An investigation of local adaptation in the model plant species *Arabidopsis thaliana*

Submitted by Nicola Krystyna Perera to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, February 2017.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

(Signature) .....

Abstract

Species extinction rates are causing alarm. Anthropogenic distortion of the climate system is rapidly altering the natural environment. *Arabidopsis thaliana* is a model species in molecular biology with widespread wild populations showing functional diversity however its ecology and evolution is poorly understood. Faced with a changing natural world, what is the adaptive potential of the model plant species *Arabidopsis thaliana*? This thesis focuses on the interactions of genotypes, phenotypes and environments to assess the current state of adaptation in this vagile species and to identify mechanisms for rapid adaptation to future stress, focusing on plant pathogens.

Here I show that *A. thaliana* populations in England exhibit evidence of local adaptation and genetic structure. A large common garden experiment using genotypes gathered in natural habitats revealed functional fitness differences in genotype-by-environment interactions. Wild populations showed differential representation of *RPM1* alleles suggesting non-random processes are responsible for the exhibited patterns. A further common garden experiment demonstrated 'home site advantage' through a correlation between fitness and home site climate, which suggests that local adaptation had occurred.

Phenotypic plasticity and mechanisms for rapid adaptation could be essential for plant survival under predicted climate change. Using *Xanthomonas* spp. as xenopathogens, I show differing levels of pre-adaptation for pathogen response exists in wild UK populations of *A. thaliana*. By using a multi-generation study, I found some evidence that epigenetic modification enabled rapid adaptation to pathogen stress.

Finally, I compared the metabolic expressions of phenotype among genotypes in two artificial environments. Environmental effects detected by this method are far greater than genetic ones, suggesting that metabolic plasticity can underpin environmental adaptation.

Taken together, my results suggest that wild populations of *A. thaliana* contain a range of mechanisms for rapid adaptation to environmental change. If these capacities are general, my work offers a note of optimism about the fate of some wild plant species in the face of global climate change. Additionally, as *A. thaliana* is a model species in genomics, my findings may facilitate future exploitation of these traits by crop geneticists.

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Author's acknowledgements

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Thanks to my supervisors James Cresswell, Murray Grant and Paula Kover for providing guidance and support throughout.

Chapter 2 Ron Yang analysed the 250K SNP data to determine if a group signal was present.
Chapter 3 Venura Perera carried out the kernel density approximation of climate data that I then used for subsequent analyses.
Chapter 4 I worked jointly with Bing Zhai on the large scale screening of infection phenotypes.
Chapter 6 Hannah Florance ran the samples through the LCMS and Venura Perera carried out the initial LCMS data processing.

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List of Abbreviations

ABA	Abscisic acid
AMOVA	Analysis of molecular variance
ANOVA	Analysis of variance
Avr gene	Avirulence gene
CAPS	Cleaved amplified polymorphic sequence
Cfu	Colony forming units
CG	Common garden
DAMPS	Danger associated molecular patterns
Dpi	Days post inoculation
EI	Epigenetic inheritance
ETI	Effector triggered immunity
Fb	Field-based
GD	Genetic distance
GH	Glasshouse
GM	Genetically modified
GR	Growth room
GWA	Genome wide association
GxE	Genotype x Environment interaction
H_0	Null hypothesis
HAE	Herbivore associated elicitors

HAMPS	Herbivore associated molecular patterns
HM	Host membrane
Hpi	Hours post inoculation
HR	Hypersensitive response
IM	Inner membrane
IPCC	International panel on climate change
JA	Jasmonic acid
KB	Kings broth
KFA	Kernel feature alignment
LC-MS	Liquid chromatography- mass spectrometry
LEA	Late embryogenesis abundant
MAMPS	Microbe associated molecular patterns
MD	Metabolite distance
mGWA	Metabolomic genome wide association
mRNA	Messenger ribonucleic acid
MSL	Mean sea level
NHR	Non-host resistance
OD	Optical density
OM	Outer membrane
PAMP	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PG	Peptidoglycan

PR	Pathogenesis related
PRR	Pattern recognition receptors
PTI	PAMP triggered immunity
QTL	Quantitative trait loci
R genes	Resistance genes
RFLP	Reverse fragment length polymorphism
RT	Reciprocal transplant
SA	Salicyclic acid
SAR	Systemic acquired resistance
SF	Self fertilising
SNP	Single nucleotide polymorphism
T3SS	Type III secretion system
tSAR	Transgenerational systemic acquired resistance

Accessions used in this thesis

This work started with a collection of 96 singleton accessions (one plant sampled per site) from across the UK donated by E. Holub, HRI, Wellesbourne. These accessions have been used in a variety of previously published studies and therefore have genetic data freely available. All have 149 SNP data also provided by E. Holub, a subset were included in the 250K SNP study (Horton et al. 2012). These accessions were used in my earliest experiments and in any were the experimental design required knowledge of genetics. In 2009 I sampled a number of sites including re-visiting many of the locations were E. Holub had sampled (from longitudes and latitudes supplied). I attempted to gather siliques from a number of plants from these locations to understand the diversity present at these sites.

Accessions used in the experimental chapters:

Chapter 2:

The population diversity study is based upon accessions gathered during my sampling.

The common gardens used Holub accessions as they had previously been characterized at Rpm1 (Atwell et al. 2009).

Chapter 3:

The common gardens used my accessions so that their performance could be correlated to historic home site climate prior to their sampling date.

Chapter 4:

The xenopathogens work used Holub accessions as this experiment started prior to my sampling.

Chapter 5:

Epigenetics work used my accessions so that they had not been previously exposed to lab work.

Chapter 6:

Metabolite work was based upon Holub accessions with 250K SNP data available for the genetic analyses.

Chapter 1: Main Introduction

"Two fundamental and interconnected themes in ecology are the development and maintenance of spatial and temporal pattern, and the consequences of that pattern for the dynamics of populations and ecosystems." (Levin, 1992)

"Organisms looked at as detectors of the universe, have a limited memory and as individuals are subject to environmental caprice. But this is true of populations and species through time, that is, evolutionary adaptation." (Lewontin, 1966)

Preamble

The research presented in this thesis focuses on the interaction between genotype and environment, evidenced through the phenotype. Considered as a state rather than a process, 'adaptation' describes an optimal correspondence between a genotype's physical manifestation and its environment. However, due to the dynamic nature of environmental systems and the inherent role of chance in the generation of novel genetic diversity, the absolute optimum may remain elusive. The perfectly adapted niche of a genotype describes the optimal environmental conditions for that genotype. When adapted genotypes are moved between environments, i.e. from their 'home site' to a new site, the success of the phenotypes may reveal the similarity of their previously adapted niche to the new environment. Whether home site conditions in actuality represent the optimum adaptive niche for a genotype depends upon a number of factors such as: founder effects, the species' breeding system, elapsed time of the genotype at the home site, and the site's environmental stability.

A fundamental requirement underpinning the evolutionary process of adaptation is differential fitness among genotypes, because without this their distribution will drift by chance. The patterns of differential fitness across two or more environments can be useful for interpreting degrees of adaptation. Changes in differential fitness across environments are formalized as

‘Genotype by environment interactions’, which are often abbreviated as GxE interactions, or GxE. GxEs are classically depicted as a reaction norm graph as shown in **Figure 1**, which displays changes in fitness hierarchy between environments. When a GxE interaction occurs, the most fecund genotypes in one environment may be the worst performers under different environmental conditions.

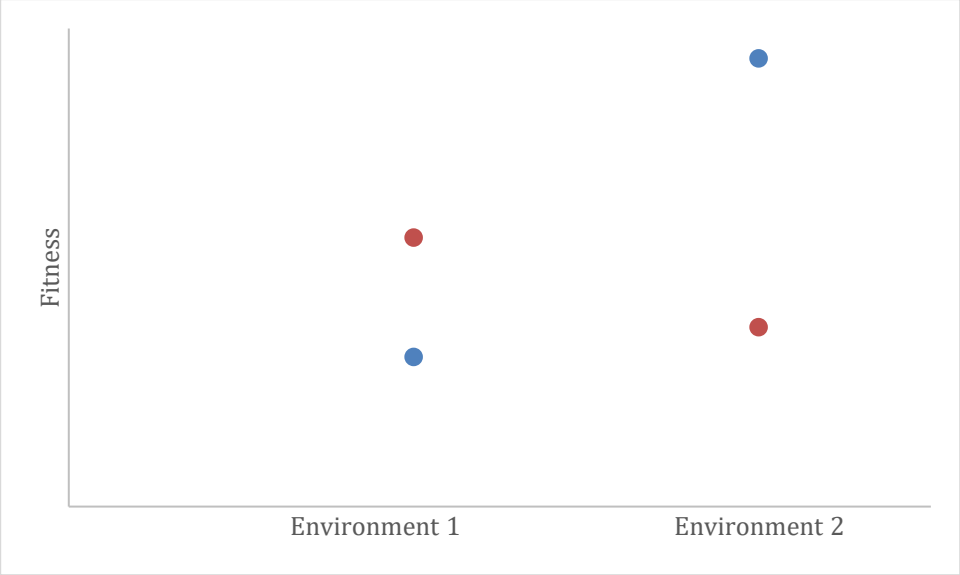

Figure 1 Example reaction norm graph showing the performance of two genotypes (orange and blue circles) between two environments. As the fitness hierarchy changes between the two environments a GxE interaction is said to have occurred.

In **Chapter 2,** I investigate GxEs in the model plant species, *Arabidopsis thaliana*, across two distinct environments using fecundity as a measure of fitness. This reveals differing selective pressures between environments which could maintain the genetic diversity that I characterize among this plant’s wild populations across the United Kingdom. Using both new and existing genomic data, I focus on loci associated with pathogen resistance (the so-called ‘R’ locus). I reduced the risks inherent in using natural accessions, such as genetic linkage, by designing a group-wise study and using putatively neutral genetic markers (SNPs).

In **Chapter 3,** I attempt to discover some of the factors that have shaped adaptation to home sites in *A. thaliana* across the UK. Specifically, I use a common garden experiment to investigate whether the climatic similarity between the garden environment and home site can predict performance in the novel garden environment. By repeating the experimental garden in successive years, I also tested the stability of selection pressures between years. In essence, I have tested GxE effects across two 'sites', but additionally here the differences in environment also have a temporal influence. Besides considering the implications for maintaining genetic diversity in the plant's collective UK gene pool, I also consider the implications of these results in the context of the predicted consequences of global climate change, and particularly with respect to the evolutionary consequences of an increased frequency of extreme weather events, which have the potential to disrupt naturally prevailing selection regimes.

Global climate change is a focus of my next study area because it is altering the geographic range of many economically important crop pathogens, which means that populations of *A. thaliana* soon may be confronted by the arrival of xenopathogens. **Chapter 4,** explores the potential for pre-adaptation to protect species from novel pathogenic threats by providing a rapid evolutionary path to resistance.

Another potential route to rapid adaptation of pathogen resistance is by epigenetic mechanisms, in which a new environment elicits heritable evolutionary responses that are transmitted as maternal effects. In **Chapter 5,** I test several genotypes that were drawn from wild UK populations for their epigenetic potential to develop rapid resistance to multiple pathogen stressors.

The bulk of the investigations reviewed thus far have focused on the manifestation of genotype at a whole-organism, ecological level of organization. Finally, in **Chapter 6,** I investigate the possible complexity in the phenotypic manifestation of a genotype at the molecular level. Specifically, I report a detailed study of the correspondences between the genome and metabolome in *A. thaliana*. In this last study, I compare genetic diversity among individuals with their molecular-level manifestations in phenotypic diversity; the comparisons among the

genotypes are made both within and between two different environments where the plants were grown. The chemical diversity of a selection of UK accessions revealed by this study captures a snapshot of evidently highly dynamic genome-phenome-envirome interactions.

As the introduction to this thesis, I will address several key areas of current knowledge relating to genome, phenome, and envirome interactions in plants (**Figure 2**). Because it is the focal species of my study, I will look in particular detail at the model plant species utilised in this work, *Arabidopsis thaliana,* and the main environmental factors that I have considered, namely climatic variables and pathogens.

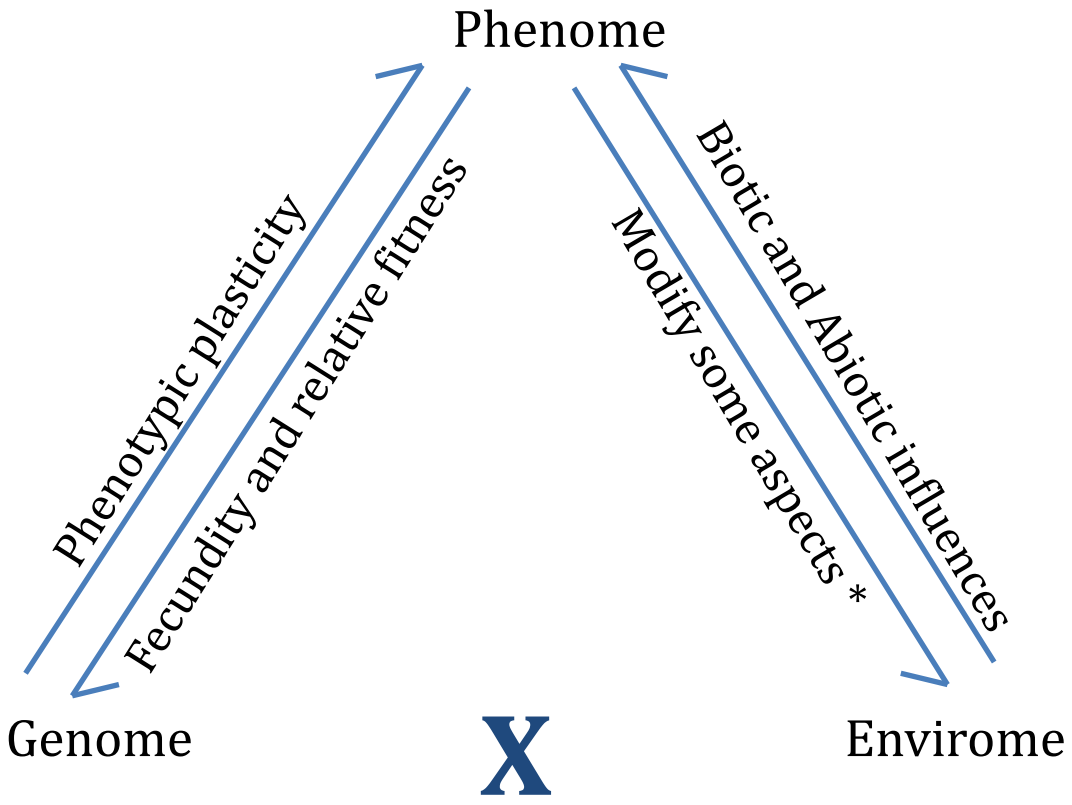

Figure 2 The Genome, Phenome, Envirome interactions triangle. The phenome is the product of the interaction between genome and the envirome. However the phenome can dynamically influence the envirome by, for example, reducing herbivore abundance by attracting natural predators.

Introduction

Our natural environment is in crisis: we are depleting species and resources at an unsustainable rate (Burns et al, 2013; Millenium Ecosystem Assessment, 2005) and we are altering fundamentally the climate of our planet (Bernstein et al, 2007). Potentially, we have become so inconsiderate of the ecosystem that supports us that we are in danger of causing irreversible harm to our life support system. Plants play a vital role in ecosystem health and function, but we do not know whether they have the adaptive potential to evolve to fit the new world that we humans are so rapidly creating. Nature is resilient (Lovelock and Margulis, 1974) however, and natural selection could potentially steer species away from evolutionary catastrophe, but can it keep pace with the unprecedented speed of anthropogenic change? In part, the answer to this question depends on the genetic diversity already present in plant species. A major objective of this thesis is to explore the genetic diversity and adaptive potential of plants.

There is a second imperative for exploring the relationship between global environmental change and the genetic diversity of plants. As the global population of humans continues to expand rapidly, there is increasing pressure on agricultural systems. Our understanding of evolutionary processes is fundamental to harnessing the power of natural genetic diversity and the deployment of GM technology through evolutionarily informed agricultural practices (Zhan et al., 2014). Specifically, it would be valuable to identify the genetic basis of adaptive traits, because GM technology could potentially enable transfer into crops, which would thereby be better suited to a changing world. Here, I address in principle the extent to which wild plant populations may harbour useful traits. Furthermore, one of the fundamental risks of GM technology is that the traits that we endow upon the plants will be superceded by evolution in the pest species. Adaptation is not static; therefore can the widespread repeated deployment of genetic ‘clones’ carrying resistance genes ever be the optimal strategy?

The modern revolution that is molecular biology has provided the opportunity to investigate the proximal consequences of evolution and to thereby fulfil scientists’ quest to explain how the diversity of life arose and is maintained. The reductionist approach of molecular biology dissects

genetic traits through employing genetic modification to, for example, 'knock out' certain genes (Carpenter & Sabatini, 2004). The recent rise of systems biology has seen the adoption of a more holistic approach, which seeks to integrate field ecology with molecular studies, thereby allowing for the characterisation of interconnected functionalities that might never have been revealed in a laboratory setting (Baldwin, 1998; Wituszynska *et al*, 2013). For example, the readily characterised phenotypic trait of flowering time in *Arabidopsis thaliana* is genetically controlled with approximately 90% heritability, but genome-wide association (GWA) studies can only identify SNPs and population structure to account for 45% of the phenotypic variation (Li et al, 2010). The 'missing half' of the heritability is thought to be found in genotype-by-environment (GxE) interactions (Brachi et al., 2011). This remarkable phenomenon means that the dynamic impact of the environment on the displayed phenotype is comparable in influence to the underlying genetic differences among individuals. Consequently, incorporating the effect of an ecologically realistic environment is essential if the intent is to unpick the inheritance of traits and the adaptive process. In my investigations, I chose the plant species *A. thaliana* to integrate molecular biology and ecology because the species is a model plant in molecular biology, but also one with wild populations and ecologically relevant diversity. Moreover, the plant is a good focal species for novel research because its field ecology is rather poorly understood.

***Arabidopsis thaliana*: a genomic natural history**

Arabidopsis thaliana was first described by Johannes Thal in 1577 in the Harz Mountains of Northern Germany. However its history as a wildflower of the mountains has been largely overshadowed by its image as a small ruderal, or opportunistic weed in areas of human disturbance, and by its scientific role as a model plant species whose genome was the first to be sequenced completely.

Molecular milestones

A collaborative program was created in 1989 by the US National Science Foundation to promote *A. thaliana* as a model plant species in genetics analogous to *Drosophila melanogaster*

and *Caenorhabditis elegans*. This international effort culminated in publishing the plant's complete genome sequence in 2000 (The Arabidopsis Genome Initiative, 2000).

Since then, scientists have undertaken the characterisation of 9% of gene functions experimentally and nearly 70% of its genes are functionally classified by sequence similarities (The Arabidopsis Genome Initiative, 2000). This annotated genome provides a valuable resource to elucidate processes in other agriculturally relevant plant species, but it also has potential to provide an understanding of plant ecology and to enable identification of the attributes that enable evolutionary adaptation.

Because of the knowledge of its genome, *Arabidopsis thaliana* rose to prominence as the species of choice for molecular biologists seeking to understand an array of features such as circadian rhythms, pathogen interactions, evolution and genetics. The genetic material of *A. thaliana* has proven to be easily mutagenized and has been artificially manipulated since the 1940s (Reinholz, 1947). Due to its rapid lifecycle and small genome (125Mb) it has proven itself to be a tractable species for molecular study. The first fifty plant genomes published range between 77Mb (*Utricularia gibba)* to 17,000Mb *(Triticum uratu)* with the mode being between 500 and 600Mb (Michael & Jackson, 2013) *Arabidopsis thaliana* has one of the smallest plant genomes published to date.
However its potential as a model organism for ecology has been less well exploited because of its weedy life history, which is perhaps perceived as 'artificial' and therefore less interesting for academic study. Additionally, many populations are considered to be transient and less likely to exhibit a gene pool whose composition is shaped by adaptation (Jorgensen and Emerson, 2008). However, the species evidently exhibits a high level of genetic variation (Shindo et al., 2007) that is maintained in complex population structure (Nordborg et al., 2005), which suggests instead that it may be a relevant species for studies of population genetics and evolutionary biology.

Additionally, perhaps the greatest value of this organism is the wealth of information available on the genome. For example, the '1001 genomes project' has sought to create a freely

available genetic resource that will facilitate the study of the genetic basis of phenotypic variation in *A. thaliana*. Incorporating this molecular information into ecological studies will begin to provide a powerful tool for understanding the ecology of *A. thaliana* and for extrapolating to other species. Potentially, therefore, a particularly fruitful research area exists in the ecology of model molecular species that have extensive molecular data freely available at the click of a computer's mouse. It is this unique opportunity that I sought to exploit in the work described here.

Evolutionary ecology

A. thaliana diverged from other *Arabidopsis* spp. approximately 5-6 million years ago (Shimizu and Purugganan, 2005). It is probably native across Europe, North Africa and Asia and it is a naturalised introduced species in America and Australasia. Genome scans suggest that its geographic range was contracted by Pleistocene glacial advances and it probably survived in isolated refugia on the Iberian Peninsula and Asia. From here, its range then expanded back into Europe approximately 17,000 years ago (Sharbel et al., 2000). Nowadays, it can be found across all continents of the world except Antarctica (Hoffmann et al., 2002; Shindo et al., 2007; Weigel & Mott, 2009) USDA website: http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?3769). It has a larger range than other *Arabidopsis* species (Hoffmann, 2005), possibly because its breeding system of self-fertilisation reduces reproductive constraints on colonisation by isolated individuals (Shimizu and Purugganan, 2005) and its ruderal lifestyle makes it a ready colonist of the ubiquitous disturbed habitats produced by modern humans.

Thus, the range expansion of *A. thaliana* is at least in part attributable to human disturbance. As *A. thaliana* is often found living in areas of human habitation, clearly it must be at least in part dispersed by human activity. However, its presence has not been frequently detected in studies looking at anthropogenic dispersal mechanisms. Multiple studies sampling the mud from underneath cars did not find *A. thaliana* (Clifford, 1959; Lonsdale & Lane, 1994; Schmidt, 1989; Zwaenepoel et al., 2006) and neither was it present when mud from walkers' footwear was sampled (Clifford, 1956). However, the movement of horticultural stocks is one proven

method of long-distance transport, because *A. thaliana* was found at low abundance growing in pots among the commercially-valued plants (Hodkinson and Thompson, 1997). Additionally its light siliques and seeds are passively dispersed by the wind and in the soil (Wender et al., 2005). Dispersal over both time and space have been shown to be important factors in shaping 36 natural populations in Norway. Falahati-Anbaran et al., 2014 found that the immigration rate was approximately 1.7% per year and in 10 of the 36 studied sites around a third of the above ground plants were from seed shed 2 or 3 years previously. Whether seeds will germinate or become dormant depends upon the temperature when the seeds develop, Springthorpe and Penfield (2015) found that if seeds developed at less that 14ºC the majority would become dormant whereas if they developed above 15ºC very few would become dormant.

A plant species, like *A. thaliana*, that is a highly vagile seed disperser and also a predominantly self-fertilising species is likely to be a successful coloniser of far-flung areas of the globe only if it also exhibits a wide environmental tolerance. This tolerance can arise from either genetic diversity, phenotypic plasticity, or, potentially, rapid epigenetic adaptation. A widespread geographic distribution in a species that is also amenable to short term studies because of its rapid life cycle provides an exciting opportunity to study not only evidence of past adaptation from genetic analysis and performance studies, such as common gardens, but also to create artificial selection experiments and follow them through multiple generations. This method has been successfully employed with bacteria (Buckling et al., 2009; Buckling & Rainey, 2002; Lenski, 1998), insects such as *Drosophila* (Reed et al., 2003; Shiotsugu et al., 1997) and in plants including *A. thaliana* (K*over et al*, 2009; Scarcelli and Kover, 2009). In fact, I use a similar approach in Chapter 5, where I assess the potential for epigenetic "rescue" of pathogen stressed plants over successive generations.

Natural genetic diversity: origin and maintenence

Bring together any collection of genotypes from across a species range and they will likely exhibit an array of developmental, morphological and physiological differences. It is easy to fall into the adaptionist trap and to attempt to explain each genotype’s specific traits by inferring

their functional benefits at the genotypes' home site. However, it would be premature to do so without good experimental evidence because random, or neutral, processes (e.g. founder effects) could also be responsible (Gould & Lewontin, 1979; Lukeš et al, 2011; Stoltzfus, 2012). Genetic diversity arises by chance, by random mutations, and this raw variation presents the opportunity for adaptation by selection. A random, spontaneous mutation can either be deleterious, positive or neutral in effect. A positive mutation will, by definition, increase the overall fitness of an organism in its environment. Whilst fitness can be measured by many parameters, the ultimate evolutionary currency is a genotype's contribution of alleles to the next generation. In self-fertilising species, like *A. thaliana,* this can be simply estimated by counting the number of progeny produced, whereas in crossing species measures should be made of both female and male reproductive output (Heil and Baldwin, 2002).

Alone, adaptation by natural selection will erode genetic diversity by sifting away alleles in the less-fit individuals. However, variation can be maintained in nature by neutral processes or by variation of the selective process, such as differential selection across a heterogeneous environment. The natural maintenance of genetic diversity potentially buffers a species against environmental changes. Mutation is slow, however, with spontaneous random mutations estimated to occur at 7×10^{9} base substitutions per site per generation for *A. thaliana* (Ossowski *et al*, 2010). Consequently, loss of genetic diversity through relentless natural selection can create a catastrophic bottleneck if the population experiences an unprecedented, novel stress. The loss of variation in the face of environmental change can be countered in part by an increase in mutation rate under certain stressors, which has been demonstrated to arise in the presence of DNA-damaging agents (e.g. UV), certain environmental stressors (e.g. NaCl) (Puchta et al, 1995) and pathogens (e.g. tobacco mosaic virus) (Kovalchuk et al., 2003). Numerous studies involving bacteria have found increases in mutation rates for advantageous characteristics, which suggests that non-random mutation is possible as a stress response, at least for some species (Cairns et al, 1988; Hall, 1990; Ninio, 1991).

If natural selection erodes genetic diversity, yet this diversity is potentially the route to adaptation, what can maintain it? Spatial (see Chapter 2) and temporal (see Chapter 3)

differences in environmental conditions can create balancing selection (manifest as sustained presence of genetic variation) because selective pressures vary in space and time. Balancing selection will maintain genetic diversity when, for example, an allele that is beneficial under certain conditions may be relatively costly in another. The differing fitness contribution of an allele in different environments could be due to a straightforward effect of spatial variation of costs and benefits. For example, genes for pathogen resistance may incur a cost when the pathogens are absent (Gillespie, 1975; Clarke, 1979), (see section 1.3.2.2 Table 2 for more details). Anderson et al, (2013) demonstrated this for quantitative trait loci (QTL) involved in flowering in the Brassica *Boechera stricta*; when plants from two populations were grown in reciprocal common gardens, individuals were more likely to flower at their home site than at the foreign site.

Whether variation in fitness is, in actuality, responsible for the maintenance of observed levels of genetic diversity is controversial. Mathematical models of population genetics have predicted that both dependence and independence from fitness costs will maintain genetic diversity across a spatially heterogeneous landscape (Bergelson & Dwyer, 2001; Bergelson & Purrington, 1996; Damgaard, 1999; Leonard, 1977; Salathé et al., 2005; Thrall & Burdon, 2006). A diverse gene pool can also persist for long periods if the majority of genes are selectively neutral or nearly neutral in effect (Kimura, 1968). If a gene is neutral it has no fitness effect for the carrier and it is therefore invisible to natural selection. Consequently gene frequencies will drift solely due to stochastic events. Proponents of neutral theory acknowledge that natural selection will be acting on a subset of genes but they argue that allele frequencies at most loci originate from random processes. In accordance with earlier observations (Zuckerkandl and Pauling, 1962), neutral theory predicts that changes in genetic sequence will be approximately linear over time, which creates a molecular clock. In effect, neutral theory provides a testable null hypothesis that makes quantifiable predictions about the rate of molecular change, which is used in comparative genomics to detect areas of the genome that diverge from the neutral rate; these divergent loci are inferred to have undergone selection. Many methods have been developed to identify genes under selection in comparison to neutral sequence (Lewontin and Krakauer, 1973; McDonald and Kreitman, 1991; Bonhomme *et al*,

2010). All loci should be similarly affected by stochastic processes and only a subset will be diverged due to selection.

Perhaps unsurprisingly, species vary considerably in the extent of positive selection that is detected by genome scans. In plant species genome scans (reviewed in Strasburg et al., 2012) reveal that the relative proportion of putative regions under selection, ranges from 0.4% (Jump et al., 2006) to 35.5% (Lexer *et al*., 2010). Overall, an average 8.9% of loci appear to have been under selection (Strasburg et al., 2012). With the increasing availability and affordability of high-throughput genetic data, genomic scans are providing a possible analysis tool for the identification of loci under selection.

