenotypic performance, which

echoed the inherent differences among them, although lifespan (Table 3.3) as well as silique number and seed weight (Table 3.4) were significantly reduced by drought stress. While there were no significant interaction effects, it did appear that there were genotypic differences in drought response for seed weight (Fig. 3.10), as R-Sweden and R-France genotypes had significantly higher weights than U-Sweden only under drought stress.

HSP Expression. I found significant differences in HSP gene expression when plants were exposed to drought stress (Fig 3.11). In HSP17.6, R-Sweden had significantly higher induction values than U-Germany, as seen by its steep increase (high plasticity) in drought conditions (Fig. 3.12). HSP101 induction was quite variable, as three genotypes (R-Germany, U-Sweden and R-Sweden) increased and the rest decreased expression (Fig. 3.13). Overall, rural genotypes expressed higher induction levels of HSP17.6 than their urban counterparts; this trend was somewhat apparent in HSP101 (Fig 3.11).

Success. Based on fitness estimates alone, it would appear that R-France and R-Sweden were most successful in drought conditions, as they produced the highest seed weight (Fig. 3.10). This does correlate with the HSP17.6 induction levels in drought; R-Sweden and R-France expressed the highest amounts of the stress protein, although for HSP101, only R-Sweden was upregulated; R-France expression actually decreased. U-Sweden again was the least successful genotype. It produced the least amount of seed and had low levels of HSP17.6 in drought conditions. Again, this was not reflected in HSP101.

Experimental Stress Treatments: Heat

Phenotype. There was strong phenotypic response to heat stress. While genotypes generally maintained their differences, as suggested by significant genotype effects, the heat stress caused major decreases in phenotypic performance in lifespan (Table 3.5), height and lateral branches (Table 3.6), as well as silique number and seed weight (Table 3.7). Lifespan, lateral branches and silique number expressed significant interaction terms, indicating differential genotypic response to heat stress. While it seemed that there was a slight genotypic partitioning in heat response for seed weight (Fig. 3.14), there were no significant interaction effects observed.

HSP Expression. In both genes, U-Sweden had significantly stronger HSP induction under heat stress than R-Germany (Fig. 3.15). In HSP17.6, R-Sweden again displayed a very steep slope, indicating high induction (plasticity) under heat stress (Fig. 3.16). HSP101 also showed differential induction across genotypes (Fig. 3.17), however, neither of these expression responses could be linked to the phenotypic performance of the genotypes.

Success. During heat stress, the best performer was R-France. This genotype again flowered early, grew the tallest, had the most branches, and produced the most siliques. Ranking second overall was R-Sweden. This genotype produced taller plants and significantly higher seed weights than the highest baseline performers, R-France and U-Germany (Fig. 3.14). This genotype, which had moderate success in the baseline assessment, performed very well when heat stress was applied. These two genotypes

expressed similar and intermediate to low induction levels of both HSP17.6 and HSP101 under heat stress (Fig. 3.15).

As in the baseline assessment, U-Sweden was by far the least successful genotype. U-Germany, a genotype that showed high performance with no stress, was also unable to tolerate heat, as was evident by its low performance, especially in seed weight. Interestingly, these genotypes expressed similar and significantly higher induction levels of HSPs than the other genotypes (Fig. 3.15).

Overall, the rural genotypes in this experiment out-performed all the urban genotypes in heat stress. This seems to have an inverse relationship of HSP expression; all urban genotypes expressed higher induction levels of HSP17.6 than the rural ones; a similar trend was apparent in HSP101.

Experimental Stress Treatments: Heat/Drought

Phenotype. Again, there was strong phenotypic response to heat/drought stress. Genotypes maintained their differences, as seen through significant genotype effects, but the heat/drought stress caused major decreases in phenotypic performance in lifespan (Table 3.8), height (Table 3.9), and fitness estimates (Table 3.10). Height and silique number expressed significant interaction terms, as there were differential genotypic responses to heat/drought stress. Again, seed weight seemed to show a partitioning of genotype response in the stress treatment (Fig. 3.14) as all rural strains had significantly higher seed weights in heat/drought stress, but there was no significant interaction effect observed.

HSP Expression. Results for expression were quite variable across genotypes and genes in heat/drought stress (Fig. 3.19). In HSP17.6, U-Sweden and U-Germany displayed significantly higher induction levels than R-France and R-Sweden, although R-Sweden again shows the steepest slope (Fig. 3.20). In HSP101, U-Sweden expressed the least amount of the stress protein while U-France expressed the most. A significant interaction effect was observed in HSP101 expression, indicating differential genotypic response to heat/drought stress (Fig. 3.21), but these expression patterns could not be correlated to plant performance.

Success. During heat/drought stress, the best performers were again R-Sweden and R-France. These genotypes maintained significantly higher values for silique number and total seed weight (Fig. 3.18). These genotypes similarly expressed low induction levels of HSP17.6, but intermediate levels of HSP101 (Fig. 3.19).

Again, U-Sweden was the least successful genotype, as well as U-France, in both siliques produced and seed weight. The gene induction data showed no clear pattern here. U-Sweden expressed significantly higher HSP17.6 induction than four other genotypes, but significantly lower HSP101 induction than the rest. U-France showed almost the opposite pattern with its HSP17.6 induction intermediate and a significantly higher HSP101 induction than the rest of the genotypes (Fig. 3.20, 3.21).

Overall, the rural genotypes in this experiment out-performed all the urban genotypes in heat/drought stress. However, unlike under heat stress, there was no clear connection between performance, gene expression and provenance.

DISCUSSION

Baseline Assessment: Natural Variation in Phenotype and HSPs

Phenotype. All phenotypic traits measured here, except for seed weight, differed significantly among the six genotypes. For some time, researchers have observed abundant phenotypic variation for morphological and physiological traits, so much so that *Arabidopsis* accessions can often be distinguished easily from those collected from different locales (Koorneef et al., 2004). The abundance of natural variation in *A. thaliana* has led to an increase of studies attempting to identify genes that produce these complex phenotypes (as reviewed in: Alonso-Blanco et al., 2005; Mitchell-Olds and Schmitt, 2006), many of which involve HSP complexes. This may allow researchers to better predict fitness of plants exposed to certain stressors. Moreover, these discoveries might lead to new methods to aid the selection of stress-resistant seed stock for ecological restoration.

HSP Expression. There was significant variation found in constitutive levels of HSP17.6. While genotypes R-France and U-Germany displayed significantly higher HSP17.6 expression levels than did R-Sweden, the difference observed was most likely inflated due to the potentially outlying low values of R-Sweden (Fig. 3.8). There were no significant differences among the genotypes when looking at HSP101 (Fig. 3.8). These two genes may be differentially stress-induced, but should remain at similar basal levels in the absence of stress, as seen by the majority of the results.

This pattern has been seen in other studies looking directly at natural genotypic variation in HSP stress response. Rampino et al. (2009) examined 16 wheat cultivars for basal and acquired thermotolerance. In doing so, they measured no significant difference among genotype in regards to small HSP (16.9, 17.6, 23.5, 26.5) expression under control conditions. Tonsor et al. (2008) also found no significant difference in basal expression of HSP101 between ten latitudinal *A. thaliana* ecotypes in control temperatures, and only when heat stress was applied found significant induction differences.

Experimental Stress Treatments

Generally, genotypes differed in their responses to stress treatments (Tables 3.3-3.10). However, in this study, there were no universal patterns found; each genotype responded very differently to the variety of stress treatments performed. For this reason, stress experiments are separately discussed below.

Experimental Stress Treatments: Drought

Phenotype. While drought stress did not affect many of the phenotypic parameters measured, it did have a profound impact on seed weight, which varied among genotypes. Control conditions showed no difference, but R-France and R-Sweden had significantly higher seed weights than U-Sweden (Fig. 3.10) under drought stress. This variation was expected, as *A. thaliana* is native to many different habitats and experiences varying drought constraints (Bouchabke et al., 2008). Population differences have been found in drought adaptation through flowering time plasticity, used as a proxy for fitness, in 39 accessions of *A. thaliana* (McKay et al., 2003) and achene number among three

populations of *Polygonum persicaria* exposed to drought conditions (Heschel et al., 2004).

HSP Expression. HSP levels were not expected to dramatically increase in the presence of drought stress, as the drought treatment primarily served as a control for the heat/drought treatment. In some cases, the pattern was anticipated: R-France, R-Germany and U-France exhibited a slight increase in induction rate for HSP17.6. However, R-Sweden had a very steep increase, whereas U-Germany and U-Sweden actually decreased HSP levels (Fig. 3.12). This divergence was even more apparent in HSP101, although some genotypes in fact switched direction; U-Sweden significantly increased its induction and both French genotypes decreased expression (Fig. 3.13). These diverse results cannot be fully explained, as the connections between drought tolerance and HSP induction are still unclear (but see Campbell et al., 2001; Rizhsky et al., 2004).

Interestingly, the genotypes that induced the strongest HSP17.6 induction, R-Sweden and R-France were the same genotypes that exhibited highest seed weights under drought stress, while the genotype with significantly lower HSP17.6, U-Sweden, performed the worst (Fig. 3.10, 3.12). This may indicate that HSP17.6 can impart drought tolerance in *A. thaliana.* Sato and Yokoya (2008) did show that transgenic rice seedlings exhibited increased drought tolerance and survival when overexpressing a similar small HSP, 17.7. However, to my knowledge, no study has found this phenomenon in *A. thaliana* or in naturally derived plants.

According to the site information, the wettest sites, as measured by annual precipitation (cm), are R-Germany (94), R-France (81), and U-France (68). This did

correlate with the slight, and assumedly appropriate, HSP17.6 response to drought stress. However, this had no bearing on which genotypes were most fit. There were also no clear induction patterns or fitness benefits seen for HSP101.

Experimental Stress Treatments: Heat

Phenotype. Heat stress decreased fitness estimates in all genotypes, yet they differed in extent. Under heat stress, the rural genotypes produced higher seed weights than the other genotypes (Fig. 3.14). As there was no difference in seed weight in the control (or baseline assessment), the differential performance in seed weight of genotypes represents an actual variation in stress response. Zinn et al. (2010) similarly found significant *A. thaliana* ecotype variation in seed set when plants were exposed to stressful conditions of hot days and cold nights. Saha et al. (2010) also found significant variation in fruit number among 12 sweet pepper genotypes exposed to heat stress.

The data trends presented here suggest that rural genotypes are generally more fit than urban genotypes under heat stress. While at first this seems counter-intuitive, as urban genotypes should be more adapted to this type of stress, the result is more likely due to the adaptive strength of rural plants. Urban plants are regularly exposed to poor air quality (McDonnell et al., 1997), high levels of soil heavy metals (Pouyat et al., 1995), low soil nutrient quality (Pickett et al., 2001), limited soil microbes and invertebrates (White and McDonnell, 1988), and hydrophobic soils (Pouyat et al., 2010). These consistent stressors may weaken the defenses of a plant, creating limitations on how much additional stress response it can exhibit. Williams et al. (2008) hypothesize that stressors in the urban environment can lead to a narrowing of functional traits. This can

affect plants directly exposed, but also influence subsequent generations through carryover maternal effects. It is conceivable, therefore, that urban genotypes are inherently weaker and less resilient under various stress conditions.

HSP Expression. HSP induction was significantly increased by heat stress in both genes. I found significant genotypic variation in HSP17.6 and HSP101 (Fig 3.16, 3.17), indicating differential response to heat. Because HSPs are so important to thermotolerance, it is often presumed that plants from heat-stressed (or simply hotter) environments have accumulated higher levels of HSPs (Feder, 1999; Barua et al., 2008). The results of actual studies are mixed. Barua et al. (2003) tested the thermotolerance of five *Chenopodium album* ecotypes while measuring chloroplast sHSPs. Plants from warmer populations induced significantly more sHSPs and had higher levels of thermotolerance than did plants from colder locations. Conversely, Knight and Ackerly (2001) found no relationship, and even a slightly negative trend, of *Ceanothus* chloroplast sHSP expression and the mean maximum July temperature. I also found little correlation between HSP levels and average summer temperatures. There are limitations on how much is known of the temperature variability of these six collection sites. Barua et al. (2008) found that variability, through daily ranges in temperature and frequency of extreme temperature events, is a better predictor of HSP content variation than mean temperatures. Such fine-grained information would be helpful in interpreting these various results, but is not yet available.

I also found there to be little to no correlation between the levels of HSP induction and fitness of genotypes under heat stress. This was contrary to my initial

hypothesis and quite surprising, since it has been observed that loss-of-function *A. thaliana* mutants in both HSP17.6 (this study, Chapter 1) and HSP101 (Tonsor et al., 2008) produce significantly lower silique numbers than wildtype strains in stress. As mentioned before, uncovering subtle natural variation is more difficult than finding the very apparent divergence between wildtype and mutant strains. This may suggest that there are thresholds of gene product under which fitness is affected, but that the genotypes studied here, varied as they were, all exceeded the minimum requirements for maintaining ample fitness.

While the data trends indicate that urban genotypes induced stronger heat responses in both genes, the only significant finding was that U-Sweden, the poorest performer overall had the highest induction levels of both HSP17.6 and HSP101 (Fig. 3.15). This may suggest that this cold-climate genotype induced an inappropriate response due to a novel heat stress that the genotype never experienced before in its evolutionary history (Ghalambor et al., 2007). Conversely, U-Sweden might have exhibited very high expression rates due to a recent mutation and what was seen here was an example of deleterious pleiotropy. For example, Sun et al. (2001) found that *A. thaliana* mutants overexpressing HSP17.6 survived better under salt stress, but I found these 'OE' mutants to have lower germination rates, decreased growth and shortened lifespans (preliminary data, not shown). Krebs and Feder (1997) similarly found that abnormally high concentration of HSPs in *Drosophila* larvae led to decreased growth and survival.

The heat stress experiment performed in this study addressed basal thermotolerance, rather than an acquired phenotype, inasmuch as there was only one heat

shock applied. While some studies have been successful in determining the importance of HSPs from one heat shock treatment (Queitsch et al., 2000; Hong and Vierling, 2000, 2001), it has been suggested that detecting genetic variability in thermotolerance of HSPs can only be successfully performed by first applying an "acclimation treatment" and then a severe stress treatment (Rampino et al., 2009). Any future studies addressing these findings should add an acquired thermotolerance component to better reveal the roles of HSPs under such circumstances.

Experimental Stress Treatments: Heat/Drought

Performance. Heat/drought stress also decreased fitness estimates across all genotypes and again variation was evident. In heat/drought stress, all rural genotypes produced higher seed weights than U-France and U-Sweden (Fig. 3.18). Again, since there were no differences in the control or baseline values of seed weight, this differentiation confirmed the presence of natural variation of heat/drought response among the genotypes. While many recent studies have highlighted the need to investigate plant tolerance under multiple stress conditions (e.g., Rizsky, 2002; 2004; Mittler, 2006, Barnabas et al., 2007), most studies still predominantly measure natural variation of *A. thaliana* in single stress responses (Zhen and Ungerer, 2008; Katori et al., 2010; Vashisht et al., 2011). Very little attention has been paid to the natural variation of response to multiple stressors (but see Vallejo et al., 2010).

Rural genotypes again significantly outperformed their urban counterparts in heat/drought stress (3.18). For the same reasons listed above, rural genotypes probably have more resources available to them to initiate appropriate stress responses.

HSP Expression. HSP induction was significantly increased by heat/drought stress for both genes (Fig. 3.20, 3.21). In fact, both HSPs had higher induction levels under heat/drought than under heat or drought alone. While breeders and farmers have known for some time that combinations of abiotic stressors are most lethal to plants (Mittler, 2006; Barnabas et al., 2007), few studies have analyzed the molecular mechanisms behind such tolerances. Using transcriptome analysis, Rizhsky et al. (2004) uncovered particular genes that were upregulated during a combination heat/drought stress, but not necessarily induced during individual heat or drought stress. HSP17.6 and HSP101 were two of the few genes they found that upregulated across all single and multiple stress conditions, so it was not surprising to see their strong expression in response to heat/drought stress in this experiment.

Again, genotype variation was significant in HSP101 (Fig 3.21). Interestingly however, the profiles of heat/drought induction differed dramatically from that of heat alone. There was neither any correlation with fitness estimates, nor noticeable partitioning between urban and rural genotypes. Essentially, these data were wide-ranging and might be unhelpful in identifying heat/drought tolerant plants.

Application to Urban Restoration Ecology and Future Considerations

This study emphasizes the importance of understanding how urban and climatic stress affects stress gene expression and reproductive development, and highlights the potential for selecting more tolerant genotypes for use in urban restoration conditions in a changing climate. For quite some time, agricultural researchers have been calling for

such of biochemical selection criteria, specifically using induction studies, as a way to identify and choose stress tolerant genotypes for use in crop production (Krishnan et al., 1989; Kumar et al., 1999; Rampino et al., 2009). This idea is slowly becoming adopted in restoration theory (though not yet in practice). Jones and Monaco (2009) argue that genetically altered plant material developed for abiotic stress tolerance is useful for restoring ecosystem structure, function and biodiversity in highly modified environments. This "assisted evolution" may be increasingly important for highly urbanized areas, and for future climatic conditions. More investigations like this study must be performed to identify other promising genes, analyze plant response to multiple stressors, and add a field component in urban habitats. These types of experiment must continue in order for successful urban genotype selection to advance.

While a pattern was sometimes found between genotype success and HSP levels, the results presented here suggest that HSP induction is but one part of a complex abiotic stress response, and that predicting plant success using this type of molecular data alone may be problematic. In light of the strong performance data and inconsistent HSP results throughout these experiments, it is most prudent to define the most stress-resistant genotypes as R-Sweden and R-France. Using performance as a predictor for future success in novel habitats can only be justified with future trials under additional stress regimes. Therefore, these six genotypes will be planted in a controlled salt and salt/heat treatment and in two site soils: brownfield soil and post-agricultural field soil. If the most successful genotypes again prevail, it will add support to performance-based screening of genotypes for use in heterogeneous urban sites, characterized by unknown combinations of stressors.

Are Stress-Resistant Genotypes Phenotypically Plastic?

Phenotype is defined as any measureable trait of an organism. As we delve more into the molecular realm of plant physiology and evolution, we gain the ability to measure more nuanced genetic response. In essence, plants that display flatter reaction norms over different stressful environments yet show extreme changes in stress gene induction rates should be considered highly plastic in the true sense of the word. In the case of the stress-resistant genotypes found in these experiments, there are many genetic and biochemical phenomena (along with HSP response) working just under the surface allowing for homeostasis in the face of unknown stressors.

Conclusion

Natural variation within a species is not only exceedingly important for populations' capacity to acclimate and adapt to constantly changing environments, but shows evidence of a species' ability to expand its range and adapt to novel habitats and conditions. This study clearly demonstrates that significant natural variation in phenotype and HSP induction exists among the six *A. thaliana* genotypes observed. Intrinsic genotypic differences were found in the absence of stress; yet even more revealing were the differential phenotypic and molecular responses specific to particular stress treatments. This work provides valuable ecological insight into the underpinnings of heat and drought response via the HSP system in *A. thaliana*. Extension to a wider array of systems would be beneficial.

Table 3.1. Information about the genotypes used in this study. CS number is a stock number created by ABRC; accession names are from the stock list. Habitat, when not provided, was mapped by latitude and longitude and determined by land-use.

