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The Influence of Drought on the Ecological and Evolutionary Genomics of Fungal Pathogenesis in an Annual Plant, *Brassica rapa*

A Dissertation Presented

by

Niamh B. O'Hara

to

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Abstract of the Dissertation

The Influence of Drought on the Ecological and Evolutionary Genomics of Fungal Pathogenesis in an Annual Plant, *Brassica rapa*

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There is growing evidence that populations are evolving rapidly in response to climate change. However, the potential cost associated with adapting, and the genetic basis of both rapid evolution and costs of adaptation remain largely unexplored. With climate change, drought is increasing in frequency and severity, widely affecting plant yield and fitness in agricultural and natural populations. For my dissertation, I integrated multiple approaches (field, greenhouse, lab and bioinformatic) to explore the ecological and evolutionary effects of drought on an agriculturally important plant-pathogen system: the annual herbaceous plant *Brassica rapa* (field mustard), and its fungal pathogen *Alternaria brassicae*.

In a field study, including multiple sites on the west coast of the U.S., I found a positive association between soil moisture and disease severity. However, the relationship between soil moisture and disease varied among locations and over time, and the factors influencing disease susceptibility in the field are complex.

In a greenhouse study with pre-drought ancestors and post-drought descendants, I found an evolutionary shift to earlier flowering, and an evolutionary increase in disease susceptibility, indicating a cost of the drought adaptation. I found that that earlier flowering

plants had thinner leaves, which are more easily invaded by fungal pathogens, indicating a potential mechanism for the increase in susceptibility observed.

I conducted whole genome shotgun sequencing on 205 ancestral and descendant *B. rapa* plants. I found considerable genetic differentiation across the genome, consistent with a rapid evolutionary response to drought at multiple loci. Many significantly differentiated genes are involved in pathogen response, indicating a genetic basis for the observed shift in disease susceptibility. Site frequency spectrum analysis indicated that selection appears to have acted on standing genetic variation (soft sweeps). The results of this study shed light on both a cost of adaptation and on the genetic basis of rapid evolutionary responses to a change in climatic conditions.

Dedication

To Dan, for his unswerving support, and for lovingly building an artistic/scientific life with me…

To my mom, for 32 years of encouragement, and unrestricted use of her home kitchen for processing biological samples…

To my dad, who had already turned me into a scientist by age 8.

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List of Abbreviations

ABA	Abscisic acid
ET	Ethylene
GLM	Generalized linear models
GO	Gene ontology
HR	Hypersensitive response
IPCC	International Panel on Climate Change
JA	Jasmonic acid
SA	Salicylic acid
SLA	Specific leaf area
SSH	Suppression subtractive hybridization
WGS	Whole genome sequencing

Acknowledgments

I have had the good fortune to have not one, but two outstanding doctoral advisors, Joshua S. Rest, and Steven J. Franks. Early in my graduate career my previous advisor left the field of science and I was challenged with finding my way as a second year graduate student. Josh, a forward-thinking and open-minded scientist, reached across biological systems to the plant kingdom and provided an intellectual home for me in his yeast genomics lab. Simultaneously, Steve reached across schools, advising me from Fordham University while I continued to attend Stony Brook University. Despite this distance, he was a incredibly accessible, inspiring me with one of the most applied and exciting evolutionary ecology systems that I had encountered, *Brassica rapa* and its rapid evolution to a natural climate fluctuation. Together, Josh and Steve have been central to my development as a scientist. Their expertise and guidance was at times overlapping and at times complementary. Much of the breadth of knowledge that I have acquired I attribute to them. Steve provided crucial guidance in the study system, fieldwork, greenhouse experiments, and analysis; while Josh provided guidance in laboratory work, genomics, and analysis. Furthermore, they helped me to think, write, and speak with greater clarity, for which I am indebted.

Together we formed a genomics group along with Nolan Kane, and his graduate student Silas Tittes, at University of Colorado. At the University of Colorado I began the process of developing genomic bioinformatic skills, learning from these masters. Nolan was incredibly welcoming and helpful, not only in all things bioinformatic, but also in providing an example of a full and balanced life as a scientist. Silas was endlessly patient, and optimistic, which was contagious in the lab.

My committee members Jessica Gurevitch and John True played a vital role in shaping this dissertation. Jessica challenged me by asking tough questions to elevate my work. She also provided guidance with experimental design and analysis. John is a true graduate student advocate, from which I, along with many other students in my department have benefitted. Additionally, his knowledge of genetics and interest in the interplay between the fields of

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I was fortunate to interact with stellar graduate students and postdocs in my program at Stony Brook and at Fordham University. In particular Megan Flenniken provided priceless comradery, and solidarity and made even the most challenging times an adventure; Aman Gill and Stephen Sabatino were always available with loads of advice and support; and the Rest lab members, especially Chris Morales, Dana Opulente, and Julius Fisher, provided constructive feedback on lab work, analysis, and presentations. At Fordham, members of the Frank's lab including Mike Sekor and Jenn Weber have provided support and feedback on manuscripts, which was greatly appreciated.

In addition to conducting research, I was also a teachers assistant and instructor for the past 5 years. During this time Marvin O'Neal was the best, most exemplary boss anyone could ask for. He was exceedingly talented at challenging and inspiring people based on their individual strengths. Marvin inspired me to conduct research on Stony Brook Undergrads as well as on my field mustard plants, opening up a totally new discipline to me.

Very early in my scientific career, as a young child, my father taught me how HIV enters the cell, why the moon has phases, and how to identify numerous migratory birds. I am a scientist because he is a scientist. Later, but still early on, Jerry Lin was my first laboratory supervisor. I'd like to thank him for being a serious stickler about my pipetting and multitasking lab skills. Additionally, Massimo Pigliucci was my first advisor at Stony Brook and helped lay a foundation on which to build my scientific thinking for which I am truly grateful.

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I received financial support during my graduate career as a Howard Hughes Medical Institute (HHMI) fellow, as well as from my department in the form of small grants. The writing of this dissertation was made possible due to an American Fellowship from American Association of University Women (AAUW), which effectively works to promote education for women and strengthen the role of women in academia.

Chapter 1. Overview

Climate change has set up an awesome and terrifying global "experiment" with complex and far-reaching ramifications. There is a large body of literature that explores the ecological and evolutionary effects of widespread climate changes (Penuelas and Filella 2001, Hoffmann and Sgro 2011, Franks et al. 2014), however, few studies examine the interplay of ecological and evolutionary variables, specifically what ecological ramifications adaptation to climate change can have. Furthermore, few studies explore the genomic basis of rapid evolution in natural populations (Mitchell-Olds and Schmitt 2006, Metcalf and Mitchell-Olds 2009, Walsh 2009). These gaps in our understanding motivated my research.

One textbook example of rapid evolution in response to a climate fluctuation is the adaptive earlier flowering of natural populations of *Brassica rapa* L. (syn. *campestris*) (Brassicaceae), or field mustard, in southern California in response to a natural extended drought (Franks et al. 2007). Drought is increasing in frequency and severity with climate change (IPCC 2007) and is having widespread effects on fitness and yield in natural and agricultural systems, for example, in many plant systems it is expected to have the ecological effect of an increase in disease susceptibility (Ayres 1984, Paul and Ayres 1987, Coakley et al. 1999). Altered temperature and precipitation could favor pathogens in three main ways: 1) by acting directly on infectious agents, such as bacteria and viruses, for example, warmer temperatures increasing bacterial growth, 2) by affecting vectors of diseases or other community interactions, such as increased precipitation increasing the populations of mosquitoes carrying malaria or dengue fever (Patz et al. 2005), or 3) by acting on host physiology, such as drought or temperature stress weakening immunity (Desprez-Loustau et al. 2006). The ecological and evolutionary effects of drought, the evolutionary-ecology dynamics, and the genomics of drought adaptation are of grave importance.

The exploration of these complex and diverse factors necessitates the integration of multiple approaches. Fortunately, in addition to traditional ecology and evolutionary biology

study approaches, the advent of affordable whole genome sequencing has allowed for the study of the underlying genetics of evolutionary-ecology questions. I explored the evolutionary-ecology of the *B. rapa – Alternaria brassicae* fungal plant pathogen system under climate change using different approaches including 1) field work to explore the ecological factors that affect the disease susceptibility of *B. rapa* to its fungal pathogen *A. brassicae*, 2) greenhouse work using a resurrection approach to determine if evolution in response to drought affects disease susceptibility, and 3) genomics work to explore the genomics of rapid evolution in natural populations.

The three interrelated goals of this dissertation are to explore the biotic factors that govern *B. rapa*'s susceptibility to *A. brassicae* in natural populations, determine how adaptation to changes in these biotic factors affect disease susceptibility, and explore the underlying genetics of these evolutionary ecology dynamics. These goals were tested in the three studies outlined in the three chapters below.

Chapter 2: In a field study, including multiple sites on the west coast of the U.S., I mapped the distribution of *B. rapa*, collected data on abiotic and biotic factors including host density, developmental stage, height, soil moisture, and Alternaria blackspot disease severity. I analyzed the association between these variables and disease severity and found a positive association between soil moisture and disease severity. However, the relationship between soil moisture and disease varied among locations and over time, and the factors influencing disease severity in the field are complex.

Chapter 3: To determine how adaptation to drought affects disease susceptibility, I used a "resurrection approach" greenhouse study (Franks et al. 2008). The *B. rapa* seed that was used in this study was collected from a natural population in southern California in 1997, before an extended drought, and then again from the same location in 2004, following the drought. An adaptive evolutionary shift to earlier flowering following the drought had been previously demonstrated in this collection (Franks et al. 2007). In the current study, ancestral and descendant seed was grown together under common conditions and inoculated with field

collected *A. brassicae*. I found an evolutionary shift to earlier flowering, and an evolutionary increase in disease susceptibility, indicating a cost of the drought adaptation. I found that that earlier flowering plants had thinner leaves, which are more easily invaded by fungal pathogens, indicating a potential mechanism for the increase in susceptibility observed.

Chapter 4: To explore the genetic basis of the evolution in disease susceptibility observed, I conducted whole genome shotgun sequencing on 205 ancestral and descendant *B. rapa* plants. I found considerable genetic differentiation across the genome, consistent with a rapid evolutionary response to drought at multiple loci. A number of significantly differentiated genes are known to be involved in necrotrophic fungal pathogen response, indicating that they might have contributed to the evolution in disease susceptibility. Additionally, a number of genes that are known to be involved in drought and pathogen response evolved, indicating that antagonistic pleiotropy might have played a role in the evolution of disease susceptibility observed. Site frequency spectrum analysis indicated that selection appears to have acted on standing genetic variation (soft sweeps). The results of this study shed light on the genetic basis of rapid evolutionary responses to a change in climatic conditions.

I took differing and complementary approaches in these studies to address the factors that influence disease in a natural system. The questions I could address, and the extent to which I could manipulate and control experimental variables differed, due to the differences among these approaches. Chapter 2 was conducted solely in the field and was observational with no factors manipulated. While I found interesting patterns, this study ultimately led me to conduct a greenhouse experiment (chapter 3) in a more controlled environment in order to experimentally address hypotheses I had generated. In my greenhouse work, I measured evolution in phenotypes and determined a possible functional role of leaf structure in the evolution of disease susceptibility. However, it was not until I examined the genomic sequences in chapter 4 that I was able to explore this evolution at the molecular level and determine that pleiotropy might play a role in the increase in disease susceptibility observed.

In addition to methodological approach, these studies differed in spatial and time scales. My field studies, chapter 2, encompassed four natural populations along the West Coast, while

the greenhouse and laboratory work, chapters 3 and 4, focused on one population. Chapter 2 also incorporated spatial heterogeneity as a factor, which adds a level of complexity to natural systems in the field (Gilbert 2002, Antonovics 2004, Alexander 2010). Chapter 2 was conducted over the course of a growing season (2011) as an ecological field study. In contrast, in chapters 3 and 4 I utilized a resurrection approach using ancestors and descendant plants, spanning 7 years, focusing on evolution in this system and the ecological ramifications of an evolution shift. This dissertation contributes to growing body of literature (Hairston et al. 2005, Pelletier et al. 2009, Basser et al. 2010) that demonstrates that evolution can take place rapidly enough to have ecological effects. As demonstrated in this dissertation, complex biological questions necessitate diverse and complementary study approaches.

Chapter 2. Factors affecting the disease severity of Alternaria blackspot in natural *Brassica rapa* populations on the California and Oregon coasts

ABSTRACT

Abundant water availability is largely cited as the driving cause of severe foliar fungal disease in many plant systems. However, other ecological factors, such as host density, host developmental stage, and microclimatic conditions also play pivotal roles in disease severity, especially in natural populations in which these factors are not artificially controlled. Predicted increases in fungal disease associated with ongoing changes in precipitation accompanying climate change necessitate resolving the role of water availability and other ecological variables in determining disease severity of foliar fungal diseases.