For species with long generation times, functional adaptive traits are slow to evolve due to their reliance on random spontaneous mutations. However this process can be accelerated if pre-existing functional traits are co-opted or recycled for different purposes, which is termed 'pre-adaptation'. Pre-adaptation is particularly beneficial for pathogen resistance because local epidemics arise suddenly and constitutive resistance is probably costly (Tian et al., 2003) (also see Chapter 4). For example, *downy mildew resistant 5 (dmr5)* in *Arabidopsis thaliana* is a functional allele of *RPM1* that has been co-opted from *Pseudomonas* resistance to provide resistance to *Hyaloperonospora parasitica* by a simple genetic change that comprises only a single base pair substitution (Huibers, 2008). When new genetic solutions to environmental challenges evolve due to the occurrence of different mutations in independent, reproductively isolated lineages, then convergent evolution may produce different solutions to the same problem. Ashfield et al., 2004 showed that *Pseudomonas* resistance genes that recognise *avrB* in soybean (*Rpg1-b)* and *Arabidopsis thaliana (RPM1)* are an example of convergent evolution as they are not orthologous, and instead lack substantive sequence similarity.

Pre-adaptations from natural genetic diversity accelerate selection for resistance and therefore help to protect natural populations from disease epidemics. Artificial agricultural systems, by contrast, often comprise only a monoculture of similar genotypes, which in effect creates substantial directional selection for virulent pathogens. Whilst pathogens are usually faster to

evolve than their hosts due to their often large population size and short generation time, their evolution can be stymied by genetic diversity among the host population. The blanket susceptibility of genetic uniformity explains why GM crops are likely to be ultimately defeated by pathogens and this invites a reconsideration of how GM technology could be more effectively deployed (Zhan et al., 2014). In contrast, natural systems can maintain functional resistance genes over millennia (**Figure 3**). For example, the resistance gene (*RPM1)* that protects *Brassica* species against *Pseudomonas syringae* has co-existed with the susceptible null allele for an estimated 9.8 million years (Stahl et al., 1999). There is substantial understanding of RPM1 and its allelic variants (Grant et al., 1995; Rose et al., 2012; Tornero et al., 2002). I take advantage of this knowledge by using the *Rpm1* locus as a focus for the work presented in Chapter 2.

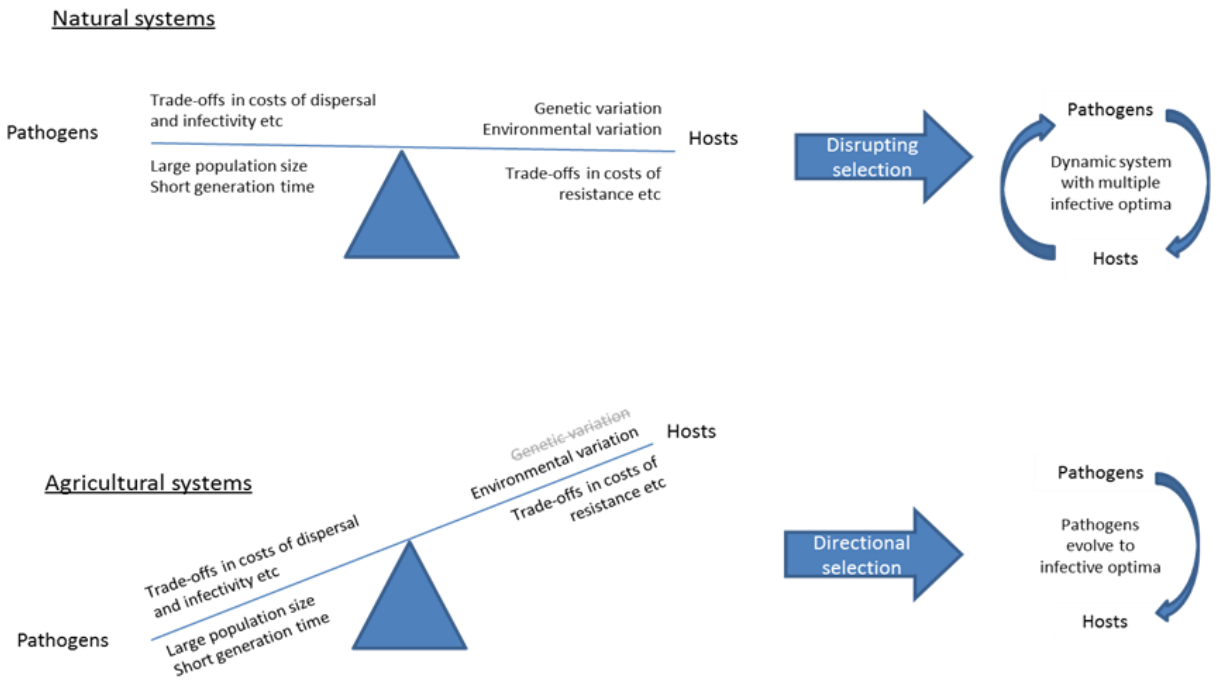

Figure 3 Evolution in natural versus agricultural systems, showing the importance of genetic diversity in pathogen interactions (developed from Zhan et al, 2014).

Population genetic structure of *A. thaliana*: Worldwide and UK-wide

Analyses of genetic structure in populations of *A. thaliana* reveal high levels (>95%) of self-fertilisation (Abbott & Gomes, 1989; Bergelson et al., 1998; Kuittinen et al. , 1997; Todokoro et al., 1995), although the results of some studies suggest that outcrossing maybe occurring more frequently than previously thought (Jørgensen and Mauricio, 2004; Bakk*er et al*., 2006). Despite self-fertilisation, populations do not comprise single inbred kinships (Nordborg et al., 2005). Instead, molecular analyses have revealed high levels of genetic variation within and among populations (Abbott and Gomes, 1989).

In part, this change of understanding of the gene pool's diversity occurred because the earlier studies had relied upon low-resolution methods: characterisation of variation in allozymes (Abbott and Gomes, 1989); RFLPs (Bergelson *et al*., 1998); and microsatellites (Kuittinen et al., 1997; Stenøien et al., 2005; Todokoro et al., 1995). Studies based upon high-resolution coverage (i.e. sequence data) are better able to reveal patterns in distribution (Nordborg et al., 2005; Schmid et al., 2006; Bakker et al., 2006) than those based on low resolution methods. The advances in sequence genetics, which has been driven principally by the increasing availability of high-throughput methods of analysis, have revolutionised this field of population genetics. The increased resolution possible from genome-wide SNP analyses makes this the method of choice for the quantification of genetic variation in *Arabidopsis thaliana* (Schmid et al., 2006; Bakker et al., 2006). In this thesis, I use high resolution SNP data to investigate correlations between genotype and phenotype (see Chapter 6).

It is important that the interpretation of spatial patterns in genetic diversity is made with due consideration to the context of sampling methodologies and area sampled, as well as genetic methods applied. The extent of a geographic pattern in the distribution of genetic diversity may in part reflect the time since colonisation. For example, studies of genetic variation across North America, where *A. thaliana* is thought to have been introduced from the UK (Nordborg *et al.*, 2005; Bakker *et al.*, 2006), typically do not find geographic differentiation in the gene pool (Bergelson et al., 1998).

A. thaliana is found abundantly across the UK, with a recorded altitudinal range from sea level

to 685 m altitude. Previously, there has been little work to specifically characterise the UK gene pool, except for a study that examined resistance to powdery mildew (*RPW8)* in populations (Jorgensen and Emerson, 2008); and in relation to specific resistance genes (Atwell et al., 2009). Surprisingly, in light of the long evolutionary history of *A. thaliana* in this country, these studies did not find evidence for natural selection at the loci that were considered. The implications of this seemed to be that these pathogens may not be actively shaping evolution in UK's *A. thaliana*. However in this thesis, unlike Atwell et al. (2009), I sampled populations repeatedly to establish their genetic composition, because a study based on singletons may not be representative of the response to selective pressures at a site.

Envirome(nt)

Just as the phenotype and the genotype are intimately linked, the environment is dynamically involved in this process. Here I use the terms Envirome, Phenome and Genome to convey the association between these factors, however the terms themselves have the same meaning as Environment, Phenotype and Genotype.

A. thaliana and its abiotic interactions

Important abiotic interactions for plants include many aspects of climate, such as temperature and drought, as well as soil conditions and nutrients.

Climate

The average weather conditions at a site affect fundamental plant processes either directly by, for example, determining photosynthetic potential (light intensity and daylight hours) and impacting on nutrient uptake (the availability of water), or indirectly by influencing pathogen abundance. In these ways, variation in climate causes many of the complex stresses that a plant experiences. If adaptation to home-site conditions has taken place, the similarity of an alien site's climate to that of the home site should be a predictor of plant performance. In this

thesis, I test this relationship experimentally (Chapter 3).

Plants are susceptible to even small changes in global mean temperature. Significant implications are predicted for common crop species based upon climate change scenarios. For example under climate change scenario A2a ("business as usual", IPCC), it is predicted that in the next fifty years the geographic range of feasible cultivation will decrease for 23 common crop species, including strawberries (32% reduction in area suitable for cultivation), wheat (18%), rye (16%), apples (12%) and oats (12%). Conversely 20 crop species will see their ranges increase (Lane and Jarvis, 2007).

Conventional climate envelope/geographic range modelling assumes that climatic parameters are the dominant driver of a species' geographic distribution (Hoffmann et al., 2002; Woodward, 1987). For the genus *Arabidopsis*, a positive correlation exists between the width of a species' climatic envelope and the spatial extent of its range (Hoffmann, 2005). This correlation strongly suggests that climate has significantly shaped the evolution of this genus. In northern Europe, low temperatures in spring and autumn restrict the range of *Arabidopsis*, whereas high temperature and low precipitation in summer determines its range across N Africa, SW Asia and Middle Asia (Hoffmann, 2005).

Plant response to climatic stress

The whole plant experiences climatic stresses and its sessile habit offers no opportunity for an individual to escape. Therefore, under climatic stress the successful plant must find a mechanism for tolerance and minimise the damage caused. When a plant experiences an abiotic stress, specific sensors will detect these stress signals. Among the first abiotic stress sensors to be characterised in plants are AtHK1 (Urao et al., 1999; Wohlbach et al., 2008) and CRE1 (Reiser et al., 2003), both of which are histidine kinases that detect changes in osmotic pressure. The sensors then stimulate various signal transduction pathways, including reactive oxygen species (Mittler 2002; Apel and Hirt 2004), calcium ion flux (Knight & Knight, 2000; Sanders et al., 1999) and phytohormones, in particular abscisic acid (ABA). ABA is a key

messenger in the abiotic stress response of plants and up-regulation of ABA synthesis occurs under drought and salinity stress (Seki et al., 2007).

These signalling pathways then trigger the expression of a specific suite of stress-induced genes, which may encode functional proteins for stress tolerance, typically chaperones and late embryogenesis abundant (LEA) proteins or regulatory proteins for further signal transduction.

ABA signalling under drought or salinity stress, ultimately results in increased stress tolerance by causing stomatal closure thus minimising water loss, altered gene expression and the accumulation of osmo-compatible solutes (Huang et al., 2007; Hubbard et al., 2010; Kim et al., 2007).

Biotic interactions

Each component of an ecosystem can, in principle, be considered in isolation, but it is often restrictive to do so - even when the focus is at the physiological or molecular level - because an understanding of interactive complexity must be added to reveal the fundamental relationships between form and function. In nature, each individual experiences different levels of stress and selection pressure, which are modulated not only by their genetic composition but also by the environment, which also includes interactions with species around them. Competition, for example, is an interaction that may occur both within and between species. For self-fertilising or clonal plants, competition will also occur between individuals of identical genotypes. Whilst competitive interactions are doubtless important ecologically, and particularly in influencing the dynamics of disease epidemics, I simplified the common garden studies by excluding competition effects by weeding and by spacing experimental plants widely. Nevertheless, some of the important and influential biotic interactions were retained, such as herbivory and disease. This action is defensible because *A. thaliana* individuals are often found singly in relatively stressful conditions that have restricted inhabitation by other plant species.

Herbivory

There are estimated to be 3 million herbivorous insect species (Schoonhoven et al., 2005) sharing 400 million years of co-evolutionary history with their plant hosts (Santamaria et al., 2013; Wheat et al., 2007). Insect herbivores have an array of morphological adaptations allowing them to chew, snip or tear plant tissue. Whilst other tissues may be targeted, leaf tissue is clearly targeted by the largest proportion of species (Santamaria et al., 2013). Furthermore, co-evolution has resulted in the development of numerous methods to sequester or metabolise plant defensive toxins (Nishida, 2002).

A. thaliana is attacked by a variety of herbivores. Like many other plant species, in *A thaliana* defence against herbivory starts with physical barriers such as the cuticle thickness and trichomes (Glas et al., 2012; Hanley et al., 2007; Tian et al., 2012) and modifications to cell walls (Divol et al., 2007; Sasidharan et al., 2011). Once mechanical damage is incurred, the injury stimulates certain generic physiological responses, but additionally the plant mounts specific responses to particular herbivores, whose actions are detected by pattern recognition receptors (PRRs). PRRs recognise the highly conserved herbivore-associated elicitors (HAEs) and herbivore-associated molecular patterns (HAMPs) found in insect saliva (Bonaventure et al., 2011; Halitschke et al., 2001; Mithöfer & Boland, 2008). Induced defences are then activated both locally and systemically, including targeted defences for the specific herbivore detected (Figure 4). Herbivore defence in *A. thaliana* causes changes in over 700 mRNAs (Schenk et al., 2000). Amongst the chemical arsenal employed by *A. thaliana* in response to herbivory are a range of glucosinolates to provide protection. There are approximately 126 glucosinolates protecting the order *Brassicales* against herbivory (Mewis et al., 2006). In my common gardens (Chapters 2 & 3), *A. thaliana* suffered extensive herbivore damage and the varied impact of herbivores among plant genotypes indicated differential adaptation to herbivore resistance and tolerance.

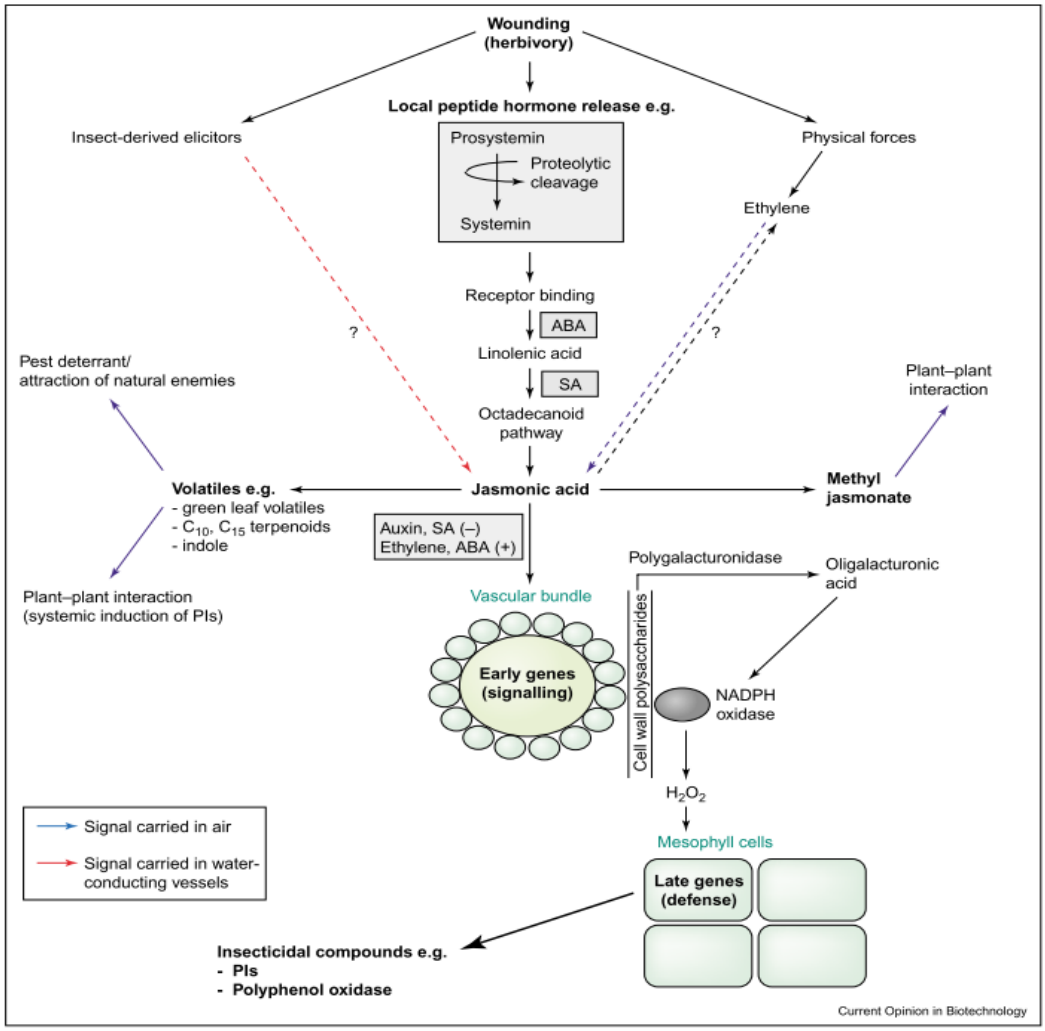

Figure 4 A generalised overview of the wounding response and signalling defence pathways. (Abbreviations: ABA- abscisic acid; SA- salicylic acid). Image from (Ferry et al., 2004).

From an evolutionary perspective, the most effective ecological defensive strategy against other species is one that cannot be undone by natural selection and co-evolutionary dynamics. For example, Bruinsma et al., (2009) studied the attraction of parasitoid wasps (natural enemies of the plant pest) to Jasmonic Acid (JA) induced in caterpillar-damaged *Brassica oleracea*. Herbivore predation by parasitoid wasps increased with herbivore damage to the plants. Similarly Rasmann et al., (2005) found that European maize varieties and some wild ancestors produce the sesquiterpene (E)-β-caryophyllene in response to damage of the roots by beetle larvae, thus attracting entomopathogenic nematodes. Beetle larvae from (E)-β-caryophyllene-

producing plants showed a five-fold higher nematode infection rate than those on control plants unable to produce this sesquiterpene. This increase in natural predation is likely to be an evolutionarily stable defence mechanism because natural selection will maintain the interactions as predation on the herbivores benefits the plants. Fundamentally, the basis of these defensive responses in physiology means that they are genetically based, and therefore there might exist in variation at relevant loci. Consequently, recognition of these kinds of interaction may enable greater understanding of differential performance in my study's outdoor common gardens.

Pathogens

Plant pathogens have so-called 'Jekyll and Hyde' impacts on host species depending upon the scale at which you consider their impacts (Gilbert 2002). For individuals, pathogens cause either mortality or fecundity losses; however, at the population level fluctuation in pathogen pressure can select for genetic diversity. For example, pathogen exposure can be responsible for inverting a fitness hierarchy, (Salvaudon et al., 2008) whereby infection of *A. thaliana* by the oomycete *Hyaloperonospora arabidopsis* increased the fecundity of the least fecund (under control conditions) accession, leading to the pathogen being dubbed the 'Robin Hood' parasite. Consequently, spatial variation in the prevalence of pathogens may have a critical role in the maintenance of genetic diversity.

The spatial distribution of pathogens is determined in part by environmental conditions and dispersal processes, but it is also closely linked to the spatial distribution and genetic structure of the host populations (Hershberg et al., 2008; Thrall & Burdon, 2003; Wirth et al., 2008). Climate change will alter the range of many pathogens (Chakraborty et al., 2000) and, if a pathogen migrates polewards with its host plant, it could result in the exposure of naive populations (either new host species or previously unexposed populations of existing hosts) to novel xenopathogens. Small populations with reduced genetic diversity create particular vulnerability to outbreaks of disease (O'Brien & Evermann, 1988). Demographic studies of natural disease outbreaks have revealed spatial and temporal variation in disease distribution

and severity (Jarosz et al., 1992). Long term studies of disease dynamics in natural and agricultural systems have shown broad predictability of the spread of epidemics at meta-population level, but this predictability is lower at smaller spatial scales, where the process is characterised by marked stochasticity at individual deme level due to the interacting dynamics of the system (Figure 5 Burdon and Thrall, 2014).

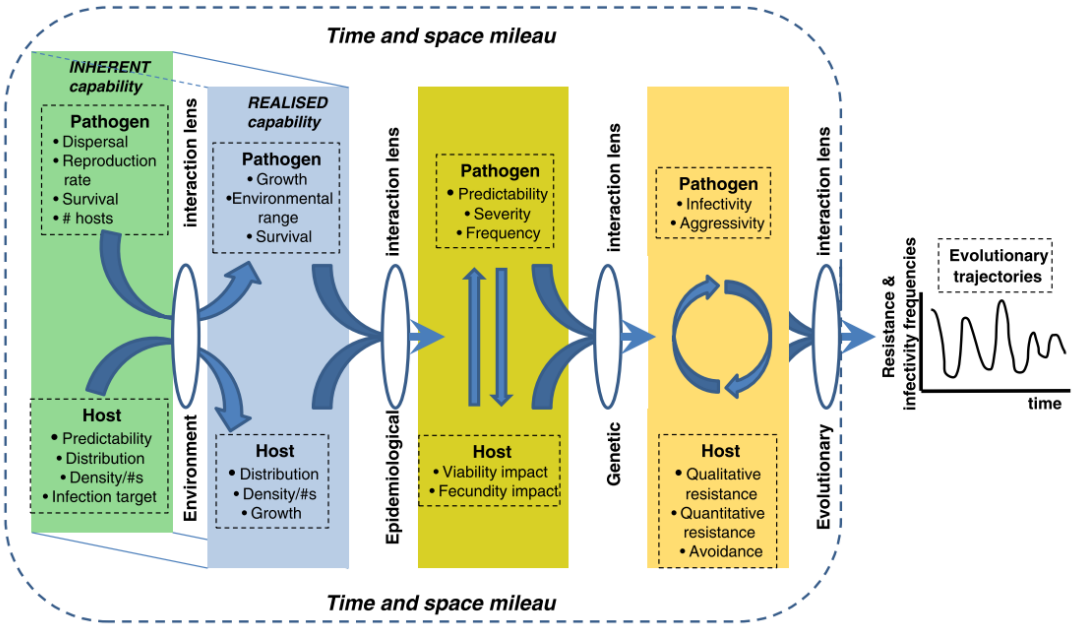

Figure 5 The interaction lens altering resistance and infectivity frequencies. The environment, epidemiology of pathogen growth and dispersal, the genetic interaction and subsequent evolutionary changes causing fluctuating dynamics. (Image from Burdon & Thrall, 2014).

The implications of this for my study of *A. thaliana* in the UK will vary among genotypes depending upon the selection pressures at their home site, the evolutionary strategy followed and its level of success. To briefly explore this further in the context of sustained genetic variation, I now consider different co-evolutionary models, such as the so-called 'arms race' and 'trench warfare' scenarios.

Host-pathogen co-evolutionary models

Pathogens and their hosts are involved in a constant co-evolutionary race. Host species evolve

either to evade their pathogens or to mitigate the effects of disease, whilst pathogens evolve to optimise their ability to infect. Losers in the battle pay the ultimate evolutionary cost of failure to reproduce. The classic model of host-pathogen interactions is known as the 'arms race' model, which is based on the theory that co-evolutionary dynamics between the partners depend upon innovation and counter-innovation, with each species seeming to be racing to not be outdone. The 'arms race' model is not one that promotes genetic diversity in the gene pools of participants, because outmoded strategies are discarded. To address this discrepancy with observed longstanding polymorphisms, the 'trench warfare' model (Stahl et al., 1999b) elaborates the theory to produce a kind of balancing selection (namely, frequency-dependent selection), whereby the fitness of alleles changes over time depending on their frequency, rather than through the uniqueness of their innovations relative to those of the countering partner. Although it originated from the analysis of energy flow (Liow et al., 2011), Van Valen's 'Red Queen' hypothesis (Van Valen, 1974) eloquently explains many cases of evolutionary conflict between species. The implication for my study is that the 'signature of selection' will vary depending upon which model has been the most applicable to the interaction considered. The signature will be evident in the distribution and fitness costs of *Rpm1* alleles, considered in Chapter 2, and I expect that this should support the trench warfare model, because the focal system is a long-standing, stable polymorphism.

Types of plant pathogen

Many organisms attempt to utilise plants as a carbon source and, like many other species, *A. thaliana* faces many pathogens that attempt to exploit its nutritive value. The organisms causing infectious diseases in plants in general and *A. thaliana* in particular include fungi, bacteria, viruses and nematodes. My study of *A. thaliana* focuses particularly on the bacterial (*Pseudomonas syringae* and an array of *Xanthomonads*) and fungal pathogens (*Botrytis cinerea*), so here I give a brief review of the physiological and molecular basis for these interactions.

Fungal plant pathogens

Plants face formidable foes from the fungal kingdom. Fungal pathogens of plants can be highly specialised at the genus, species or even cultivar level. For example, there are the different strains of *Puccinia graminis* that each cause black stem rust in a different cereal crop. Furthermore, they can be specific to the plant tissue that they can infect. For example, *Gaeumannomyces graminis* is a pathogen only of the plant root. Fungal pathogens can be: biotrophic, existing on living tissue and diverting resources from the plant (for example *Sphaerotheca pannosa causing powdery mildew*); hemibiotrophic, feeding on living and dead tissue at different life stages; or necrotrophic, where the host is killed and the pathogen utilises the dead tissue (for example *Botrytis cinerea* causing grey mould). Fungal pathogens have evolved sophisticated survival and dispersal mechanisms via sporulation.

The fungal infection process starts with the initial inoculum of fungal spores on the surface of a plant. These will germinate as a result of the uptake of water or a chemical stimulant. To penetrate the plant tissue the fungus must either find an opening (such as stomata or wounds) or force entry through direct penetration. This is achieved by applying pressure from an appressorium (de Jong et al., 1997) and the secretion of enzymes to degrade plant tissue (van Kan, 2006). How infection proceeds then depends upon the life history of the pathogen. Necrotrophs will secrete toxins to kill the plant tissue and thus release the nutrients it requires for growth and reproduction,whereas biotrophs will produce specialised hyphae known as haustoria, to extract nutrients from plant tissue (Mendgen and Hahn, 2002).

Known fungal pathogens of *Brassicaceae* and *A. thaliana* include *Botrytis cinerea* (causing grey mould), *Peronospora parasitica* (downy mildew), *Albugo candida* (white rust) and *Leptospaeria maculans* (stem canker),all of which are known to occur in the UK. So the plant populations from which I collected my study accessions will therefore doubtless have encountered them, as well as potentially occurring in the common garden experiments.

Bacterial plant pathogens

The majority of bacteria co-exist with plants without causing evident harm. Globally, bacteria

cause less plant disease than fungi and viruses (Bebber, Ramotowski, and Gurr, 2013), although they are still responsible for a wide variety of economically important losses, particularly in sub-tropical and tropical regions.

Like their fungal counterparts, bacterial plant pathogens can be necrotrophic (for example *Pectobacterium carotovorum,* formerly *Erwinina carotovora,* (Barras et al., 1994), biotrophic (for example all *Xanthomonad* species, Gabriel 1997) or hemi-biotrophic, which exhibit properties of both living parasitically on live tissue and then persisting on the dead tissue (for example *Pseudomonas syringae*, Lee & Rose 2010). In this research I use both *Xanthomonads* and *Pseudomonas syringae*.

Plants are exposed because the initial bacterial inoculum to start infection is believed to be frequently carried from the previous generation within the seed coat itself. Alternatively, it can be spread by rain splash or also by insect vectors, such as aphids. Bacteria may passively enter the plant via stomata, wounds or hydathodes.

Once the bacteria encounter living plant tissues, the plant may detect the highly conserved pathogen associated molecular patterns (PAMPS) or microbe associated molecular patterns (MAMPS) found on the bacterial cell surface and this triggers the generic basal defence response. Concurrently the type III secretion system of the bacteria is assembling the 'hypersensitive response and pathogenicity' (*Hrp*) pilus through which type III effector proteins will pass into the plant cell. The effector proteins are one of a variety of virulence factors (see Table 1) deployed by the bacteria as they attempt to optimise bacterial growth conditions and disable plant defences by reprogramming the plant. The type III effector proteins include the avirulence (*avr*) proteins, which may be detected in the gene-for-gene interaction and thus initiate the hypersensitive response to prevent bacterial proliferation. Certain genetic loci are critical for this defence. Specifically, recognisable *avr* genes that stop disease clearly have a high fitness cost for the bacteria therefore their continued persistence in the bacterial population is either linked to fluctuating co-evolutionary dynamics, drift or some underlying important function. For example Ritter & Dangl (1995) found that *avrRpm1* was essential for virulence of

Pseudomonas syringae pv. maculicola on *A. thaliana*.

Bacterial virulence factor	**Description**
Effector proteins	Injected into the cell via the type III secretion system. Essential for the virulence of *Pseudomonas syringae, Xanthomonas* spp*., Ralstonia solanacerum* and *Erwinia* species (d*e Torres et al*>, 2006). They function to suppress host defences and modify cellular conditions for bacterial growth. Also includes avirulence genes involved in the gene-for-gene resistance response (Abramovitch and Martin 2004).
Toxins	Coronatine, secreted by several pathovars of *Pseudomonas syringae,* mimics methyl jasmonate and therefore disrupts plant defence signalling (Nomura et al., 2005). Coronatine has been shown to significantly increase bacterial multiplication in infected leaves (Bender et al., 1987; Brooks et al., 2004; Mittal & Davis, 1995).
Cell wall degrading enzymes	Pectic enzymes produced by *Erwinia chrysanthemi* and *E. carotovora* causing soft rot (Bateman and Millar 1966; Collmer and Keen 1986).
Phytohormones	Typically auxins that are involved in growth and repair, they therefore cause various neoplastic diseases when secreted by bacteria. Indole-3-acetic acid (IAA) secreted by *Pseudomonas syringae pv. savastanoi* causes olive and oleander knot (Yamada 1993).
Exopolysaccharides (EPSs)	Functionality still uncertain however EPSs are believed to act to avoid or delay host defences (Denny, 1995; Jahr et al., 1999; Leigh & Coplin, 1992; Yu et al., 1999).