Genotype	Accession	CS Number	Town	Habitat	Latitude	Longitude	Average Sum/Win Temp. (C)	Annual Precipitation (cm)	Altitude (m)
R-France	Gy-0	CS76139	Guyancourt	Farmland	N48.7667	E2.0833	18/6	81	154
U-France	Ange-1	CS28020	Angers	RR station	N47.4784	W0.5473	17/5	68	41
R-Germany	Ak-1	CS28011	Achkarren	Vineyard	N48.0667	E7.6333	23/-2	94	200
U-Germany	Si-0	CS28739	Siegen	City roadside	N50.8667	E8.0333	16/2	64	305
R-Sweden	Ull2-3	CS76293	Ullstorp	Farmland	N55.5333	E13.9833	13/1	57	57
U-Sweden	St-0	CS76231	Stockholm	City roadside	N59.3350	E18.0667	15/1	54	52

Table 3.2. Transformation of ddCt data from qPCR. To standardize the replicates without changing relationship, data were log transformed, mean centered and then autoscaled. This table represents the R-France genotype. Figure 3.4 shows the subsequent variance reduction across control, heat, and drought values.

R-France genotype	Ct HSP17.6	Ct ACT2	dCt	ddCt	Fold	Fold (Log trans.)	Exp. Average	Mean Centered	Exp. SD	Autoscaled	Autoscaled X Mean Exp. SD
C (1.1)	36.5	33.1	3.4	0.0	1.0	0.0000	1.1294	-1.1294	0.9553	-1.1823	-1.3499
H (1.1)	28.3	30.8	-2.6	-6.0	62.7	1.7972		0.6679		0.6992	0.7983
D (1.1)	33.2	32.1	1.1	-2.3	4.8	0.6857		-0.4436		-0.4644	-0.5303
HD (1.1)	33.2	36.6	-3.4	-6.8	108.3	2.0345		0.9051		0.9475	1.0819
C (1.2)	35.8	33.0	2.8	0.0	1.0	0.0000	0.9230	-0.9230	1.4575	-0.6333	-0.7231
H (1.2)	28.6	31.4	-2.8	-5.6	48.0	1.6811		0.7581		0.5202	0.5939
D (1.2)	36.2	31.6	4.7	1.9	0.3	-0.5650		-1.4880		-1.0209	-1.1657
HD (1.2)	28.0	33.7	-5.8	-8.6	376.6	2.5759		1.6529		1.1341	1.2949
C (1.3)	36.1	33.8	2.3	0.0	1.0	0.0000	0.9590	-0.9590	1.0128	-0.9469	-1.0812
H (1.3)	28.4	31.3	-2.9	-5.2	37.2	1.5700		0.6110		0.6033	0.6888
D (1.3)	35.4	33.8	1.6	-0.7	1.6	0.2047		-0.7543		-0.7448	-0.8504
HD (1.3)	30.2	34.8	-4.6	-6.8	115.2	2.0613		1.1023		1.0884	1.2428

Table 3.3. Lifespan; Generalized linear model (log-linked, Poisson) results of the drought experiment: Whole model results and effect likelihood ratio analysis. Only drought (D) treatment significantly affected lifespan of *Arabidopsis thaliana*.

MODEL	**-LogLikelihood**	**df**	**Chi-Square**	**P>Chi-Square**
Difference	14.306	11	28.612	0.0026*
Full	556.979			
Reduced	571.285			
STAT	**Chi-Square**	**df**	**P>Chi-Square**	**Overdispersion**
Pearson	10.3610	38	1.000	0.2727
	SOURCE	**df**	**L-R Chi-Square**	**P>Chi-Square**
	Genotype	5	6.461	0.2639
	D Treatment	1	18.459	<0.0001*
	D*Genotype	5	4.917	0.4261

Table 3.4. Fitness estimates **(A)** silique number and **(B)** total seed weight (log transformed); 2-way ANOVA results of the drought experiment: Whole model results and effect likelihood ratio analysis. Only genotype and drought (D) treatment significantly affected the amount of siliques produced by *Arabidopsis thaliana*; there were no significant interaction effects found.

(A)

R-Square	0.6649
Observations	50

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	9539.217	867.202	6.8528	
Error	38	4808.783	126.547	**Prob > F**	
Total	49	14348.000		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	8560.604	13.5295	<0.0001*
D Trt	1	1	614.503	4.8559	0.0337*
D*Geno	5	5	148.467	0.2346	0.9448

(B)

R-Square	0.6919
Observations	50

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	12.984	1.180	7.7569	
Error	38	5.783	0.152	**Prob > F**	
Total	49	18.767		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	2.289	3.0089	<0.0220*
D Trt	1	1	9.024	59.3015	<0.0001*
D*Geno	5	5	0.689	0.9051	0.4879

Table 3.5. Lifespan; Generalized linear model (log-linked, Poisson) results of the heat experiment: Whole model results and effect likelihood ratio analysis. Genotype and heat (H) treatment significantly affected lifespan of *Arabidopsis thaliana*. A significant interaction effect was observed.

MODEL	**-LogLikelihood**	**df**	**Chi-Square**	**P>Chi-Square**
Difference	39.762	11	79.524	<0.0001*
Full	1215.494			
Reduced	1255.256			

STAT	**Chi-Square**	**df**	**P>Chi-Square**	**Overdispersion**
Pearson	5.1026	40	1.000	0.1276

	SOURCE	**df**	**L-R Chi-Square**	**P>Chi-Square**
	Genotype	5	37.539	<0.0001*
	H Treatment	1	31.427	<0.0001*
	H *Genotype	5	14.356	0.0135*

Table 3.6. Morphology; **(A)** height (log transformed) and **(B)** number of lateral branches; Generalized linear model (both datasets identity-linked for normal distribution) results of the heat experiment: Whole model results and effect likelihood ratio analysis. Genotype and heat (H) treatment affected the morphology of *Arabidopsis thaliana*. There was a significant interaction found for lateral branches produced.

(A)

MODEL	-LogLikelihood	df	Chi-Square	P>Chi-Square
Difference	38.236	11	76.4717	<0.0001*
Full	8.756			
Reduced	49.992			

STAT	Chi-Square	df	P>Chi-Square	Overdispersion
Pearson	4.264	40	1.000	0.0820

	SOURCE	df	L-R Chi-Square	P>Chi-Square
	Genotype	5	68.432	<0.0001*
	H Treatment	1	22.180	<0.0001*
	H*Geno	5	7.088	0.2142

(B)

MODEL	-LogLikelihood	df	Chi-Square	P>Chi-Square
Difference	23.819	11	47.6376	<0.0001*
Full	39.730			
Reduced	63.549			

STAT	Chi-Square	df	P>Chi-Square	Overdispersion
Pearson	14.033	40	1.000	0.2699

	SOURCE	df	L-R Chi-Square	P>Chi-Square
	Genotype	1	35.820	<0.0001*
	H Treatment	2	6.182	0.0129*
	H*Geno	2	13.626	0.0182*

Table 3.7. Fitness estimates **(A)** silique number and **(B)** total seed weight (log transformed); 2-way ANOVA results of the heat experiment: Whole model results and effect likelihood ratio analysis. Genotype and heat (H) treatment significantly affected the amount of siliques produced by *Arabidopsis thaliana*. There was a significant interaction effect found for siliques produced.

(A)

R-Square	0.7585
Observations	52

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	11052.603	1004.78	11.4207	
Error	40	3519.167	87.98	**Prob > F**	
Total	51	14571.769		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	8071.142	18.3478	<0.0001*
H Trt	1	1	1555.642	17.6819	<0.0001*
H*Geno	5	5	1494.494	3.3974	0.0019*

(B)

R-Square	0.7731
Observations	52

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	18.838	1.713	12.3923	
Error	40	5.528	0.138	**Prob > F**	
Total	51	24.366		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	2.342	3.3892	<0.0120*
H Trt	1	1	17.126	123.9224	<0.0001*
H*Geno	5	5	0.439	0.6355	0.6738

Table 3.8. Lifespan; Generalized linear model (log-linked, Poisson) results of the heat/drought experiment: Whole model results and effect likelihood ratio analysis. Genotype and heat/drought (HD) treatment significantly affected lifespan of *Arabidopsis thaliana*. There was no significant interaction effect found.

MODEL	**-LogLikelihood**	**df**	**Chi-Square**	**P>Chi-Square**
Difference	20.952	11	41.9046	<0.0001*
Full	-81.884			
Reduced	-60.932			

STAT	**Chi-Square**	**df**	**P>Chi-Square**	**Overdispersion**
Pearson	0.1640	43	1.000	0.0030

	SOURCE	**df**	**L-R Chi-Square**	**P>Chi-Square**
	Genotype	5	18.378	0.0025*
	HD Treatment	1	23.335	<0.0001*
	HD*Genotype	5	10.330	0.0664

Table 3.9. Height; 2-way ANOVA results of the heat/drought experiment: Whole model results and effect likelihood ratio analysis. Genotype and heat (HD) treatment significantly affected the height of *Arabidopsis thaliana.* There were significant interaction effects observed.

R-Square	0.7902
Observations	55

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	3854.322	350.393	14.7227	
Error	43	1023.377	23.799	**Prob > F**	
Total	54	4877.699		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	3217.658	27.0398	<0.0001*
HD Trt	1	1	224.452	9.4310	0.0337*
HD*Geno	5	5	458.911	3.8565	0.0056*

Table 3.10. Fitness estimates **(A)** silique number and **(B)** total seed weight (log transformed); 2-way ANOVA results of the heat/drought experiment: Whole model results and effect likelihood ratio analysis. Genotype and heat/drought (HD) treatment significantly affected the amount of siliques produced by *Arabidopsis thaliana*. There was a significant interaction found for siliques produced.

(A)

R-Square	0.7328
Observations	55

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	10122.632	920.239	10.6637	
Error	43	2710.750	86.297	**Prob > F**	
Total	54	13833.382		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	6151.699	14.2571	<0.0001*
HD Trt	1	1	2903.706	33.6480	<0.0001*
HD*Geno	5	5	1361.648	3.1557	0.0163*

(B)

R-Square	0.7378
Observations	55

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	16.342	1.486	11.0038	
Error	43	5.806	0.1350	**Prob > F**	
Total	54	22.148		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	2.329	3.4505	<0.0104*
HD Trt	1	1	13.251	98.1466	<0.0001*
HD*Geno	5	5	0.441	0.6520	0.6615

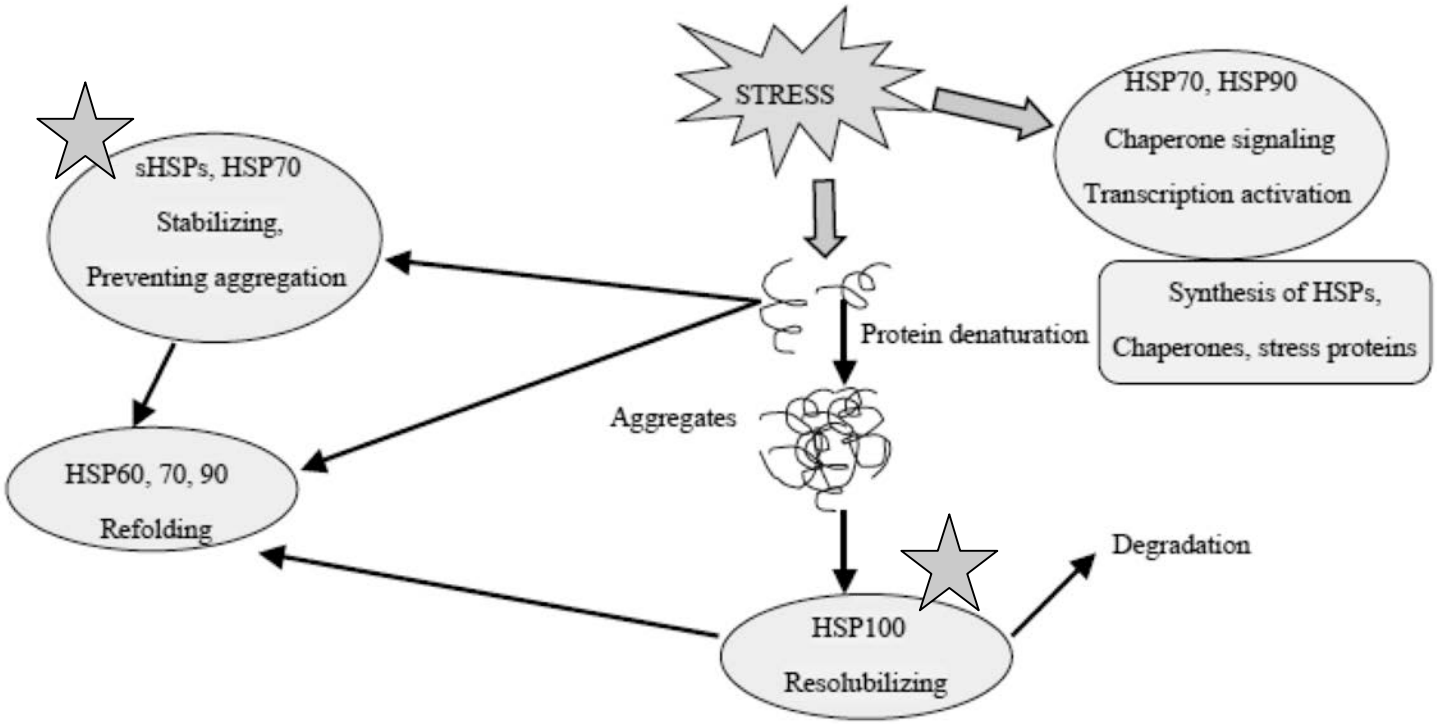

Figure 3.1. Heat-shock protein (HSP) network during abiotic stress response. HSP 17.6 (sHsp family) and HSP 101 (Hsp100 family) and their roles are starred. Abiotic stress denatures proteins and can form aggregates. HSPs at all levels work to prevent aggregation, refold and resolubilize proteins, or degrade irreparably damaged proteins (illustration modified from Wang et al., 2004).

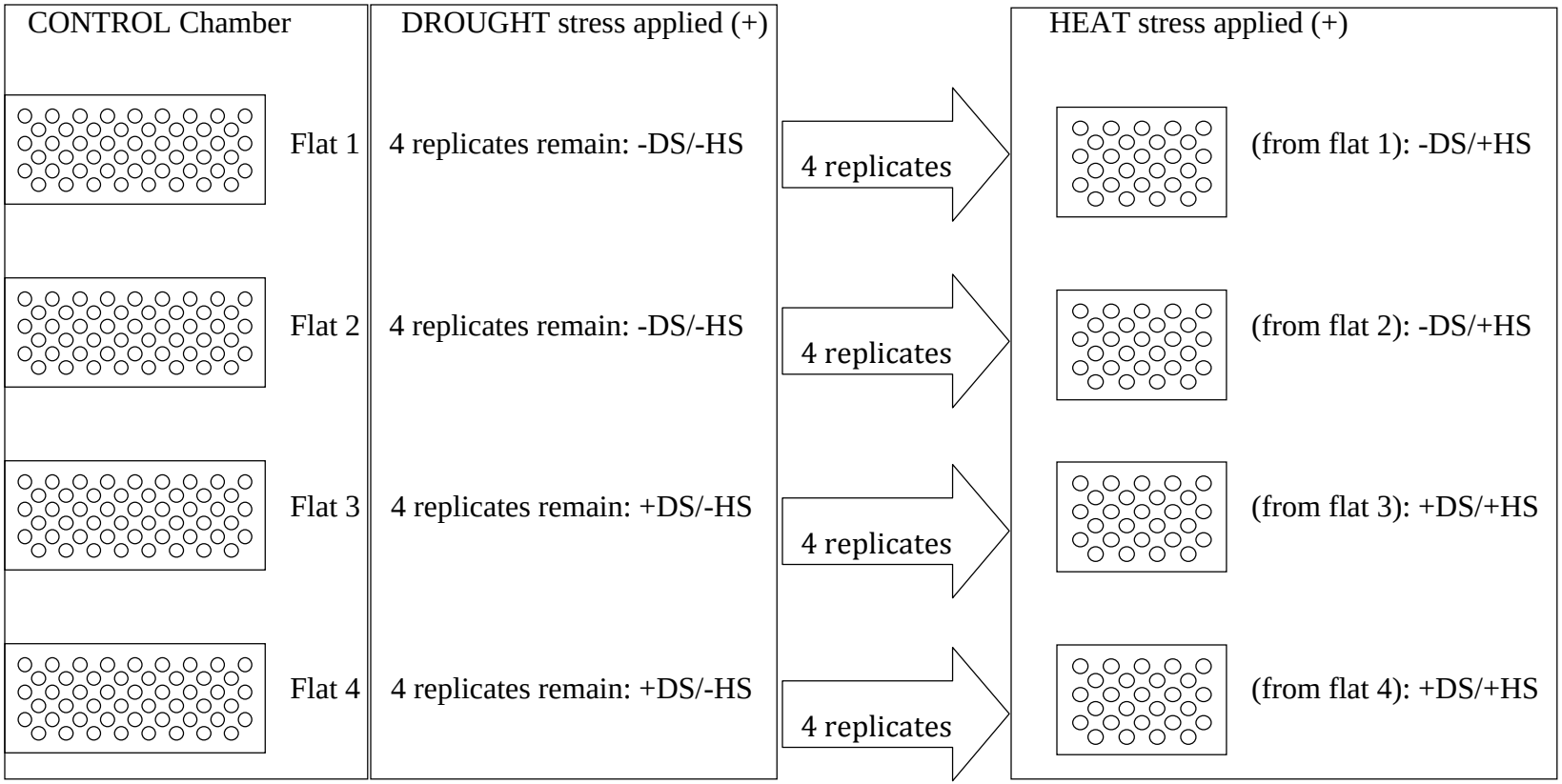

Figure 3.2. Experimental design of stress experiment: 32 reps of six genotypes (192 plants total; 48 plugs per flat) germinated in control chamber. Three weeks past germination, drought stress was applied to two flats (in control chamber). One week later, four random reps of each genotype from each flat (96 plants) were transferred to hot chamber for six hours and then either collected or returned to CONTROL.