The West Coast of United States is an agriculturally important region, which displays a gradient of water availability from wet to dry moving from north to south. In this study, I took advantage of this gradient to study an agriculturally important annual, herbaceous plant, *Brassica rapa*, in natural, feral populations. Using this plant system, I explored the spatial, host, and environmental variables, including soil moisture, throughout the growing season that affect disease severity of a *B. rapa* foliar fungal disease, Alternaria blackspot. Data were collected in a spatially structured manner at two time points during the summer of 2011 from four *B. rapa* plant populations along the California and Oregon coasts. Generalized linear models (GLM) were built to determine the factors that vary with disease severity in these populations.

In agreement with much of the literature I found that soil moisture and disease severity were positively associated, however, this was only early in the growing season. Later in the growing season, I found that other factors such as sun exposure, herbivory, and host density drove disease severity. I also found that the relationship between soil moisture and disease varied among locations at both the early and late time points. Together, these findings suggest that there are many factors influencing fungal disease in this natural, herbaceous plant system and timing of data collection can be of great importance in unraveling these factors. Predicting

the effects of climate change on plant disease should be based on many factors in addition to water availability.

INTRODUCTION

Plant pathogens are detrimental to plants, substantially reducing crop yield in agricultural systems, as well as influencing species distributions, population dynamics, community structure and evolution in natural systems (Burdon et al. 2006). The disease severity of foliar fungal pathogens is largely thought to be driven by environmental factors, primarily water availability. However, disease dynamics in natural populations are exceedingly complex and other factors such as host density, host developmental stage, and other microclimatic variables such as sunlight have also been cited (Agrios 2005). Resolving the factors that drive disease severity is an ongoing and high priority endeavor for both agricultural and natural plant systems.

Since the environment plays a pivotal role in plant disease prognosis, recent research has focused on how climate change, including how increases in temperature and altered precipitation patterns (IPCC 2007) will affect plant disease dynamics. Generally, climate change is expected to increase infectious disease in many plant ecosystems (Ayres 1984, Paul and Ayres 1987, Coakley et al. 1999); warmer temperature or precipitation might increase the rate of pathogen growth, increased precipitation might increase vector populations (Patz et al. 2005), and water or temperature stress might weaken host immunity (Desprez-Loustau et al. 2006). Furthermore, increased susceptibility to pathogenic disease can occur as a cost of adapting to climatic changes such as drought (chapter 3).

In this study, I utilized natural plant populations, growing along a water availability gradient down the West Coast of the United States, to determine the relative importance across the growing season of water availability versus other host and ecological factors (i.e. host density, host developmental stage, herbivory and sunlight) in determining disease severity

of a foliar fungal pathogen. I focused on an economically important plant species, *B. rapa* L. (syn. *campestris*)(Brassicaceae, field mustard) and its common foliar fungal disease Alternaria blackspot, which is caused by *Alternaria brassicae* (Conn et al. 1990). This plant-pathogen was chosen because the host, *B. rapa,* is an important crop species (bok choi, napa cabbage, oilseed, turnip), which forms feral populations that inhabit a wide variety of environmental conditions. The pathogen, *A. brassicae,* is an important pathogenic sac fungus that causes damping off, leaf spots, and defoliation, and infects most cruciferous crops worldwide, severely decreasing crop yield (Rotem 1994, Meena et al. 2010). Many ecological factors affect Alternaria blackspot disease. Host density has been shown to have a positive relationship with disease levels, as has host developmental stage, with older plants being more diseased. In addition, rainfall/moisture has been shown to have large effects, with the highest levels of disease reported in areas of high rainfall (Humpherson-Jones and Phelps 1989, Rotem 1994, Agrios 2005, Meena et al. 2010).

Utilizing natural populations of *B. rapa* growing along the West Coast moisture gradient I asked: does water availability drive disease severity in this natural plant pathogen system? Or do other ecological, host, or spatial factors drive disease severity? Specifically, I based on the literature, I expected three main factors to drive disease severity, which I tested in the following hypotheses: (1) I hypothesized that disease severity patterns for this fungus are driven by water availability to a greater extent than other ecological variables (e.g. herbivory and sunlight) and I predicted that disease severity is greater in regions with greater rainfall, while within field locations disease severity is greater in the plots with higher soil moisture than the drier plots (Humpherson-Jones and Phelps 1989, Rotem 1994). (2) I hypothesized that disease severity increases with plant density with larger, denser patches that are closer together supporting greater disease severity (Burdon and Chilvers 1982). (3) I hypothesized that plant developmental stage is positively correlated with disease severity, and predicted that plant quadrats that are older on average also have greater disease severity (Rotem 1994). To explore these hypotheses, I collected data on host and ecological factors and measured

Alternaria blackspot disease severity in four populations of *B. rapa* at two time points during the growing season of 2011.

MATERIAL AND METHODS

FIELD LOCATIONS

Field locations were chosen for this study along the California and Oregon coasts to represent a range of environmental variables, including rainfall. Two locations in central California (Muir Beach and Bodega Bay), and two locations in Oregon (Newport and Cape Perpetua) were chosen. All field locations were on the coast and ranged from relatively dry in central CA (~100 cm/year), to wetter in central Oregon (~200 cm/year) based on precipitation data from 1961 to 1990 from NOAA Cooperative stations and USDA-NRCS SNOTEL stations (Daly et al. 1994)(Table 1). These locations all also had considerable coastal fog. Soil at all locations was well drained (USDA web soil survey Feb 22, 2012).

SAMPLING

Sampling was conducted in May and July during the 2011 growing season, in a spatially structured manner (as described below), at all field locations, hereafter referred to by location codes CA1, CA2, OR1, and OR2 (Table 1). Each population was mapped using an engineering compass and meter tape. Size of host patches and populations were determined by measuring patch sizes in ImageJ (Schneider et al. 2012). To facilitate the analysis of patch size data, which had a few extreme outliers, patches were categorized into four size bins.

To explore how ecological variables affect disease severity, I collected the following data from at least 10 quadrats in each population, selected at random, from within host patches: Alternaria blackspot disease severity (% of quadrat with host tissue displaying symptoms) was assessed visually by two independent researchers and averaged. Alternaria blackspot symptoms were identifiable in the field, characterized by brown necrotic spots surrounded by chlorosis (Figure 1). Plant tissue was also collected from all field locations and symptoms were

verified to be caused by *A. brassicae* by identification of spore morphology by the Plant Pathology Center at Oregon State University. I also collected data on host density (% cover in each quadrat assessed visually), stems per meter (count of the number of plants in each quadrat), height of host (average height in each quadrat), developmental stage of host (average developmental stage in each quadrat classified as either young, flowering, fruiting, or senescing assessed visually), herbivory, (damage visually assessed), level of sun (visual assessment of amount of shade), and soil moisture in each quadrat (measured by TDR and verified gravimetrically as described below). Visual assessments were independently collected by two researchers and averaged. Distance between host patches was a spatial factor that was used in the analysis that was determined by measuring the area of neighboring patches that fell within 3 m of the edge of each patch. The 3 m distance was chosen because *Alternaria brassicicola*, a closely related fungus with similar means of dispersal, spreads by water splash primarily within that distance (Chen et al. 2003). Similarly to patch size, distance between host patches was grouped into 5 bins for analysis: less than $12m^2$ of neighboring patches fell within 3m of the patch of interest, $12m^2$ - $15m^2$, $15m^2$ - $18m^2$, $18m^2$ - $20m^2$, and greater than $20m^2$.

To obtain measurements of soil moisture in each quadrat, I used a Field Scout TDR 100 Soil Moisture Meter (Spectrum Technologies, Inc.) with 10 cm probes. TDR readings were verified gravimetrically using a 25 cm soil core (Black 1965). All field data including soil moisture were collected on the same day at each location. Since time since last rainfall varied between field locations, soil moisture measurements were not used to compare between populations. A comparison of soil drainage by USDA web soil survey showed that all field locations had similarly well-drained soils (Table 1).

ANALYSIS

My goal was to determine the effects of multiple factors on plant disease, therefore I conducted analyses using generalized linear models. I first created one model including all factors measured and all locations (full model). Based on this full model, I ran a stepwise regression to determine the factors that significantly improved the fit of the model to these

data (reduced model). I then conducted additional analyses to look at specific factors of interest in greater depth. My experimental design was hierarchical, and I took this into account in my analyses by nesting location within region. However, region did not improve the fit of the model so it was dropped from the reduced model.

ENVIRONMENTAL, SPATIAL, AND HOST VARIABLES RELATIONSHIPS WITH DISEASE SEVERITY

To determine how disease severity was influenced by the environmental, spatial, and host factors for which data was collected, two generalized linear models (GLM) with a Gaussian distribution were constructed, one for the early season collection, and one for the late. Separate early and late models were constructed since disease severity patterns and explanatory variables varied greatly between time points, and I was interested in exploring these patterns individually. All analyses were conducted using R 3.0.1 (R Core Team 2013). Preceding analysis, data were transformed to meet the assumptions of an ANOVA (Table 2). A Shapiro-Wilk Normality test was performed on all data following transformation to verify a normal distribution.

To determine the importance of each explanatory variable in predicting the response variable (disease severity), the model was fitted with all explanatory variables for which data were collected (location, region, stage, stems per meter, coverage, level of sun, soil moisture, herbivory, height, size of patch, distance between host patches). Interactions were included in the model between location and soil moisture, since I expected the distribution of soil moisture to vary with local rainfall. I then ran a bidirectional stepwise regression using the Akaike Information Criterion (AIC) score to select the best, reduced model.

For the early time point, the explanatory variables that were included in the reduced model were location, soil moisture, host height, herbivory, size of host patch, level of sun exposure, distance between host patches, with an interaction between soil moisture and location. For the late time point, the explanatory variables included were location, host density

(cover), host height, herbivory, level of sun exposure, developmental stage of host, and size of host patches.

THE EFFECTS OF TIME AND SOIL MOISTURE ON DISEASE SEVERITY

I predicted that disease severity increased with soil moisture and also increased over the course of the growing season. To test this I conducted additional analyses to look at the relationship between time in the growing season, soil moisture and disease severity individually. To determine how disease severity varied over time and across locations, I conducted a 2-way ANOVA with location and time as factors, and log transformed disease severity data as the independent variable. A Tukey HSD post-hoc analysis was used to determine which locations were significantly different from each other. To determine if disease severity varied with soil moisture within locations, I regressed disease severity on soil moisture for each location using transformed TDR values (Table 2), with early and late time points analyzed separately.

RESULTS

MULTIPLE FACTORS INFLUENCE DISEASE

I found that multiple factors were associated with disease severity in this natural plant pathogen system. Surprisingly, I found that region did not influence disease severity, so this term was dropped from further analysis. However, time did influence disease severity, with severity increasing later in the season as predicted, thus, the two time points were analyzed separately. The early time point model explained 64.4% of the disease severity variation observed. Early in the season, I found that soil moisture was significantly correlated to disease severity (GLM: $p=0.041$), with wetter soil supporting greater disease severity (Table 3, Figure 2A). I also found that location did not play a role in disease severity, although I found an interaction between location and soil moisture (GML: $p=0.006$, Table 3). Host height and size of the host patch were found to show a trend of an effect on disease severity (GLM: $p=0.097$ and $p=0.067$ respectively, Table 3, Figure 2A).

The late season model explained 65.7% of the disease severity variation observed. For this model I found that host density (% cover of host) was significantly correlated with disease severity, with denser areas supporting greater disease severity (GLM: p=0.013, Table 3, Figure 2B). I also found that location played a significant role in disease severity (GLM: p=0.013, Table 3, Figure 2B). Host height was found to have a significant, inverse relationship with disease severity (GLM: p=0.043), while herbivory was found to have a highly significant inverse relationship with disease severity (GLM: p=0.001, Table 3, Figure 2B). Level of sun exposure had a significant effect on disease severity (GLM: p=0.001), with the highest disease severity being found at the extreme levels of sunlight (either very shaded or very high levels of sun)(Table 3, Figure 2B). Finally, host developmental stage was found to show a trend correlated with disease severity, with older plants being more diseased (GLM: p=0.075, Table 3, Figure 2B).

TIME AND DISEASE SEVERITY

I found considerable variation in disease severity across all locations and time points. While Alternaria blackspot symptoms were widespread, with 22.2 % of *B. rapa* showing symptoms (Table 4), the percent of host vegetation that was infected at the late time point varied considerably from 21.9% at CA2 to 35.2% at CA1. OR1, which had by far the largest population of *B. rapa* (8,081.79 m^2), also had by far the largest pathogen load with 1,681.2 m^2 of host vegetation showing disease symptoms.