Table 1 Bacterial virulence factors

Pathogens are an important evolutionary force that act on plants. There are clearly costs of disease, yet both resistance and susceptibility may occur in host plant populations. For example, in UK *A. thaliana*, there is widespread variation in the local frequencies of various *Rpm1* alleles, which I characterise at the population level for the first time in Chapter 2. Spatial variation in genetic diversity, or population genetic structure, potentially can in part be explained

by pathogen distribution. But there is a problem for explaining any such pattern by reference to optimizing selection: if resistance was cost-free then alleles for resistance should occur ubiquitously at the highest frequency. Experimental evidence suggests that fitness costs of resistance can indeed be significant, and thereby driving population genetic diversity in resistance (*Graham et al*., 2010), because non-resistance genotypes will thrive wherever pathogen outbreaks are rare. The basis of fitness costs can be divided into three main categories (Table 2).

Fitness cost	Description	Examples
Allocation costs	Caused by diverting limited resources from growth to defence.	(Chew and Rodman, 1979) (Rhoades, 1979) (Bazzaz et al., 1987) (Heil et al., 2000) (Simms and Fritz, 1992)
Autotoxicity costs	Caused by toxic defence compounds.	(Gog et al., 2005) (Baldwin and Callahan, 1993)
Ecological costs and trade offs	Caused by the disruption of natural interactions.	Increased chemical defence compounds reduced pollinator visit time (Strauss et al., 1999) Decreased competitive potential.

Table 2 Examples of the different types of fitness costs for resistance traits.

There are special aspects of the *A. thaliana –Pseudomonas syringae* pathosystem that are relevant to the studies in this thesis and I discuss them next.

Arabidopsis- Pseudomonas pathosystem

In the 1980s, large-scale screening experiments showed that the Gram-negative bacterial species *Pseudomonas syringae,* the causative agent of bacterial speck in tomatoes, was also capable of causing disease in *A. thaliana* (Dangl et al., 1992; Dong et al., 1991; Innes et al., 1991; Whalen et al., 1991). This important discovery led to an intense period of research into characterising the mechanisms of the underlying plant-pathogen disease processes.

Plant defence responses

A disease outcome depends upon the successful evasion and suppression of a plant's recognition and defence responses. There are two classes of defence response: PAMP triggered immunity (PTI) and effector triggered immunity (ETI) (Figure 6).

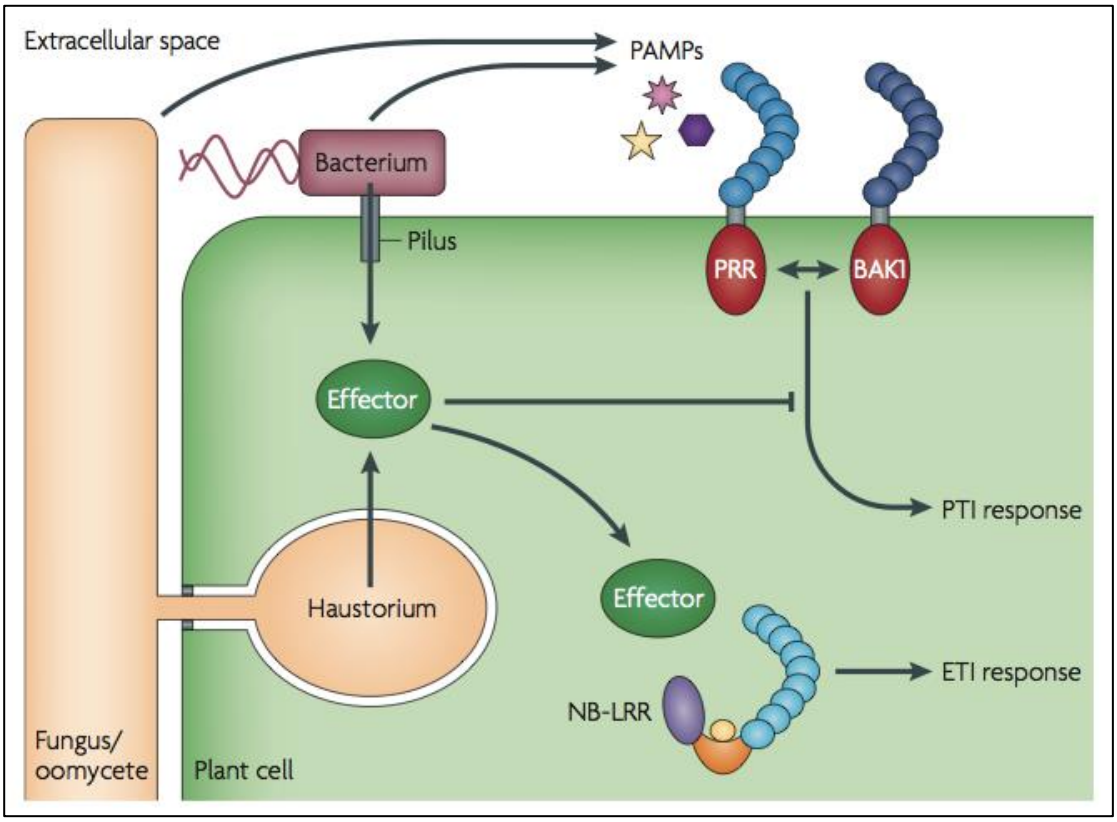

Figure 6 The mechanisms of plant immunity from (Dodds and Rathjen, 2010)

Plant defence responses occur in dynamic layers as shown in the zig-zag model (Figure 7).

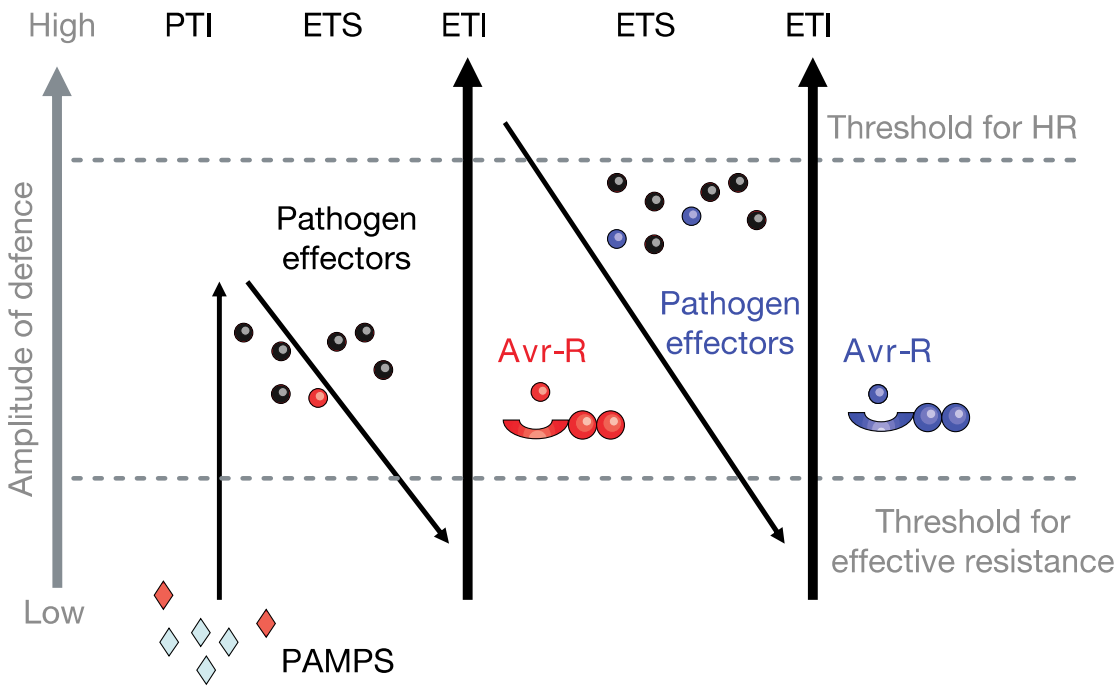

Figure 7 Zigzag model of disease defence (Jones and Dangl, 2006). The amplitude of defence relates to the amount of resources deployed by the plant for defence, the x axis is subsequent generations over evolutionary timescales. The recognition of PAMPS induces the basal defence response or PAMP triggered immunity (PTI). The pathogen effectors then overcome PTI but in so doing may themselves be recognised thus inducing the stronger targeted effector triggered immunity (ETI).

When pattern recognition receptors (PRRs) with ligand-binding domains outside the plant cell detect ‘pathogen associated molecular patterns’ (PAMPS) or ‘microbe associated molecular patterns’ (MAMPS) (Boller and He, 2009) the generic basal defence responses are initiated. PAMPS are highly conserved features, often essential to the pathogen such as bacterial flagellin and fungal chitin. This means that pathogen evasion of PTI is limited. Plants also mount a defence response when danger associated molecular patterns (DAMPS) are detected, such as the breakdown products of the cell wall. Concurrently a *Pseudomonas* pathogen will haveformed the type III secretion system (T3SS) consisting of membranous rings and a needle-like structure, (Figure 8), together creating a continuous path from the bacterial cytoplasm to the host cytoplasm.

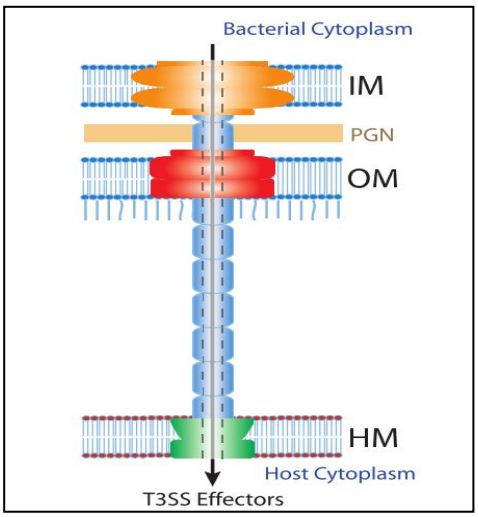

Figure 8 The rings of the T3SS provide a continuous route from the bacterial cytoplasm to the host cytoplasm. Crossing the IM: bacterial inner membrane, PGN: peptidoglycan layer, OM: bacterial outer membrane and the HM: host membrane. Image from Coburn et al., (2007).

Through this structure, pathogens insert an array of effector proteins into the plant cell, thereby bypassing extracellular defences. Some of these proteins may be recognised by the plant in the specific gene-for-gene interaction known as effector triggered immunity (ETI) often leading to the hypersensitive response to halt pathogen spread. The recognition of effector proteins produces evolutionary pressure for pathogen innovation and turnover in both bacterial effector proteins and plant avirulence genes.

Non-host resistance and basal defence

Non-host resistance (NHR) is the most common type of plant resistance and stops the majority of microbial interactions from becoming pathogenic. NHR describes the resistance shown by an entire species to a specific pathogen (Heath, 1987). It can be divided into two categories, type I where disease is halted without the development of any visible symptoms, and type II, which relies upon the hypersensitive response (HR) (Mysore and Ryu, 2004). Basal defences have limited strength (Figure 7) and can be defined as the defence response mounted by a susceptible host to a virulent pathogen (Jones and Dangl, 2006). They are both generic responses involving broad-spectrum defence.

The first line of defence in any interaction involves passive protection by physical barriers. If these physical barriers are breached then the constitutive prophylactic production of secondary metabolites offers broad-spectrum non-specific protection. Later the induced defence responses include the up-regulation of a range of antimicrobial compounds.

Basal defence responses are triggered by the recognition of physical damage (Russo & Bushnell 1989), conserved features like bacterial flagellin (Asai et al., 2002; Gómez-Gómez and Boller, 2002; Nürnberger and Brunner, 2002) or the breakdown products of cell wall components by fungi (Heath et al., 1997). The defence response includes many modifications of the cell wall to increase the physical barrier to infection and compartmentalise pathogens that have already invaded, so that they cannot spread further into the plant (Xu & Heath, 1998; Mould & Heath, 1999). This can be achieved by rapid oxidative cross-linking (Lamb & Dixon 1997) or by the deposition of silica (Tatagiba et al., 2014) or callose containing papillae (Voigt, 2014). The reorganisation of the plant cytoskeleton (microfilaments and microtubules) has been shown to be crucial in NHR of barley to *Erysiphepisi* (Kobayashi et al., 1992; Kobayashi et al., 1994; Kobayashi et al., 1997), with extensive modifications occurring at the point of contact between plant and fungi (Gross et al., 1993).

When pathogen ingress is detected, a range of pathogenesis-related (PR) genes are upregulated (Heath, 1997). Unlike the genotype specific resistance (R) genes, these PR genes provide non-specific defence. In *Arabidopsis thaliana*, the non-host resistance gene *NHO1* confers resistance against a range of *Pseudomonads* (Lu et al., 2001) whilst the virulent DC3000 is able to evade this defence by suppressing *NHO1* expression (Kang et al., 2003). This gene has also been shown to be involved in resistance to *Botrytis cinerea* (Kang et al., 2003). The loss of *NHO1* not only impacted NHR but also reduced the efficacy of R-gene defence (Lu et al., 2001).The *PEN* genes were found to be essential for NHR in *Arabidopsis thaliana* to prevent penetration of the barley pathogen *Blumeria graminis* (Stein et al., 2006).

Other responses target key pathogenicity processes, for example proteases are used by a

variety of pathogens to aid in the suppression of host defences and by herbivores to digest plant tissues (Botella *et al*., 1996). Protease inhibitors produced by plants have been shown to have both anti-microbial and anti-feedant properties (Joshi et al., 1999; Joshi et al., 1998; Laluk & Mengiste, 2011).

Resistance genes

Genotype-specific defence responses are initiated by the recognition of specific microbial genes allowing the plant to successfully combat the infection process. The gene-for-gene interaction (Flor, 1971) relies upon specific genes being present in both host and pathogen for the recognition of infection and for successful defence. Recognition of the pathogen avirulence gene results in programmed cell death in the form of the hypersensitive response (Klement et al., 1964; Klement, 1963).

Chemical defence

Both basal and R-gene mediated defence responses have chemical defence embedded in them. This starts with the propagation throughout the plant of the recognition event by chemical signalling. A co-ordinated defence is then mounted by the expression of the phytohormones: salicyclic acid (SA), jasmonic acid (JA), abscisic acid (ABA) and aromatic amino acids.

For example, phytoalexins are a diverse range of secondary metabolites that inhibit or kill fungal and bacterial pathogens (Kuc, 1995; Nicholson & Hammerschmidt 1992; Hammerschmidt 1999; Dixon 1986; Poloni & Schirawski 2014). Phytoalexins tend to occur locally at the site of infection and the disease outcome is often determined by the speed at which they accumulate. They may exist in their active form, or as inactive precursors waiting for the detection of damage or attack (Osbourn, 1996). Examples include phenolics and saponins. Phenolics are antimicrobials that have been extensively characterised since the 1940s concurrent with developments in chromatographic methodology (Martin & Synge 1941; Gordon, Martin & Synge, 1943). Phenolics are present in all higher plants (Levin, 1971) and are involved in constitutive and induced defence responses. Saponins are potent antifungals and have been

shown to be part of the generic NHR response as they occur at high concentrations in healthy plants (Osbourn, 1996). Saponin deficient mutants are highly susceptible to infection (Papadopoulou et al., 1999).

Tolerance

Whilst resistance may invariably seem like the optimal response to disease, there are potential trade-offs. False induction of resistance mechanisms by abiotic conditions may be costly (Wang et al., 2001), possibly through diversion from critical processes of limiting resources such as proteins or metabolites. Additionally, environmental exposure to pathogens may occur to varying degrees resulting in a cost-benefit spectrum. For example, at low levels of pathogen inoculum, the initiation of resistance pathways may exceed the benefits, but to various degrees (Roux et al., 2010). An alternative evolutionary response to pathogens is tolerance by the host, defined as the ability of a host organism to minimise the cost of disease (Kover & Schaal, 2002). In plants this may be achieved, for example, by early initiation of flowering (Korves & Bergelson, 2003) or by increased branching (Korves & Bergelson, 2004). Both of these responses offset the impact of anticipated reduction in life expectancy by increasing the immediate reproductive output.

Biotic and Abiotic Factors Interact

If we have a wet summer, as in 2012, this can increase the prevalence of pathogens, especially fungi. Additionally, the rainfall will increase soil erosion and nutrient leaching, increase costs of drying crops and increase spoilage of stored crops. The effect of climate on plants is felt by all of us as crop losses correlate with the prices that we pay for the food that we eat. Extreme weather events are predicted to become more commonplace under climate change scenarios (IPCC). This could have devastating impacts for agriculture, for example the extreme weather events of 2012 caused $17.3 billion in crop losses in the USA alone, $4.1 billion being the average for the preceding decade (http://www.nrdc.org/media/2013/130827.asp). Whilst the 2003 heat wave in Europe is estimated to have reduced crop production by nearly a third (*Ciais et al.,* 2005). Even without these extreme weather events losses due to drought in maize and

rice are estimated at $5 billion per annum (Xiong, 2013).

Clearly, biotic and abiotic factors do not produce their effects in isolation from each other. Laboratory studies combining biotic and abiotic stresses have shown that they can have non-additive impacts. For example, abiotic conditions may affect the distribution and growth of pathogens (Ghini et al., 2011; Luck et al., 2011). Cross-tolerance, where exposure to one stress provides protection from a different stress, reveals a complex cross-talk between defence responses (Bian & Jiang, 2009; Janda et al., 2003; Plazek & Zur, 2003). For example Xu et al. (2008) tested the drought tolerance of 10 plant species after inoculation with RNA viruses and all infected plants showed increased drought tolerance. Therefore, under some circumstances being diseased could be beneficial for the plant. This means that single stress response screenings can be unreliable for the prediction of field-relevant performance. Consequently, field experiments in outdoor common gardens are a good system for examining the performance of genotype in more natural environments.

Phenome

The phenome is the dynamic expression of the interaction between genotype and environment.

Phenotypic plasticity

Phenotypic plasticity defines the range of potential phenome responses for a given genotype. The plastic limit of a genotype is the environmental perturbation beyond which an individual cannot survive and reproduce. The phenotype expressed will have an evolutionary fitness associated with it. The fitness of the phenome range of a genotype, in relation to a continuous environmental parameter, can be considered simplistically (Figure 9). Among real systems the shape of the curve will vary as selection will depend upon the stability of conditions, timing relative to reproduction and genetic competition. Fitness costs associated with different phenotypes, including those arising from plasticity, along an environmental gradient create GxE interactions.

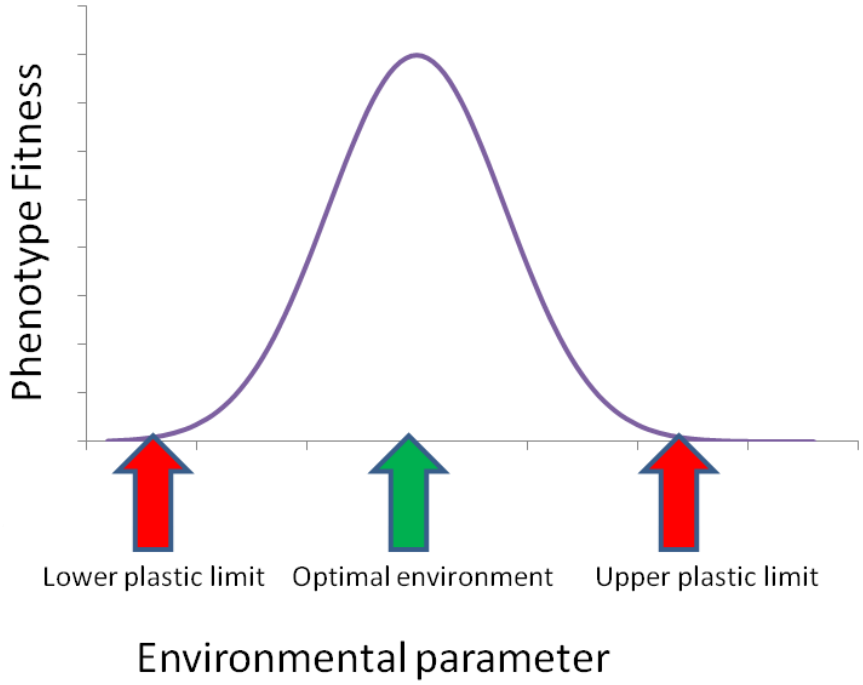

Figure 9 Fitness across a simplified phenome range along an environmental gradient. The plant can survive and reproduce between the red arrows but only produces maximum seed set when conditions correspond with the green arrow.

Phenotypic plasticity is itself an evolved characteristic that buffers organisms against environmental fluctuations. Understanding the phenome range could aid agricultural productivity under climate change scenarios and concurrent predicted increased extreme weather events.

Chemical diversity in the phenome

Understanding the link between genetics, environment and phenotype is a continuing goal in the quest to enhance crop production and food security. If the genetic sequence is seen as the blueprint from which the functioning organism is built, the metabolome can then be seen as the bricks and mortar from which the design is built. The expressed genetic diversity is enacted by its chemical constituents, modified by environmental stimuli. An organism's metabolome is the ultimate phenotype, capable of encapsulating genetic and environmental information, however our ability to understand the huge datasets available is lagging behind the technology able to

rapidly generate them. Therefore it is important that researchers from across all fields of biology explore the opportunities presented by metabolome data.

Simplistically, metabolism can be divided into primary and secondary (Kossel, 1891) although it is now recognised that there is some overlapping functionality (Schwachtje & Baldwin, 2008; D'Auria & Gershenzon, 2005). Primary metabolism typically refers to those compounds essential for cellular maintenance and replication, whilst secondary metabolism refers to compounds that mediate an organism's environmental response.

Over 200,000 secondary metabolites are believed to occur in vascular plants (Wink et al., 1988), and over 5,000 in *Arabidopsis thaliana* alone (Roessner et al., 2001). An array of adapted processes rely upon this diverse chemical arsenal. Secondary metabolites have been shown to have an intrinsic role in herbivore defence (Hartmann, 2008; Mewis et al., 2006; Kessler & Baldwin, 2002), pathogen interactions (Hahlbrock et al. 2003; Wittstock & Gershenzon 2002; Rojas et al., 2014) and abiotic stress responses (Shulaev et al., 2008; Bowne et al., 2011).

In order to estimate the genetic influence on metabolite expression initial studies focused on quantitative trait loci (QTL) mapping of crossed populations. More recently genome wide association study coupled with metabolomics analysis (mGWA) is providing a faster more flexible approach (Chen *et al*., 2014). Just as the genetic code has redundancy to preserve function it appears that metabolome could be similarly protected. Chan et al., (2010) found that the *Arabidopsis thaliana* metabolome did not fully reflect the high level of genetic diversity revealed by SNP analysis. Despite this the metabolic fingerprint is still highly heritable: Chen et al., (2014) found 840 distinct metabolic traits and of these 83.1% had over 50% broad sense heritability. Genetic differences can be detected by both presence/ absence and also by differential expression of compounds. Metabolite fingerprinting is sensitive enough to detect differences between GM lines (DiLeo et al., 2014) and has shown some potential for phylogenetic clustering (Wahyuni et al., 2012). Metabolite fingerprinting has the potential to provide high resolution rapid expression of GxE interactions. Understanding an organism's

metabolite fingerprint is compounded by its transient ephemeral nature, the metabolome of *Arabidopsis thaliana* has shown conditional effects in relation to both environment and developmental stage (Chan et al., 2011; Wentzell & Kliebenstein, 2008). Despite these difficulties the high level of heritability suggests that it should be possible to infer phylogeny from metabolite data (Chapter 5).

Epigenetics and adaptation

Under environmental stress plants can prime themselves to reduce further impacts. For example in systemic acquired resistance (SAR) pathogen detection on one leaf produces a whole plant resistance response (Truman et al., 2007). It has now been shown that a priming response can be heritable across multiple generations under pathogen challenge (Slaughter et al., 2012; Luna et al., 2011) and abiotic stress (Molinier et al., 2006), although this is not a universal response (Pecinka et al., 2009). Transgenerational changes in phenome expression that provide evolutionary advantage under constant environmental stress can be considered adaptive.

Epigenetic modifications or transgenerational stress memory provide a potential route to rapid adaptation (see Chapter 6). These are modifications that alter gene expression without changing the underlying genetic sequence (Jablonka et al., 1998). Epigenetic mechanisms can produce new phenotypes that are then filtered by natural selection. The interconnected mechanisms known to be involved in this are DNA methylation (Griffith & Mahler 1969- from (Holliday 2006) small RNAs (Bond & Baulcombe, 2014; Heard & Martienssen, 2014; Matzke et al., 2009; Peschansky & Wahlestedt, 2014; Zhao & Chen, 2014) and histone modifications.

Epigenetically produced phenotypes may be stable only over short timescales or inherited over multiple generations. Small isolated populations of plants are particularly vulnerable to genetic bottlenecks where the population may lack a successful genotype in the face of environmental stress. Epigenetic mechanisms provide phenotypic diversity without genotypic diversity (Figure 10).

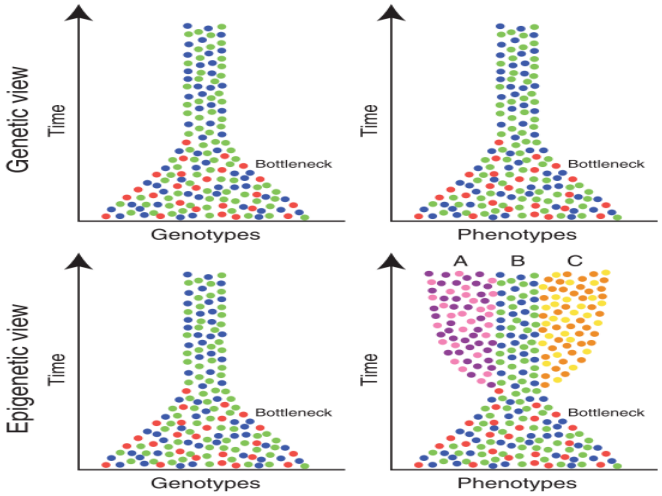

Figure 10 How epigenetics can mitigate for genetic bottlenecks in populations, where phenotypes A and C are produced by epigenetic innovation, (image from Rapp et al, 2005).

Genotype by environment interactions (GxE)

In this thesis, I explore principally GxE interactions as a mechanism for maintaining genetic diversity. To quantify the impact of environment on performance genotypes must be grown in alternate environmental conditions. The classic methods for measuring GxE interactions in plants are reciprocal transplant (RT) and common garden (CG) experiments. RT experiments are logistically more challenging as they are effectively a CG at each home site. Common garden experiments have been an integral research tool in ecology since the 18th century (Langlet, 1971) and the comparison of performance of lines between gardens has been crucial in demonstrating the environment-specific adaptedness of ecotypes (Turesson, 1922; Linhart & Grant, 1996; Heschel & Paige 1995). With data on current and historic environmental variables increasingly available, performance in a single CG can be accurately compared to the historic home site.

Reaction norms are typically used to reveal GxE interactions. Figure 11 shows a GxE between genotypes A and B. In panel (a), the environment is having different effects upon the two genotypes as the fitness hierarchy changes between environments. Whereas genotypes C and

D on the right despite changes in fitness due to the different environments do not show GxE, as the lines remain parallel showing that environmental differences are affecting them in the same way.

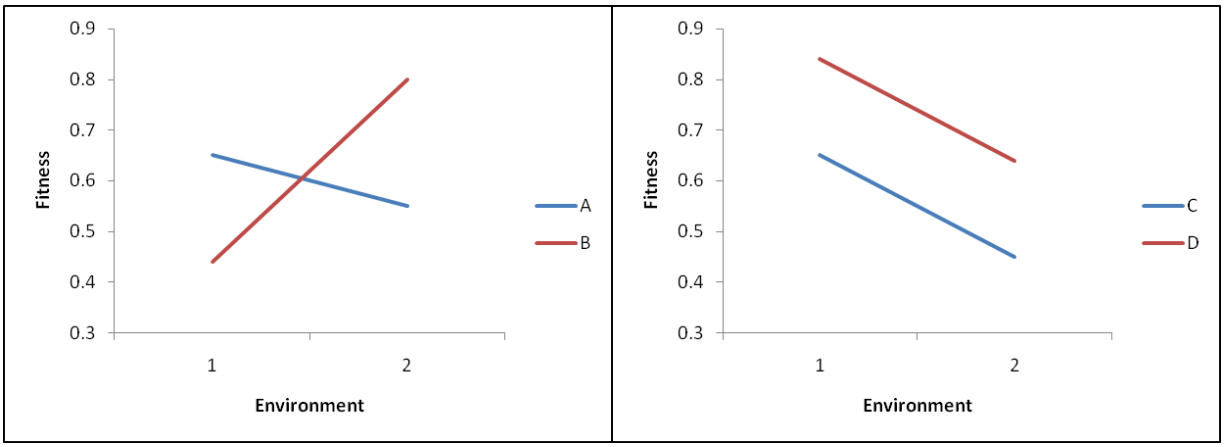

Figure 11 Reaction norms between two environments. The lines are used for illustration only and does not imply that environment is a continuous variable.