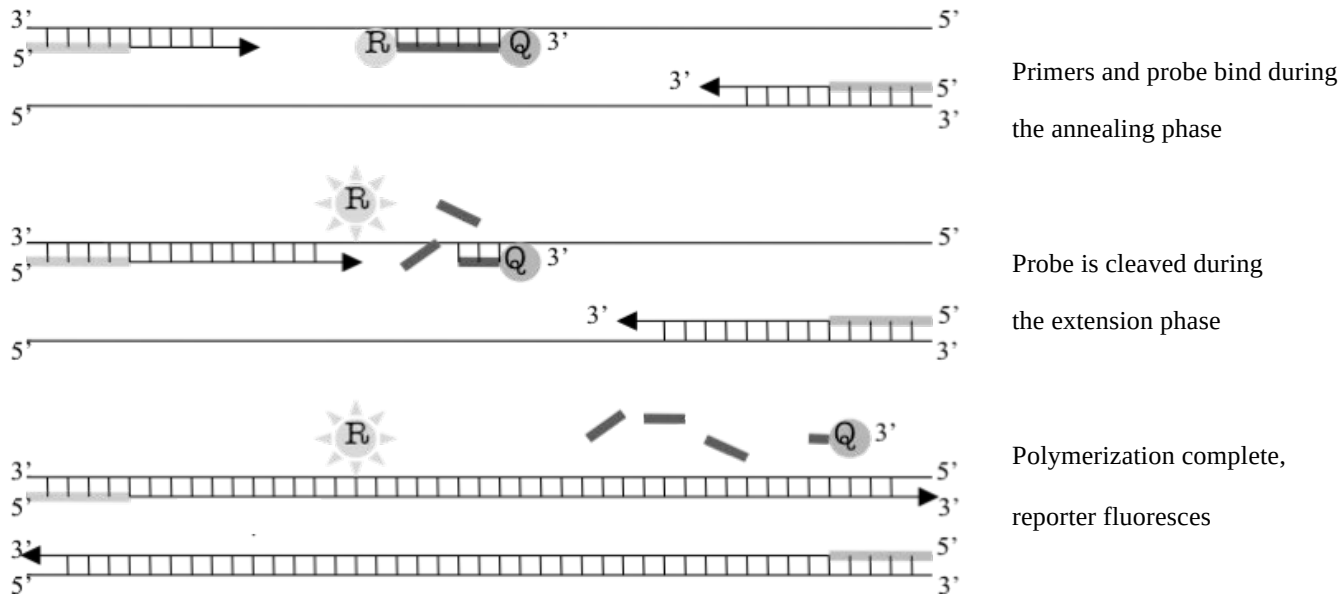

Figure 3.3. As in conventional PCR, temperatures increase to denature the cDNA and two primers attach to the 3' ends of the gene of interest on both the sense and anti-sense strand. In TaqMan reactions, a fluorescent DNA probe anneals inside the gene, between the forward and reverse primers. This probe contains both a fluorescent reporter (R) and a quencher molecule (Q) in close proximity; the quencher prevents any detectable fluorescence to be emitted. Taq DNA polymerase, the enzyme responsible for extending the primers and amplifying the gene of interest has 5' → 3' exonuclease activity; as Taq polymerase approaches the probe, it cleaves the molecule and separates the quencher from the fluorescent reporter. The subsequent increase in fluorescence is proportional to the amount of transcript present (Bustin, 2000; VanGuilder et al., 2008). This primer/probe technology increases accuracy and specificity of the PCR product because three (forward primer, reverse primer, probe) independent nucleotide sequences must match (Wang and Brown, 1999, Bustin, 2000).

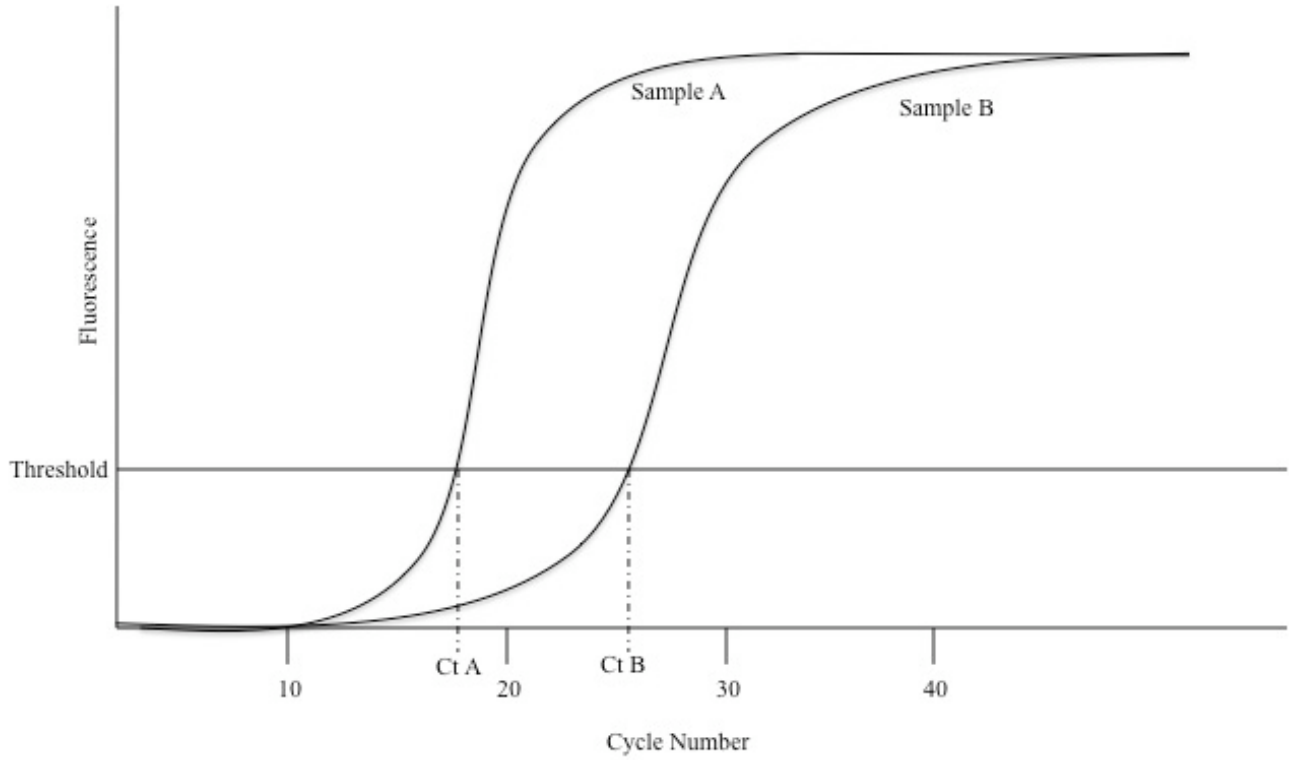

Figure 3.4. In Real-time quantitative PCR, fluorescence is plotted on the Y axis against the number of PCR cycles on the X axis. A threshold fluorescence level is set just above background levels. The number of cycles at which a sample's fluorescence crosses that threshold is defined as the cycle threshold, or Ct. The difference of Ct levels between samples indicate how much DNA is in the sample. Low Ct values represent a larger amount of DNA (takes quicker to fluoresce) and a higher number represents a smaller amount (takes a longer time to fluoresce).

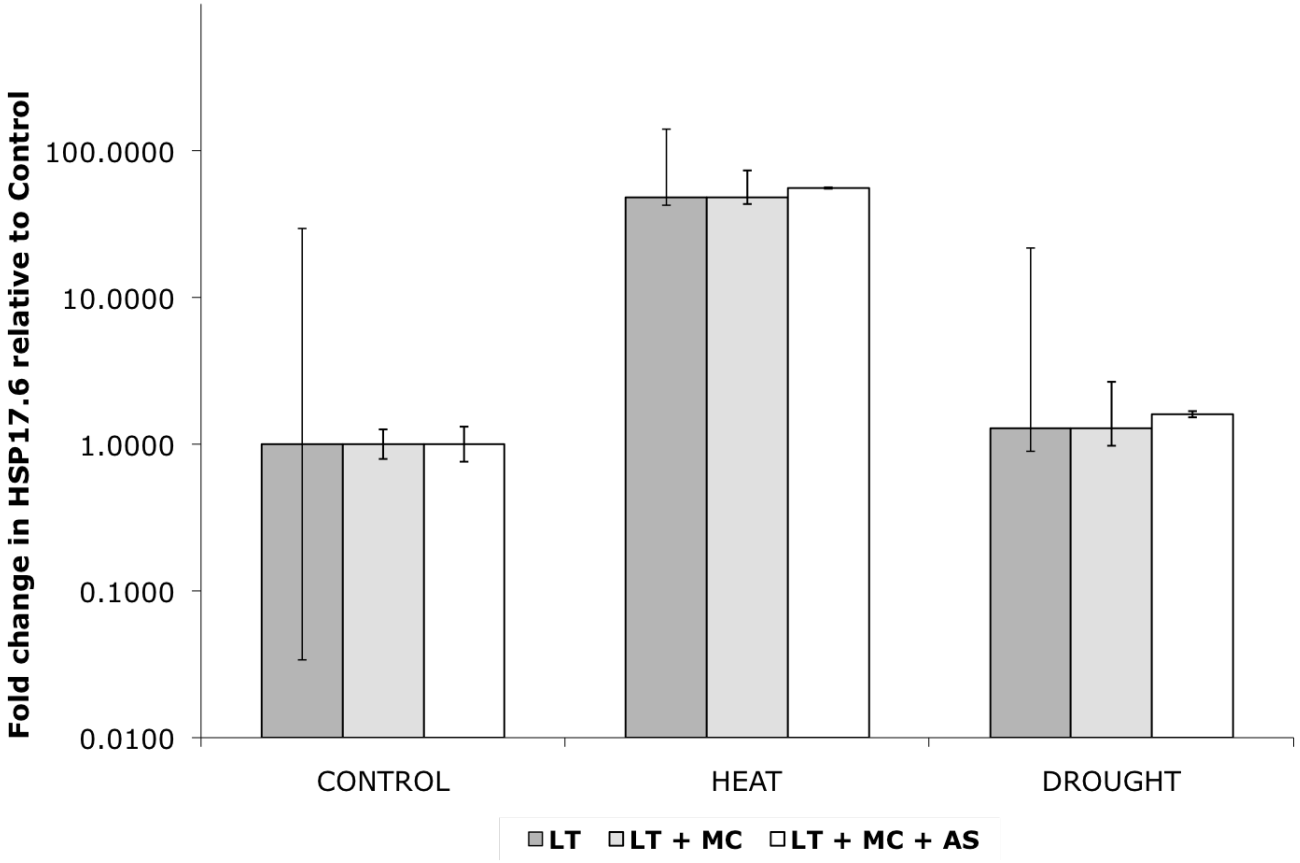

Figure 3.5. Results of data transformation for HSP17.6 induction in R-France genotype in heat and drought stress. Each sequential transformation (LT - log, MC - mean-centering, and AS - autoscaling) minimizes variance yet maintains relationship and overall value. For dataset on which this graph is based, see Table 3.2. (Modified from Willems et al., 2008).

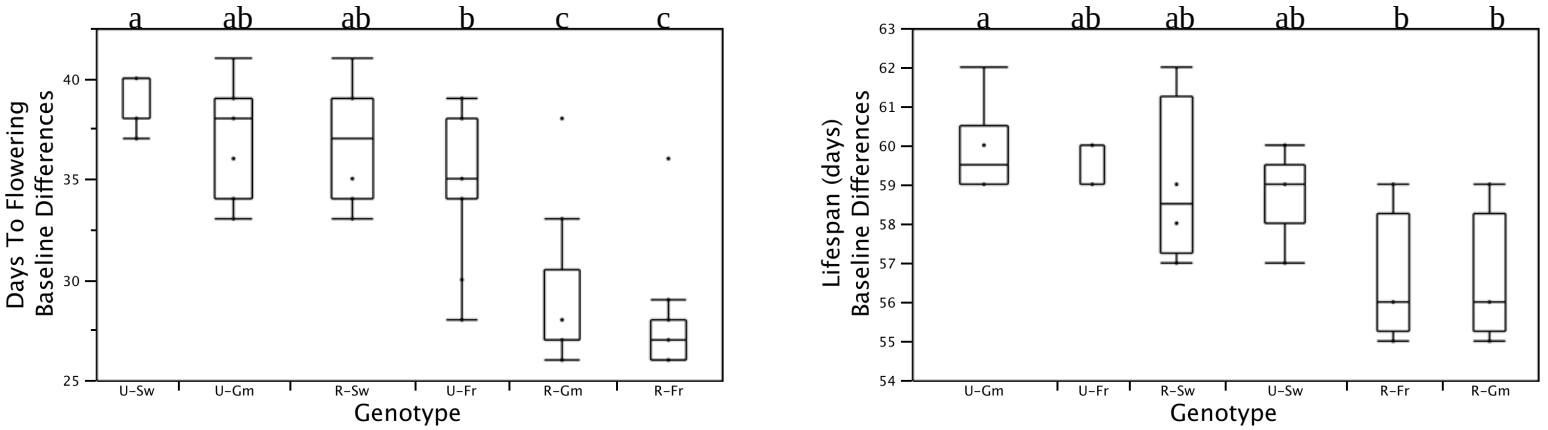

Figure 3.6. Phenology results of one-way Analysis of Variance and means comparisons of baseline differences among genotypes. Genotypes R-Germany and R-France flowered significantly earlier ($F_{(5,94)}$= 31.5611; p<0.0001) and had significantly shorter lifespans ($F_{(5,20)}$= 4.1577; p=0.0094) than the other genotypes. Genotypes not connected by the same letter are significantly different (Tukey-Kramer HSD (α=0.05)).

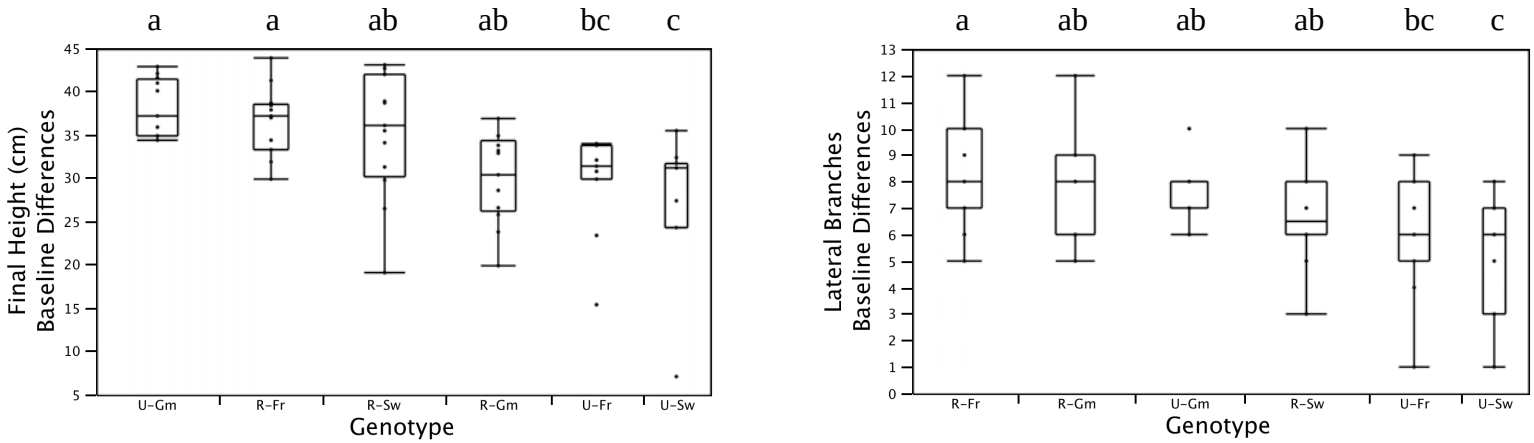

Figure 3.7. Morphology results of one-way Analysis of Variance and means comparisons of baseline differences among genotypes. Genotypes U-Germany and R-France grew significantly taller than U-France and U-Sweden ($F_{(5,94)}$= 9.1800; $p<0.0001$). R-France genotypes produced significantly more lateral branches than U-France and U-Sweden ($F_{(5,94)}$= 4.8694; $p=0.0005$). Genotypes not connected by the same letter are significantly different (Tukey-Kramer HSD ($\alpha=0.05$)).

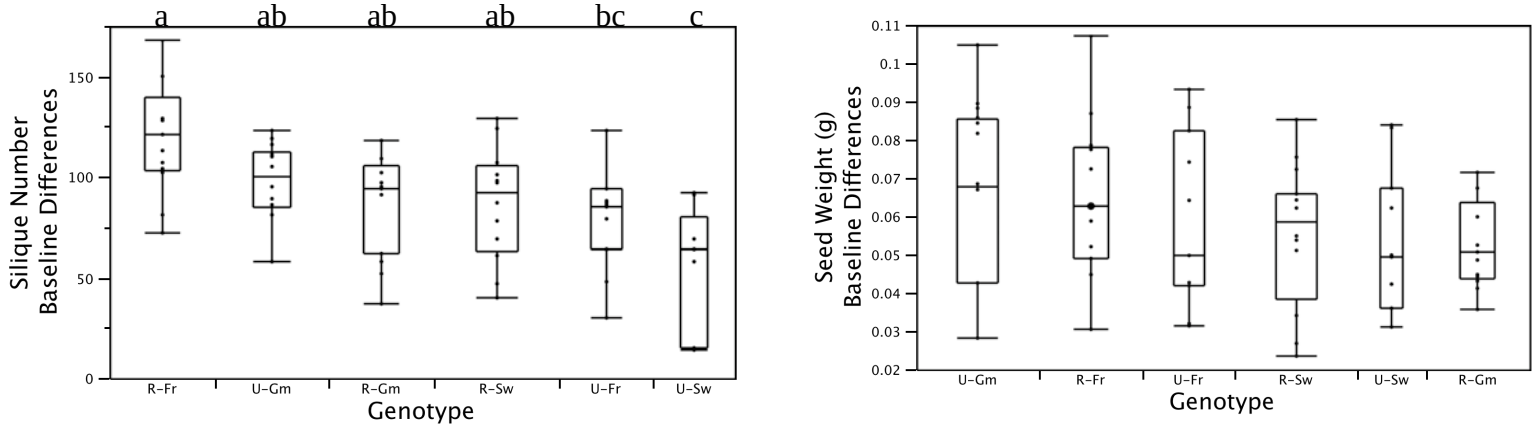

Figure 3.8. Fitness results of one-way Analysis of Variance and means comparisons of baseline differences among genotypes. For silique number, R-France and U-Germany display significantly higher values than U-Sweden (Silique number: $F_{(5,94)}= 9.0202$; $p<0.0001$). There were no significant differences found in baseline seed weight. Genotypes not connected by the same letter are significantly different (Tukey-Kramer HSD ($\alpha=0.05$)).

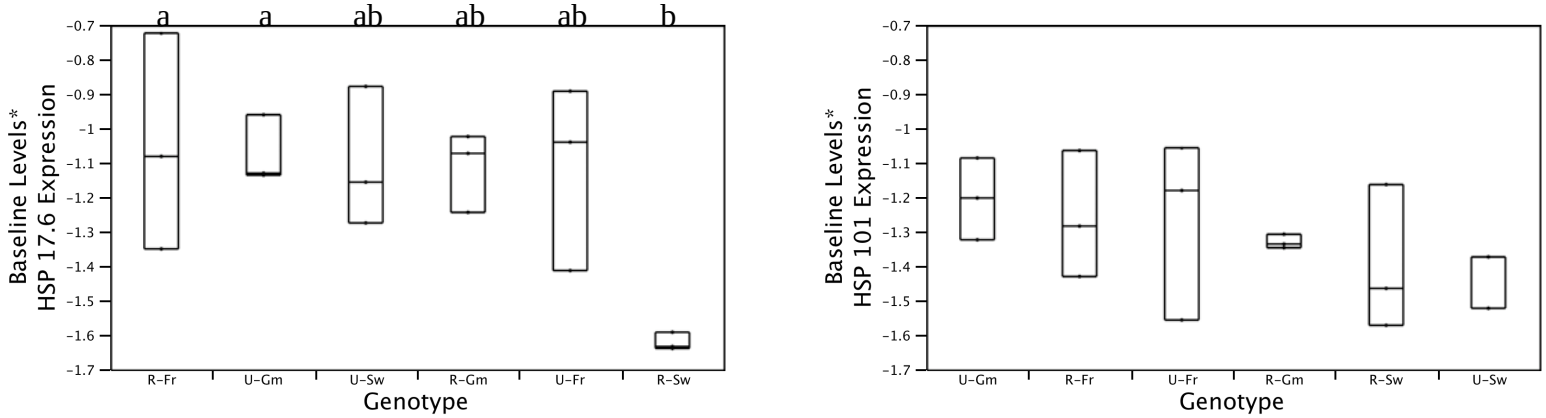

Figure 3.9. Constitutive levels of gene expression for HSP17.6 and HSP101 across genotype. There was variation found in constitutive levels of HSP17.6. R-France and U-Germany genotypes expressed significantly higher levels of the gene than R-Sweden ($F_{(5,12)}$= 3.6094; p=0.0318). Genotypes not connected by the same letter are significantly different (Tukey-Kramer HSD (α=0.05)). While slight variation in HSP101 expression was present, there were no significant differences found in control plants. *Data presented are transformed as described in Methods.