In determining the effect of time on disease severity, I found that disease severity was significantly higher at the later time point (2-way ANOVA and a post hoc Tukey's HSD test: p=0.003), with an increase in average disease severity from 17.1% tissue infected at the early time point to 28.0% at the later time point (Figure 3A). While there was considerable variation between locations, location and the interaction between time and location did not have a significant effect on disease severity (Figure 3B).

SOIL MOISTURE AND DISEASE SEVERITY

Since microscale variation in soil moisture and disease were of particular interest, I examined the relationship between soil moisture and disease severity at each location separately. The correlation between disease severity and soil moisture was determined by regressing disease severity on soil moisture. This relationship varied across field locations and time points (Table 5, Figure 4). At the two locations in California, CA1 and CA2, for both time points this relationship was not significant, however, for the two locations in Oregon for the early time point, disease severity showed a trend, and was significantly correlated to soil moisture (regression; $p=0.086$ and $p=0.015$ respectively). For OR1, soil moisture explained 24.4% of the variation and the relationship was inverse, while for OR2, soil moisture explained 54.7% of disease variation and the relationship was direct.

DISCUSSION

In this study, I found that Alternaria blackspot disease symptoms were widespread at all of my field locations throughout the growing season (22.2% *B. rapa* tissue infected, Table 4). However, I found that disease severity varied widely with time, and location, as well as environmental and host factors. I collected data on variables determined in other studies to affect disease severity of foliar fungal pathogens and found that the factors that had the greatest effect on disease severity of this fungal disease were time in the growing season, location, soil moisture, host height, sun exposure, herbivory, and host density (cover). These factors explained 64.4% of the variation in disease severity observed at the early time point, and 65.7% at the late time point (Table 3). Unexplained variation in disease severity could be due to other environmental variables that were not measured. This was an observational study, with environmental factors not manipulated, so the results are suggestive of relationships between specific factors and disease severity but do not definitively establish causation. The explanatory variables with significant association with disease severity are each addressed below.

DISEASE SEVERITY OVER TIME

I found that disease severity was significantly worse later in the season (Figure 3), with an increase in average disease severity from 17.1% tissue infected at the early time point to 28.0% of tissue infected at the later time point, a pattern common in disease studies (Madden and Hughes 1995). More specifically, studies on *Alternaria* fungal infection in *Brassica* species show that older plant tissue exhibits higher disease severity (Mridha and Wheeler 1993). Studies have shown that increases in disease severity occur later in the season due to weakened defenses at later developmental stages of the host plant (Rotem 1994), which I considered as one possible explanation for the pattern observed. Alternatively, an increase in disease severity over time could simply be due to the prolonged length of time that the fungus has to establish and spread. I did not find a significant correlation between developmental stage and disease severity, which indicates length of time spreading in the populations might have been responsible for this temporal disease severity pattern, however, experimental studies would need to be conducted to test this.

I found that disease severity diverges between locations over the course of the season, so that location has a much more significant effect on disease severity later in the season. In particular, there was a pronounced increase in average disease severity from the early to late time point for my two largest field locations, CA1 and OR1 (Figure 3). These larger populations increased in average disease severity by 15.2% (CA1) and 15.0% (OR1), while the smaller populations only increased by 7.7% (CA2) and 5.5% (OR2). These findings, along with the finding that my two largest field locations, CA1 and OR1, had the greatest area of infected tissue (Table 4), indicate that a larger host population might support a greater amount of infection. This is important when considering that feral crop plants, such as *B. rapa* can act as a disease reservoir for closely related crops, such as canola (Burdon and Thrall 2008). My findings support the possibility that the size of neighboring feral populations could be important in the amount of disease they can harbor.

ENVIRONMENTAL VARIABLES INCLUDING SOIL MOISTURE AND DISEASE SEVERITY

I found a significant positive correlation between disease severity and soil moisture for the early time point (Table 3, Figure 2). My findings are in agreement with many drought- foliar disease studies, and might be due to the fact that moisture is needed for the dispersal and growth of such fungal spores. Alternaria species have been shown to need high relative humidity in order to germinate as well as splash to disperse (Humpherson-Jones and Phelps 1989, Rotem 1994). Furthermore, I found that the interaction between location and soil moisture had a significant effect on disease severity for the OR1 location (Table 3). Interestingly, soil moisture dropped out of the model for the later time point, which suggests that soil moisture mediates disease severity to a greater extent earlier in the growing season.

I looked at the relationship between soil moisture and disease severity at each location by regressing disease severity on soil moisture, and I found that this relationship varied across location and time point (Table 5, Figure 4). At three of the locations, CA1, CA2, and OR1 this relationship was not significant, however, for one location, OR2, disease severity was positively significantly correlated to soil moisture explaining 54.7 % of the variation seen. These findings suggest that the relationship between moisture and disease depends on multiple factors, which vary at each location, and suggests that water availability might not be driving disease severity in this plant pathogen system in many locations.

The level of sun exposure had a significant effect on disease severity at the later time point (Table 3, Figure 2), but was not significant for the early time point. For the later time point it was found that disease severity was highest for plants that received extreme amounts of sunlight (either very shaded or very high levels of sun). The increase in disease severity under low sun exposure could be explained by the inverse relationship between moisture and sun exposure, with a high level of moisture at the microclimate level supporting spore germination and spread. The increase in disease severity under high levels of sun exposure could be explained by the fact that an excess of light can stress plants and weaken their defenses (Agrios 2005). However, these factors need to be explored further experimentally.

I expected that disease severity and herbivory would be positively associated, in agreement with many plant pathogen studies (Kennedy and Barbour 1992, Simms and Rausher 1993). In contrast, I found that herbivory had a highly significant and negative correlation with disease severity at the later time point (Table 3, Figure 2). It is important to note that the relationship between disease severity and herbivore damage is complex and is mediated by many factors, including defensive compounds produced by the plant, and food preference of the herbivores (Taiz and Zeiger 2006). In agreement with my finding, another study in *B. rapa,* exploring the relationship between herbivory by the gall midge, and Alternaria fungal infection, found that plants that had the highest level of fungal infection were preyed upon the least, which might have been due to food choice (Nakamura et al. 1995). An experimental study would need to be conducted to determine whether I am seeing a pattern produced by food choice, or the effects of the microenvironment on both the pathogen and herbivores in this plant pathogen system.

SPATIAL FACTORS AND DISEASE SEVERITY

I found that host distribution played a role in disease severity. For the early model there was a trend of an effect of host patch size on disease severity (Table 3, Figure 2). Additionally, there was a significant positive correlation between disease severity and host density (% cover) for the late time point (Table 3, Figure 2), which supports the findings in the literature and could be due to the fact that an increase in the density of the host eases transmission of the pathogen (Burdon and Chilvers 1982). The density of the host dropped out of the early model, which suggests that density plays a greater role once infection has been established and is spreading, as one would expect later in the season.

HOST VARIABLES AND DISEASE SEVERITY

I did not find considerable effects of host variables on disease severity at the early time point. For the later time point I found that host height was significantly negatively correlated

with disease severity, while host developmental stage only showed a trend, which was positively correlated with disease severity (Table 3, Figure 2). The inverse relationship between host height and disease severity observed could be due to the fact that the tallest plants lost leaves once they reached a late developmental stage, which made collecting disease severity data at the very late stage and height difficult. This collection issue might also explain the lack of a significant correlation between disease severity and developmental stage, which I expected to see based on the literature (Rotem 1994, Vloutoglou and Kalogerakis 2000). This relationship is being explored further in greenhouse studies. However, these initial host findings indicate that the host state is not driving disease severity but the system is instead responding to environmental variables and time. This indicates that the effects of climate change might work through the environment rather than stressing the plant and working through the host.

SUMMARY

I found that over the course of a growing season the factors that drive disease severity of this fungal foliar pathogen vary, with soil moisture playing a greater role earlier in the season and level of sun exposure, herbivory, host density, and host height playing a greater role later in the season, when disease severity increased. This study illustrates that factors influencing disease severity in this natural plant pathogen system are complex, and that the timing of data collection can be of great importance in unraveling these factors. It also indicates that predicting the effects of climate change on plant disease should be based on many factors in addition to water availability.

Table 1. Characterization of field locations including precipitation and soil type. Precipitation data averaged over 10 years was acquired from NOAA Cooperative stations and USDA-NRCS SNOTEL stations. Soil data was obtained from the USDA.

Location Info	Location	Region	Precipitation (cm/year)	Soil type	Soil drainage
Muir Beach	CA1	Central CA	101	Cronkhite (40%) and Barnabe (30%)	Moderately well-drained
Bodega Bay	CA2	Central CA	101	Baywood loamy sand (85%)	Very well-drained
Newport	OR1	Central OR	203	Neskowin-Salander silt loams (90%)	Well-drained
Cape Perpetua	OR2	Central OR	203	Neskowin-Salander silt loams (90%)	Well-drained

Table 2. Description of data and transformations conducted to meet assumptions of an ANOVA. Conducted a Shapiro-Wilk Normality test on all data following transformation to verify normal distribution.

Variable	Method of Collection	Transformation	Type of data
Disease severity	Visually- % host tissue in quadrat with symptoms	Log	Continuous
Location		None	4 classes
Region		None	2 classes
Host Devel. Stage	Visually-average stage of host in quadrat	Log	Continuous
Stems per meter	Counted per quadrat	Log	Count
Soil moisture (a)	TDR	Arcsine square root	Continuous
Soil moisture (b)	Gravimetric water content	Log	Continuous
Level of Sun	Visually- by amount of shade on quadrat	None	4 classes
Host density (% cover)	Visually- per quadrat	Arcsine square root	Continuous
Host height	Averaged up to 5 host plants per quadrat	None	Continuous
Herbivory	Visually- per quadrat	Log	Continuous
Size of Host Patch	Mapped and measured by Image J	None	6 classes
Distance between patches	Calculated based on area of neighboring patch in 3 meter radius around each patch	None	4 classes

Table 3. Results of GLM models for early and late season data exploring the factors affecting disease severity. R^2 reported is Nagelkerke R^2 index. Only variables with trends and significant values are shown.

	Df	Chisq	P value	Regression coefficients
Early Season Model				
Soil moisture (gravimetric)	1	4.195	0.041 *	0.582
Height	1	2.749	0.097.	0.446
Size of host patch	3	7.156	0.067.	
Location x soil moisture	3	12.297	0.006 **	
Null deviance: 6.016 on 49 degrees of freedom; residual deviance: 2.862 on 30 degrees of freedom; R^2 = 0.644				
Late Season Model				
Host density	1	6.172	0.013 *	0.011
Location	3	10.738	0.013 *	
Host height	1	4.114	0.043 *	-0.565
Herbivory	1	10.988	0.001 ***	-0.627
Level of sun	3	15.615	0.001 **	
Host developmental stage	1	3.166	0.075 .	5.764
Null deviance: 3.605 on 34 degrees of freedom; residual deviance: 1.266 on 21 degrees of freedom; R^2 = 0.657				

Table 4. Characterization of populations by area. Area of population determined by mapping population and measuring in ImageJ. Host stems per m^2 and disease severity were calculated by averaging across quadrats at each location at the late time point.

Location	Total Area of *B. rapa* (m^2)	# of Host Stems per m^2 (± SD)	Disease Severity at Late Time (± SD)
CA1	2,502.36	13.5 (± 7.4)	35.2% (± 32.7)
CA2	1,415.94	20.6 (± 15.3)	21.9% (± 21.1)
OR1	8,081.79	7.8 (± 4.7)	26.5%(± 21.1)
OR2	139.43	4.3 (± 2.1)	28.0%(± 13.9)

Table 5. Disease severity regressed on soil moisture taken by TDR. Regression was run on transformed data (Table 2).

Location	Time	DF	F value	P value	Correlation (multiple R-squared)
CA1	Early	1	1.161	0.297	0.068
	Late	1	1.583	0.237	0.137
CA2	Early	1	0.613	0.452	0.058
	Late	1	0.122	0.734	0.013
OR1	Early	1	3.553	0.086 .	0.244
	Late	1	0.030	0.866	0.003
OR2	Early	1	9.647	0.015 *	0.547
	Late	1	0.219	0.652	0.027

Figure 1. Characteristic Alternaria blackspot lesions on a *B. rapa* leaf.