Summary and aims

The principal aim of this research was to explore adaptation and the maintenance of genetic variation in UK *Arabidopsis thaliana*. My main approach was to use outdoor common garden experiments to characterise phenotype differences and performance hierarchies among genotypes and thereby to test for GxE interactions. The specific objectives were:

- Can spatial heterogeneity maintain diversity within a long established gene for gene interaction? (Chapter 2).
- Do UK populations of *A. thaliana* show adaptation to home site climate? (Chapter 3).
- Does temporal variation in climate favour the maintenance of genetic diversity? (Chapter 3).
- Can existing genetic diversity protect against xenopathogens through pre-adapted resistance traits? (Chapter 4).

- Can metabolite fingerprinting recreate phylogeny and reveal the chemical basis of a GxE interaction? (Chapter 5).
- What is the adaptive potential of epigenetic modification to multiple pathogen challenge? (Chapter 6).

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Chapter 2: The maintenance of genetic diversity at *RPM1*, a locus for pathogen defence in *Arabidopsis thaliana*

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Abstract

Substantial genetic diversity has been characterised in the model plant species *Arabidopsis thaliana.* For functional traits like pathogen resistance, an optimal genotype maybe elusive due to variation in selection over space and time, which could occur if resistance carried fitness costs in pathogen absence. The resistance gene *RPM1* and its susceptible counterpart *rpm1-null* are thought to have co-existed for 9.8 million years as selective pressures advance and retreat, other more recently evolved *rpm1* alleles have also been characterised. We first screened genetic diversity in populations across the UK to determine the level of variation at this locus. We used a group-wise common garden experiment to look for *Rpm1*-related differential performance (i.e. fitness costs) in two contrasting environments. We found variation in performance both within and between populations. The common gardens revealed a classic genotype-by-environment (GxE) interaction in both *RPM1* and *rpm1*. However the fitness differential was lower between the longstanding *RPM1* and *rpm1-null*, which suggests that ameliorating modifiers have arisen during evolution.

Introduction

Evolution by Darwinian natural selection erodes the diversity of alleles in a species' gene pool as adaptation proceeds towards an optimum (Fisher, 1930; Falconer, 1981). However, substantial levels of genetic variation are found in many species, including plants (Linhart &

Grant, 1996), which requires explanation. Particularly intriguing challenges are presented by longstanding genetic polymorphisms that are demonstrably associated with a functional phenotype (i.e. where allelic variation is not selectively neutral). Polymorphisms are common in plant *R*-genes (Bergelson et al. 2001), which are loci involved in gene-for-gene interactions with microbial pathogens. In these interactions, a single *R*-gene can confer qualitative resistance to a particular pathogen by enabling the plant to recognise one or more microbial effector proteins (or their respective activities), which are encoded by specific avirulence (avr) genes. Among the *R* genes of the model plant *Arabidopsis thaliana*, there is an ancient genetic polymorphism at the *Rpm1* locus (Stahl et al., 1999) which provides resistance to pathogenic microbes carrying either of two *Pseudomonas syringae avr* genes, *avrRpm1* or *avrB*. The susceptible *rpm1-null* is a complete deletion of the functional *RPM1* resistance gene (Grant et al., 1995; Grant et al., 1998). The degree of divergence in the flanking sequence of the two alleles suggests that they have co-existed for 9.8 million years (Stahl et al., 1999). More recently, other susceptible alleles (rpm1) have been characterised that code for non-functional *RPM1* proteins (Rose et al., 2012). These non-functional alleles of *RPM1,* which were found by phenotype screening and sequence analysis, have lost their defence function due to the presence of both premature STOP codons and single base pair insertions (Rose et al., 2012). Susceptible non-functional alleles have therefore emerged multiple times at this locus and have evidently persisted despite seemingly maladaptive consequences for the individuals involved, but what can explain this?

Mechanisms that could maintain genetic diversity at the RPM1 locus

One mechanism that can maintain genetic diversity in functional traits operates when the balance of costs and benefits associated with each allele varies among the patches of a heterogeneous environment (Roux & Bergelson, 2016). In essence, the ranking of alleles in the hierarchy of evolutionary fitness is subject to spatial variation. Population geneticists refer to spatial variation in fitness hierarchies as 'genotype × environment' interactions, denoted 'G×E', which are so called because the relative fitness of a genotype depends on its environment (Tabery, 2008). G×E interactions are widespread under natural conditions (Hedrick, 1986; Hedrick et al., 1976; Linhart & Grant, 1996). It is straightforward to envisage

how G×E interactions could arise in plant-pathogen interactions as follows. There may be a high cost to a plant for having a pathogen recognition system through either the metabolic cost of chemical defences or the sacrifice of plant tissue through programmed cell death. Whilst these responses maybe defensively beneficial under pathogen challenge, conversely they may be detrimental when the pathogen is largely benign or at low abundance and when defence responses can be inappropriately induced by environmental conditions, or 'false induction' (Wang et al., 2001). In these ways, the relative fitness of alleles that code for pathogen resistance could vary spatially. Could some of these conditions apply to *RPM1* in *A. thaliana*?

Pseudomonas syringae is a Gram-negative bacterium that is a pathogen of a wide range of plant species, including *A. thaliana.* Disease severity due to infection by *P. syringae* varies among *A. thaliana* genotypes (Kover and Schaal, 2002). For example, infection decreases the reproductive output of resistant genotypes of *A. thaliana* (both transgenic and natural ecotypes) by approximately 50% at high inoculums (10^9cfu/ml), while the decrease is 75% for susceptible genotypes (Roux et al., 2010). Moreover, resistance is proposed to be costly in the absence of the pathogen possibly because basal levels of *RPM1* expression indirectly induce other costly plant defence responses (Tian et al, 2003), which could provide the basis for G×E interactions to maintain allelic diversity at *RPM1*. Specifically, spatial variation in damage to host plants by frost or herbivory could create spatial patterns in the intensity of pathogen challenge while variation in moisture and the flow of surface waters could create patterns in pathogen abundance.

However, the viability of a role for a G×E-based mechanism in maintaining allelic diversity at *RPM1* in *A. thaliana* is contraindicated by an experiment that found no difference in reproductive performance between transgenic *RPM1*$^+$ and *RPM1*$^-$ plants that were challenged with a mock inoculum (i.e. benign pathogen) in the glasshouse (Roux et al., 2010). However, unintended consequences of the genetic modification process used to produce the experimental genotypes could have confounded the experiment, for example genetic linkage or disruption to other functions that are as yet not characterised for this gene. Furthermore, it is unclear whether the result is relevant to naturally occurring genotypes under field conditions. Consequently, the

importance of a G×E-based mechanism remains to be established. Common garden experiments are a research approach that is well suited to testing the relative performance of genetic variants under environmentally realistic conditions (Turesson, 1922). In this study common gardens were created in a field based environment and a glasshouse, which although it is less realistic provides a distinctly different environment for comparison.

Our current study therefore addressed two objectives. First, in order to better establish the extant diversity in the representation of allelic variants of *rpm1* in *A. thaliana* across the United Kingdom, we carried out extensive population sampling in 30 sub-populations distributed across the United Kingdom, which makes our survey the most extensive conducted to date. Second, in order to determine whether G×E interactions contribute to the maintenance of diversity at RPM1, we assembled a collection of UK accessions of *A. thaliana* that were genotyped at the *Rpm1* locus and we compared their relative performance in two distinctly different common gardens.

Methods

Establishing the genetic diversity of UK *A. thaliana*

Sampling of natural plant populations

We identified populations of *Arabidopsis thaliana* across the UK that ranged geographically from Inverness, Scotland (57.46658°N, -4.22596°W) to Mithian, Cornwall (50.31152°N, -5.16935°W). Here we define a population as any collection of plants growing in an area clearly delineated by boundaries that would impede gene flow. However this term is used loosely here to distinguish different areas of origin as gene flow could be occurring over much greater scales. The populations ranged in size from a couple of square metres on a single wall to a large graveyard. At each site during the summer of 2009, we gathered mature siliques from haphazardly chosen individual plants and each individual's seed was designated as an accession (*sensu* Weigel, 2012). At each location, we collected seed from plants that were found growing on any of various substrates including stone walls, kerb stones, grass and soil.

Overall, we collected 497 accessions from 31 different geographic locations to provide the basis for studying *Rpm1* diversity.

Plant preparation

For molecular characterisation, seeds of each accession were separately sown in a medium of one part vermiculite to four parts of Levington F_2 peat free compost (JFC Munro, http://www.jfcmunro.co.uk) and stratified at 4°C for up to seven days before being transferred to a short-day light regime in a controlled growth chamber (10hr light, ~120 μE, 22°C day, 20°C night). After germination, individual plants were pricked out into similar soil after the appearance of their first true leaves and grown to approximately five weeks old or until they had attained a suitable leaf size for pathogen infiltration.

Determination of Rpm1 genotypes

We used polymerase chain reaction (PCR) and cleaved amplified polymorphic sequence (CAPS) genotyping was used to identify *Rpm1* alleles in each accession (Rose *et al.*, 2012). Genomic DNA was extracted from leaf tissue using the 'Shorty' technique. Two specific sections of *RPM1* where novel polymorphisms have been identified (Rose et al. 2012) were amplified by PCR using previously identified primers (Table 3) with annealing at 55°C. The results were then visualised by electrophoresis on a 1.2% agarose gel.

Primer	Forward	Reverse
2	5'GAAGATGGCTTCGGCTA3'	5'CTACACTCTCAGCGAATATC TTC3'
5F-6R	5'GATGACAGTGATGGTGATGA TG3'	5'CTCTCATCTTGGCGGCCAAG GC3'
rpm1-null	5'GTCCTGGAGTTGTGATGTTG 3'	5'GATAGATACCAGAGAGTCG C3'

Table 3 Primer pairs used in the PCR screening of Rpm1 alleles (Rose et al., 2012)

Samples that gave a negative PCR result (relevant gene section not present) were then tested for the presence of *rpm1-null* (sequence homologous to Nd-1). CAPS markers were used (Rose *et al.*, 2012) to identify the *rpm1* allele (Table 4).

PCR product used	**Restriction Enzyme**	**Product**
Rpm1-2F Rpm1-2R	ScrFI	9bp deletion: 400, 200 and 50bp Homologous to Col-0: 600 and 50bp
Rpm1-5F Rpm1-6R	AvaI	Tryptophan: undigested PCR product 1332bp Homologous to Col-0: 800 and 530bp

Table 4 CAPS markers used (Rose *et al.*, 2012)

Plants functional phenotyping

We used the hypersensitive response (HR) to pathogen inoculation, which is a form of localised cell death that prevents bacterial proliferation (Klement et al., 1964; Klement, 1963), to infer functional *RPM1* phenotype. Bacterial inoculations were prepared from cultures of *Pseudomonas syringae M4 avrRpm1* and suspended in 10mM $MgCl_2$ at OD_{600}0.2 (~ 2 x 10^8 c.f.u. ml^{-1}). Plants were inoculated using a blunt-ended syringe to deliver the bacterial

suspension through stomata on the underside of a leaf. Each plant's HR response was scored as either present or absent 16 hours post inoculation (hpi).

Assignment of accessions to genotype

Accessions were assigned to a genotype based on their response to the bacterial challenge and the PCR product identified, as follows. Assignation to *RPM1* if the accession displayed HR phenotype and *RPM1* PCR product; to *rpm1* if the accession displayed no HR and *RPM1* PCR product ; to *rpm1-null* if the accession displayed no HR with *rpm1-null* PCR product .

Statistical methods

We evaluated whether the representation of alleles varied among the 30 UK populations by a Monte Carlo simulation based on the chi-squared goodness-of-fit test. We simulated the null hypothesis (H_0) that the frequency of different alleles at individual sites was a random sample from a gene pool containing the national average frequencies. If a particular allele *i* occurred with frequency F_i in the gene pool, then the expected number of individuals with this genotype in the jth population that contains n_j individuals is n_jF_i. The overall deviation of the observed allele frequencies from their expected frequencies under H_0 is given by $\chi = \sum_j \left(\frac{(O_j - n_jF_i)}{n_jF_i}\right)$. The sampling distribution of χ cannot be reliably approximated by a parametric chi-squared distribution because small populations produce expected values of $n_jF_i < 5$. We therefore used the Monte-Carlo approach based upon 2000 replicates to obtain the critical P-value, which we implemented in R 2.8.0.

Characterisation of fitness hierarchies and G×E interactions

In order to investigate the existence of G×E interactions, we grew multiple natural accessions of each *Rpm1* variant in each of two common gardens. Vila-Aiub et al., 2009, found in their review of fitness cost studies that only 25% attempted to control genetic background, they suggested that comparing functional differences in multiple natural backgrounds was an acceptable alternative to genetic modification. To reduce the risks that the fitness hierarchies

associated with *Rpm1* were influenced by other genes we therefore compared 30 natural accessions grouped into three functional groups. Furthermore we analysed sequence data (SNP polymorphisms) to quantitatively test for genetic relatedness within our groups. Some accessions used had 250K SNP data available which gives coverage of approximately one SNP every 480bp, which is predicted to represent around 90% of all non-singleton SNPs in *Arabidopsis thaliana* (Weigel & Mott, 2009; Kim et al., 2007).

Two Common Gardens

We established two common gardens, one in a glasshouse, denoted GH-CG, and one outdoors, the field-based common garden, denoted Fb-CG, which we now describe as follows. Each common garden experiment comprised 30 accessions, or 10 from each of the three RPM1 genotypes (Table 5). These accessions supplied by E. Holub had been previously been characterized at *Rpm1* (Atwell, 1997).

To establish GH-CG, ten plants of each accession were pricked out into 7.6cm pots containing 2 parts Levington F_2 peat free compost (JFC Munro, http://www.jfcmunro.co.uk) and one part sand. The plants were grown under long day conditions (16hr L: 8hr D) and individuals were randomly assigned to locations in a glasshouse at the University of Exeter. When the first siliques began to brown, paper bags were positioned over the inflorescences to capture falling seed. Plants were harvested at death and inflorescences left to dry before threshing and measuring total seed mass.

We established the Fb-CG in Somerset, UK (50.999568° latitude and -3.366754° longitude), this location was selected for convenience for ongoing maintenance and monitoring. The experimental plot was located in permanent pasture with no evident *Arabidopsis thaliana* growing within the sward. Three experimental beds (total area = 90 m^2) were created each of 10 × 1 m and divided by two 1 m wide access strips. The plot was screened with mesh to prevent damage by birds and mammals. In late March, each accession was sown three times at discrete locations within each square metre (i.e. 90 replicate sowings). Each sowing

consisted of a sprinkle of seeds that was thinned to one focal plant post-germination. Within the experimental beds, individuals were grown in a grid at a separation distance of 0.1 m and weekly weeding was carried out to minimise the effects of interspecific competition. Each week, for each plant we recorded its developmental stage (list of stages), rosette diameter and stem height as well as qualitative descriptors of plant status and health. The fate of each plant that disappeared during the experimental period was categorised based on these comments; for example, a plant showing signs of sustained herbivore attack in the weeks preceding its disappearance was presumed to have died from herbivory. Weekly reductions in rosette diameter were used to quantify herbivore damage. For each plant, we recorded the ultimate life-stage attained from *'not germinated',* to *'reproductive maturity'.* If a plant reached silique production, it was recorded as having survived to reproductive maturity even if the plant was lost prior to harvesting. Plants were harvested after senescence and the total reproductive output was estimated for thirty individuals per accession, if available. Total reproductive output was estimated by counting the number of siliques (or pedicels, if the silique was missing) across the entire plant and multiplying by the average number of seeds per silique (obtained from counting the number of seeds per silique of approximately 25 randomly selected siliques from each accession).

To test for the presence of a GxE interaction in relation to *Rpm1*, we chose ten genetically dissimilar accessions in each of three *Rpm1* groups (Table 5). The natural UK accessions used in this study were chosen from a collection supplied by E. Holub (HRI, Wellesbourne). Previous characterisation of their *Rpm1* genotype (Ro*se et al*., 2012) allowed us to group them by resistance phenotype. Using 149 SNP data also supplied by E. Holub (HRI, Wellesbourne), the ten most genetically disparate accessions within each resistance phenotype were identified for inclusion in the experiment based on the genetic distances calculated in GenAlEx (Peakall & Smouse, 2006).

Rpm1 Group	Description	Accessions

Resistant *RPM1*	Functional *RPM1*	Bek-1, Cal-2, Chi-1, Edi-1, Haw-1, Ksk-1, Roy-1, Sma-1, Wim-1, Wma-1
Susceptible *rpm1-null*	No *RPM1* sequence (homologous to Nd-1)	Chr-1, Cnt-2, Igt-1, Kil-1, Kyl-1, Rip-1, Rot-1, Tea-1, Unt-1, Wis-1
Susceptible *rpm1*	Residual non-functional *RPM1* sequence	Amb-1, Asp-1, Chs-1, Cnt-1, Cnt-3, Edi-2, Hil-1, Laz-1, Su-1, Ty-0

Table 5 The Rpm1 groups used in this study and the ten accessions selected for each group. The glasshouse common garden did not include Asp-1 and Chs-1 due to non-germination.

Statistical methods

To test for a G×E interaction, we analysed variation in reproductive performance using a two-way analysis of variance (ANOVA) with the three classes of RPM1 variants as the genetic factor and the two common gardens (GH-CG, Fb-CG) as the environmental factor. All plants were included in the dataset of reproductive output and any plants that died before setting seed were given a fitness of zero. When testing for the effect of *RPM1* genotype on fitness, we used the means for each accession under each treatment in order to avoid bias due to unequal sample sizes. To allow comparison between the two common gardens for estimating total we scaled the mean reproductive output of each accession in each environment as the proportion of the best output achieved in that environment. The dataset did not meet parametric assumptions so we obtained the critical values of the test statistic *F* using Monte Carlo ANOVA (resampling based on 1000 simulations) that was implemented in R 2.8.0. We used a similar approach to analyse variation in 'resistance to herbivory', which was defined as the number of weeks between germination and the total loss of rosette tissue.

To assess whether the three *Rpm1* groups varied significantly in their survival rates, survival analysis was carried out using Cox's proportional hazards in R 2.8.0. This analysis was

appropriate because there was no censorship in the data (as all individuals were followed to death) and the likelihood of death was related to age.

To determine whether allelic variation at non-*RPM1* loci was associated with our three groups and thereby have confounded the interpretation of our experimental results, we analysed SNP-based data using analysis of molecular variance (AMOVA) in GenAlEx (Peakall & Smouse, 2006). We found no evidence of genetic differentiation among our groups (99% of the molecular variation was found to occur across groups based on 9,999 permutations). Using 250K SNP data that was available for 14 of the 30 accessions in our common garden experiments (Horton *et al*., 2012), we again used AMOVA (implemented in Java to accommodate a large dataset) and we again found no genetic differentiation among the groups.

Results

Rpm1 diversity of *A. thaliana* across the United Kingdom

We observed that 37% of the accessions that we collected carried functional alleles at the *RPM1* locus (Table 6).

Rpm1 genotype	Number of individual accessions	Sample size	Weighted percentage
RPM1	121	24	37
rpm1	186	25	52
rpm1-null	89	9	11

Table 6 UK-wide frequency of RPM1 functionality in response to M4 avrRpm1. The weighted percentage takes account of differences in sample size, giving the probability of genotypes occurring in a population.

The representation of the RPM1 variants in local populations differed significantly from the national gene pool distribution (Monte Carlo Chi-squared goodness-of-fit test, χ^2= 352.1, p < 0.001; Figure 12).

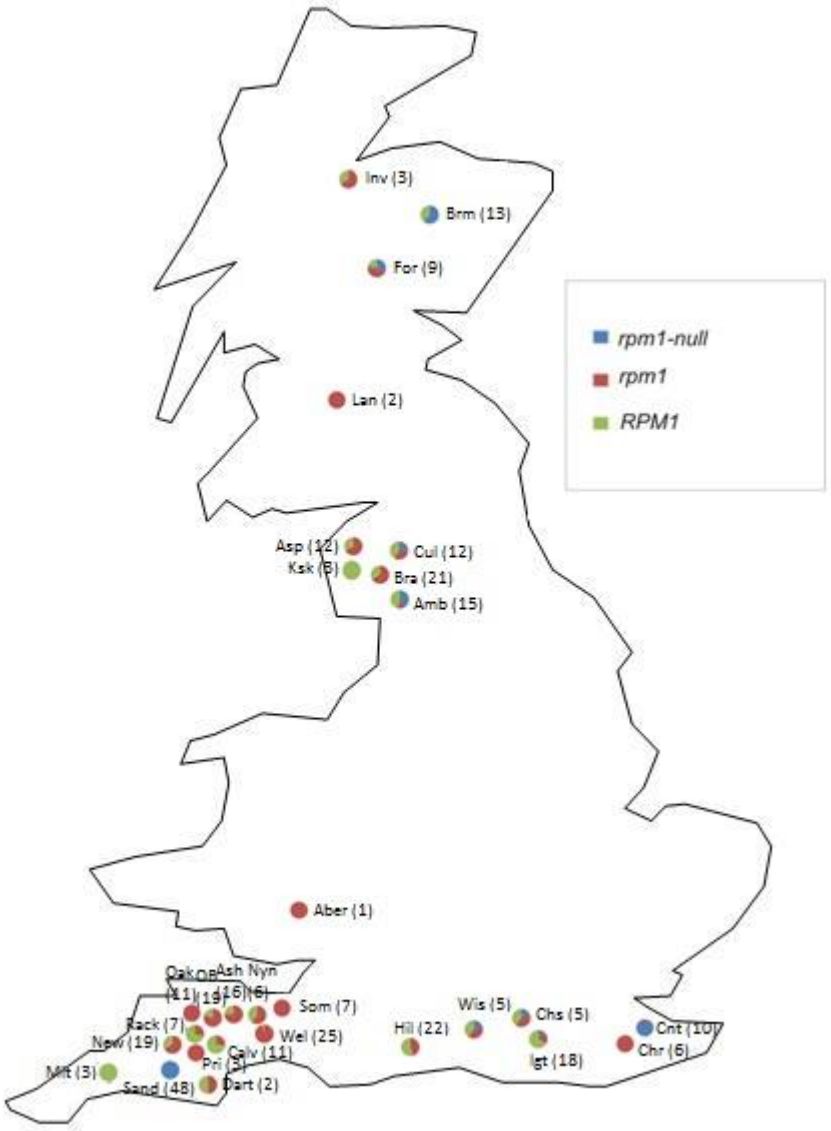

Figure 12 Population differentiation at Rpm1 locus for 396 individuals from 30 populations, map showing approximate locations. Each pie represents the proportion of individuals belonging to each Rpm1 group (blue= *rpm1-null*, red= *rpm1*, green= *RPM1*).

Common garden experiments

Variation in the reproductive output of 30 accessions of *A. thaliana* in three *Rpm1* groups grown in two common gardens revealed a genotype by environment (G×E) interaction (Monte Carlo ANOVA, F= 4.71, 95% critical F_{MC} = 3.10, p<0.05). Environmentally mediated differences in the fitness were most evident in the resistant (*RPM1)* and susceptible accessions (*rpm1)* carrying residual sequence of *RPM1* (Figure 13).

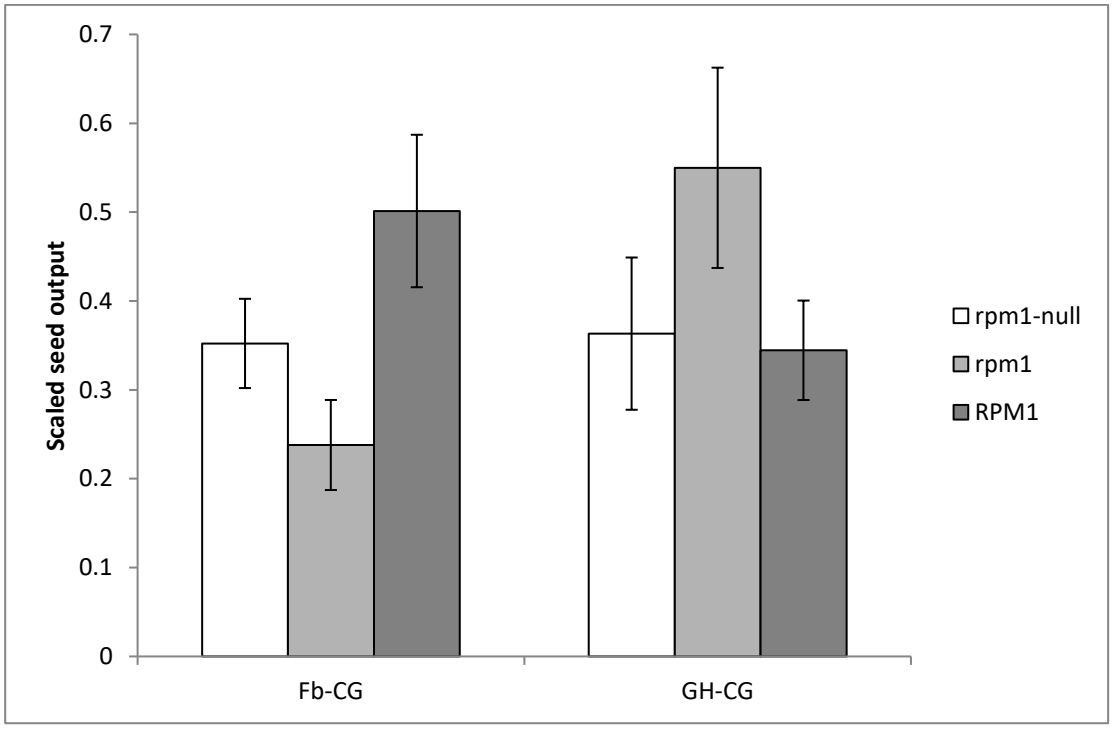

Figure 13 Group mean of scaled seed output for constituent accessions. Scaling involved dividing accession mean by the maximum value for each environment. This ranked performance measure allows comparison between the two methods of estimating reproductive output. Actual values shown in bars above. (Fb-CG= Field based common garden, GH-CG= Glasshouse common garden). Error bars represent +/- 1SE.

Individual accessions varied in their resistance to herbivory (Monte Carlo ANOVA, F=6.00, 95% critical F_{MC} = 1.46, p<0.05), but these effects were not specific to *Rpm1* group (Monte Carlo ANOVA, F=0.40, 95% critical F_{MC} = 3.73, p>0.05). Irrespective of individual accessions, the

mean changes in rosette diameter across the three groups followed a similar pattern (Figure 14) and all accessions had no rosette remaining at harvest. This suggests that herbivory was not responsible for the differential performance in reproductive output exhibited by the *Rpm1* groups.

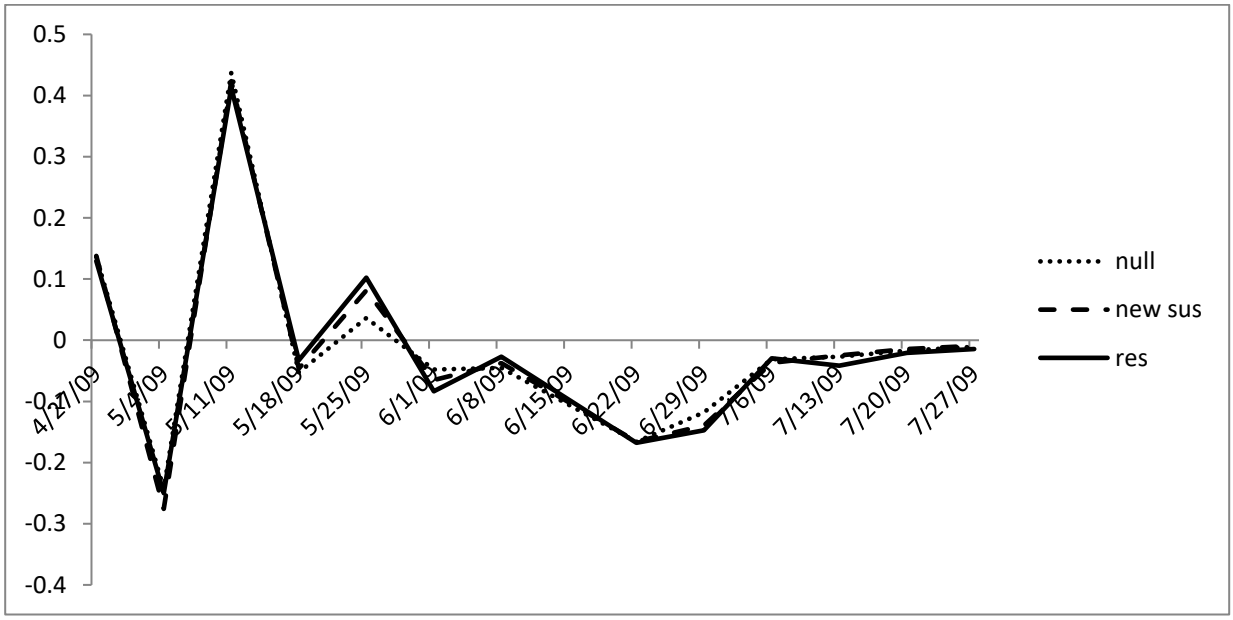

Figure 14 Mean change in rosette diameter during the main growing season, as a percentage of the maximum and minimum diameter achieved by each accession to remove intrinsic differences in growth form between accessions in Fb-CG.