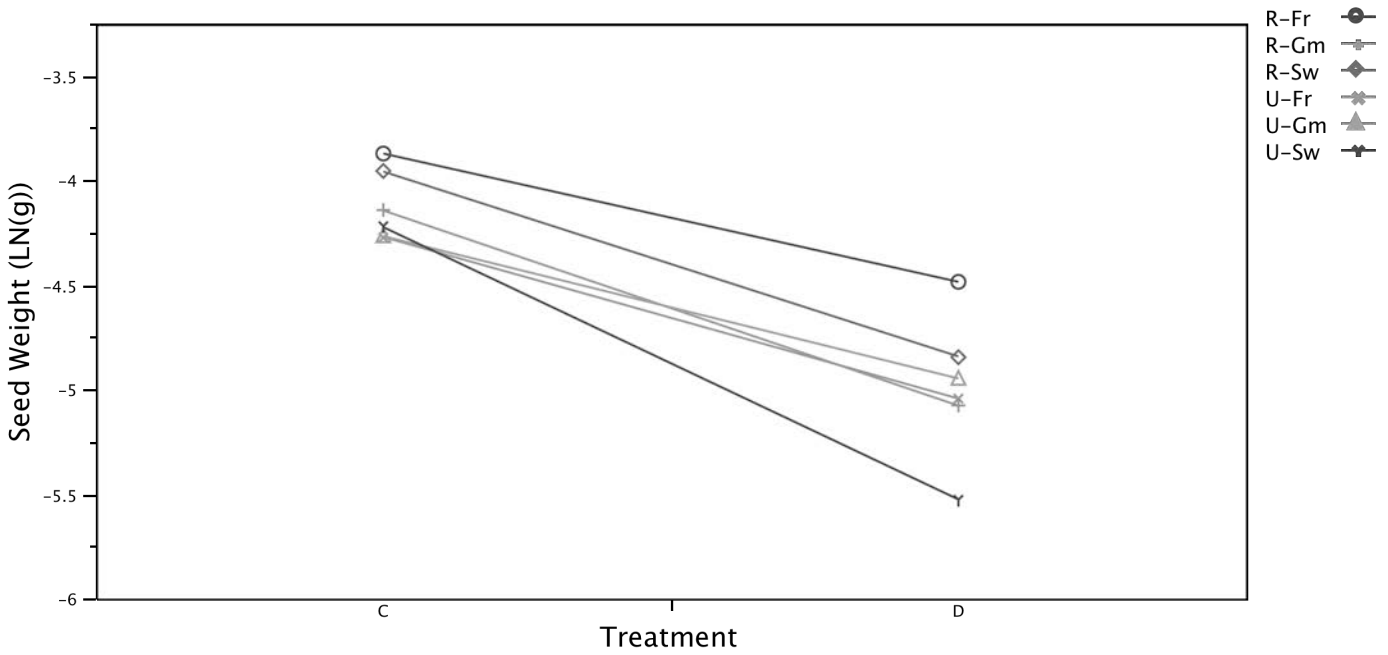

Figure 3.10. Reaction Norms of seed weight (LN(g)) in six genotypes exposed to drought stress. There was no difference between the genotypes in control conditions, but in drought stress, R-France and R-Sweden had significantly higher seed weights than U-Sweden ($F_{(5,38)}$= 3.0089; p=0.0220). Values plotted are least-squares means of raw data.

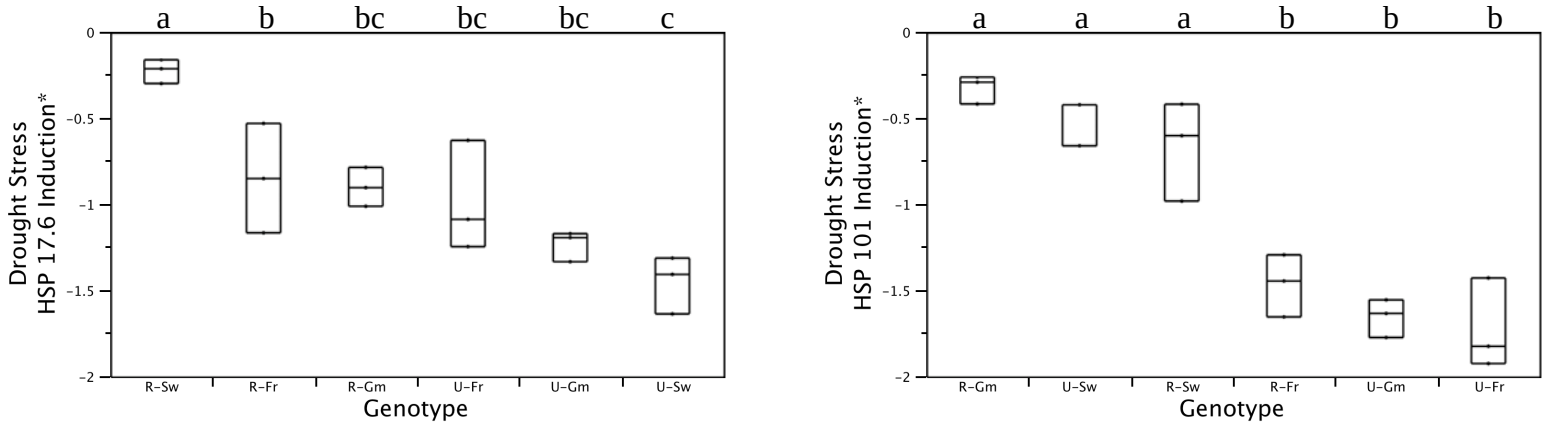

Figure 3.11. Induced levels of HSP17.6 and HSP101 in drought stress. In both cases, R-Sweden had significantly higher induction values than U-Germany (HSP17.6: $F_{(5,12)}= 12.2279$; $p=0.0002$; HSP101: $F_{(5,12)}= 30.5441$; $p<0.0001$). Genotypes not connected by the same letter are significantly different (Tukey-Kramer HSD ($\alpha=0.05$)). *Induction data presented are transformed as described in Methods.

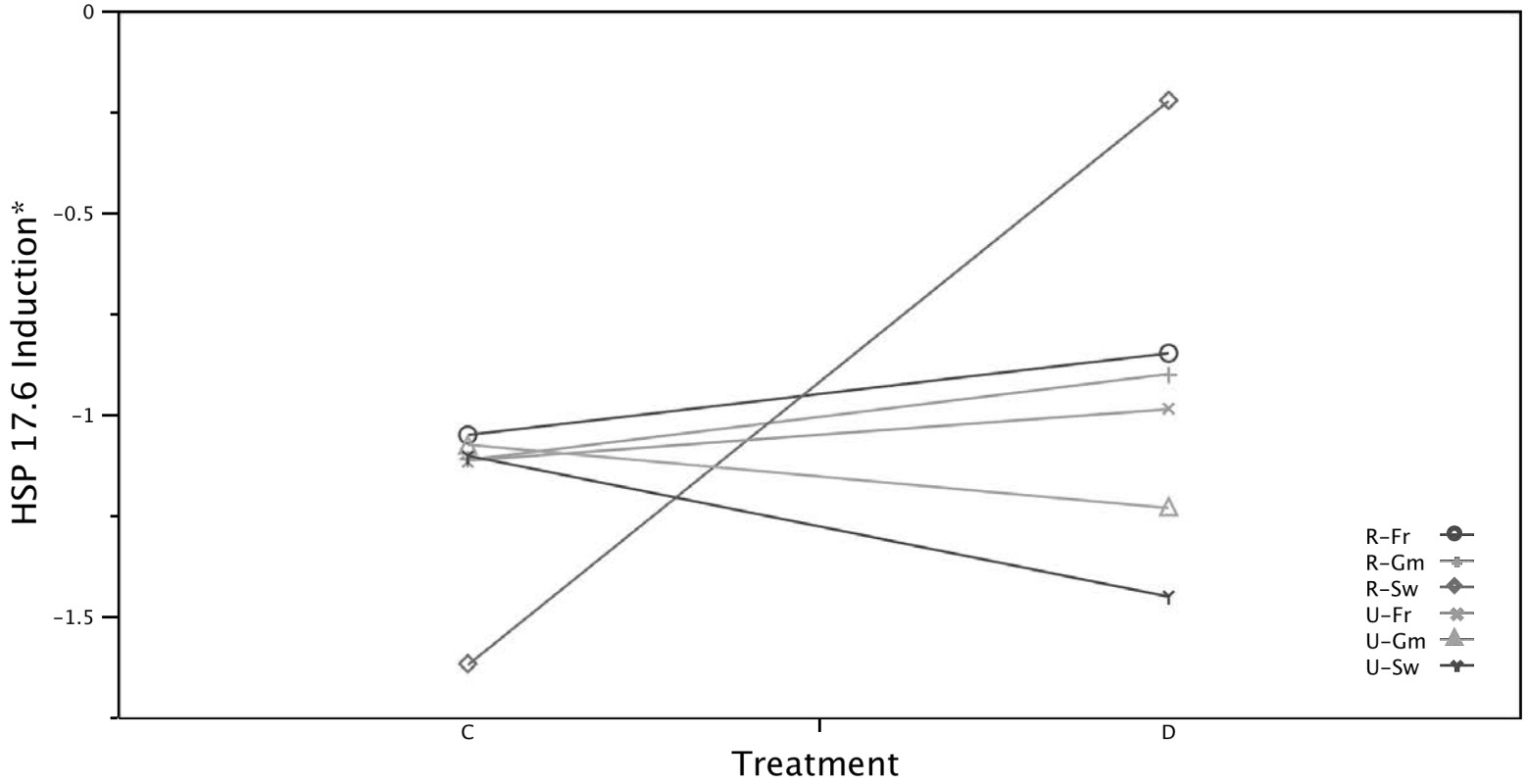

Figure 3.12. Differential induction of HSP17.6 in drought stress. Two way ANOVA effect test results: genotype ($F_{(5,24)}$= 2.6260; p=0.0496); treatment ($F_{(1,24)}$= 12.5121; p=0.0017); genotype x treatment ($F_{(5,24)}$= 13.5710; p<0.0001). *Induction data presented are transformed as described in Methods.

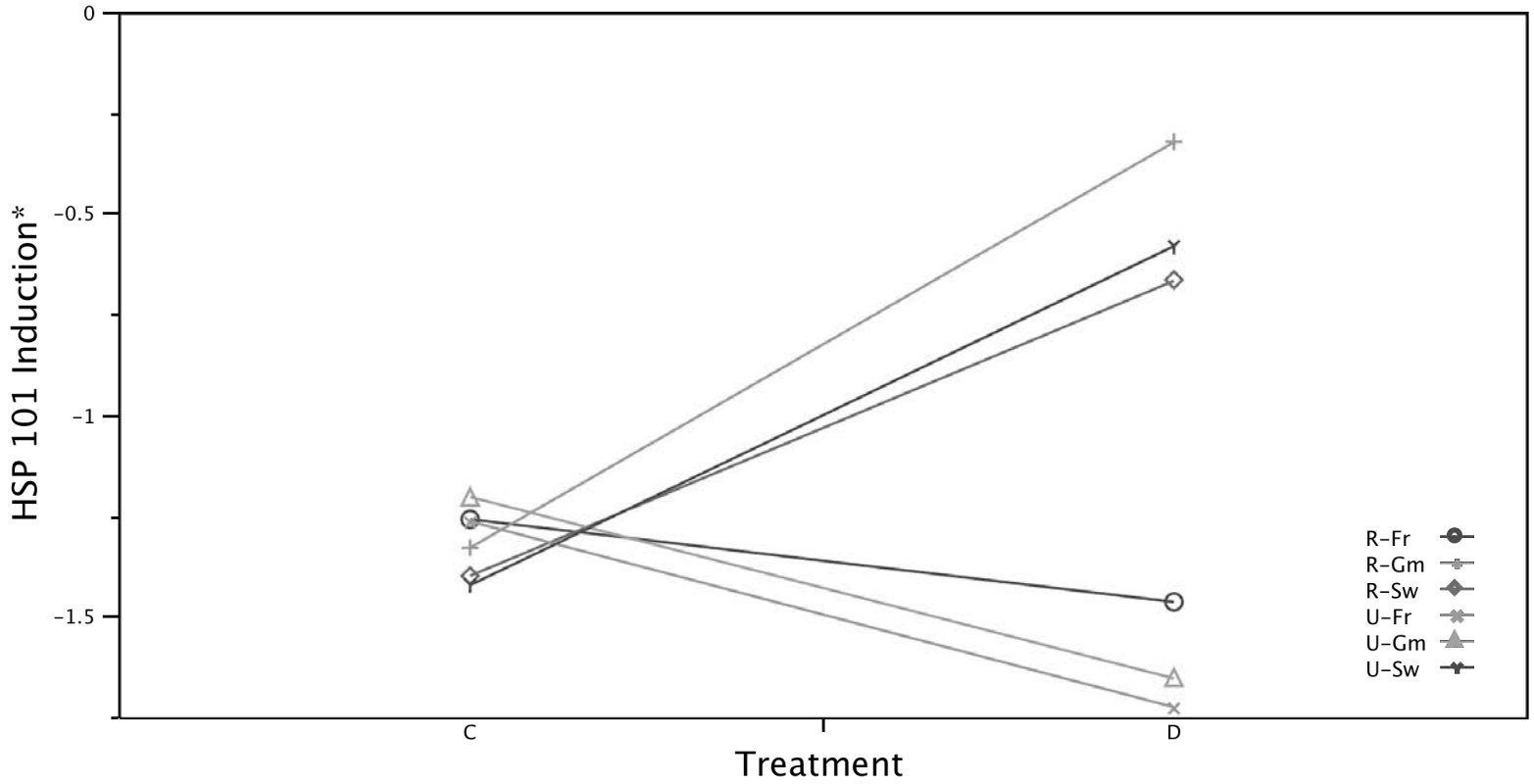

Figure 3.13. Differential induction of HSP101 in drought stress. Two way ANOVA effect test results: genotype ($F_{(5,24)}$= 13.6925; $p<0.0001$); treatment ($F_{(1,24)}$= 16.4511; $p=0.0005$); genotype x treatment ($F_{(5,24)}$= 21.7321; $p<0.0001$). *Induction data presented are transformed as described in Methods.

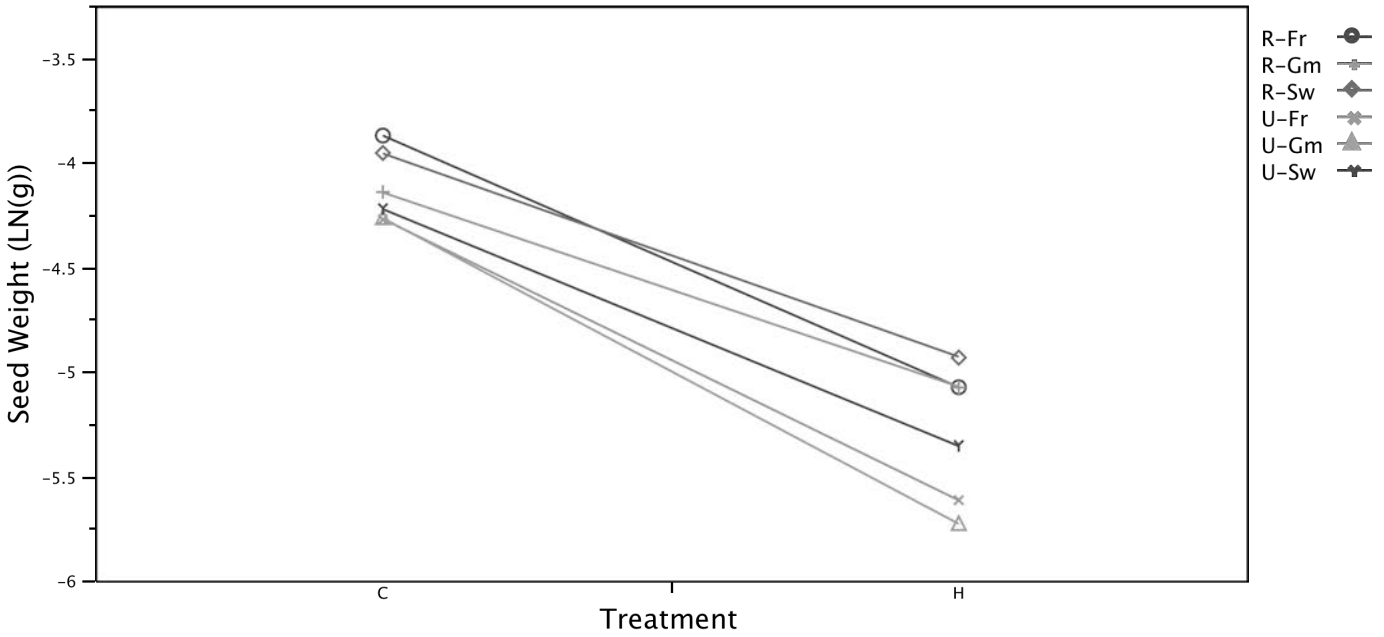

Figure 3.14. Reaction Norms of seed weight (LN(g)) in six genotypes exposed to heat stress. There was no difference between the genotypes in control conditions, but in heat stress, all rural strains produced significantly higher seed weights than U-France and U-Germany ($F_{(5,20)}$= 7.0946; p=0.0006). Values plotted are least-squares means of raw data.

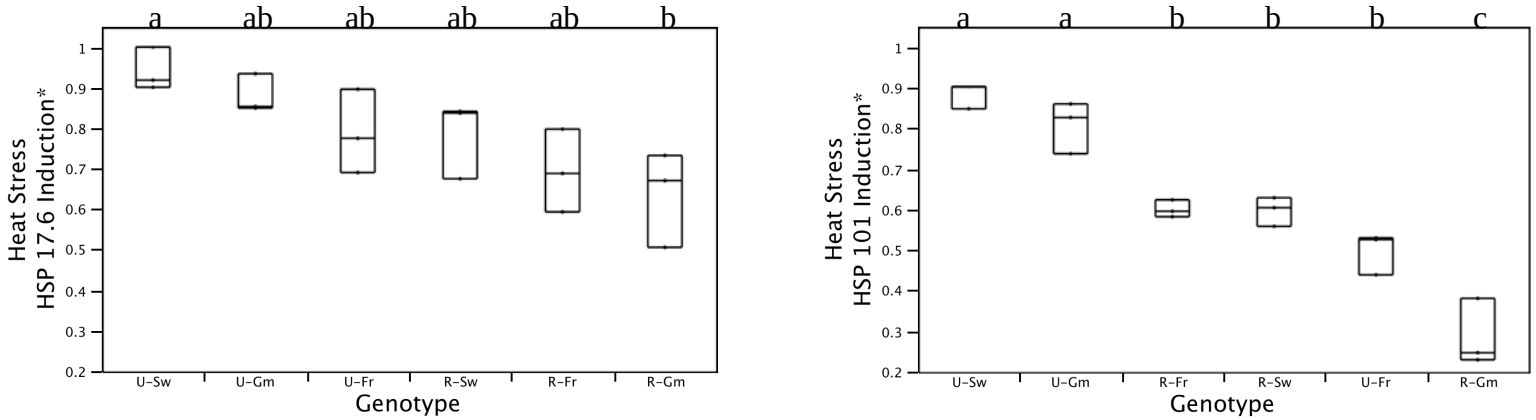

Figure 3.15. Induced levels of HSP17.6 and HSP101 in heat stress. In both cases U-Sweden had significantly stronger HSP induction in heat stress than R-Germany (HSP17.6: $F_{(5,12)}= 4.6611$; $p=0.0135$; HSP101: $F_{(5,12)}= 51.5379$; $p<0.0001$). Genotypes not connected by the same letter are significantly different (Tukey-Kramer HSD ($\alpha=0.05$)). *Induction data presented are transformed as described in Methods.