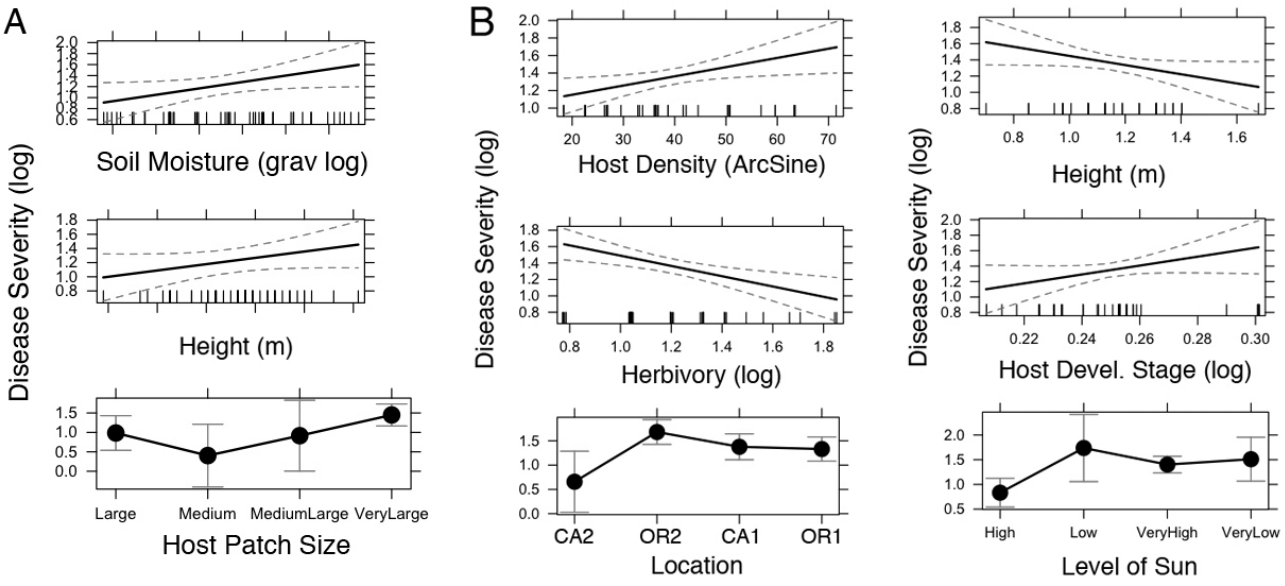

Figure 2. The effect of explanatory variables, which are significant or showing a trend, on disease severity for early (A) and late (B) GLM models. Dashed lines show the 95-percent confidence interval.

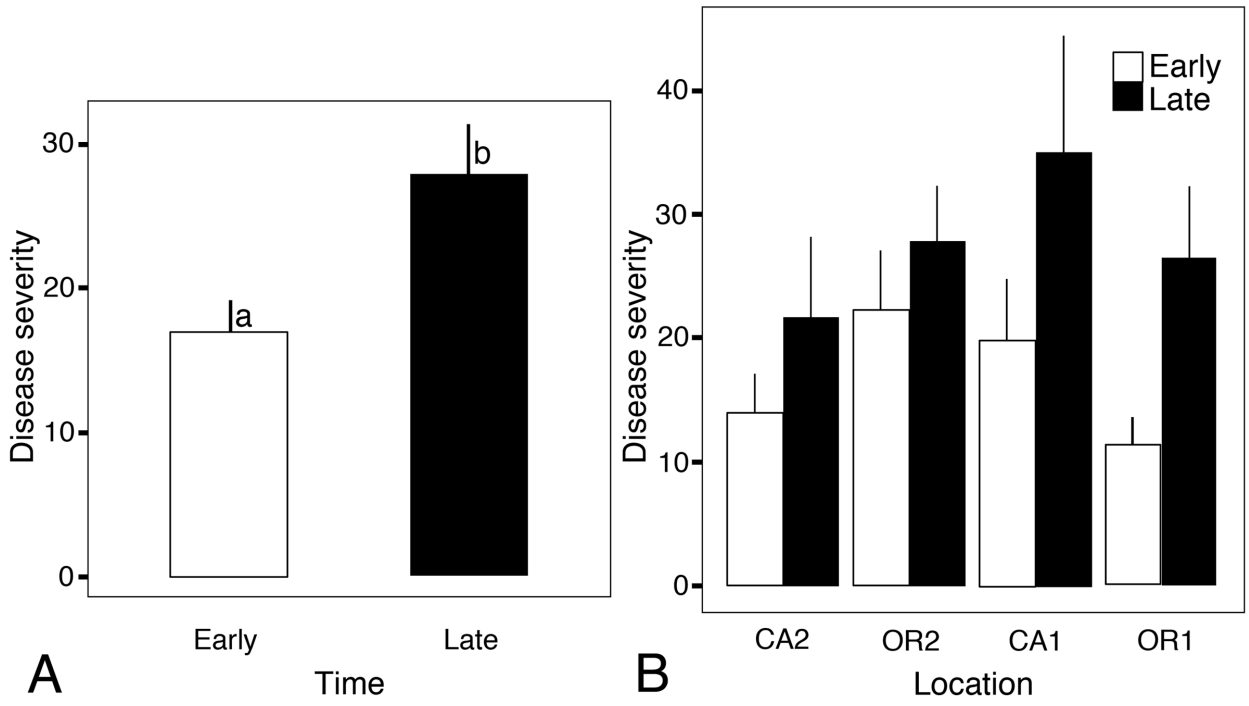

Figure 3. Results of 2-way ANOVA with disease severity as independent variable and time and location as factors, followed by a post hoc Tukey's HSD test. Analysis conducted on log transformed disease severity data with untransformed data shown with (±SEM) A) Disease severity was significantly higher at the later time point, as indicated by the letters over the bars ($p=0.003$). B) Location at both early and late time points had no significant effect on disease severity.

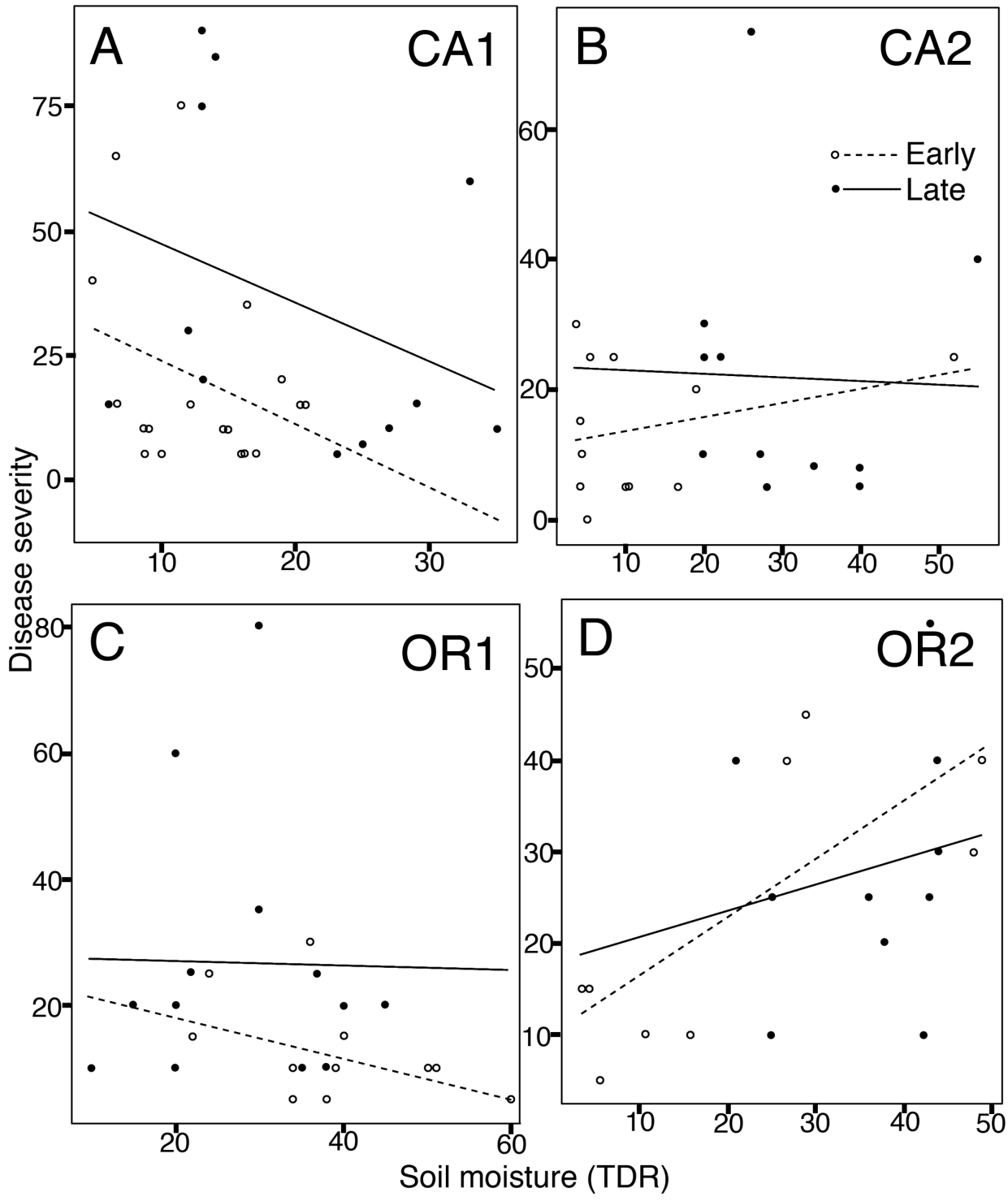

Figure 4**.** Disease severity regressed on soil moisture (taken by TDR) at each field location with early and late time points shown. Scatter plot shows untransformed data. Correlation and significance values were calculated using transformed data (Table 2). A) Shows CA1, for which the relationship was not significant. B) Shows CA2, for which the relationship was not

significant. C) Shows OR1, for which the relationship showed a trend for the early time point (p=0.086). D) Shows OR2, for which the relationship was significant (p=0.015)

Chapter 3. Increased susceptibility to fungal disease is a cost of climate change adaptation in *Brassica rapa*

ABSTRACT

Although populations can adapt to climate change, costs of adaptation could imperil persistence. Using a 'resurrection' experiment of preserved ancestral and descendant seeds in a greenhouse, I tested whether increased susceptibility to disease was a cost of adaptation to a climatic change in a population of *Brassica rapa* (field mustard) that evolved to flower earlier following a natural drought. I found that the evolutionary shift to early flowering resulted in an increase in susceptibility to a fungal pathogen, *Alternaria brassicae*, indicating an ecological cost of adapting to drought. This cost is likely due to a trade-off between escaping drought and disease. I found evidence of selection for earlier flowering but no direct selection for disease susceptibility. The mechanism of the trade-off appears to relate to leaf structure; plants that flowered earlier had significantly higher specific leaf area (SLA) with broader, thinner leaves that were more susceptible to infection. I provide evidence that even if populations are able to adapt to changes in abiotic conditions caused by climate change, these adaptation can incur costs, such as increased susceptibility to diseases, which could make populations increasingly vulnerable.

SIGNIFICANCE

Climate change is fundamentally altering ecosystems worldwide. Populations might be able to adapt to climate change, but evolutionary responses might have costs. I used the 'resurrection approach' to investigate evolution and costs of adaptation in a well-studied case of rapid drought adaptation. I found that an evolutionary shift to early flowering in annual *Brassica rapa* plants following drought co-occurred with an evolutionary increase in susceptibility to disease caused by the pathogenic fungus *Alternaria brassicae*. This cost of adaptation appears due to a trade-off between drought and pathogen escape, caused by early flowering plants having leaves that are thinner and more easily infected by the fungus. The

global trend toward earlier flowering might thus leave plant populations more susceptible to disease.

INTRODUCTION

Ongoing changes in climate include increasing temperature and altered precipitation patterns, with drought increasing in severity and frequency over the past 50 years (IPCC 2007). These changes in climate are having widespread effects on many populations, with changes in phenology commonly observed (Parmesan and Yohe 2003). In some cases, such responses appear adaptive (Penuelas and Filella 2001, Hoffmann and Sgro 2011, Franks et al. 2014), indicating that evolution could help populations mitigate the effects of climate change. Limits to adaptation include the finite availability of genetic variation and constraints on combinations of traits imposed by genetic architecture (Etterson and Shaw 2001, Parmesan and Yohe 2003, Willi et al. 2006). Even when populations overcome limits and constraints, there might be costs of adaptation that hinder the long-term ability of populations to accommodate changes in climate. Depending on the severity of these costs, they might in turn constrain evolution. Such costs are particularly likely when there are trade-offs involving traits that are adaptive in one context and maladaptive in another (Berenbaum et al. 1986, Brodie III and Brodie Jr 1999). One such trade-off can occur between defense against natural enemies such as herbivores and pathogens, and growth or reproduction (Simms and Rausher 1987). This trade-off between growth and defense has been well studied and underlies much of plant defense theory (Mole 1994). For example, plants are thought to be under natural selection for increased growth and competitive ability at the expense of defense allocation in introduced ranges where specialist natural enemies are absent (Blossey and Notzold 1995). In this case, the adaptation conferring greater competitive ability results in a cost of decreased defense, which is only paid if natural enemies are present. Though rarely considered, costs of adaptation could also hinder the ability of populations to respond to climate change.