Mortality occurred most frequently among young plants (when rosettes <2 cm diameter and prior to bolting) or natural senescence at reproductive maturity (from first silique formation) (Figure 15). Whilst individual accessions varied in their herbivory tolerance and lifestage at death once this data was combined by RPM1 group there were no distinct differences (Figure 17). The longevity of individual plants (defined as the number of weeks an individual survived between germination and death) varied significantly among the Rpm1 groups (Cox's proportional hazards test, $p < 0.05$), (Figure 16). In the GH-CG all germinants survived to reproductive maturity.

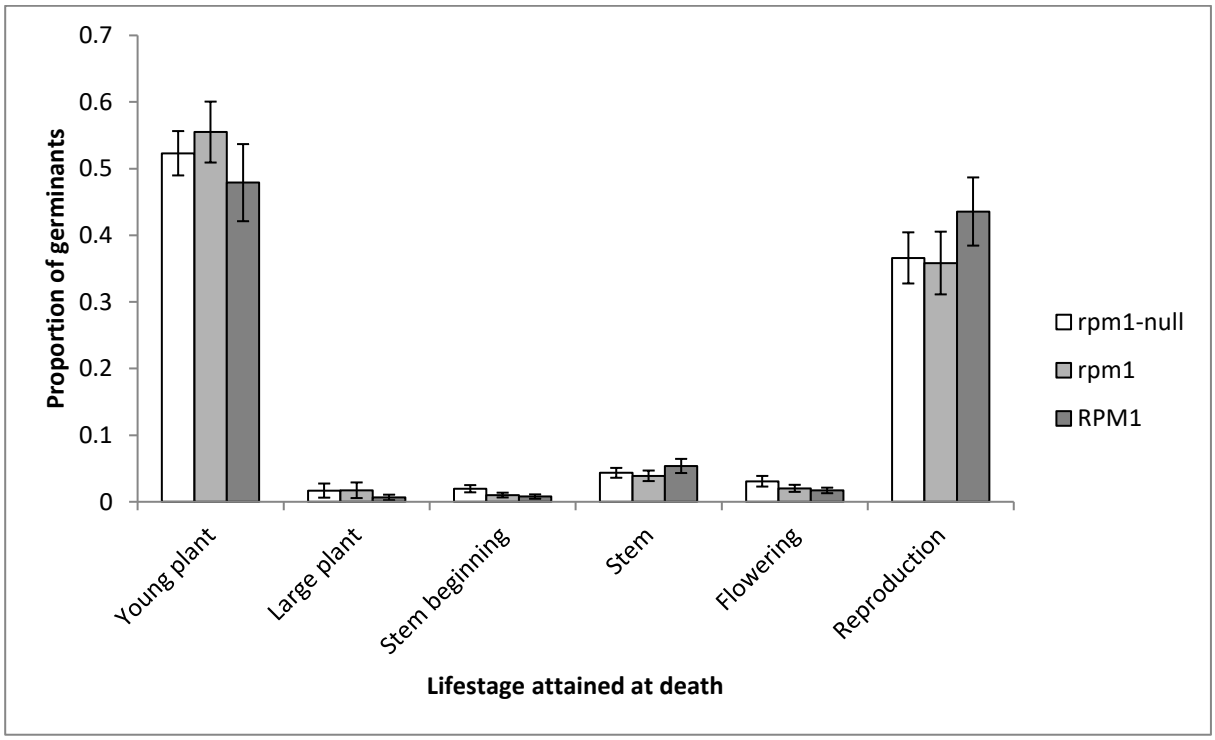

Figure 15 Comparison of the life-stage attained at death by individuals within each Rpm1 group as a proportion of the total number of germinants, in the Fb-CG.

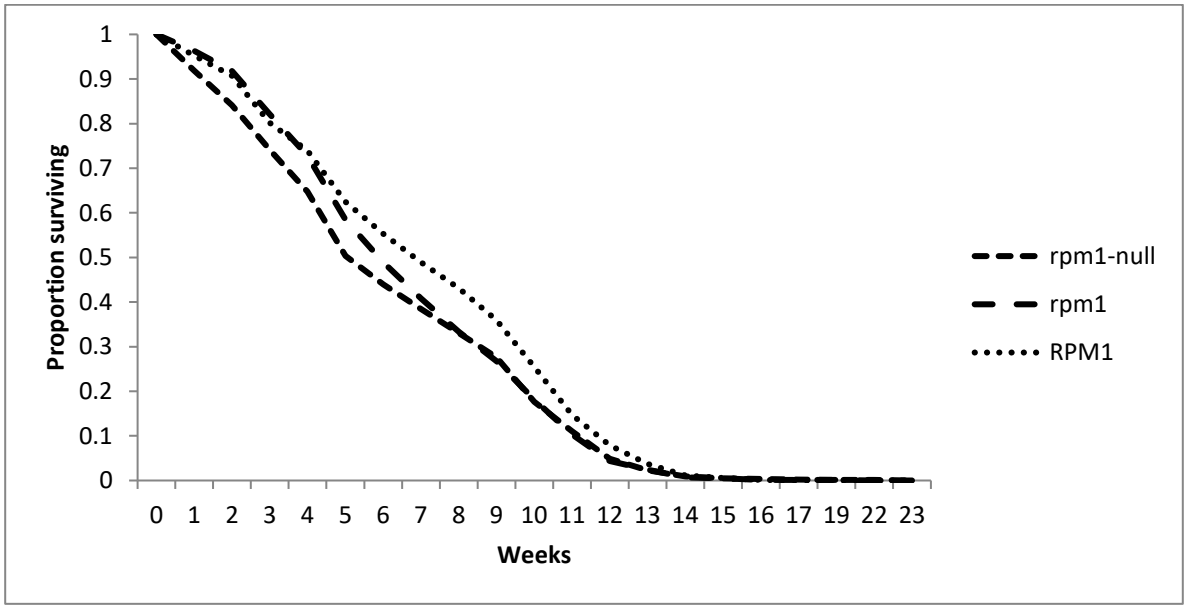

Figure 16 Survivorship curve of individuals within the Rpm1 groups during the experiment, only individuals that germinated were considered in the Fb-CG.

Discussion

Demography of *Arabidopsis thaliana*

Genetic diversity varies in space and time due to a combination of natural selection and stochastic processes. Consistent with studies of genetic diversity of *A. thaliana* in other parts of the world (Picó et al., 2008; Innan et al., 1997; He et al., 2007; Kuittinen et al., 1997; Bakker et al., 2006), we have shown that the spatial pattern of *Rpm1* alleles across the UK varies widely within and between populations. We use the *Rpm1* locus both as a marker of genetic variation and to consider its evolution. We have demonstrated a detectable interesting pattern that does not appear to represent purely drift as there is no evidence of a geographic cline. The resistant *RPM1* allele was found at the lowest frequency (37%). Despite the obvious benefit of resistance other surveys have similarly characterised resistance as the rarest allele across multiple sites (Atwell et al,, 2012; Jorgensen & Emerson, 2008). This could suggest that the pathogens in question are themselves rare and/or that fitness costs of resistance are significant in the absence of disease. The allele occuring at the highest frequency (52%) was *rpm1,* this allele is thought to represent a more recent loss of function than the ancient *rpm1-null* (Atwell et al., 2012).

The significant differentiation among gene pools of natural populations in the UK at *Rpm1* could be consistent with the action of selection at this locus, however it could also be attributed to localised founder effects. Further genome wide screening of molecular markers is required to determine the proximal cause of the observed patterns.

GxE and the role of selection in maintaining *Rpm1* polymorphism

As in many other systems (Boelling, 2003; Wallenbeck et al., 2009; Lazzaro et al., 2008; Santos et al., 2011; Breese, 1969) we have established in principle that a GxE interaction could help maintain polymorphism at *Rpm1* and could explain the observed pattern across the UK. We did not attempt to duplicate real conditions however we have demonstrated between two

contrasting environments that this mechanism could support the maintainance of genetic diversity at this locus. The GxE between our two experimental environments showed that environmental heterogeneity could maintain the resistant *RPM1* and susceptible *rpm1* alleles. In the Fb-CG the resistant group outperformed the susceptible *rpm1* group suggesting that pathogen pressure could have been present during the growing season. In the GH-CG the resistant accessions underperformed relative to the *rpm1* accessions.

The relative fitness of *rpm1-null* was unaffected by the two environments in our study. It is possible that a broader range of common gardens would produce the GxE required to explain the long term maintenance of these alleles. However the differential performance between the two susceptible groups offers a potential insight into the long-term maintenance of *rpm1-null* where the entire *RPM1* gene has been deleted. Susceptible accessions with residual *RPM1* sequence whilst outperforming both other groups in the glasshouse were less able to tolerate the field conditions and consequently saw a marked fall in performance. One hypothesis for the presence of fitness costs of *RPM1* in the absence of pathogens is that basal expression levels could incur an allocation cost by diverting limited resources. In line with this hypothesis it is possible that susceptible accessions carrying residual sequence of *RPM1* could be incurring a metabolic cost of the induction of a non-functional protein in the presence of pathogen pressure. This could in part explain the long-term maintenance of *rpm1-null* despite selective sweeps creating new susceptible alleles with higher relative fitness under some conditions.

We note that we have not yet established the precise cause of the GxE in our common gardens. The group-wise method we employed allows the use of natural accessions whilst minimising the confounding effect of genetic linkage. Our SNP analyses give a degree of confidence that the accessions within each *Rpm1* group are genetically disparate and as such are less likely to exhibit a false positive result. We discount the potentially confounding effect of herbivory as although it was evident and contained a strong accessional signal there was no significant interaction with fitness at the group level. Competitive interactions were minimised by sowing in individual pots (GH-CG) and at 10 cm intervals with weekly weeding throughout the growing season (Fb-CG). Leaf sampling of experimental plants for bacterial presence was not carried

out as this would itself have resulted in fitness consequences for the plants. We therefore hypothesise that resistance traits were invoked by the presence of *P. syringae* carrying *avrRpm1* or *avrB*, both of which can elicit the resistance response of *RPM1*, therefore contributing to the increased fitness of resistant accessions. In the GH-CG no signs of bacterial infection were observed. Clearly, further work is needed to finally establish the true basis as we cannot rule out the possibility that *R*-genes play subtle multifunctional roles in other developmental processes that confer a fitness advantage in the common garden experiments. For example, the downy mildew resistance gene 5 (*dmr5*) has been mapped to a single mutation in *RPM1,* which causes a loss of *RPM1* resistance but a gain of a different function (Huibers, 2008). Similar mechanisms could be occurring in the *rpm1* alleles we considered.

The ancient susceptible *rpm1-null* did not significantly differentiate in either environment from resistant *RPM1.* If a genotype carries a large fitness cost then selection pressure is strong to reduce that cost by subsequent ameliorating mutations. Fitness costs between genotypes can be mitigated by optimizing resource allocation through inducible defences, priming and/or transgenerational defence strategies (Karasov et al., 2017). It is well established in microbes that compensatory evolution can quickly and efficiently reduce fitness costs associated with, for example antibiotic resistance (Maisnier-Patin and Andersson, 2004; Schrag et al., 1997; Lenski, 1998). It is hypothesised that initially costly plant resistance could be ameliorated by, for example, changes in secondary metabolism (Neilson et al, 2013). Equally selection will also be present for tolerance mechanisms (Kover & Schall, 2002). Tolerance could be a particularly important in ecological interactions where defence responses may incur ecological costs due to interactions with other species (eg herbivory papers) or photosynthetic capability. As these two alleles have co-existed for an estimated 9.8 myrs there has been the opportunity for compensatory evolution to erode some of the initial fitness differences between these alleles. We therefore hypothesise from studying natural accessions that compensatory evolution has reduced the fitness differences between *RPM1* and *rpm1-null*.

Wider implications

Understanding how hosts and pathogens evolve and coexist in nature can assist in the development of evolutionary informed farming practices (Zhan et al., 2014). The development of pathogen resistant crop species can halt disease progression however the large scale monoculture of these varieties is concerning as this favours rapid counter-innovation in pathogen species. Understanding fitness costs of resistant alleles under different conditions is essential for the successful deployment of GM technology. Future sustainable food production must embrace the evolutionary ecology of the species involved. Just as in the natural environment across the agricultural landscape there is not one single optimum solution that can be engineered. Genetic diversity is the key to sustainability by disrupting selection and maintaining resilience.

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Appendix A: Quantitative RT-PCR

The large fitness cost incurred by *rpm1* alleles in the field-based CG led us to hypothesise that this could be due to a cost of false induction of the non-functional resistance gene. We used quantitative RT-PCR to determine whether non-functional *rpm1* alleles could incur a fitness cost due to false expression in pathogen presence.

Plants

Previous screening (Rose *et al*., 2012) identified 3 different non-functional *Rpm1* alleles. One accession was randomly chosen carrying each allele (Hil-1: Sus-1 (susceptible group); Laz-1: Sus-2; Ty-0: Sus-3), Col-0 was used as the positive control carrying a fully-functional *RPM1* gene and Nd-1 was used as the negative control carrying the *rpm1-null* gene with total deletion of the *RPM1* gene. Plants were grown under controlled conditions as described (de Torres et al., 2003).

Three replicates per accession were inoculated with *Pseudomonas syringae* DC3000 carrying *avr-Rpm1,* three leaves per plant were sampled and flash frozen in liquid nitrogen at 3 hours post inoculation. Concurrently three non-inoculated replicates were also sampled from each accession.

Bacteria

Pseudomonas syringae DC3000 carrying *avr-Rpm1* was used to trigger the resistance response. Bacterial cultures were maintained and used as described (de Torres *et al*>, 2003). For RNA expression bacterial cell density was adjusted to OD_{600} 0.2 (~10^8cfu ml^{-1}) in 10mM $MgCl_2$.

RNA extraction and RT-PCR

Total RNA was extracted as described (de Torres *et al*., 2003). cDNA was made from 1ug of total RNA with Superscript III (Invitrogen Corporation, California, USA) following the manufacturer's instructions. Quantitative PCR was performed on the resulting cDNA using Agilent Technologies' Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix (cat. no. 600883; Agilent Technologies LDA UK Ltd, Life Sciences & Chemical Analysis Group, Stockport, UK) on a Rotor-Gene 3000 (Corbett Research, Cambridge, UK). *Actin 2* was the internal standard used to normalise cDNA quantity between samples. Expression levels were calculated relative to *Actin 2* expression. Primers were designed within a region of the *Rpm1* gene that was conserved between all susceptible alleles. Primers used are shown in Table 7.

Primer name	Sequence
rpm1-RT-1F	TGCGTGGATCTCAGCTACAG
rpm1-RT-1R	CTTGTGCAAAACGCAACCTA
rpm1-RT-2F	GCGTTTTGCACAAGGATTTC
rpm1-RT-2R	ACCCTCTACAAGCCCTGACA
Actin2-F	AGTGGTCGTACAACCGGTATTGT
Actin2-R	GATGGGCATGAGGAAGAGAGAAAC

Table 7 RT-PCR primers used

Relative expression values were calculated as per (Pfaffel, 2001).

Results

Rpm1 expression is triggered by infiltration with *DC-avrRpm1* in all accessions tested except the negative control Nd-1 as shown in Table 8. The results support the hypothesis that fitness costs due to false induction could be incurred under pathogen presence.

Accession	DC Ratio vs Col0	NI Ratio vs Col0

Col-0 RPM1	1	5.73458E-06
Ty-0 Sus-3	1.24	4.24157E-06
Laz-1 Sus-2	0.85	1.6861E-06
Hil-1 Sus-1	0.68	2.1272E-06
Nd-1 rpm1-null	0.05	6.37861E-09

Table 8 Average relative gene expression across 3 biological replicates and 3 technical replicates

Appendix B: Natural fauna screening

We have established in principle that GxE could maintain genetic diversity at *Rpm1,* however we did not attempt to identify the nature of the fitness cost. Balancing selection at an *R* locus could arise due to varying pathogen abundance or false induction of resistance. Co-evolutionary models predict that frequencies of resistance and avirulence genes will fluctuate in synchrony with each other (Brown and Tellier, 2011; Laine and Tellier, 2008). However whilst resistance genes have been extensively characterised across the species range there is minimal literature looking at the prevalence and distribution of the corresponding avirulence genes (but see Dunning, 2008). We therefore surveyed the natural fauna of wild populations of *A. thaliana* across the South-West UK for the presence of the avirulence genes known to interact with *RPM1*.

Natural fauna population sampling

For local south-west UK sites during early summer 2010 leaf samples were harvested haphazardly and put on ice for transportation back to the laboratory. They were then flash frozen in liquid nitrogen before being stored at $^{-}$80°C.

Natural fauna PCR phenotyping

50µl sterile water was added to each sample of frozen tissue. Samples were then placed in tissue lyser for 2 minutes at 25 Hz (Qiagen, http://www.qiagen.com/). The resulting homogenate was spread plated onto KB (Kings broth) media with no selective antibiotics. The plates were left to grow at 27°C for 4-5 days. A sterile tip was then passed across the array of colonies formed and the collected cells suspended in sterile water. 25µl was then placed in a well plate and the cells lysed at 95°C for 3 minutes to release DNA from the bacterial cells. These samples were then analysed by PCR for the presence of genus specific DNA identifying the presence of *Pseudomonas* (Widmer et al., 1998) and *Xanthomonas spp.* (Goncalves and Rosato, 2002) and for the presence of the avirulence genes involved in the gene-for-gene interaction with *RPM1: avrB* and *avrRpm1* (Table 9).

Primer	Sequence
Pseudomonas- F	GGTCTGAGAGGATGATCAGT
Pseudomonas- R	TTAGCTCCACCTCGCGGC
Xanthomonas- F	ATCACCTCCTTTTGAGCATGACR
Xanthomonas- R	GGTTCTTTTCACCTTTCCCTC

Table 9 Primers used in natural fauna sampling

Natural fauna screening

Samples from 7 locations in the South-West UK were screened as shown in Table 10. Both *Pseudomonas* and *Xanthomonas* species were found extensively across these locations as shown in Figure 17.

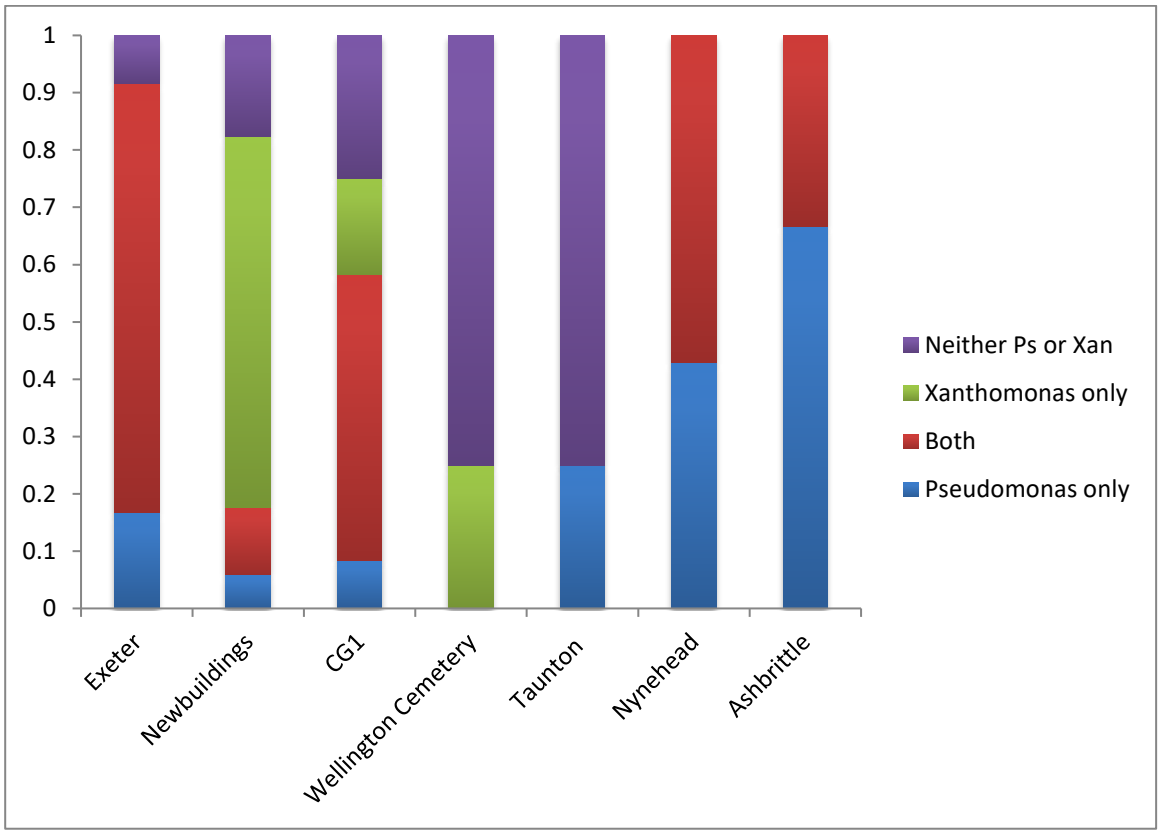

Figure 17 Stacked bar chart showing the proportion samples from each site that had PCR products of genus specific primers for *Pseudomonas* and *Xanthomonas* species. Across all sites n=68.

Location	**Site characteristics**
Exeter	Edge of pavement
Newbuildings	Gravel carpark
CG1	Grass field site of Fb-CG
Wellington Cemetery	Grass and around graves
Taunton	Flowerbed
Nynehead	Gravel paths and edge of concrete paths
Ashbrittle	Sand

Table 10 Site characteristics and sample size of natural fauna sampling

Of the avirulence genes involved in the gene-for-gene interaction with *RPM1*, *avrB* was not found in these samples and *avrRPM1* was found in only one sample at the Exeter site as shown in Figure 18. Results shown as percentage of samples across all sites are shown in Table 11Table 11 PCR results as percentages of all samples screened across all sites (n=68 and 77 for genus specific and avirulence genes screenings respectively).

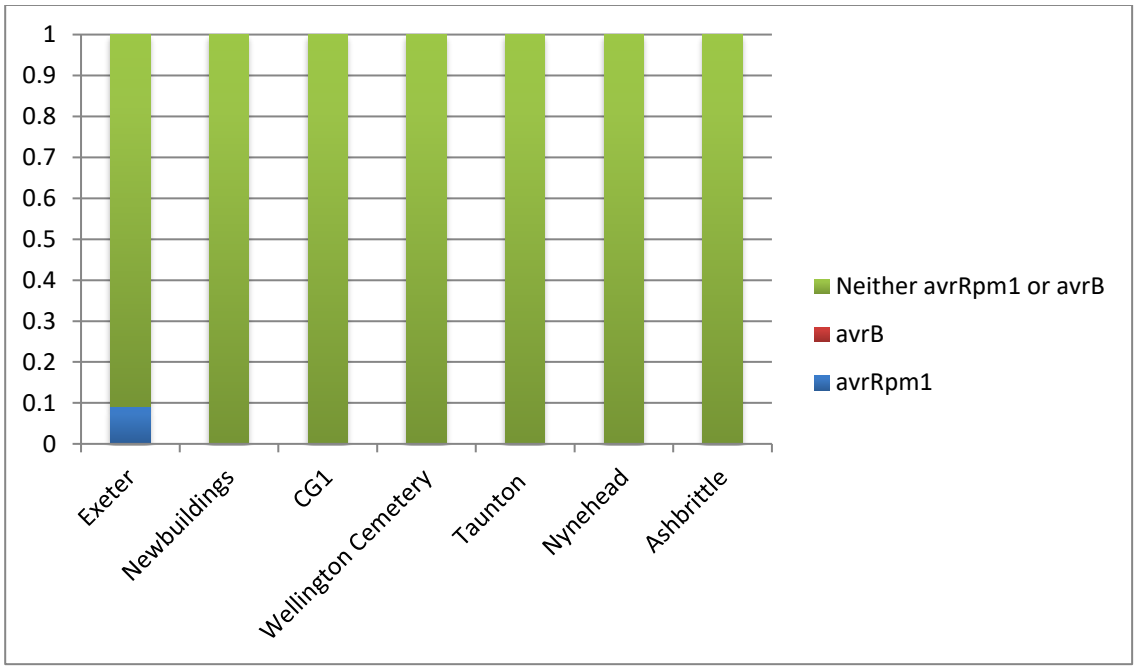

Figure 18 Stacked bar chart showing the proportion of samples at each site that had pcr products of avrB (n=0) and avrRpm1 (n=1). Across all sites n=77.

PCR product	**Percentage of all samples screened**
Pseudomonas species present	60%
Xanthomonas species present	57%
Only *Pseudomonas* species present	23%
Only *Xanthomonas* species present	20%

Both *Pseudomonas* and *Xanthomonas* species present	37%
Neither *Pseudomonas* or *Xanthomonas* species present	19%
Neither *avrRpm1* or *avrB* present	99.9%
avrRpm1 present	0.1%

Table 11 PCR results as percentages of all samples screened across all sites (n=68 and 77 for genus specific and avirulence genes screenings respectively).

This preliminary screening suggests that whilst both *Pseudomonas* and *Xanthomonas* species are common on *Arabidopsis thaliana* across this region there is little evidence for selective pressure at *RPM1* exerted by the presence of the corresponding avirulence genes. No attempt was made to screen pathogenicity so all species found could have been epiphytic. The low frequency that the avirulence genes involved in the gene-for-gene interaction with *RPM1* occur at in the South-West UK may simply reflect temporal variation, however this is an intriguing finding and further work is required to unravel the basis of selection at this locus.

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Chapter 3: Performance of UK-wide accessions of *Arabidopsis thaliana* in a common garden varies annually in relation to home-site climate

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Abstract

Despite the action of natural selection in eliminating suboptimal variants, many species contain large amounts of genetic variation. In sessile organisms particularly, spatial variation in the microclimates experienced by individuals may present a wide range of optima that would sustain genetic variation by diverse local adaptation that, if it existed, would be manifest as ecotypic variants. While classic studies have demonstrated ecotypic variation in plants that occupy along alpine clines, there a few known examples among highly vagile weedy species. Here we show that local populations of a weedy thale cress, *A. thaliana*, are ecotypes that are adapted to the climates of their home sites. We collected genotypes from local populations across the United Kingdom and monitored their reproductive performance in a common garden over two successive years. We found that local populations varied consistently in our common garden and that their relative success was explained by the degree to which their home site climatically resembled the common garden. Our study demonstrates the existence of adaptive ecotypic variation in the national gene pool of a widely studied model plant. We anticipate that our work contributes to the understanding of genetic diversity in plants generally and also may have application to the identification of traits useful in crop development.

Introduction

For many plant species, their geographic range may include habitats that span a substantial degree of climatic variation. Being sessile, individual plants cannot escape unfavourable climatic conditions. Consequently spatial variation in phenotypes in natural plant populations is widespread among plant species. This may be a manifestation of adjustments in traits that suit them to local environmental conditions. For example, well studied plant attributes that are climatic adaptations include mechanisms of drought tolerance (Knight et al., 2006; McKay et al., 2003; Seki et al., 2007), cold and freezing tolerance (Hasdai et al., 2006; Kim et al., 2007) and photosynthetic responses to light fluctuations (Külheim et al., 2002). In this way, a single species may occur across a wide range of habitats that differ in environmental conditions.

While many plants appear to be well suited to the environment in which they grow, this 'suitedness' may arise in two ways. First, the phenotype could adjust to the habitat by plasticity, which requires that the individual's experience of its environment alters its phenotype within the genetically constrained parameters. In effect, phenotypic plasticity allows the same genome to produce different phenotypes depending upon the environment in which it develops (Schlichting, 1986). Second, a plant may be suited to its habitat because of local adaptation, were natural selection based on relative performance creates a local preponderance of genotypes that express functionally useful traits, which results in ecotypic variation (Turesson, 1922).

Plant adaptations have been characterised to both biotic and abiotic forces. The adaptation hypothesis is supported by evidence of natural plant populations showing geographic structure to climatic conditions (Jum*p et al.*, 2006; Leinonen et al., 2011). It is important to separate these two mechanisms and so understand the basis by which plants are suited to particular environments because the global climate is changing and at a rate that will probably challenge many species to keep pace (IPCC). Wild plant populations are already exhibiting plastic and evolutionary changes in response to climate change (Franks et al.,2014; Peñuelas and Filella, 2001; Parmesan and Yohe, 2003). Understanding the mechanisms by which plants accommodate climate change within the constraints of their genetics and the accompanying evolutionary processes, will better assure global food security by enabling plant breeders to

continually suit crops to their changing regional climate. It is likely that evolutionarily informed agriculture (Zhan et al., 2014) will be of increasing importance as both the biotic and abiotic environment changes (Bebber et al., 2013).