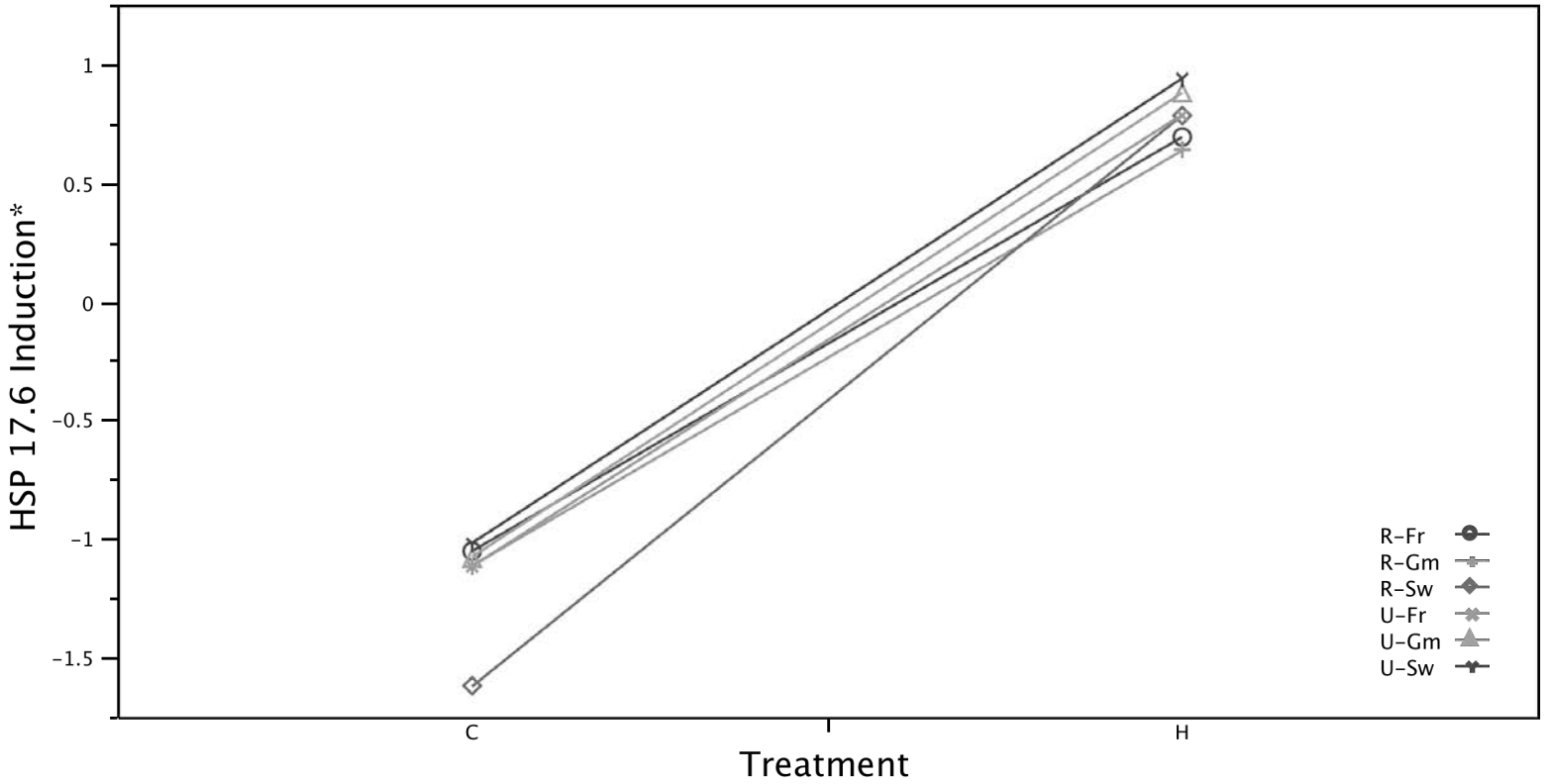

Figure 3.16. Differential induction of HSP17.6 in heat stress. Two way ANOVA effect test results: genotype ($F_{(5,24)}$= 3.7999; p=0.0112); treatment ($F_{(1,24)}$= 1464.557; p<0.0001); genotype x treatment ($F_{(5,24)}$= 3.7801; p=0.0115). *Induction data presented are transformed as described in Methods.

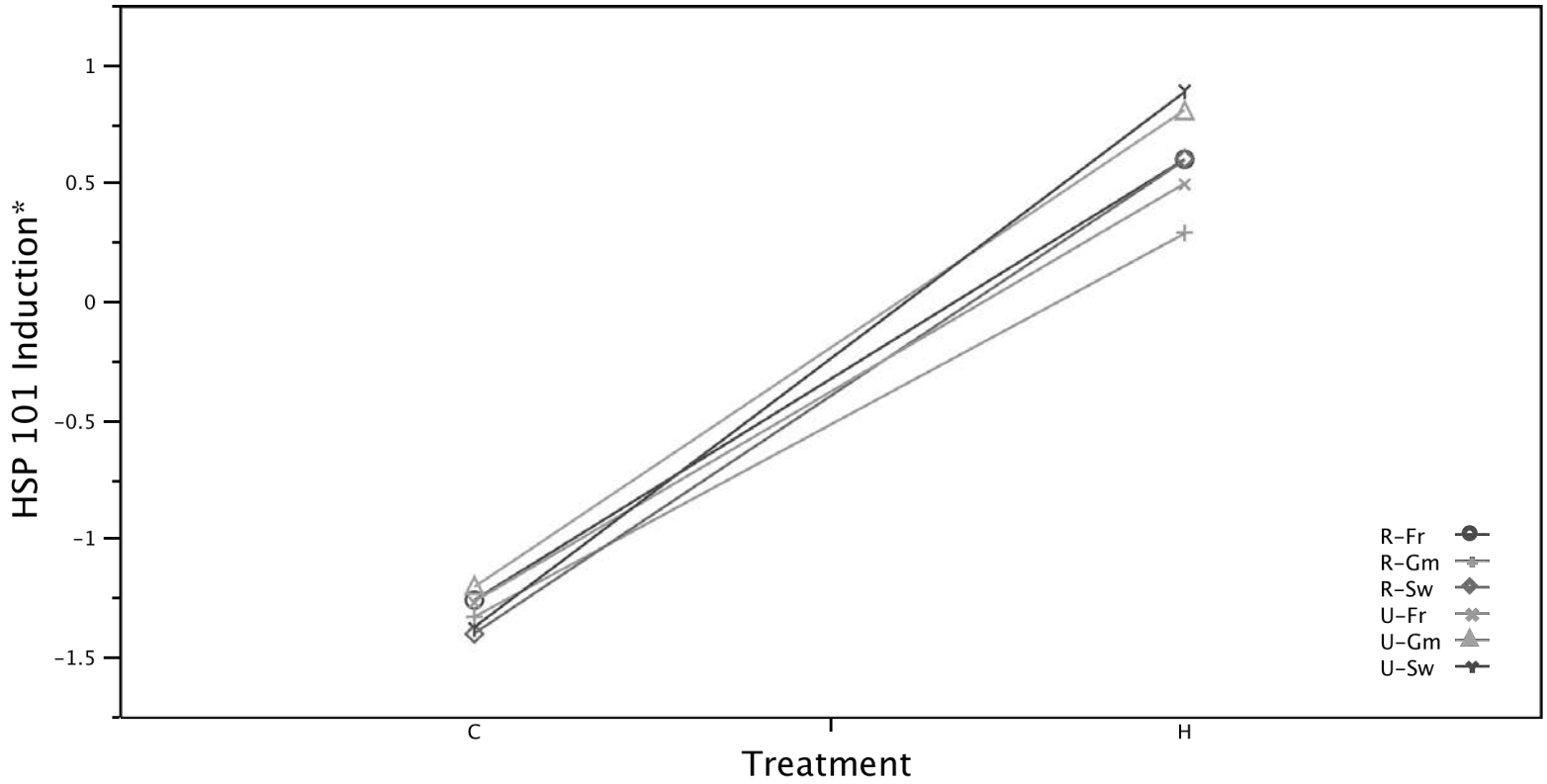

Figure 3.17. Differential induction of HSP101 in heat stress. Two way ANOVA effect test results: genotype ($F_{(5,24)}$= 4.9264; p=0.0030); treatment ($F_{(1,24)}$= 2167.292; p<0.0001); genotype x treatment ($F_{(5,24)}$= 5.5447; p=0.0016). *Induction data presented are transformed as described in Methods.

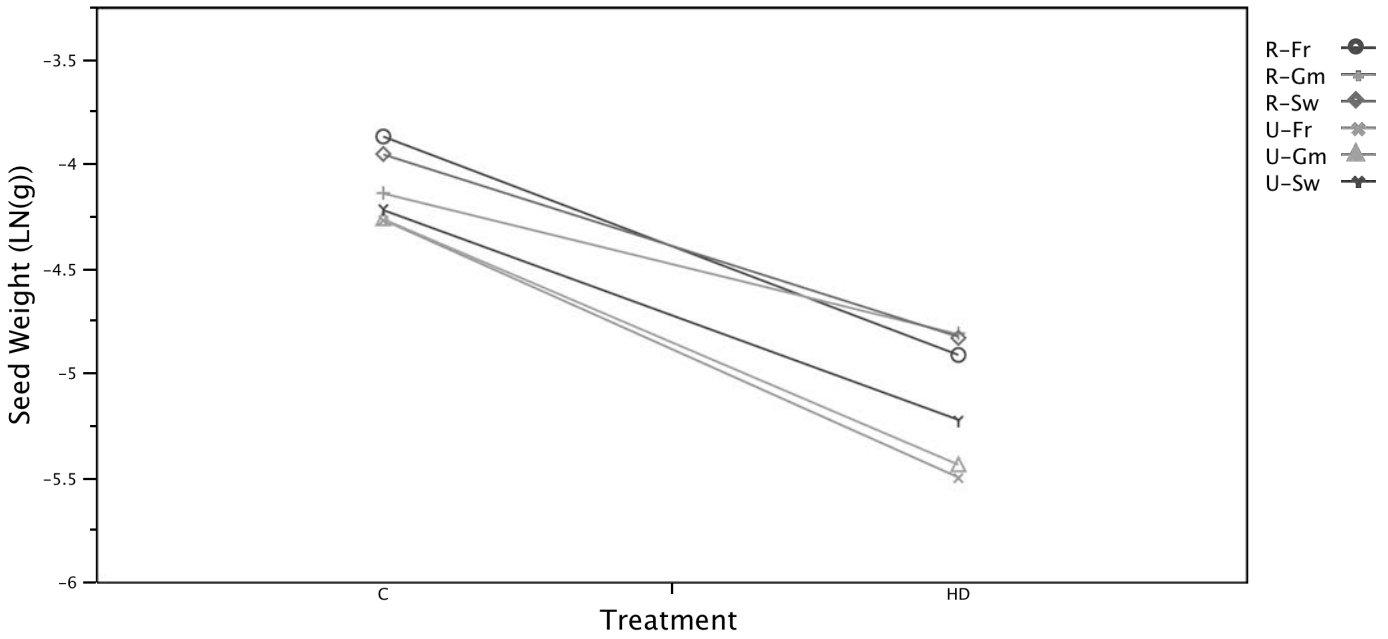

Figure 3.18. Reaction Norms of seed weight (LN(g)) in six genotypes exposed to heat/drought stress. There was no difference between the genotypes in control conditions, but in heat/drought stress, all rural genotypes had significantly higher seed weights than U-Germany and U-France ($F_{(5,43)}$= 3.4505; p=0.0104). Values plotted are least-squares means of raw data.

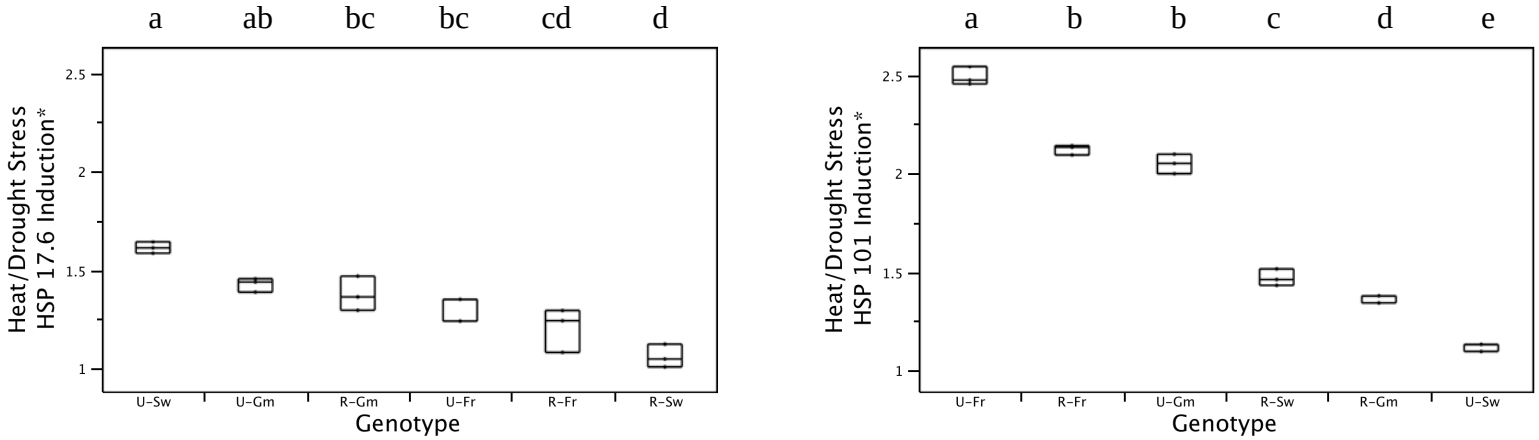

Figure 3.19. Induced levels of HSP17.6 and HSP101 in heat/drought stress. In HSP17.6, U-Sweden and U-Germany had significantly higher induction levels than R-France and R-Sweden ($F_{(5,12)}$= 22.0606; $p<0.0001$). For HSP101, U-France displayed the strongest induction in heat/drought stress, whereas U-Sweden displayed the weakest ($F_{(5,12)}$= 639.2536; $p<0.0001$). Genotypes not connected by the same letter are significantly different (Tukey-Kramer HSD (α=0.05)). *Induction data presented are transformed as described in Methods.

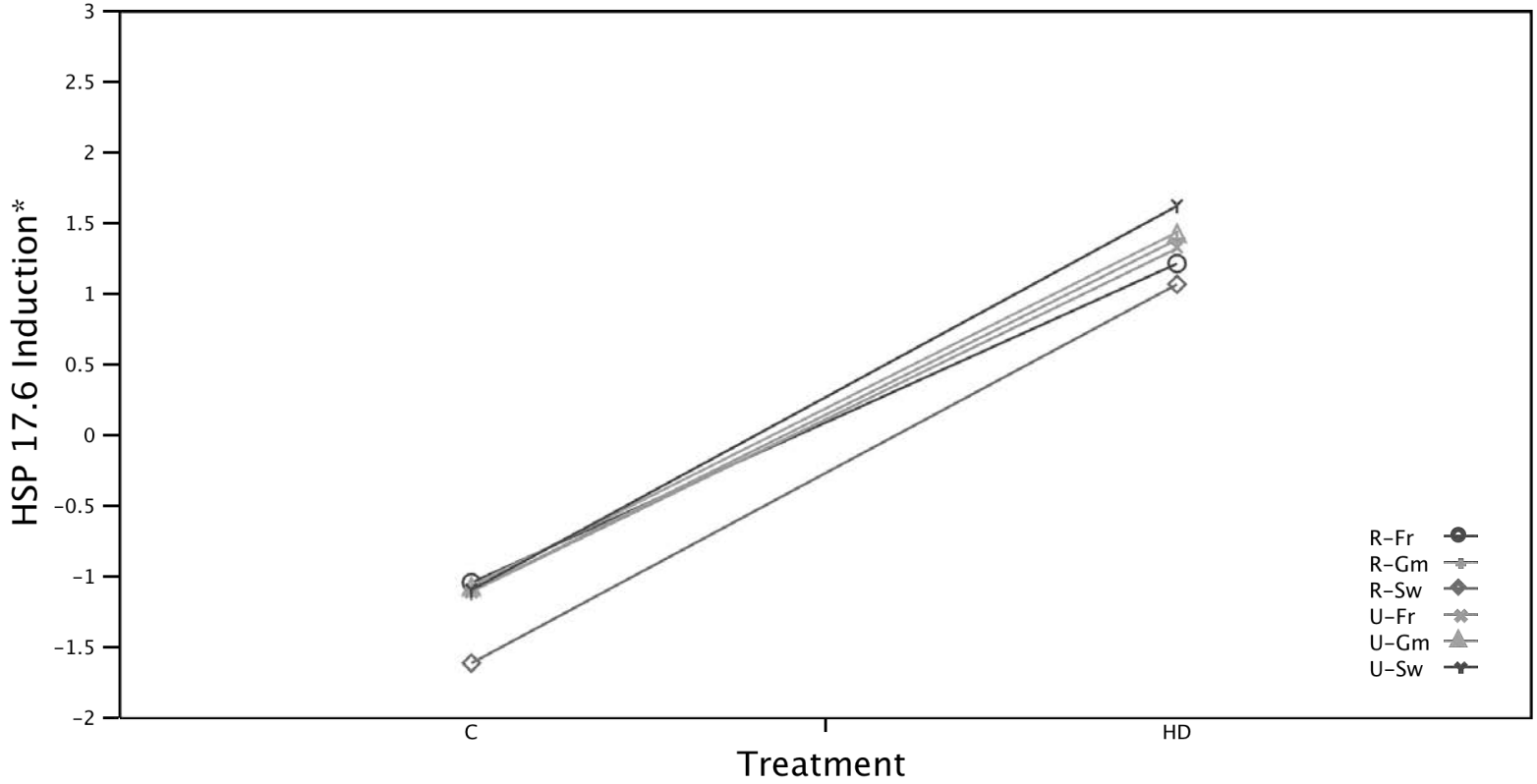

Figure 3.20. Differential induction of HSP17.6 in heat/drought stress. Two way ANOVA effect test results: genotype ($F_{(5,24)}$= 9.3838; p<0.0001); treatment ($F_{(1,24)}$= 2565.209; p<0.0001); genotype x treatment ($F_{(5,24)}$= 1.9325; p=0.1261). *Induction data presented are transformed as described in Methods.