Prior research found a rapid adaptive evolutionary shift to earlier flowering and changes in other traits in response to a change in climate (drought) in southern Californian populations

of the annual plant *Brassica rapa* (Franks et al. 2007, Franks and Weis 2008). Here, I investigate whether these adaptive evolutionary changes have come at a cost. This is particularly likely because the early flowering plants, which were able to escape drought, allocate resources to rapid growth and development (Franks 2011), potentially leaving fewer resources available for defense. I focused on defense against a fungal pathogen, *Alternaria brassicae*, which was commonly observed in my field sites and which is known to have important effects on both wild populations and agricultural varieties of crucifers (Tewari and Conn 1993). I used a resurrection approach (Franks et al. 2008), measuring pathogen response in ancestral pre-drought (seeds collected in 1997) and descendant post-drought (seeds collected in 2004) *B. rapa* populations grown under the same conditions in the greenhouse. Each ancestral or descendant plant was grown either with or without water restriction (drought) and was either inoculated with the pathogen or given a mock inoculation. I assessed phenotypes including flowering time and fitness to determine how the adaptive evolution in response to drought in the host plant, *B. rapa*, affected disease susceptibility.

MATERIAL AND METHODS

STUDY SYSTEM

The plant-pathogen system I used is the foliar fungal pathogen *A. brassicae*, which causes Alternaria blackspot in its host *B.* rapa L. (syn. *campestris*)(Brassicaceae, field mustard) (Conn et al. 1990). The pathogen *A. brassicae* is a necrotrophic fungus that causes damping off, leaf spots, defoliation and reduced seed yield in *B. rapa* (Tewari 1991, Koike et al. 2006). Because *B. rapa* is an important crop species (bok choi, napa cabbage, oilseed, turnip, polish canola), its response to this costly and destructive pathogen has been extensively studied in agriculture (Rotem 1994, Meena et al. 2010).

B. RAPA PROPOGATION

Previous to this study, a large number of seeds (>10,000) were collected from ripened seedpods (siliques) along a transect in a natural population of *B. rapa* located on the University of California Irvine campus in May of 1997 (ancestors) and June of 2004 (descendants). Plants

were grown for a generation (about 90 days) and crossed within populations under greenhouse conditions to remove storage and maternal effects (Franks et al. 2007).

A. BRASSICAE CULTIVATION

B. rapa tissue infected with *A. brassicae* was collected from Bodega Bay, California. *A. brassicae* fungal spores were isolated from the plant tissue and identified by the Oregon State University Plant Clinic. Spore plugs were grown on carrot dextrose agar plates for one week followed by another week on carrot agar plates under 12 hrs of light and 12 of dark to encourage sporulation. Fresh spores were collected the day of inoculation, strained through gauze to remove hyphae, and adjusted to a concentration of $1X10^6$ spores/mL in distilled water and 0.05% Tween. All fungal work was conducted under sterile conditions, and was permitted under APHIS license #P526P-11-00130.

EXPERIMENTAL DESIGN

Using the seeds from the *B. rapa* crosses, I conducted a greenhouse experiment including plants grown from ancestor (N=288) and descendant (N=288) seeds. Both populations were subjected to a full-factorial combination of a pathogen treatment (either mock or with spores) and a drought treatment (either watered or drought stressed).

For cultivation, seeds were planted individually in separate 3x3x5 inch pots filled with Sunshine Mix #1 growth media (Sun Gro Horticulture, Vancouver, BC, Canada), with 14 g of slow release 14-14-14 Osmocote fertilizer and fertilized with Miracle Gro All Purpose 20-20-20 fertilizer weekly (Scotts, Marysville, OH, USA). To avoid room position effects, plants were moved around the greenhouse every 5 days. Plants were watered daily to saturation. Light hours were gradually lengthened from 12 hrs to 14 hrs to mimic the growing season. Because *B. rapa* is self-incompatible, plants were hand pollinated every three days, once they started flowering.

Plants were inoculated with *A. brassicae* by wounding 2-week-old leaves with a sterile pipette tip and then placing 10 µL of a fresh spore solution on the wound. Control plants were wounded and treated with 10 µL of 0.05% Tween. Immediately following inoculation, plants

were kept at 90% humidity for 3 days and then placed at ambient humidity and either watered or drought treated. This pattern of humidity followed by drought stress mimics conditions in the field, which are characterized by a relatively wet winter and spring and dry summers. Plants that received a drought treatment were watered to saturation every 4 days.

TRAIT MEASUREMENTS

Host susceptibility was assessed in terms of disease severity, with plants showing greater damage scored as more susceptible. The disease severities of the inoculated leaves were scored 21 days post inoculation, using a visual index (Figure S1) which ranged from 1 to 10 based on the amount of chlorosis and necrosis (Buchwald and Green 1992). Generally, disease severity scores were independently verified by two researchers and researchers were blinded to whether they were assessing ancestral or descendant plants. Infected leaves displayed a highly significant decline in health (ANOVA: $F_{1,117} = 41.34$, $p= 1.98x10^{-9}$).

I quantitatively validated the visual index with a detached leaf assay (N = 50). Prior to inoculation, fully expanded leaves were detached from plants and placed in petri dishes on filter paper pre-moistened with distilled water and inoculated following the same procedure previously described. Four days post-inoculation, leaves were cleared, stained, and visualized through a microscope. Leaves were cleared using a 1:3 acetic acid to ethanol solution and shaken overnight at a low speed, at which point the clearing solution was replaced with a second clearing solution of acetic acid, ethanol, and glycerol in a 1:5:1 ratio. After rinsing in tap water, leaves were boiled for 3 minutes in a solution of 5% Parker black ink and distilled white vinegar, and then destained using tap water that was acidified with a few drops of vinegar, followed by a 5% vinegar wash (Vierheilig et al. 1998) (Figure S2). The number of spores invading leaf tissue was counted at 100x magnification. Infected, stained leaves had an average of 9.5 (± 8.7) spores per wound, while uninfected plants were free of symptoms and spores. I also found that spore counts were correlated with the disease severity scores (Pearson correlation: $r = 0.784$, $p = 0.0002$).

Plants were monitored daily and date of flowering was recorded. The fitness of plants was measured by counting the total number of seeds produced by each plant throughout its life.

Specific leaf area (SLA), the ratio of the light capturing surface area of a leaf per unit of dry leaf mass, was also measured (Milla and Reich 2007). SLA is often altered in response to stresses and is informative of resource allocation (Cornelissen et al. 2003). In order to calculate SLA, the newest fully expanded leaf was collected from plants 58 days post planting, scanned and desiccated with silica beads. Leaf area in scanned images were measured using ImageJ (Schneider et al. 2012). SLA was calculated by dividing the leaf area of each leaf by its dry weight.

DATA ANALYSIS

To determine if evolutionary shifts occurred in flowering time and disease susceptibility, I compared ancestral and descendant plants using fixed-effect one-way ANOVAs under the optimal, well-watered condition. I then used Pearson correlation to test for correlations, and trade-offs between flowering time, disease severity, and SLA that might indicate underlying mechanisms for any shifts in traits. Previous to analysis, data were transformed (Table S1) and the residual distributions were examined using the Shapiro Test. To determine the strength and direction of selection, univariate and multivariate phenotypic selection analyses (Lande 1979, Lande and Arnold 1983) were conducted using relative seed count as an estimate of fitness and standardized values of flowering time, disease susceptibility and SLA as the traits.

All analyses were conducted using R 3.0.1 (R Core Team 2013), using the following packages: stats (R Core Team 2013) for ANOVA and Shapiro tests for normality, Hmisc (Harrell 2013) for correlations, graphics (R Core Team 2013) for drawing histograms to test for normality, ggplot2 (Wickham 2009) for drawing figures, and car (Fox and Weisberg 2011) for selection analysis.

RESULTS

I found the evolutionary shift to earlier flowering after seven generations of a natural drought was accompanied by greater disease susceptibility. Post-drought descendants, under

fully watered conditions, flowered an average of 3.2 days earlier than pre-drought ancestors (ANOVA: $F_{1, 254} = 7.628$, p= 0.006)(Figure 1*A*). This finding confirms previous studies on these populations showing an adaptive shift to earlier flowering, allowing drought escape (Franks et al. 2007, Franks and Weis 2008, Franks 2011). I also found that the post-drought descendants under fully watered conditions were 16.3% more susceptible to the pathogen than the ancestors (ANOVA: $F_{1, 130} = 22.0$, p= 6.48×10^{-6}; Figure 1*B*), indicating that an evolutionary shift also occurred in disease susceptibility. This increase in disease susceptibility appears to be a cost of adapting to drought.

My results are consistent with the hypothesis that the increase in disease susceptibility was caused by a trade-off between escaping drought (early flowering) and disease. Across all populations and treatments, flowering time and disease susceptibility are significantly negatively correlated (Pearson correlation: r = -0.35, p = 2.70×10^{-9}), with earlier flowering plants being more susceptible to disease (Figure 2*A*). I found positive total selection on both early flowering and increased disease susceptibility, as shown by significant selection differentials for both traits. However, there was direct selection (significant selection gradient) on flowering only (Table 1). This indicates that the evolutionary shift to increased pathogen susceptibility was likely a byproduct of natural selection for early flowering.

I found evidence that the mechanism of this trade-off between growth and defense relates to leaf structure. Across all populations and treatments, I found a negative correlation between specific leaf area (SLA) and flowering time (Pearson correlation: r = -0.46, p = 7.66×10^{-16}) (Figure 2*B*), and a positive correlation between disease susceptibility and SLA (Pearson correlation: r = 0.22, p = 2.70×10^{-9}) (Figure 2*C*). Therefore, plants that flower earlier have greater SLA and are more susceptible to disease.

DISCUSSION

In this study, I found that a natural population of *B. rapa* evolved earlier flowering and increased susceptibility to disease within seven generations following a natural drought. This

work adds to a growing body of evidence that rapid adaptive evolution can occur in natural populations and contribute to climate change responses (Penuelas and Filella 2001, Hoffmann and Sgro 2011, Franks et al. 2014), and that contemporary evolution can play an important role in shaping ecological interactions (Pelletier et al. 2009). As with prior work in this system (Franks et al. 2007) and other recent studies (Nevo et al. 2012, Sultan et al. 2013), the resurrection approach (Franks et al. 2008) allowed direct assessment of evolutionary change.

Unlike the shift to earlier flowering, which allowed these populations to escape drought (Franks et al. 2007), the increase in disease susceptibility did not appear to be an adaptation but rather a cost of the change in flowering time. Thus a trait (early flowering) that was an adaptation to one environmental factor (drought) directly resulted in a cost (increased disease susceptibility) in the presence of a biotic factor (pathogens). I expect that this cost of increased susceptibility is likely to be substantial in natural populations where pathogens often have severe negative effects on fitness (Garrett et al. 2006). In contrast to other limits and constraints to evolution, which have been widely studied (Etterson and Shaw 2001, Conner and Hartl 2004, Willi et al. 2006, Bridle and Vines 2007), there has been little research on such costs of adaptation (e.g. Brodie III and Brodie Jr 1999).

As previously described (Franks et al. 2007, Franks 2011), the plants in this population adapted to drought via escape by early flowering rather than tolerance through increased water use efficiency. Because early flowering plants increase their rate of development (Franks and Weis 2008), this can leave fewer resources available for defense. The trade-off between growth and defense is a central tenant of plant defense theory (Simms and Rausher 1987, Redman et al. 2001). However, unlike nutrients, which are widely considered to play a key role in patterns of allocation to growth and defense (Coley et al. 1985), the role of drought is less often considered. I show here that water availability can also be important in determining the extent to which allocation to growth or defense is favored, and that shifts in allocation patterns caused by drought could have important implications for plant defense and susceptibility to disease.

Brassicas have multiple lines of defense against Alternaria fungi, including a waxy cuticle that forms a barrier to invasion (Tewari and Skoropad 1976) and induced defense upon successful invasion, governed by multiple genes, including phytoalexins, which can impart partial, but not total resistance to the disease in *B. rapa* (Nowicki et al. 2012). I found evidence that the mechanism of this trade-off between escaping drought and disease relates to leaf structure—plants that flowered earlier had significantly higher SLA (thinner leaves). I hypothesize that plants that flowered earlier allocated fewer resources to leaves, causing them to be thinner, and consequently, more susceptible to pathogen infection. The fungal pathogen *A. brassicae* enters leaves by enzymatically degrading the cuticle and cell wall and forming specialized penetration structures, as well as entering through stomata and wounds (Tsuneda and Skoropad 1978), in fact I observed penetration of leaves at sites outside of the wound. Thinner leaves are more susceptible to infection because they present a reduced mechanical barrier to invasion (Taiz and Zeiger 2006). Furthermore, previous work (Franks 2011) has found that these early-flowering plants keep their stoma open more to increase stomatal conductance, providing another route for increased invasion. While is possible that other traits, such as induced defenses played a role in the increase in disease susceptibility observed (Siemens et al. 2009, Siemens et al. 2012), my results provide compelling evidence that leaf structure was involved in the trade-off between escaping drought and disease. This finding is particularly significant in light of the fact that trade-offs are central to ecological theory, but the underlying physical mechanisms are often unknown.