Among ecologists, common gardens (CGs) are a classic method to assess whether population differentiation is predominantly the result of local adaptation or phenotypic plasticity. While a single CG can be used to reveal ecotypes, it takes further evidence to demonstrate that variants are indeed suited to their home site. One way to achieve this is to undertake reciprocal transplants (Hereford 2009; Ågren and Schemske, 2012). Another method, however, is to evaluate statistical associations between fitness exhibited in the CG and home site climate. For example, climate adaptation has been demonstrated in *A. thaliana* by correlating home site climate and plant fitness in a common garden (Rutter and Fenster, 2007). However, Rutter and Fenster's study has three important limitations, which means that the relative importance of these two mechanisms is not yet fully resolved.

First, Rutter & Fenster employed accessions from across the species range to test for broad scale adaptation, their study used a sizeable proportion (14%) of accessions that were long standing laboratory accessions, which were probably at risk of genetic contamination. Anastasio et al. (2011) identified 286 accessions from stock centre and natural collections that are potentially misidentified and 14% of the accessions used in Rutter and Fenster's study occur on this red list (Anastas*io et al*., 2011). Second, for accurate comparison between performance in the common garden and climate at the home site, the year sampled should also be considered so that the appropriate historic climate data can be used for that accession. Third, the choice to transplant seedlings after 10 days stratification and 15 days growth in the greenhouse removes a potentially important adaptive fitness stage, of germination and early establishment, and artificially accelerates development. It has been shown that direct sowing versus the transplantation of seedlings can invert the fitness hierarchy between cultivars (Naklang et al., 1996). To ameliorate these limitations, we employ accessions from across the UK to test for finer scale adaptation, and we avoid some of the preceding limitations by gathering our own seed and germinating *in situ*.

In this study we investigated whether *Arabidopsis thaliana* from 30 sites across the UK show evidence of being suited to home site climate. *A. thaliana* is a model species in which observable phenotypic variation is widespread in its wild populations. As a short-lived mobile plant species, evolutionary theory suggests that *A. thaliana* should have high potential to adjust to local conditions because it will encounter many novel environments while colonising new areas. The broad tolerance of *A. thaliana* as a species may be aided by both phenotypic plasticity of individuals and local adaptation in the gene pools of populations (Richards et al., 2006). *A. thaliana* is probably a native species in the United Kingdom (Sharbel et al., 2000), so there has been extensive evolutionary opportunity for local adaptation to have occurred. UK accessions collected from its widespread distribution and from a variety of habitats therefore provide an ideal system for studying the evolutionary ecology of local adaptation. The vast array of *A. thaliana* collections available is one of its many strengths as a model organism, but these collections have often been developed principally for molecular biology and are often missing ecological data that describes each genotype's the home site. Here, we therefore did not use laboratory accessions but instead took seed from natural populations across the UK, obtained climatic description of the home site, and then assessed performance by directly sowing seeds into our common garden allowing for natural germination and development.

Common gardens and reciprocal transplant experiments are classic methods in ecology to study adaptation. Common gardens typically only capture the correlation between genetic variation and fitness, whilst reciprocal transplants incorporate environmental variation, and when compared they tend to find more evidence of adaptation (Nuismer and Gandon, 2008). However RT experiments present a greater logistical challenge as maintenance and monitoring of diverse sites may not be possible. By combining CGs with knowledge of home site climate then it is possible to reduce the logistical burden to one field site and to still analyse the impact of environmental variables. Furthermore, whilst a single year common garden will provide a snapshot of evolutionary fitness of accessions in that environment, temporal changes at the same site can create year-to-year variation in the selection regime, thereby promoting the maintenance of variation. A single year CG could be biased by an anomalous year. Seemingly,

a disconnection is now evident between the climate of the optimum adapted form and native accessions (Wilczek et al., 2014), whether this disconnect is indeed due to the rapidity of climate change or unstable temporal selection is unknown. Here, we use a multiple year common garden experiment to both test for the existence of ecotypic variation and, further, to investigate the temporal stability of relative fitness, which provides a strong basis for inferring adaptive significance of the detectable ecotypic variation.

In summary, the objectives of our study are: (1) to determine whether the phenotypic variants from across the UK are ecotypes; (2) to evaluate whether these ecotypes are adapted to their home sites; and (3) to test whether our measures of relative fitness are resilient to year-to-year variation in weather.

Methods

Plant material

One accession per sample site, from the summer 2009 UK sampling (described in Chapter 2), was chosen to represent the diversity of the UK gene pool. Each wild collected sample was selfed twice prior to use in this experiment, to reduce the impact and standardise any short-term epigenetic modifications.

The common garden

The Patio Common Garden (PCG) was located in the grounds of the University of Exeter Geoffrey Pope Glasshouses at 50.738148° latitude and -3.534754° longitude. The area was formerly rough grass. The PCG was created during summer 2011. The area was leveled and land drains were laid to prevent flooding. A frame of slabs was constructed on foundations of hardcore and coarse yellow sand, leaving two beds of 1m by 7.5m for the construction of the patio. The patio consisted of approximately 2” square granite blocks laid tightly together. To serve as a soil medium for experimental plants, kiln dried sand was then brushed into the gaps between the blocks as the growth substrate for the plants (Figure 19). The interstices among the granite blocks served as a grid, which provided x-y referenced sowing locations so that

individual plants could be located and monitored. The plot was then perimeter fenced to exclude damage by mammals.

Figure 19 The patio common garden located in the grounds of the University of Exeter glasshouses

The experimental sowings

PCG1 was sown in October 2011. Each accession was sown 90 times at randomly chosen locations across the plot. Individual seeds were sown at each x-y coordinate (i.e. in the soil at a point adjacent to the corners of four of the granite blocks) by careful placement using the dampened end of a matchstick. Weeding was not routinely carried out because this would have disrupted the roots of experimental plants. However, if a weed was significantly overgrowing an experimental plant, it was cut back. Interspecific competition effects were not likely to be

having a significant impact as only a few other plant species were observed growing during the experimental period.

In order to repeat the experiment in a second year after harvesting the siliques produced by the experimental plants of PCG1, the granite blocks and sand were removed (to prevent seed contamination of the next generation), the blocks were scrubbed and autoclaved and then relaid with a new consignment of kiln dried sand brushed between them, which we denote PCG2. PCG2 was then sown in November 2012 using the same collection of twice-selfed seed, but in a differently randomised spatial pattern.

Data collection

For the first three months the plot was observed weekly in order to record germination dates and each plant's progressive developmental stage. During the main growing period, measurements were recorded fortnightly of rosette diameter, stem height and side stem formation along with qualitative records of plant status (e.g. evidence of herbivory). By May/June, all plants had reached reproductive maturity and thereafter until harvesting recordings were made approximately fortnightly of the presence or absence of each plant in order to determine plant losses.

Harvesting, reproductive performance and terminal measurements

As described in Chapter 2, plants were harvested when they had finished flowering and were predominantly dried out. Similar to the field-based common garden, maximum stem height and branching architecture (the number of primary, secondary, tertiary, quaternary and basal branches) were recorded. The total reproductive output was then estimated for all surviving individuals by counting the number of siliques or pedicels and multiplying this by the mean number of seeds per silique, which we estimated by sampling ~25 siliques from each accession. Additionally as the majority of the taproot was found in most cases to lift cleanly out of the sand the vertical root length was also noted.

Ultimate life-stage was recorded for all individuals from *'not germinated'* to *'reproductive maturity'*. All seed used was of the same age so analysis of survivorship included the

germination stage. Herbivory was not found to be a significant factor in this environment. By the harvesting date the majority of plants had no rosette remaining but this was observed to be rotting off the plant rather than consumed. Speed of life was defined as the weeks from germination to bolting.

Historical climate data

Historical climate data was obtained from the UK's national weather centre (Met Office archives). The Met Office dataset is divided into 5km squares across the UK and the conditions at each locality are calculated from all nearest weather stations for the position central to each square (Perry and Hollis, 2005). The data used to characterise home site conditions for our analyses were monthly averages (means) for the period January 2000 to December 2006. To carry out univariate analyses of temporal climate data, we need to transform an n-dimensional time series dataset to a single dimension without losing too much information. To achieve this we used a kernel density approximation (Shawe-Taylor and Cristianini, 2004) implemented in Matlab. This is a dimensionality reduction technique similar to principle component analysis or self-organising maps that is commonly used for complex data to be processed by simple statistical methods. For the purpose of this work, a Gaussian kernel was used with the assumption that any inter-dependencies between the parameters would be negated by the approximation. The kernel value calculated was used in the subsequent analyses. The climatic variables are detailed in Table 12.

Title	**Met Office definition**	**Description**
Mean Sea Level (MSL) Pressure	Hourly (or 3 hourly) mean sea level pressure (hPa) averaged over the month.	Conventionally pressure at all locations is referred to on this scale for ease of comparison. MSL is an important predictor in weather models as it encapsulates much climatic information: for example correlating with both temperature

		and wind speed. Weather reports include areas of high and low pressure for this reason.
Mean Relative Humidity	Hourly (or 3 hourly) relative humidty (%) averaged over the month.	The percentage presence of water vapour in the atmosphere. This contributes to water availability and photosynthesis.
Raindays ≥1mm	Number of days with ≥1mm precipitation.	Water availability is significantly related to both plant growth and yield.

Table 12 Description of climatic factors used in further analyses

Statistical analyses

Statistical analyses of the fitness effects at this location were examined for each year using the resampling Monte Carlo method described in Chapter 2. In order to test whether the reproductive performance of accessions was associated with the resemblance between home site and common garden conditions, analyses were carried out separately for each climate variable in Table 12. Bonferroni correction was applied and the critical p-value for significance was lowered to 0.016. In order to consider temporal variation at the common garden site, we combined the datasets from both years (i.e. PCG1 and PCG2) and analysed with 'year' as a potential explanatory factor.

Additionally, survival analysis (Cox's proportional hazards in R) was carried out to determine whether accessions were significantly differentiated in their survival rates and whether these differences were correlated to variation in the climatic variables.

Reproductive output

Reproductive output in the common garden was related to resemblance to home site conditions as characterised by various climatic factors in both years of our study. In Year 1, seed output correlated with the home site's mean sea level (MSL) pressure (an important predictor of weather patterns) (Monte Carlo ANOVA, $F=3.7$, 95% $F=3.5$, $p<0.05$) and in Year 2 it also correlated with days-of-rainfall (Monte Carlo ANOVA, $F=7.3$, 95% $F=3.7$, $p<0.05$). Geographic variation in common garden reproductive performance showed no clear clinal pattern Figure 20.

Performance for all accessions plummeted in Year 2, which was the brighter, dryer year when many plants showed evidence of water stress. Estimated total reproductive output in both years shows the change in fitness hierarchy.

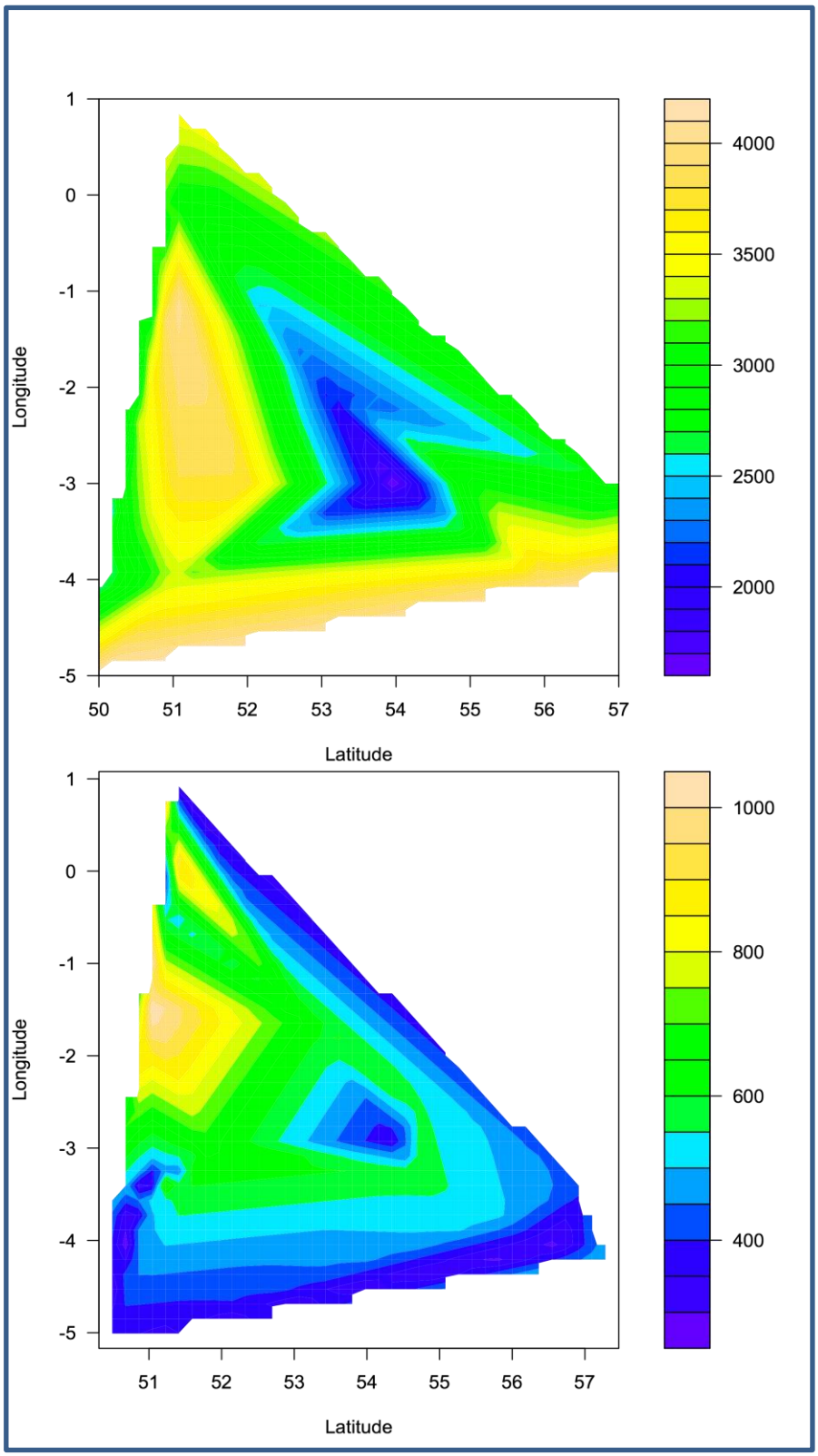

Figure 20 The average number of seeds produced against latitude and longitude of home site. Top: year 1, Bottom: year 2.

Survival

There was no evidence of herbivory or disease during either year. Different climatic factors were significantly related to survivorship in the two years; in year 1 MSL pressure (Monte Carlo ANOVA p= 0.013*) and in year 2 relative humidity (Monte Carlo ANOVA, p= 0.013*). Individual accessions followed a similar trajectory) however they differed in their rate of survival (Cox's proportional hazards; Figure 21 and Figure 22).

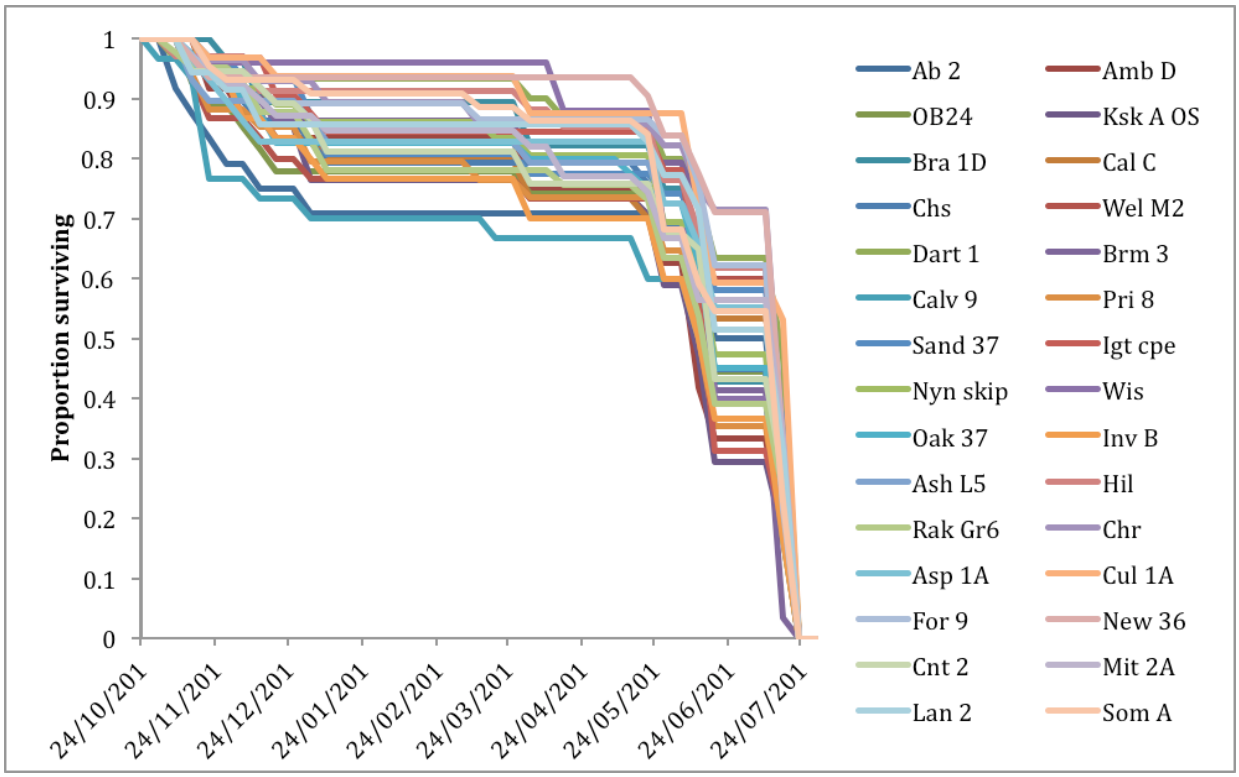

Figure 21 Survivorship curve for accessions in year 1

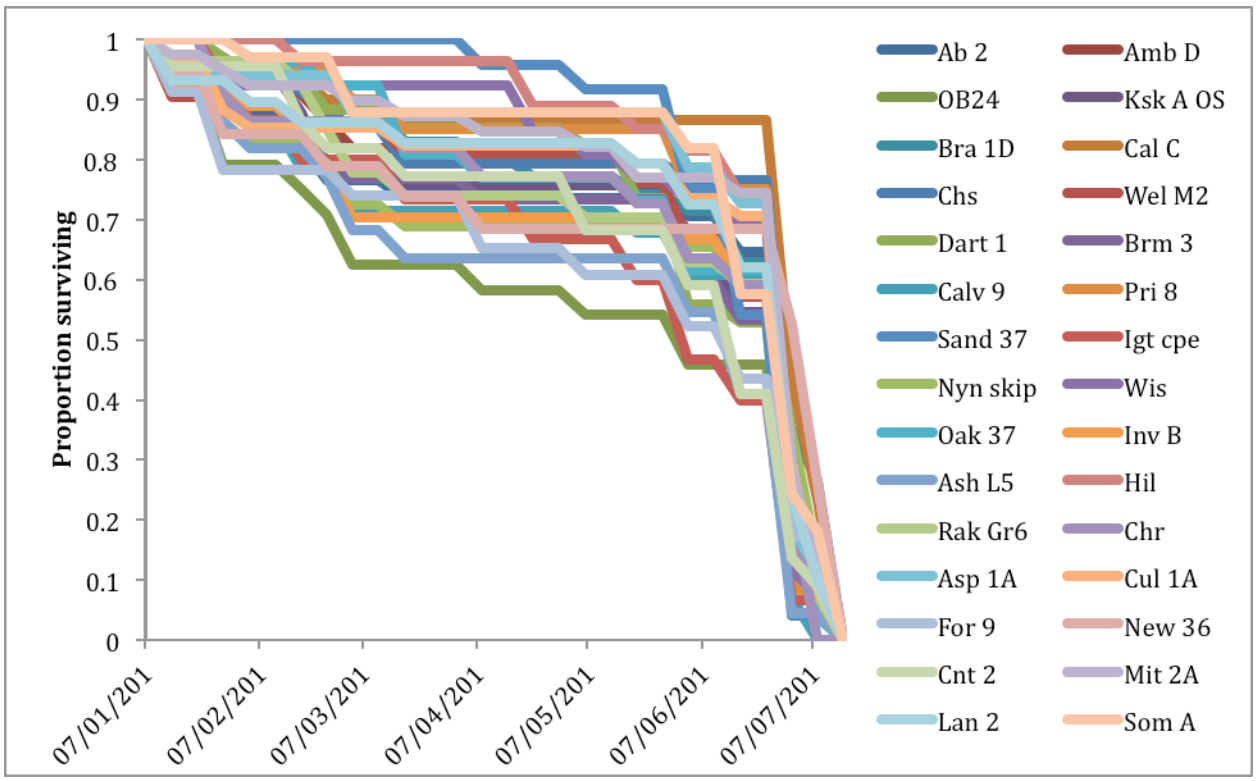

Figure 22 Survivorship curve for accessions in year 2

Discussion

Our results demonstrate that there is considerable ecotypic variation in the UK *A. thaliana* gene pool, with both evolutionary fitness and survivorship showing significant accessional variation. All accessions were able to survive and reproduce at our CG, but with varying degrees of success. These distinct differences in performance reveal the ecotypic variation present across the UK. *A. thaliana* is a highly mobile weedy species and there is a belief that many populations close to human habitation represent merely transitory migrants that do not exhibit local adaptation (Jorgensen and Emerson, 2008). The presence of gene flow does not preclude adaptation therefore we sampled from diverse habitats, many potentially impacted by human disturbance, as an intimate association with humans is now integral to the evolutionary trajectory of many wild populations.

We found that the ecotypic variation exhibited in our CGs can be attributed to local adaptation as both reproductive fitness and survivorship was correlated to aspects of home site climate. We show that a weedy life history does not preclude adaptation, indeed weeds could be seen as masters of the art, rapidly adjusting

to novel environments and stresses. Our results are consistent with findings from across the species range (Rutter and Fenster, 2007; Wilczek et al., 2014; Ågren and Schemske, 2012; Olivas et al., 2017), but to our knowledge this is the first study to search for climate adaptation in *A. thaliana* at such a fine spatial scale. Fine scale adaptive variation to herbivory has been demonstrated using 288 accessions from across Europe (including UK accessions) (Gloss et al., 2017) Previously, researchers looking for local adaptation have concentrated on larger geographic scales often spanning the species range. However it is now becoming recognised that local adaptation can occur even at a very small spatial scale, as long as selection strength exceeds gene flow (Richardson et al., 2014). We show that even in areas of potential disturbance at a spatial scale ranging from under 1km to nearly 800km *A. thaliana* has evidently adapted to prevalent home site environmental conditions. Using knowledge of fine-scale adapation, conservationists and crop breeders can optimise both conservation efforts and agricultural productivity. Genetic analysis of the accessions used could be combined with this dataset to reveal the evolutionary history of this species across the UK and could further be used to look for QTL associated with climatic adaptation.

The second year of our study revealed an evolutionary balancing act as relative fitness, and hence selection, varied over time. Different climatic features were important in year 2 and performance hierarchies were different between years. However performance could still be linked to home site conditions consistent with adaptation. This shows how easy it would be to draw erroneous conclusions about the adaptive climatic landscape if only one year's study was employed. The two years of our common gardens show clearly a capricious side to selection. A single common garden reveals a static fitness landscape whereas repeat experiments provide a window on the dynamic fitness better described as a seascape of shifting optimas than a landscape of fixed optima (Merrell 1994; Mustonen and Lässig, 2009). Time-shift experiments bring different generations of seed together to quantify the adaptive process. In a similar way repeat common gardens measure the impact of actual environmental differences. We show that this method provides a robust technique to detect adaptation whilst reducing the experimental burden of multiple common garden sites. Extreme weather events

are predicted to become more commonplace under climate change scenarios (IPCC) this will increase the role of stochastic processes in natural populations as the adapted state may prove to be an evermore elusive optima.

Climate change coupled with our rapidly expanding world population, predicted to reach 9.6 billion by 2050 and 10.9 billion by 2100 (United Nations, 2013), is causing increasing concern about the resilience of agricultural crop production and global food security. Extreme weather events are already causing significant agricultural and economic consequences: the European heatwave of 2003 is estimated to have reduced crop productivity by a third (Cia*is et al*., 2005) and the widespread drought in the USA in 2012 resulted in an increase of over 400% to $17.3 billion in insurance claims for agricultural crop losses (NRDC, 2013). We do not know whether species harbour sufficient adaptive resilience to survive and prosper under climate change scenarios. Plant species that could be the best able to cope are generalist species known to thrive across a range of conditions and able to quickly colonise new areas but even these species may struggle with the climatic challenges ahead. In our study the crash in fitness of all accessions in year 2 versus year 1 reveals the inherent vulnerability to climatic differences of a fairly resilient weedy species. Agricultural crops with lower genetic resilience than wild species, due to the commercial selection restricting genetic potential, will be poorly placed to cope under climate change scenarios. Although extensive studies have explored fitness differences between genotypes within a single season, little attention has been given to the impact of climatic differences between years and how this affects fitness hierarchies. The best performer across the two years of this experiment was not the highest overall performer.

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Chapter 4: Susceptibility of British accessions of *Arabidopsis thaliana* to infection by xenopathogens

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Abstract

Global climate change is already causing range shifts in pathogenic species. Consequently, organisms will experience novel pathogens with whom they have no shared evolutionary history. These so-called 'xenopathogens' may be disarmed by the host's pre-existing generic defence strategies, or instead they could cause considerable disease losses. We screened a collection of *Arabidopsis thaliana* from across the UK for their responses to a range of xenopathogens. We found a wide diversity of susceptibility and resistance that was not always correlated to conventional phenotypic indicators. We identified one accession (God-1) that consistently showed the lowest levels of bacterial growth when challenged by a xenopathogen. We show that genetic diversity in the host population can buffer against xenopathogen invasion.

Introduction

Global climate change is causing average temperatures to rise and many species are tracking their preferred habitats by northward migration (Parmesan and Yohe, 2003). Pathogenic microbes are among those whose geographic ranges are changing and hundreds of species of pathogens and pests have shifted polewards since 1960 by an average of approximately 3 km yr^{-1} (Bebber et al., 2013). Communities do not move in concert and the result of this can be pathogen release where plant species experience reduced pathogen pressure, however some plant species will encounter novel pathogens that have migrated northwards into the southern margin of the plant's geographic range. Thus, plants are increasingly likely to become threatened by potentially devastating diseases caused by these so-called 'xenopathogens'. It is therefore important to

understand interactions between plants and xenopathogens because agriculture and global food security will increasingly face threats when crops encounter new pathogens.

Xenopathogens could be particularly virulent to plants because the potential hosts have not experienced natural selection for pathogen-specific defensive adaptations (Altizer et al., 1995). However, plants are routinely exposed to a wide diversity of potential pathogens and the majority are rendered harmless by a combination of robust structural defences, generic basal defences and inducible defences based on specific resistance genes (Veronese et al., 2003). Structural defences include thickened cuticle (Martin et al., 1964; Jorosz et al., 1982) or dense trichomes that prevent microbial spores from contacting the plant surface (Levin, 1973). Basal defences include the production of broad-spectrum antimicrobial secondary metabolites that are triggered by the recognition of microbe associated molecular patterns (MAMPS) (Mackey & McFall, 2006) and other nonspecific immune responses (Gill et al., 2015) known as non-host resistance (NHR), which confer broad resistance to non-adapted pathogen species (Fan & Doerner, 2012; Heath, 2000; Poland et al., 2009). In contrast, specific resistance genes elicit inducible defences following detection of avirulence genes present in a co-evolved pathogen (Jones & Dangl, 2006). As a result of these potential defensive capabilities, the vulnerability of a specific plant species to xenopathogens is not immediately obvious. We therefore investigated the resilience to xenopathogens of wild-collected accessions of the model plant species *Arabidopsis thaliana*.

One major mode of origin for resistance to xenopathogens is cross-resistance. Cross-resistance arises when defences that evolved in response to a previously co-occurring natural pathogen (Kraaijeveld et al., 2012) are also effective against the xenopathogen. In effect, the similarity of the xenopathogen to a previously encountered pathogen modulated either by MAMPs or other pathogen-encoded effectors enables the host plant to recognise and combat the xenopathogenic attack. In plants, cross-resistance is widely involved in resilience to both herbivores (Armbruster, 1997; Armbruster et al., 1997; Armbruster et al., 2009) and foliar pathogens (Newcombe, 1998). In addition, resistance can be attributed

to pre-adaptation if it arises by repurposing an existing functional attribute (Gould & Vrba, 1982). For example, the signalling pathways for general stress responses such as to wounding could become co-opted to also recognise infection by pathogens (Lu et al. 2011; Odjakova & Hadjiivanova, 2001). To better evaluate the potential threats posed by xenopathogens to crops and wild plants, it is valuable to understand the extent and basis of cross-resistance and pre-adaptation. The experimental tractability and extensive molecular characterisation of *A. thaliana* provides a suitable system to test for pre-adaptation, particularly because the species shows competent defences against a wide range of microbial pathogens.