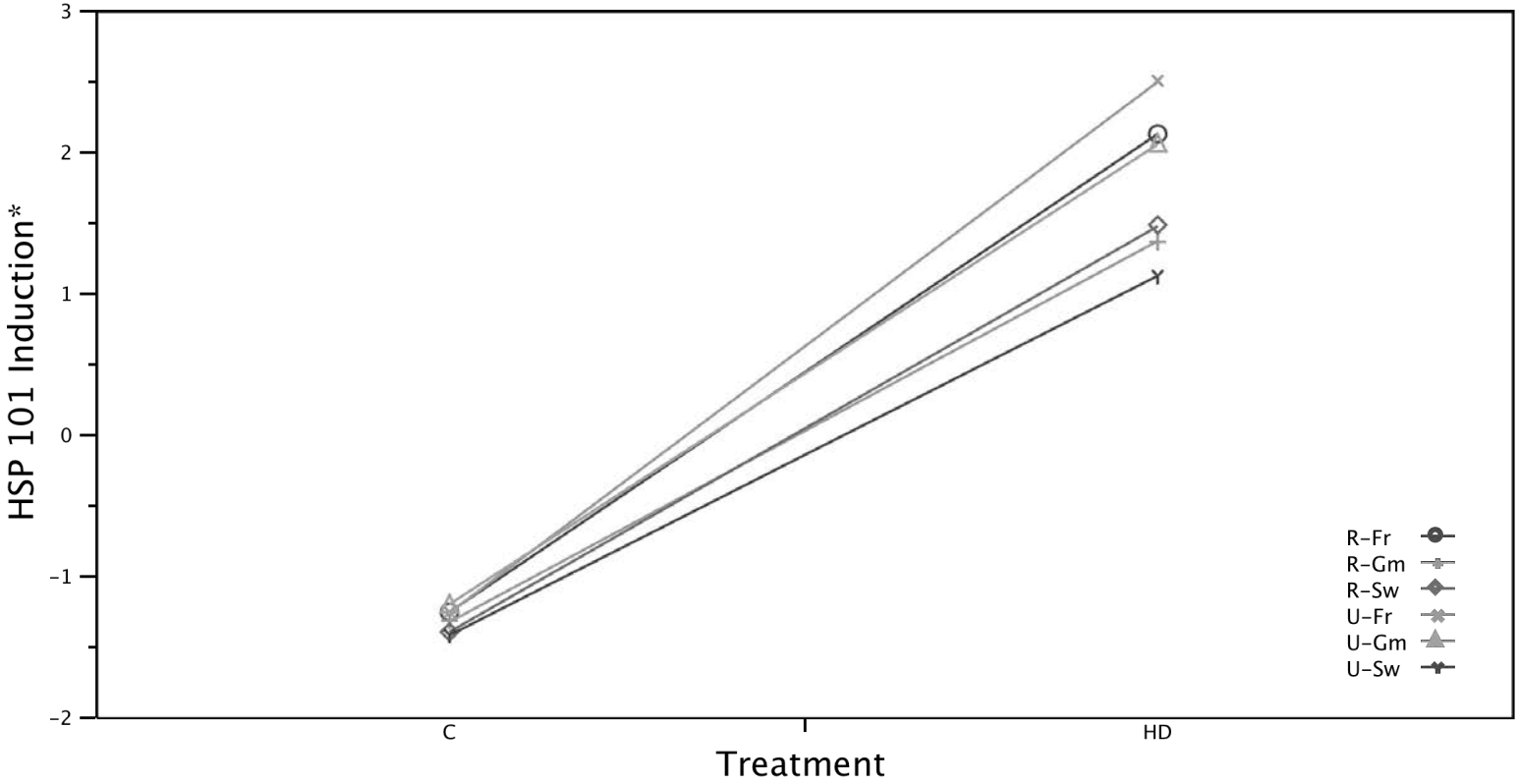

Figure 3.21. Differential induction of HSP101 in heat/drought stress. Two way ANOVA effect test results: genotype ($F_{(5,24)}$= 37.0397; $p<0.0001$); treatment ($F_{(1,24)}$= 5819.459; $p<0.0001$); genotype x treatment ($F_{(5,24)}$= 21.6106; $p<0.0001$). *Induction data presented are transformed as described in Methods.

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CHAPTER IV

ANALYSIS OF STRESS PERFORMANCE GENOTYPE SCREENING IN *ARABIDOPSIS THALIANA* AND ITS ROLE IN ECOLOGICAL RESTORATION

ABSTRACT

There is a need for simpler, less-demanding plant genotype screening methods for ecological restoration purposes. The effort to restore human-influenced land is timely and essential, given the unprecedented increase in urbanization. Highly stressful and constantly changing environments should be restored with stress-resistant and flexible plant genotypes, but little attention is paid to the stress tolerance of plants slated for urban restoration. Genetic screening procedures for stress tolerance are thought to be cost-prohibitive, unreliable and difficult, so these techniques are often ignored. Since a failure to intervene could be devastating, there is a critical need for more practical genotype screening. The work described in this chapter provides a straightforward and cost-effective method of performance-based genotype screening. I previously identified "stress-resistant" genotypes, which consistently performed best across various stress treatments. Here, I tested whether the same genotypes exhibited higher fitness under novel stress conditions. I performed a controlled salt and heat/salt stress experiment and a germination trial and fitness analysis of plants growing in urban restoration site soils in which levels of heterogeneity and types of stressors were unknown. In all cases, the two previously identified genotypes performed significantly better than four others studied. I conclude that simple preliminary stress tests can provide a reasonable and quick method of genotype selection, especially for practitioners restoring urban and degraded land.

INTRODUCTION

Ecological restoration in the face of urbanization

For the first time in history, more than half of the global human population lives in urban areas (UNFPA, 2007; van Ginkel, 2008). Urbanization has been positively correlated with increased ecological disturbance (Pyke and Knick, 2005), enhanced spatial heterogeneity (Zipperer et al., 2000; Cadenasso et al., 2007) and the existence of multiple biotic and abiotic stressors (e.g., Alberti, 2005; Williams et al., 2008). This demographic shift affects both natural habitats surrounding cities (Vitousek et al, 1997) and novel habitats created within the urban matrix (Effland and Pouyat, 1997; Lugo, 2010). Considering the unprecedented increase in urbanization and its impact on the rapid degradation of ecosystem integrity (McDonald et al., 2008; Hahs et al., 2009), action to restore human-influenced lands is both timely and essential.

Ecological restoration of urban land is both complex and an ambitious challenge. A scientific understanding of multiple ecological levels and evolutionary trajectories must be superimposed over constant modifications of land-use and environmental integrity. For this reason, complex urban sites may require more appropriate techniques to match suitable plants with their conditions. That is, highly stressful and dynamic environments call for highly stress-resistant yet flexible genotypes.

The call for appropriate genotypes is not new; Montalvo et al. (1997) addressed the importance of applying a genetic framework when approaching ecological restoration. Soon after, Falk et al. (2001) published “An introduction to restoration genetics,” formally combining the disciplines of restoration ecology and population

genetics. The field has predominantly focused on the value of using local genotypes in restoration sites to maximize local adaptation and prevent outbreeding depression (Hufford and Mazer, 2003; McKay et al., 2005; Edmands, 2007).

Restoring natural habitats with a genetically diverse array of local genotypes has empirically been shown to be successful (e.g., Gustafson et al., 2005; Ramp et al., 2006; Bischoff et al., 2008; Cremieux et al., 2010), but it is often impractical. In highly modified sites, this "local only" approach may restrict collection to small, potentially genetically depauperate populations of poor quality seed (Broadhurst et al., 2008, Gustafson et al., 2008). In more extreme cases, local seed may not even be available, as remnants of native vegetation have already disappeared (Hahs et al., 2009).

Recent papers (Jones and Monaco, 2009; Jones et al., 2010; Jones and Robins, 2011) take this argument a step further and reason that certain human-influenced ecosystems have passed a threshold beyond which local genotypes may no longer be adapted. For these situations, attempting to restore locally adapted native plants can be ineffective. Jones and Robins (2011) claim that using genetic manipulation (i.e., artificial selection, hybridization, chromosome doubling) to develop plant material able to tolerate biotic and abiotic stressors may be most the practical solution for the most demanding restoration challenges. Agricultural research has been exploiting genetic screening of stress tolerance and crop development for some time (Krishnan et al., 1989; Kumar et al., 1999; Rampino et al., 2009). However, the goal of most ecological restoration projects is to create natural communities, which are far more complex than crop fields, and therefore more difficult to genetically engineer successfully (Handel et al., 1994).

While there have been some advances in genetic screening for site remediation and restoration of contaminated land, predominantly in metal-resistance (e.g., Whiting et al., 2004; Pauwels et al., 2008), there is still a disconnect between the theoretical restoration ecology and genetics, on the one hand, and real-world restoration practice, on the other (Young et al., 2005). In particular, there has been very little progress in translating general stress tolerance (e.g., heat, drought, salt) into urban planting practice in spite of the fact that large-scale ecological restoration activities are becoming increasingly common in urban settings (Ingram, 2008; Handel, 2011). Moreover, genetic and biochemical screening procedures are thought to be cost-prohibitive (Namkoong et al., 1996, Cook and Suski, 2008), potentially unreliable (Lawrence and Kaye, 2009; Gibbs et al., 2011; this study, Chapter 3), and difficult for restoration practitioners to execute (Jones, 2003; Cook and Suski, 2008). Subsequently, these techniques are frequently not employed. There is a serious need for simpler, less-stringent plant genotype screening for ecological restoration (Weeks et al., 2011), especially since inaction may be more detrimental than applying a sub-optimal screening procedure (Jones, 2003).

Objectives and Hypotheses

This study evaluates performance-based screening and whether highly stress-resistant plants (tested in the lab or greenhouse), regardless of provenance or genetic identity, constitute a reliable option for establishing plant populations under novel (and not previously tested) stress conditions. In the preceding chapter, I examined natural variation in phenotype and HSP expression among six *A. thaliana* genotypes exposed to

various stressors. I found that although natural variation in HSP induction existed, its use in determining the success of a genotype was limited. Throughout the study, however, I found that certain genotypes (R-France and R-Sweden) consistently performed well over a variety of stressors. I identified those as “stress-resistant” genotypes. To establish the principle that performance-based screening is a valid option for restoration practitioners, here I tested whether those same stress-resistant genotypes exhibited higher fitness than the others when exposed to novel stress conditions.

This study was designed to test the performance of stress-resistant genotypes in: (1) two controlled urban stress treatments and (2) a variety of field-collected site soils in which heterogeneity and stress combinations are unknown. Stress-resistant plant genotypes, as defined by consistent performance across an array of previous stress treatments, will be best able to tolerate the heterogeneous and unknown stressor combinations in a variety of novel sites.

METHODS

Study Species

Arabidopsis thaliana (*L.*) *Heynh.* (Brassicaceae) is a cosmopolitan, highly-selfing annual, native to western Eurasia and northern Africa and found in many disturbed sites throughout the world (Hoffmann, 2002). This small mustard plant is widely used as an experimental model for higher plants (Swarbreck et al., 2008) because of its quick and prolific reproduction, small genome and successful sequencing (Koorneef and Meinke, 2010), as well as an extensive collection of accessions available from the 1001 Genomes Project (Weigel and Mott, 2009).

The six genotypes in this study were selected from that database, maintained at the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. They represent one urban and one rural population from France, Germany and Sweden (hereafter: U-France and R-France, U-Germany and R-Germany, U-Sweden and R-Sweden). I selected the urban and rural counterparts to determine whether there were any inherent differences between provenance and performance. Each pair was roughly matched for geography, elevation and climate (Table 3.1). The 1001 Genomes Project maintained that each available accession represented a single inbred line, so for the purposes of this study, I refer to the six accessions as genotypes.

Soil Collection Sites

I selected five field soils for the seed germination experiment, varying in disturbance and land-use: (1) soil from meadows within the Duke Farms Estate (DUK) in

Hillsborough, Somerset County, New Jersey (lat 40.55° N, long 74.62° W); (2) a former arsenal and superfund site situated in the Environmental Protection Agency Region 2 Compound (EPA) in Edison, Middlesex County, New Jersey (lat 40.51° N, long 74.36° W); (3) an urban park currently going through a major renovation and restoration (DOP) in Brooklyn, New York (lat 40.58° N, long 73.99° W); (4) a closed, capped and covered landfill (FKL) located in Staten Island, New York (lat 40.58° N, long 74.18° W); (5) an abandoned railroad site adjacent to a closed oil refinery (BAY), located in Bayonne, Hudson County, New Jersey (lat 40.66° N, long 74.10° W).

DUK and EPA soils were used in the site soil performance experiments. For all experiments involving field site soil, I preserved the heterogeneity of the field sites by planting seeds among 10 individual soil samples, which were randomly collected from a 10mx10m plot within each site. Additional site information and a summary of soil properties can be found in Table 2.1 and Figures 2.1 to 2.3. Potting medium (PMP High Organic Arabidopsis Medium; Lehle Seeds, Round Rock, TX) was used for the salt and heat/salt stress experiments.

Experimental Design

I first tested plant performance of six *Arabidopsis thaliana* genotypes in two controlled stress treatments, salt and heat/salt, selected because they are typical stressors that affect flora in the urban environment, as a consequence of road salting (Cunningham et al., 2008) and urban heat islands (Williams et al., 2008), and because they were not examined in the previous genotype study (Chapter 3).

I then performed a seed germination study using the six genotypes in soils from five sites, one reference and four degraded, from the New York metropolitan region to determine establishment success. The question was which genotypes had a significant germination advantage, since seedling establishment is often limited, if it occurs at all, in highly degraded urban soils (Pavao-Zuckerman, 2008).

I finally chose two of the aforementioned site soils from the New York metropolitan region, representing post-agricultural field and brownfield soils, to investigate whether stress-resistant genotypes could consistently perform well across real restoration site soils for which levels of heterogeneity and composition of stressors are unknown.

Throughout all experiments, unless noted otherwise, germination protocol, growth chamber conditions and data collection methods were as follows. An illustration of the experimental design, flats and treatments can be found in Figures 4.1 and 4.2.

Germination and growth protocol. I soaked *A. thaliana* seeds of the six genotypes on filter paper, cold stratified them in the dark at 4°C for three days and then transferred them into site or potting soil in flats, maintained under controlled growth chamber temperature and light conditions (see below) to stimulate and synchronize germination (Pigliucci and Schlichting, 1996). The majority of seeds germinated within the first 48 to 96 hours. I recorded germination date and replaced non-viable seeds immediately (except in the germination experiment described below). To prevent desiccation, I covered the flats with plastic domes, misted seeds and sub-irrigated daily until bolting (emergence of the stalk). After which I removed the covers and watered plants as needed, typically

every 2-3 days, with distilled water. Throughout the experiment, I used an Arasystem (Betatech, Gent, Belgium), which is a series of flats and plastic tubes designed specifically for growing *Arabidopsis* and seed collection.

Controlled Growth Chamber Conditions. Conditions in the growth chamber (Model #GC15-31-CW-C3-X-HL-PW-CF, Environmental Growth Chambers, Chagrin Falls, OH), consisted of a 14-hour day (~ 140 µE/m2/sec) with 25°C daytime temperature and 70% humidity, and with 23°C nighttime temperature and 60% humidity (Scholl, 1996; Weigel and Glazebrook, 2002). Flats were rotated every three or four days to minimize any effects of growth chamber position (Potvin et al., 1990).

Stress Treatment Experiment. I planted 30 replicates per genotype in six flats of potting medium under control conditions (180 plants; individual flats were randomized). Each flat contained five replicates of each genotype; each treatment had a total of 10 replicates. At three weeks after germination, before plants bolted, I began the two experimental treatments: Salt stress (S), and Heat/Salt stress (HS) (Fig. 4.1).

Salt stress: At three weeks past germination, I continued the control watering regime (above), but replaced distilled water with a 100 mM NaCl solution for one week (modified from Sun et al., 2001) in four of the six flats; the other two flats remained control (C) plants. Salt stress required blocking by flat since watering was done by sub-irrigation. After the stress treatment was complete, I resumed using distilled water until harvesting. Plants from the salt-watered flats then either remained in the control chamber to become control salt (S) stressed plants or were transferred to the hot chamber to

become heat/salt (HS) stressed plants, two flats for (S), two flats for (HS).

Heat/Salt Stress: At four weeks past germination, immediately after the salt stress, I transferred 10 random replicates from each genotype from each salt stress flats (as explained above; 60 plants) to 38°C for 6 hours (Rizhsky et al., 2004). At the end of the heat-shock period, I returned the plants to the control chamber. They remained there until senescence for performance data collection.

Seed Germination Experiment. To determine germination rate in field-collected soil, I planted a total of 300 seeds (10 seeds of six genotypes in each of the five different site soils). There were 50 pots of soil, as each of the five soils was comprised of 10 samples to maintain site heterogeneity. I planted single seeds of each genotype (6 seeds) in each of the 50 pots, in randomly assigned positions (Fig. 4.2). One week after sowing seeds, I recorded whether germination had occurred, by noting the presence or absence of the first two leaves.

Site Soil Experiment. I planted 10 replicates per genotype between two flats of two site soils (DUK and EPA) under control conditions (60 plants; site soils and genotypes were completely randomized over both flats). Plants remained in the control chamber until senescence for performance data collection.

Data Collection and Analysis

All statistical analyses were performed using JMP version 8 for Macintosh, (SAS Institute Inc, Cary, NC, USA). Specified data sets were log and square root transformed

to homogenize variances and increase normality of residuals for 2-way ANOVA.

Germination. Germination data were binary; I performed a nominal logistic regression to compare proportions of germinated seeds. To address effects of soil, genotype and soil*genotype interaction, I performed a fixed effects likelihood ratio analysis. I then calculated odds ratios to reveal the strength of association of germination rates between particular genotypes.

Performance Data. I collected data on two fitness estimates: number of siliques (counted at senescence date) and total seed weight produced (by the whole plant, measured after harvest). Both were analyzed for salt and heat/salt stress treatments; only total seed weight was analyzed for site soils. In all cases, total seed weight produced per plant was used as a proxy for seed number because they were highly correlated (r^2=0.85, $p<0.0001$) and seed weight could be more accurately determined. One- and two-way ANOVA were used in both experiments and two-way analyses were illustrated using least squares plots of the means. To determine whether certain genotypes exhibited significantly higher fitness estimates than others, Tukey HSD post-hoc tests were employed.

RESULTS

Stress Treatment Experiments

Salt Stress. Salt stress significantly decreased silique number and seed weight as expected, but the relative effect on each genotype differed, more so in seed weight as indicated by a significant interaction effect (Table 4.1). In both silique number (Fig. 4.3) and seed weight (Fig. 4.4) plots, the two stress-resistant genotypes, R-France and R-Sweden, displayed almost parallel and shallow response curves to salt stress. Subsequently, they were able to maintain high fitness values, while other genotypes exhibited a steeper decline of silique number and seed weight in the presence of salt stress. Interestingly, the only other genotype showing a very modest decline in silique number was U-Sweden, the worst performer, which started low and remained low. A strong convergence was seen among seed weights for four genotypes in salt stress, while the two most resistant genotypes displayed much higher values.

Heat/Salt Stress. Heat/salt stress significantly decreased silique numbers and seed weights of all plants; again there was differential genotypic response, as indicated by significant interaction effects (Table 4.2). When exposed to stress, silique numbers decreased, albeit to a lesser extent in the stress-resistant genotypes, R-Sweden and R-France. However, only R-Sweden had significantly more siliques in the heat/salt treatment than the worst performing genotype R-France (Fig. 4.5). R-France and R-Sweden showed a significant seed weight advantage in heat/salt stress (Fig. 4.6). All of

the other genotypes displayed much steeper response curves, which converged considerably below the values for R-France and R-Sweden.