Earlier flowering is a ubiquitous response to both drought and the longer growing seasons seen globally as the climate warms (Penuelas and Filella 2001, Parmesan and Yohe 2003, Miller-Rushing and Primack 2008, Munguia-Rosas et al. 2011). My study indicates that shifts to earlier flowering and other responses to climate change might come at a substantial cost. The cost of adaptation could potentially impose a severe constraint on the long-term ability of populations to persist as climatic conditions continue to change.

Table 1. Estimates of total and direct selection on flowering time, disease susceptibility, and specific leaf area (SLA) in *B. rapa.*

Trait	**Total selection, S (S.E)**	**Direct selection, β (S.E.)**	**Quadratic selection, γ (S.E.)**
Flowering time	-0.346 (±0.045) ***	-0.377 (±0.071) ***	0.283 (±0.310)
Disease Susceptibility	0.105 (±0.047) *	0.133 (±0.074)	-0.512(±0.453)
SLA	0.115 (±0.059)	0.084 (±0.088)	-0.045(±0.457)

Shown are the total linear selection differentials (S), direct linear selection gradients (β), and quadratic selection gradients (γ; ± 1 standard error). Fitness was estimated as seed count, and traits were normalized. Parameters significantly different from zero are indicated as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table S1: Fixed factors and response variables including transformations conducted.

	Data Type	Method of Collection	Transformation	Variable Type
Fixed Factors				
Inoculation	Categorical	NA	NA	Treatment
Population	Categorical	NA	NA	Treatment
Watering	Categorical	NA	NA	Treatment
Response Variable				
Health Inoc Leaf A-disease susceptibility	Continuous	Using verified visual disease scale	Log 10	Health response
Health Inoc Leaf B	Count	Staining of fungal bodies and count using a microscope of spores invading plant tissue	None	Health response
Flowering Time	Continuous	Flowering marked daily	Log 10	Fitness/phenological
Number of Seeds	Count	Counted	Square root	Fitness response
Specific Leaf Area	Continuous	Area of leaf measured by scanning leaf and using Image J. Leaves then dried and weighed.	Square root	Physiological response

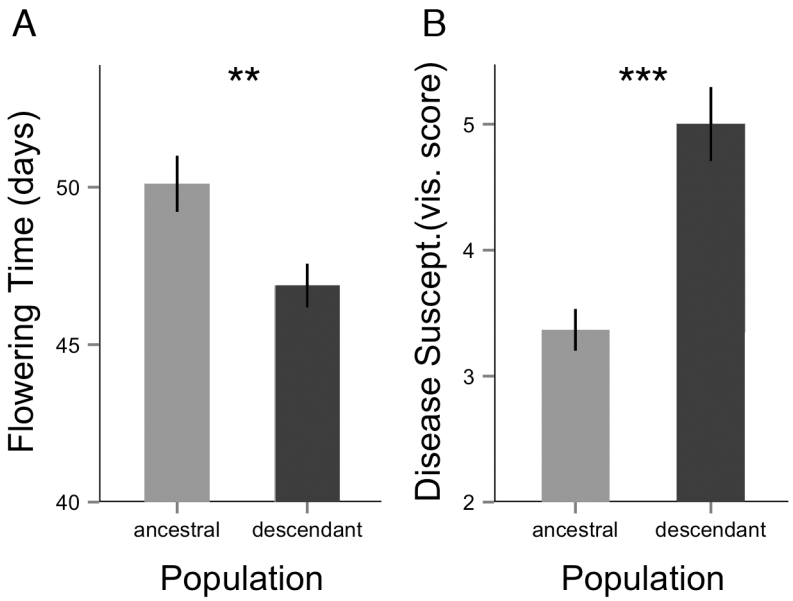

Figure 1. Concurrent evolution of time to first flowering (N = 267) (A) and susceptibility to *A. brassicae* (N = 139) (B) in *B. rapa* over seven generations in response to a drought. Shown are average trait values for ancestral (pre-drought) and descendant (post-drought) populations grown under greenhouse conditions in the fully-watered treatment. Statistical analyses were conducted on log-transformed data, with raw data shown (±SEM). Disease susceptibility was scored along a validated visual score (see methods). Significance levels determined by ANOVA: **, $P < 0.01$; ***, $P < 0.001$.

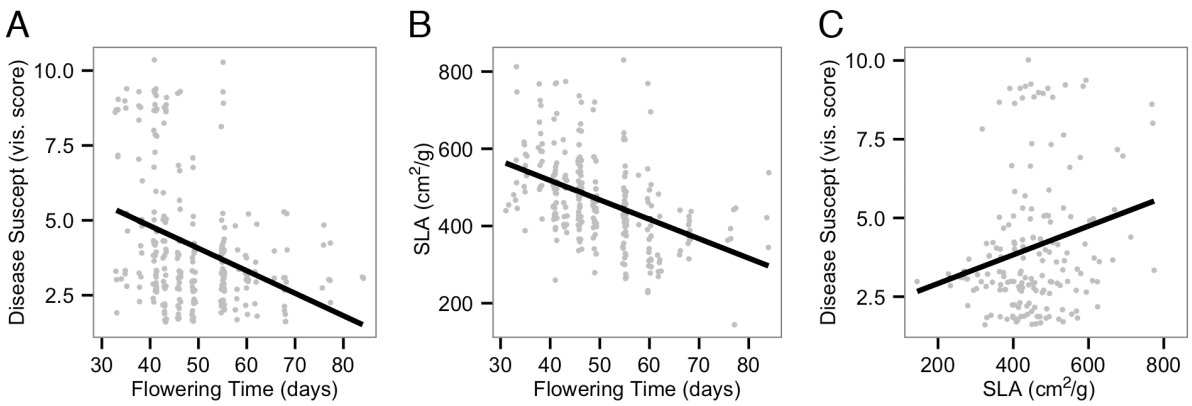

Figure 2. Plants that flower earlier are more susceptible to disease, and have higher specific leaf area (SLA). A) Relationship between disease susceptibility and flowering time for plants that flowered (N = 277), B) relationship between SLA, and flowering time (N = 280), and C) relationship between disease susceptibility and SLA (N = 277). All relationships shown have significant ($P < 0.05$) Pearson correlations on transformed data (log transformed for disease susceptibility and flowering time and square root transformed for SLA), however untransformed data are plotted.

Figure S1. The visual index that was used to measure disease severity. Generally, two researchers collected data on disease severity independently. This visual index was tested by an independent quantitative study in which inoculation sites were stained and invading spores counted. Disease severity was equated with disease susceptibility.

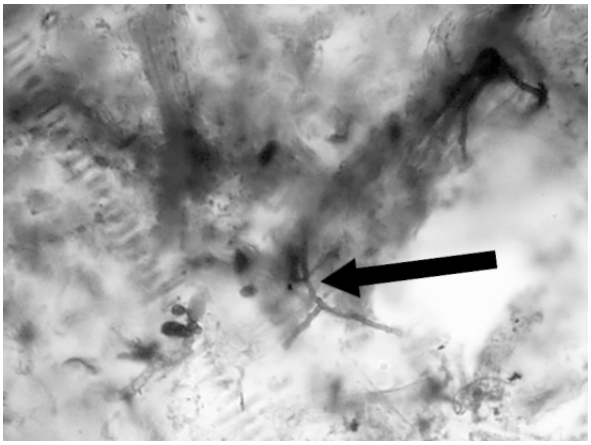

Figure S2. Image of *A. brasicae* spore invading *B. rapa* host tissue. The arrow points to a spore with an invading hypha. Other spores are visible in black. Leaves were cleared using an acetic acid solution and stained using a black ink vinegar solution. Image of cleared leaf on glass slide in distilled water taken at 100X magnification.

Chapter 4. Genome-wide signatures of an evolutionary increase in disease susceptibility in *Brassica rapa*

ABSTRACT

While populations commonly evolve in response to climate change, little is known about the genetic basis of evolution in natural populations. I took advantage of a naturally occurring drought and studied ancestral (pre-drought) and descendant (post-drought) *Brassica rapa* plants that had previously been shown to adaptively evolve earlier flowering, with a correlated non-adaptive increase in disease susceptibility to the necrotrophic fungal pathogen, *Alternaria brassicae*. Complementing previous work that explored the genetic basis of the shift in flowering time, I explored the genetic basis of the evolutionary increase in disease susceptibility by analyzing the whole genome shotgun sequences of 205 ancestral and descendant plants. I conducted outlier F_{ST} analysis to determine if known necrotrophic fungal pathogen related genes were differentiated between ancestors and descendants. I also used site frequency spectrum analysis to investigate evidence for recent selection. I found that many known necrotrophic fungal pathogen related genes evolved, and were under selection, indicating they might have contributed to the shift in susceptibility to fungal pathogens. I also found evidence for evolution and selection on known drought and pathogen response genes, indicating that pleiotropy might have played a role in the observed rapid evolutionary increase in disease susceptibility.

INTRODUCTION

Climate change has altered the evolutionary trajectory of natural plant populations worldwide (Penuelas and Filella 2001, Hoffmann and Sgro 2011, Franks et al. 2014). Despite these widespread changes, we know little about the genetic basis of evolution in natural populations (Mitchell-Olds and Schmitt 2006, Metcalf and Mitchell-Olds 2009, Walsh 2009,

Rosas et al. 2014). While many candidate genes associated with particular traits have been identified from a variety of different types of studies (e.g. association and gene expression studies), we often do not know which, if any, of these genes are responsible for phenotypic variation or responses to selection in natural populations. This limits our ability to predict evolutionary responses at individual loci. By taking advantage of evolutionary events, we can compare the genetic composition of ancestors and descendants and gain substantial information on the genes potentially involved in evolutionary changes of particular traits.

One well studied example of adaptation in natural populations is the evolution of earlier flowering in the annual herbaceous plant, *B.* rapa L. (syn. *campestris*)(Brassicaceae, field mustard) in response to a natural, extended drought (Franks et al. 2007). In addition to evolution in flowering time, I previously demonstrated that these populations experienced a genetically-based evolutionary shift to increased disease susceptibility (chapter 3). In this previous work, I used a resurrection approach (Franks et al. 2008) in which *B. rapa* seed fortuitously collected in southern California in 1997, before an extended drought, and then again in 2004, post-drought, was grown under common conditions in the greenhouse and challenged with a fungal pathogen, *Alternaria brassicea,* which causes Alternaria blackspot disease. In this way I was able to directly measure evolutionary changes and their effects. In the current study I explore the genetic basis of this evolutionary shift in disease susceptibility.

Plant pathogens can be divided into two categories: biotrophs, which feed on living tissue, and necrotrophs, which first kill tissue and then extract nutrients from dead or dying cells (Agrios 2005). While there is overlap in plant responses to these two kinds of pathogens, there are also considerable differences (Glazebrook 2005), with the plant hormone salicylic acid (SA) generally mediating defensive responses to biotrophs, and jasmonic acid (JA) and ethylene (ET) generally mediating responses to necrotrophs (McDowell and Dangl 2000). However, these hormones play a role in complex pathways involving both synergistic and antagonistic interactions. Additionally, these biotic stress response pathways have been shown to interact with abiotic stress response pathways. In particular, abscisic acid (ABA), which regulates growth and development in response to water availability, has been shown to suppress biotic stress

response pathways when highly expressed, often leaving plants more susceptible to both biotrophic and necrotrophic pathogens (Mauch-Mani and Mauch 2005). While studies have shown that ABA dependent genes are involved in adaptation to drought (Shinozaki et al. 2003), no study has yet explored how adaptation to drought affects biotic stress responses.

The fungus used in this study, *A. brassicae* is a necrotrophic pathogenic sac fungus that causes damping off, leaf spots, defoliation and reduced seed yield (Koike et al. 2006). All *Brassica* species are susceptible to *A. brassicae* (have a compatible interaction), although some species show partial resistance (an incompatible interaction). The molecular mechanisms of pathogen response in this plant-pathogen system is not well understood (Lawrence et al. 2008), however, extensive research has been conducted on the closely related plant pathogen system *Arabidopsis thaliana-Alternaria brassicicola*, which is a model system used for studying diseases caused by necrotrophs (van Wees et al. 2003, Glazebrook 2005, Mukherjee et al. 2009). Studies in the *A. thaliana-A. brassicicola* system have identified a large number of necrotrophic fungal pathogen related genes, approximately 1,100 (van Wees et al. 2003, Glazebrook 2005, Mukherjee et al. 2009), and have found that JA is required for resistance, indicated by susceptibility in *coi1*, as well as by other mutants (see Glazebrook 2005 for further details). JA activity is dependent on a functional copy of *COI1,* which encodes an F-box protein involved in proteolysis (Xie et al. 1998). Additionally, camalexin was found to play a role in resistance indicated by susceptibility of *pad3*. Based on these results, we hypothesize that polymorphism in genes that mediate JA, and perhaps ET responses, may be involved in evolutionary change to pathogen susceptibility.