In this study we chose Xanthomonads to serve as xenopathogens. *A. thaliana* is probably a natural host of *Xanthomonas campestris pv. campestris (Xcc)*, because it causes the classic symptoms of blackened veins (Simpson & Johnson, 1990; Tsuji et al., 1991) and the development of systemic infection through the vascular system (Buell and Somerville, 1997). Both tolerance and susceptibility to *Xcc* infection have been reported in *A. thaliana* (Buell & Somerville, 1997; Tsuji et al., 1991), where tolerance is defined as bacterial proliferation without causing visible disease symptoms. Certain xanthomonads are found on *A. thaliana* plants in nature (Tsuji & Somerville, 1992; Chapter 2 natural fauna sampling). We therefore expected that some of the accessions of *A. thaliana* that we used in our experiments would have evolved resistance to *Xcc*, which provided the potential for pre-adaptation to foreign Xanthomonads. Additionally, various *Xanthomonas campestris* isolates that target different hosts probably share a wide range of similar biochemical and physiological attributes. For example, c. 96% of the genome of the banana wilt pathogen *Xanthomonas vasicola pv. Musacearum (Xvm)* 4381 is also found in the sugarcane wilt pathogen *Xanthomonas campestris pv. vasculorum (Xvv)* 702. Further, there is a 60-80% overlap between *Xvm* 4381 and *Xvv* 702 with other sequenced Xanthomonads (Studholme et al., 2010). These fundamental similarities provide the potential for cross-resistance against a xenopathogen. In the present study, we specifically employed exotic strains of *Xanthomonas*, such as the banana wilt pathogen, that British accessions of *A. thaliana* are unlikely to have encountered previously. Inoculation with a single pathogen allows us to study direct causation however

this is unlikely to accurately reflect plant pathogen exposure in the field, where typically a plant would be faced with an array of pathogens. This would potentially weaken the plants defence response to an individual pathogen.

Specifically, the *Xanthomonads* that we chose as xenopathogens included two strains that cause economically important and socially devastating crop diseases, namely banana wilt and rice bacterial blight. Banana *Xanthomonas* wilt (BXW) is caused by *Xanthomonas vasicola pv. musacearum (Xvm*), which is currently devastating banana crops across Africa. BXW probably originated in the Ethiopian banana, Ensete, before spreading to the widely cultivated species of Ethiopian banana's sister genus, Musa. BXW is capable of reducing the yields of banana crops by 80-100% and no banana cultivars are currently resistant (Jogo et al., 2011). Rice bacterial blight is caused by *Xanthomonas oryzae pv. oryzae (Xoo)* and outbreaks typically result in crop losses of 50% or more (Gnanamanickam et al., 1999; Mew, 1987). *Xoo* was first recorded in Japan in 1884, and is now endemic in many parts of the world, having spread across Asia, northern Australia, Africa and USA (Gnanamanickam et al., 1999).

Our present study involved screening the susceptibility of a variety of British accessions of *A. thaliana* to *Xanthomonas* xenopathogens with three main aims as follows. First, we used the outcome of the screening to investigate the diversity of the British gene pool of *A. thaliana* in susceptibility to infection by five strains of *Xanthomonas*. Second, we evaluate the potential for pre-adaptation in the *A. thaliana-Xanthomonas* interaction. Third, we identify accessions that show greater tolerance or resistance to *Xanthomonads* because they could be the focus of further comparative study using molecular techniques to identify the mechanistic basis for host resistance to xenopathogens.

Methods

Pathogens used and bacterial preparation

In order to test the response of *A. thaliana* to xenopathogens, we used a varied selection of *Xanothomonad* strains Table 13.

Ref.	Species	Abbrev.	Host group	Host Species	Country of Origin	Tissue specificity
702	*Xanthomonas campestris pv. vasculorum* (formerly *Xanthomonas vasicola pv. vasculorum*)	Xvv	Monocot	Sugarcane	Uganda	Vascular tissue
4381 & 4383	*Xanthomonas vasicola pv. musacearum* (formerly *Xanthomonas campestris pv. musacearum)*	Xvm	Monocot	Banana	Uganda	Vascular tissue
372 & 1329	*Xanthomonas oryzae pv. oryzae*	Xoo	Monocot	Rice	China	Vascular tissue
Race 3 HRI 5212	*Xanthomonas campestris pv. campestris*	Xcc	Dicot	Brassica (Host: B. oleracea var. gemmifera)	UK 1957	Vascular tissue
Race 4 HRI 1279A	*Xanthomonas campestris pv. campestris*	Xcc	Dicot	Brassica (Host: B. oleracea var. capitata)	Cornwall, UK 1984	Vascular tissue

Table 13 Details of pathogens used

In order to produce a separate inoculum for each pathogen strain, bacteria were cultured overnight in King's Broth (KB) media. The overnight cultures were centrifuged for 5 minutes at 2,500 g. To wash the bacteria, the supernatant was poured off and the bacteria resuspended in 10mM $MgCl_2$ and centrifuged again. The bacteria were again resuspended in 10mM $MgCl_2$ and diluted to the required concentration using a spectrophotometer to estimate the colony forming units (cfu) per ml by the optical density measured at 600nm.

Plant material

In order to establish the level of variation in responses to xenopathogens in the British gene pool of *A. thaliana*, we conducted an initial phenotype screening of

91 natural UK accessions supplied by E. Holub (HRI) and we then chose a representative subset to investigate further, the subset was chosen from those that gave a strong response to inoculation. Additionally, two commonly used laboratory accessions, Col-0 and Col-5, were investigated for comparison.

Plants were grown in 1:4 vermiculite and Levington F2 peat-free compost (JFC Munro, http://www.jfcmunro.co.uk). Seeds were stratified at 4°C for three days before being transferred to a short-day controlled growth chamber (10 hr light, 120 µE, 22°C day, 20°C night). We pricked out individual plants after the establishment of their first true leaves and the plants were then grown for approximately five weeks or until they had attained a suitable leaf size for infiltration of a xenopathogenic inoculum.

Xenopathogen exposure by leaf infiltration

A response phenotype from 1-5 was established for each accession of *A. thaliana* by a screening process that utilised qualitative scoring of differential responses to pathogen infiltration among of our collection of UK accessions.

We chose two modes of pathogen exposure by infiltration because different methods can produce contrasting results (Meyer et al., 2005) possibly due to tissue-specific defence responses. *Xanthomonas* typically invades via natural openings such as hydathodes, stomates or wounds (Williams, 1980), but the realism of a purely stomatal mode of infection has been debated (Melotto et al., 2006) (Gudesblat et al., 2009) and the reliability of phenotype assignment based on stomatal infiltration has been questioned due to variable screening results (Meyer et al., 2005). In order to strengthen our phenotype assignations, we also used infection of the vascular system following stem abrasion, which is a realistic mode of infection that causes the characteristic 'black rot' disease symptoms.

For stomatal infiltration, bacteria were infiltrated on the abaxial side of a pre-marked leaf using a blunt syringe with a bacterial solution (0.2 OD_{600}; 2 x 10^7 c.f.u.ml^{-1}). In order to simulate infection following natural stem wounding, we nicked the main stem of the leaves with 'rats-teeth' tweezers that had first been dipped in a concentrated bacterial solution. Each technique was applied to

separate plants and in both techniques, four leaves were treated on each individual plant.

In order to assign phenotypes, the response of treated leaves were scored ten days post-inoculation (dpi) visually using a 5-point scale (Figure 23). Accessions showing no sign of disease at 10 dpi were considered to be tolerant or resistant. To attempt to distinguish between chlorosis due to either a hypersensitive response (HR) or disease, a score was also recorded for each plant at 5 dpi, because HR is usually evident earlier than disease.

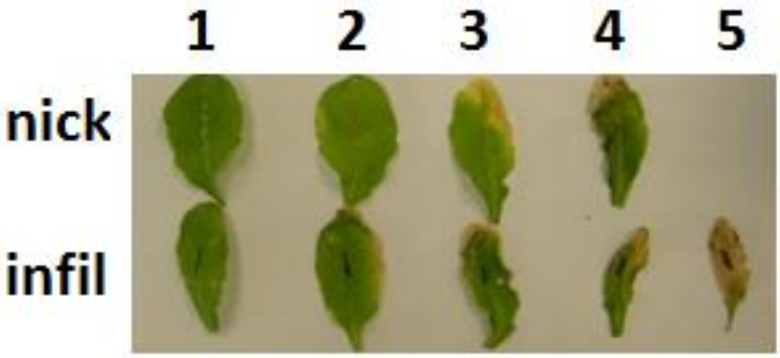

Figure 23 Phenotype scoring scale used for screening differential responses of the accessions. A score of 1 was given to plants that showed no response to treatment, minimal chlorosis was given a score of 2, increasing to 5 for a totally chlorotic leaf.

In order to determine whether accessions of *A. thaliana* differed in their responses to xenopathogen exposure, we analysed variation among the quantitative phenotype scores by Analysis of Variance (ANOVA) in R v 3.1.2.

Pre-score (5dpi) did not significantly differentiate from the scores (10dpi) the two were highly correlated ($p = <2 \times 10^{-16***}$), so for all subsequent analyses score at 10 dpi was used. It was not possible to distinguish HR from disease by this method, therefore for the phenotype screening we will refer to responsiveness rather than resistance or susceptibility. There was a significant effect of batch ($F=6.559, p=<2 \times 10^{-16***}$), excluding the effect of accession differences within batch, this could be explained by subjective error between days or inaccuracy of

estimates of bacteria infiltrated. To control for this batch effect the scores were transformed to be relative to the control (Col-0) within each batch.

Bacterial growth assay

In order to determine whether host symptoms used in our protocol for phenotype scoring reflected differences in bacterial growth *in planta*, we identified a subset of the accessions for further investigation. Initial tests were made on a subset that comprised nine accessions (Col-0, Ty-0, Wis-1, Sit-1, Laz-1, God-1, Wye-1, Fab-1, Big-1), but logistically detailed analysis was only carried out on Col-0, God-1 and Fab-1, as God-1 and Fab-1 showed strong responses and Col-0 as a control.

In order to test experimentally the relationship between symptoms and bacterial proliferation, we used the banana wilt (*Xvm)* and sugar cane (*Xvv)* pathogens, because they invoked more responses among our accessions than the rice pathogens (*Xoo).* For comparison, two native pathogens (*Xcc)* originally isolated from *Brassica* in the UK were also tested for their ability to proliferate in our accessions.

In the proliferation tests, the bacteria were prepared as described above. We initially trialled different dilutions for the pathogens from OD_{600} 0.0002 to OD_{600} 0.05 and used the concentration that best differentiated among the accessions (OD_{600} 0.0002). On each plant tested, we inoculated the underside of four suitably sized leaves using a blunt ended syringe.

Bacteria *in planta* were counted at 0, 3 and 7 days post inoculation (dpi). Typically six replicate plants were inoculated per accession and three 3 mm leaf discs were taken from each plant for bacterial counting. In order to assay levels of pathogen proliferation, the leaf discs were (pooled/separately) ground in 10mM $MgCl_2$ before serial dilutions were plated onto KB. The plates were then incubated at 28°C for either one or two days until colonies attained sufficient size to be counted under a light microscope.

Bacterial growth was measured to try to determine whether strong phenotype scores correlated with resistance or susceptibility. For this screening the xenopathogens chosen were Xvv 702 and Xvm 4383 (which is very closely related to 4381 but carrying antibiotic resistance) and these were compared to the known pathogens of *Brassica sp.* Xcc Races 3 and 4*.*

First, the optimum concentration of innoculum for Xvv and Xvm was determined and it was found that the lower concentration 0.0002OD revealed greater differentiation between the accessions, (Figure 26).

Results

Phenotype screening

UK accessions showed ecotypic variation in response to *Xanthomonas* challenge as measured by phenotypic responses to exposure to *Xanthomonas* xenopathogens whether by infiltration (ANOVA, $F=2.25$, $p < 0.001$; Figure 24 or nicking (ANOVA, $F=4.45$, $p < 0.001$).

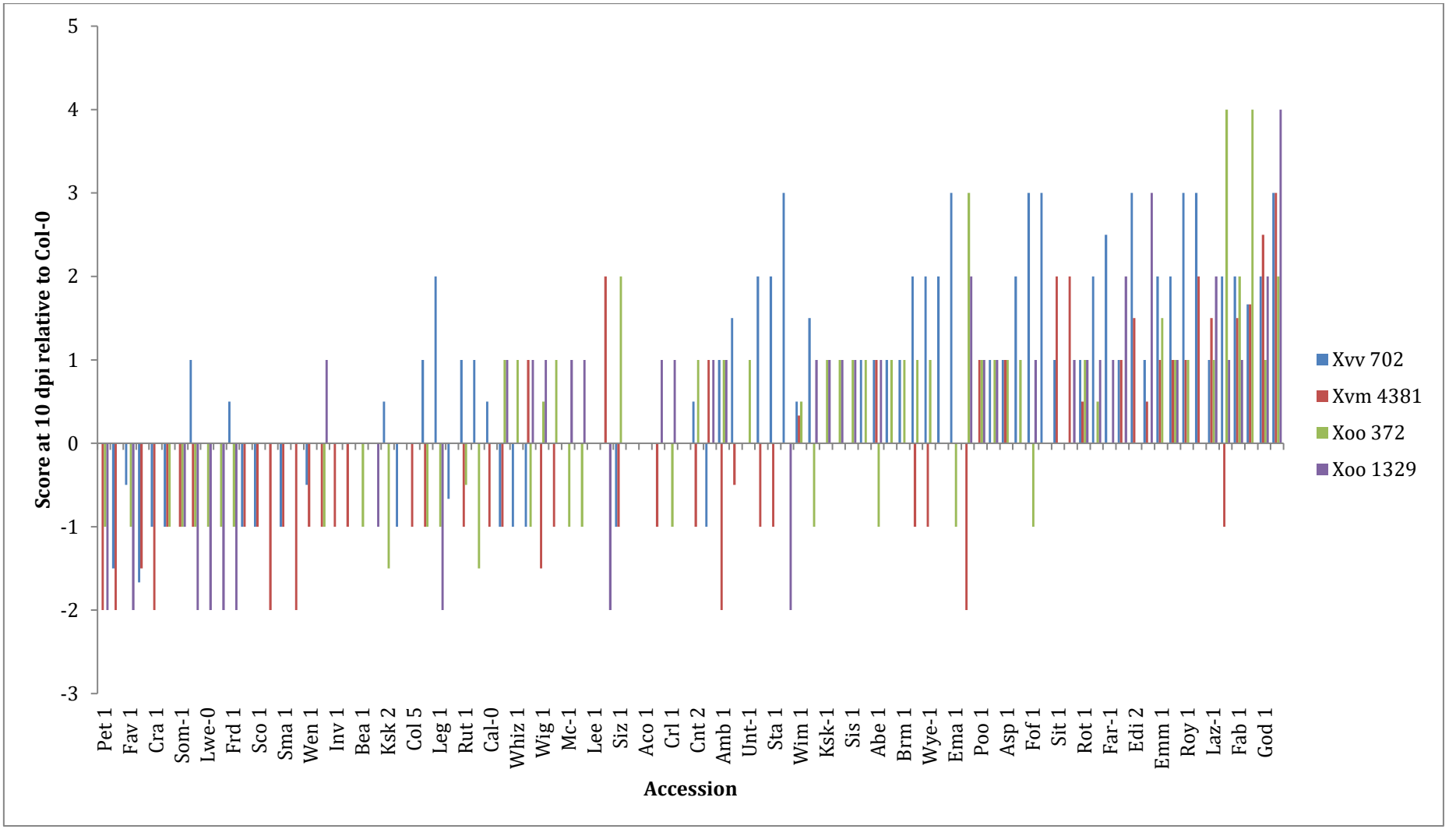

Figure 24 Phenotypes relative to Col-0 (control) showing the different responses of infiltrated plants 10 days post inoculation, based on a score of 1-5. Accessions on the left (low scores relative to Col-0) are considered to be tolerant or resistant. Accessions on the right could be showing either HR or disease.

The responses depended significantly on the method of inoculation ($p= 5.21 \times 10^{-5}$, F= 16.54). We focus on the infiltration results as this resulted in greater range in phenotype scores relative to control (Figure 25a). The magnitude of response to a particular pathogen strain depended significantly on the inoculation method ($p=2.57 \times 10^{-4}$, F=28.795; Figure 25b) except in the banana wilt pathogen *Xvm* (4381).

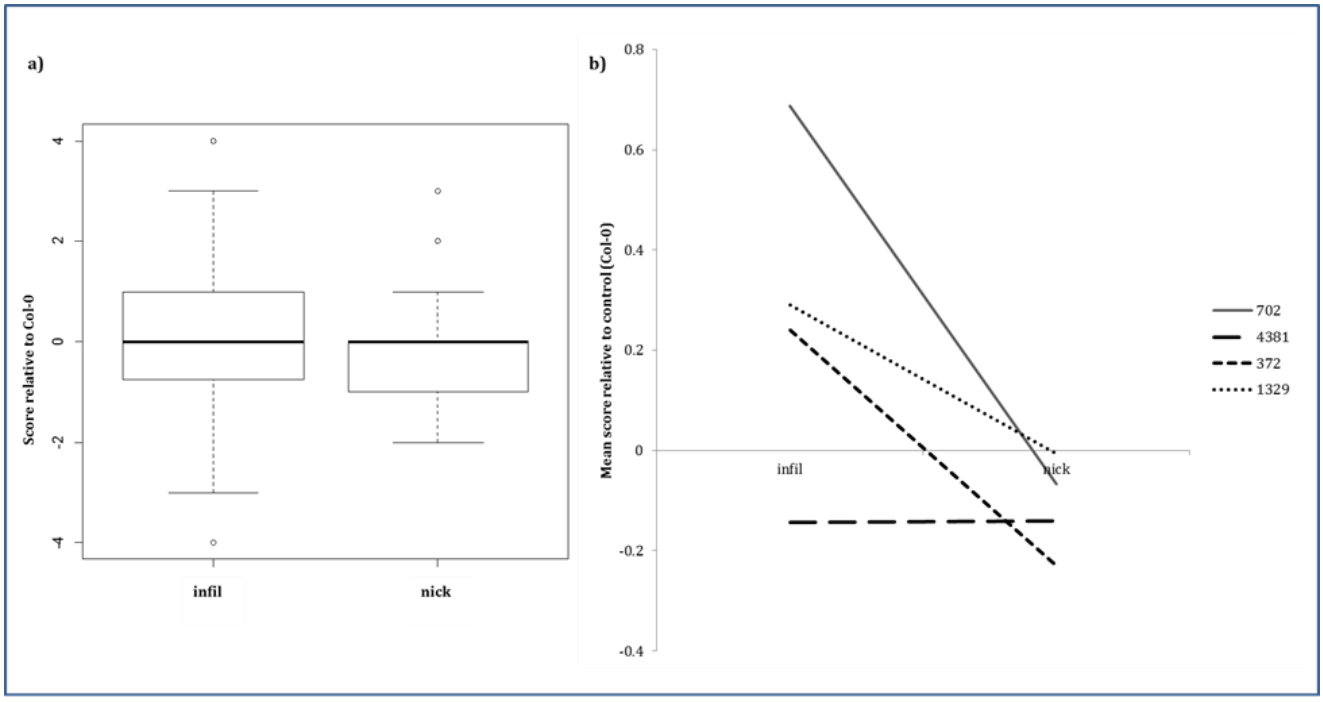

Figure 25 Effect of method of inoculation on phenotype displayed at 10 dpi relative to control (Col-0). a) Boxplot showing range of phenotype displayed by method of inoculation; b) the differential effects on phenotype displayed by method of inoculation by pathogen strains. Individual pathogens shown: Sugarcane wilt 702 (solid line), Banana wilt 4381 (long dashed line), Rice wilt 372 (short dashed line) and Rice wilt 1329 (dotted line).

Bacterial growth screening

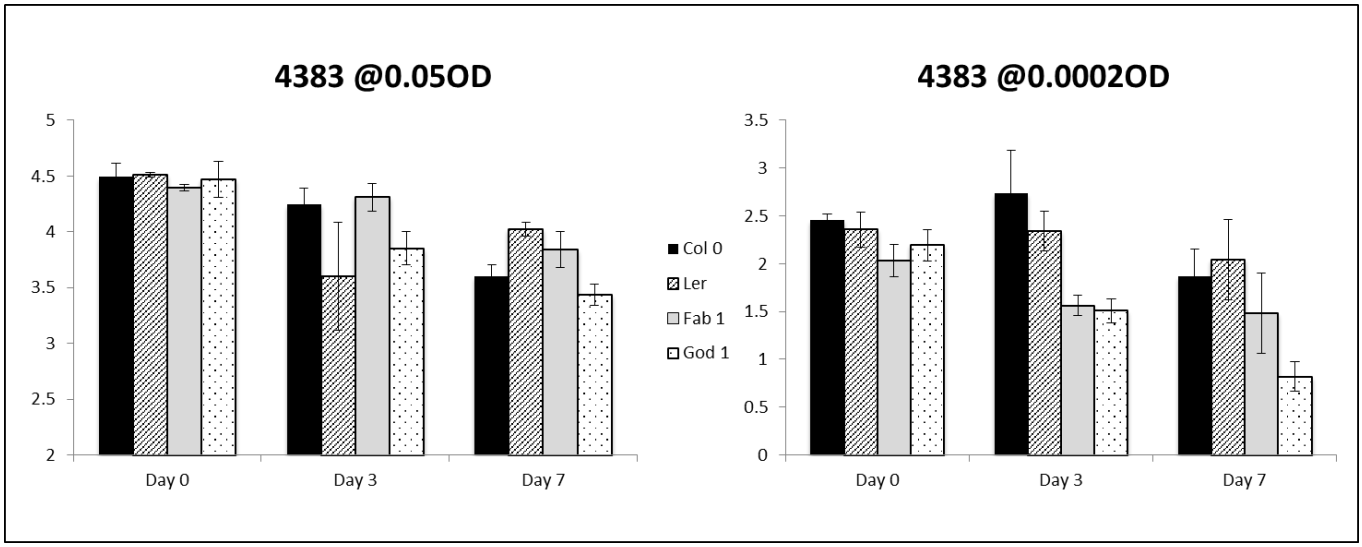

Figure 26 Impact of bacterial infiltrate concentration for Xcc 4383 on final bacterial concentration in the leaves of four different accessions of *A. thaliana* (as indicated by bar pattern)

Testing of the bacterial inoculum concentration revealed that at lower initial inoculum the accessions showed greater differentiation in defence Figure 26 therefore we used lower concentration for screening. Sugarcane wilt proliferated in all accessions, which indicates a broad compatibility in *Arabidopsis* (Figure 28). In contrast, the banana wilt pathogen was unable to proliferate in the strains of *A. thaliana* that we tested (Figure 27).

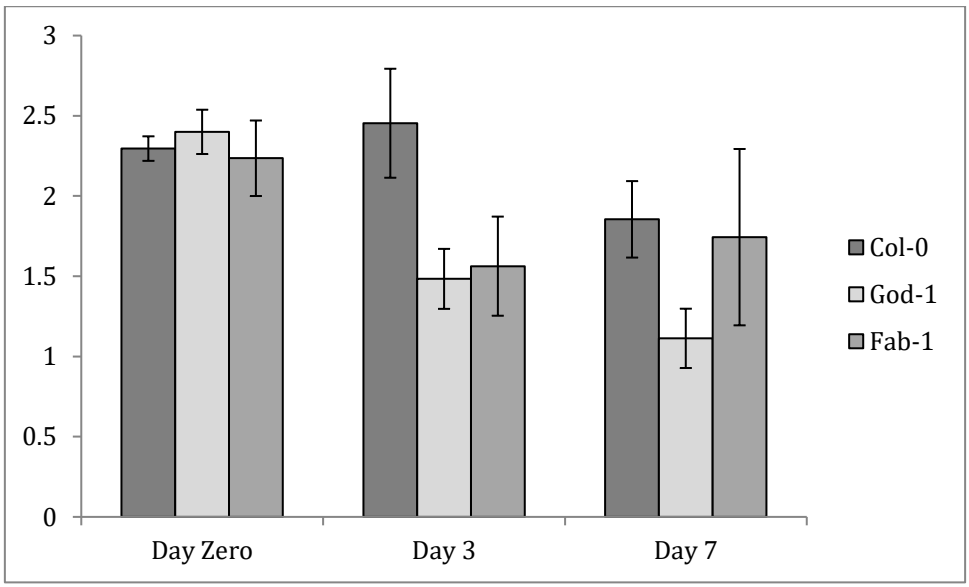

Figure 27 Bacterial growth assay for Xvm 4383 (Banana wilt) at 0.0002OD. Significant relationship over time for Col-0 (F= 10.19, p= 0.00368**) and God-1 (F= 22.37, p= 8.26e-05***). Fab-1 does not significantly change over time (F= 1.497, p= 0.232).

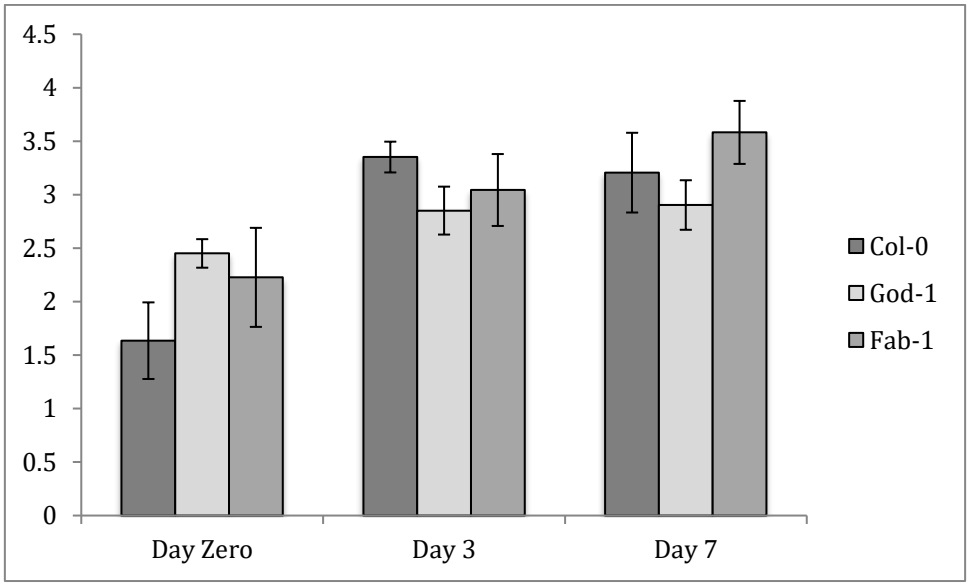

Figure 28 Bacterial growth assay for Xvv702 (Sugarcane wilt) at 0.0002OD. All accessions show a significant change over time, Col-0 (F= 6.273, p= 0.0221*), God-1 (F=5.944, p=0.068*) and Fab-1 (F= 21.67, p=0.000264***).

Our bacterial growth assays demonstrate that phenotype is not consistently correlated with bacterial growth. For example, one of our accessions, Fab-1, consistently exhibited a strongly chlorotic phenotype whether or not bacteria had proliferated in its tissues (Figure 29, Figure 30, Figure 31, Figure 32). Indeed,

this accession produced a chlorotic phenotype even in response to the sham inoculation ($MgCl_2$ control), which exhibited a phenotype score of 3 for both infiltration and nicking exposures.

Figure 29 Phenotyping images of the response of UK accession Fab-1 to the Xanthomonas pathogens 702 and 4381 at 10dpi following nick inoculation.

Natural *Xanthomonas* pathogens of UK *A. thaliana* also elicit a range of susceptible responses with Fab-1 showing the greatest susceptibility (Figure 31, Figure 33).