Seed Germination Experiment

Both genotype and site soil affected germination rates significantly, yet there was no significant interaction effect (Table 4.3). R-Sweden and R-France, the two stress-resistant genotypes exhibited highest germination rates. Over all site soils, R-Sweden was over 100 times and R-France over 10 times more likely to germinate than the two least successful genotypes, U-France and U-Sweden. While R-Sweden and R-France appeared to have higher proportions of germinated seed in the more stressful sites, this effect did not significantly strengthen as sites became more stressful, which may indicate strong inherent genotypic differences that were only slightly affected by soil type.

Site Soil Experiments

There were no genotypic differences observed in DUK soil, which was not surprising, since the soil is not contaminated and quite homogenous (this study, Chapter 2). All plants in EPA soil experienced a significant decrease in total seed weight compared to DUK soil; the stress-resistant genotypes, R-Sweden and R-France were the least affected by the decrease in soil quality (Fig. 4.7), although there were no significant interaction effects (Table 4.4).

DISCUSSION

Stress-Resistant Genotypes for Successful Population Establishment

In severely degraded areas, stress-resistant genotypes may establish vegetative cover quickly (Lesica and Allendorf, 1999). Successful germination and subsequent rapid establishment of plants is a critical first step in successful restoration that controls erosion and prevents biotic invasion (Waldron et al, 2011). In this study, the seeds of stress-resistant genotypes had significantly higher germination rates, and were able to tolerate the most stressful restoration soils.

Natural variation in stress tolerance to salt and heat has previously been observed at the germination and young seedling stages (Quesada et al., 2002; Wang et al., 2003; this study, Chapter 3) and researchers are currently investigating quantitative trait loci (QTL) responsible for these responses (Katori et al., 2010; Mason et al., 2010; Ren et al., 2010). In many cases, the actual loci controlling quantitative genetic variation in abiotic stress tolerance are still unknown (DesMarais and Juenger, 2010). Hancock et al. (2011) have recently identified "climate adapted" loci in *A. thaliana* and were able to predict differences in fitness for different accessions (genotypes) in a common environment trial. As more and more QTL are identified for stress response, they may provide new genetic material for the selection of stress-resistant seed stock (Pauwels et al., 2008; Ruan and Teixeira da Silva, 2010). At that point, it may be prudent to slowly incorporate into restoration practice certain genetic manipulations such as artificial selection of certain genomic regions associated with improved stress tolerance (Mason et al., 2010).

While the experiments presented here demonstrate germination advantages of using particular genotypes in stressful soils, stress-resistance that facilitates plant establishment is just one part of the overall success of a restoration project. The goal of ecological restoration is not only rapid establishment, but also long-term viability of populations (Lesica and Allendorf, 1999).

Population Persistence in Constantly Changing Environments

The ability of restored plant populations to evolve and adapt to changing environments depends primarily on the level of genetic diversity within the installed populations (Montalvo et al., 1997; Hufford and Mazer, 2003; Vander Mijnsbrugge et al., 2010), as this diversity provides the raw material for evolution by natural selection (Fisher, 1930). While genetic diversity was not measured directly in this study, I believe the findings here compliment the widely held approach that diversity be maintained in any plant installation. Practitioners must seriously consider the genetic diversity of these populations (McKay et al., 2005), especially now, as future global change can affect our restoration efforts (Rice and Emery, 2003; Harris et al., 2006). Finding appropriate genotypes and increasing genetic diversity, through hybridization or bulking (Jones and Robins, 2011), are two processes that can and should occur simultaneously. Only together will they ensure sufficient establishment and persistence necessary for successful ecological restoration.

Potential Objections to Using Selected Stress-Resistant Genotypes in Restoration

The stress-resistant genotypes used may not be local. Both theoretical and empirical studies have challenged the idea that species of local provenance will necessarily exhibit higher fitness (e.g., Wilkinson, 2001; Smith et al, 2007; Broadhurst et al., 2008). Matching habitat and ecological conditions can be more important than minimizing the geographical distance between a source population and restoration site (Lesica and Allendorf, 1999; Montalvo and Ellstrand, 2000; McKay et al., 2005).

Additionally, insisting on exclusively local plant material can be limiting and/or impossible and may commit populations to a "genetic dead end" that will not allow for adaptation to changing conditions (Harris et al., 2006). This leads to the practical question, why are we so concerned about using local genotypes if presumably natural selection will eventually eliminate any poorly adapted individuals from the population? In their 2003 paper, Hufford and Mazer admit this possibility, and suggest the need to consider outbreeding depression in restoration may be less important than once thought. This sentiment is echoed in Falk et al. (2006) and Broadhurst et al. (2008).

The potential risks of translocations are currently being examined, yet many empirical and theoretical experiments have not observed the anticipated detrimental effects of outbreeding depression (e.g., Luijten et al., 2002; Frankham et al., 2011; Jones and Robins, 2011; Muola et al., 2011). Cremieux et al. (2010) did find a temporary decrease in fitness of inter-population hybrids among *Plantago lanceolata* out-crossed with geographically distant individuals, but by the end of one growing season, most fitness estimates returned close to the average of the parent generation. Thorpe and Stanley (2011) and Weeks et al. (2011) have cautioned that the generally unsubstantiated

risks of outbreeding depression restrain current management options and commonly lead to inaction.

Artificially selected genotypes are maladapted. In their guidelines for choosing appropriate genetic plant material, Lesica and Allendorf (1999) recommend avoiding strongly selected cultivars. They argue that plants specifically bred for particular traits may perform well immediately, especially in small and highly degraded sites, but as conditions change, these highly specific cultivars will become maladapted. While the stress-resistant genotypes defined in this study may qualify as cultivars, these have been selected for *general* stress resistance. While the strains used in this study lack the necessary genetic variation for long-term approaches, they act to establish the principle that these types of plants (a large set, optimally) can be used for restoration. The focus of these experiments was to find genotypes that could tolerate and successfully reproduce in a variety of unknown and novel stressors, and in that case, they should be better adapted for changing conditions.

Artificially selected genotypes have low levels of genetic diversity. Regardless of how scientists define "local", they have long agreed that high genetic diversity should be maintained throughout any restoration process to prevent founder effects (Hufford and Mazer, 2003) and ensure successful adaptation through evolution (Ellstrand and Elam, 1993; Montalvo et al., 1997; Sinclair and Hobbs, 2009). It is usually assumed that artificially selected collections of genotypes contain less genetic diversity than unselected sets. Jones and Robins (2011) argue that this is not necessarily true, as genetic diversity

can be retained by maintaining a large enough effective population size to minimize inbreeding depression (Falconer, 1960).

Conclusion

This study tested performance-based screening and whether highly stress-resistant plants (tested in the lab or greenhouse), regardless of provenance or genetic identity, constitute a reliable option for establishing plant populations under novel (and not previously tested) stress conditions. In this series of experiments, I have shown that the best-adapted genotypes, as evidenced by previous tests of heat, drought and heat/drought stress, also perform well on novel and untested combinations of stressors. In particular, genotypes R-Sweden and R-France consistently exhibited higher germination rates, silique numbers and seed weights than the other genotypes under salt stress, heat/salt stress and a variety of restoration site soils.

While there is still a healthy debate occurring among restoration geneticists about the best option for genotype selection, restoration practitioners need straightforward and uncomplicated advice on what plants to use, especially when working in highly urbanized and degraded sites. This study adds support to the idea that genotype selection for urban restoration need not be costly or difficult. Simple preliminary stress tests using an array of stressors characteristic of any restoration site can be a reliable option to predict planting success for genotypes, under situations where local seed is unavailable or poorly adapted because of changing environmental conditions.

Moreover, these tests can provide effective, low-cost alternative to highly complex molecular analyses, whose connections to actual restoration success are still

tenuous at best. The goal of any restoration project is to recreate functioning ecosystem processes as best we can. This study offers that potential by introducing an uncomplicated genotype screening process, which is accessible to all practitioners interested in reliable plant establishment, performance and persistence.

Table 4.1. Fitness estimates **(A)** silique number and **(B)** total seed weight (square root transformed); 2-way ANOVA results of the salt experiment: Whole model results and effect likelihood ratio analysis. Genotype and salt (S) treatment significantly affected the amount of siliques produced by *Arabidopsis thaliana*. There was a significant interaction effect found for total seed weight.

(A)

R-Square	0.4248
Observations	114

SOURCE	**df**	**SS**	**MS**	**F-Ratio**
Model	11	24180.715	2198.25	6.8483
Error	102	32741.144	320.99	**Prob > F**
Total	113	56921.860		<0.0001*

SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	13983.936	8.7130	<0.0001*
S Trt	1	1	7043.163	21.9419	<0.0001*
S*Geno	5	5	3188.054	1.9864	0.0869

(B)

R-Square	0.7588
Observations	115

SOURCE	**df**	**SS**	**MS**	**F-Ratio**
Model	11	0.502	0.046	29.4584
Error	103	0.160	0.002	**Prob > F**
Total	114	0.662		<0.0001*

SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	0.079	10.1809	<0.0001*
S Trt	1	1	0.398	256.6623	<0.0001*
S*Geno	5	5	0.028	3.6477	0.0044*

Table 4.2. Fitness estimates **(A)** silique number (square root transformed) and **(B)** total seed weight (log transformed); 2-way ANOVA results of the heat/salt experiment: Whole model results and effect likelihood ratio analysis. Genotype and heat/salt (HS) treatment significantly affected the amount of siliques produced by *Arabidopsis thaliana*. There was a significant interaction effect found for silique number and total seed weight.

(A)

R-Square	0.6801
Observations	107

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	354.272	32.307	18.3568	
Error	95	166.675	1.755	**Prob > F**	
Total	106	520.947		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	42.019	4.7899	0.0006*
HS Trt	1	1	268.406	152.9838	<0.0001*
HS*Geno	5	5	27.883	3.1785	0.0107*

(B)

R-Square	0.8143
Observations	103

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	79.216	7.201	36.2820	
Error	191	18.062	0.198	**Prob > F**	
Total	102	97.278		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	5.944	5.9890	<0.0001*
HS Trt	1	1	73.562	370.6161	<0.0001*
HS*Geno	5	5	2.622	2.6420	0.0282*

Table 4.3. Nominal logistic regression results for germination experiment: Whole model results and effect likelihood ratio analysis. Both soil and genotype significantly affected germination rate of *Arabidopsis thaliana*. There were no significant interaction effects.

		MODEL	**-LogLikelihood**	**df**	**Chi-Square**	**Prob>Chi-Square**
R-Square (U)	0.1534					
Observations	300	Difference	28.249	4	32.285	<0.0016*
		Full	155.850			
		Reduced	184.099			
		SOURCE	**N**	**df**	**L-R Chi-Square**	**Prob>Chi-Square**
		Soil	4	4	32.285	<0.0001*
		Genotype	5	5	24.290	0.0002*
		Soil*Genotype	20	20	10.168	0.96550

Table 4.4. Two-way ANOVA and effect test results for SR seed weight (g) in *Arabidopsis thaliana* in DUK and EPA soils. Genotype and soil treatment significantly affected seed weight of *Arabidopsis thaliana.* There were no significant interaction effects observed.

R-Square	0.3339
Observations	120

SOURCE	**df**	**SS**	**MS**	**F-Ratio**	
Model	11	0.082	0.007	4.9212	
Error	108	0.163	0.002	**Prob > F**	
Total	119	0.245		<0.0001*	
SOURCE	**N**	**df**	**SS**	**F-Ratio**	**Prob > F**
Genotype	5	5	0.045	5.9420	<0.0001*
Soil Trt	1	1	0.028	18.2931	<0.0001*
Soil*Geno	5	5	0.009	1.2260	0.3019

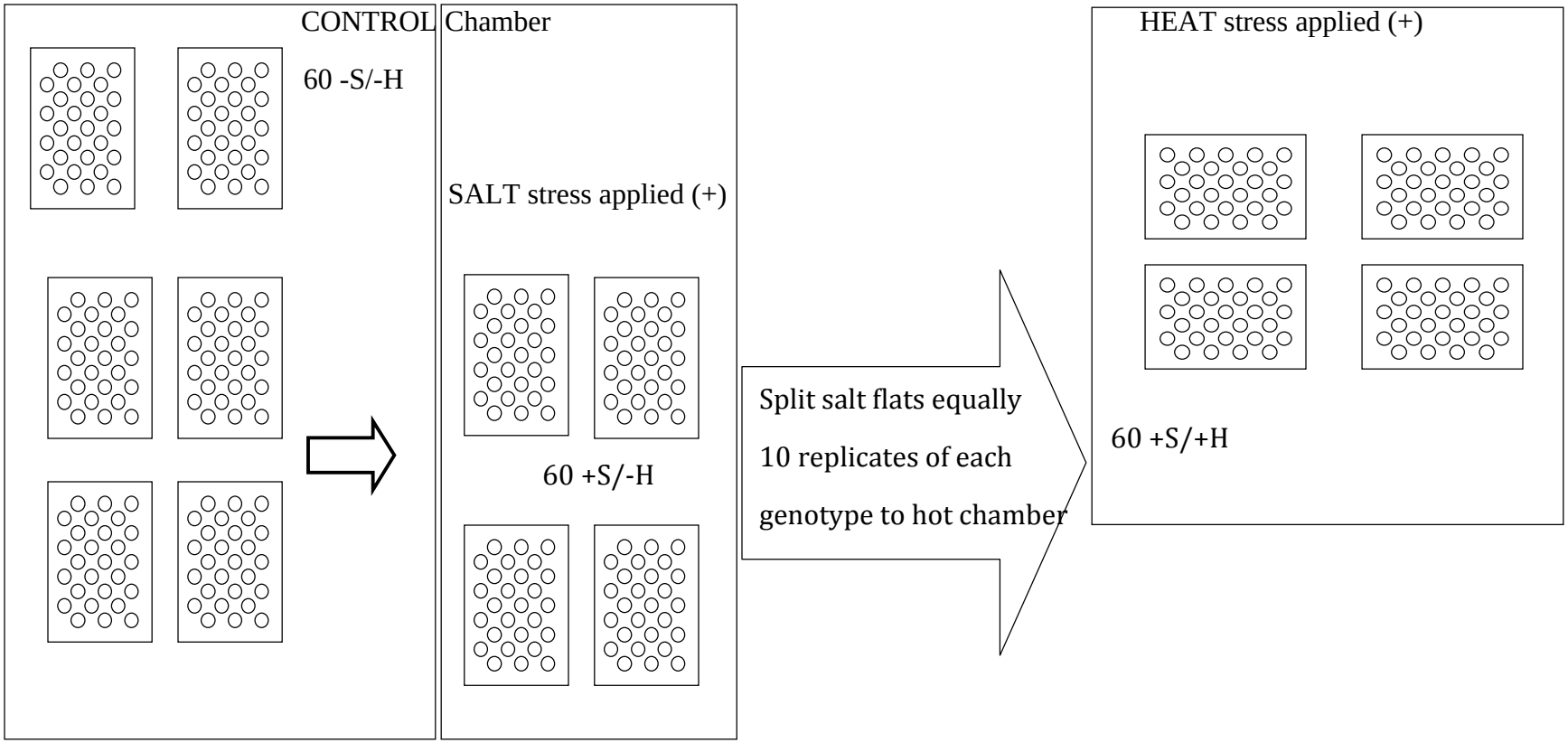

Figure 4.2. Experimental design of stress treatment experiment: 30 replicates of six genotypes (180 plants total; 30 plugs per individually randomized flat) germinated in the control chamber. Three weeks later, salt stress was applied to four flats (in control chamber). After one week, ten random replicates of each genotype from the salt flats (60 plants) were transferred to the heat chamber for six hours and then returned to the control chamber for the duration of the experiment.

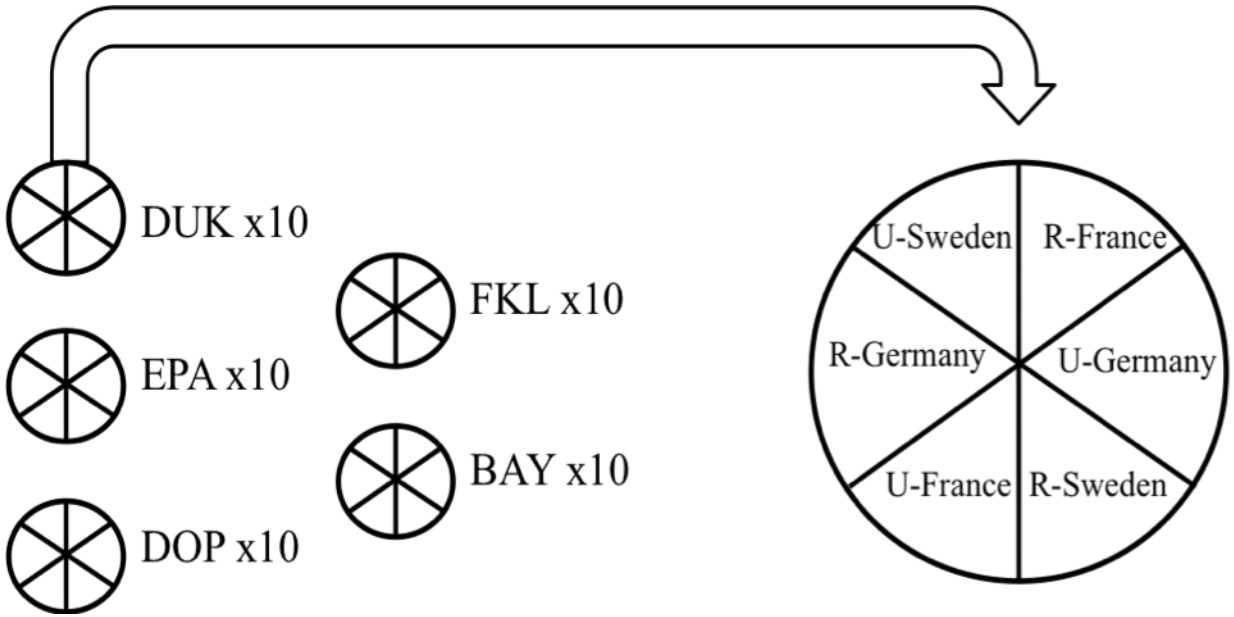

Figure 4.2. Seed germination experiment design. Ten seeds from six genotypes were planted within fifty pots representing ten individual soil samples from five field sites. Single seeds from each genotype were randomly placed within six positions for each pot, as shown by the larger illustration.