In this study I aim to identify the genetic signatures of a shift in disease susceptibility associated with drought adaptation by conducting a genome-wide analysis of ancestral (pre-drought) and descendant (post-drought) *B. rapa* natural populations. I assessed signatures of selection in two complementary ways. First, I conducted outlier F_{ST} analysis to determine if allele frequencies of previously identified necrotrophic fungal pathogen response genes are differentiated between ancestors and descendants, indicating rapid contemporary evolution at these loci. Second, I used site frequency spectrum analysis to look for signatures of selective

sweeps or other strong or recurrent selective patterns at necrotrophic fungal pathogen related genes.

MATERIAL AND METHODS

PLANT SAMPLES

Previously, a large number of seeds (>10,000) were bulk collected from two natural feral *B. rapa* populations (Arboretum and Back Bay) in Southern California near the University of California Irvine campus. Seeds were collected along a transect from ripened seedpod (siliques) in 1997, before an extended natural drought, and then again in 2004, post-drought. Plants were grown up from seed and cross-pollinated in the greenhouse within population and year to control for maternal and storage effects. F1 seeds were used for all further studies.

SAMPLE PREPARATION AND SHOTGUN SEQUENCING

In this study, 500 ancestral and descendant plants from Arboretum and Back Bay populations were grown up from F1 seeds in the greenhouse (N = 2,000) and tissue was collected from first true leaves and dried. Genomic DNA was extracted, checked for quality and quantified by real-time PCR using GAPDH as a reference gene (Qi et al. 2010). Samples with high molecular weight were pooled in equimolar amounts within year and population (ancestral N = 50, descendant N = 74 for Arboretum; ancestral N = 22, descendant N = 59 for Back Bay). Duplicate pools were created, to verify that pooling was precise, for a total of 8 pools. Pools were used to make Nextera (Illumina) libraries using standard protocols. Each pool was tagged and sequenced in three lanes of a HiSeq 2000 (Illumina).

BIOINFORMATIC ANALYSIS AND VALIDATION

BWA (Li and Durbin 2009) was used to align sequence reads to the *B. rapa* genome v1.18 (Wang et al. 2011). Sequence variants were called using the Bayesian mpileup algorithm in Samtools (Li et al. 2009, Li and Durbin 2010). Population genetic estimates were calculated using the packages Popoolation (Pandey et al. 2011) and Popoolation2 (Kofler et al. 2011), including divergence (F_{ST})(Hartl and Clark 2007, Karlsson et al. 2007), and Tajima's D (Tajima 1989). Population genetic estimates were compared between replicates and found to be highly correlated so technical replicates were grouped for analyses reported. Additionally only results from the Arboretum population are reported here because I focused on that population in my disease susceptibility studies (chapter 3) due to the greater evolutionary shift in flowering time that that population experienced (Franks et al. 2007).

To validate SNPs and population genetic estimates determined using these pooled whole-genome sequences, I selected 10 genes that were found to be highly differentiated between ancestors and descendants. I assayed a single SNP within each gene for 96 individuals using KASP genotyping (LGC Genomics).

Statistical significance was determined for the F_{ST} values of genes by comparing the experimental F_{ST} values to a null distribution created by resampling 1000x with bootstrapping, followed by outlier analysis (Fox and Weisberg 2011, R Core Team 2013). Multiple correction was conducted using fdrtool (Klaus and Strimmer 2014) and genes were considered significant if they had a q value of less than 0.05. The q value is a more appropriate measure of statistical significance for large genomic data sets than the p value because it describes false discovery rate rather than just conducting a stringent correction that can result in many missed findings (Storey and Tibshirani 2003). A q value cutoff of 0.05 implies that about 5% of the genes deemed significant are false positives. I also report the top 50 most differentiated necrotrophic fungal pathogen related genes, including those with a q value of >0.05, because statistical measures for genomic data should be interpreted with caution.

EXPLORATORY FUNCTIONAL ANALYSIS

A list of 1,182 known necrotrophic fungal pathogen related genes was assembled from previous *A. thaliana-A. brassicicola* expression and suppression subtractive hybridization (SSH) mutant studies. These necrotrophic fungal pathogen related genes include genes known to be involved in both compatible interactions (Mukherjee et al. 2009) and incompatible interactions (van Wees et al. 2003) because there is a range of compatibility between *B. rapa-A. brassicae* (Nowicki et al. 2012). I also conducted a functional analysis by analyzing gene ontology (GO) using ErmineJ (Gillis et al. 2010).

RESULTS

I found genetic evidence of evolution in response to drought, demonstrated by a change in allele frequencies at many loci between ancestor and descendant populations. I validated these pooled whole-genome sequencing results by determining alleles present in individual plants using KASP genotyping for 10 loci (Franks et al. in prep). Some of the loci that evolved have been implicated in pathogen response, flowering time, and drought response, and a few of these loci are pleiotropic and have been implicated in both abiotic and biotic stress response. Global shifts and evolution of flowering time genes observed have been discussed elsewhere (Franks et al. in prep). Here I will focus on evolution of known necrotrophic fungal pathogen related genes.

Across the genome, I found that 11 of the 1,182 known necrotrophic fungal pathogen related genes were significantly differentiated between ancestors and descendants (Figure 1), with an F_{ST} above background ($q < 0.05$). I also report the 20 most differentiated necrotrophic fungal pathogen related genes because these might be involved in the shift in susceptibility noted, despite not all being statistically significant after false discovery correction (Table 1). To determine if the genes that were highly differentiated were under selection I also calculated Tajima's D. A heat map for the 50 most differentiated (high F_{ST}) necrotrophic fungal pathogen related genes also illustrates levels of Tajima's D in the pre and post-drought populations for the same genes (Figure 2). Eight necrotrophic fungal pathogen related genes which were

differentiated between ancestors and descendants (high F_{ST}) and showed evidence of being under selection (low Tajima's D) were identified of being of particular interest.

I conducted Gene Ontology (GO) analysis to determine if pathogen response functional categories were overrepresented among all genes when ranked by their level of evolutionary differentiation (ranked by F_{ST}). After a false discover correction (Bejamini-Hochberg FDR), I did not see enrichment of pathogen response categories, but I did see enrichment of two other categories: regulation of circadian rhythm (GO:0042752; p = 0.02) and protein phosphatase type 1 (GO:0000164; p = 0.05).

DISCUSSION

This is one of few studies to examine the genetic basis of evolution in a natural plant population (Mitchell-Olds and Schmitt 2006, Metcalf and Mitchell-Olds 2009, Walsh 2009, Rosas et al. 2014), and the first, to my knowledge, to examine evolution in disease susceptibility following a drought. I found that a number of known necrotrophic fungal pathogen related genes evolved over the course of just 7 years of drought. This is consistent with selection acting on a trait that is controlled by many loci, such as defense response to a necrotrophic fungus, which generally is more diffuse than response to a biotrophic pathogen, which in some cases is controlled by a single gene (Glazebrook 2005, Nowicki et al. 2012).

I found that a number of COI1 dependent genes evolved over the course of the drought (Table 1). In fact 18 of the top 50 most differentiated necrotrophic fungal pathogen related genes were COI1 dependent. JA activity is dependent on *COI1,* which encodes an F-box protein involved in proteolysis (Xie et al. 1998). Studies in the *A. thaliana-A. brassicicola* plant pathogen system have similarly identified COI1 dependent genes as playing a central role in defense suggesting that JA signalling mediates the defense response. One study found that 265 out of 645 genes induced by fungal infection were dependent on COI1, indicating that JA signalling plays a role in resistance to *A. brassicicola* (van Wees et al. 2003). The evolution of COI1 dependent genes reported here indicates that JA signalling might play an important role in the

evolution of susceptibility in addition to its known role in a plastic response to necrotrophic fungal pathogens.

Evolution in one trait is often correlated with evolution in other characters. This can be due to correlated selection, or genetic correlation. Genetic correlation, in turn, can be due to linkage disequilibrium between genes that affect the evolved characters, or pleiotropy (Futuyma 2009). I found evidence that selection on pleiotropic genes might play a role in the in the correlated evolution observed between earlier flowering time and increased pathogen susceptibility in response to drought. A number of genes that evolved (high F_{ST}) and were under positive selection (low negative Tajima's D) are known to be involved in both drought and pathogen response. Specific candidate pleiotropic genes are discussed in greater detail below. It is possible that this is an example of antagonistic pleiotropy playing a role in evolution (Williams 1957), with genes evolving that are responsible for both adaptation to drought (under positive selection) and increased susceptibility (possibly under negative selection but not strong enough to overcome the positive selection). Since pleiotropy can often limit adaptation (Futuyma 2009), the rapid rate of adaptation to drought in this system is especially surprising and could be due to either the strength of drought selection or a release from pathogen stress during drought years. It is also possible that correlated selection on linked genes might have contributed to the increase in disease susceptibility observed, however I was unable to determine this from this pooled genomic data set. I do expect that the observed patterns are primarily due to selection and not genetic drift because these populations were sufficiently large both before and after the drought, and I did not see evidence of a bottleneck in my genomics work (Franks et al. in prep). Additionally, the short time span over which evolution occurred makes genetic drift an unlikely cause.

The most highly differentiated gene in this study was Sec14p-like phosphatidylinositol transfer family protein (Bra030295) (F_{ST} = 0.21; q = 1.75 x 10^{-5}) which has been found to be differentially expressed in Arabidopsis in response to infection with *A. brassicicola* (van Wees et al. 2003) and cabbage leaf curl virus (Ascencio-Ibanez et al. 2008). Little is known about the specific function of this gene, although it is thought to play a role in the movement of

substances across cell membranes and has been found to be expressed widely in 23 plant structures (TAIR).

I found that the second most highly differentiated known necrotrophic fungal pathogen related gene was the COI1 dependent gene ERF4 (Bra038107)(F_{ST} = 0.20; q = 7.30 x 10^{-5}). In addition to a demonstrated role in the antagonistic regulation of JA and ET, this gene is also induced by ABA (Hoth et al. 2002, Nemhauser et al. 2006). This is interesting because this gene, along with other genes mediated by ABA, including Bra012746 and Bra033968 which are discussed below, and the WRKY DNA-binding protein 11 (Bra011282)(F_{ST} = 0.16; q = 0.02) have pleiotropic roles in both abiotic and biotic stress response (Journot-Catalino et al. 2006, Huang et al. 2008, Liu et al. 2011). Studies have shown that ERF4 is induced by ABA, and ERF4 suppresses expression of PDF1.2 (McGrath et al. 2005), a defense effector that is elicited by JA signaling (Glazebrook 2005).

I identified 8 necrotrophic fungal pathogen related genes that had relatively high F_{ST} (are in the top 50 most differentiated, but not all significant) and experienced a shift from an already negative (or low) Tajima's D in ancestors to a more negative Tajima's D in descendants, indicating that these genes both evolved and are under selection. I expected this ancestral-descendant Tajima's D pattern to be particularly interesting because the study populations are in a geographic region that is cyclically affected by climate fluctuations, including drought, corresponding to El Nino—Southern Oscillation cycles. While the period studied was an extreme drought event for this region (Hoerling and Kumar 2003, McCabe et al. 2004), a negative Tajima's D in ancestors could indicate previous selection due to past drought events. Genes of interest with high F_{ST} and a shift to lower negative Tajima's D are Bra033968 (F_{ST} = 0.16; q = 0.03), Bra003820 (F_{ST} = 0.15; q = 0.07), Bra012746 (F_{ST} = 0.14; q = 0.11), Bra013794(F_{ST} = 0.13; q = 0.40), Bra006015(F_{ST} = 0.13; q = 0.40), Bra022445(F_{ST} = 0.13; q = 0.41), Bra027940(F_{ST} = 0.13; q = 0.45), and Bra000330(F_{ST} = 0.13; q = 0.46; Table 1; Figure 2, indicated by arrow). Note that, genome-wide, there is no association between F_{ST} and Tajima's D, and further there are no genes that are outliers for both high F_{ST} (>0.2) and low Tajima's D (<-2) (Franks *et al.* in

prep). This suggests that F_{ST} and Tajima's D are detecting selection operating at different time scales or to different extents (e.g. fluctuating).