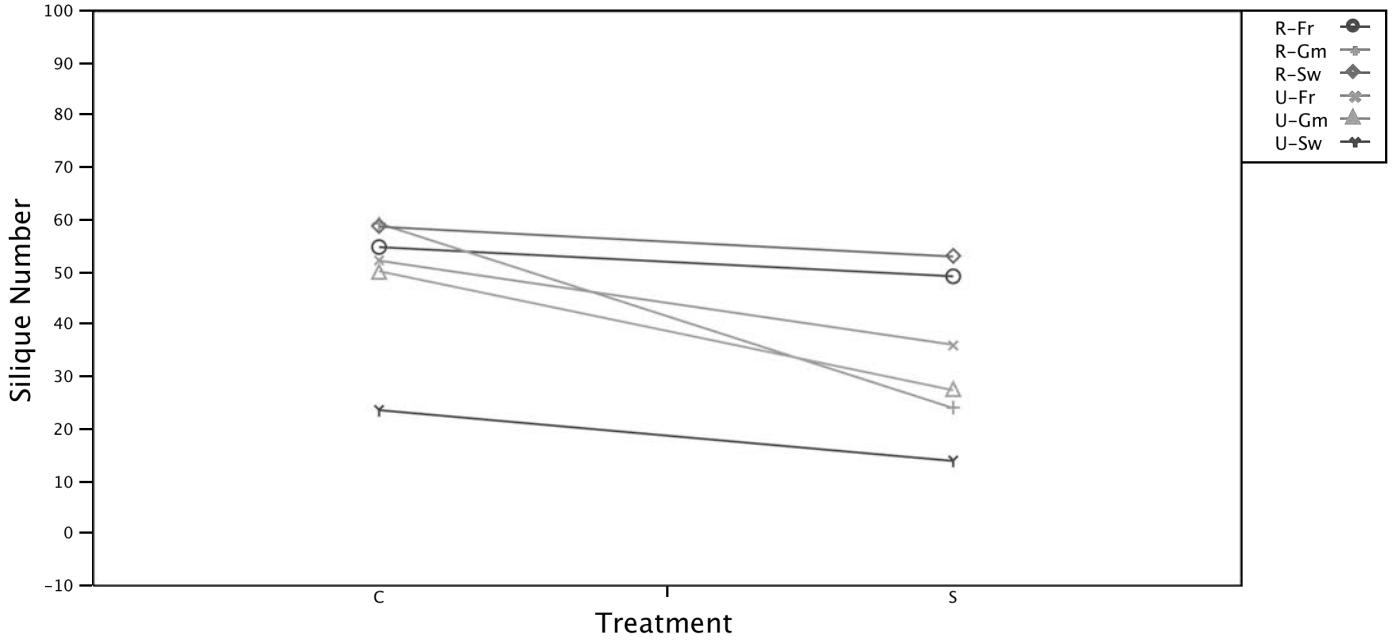

Figure 4.3. Silique number in *Arabidopsis thaliana* in control and salt stress treatments. R-Sweden and R-France had significantly higher silique numbers than the least successful genotype U-Sweden in control conditions ($F_{5, 54} = 4.1091$; $p=0.0031$), however in salt stress, these two stress-resistant genotypes significantly surpassed U-Germany, R-Germany, and U-Sweden in silique production ($F_{5, 84} = 9.4494$; $p<0.0001$). Values plotted are least-squares means.

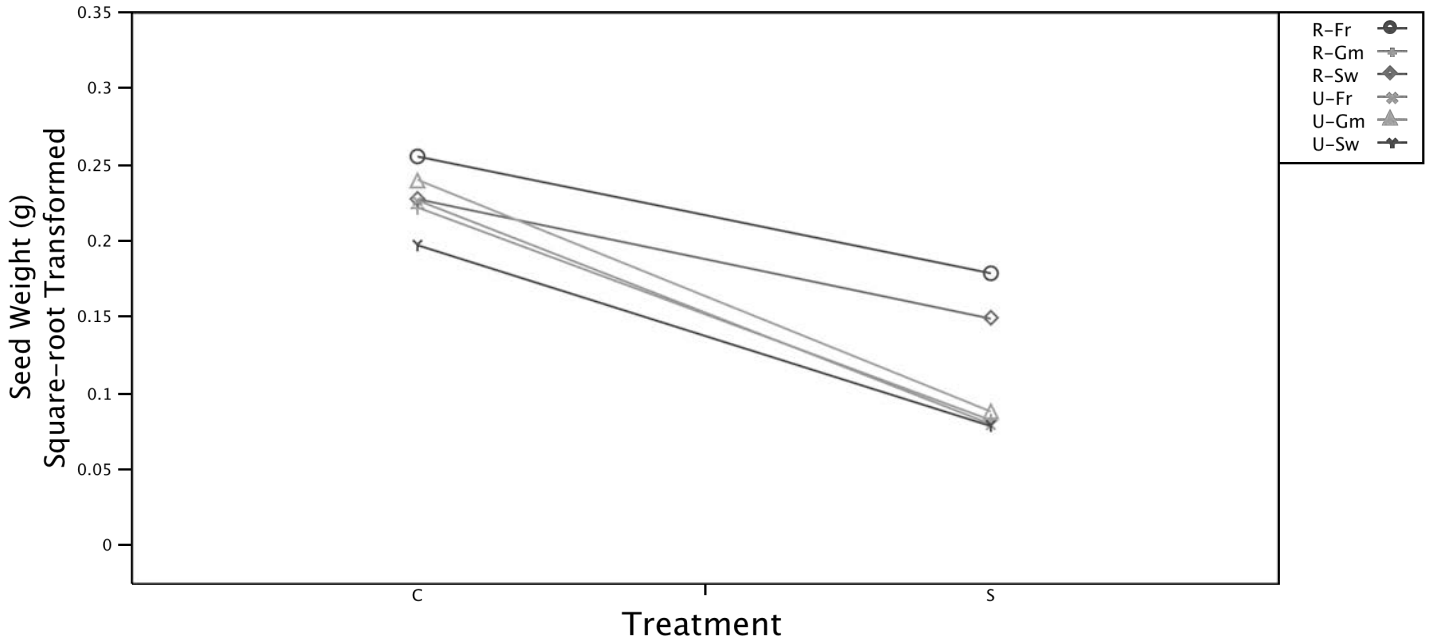

Figure 4.4. Total seed weight in *Arabidopsis thaliana* in control and salt stress treatments. There was no significant difference among genotypes in control conditions. In salt stress, the two stress-resistant genotypes, R-France and R-Sweden produced significantly more seed than all other genotypes ($F_{5, 49} = 16.7610$; $p<0.0001$). Seed weight data were square root (SR) transformed to normalize residuals. Values plotted are least-squares means.

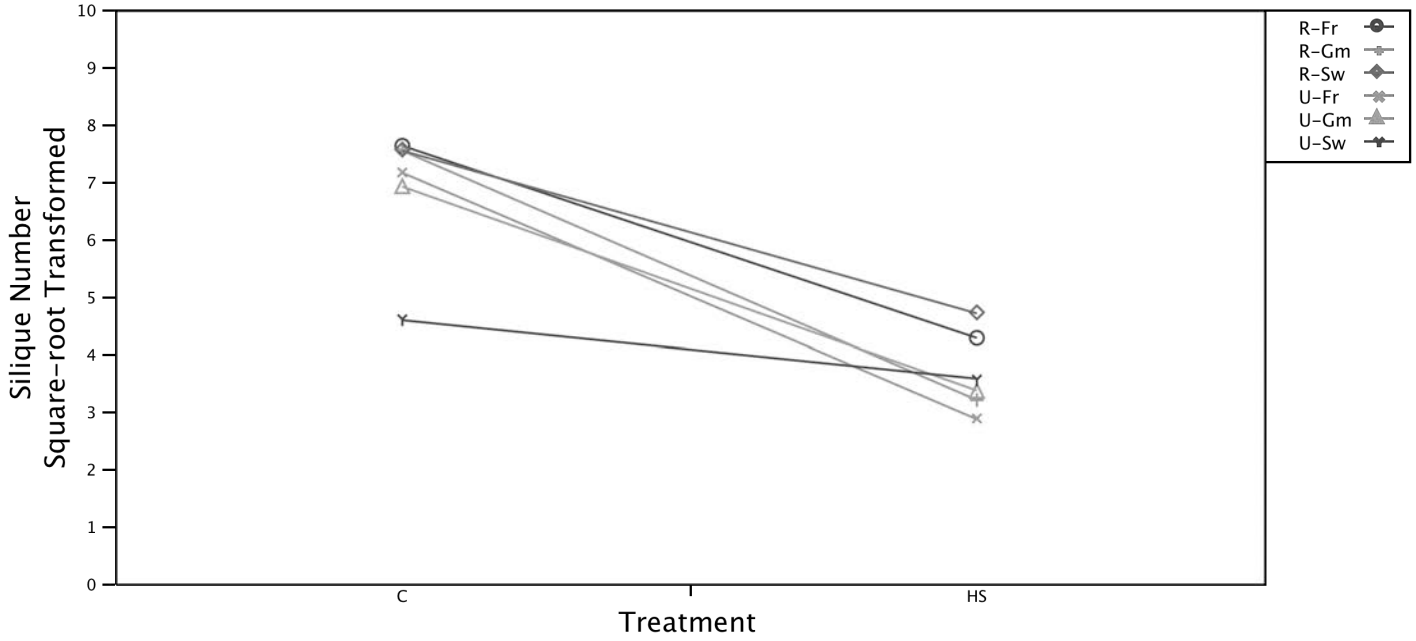

Figure 4.5. Silique number in *Arabidopsis thaliana* in control and heat/salt stress treatments. All genotypes produced significantly higher silique numbers than the least successful genotype U-Sweden in control conditions ($F_{5, 53} = 6.1903$; $p<0.0001$), however in heat/salt stress, only the R-Sweden genotype had significantly higher numbers of siliques than U-France ($F_{5, 42} = 3.2818$; $p<0.0137$). Silique number data were square root (SR) transformed to normalize residuals. Values plotted are least-squares means.

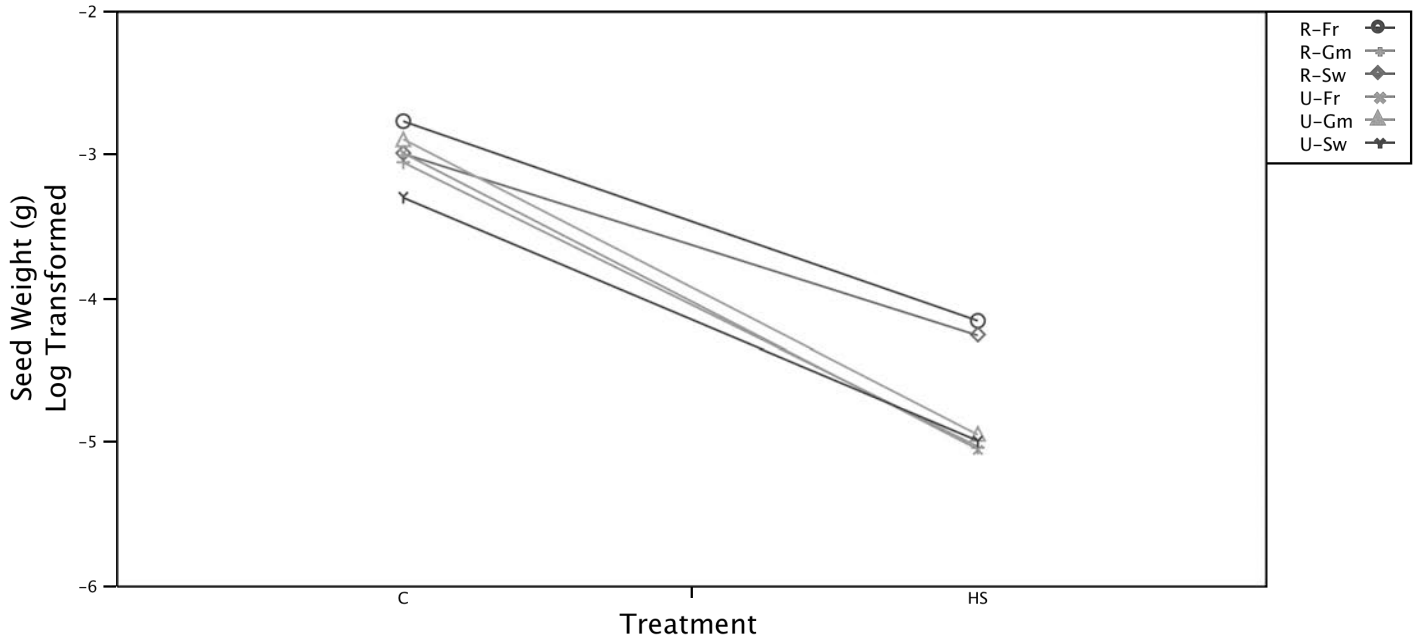

Figure 4.6. Total seed weight in *Arabidopsis thaliana* in control and heat/salt stress treatments. There was no significant difference among genotypes in control conditions. In heat/salt stress, R-France had similar values as R-Sweden, but significantly higher seed weights than all the other genotypes ($F_{5, 37} = 5.0009$; $p=0.0013$). Seed weight data were log (LN) transformed to minimize heteroscedacity and normalize residuals. Values plotted are least-squares means.

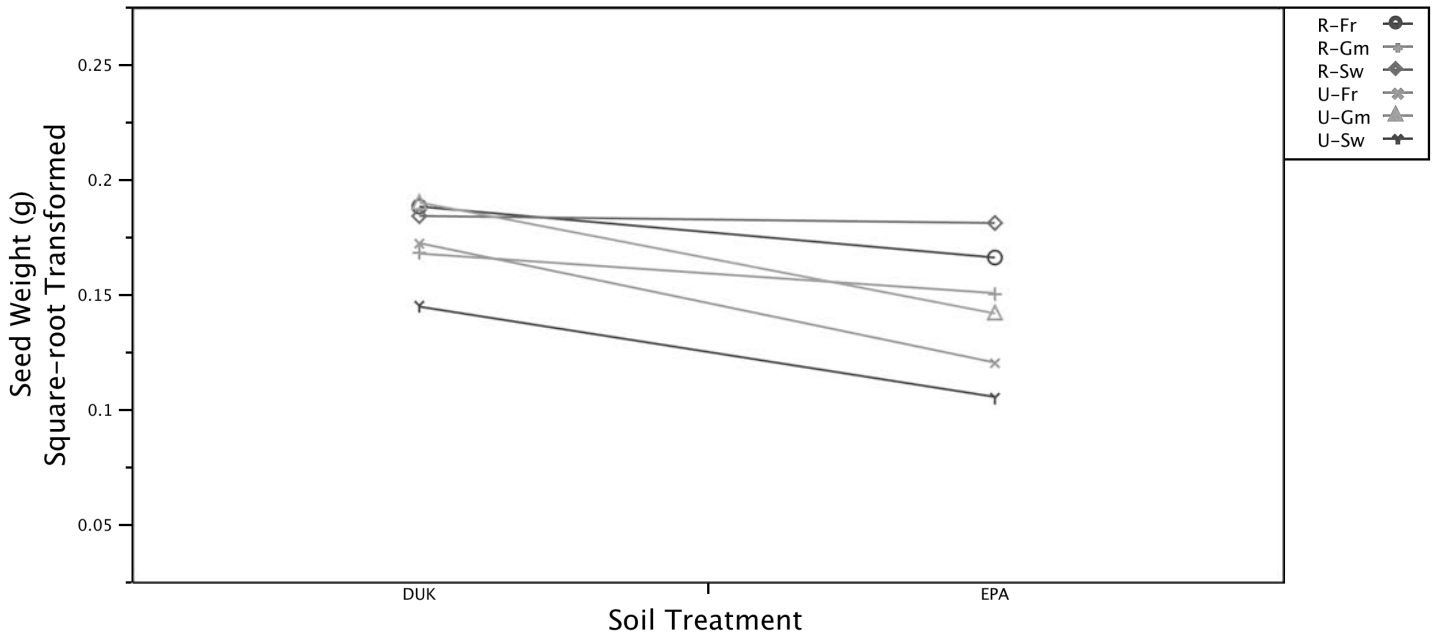

Figure 4.7. Total seed weight in *Arabidopsis thaliana* in DUK and EPA soil treatments. There was no significant difference among genotypes in DUK soil. In EPA soil, R-Sweden and R-France produced significantly more seed than the U-Sweden genotype ($F_{5, 54}$ = 5.1371; p=0.0006). Seed weight data were square root (SR) transformed to normalize residuals. Values plotted are least-squares means.

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CHAPTER V

SUMMARY AND CONCLUSIONS

Research Summary and Conclusions

The major conclusions of this dissertation are addressed below under the research questions presented in Chapter I.

1) Can the success of a plant genotype be determined by the presence of an induced stress response and will the benefit of this response be more pronounced as stressful conditions increase?

Overall, this study showed an adaptive benefit for *Arabidopsis thaliana* in possessing a working HSP17.6 response. Wildtype (WT) genotypes had longer lifespans, produced larger plants, and, most importantly, displayed increased fitness estimates (silique number and seed weight). Conversely, mutants lacking a plastic HSP17.6 response generally showed an inability to cope with various types of abiotic stress. This difference was generally more pronounced in high stress conditions, providing evidence of adaptive plasticity for *A. thaliana* HSP17.6 induction.

While this model system was necessary to test the adaptive nature of a plastic response, it is difficult to take these results and make inferences to the highly applied discipline of ecological restoration. However, this tractable and manageable model system allowed me to perform one of the first studies of the potential utility of phenotypic plasticity. My results indicate that heat shock proteins in plants can provide

adaptive plasticity in the face of urban (salt and heat/drought) conditions and that they can have an impact on plant growth and fitness. This study highlights the importance of understanding how urban and climatic stress affects reproductive development in the context of stress response, and highlights the potential for selecting more flexible and tolerant genotypes for use in ecological restoration of urban and degraded land

2) Is there significant natural variation in stress response? Can that information predict plant performance and success in stressful environments?

In the absence of stress, natural variation was observed in phenotype (all genotypic differences were significant except for seed weight) and HSP17.6 expression, but not HSP101 expression. When various urban stressors were applied, genotypes often expressed differential phenotypic and genetic responses. However, these genetic responses were only occasionally adaptive, predominantly in HSP17.6, and strong correlations between stress response (HSP induction) and fitness were not evident. The results from this study suggest that HSP induction is but one part of a complex abiotic stress response, and that predicting plant success using this type of molecular data alone may be problematic. While this work provides valuable ecological insight into the underpinnings of heat and drought response via the HSP system in *A. thaliana*, extension to a wider array of systems would be beneficial.

Through these experiments, I unexpectedly identified two genotypes (R-France and R-Sweden) that consistently performed well over a variety of urban stressors. I identified those as “stress-resistant” genotypes. It seemed as though genotype and

previous performance, rather than gene expression, was a better indicator of success in stressful urban conditions.

3) Will stress-resistant plant genotypes, as defined by consistent performance across a broad array of previous stress treatments, successfully tolerate heterogeneous and unknown stressor combinations in a variety of new sites?

The results of these experiments showed that the best-adapted genotypes, as evidenced by previous tests of heat, drought and heat/drought stress consistently performed better than other genotypes in novel stress treatments, and more importantly, in restoration field soils, which contained unknown levels of heterogeneity and combinations of stressors. In particular, the stress-resistant genotypes consistently exhibited higher germination rates, silique numbers and seed weights than the other genotypes under salt stress, heat/salt stress and a variety of restoration site soils.

This study suggests that genotype selection for urban restoration need not be costly or difficult through genetic screening. Simple preliminary stress tests using an array of stressors characteristic of any restoration site can be successfully used to predict planting success for genotypes, under situations where local seed is unavailable or poorly adapted.

Future Research

This research leads to many more questions about the selection of stress-resistant genotypes for use in ecological restoration, particularly of urban and degraded land. Future studies should address plant stress response and resistance by exploring the expression of multiple genes and pathways (through the use of QTL analyses) and multiple stressors simultaneously. While advancements in molecular genetics have facilitated the emergence of this type of work (e.g., Pauwels et al., 2008; Ruan and Teixeira da Silva, 2010; Hancock et al., 2011), future research needs to move out of the lab and into the field. Studies need to focus on more restoration relevant species rather than model organisms and within an ecological context. This will allow restoration geneticists to better understand how this stress resistance will play out in "natural" urban ecosystems.

While incorporating the ideas of evolution, genetics and plasticity into the existing template of restoration practice can foster new ideas regarding genotype selection and the restoration of human-influenced lands, we must ensure that these ideas are shared with the restoration practitioners on the ground. Scientists must bridge the gap between restoration goals and practices (Palmer, 2008; Christian-Smith and Merenlender, 2010) by continuing to evaluate trends in ecological restoration and offer fresh ideas to expand upon and improve current methodologies.

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