Caffeoyl-CoA 3-O-methyltransferase (Bra033968) was the only gene among the 50 considered here that had a significant F_{ST} value and shifted from a low Tajima's D to a negative Tajima's D. It is a lignin biosynthesis gene, which is pleiotrophic, playing a role in both drought and pathogen response. Lignification is a primary defense mechanism against pathogens (Vance et al. 1980), which works primarily through leaf structure (Taiz and Zeiger 2006). More specifically, this gene is a known necrotrophic fungal pathogen related gene that is COI1 dependent (van Wees et al. 2003). It has also been shown to be suppressed in Arabidopsis by ABA application, along with other pathogen related genes, resulting in a decrease in lignin accumulation and an increase in disease susceptibility to *Pseudomonas syringae pathovar (pv.) tomato* (Mohr and Cahill 2007). This gene is further interesting because lignin plays a key role in leaf structure, and I found evidence that leaf structure played a role in the increase in disease susceptibility seen (chapter 3), with earlier flowering plants having thinner leaves that displayed greater disease severity.

Another gene on this list of relatively high F_{ST} and low Tajima's D with an especially interesting function is NUDT7 (Bra012746)($TajD_{ancestor}$ = -0.54; $TajD_{descendant}$ = -0.91), which is pleiotropic, playing a role in both pathogen and drought response. NUDT7 is a negative regulator of basal resistance thought to prevent an excessive defensive response, indicated by a loss of function mutation in this gene in Arabidopsis resulting in activation of the cell death pathway (the hypersensitive response (HR))(Bartsch et al. 2006), and increased disease resistance to the bacterial pathogen *Pseudomonas syringae* (Jambunathan and Mahalingam 2006). The *nudt7* mutant induces both SA-independent and SA-dependent pathogen response pathways (Ge et al. 2007). Since necrotrophic pathogens, unlike the biotrophic *P. syringae*, take advantage of cell death, evolution in this gene could contribute to an increase in susceptibility to a necrotrophic fungal pathogen.

My results demonstrate that many known necrotrophic fungal pathogen related genes evolved, with a change in allele frequency, in just 7 years of drought. These results, along with

the reduction in genetic variation in specific regions of the genome and knowledge of the function of these genes, suggests a number of candidate genes for future work. Further, several implicated genes are potentially involved in both defense and drought resistance, indicating that antagonistic pleiotropy might play a role in the observed evolutionary increase in disease susceptibility following a natural drought.

Table 1. The 20 most highly differentiated (high F_{ST}) necrotrophic fungal pathogen related genes. Significance levels determined by resampling with bootstrapping 1000x and outlier analysis: *, q < 0.05; **, q < 0.01; ***, q < 0.001.

***A. thaliana* ID**	***B. rapa* ID**	**F_{ST}**	**q value**	**Gene Name**	**Notes from previous studies**	**Description**
AT2G21520	Bra030295	0.21 ***	1.75×10^{-5}		Altered by infection with *A. brassicicola* known by expression analysis	Sec14p-like phosphatidylinositol transfer family protein
AT3G15210	Bra038107	0.20 ***	7.30×10^{-5}	ATERF-4,ATERF4,ERF4,RAP2.5	COI1 dependent genes; Altered by infection with *A. brassicicola* known by expression analysis	ethylene responsive element binding factor 4
AT5G54160	Bra026320	0.20 ***	1.45 x 10-^{4}	ATOMT1,OMT1	Altered by infection with *A. brassicicola* known by expression analysis	O-methyltransferase 1
AT2G02390	Bra017417	0.19 ***	4.16×10^{-4}	ATGSTZ1,GST18,GSTZ1	Altered by infection with *A. brassicicola* known by expression analysis	glutathione S-transferase zeta 1
AT4G01960	Bra036302	0.17 **	4.48×10^{-3}		Altered by infection with *A. brassicicola* known by expression analysis	
AT4G39270	Bra033611	0.17 **	5.63×10^{-3}		Altered by infection with *A. brassicicola* known by expression analysis	Leucine-rich repeat protein kinase family protein
AT4G31550	Bra011282	0.16*	0.02	ATWRKY11,WRKY11	Altered by infection with *A. brassicicola* known by expression analysis	WRKY DNA-binding protein 11
AT1G23040	Bra012356	0.16*	0.02		Altered by infection with *A. brassicicola* known by expression analysis	hydroxyproline-rich glycoprotein family protein
AT2G33120	Bra021828	0.16*	0.02	ATVAMP722,SAR1,VAMP722	Altered by infection with *A. brassicicola* known by expression analysis	synaptobrevin-related protein 1
AT1G67980	Bra033968	0.16*	0.03	CCOAMT	COI1 dependent genes; Altered by infection with *A. brassicicola* known by expression analysis	caffeoyl-CoA 3-O-methyltransferase
AT2G17720	Bra024495	0.15*	0.05		Altered by infection with *A. brassicicola*	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase

					known by expression analysis	superfamily protein
AT1G45145	Bra036335	0.15	0.06	ATH5,ATTRX5, LIV1,TRX5	COI1 dependent genes; Altered by infection with *A. brassicicola* known by expression analysis	thioredoxin H-type 5
AT1G74020	Bra003820	0.15	0.07	SS2	COI1 dependent genes; Altered by infection with *A. brassicicola* known by expression analysis	strictosidine synthase 2
AT1G23730	Bra012384	0.15	0.07	ATBCA3,BCA3	Altered by infection with *A. brassicicola* known by expression analysis	beta carbonic anhydrase 3
AT4G12720	Bra012746	0.14	0.11	AtNUDT7,ATNUDX7,GFG1,NUDT7	Altered by infection with *A. brassicicola* known by expression analysis	MutT/nudix family protein
AT2G02930	Bra018543	0.14	0.14	ATGSTF3,GST16,GSTF3	Altered by infection with *A. brassicicola* known by expression analysis	glutathione S-transferase F3
AT1G10140	Bra031702	0.14	0.16		COI1 dependent genes; Altered by infection with *A. brassicicola* known by expression analysis	Uncharacterised conserved protein UCP031279
AT3G02520	Bra040592	0.14	0.17	GF14 NU,GRF7	Altered by infection with *A. brassicicola* known by expression analysis	general regulatory factor 7
AT5G65750	Bra024417	0.14	0.19		Altered by infection with *A. brassicicola* known by expression analysis	2-oxoglutarate dehydrogenase, E1 component
AT1G16520	Bra026043	0.14	0.22		Altered by infection with *A. brassicicola* known by expression analysis	

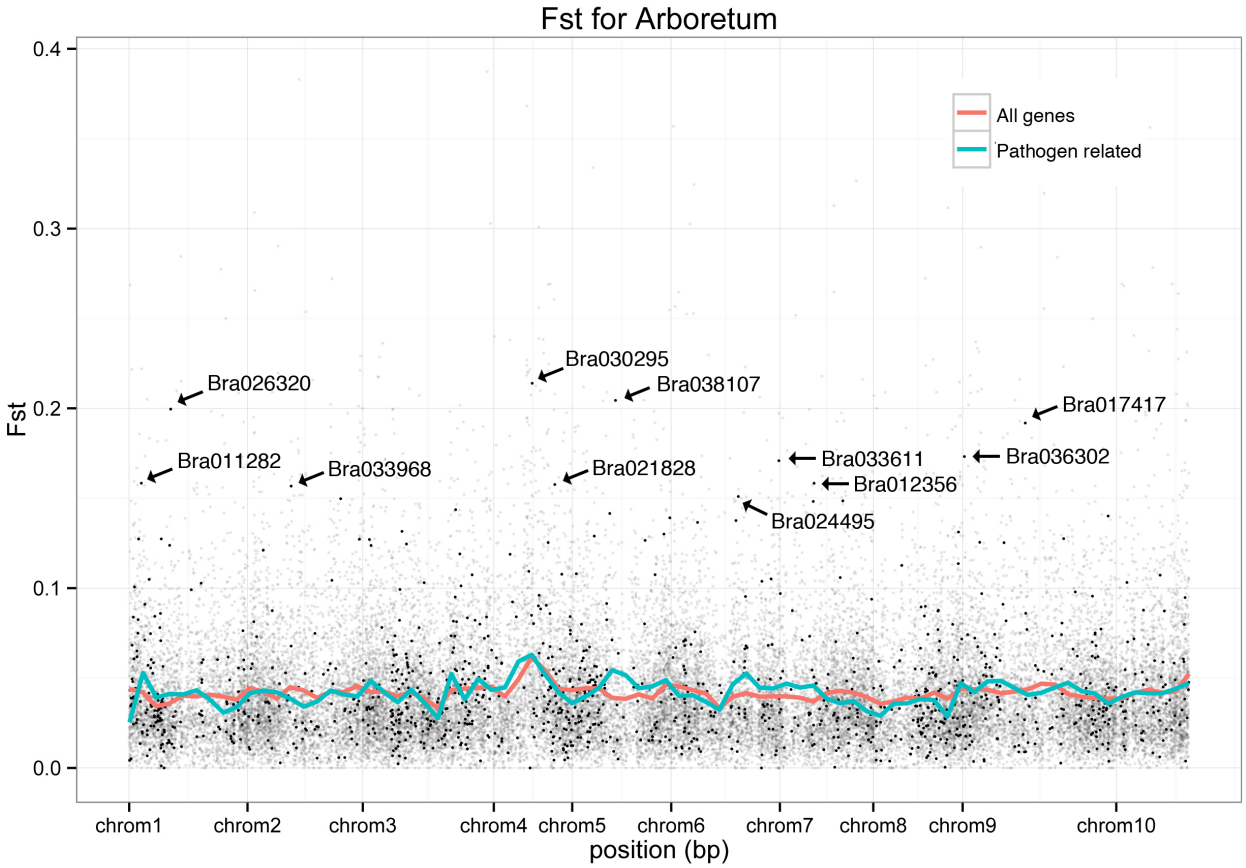

Figure 1. Population genetic estimates of differentiation (F_{ST}) across the *Brassica rapa* genome over 7 years of drought. Average F_{ST} calculated for the Arboretum population for each gene (dots) calculated using 100kb sliding windows across the genome. Genes identified elsewhere as involved in response to necrotrophic fungal infection (*A. brassicicola*) are shown in black. All other genes are shown in grey. Among fungal response genes, significantly differentiated genes are labelled. Trend lines shown for all genes (pink) and necrotrophic fungal pathogen related genes (green).

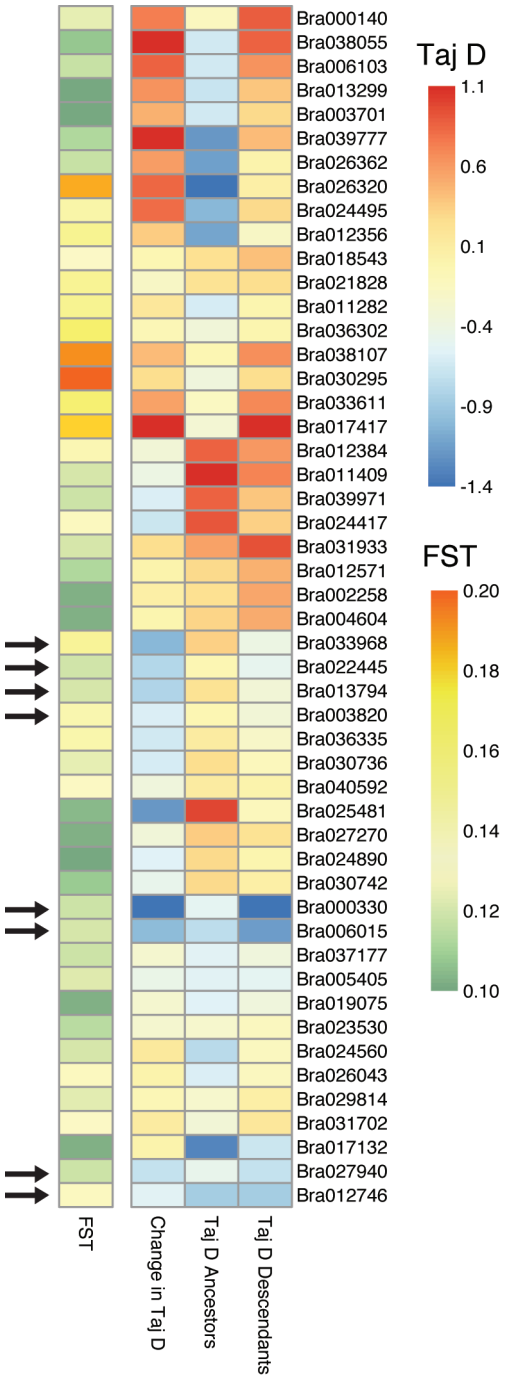

Figure 2. Patterns of evolution (high F_{ST}) and selection (low Tajima's D) for necrotrophic fungal pathogen related genes. Shown is a heatmap of F_{ST} (column 1), change in Tajima's D between ancestor and descendants (column2), Tajima's D for both ancestors and descendants (columns 3 and 4) for 50 necrotrophic fungal pathogen related genes with the highest F_{ST}. Arrows indicate genes with relatively high F_{ST} and a decrease in Tajima's D over the course of the 7 year drought.

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