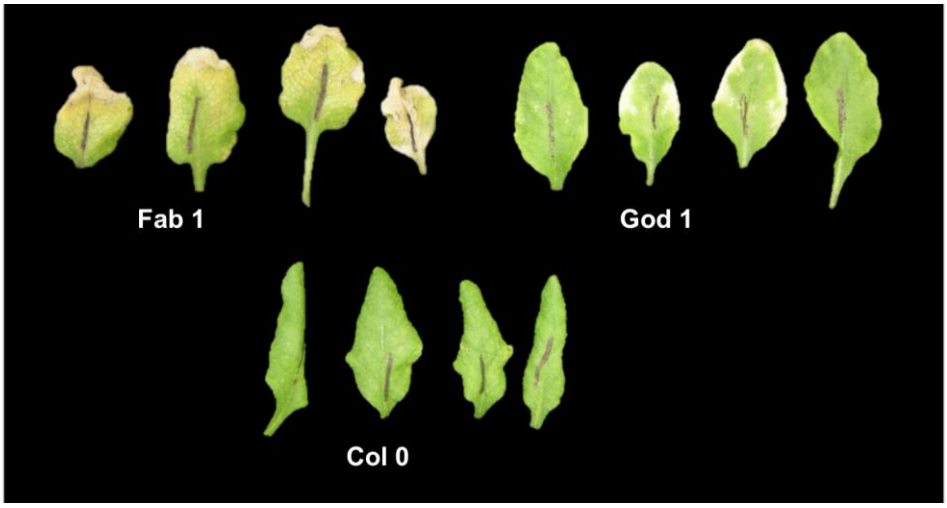

Figure 30 Phenotype exhibited 8 days after infiltration with *Xcc* Race 3 at 0.0008OD

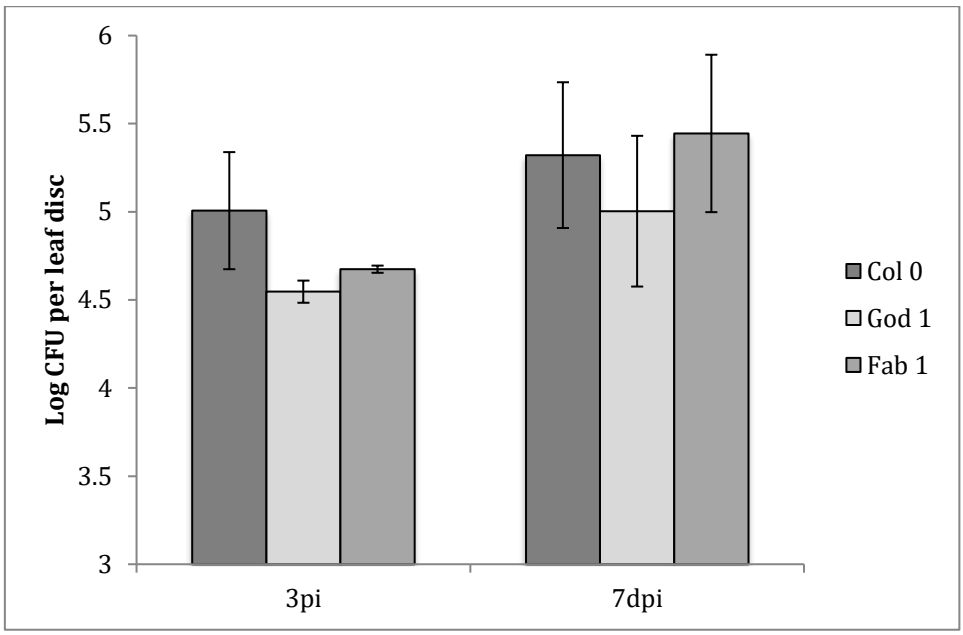

Figure 31 Bacterial growth assay of *Xanthomonas Xcc* Race 3 at OD_{600} 0.0008. All accessions are susceptible, as at day 0 the inoculum would have been below approximately log 3 cfu.

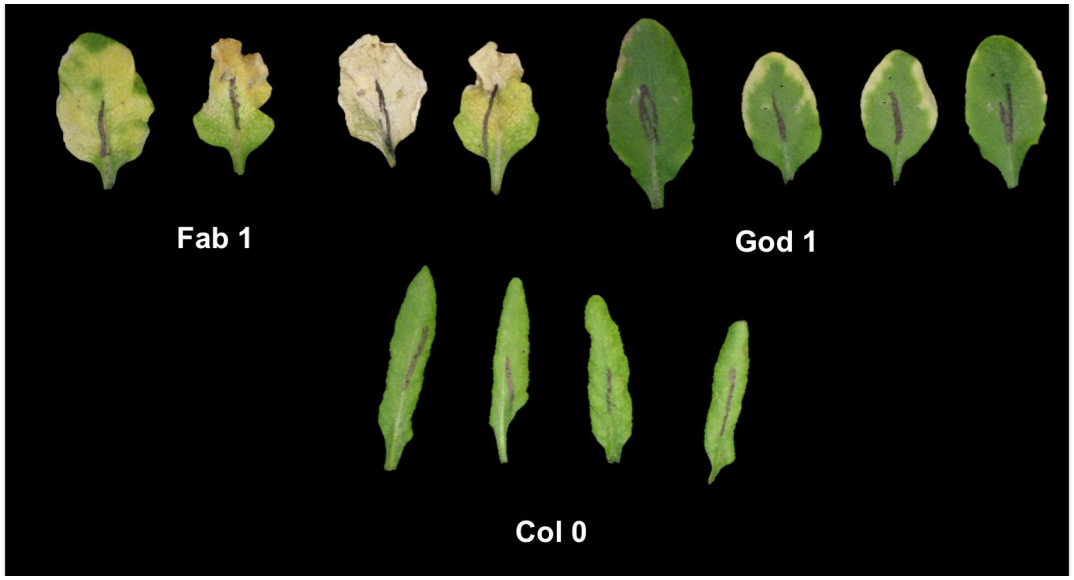

Figure 32 Phenotype exhibited 8 days after infiltration with *Xcc* Race 4 at OD_{600} 0.0008

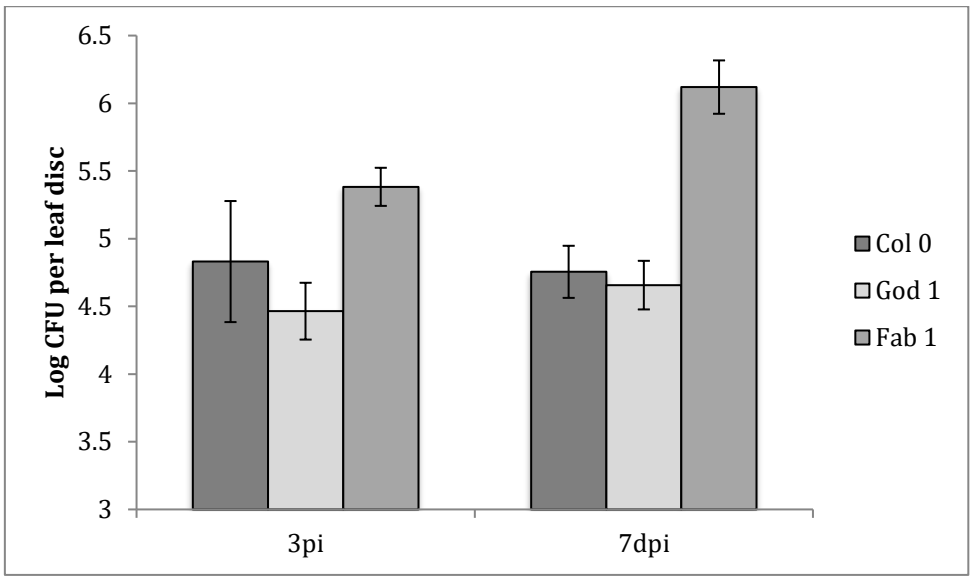

Figure 33 Bacterial growth assay of *Xcc* Race 4 at OD_{600} 0.0008. All accessions are susceptible as at day 0 the initial inoculum would have been below ~ log 3 cfu.

Discussion

We found that wild populations of *A. thaliana* show a wide diversity of resistance and susceptibility to xenopathogens. Specifically, when exposed to any of the four xenopathogens, some accessions showed no symptoms of disease (e.g. Pet-1, Che-1, Cra-1) and others were asymptomatic in exposures to at least several of the xenopathogens (Bet-1, Bog-1, Fav-1, Som-1). It is likely that the asymptomatic responses result from either pathogen incompatibility or pre-adaptation to these xenopathogens, which are phylogenetically closely related to native pathogen strains. We therefore hypothesise that genetic diversity in resistance responses in UK accessions can provide protection against xenopathogens. Potentially, further investigation of these asymptomatic accessions could provide molecular information for engineering resistance to xenopathogens in crops.

We identified one accession (God-1) which gave asymptomatic responses to all of the Xanthomonad pathogens that we investigated. God-1 consistently showed the lowest levels of bacterial growth being able to inhibit or halt proliferation more effectively than the other accessions screened. Future molecular studies could investigate God-1 for the genetic basis of these characteristics. In particular, it

may be valuable to establish the QTL changes occurring to stop infection. This could yield useful insight into the infection and resistance mechanisms between *Xanthomonads* and *Arabidopsis thaliana*.

All xenopathogen strains that we tested were able to proliferate in at least some of the wild-collected accessions of *A. thaliana* that we studied, except the banana wilt pathogen. If plant pathogens continue their poleward advance (Anderson et al., 2004; Bebber et al., 2013), the wild populations of *A. thaliana* populations will experience an onslaught of novel challenges. If xenopathogens were to invade natural populations in the UK, our findings indicate that some populations could be susceptible to novel diseases, but that collectively the British gene pool contains variation that could provide the basis for the rapid evolution of resistance by cross-resistance. We therefore suggest that genetic diversity will provide an evolutionary buffer against xenopathogens.

The banana wilt pathogen was not able to proliferate in the three accessions screened however it did not significantly decline in Fab-1, so whilst not able to cause disease it was able to survive within this accession. The sugarcane pathogen *Xvv* and banana wilt pathogen *Xcm* are closely related and have been the subject of a comparative genomic study to identify factors associated with virulence on bananas (Studholme et al., 2010). This study identified differences in the effector proteins known to be used by plant pathogens to reprogram plant cells for optimal bacterial proliferation. *Arabidopsis thaliana* appears to show the inverse relationship, *Xvv* can cause disease whereas *Xcm* cannot. To our knowledge this is the first time that *Xvv* has been reported to be capable of proliferating within *A. thaliana* providing a potentially useful pathosystem for further study.

Visual phenotype screening on a discrete scale is a simple low cost method to compare the responses of a batch of accessions. However this work demonstrates some the shortcomings of this method, including variability between observations and observers and the accuracy of correlation to disease state (Bock et al., 2008; Bock et al., 2010; Nutter et al., 1993). Alternative imaging

methods are proving more accurate and can be used in conjunction with visual inspection of phenotype to enhance the interpretation of symptoms. For example fluorescence imaging can detect changes in chlorophyll fluorescence emission (Baker, 2008), unstressed plants maintain a stable maximum quantum efficiency of photo-system II (PSII) whilst stressed plants show decreases in this value before symptoms are visible to the naked eye. Similarly hyperspectral and thermal imaging can also be used for some diseases (Mutka & Bart, 2015). Thermal imaging measures temperature changes due to pathogen impacts on stomatal closure and hence transpiration rate (Lindenthal et al., 2005). *Xanthomonas campestris pv. campestris* infection suppresses stomatal closure (Gudesblat et al., 2009) so thermal imaging could be useful for quantification of disease by this pathogen.

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Chapter 5: Artificial selection for pathogen resistance in *Arabidopsis* thaliana causes adaptation by epigenetic inheritance.

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Abstract

Epigenetic inheritance mechanisms have been characterised across many species and differing inducing stressors. Epigenetic inheritance could have an adaptive role if it reduces fitness costs of defence against pathogens in changeable environments, by allowing the rapid induction of defence responses. In plants, it is unknown how widespread epigentically induced defence is, the scale of possible fitness benefits and whether epigenetic potential varies among genotypes. We therefore exposed five accessions of *Arabidopsis thaliana* to virulent (disease), avirulent (systemic acquired resistance) or no pathogens (control) for three generations and we monitored seed set as a measure of 'fitness'. We then screened resistance to virulent pathogens. Our results suggest that *Arabidopsis thaliana* can begin to adapt epigenetically to sustained pathogen stress within three generations.

Introduction

For plants, the environment imposes various biotic and abiotic stresses that restrict performance. For example, herbivores and pathogenic organisms attack and damage plants for their nutritional content. Being sessile and lacking the means to flee, plants have evolved various defensive responses to these attacks. Constitutive defences include surface features that resist attack, such as thickened cuticles or trichomes, and secondary chemicals that render plant tissues unpalatable or indigestible. For the plant, investment in constitutive

defences may be costly in benign environments where attack is rare. If the risk of attack varies in space and time, it is beneficial to possess inducible defences that are initiated only when an attack is recognised (reviewed in Agrawal 2011). When the attacks are by microbial pathogens, systemic acquired resistance (SAR) is one mode of inducible defence whereby the plant initiates physiological resistance throughout its tissues after detecting damage to a single leaf. By implementing SAR, the individual plant in effect exhibits phenotypic plasticity within its own lifetime and this phenomenon is well studied. However, transgenerational SAR (tSAR) is also possible, but the extent to which this occurs is less well understood. Here, we investigate the proposition that tSAR occurs in a ruderal weed, *Arabidopsis thaliana*.

Transgenerational SAR occurs when a maternal plant encounters a pathogen and responds by priming its offspring for more rapid or stronger defence (Luna et al., 2012; Slaughter et al., 2012). In some cases, the defensive responses can resemble a constitutive system because they extend over an individual's lifetime and, furthermore, they can be inherited over several generations (Kovalchuk et al., 2003; Boyko et al., 2007; Stokes et al., 2002; Molinier et al. 2006; Durrant, 1962). It is adaptive for plants to possess tSAR when the pathogen is highly virulent and when the implementation of a systemic defence response is slow both within the lifetime of the plant and relative to the speed of pathogenesis. Epimutations could be an adaptive response increasing phenotypic plasticity of genotypes in a changeable environment (Verhoeven et al., 2016) The inheritance of environmentally acquired attributes is known as transgenerational stress memory (Pecinka et al. 2009), or epigenetic inheritance (Jablonka and Raz, 2009), denoted EI.

Mechanisms of epigenetic inheritance

In plants, as in other organisms, DNA mutations underlie much of the observed natural variation in wild populations, but it is increasingly recognised that heritable, non-sequence-based variation exists which can be triggered by some aspect of the environment, typically a stress. Here, we adopt the usage advocated by Jablonka & Raz, (2009), who propose that epigenetic inheritance

refers specifically to the processes responsible for non-sequence-based variation that persists across generations.

Epigenetic phenomena have been demonstrated in various species including humans (Egger et al., 2004; Pembrey et al., 2006; Whitelaw, 2006), other mammals (Peaston and Whitelaw 2006) and plants (Akimoto et al. 2007; Bond & Baulcombe 2014). Many mechanisms may be responsible for epigenetic variation, which include DNA methylation, histone modification, micro RNA (miRNA) and small interfering RNAs (siRNA) (Bond & Baulcombe, 2014; Rapp & Wendel, 2005; Richards, 2006; Peschansky & Wahlestedt, 2014). DNA methylation is the most studied and it has been shown to silence gene expression, thus altering the phenotype (Chiang et al., 1996; Kato et al., 2003). The effects of methylation are passed to progeny following conventional Mendelian segregation (Finnegan et al., 1998).

In plants, epigenetic inheritance has been demonstrated to occur after experimental manipulation (Anway et al., 2005; Newbold et al., 2000; Shiao et al., 2005; Verhoeven et al., 2010) and also by natural selection (Herrera & Bazaga, 2010). The process is probably widespread and it can control major aspects of phenotype. For example, a naturally occurring variation in the floral architecture of toadflax *Linaria vulgaris* first noted by Linnaeus in 1742 is epigenetically programmed (Cubas et al., 1999). These toadflax flowers are radially or bilaterally symmetrical depending on the methylation state of the *CYCLOIDEA* gene (Cubas et al., 1999). Biochemical traits can be also controlled epigenetically. In the violet, *Viola cazorlensis*, vulnerability to mammalian herbivory in the field is associated with changes in DNA methylation (Herrera & Bazaga, 2011). Indeed, virtually any trait appears to be susceptible to epigenetic influence. In corn, *Zea mays*, so-called ‘epialleles’ have been characterised that affect pigmentation in seed and vegetative tissues (Das & Messing, 1994; Stam et al., 2002). Whether these natural epialleles are maintained by drift or natural selection due, for example, to beneficial effects on pollination and herbivory respectively is not always clear, but their occurrence indicates that epigenetic phenomena are important in shaping plant adaptation.

Artificially induced epialleles can influence a wide array of responses from floral morphology of *Arabidopsis thaliana* (Jacobsen & Meyerowitz, 1997) to bolting timing of *Brassica napus* (*Soppe et al.*, 2000) and pathogen resistance of *Oryza sativa ssp. japonica* (Akimoto et al., 2007). When *Brassica napus* was grown under divergent selection for flowering time over six generations, the progeny differed in their flowering time by over 12 days, but recombination alone could only account for 30% of this effect variation and epigenetic mechanisms probably underlie this rapid divergence under selection (Pires et al., 2004).

However, it remains unclear the extent to which SAR can occur by EI in plants. For a ruderal colonist like *A. thaliana*, EI could be vital in enabling a rapid adaptive response by a normally self-fertilising species. Self-fertilising (SF) plants do not receive the potential adaptive benefits of variation afforded by genetic recombination through sexual reproduction. Therefore, they are vulnerable to environmental change and, compared to their sexually reproducing counterparts, selection is likely to be strong for mechanisms that increase phenotypic flexibility. In SF plant species that have evolved phenotypic flexibility, EI is adaptive because it is a mechanism that potentially could deliver rapid, inducible responses to pathogen attack. The model plant *A. thaliana* is a SF species whose wild populations are exposed to diseases that create selection pressures that could favour the evolution of epigenetic inheritance of SAR. Indeed, there is already some evidence for this, as follows.

Transgenerational systemic acquired resistance, tSAR, in *A. thaliana* occurs under artificial conditions after only one generation of exposure to the virulent *Pseudomonas syringae pv. tomato* DC3000 (Luna et al., 2012) and the avirulent *Pseudomonas syringae pv tomato avrRpt2* or β-amino-butyric acid (Slaughter et al., 2012). However, whether epigenetic inheritance is involved in responses to disease in wild populations of *A. thaliana* remains unclear. After studying a range of biotic and abiotic selective pressures Pecinka et al., 2009 concluded that epigenetic responses did not appear to be a general stress response. Moreover, if epigenetic ‘rescue’ from environmental stress were widespread, then pathogen

virulence would be unrealistically short-lived and epidemics be shorter than actually observed. Consequently, while it has been valuable to make initial investigations on laboratory strains, it is now important to extend investigations to naturally occurring genetic variation.

The investigation of EI in beneficial plant traits has not just fundamental value, but it may generate useful applications in agriculture. Highly virulent pathogens can decimate crops year on year (Biruma et al., 2007; Savary et al., 2006), so understanding EI could provide a possible basis for crop protection. Furthermore experimental induction of epigenetic phenomena often involves challenge with a single stress, however in nature organisms are rarely exposed to a single stress and multiple stresses can have non-additive effects. Clearly, whether EI enables inducible response to pathogens in *A. thaliana* requires investigation.

In order to test for EI, we grew *A. thaliana* for three generations in the laboratory under pathogen stress and then used a cross-generation common garden to evaluate the impact of selection. Increases in pathogen resistance over successive generations can be attributed to an epigenetic basis because *A. thaliana* is principally self-fertilising, which means that evolution through DNA-based inheritance is unlikely under controlled laboratory conditions.

The plant's strongest defence response relies upon the presence of a resistance gene in the plant that corresponds with the avirulence (*avr*) gene in the pathogen (Flor, 1971), which invokes a rapid hypersensitive response to compartmentalise the pathogen and prevent further ingress and primes the plant by SAR to reduce the risk of further attack. In order to determine whether pathogen recognition is important in EI, we challenged one treatment group of plants with pathogens carrying recognisable *avr* genes that will activate a classical SAR response pathway (Grant & Lamb, 2006) and another replicate group with virulent disease-causing pathogens. In order to determine the capacity for EI to arise in natural variants, we collected *A. thaliana* from wild populations (Chapter 2) and we also included the standard lab genotype Columbia for comparison. Using this array of

genotypes allowed us to explore genetic differences in potential for epigenetic adaptation.

Specifically, our investigation aimed to answer three questions as follows. First, where *A. thaliana* encounters an environment that presents a sustained pathogenic challenge, does plant fitness increase over successive generations in a manner that is attributable to EI? Second, do genetic variants of *A. thaliana* differ in their capacity to respond to sustained pathogen challenge by EI? Third, does the strength of EI depend on the capacity of maternal plants to recognise and compartmentalise the pathogen?

Methods

To test for EI, we grew five genetically distinct lines (accessions) of *A. thaliana* for three generations in environments that presented sustained pathogen challenges (either virulent or avirulent strains) for comparison with control conditions.

We established three treatments as follows. Two treatment groups were exposed to pathogen challenges: P_R and P_D. Treatment P_R involved a pathogenic challenge using bacterial pathogens known to invoke systemic acquired resistance (SAR), whilst the P_D treatment involved a pathogenic challenge that caused disease symptoms because the plants had no R-gene defence against the bacterial and fungal pathogens used. The third treatment was a control which were plants grown alongside the other groups but with no inoculations.

Each treatment group consisted of six replicate individual plants of five accessions (the standard laboratory line, Columbia, and four collected from wild populations located in the United Kingdom).

After three generations under the three types of pathogen exposure (P_R and P_D and unchallenged control), the seed progeny and seed of the original parents were grown and the adult plants screened for resilience to pathogens.

Plant provenance and husbandry

We studied the common laboratory strain Col-0 and four natural accessions from across the UK: Igt cpe B32 (source location – Igtham, Kent); Sand 37 F37 (Sandford, Devon); Lan 2 L74 (Lanark, Scotland); and Inv B N48 (Inverness, Scotland). The wild accessions were collected as seed during summer 2009 (see Chapter 2). In order to reduce the risk of heterozygosity or unstable epigenetic phenomena in the wild-collected plants, these lines were self-fertilised for two generations without experimental challenge in the glasshouses at the University of Exeter prior to use in the present study.

For each generation in our trial, the accessions were grown from seed under controlled conditions in an environmental chamber. For sowing, seed was stratified in 0.1% agarose solution for three days prior to sowing into 1:4 vermiculite and Levington F2 peat free compost (JFC Munro, http://www.jfcmunro.co.uk). Approximately five seeds were sown per pot and the germinating seedlings were thinned to leave one individual. Plants were grown in a Snidjer controlled growth chamber under short day conditions (12hr light, 100-150 µE, 22°C day, 20°C night) for the first five weeks at which point the day length was increased to 16 hours to encourage flowering. Pots were spatially randomised within the cabinet weekly. Once the primary flowering stem had reached approximately 100 mm height, an Aracon tube (Beta Developments, Gent, Belgium) was used to prevent cross-fertilisation and to collect seed. After natural senescence, fitness of each plant was quantified by the seed mass produced.

Pathogen provenance, propagation and challenge

In order to simulate the mixed microbial flora of wild conditions and to induce pathogen resistance systems, we used challenges comprising mixed infections. The challenges applied and relevant concentrations are shown in Table 14. The pathogen mixtures also utilised: *Pseudomonas syringae* (DC3000 avrRpm1,

avrRps4 and avrRpt2); *Xanthomonas campestris pv campestris* (races 3 and 4)*;* and *Botrytis cinerea*.

P. syringae DC3000 was grown in Kings Broth (KB) media containing Rifampicillin (50µg/ml) and Kanamycin (25µg/ml). *X. campestris pv campestris* was grown in KB media with no antibiotic selection. *B. cinerea* was grown on apricot halves. Bacteria were grown overnight in liquid media before being washed and resuspended twice in 10mM $MgCl_2$. Bacteria were then diluted to the required concentration, estimated by the optical density of the solution at 600nm using a spectrophotometer. Fungal spores of *B. cinerea* were harvested at 7-10 days and suspended in grape juice. A haemocytometer was used to estimate the number of spores per aliquot (ml) before the suspension was diluted to the required concentration.

Bacterial challenges were conducted by using a small needleless syringe to introduce the microbial suspension via stomata on the abaxial side of a premarked leaf. Each successive treatment was applied to a new and previously untreated leaf except in rare instances where virulent pathogens had retarded plant growth to such an extent that no new leaves were available; in these cases, leaves were infiltrated a second time.

Fungal challenges involved placing a 5µl drop of suspended spores on either side of the leaf midvein. Treated leaves were lightly restrained to reduce the nyctinastic movement and prevent cross-contamination of other leaves. Treated plants were bagged to maintain high humidity, which favours infection. After four days the treated leaves were removed so that sporulation did not contaminate other plants.

During the two-week period of pathogen challenge, plants in each treatment group were segregated in order to prevent infections among groups; they were randomly distributed otherwise.

The control group were grown alongside the experimental plants but were left unchallenged for the course of the experiment.

Week post sowing	**Treatment**	**PD**	**PR**	**Control**
4	1	*Pseudomonas syringae* DC3000 + pvsp61 3 leaves @ 0.002 OD	*Pseudomonas syringae* DC3000 equal mixture of *avrRpm1*, *avrRps4* and *avrRpt2* 3 leaves @ 0.0002 OD	Grown alongside other plants but with no challenge
	2	*Xanthomonas campestris pv campestris* equal mixture of races 3 and 4. 3 leaves @ 0.008 OD	*Pseudomonas syringae* DC3000 equal mixture of *avrRpm1*, *avrRps4* and *avrRpt2* 3 leaves @ 0.002 OD	Grown alongside other plants but with no challenge
5	3	*Botrytis cinerea* 3 leaves, 2x 5µl drops@ 10^5 spores/ml	*Pseudomonas syringae* DC3000 equal mixture of *avrRpm1*, *avrRps4* and *avrRpt2*	Grown alongside other plants but with no challenge

			3 leaves @ 0.002 OD	
	4	*Pseudomonas syringae* DC3000 + pvsp61 3 leaves @ 0.002 OD	*Pseudomonas syringae* DC3000 *equal* mixture of avrRpm1, *avrRps4* and *avrRpt2* 3 leaves @ 0.002 OD	Grown alongside other plants but with no challenge

Table 14 The treatment groups

Measurements of plant performance and adaptation

After natural senescence, siliques were collected, threshed and sieved to gather seed. Evolutionary fitness was characterised by the seed mass produced per plant. We investigated the occurrence of adaptation over the course of the experiment by testing for increases in seed production over successive generations. We relativized the performance of the pathogen-treated groups against that of the control group in the same generation because seed production declined over successive generations for undetermined causes (see Results). In effect, we tested for increasing differences between experimental treatment and the control.

We do not know whether EI mechanisms are consistent between replicates or whether chance events could occur in just one replicate. Therefore seed from each replicate in each generation was used to sow that replicate in the preceding generation, in effect treating each replicate as a separate line.

To determine whether increased resilience to pathogens contributed to adaptation, we compared pathogen growth among the original parental lines and

the treatment groups after three generations. Specifically, we investigated the levels of growth achieved by *Pseudomonas syringae* DC3000 and *Botrytis cinerea* in challenges that were conducted as described above with the additional provisos that *P. syringae* DC3000 was infiltrated at 0.0002OD and bacterial growth measured at three days post inoculation (3 dpi). The lower concentration was used for this assay or the bacterial levels would be too high to count. Bacterial growth was measured as described elsewhere (Chapter 4). To investigate the level of infection achieved by *B. cinerea*, experimental infections were conducted on four leaves per plant that were removed and placed on 0.1% agar. Plants were grown for approximately 5 weeks before the leaves were harvested for testing. On each leaf, two 5µl drops of spore suspension were placed either side of the mid-vein of each leaf. Maximum lesion size was calculated from digital photographs using Image J (Schneider et al., 2012). These assays were conducted only in two accessions from wild populations, namely Lanark and Inverness. These accessions were chosen for further study because the performance of Lanark improved under pathogen challenge over the three generations whereas that of the Inverness accession remained stable (see Results). We therefore investigated whether the variation in performance was attributable to differential permissiveness for microbial proliferation.

Statistical analyses

To determine whether the exhibited patterns were statistically significant we used ANOVA in R (v.3.2.1). Where the assumptions of parametric ANOVA were violated, we performed Monte Carlo ANOVA as described in Chapter 2.

Results

Adaptation to sustained pathogen challenge

Collectively, the performance of *A. thaliana* under pathogen challenge increased over three successive generations whether the pathogen was virulent or not (ANOVA, interaction term for Accession* Generation*Treatment, $F = 2.6299$, $p < 0.001$; Figure 34), which demonstrates adaptation. At the accession level and prior to normalisation the pattern is noisy (Figure 36). Due to time constraints we

selected two accessions to focus on for more analysis: Inv as it showed high resilience to the challenges applied (stable fitness) and Lan as it showed a marked fitness differential disease and SAR challenges compared to control group plants (Figure 36).

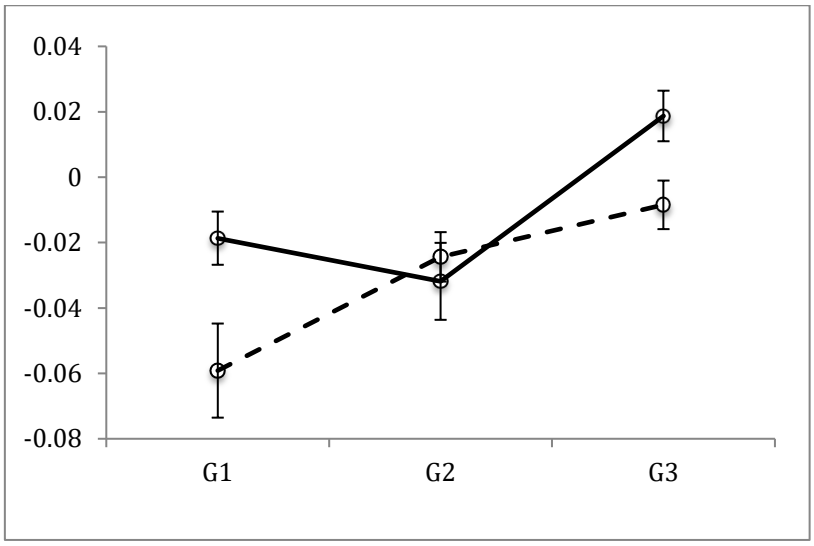

Figure 34 Relative reproductive performance (mean seed mass in g normalised to control levels) of five accessions of *Arabidopsis thaliana* under sustained exposure to mixed pathogenic challenge over three successive generations *(x*-axis: generation). Means (open circles) are interpolated for inspection purposes only; dashed line: plants challenged with virulent pathogens; and solid line: avirulent pathogens.

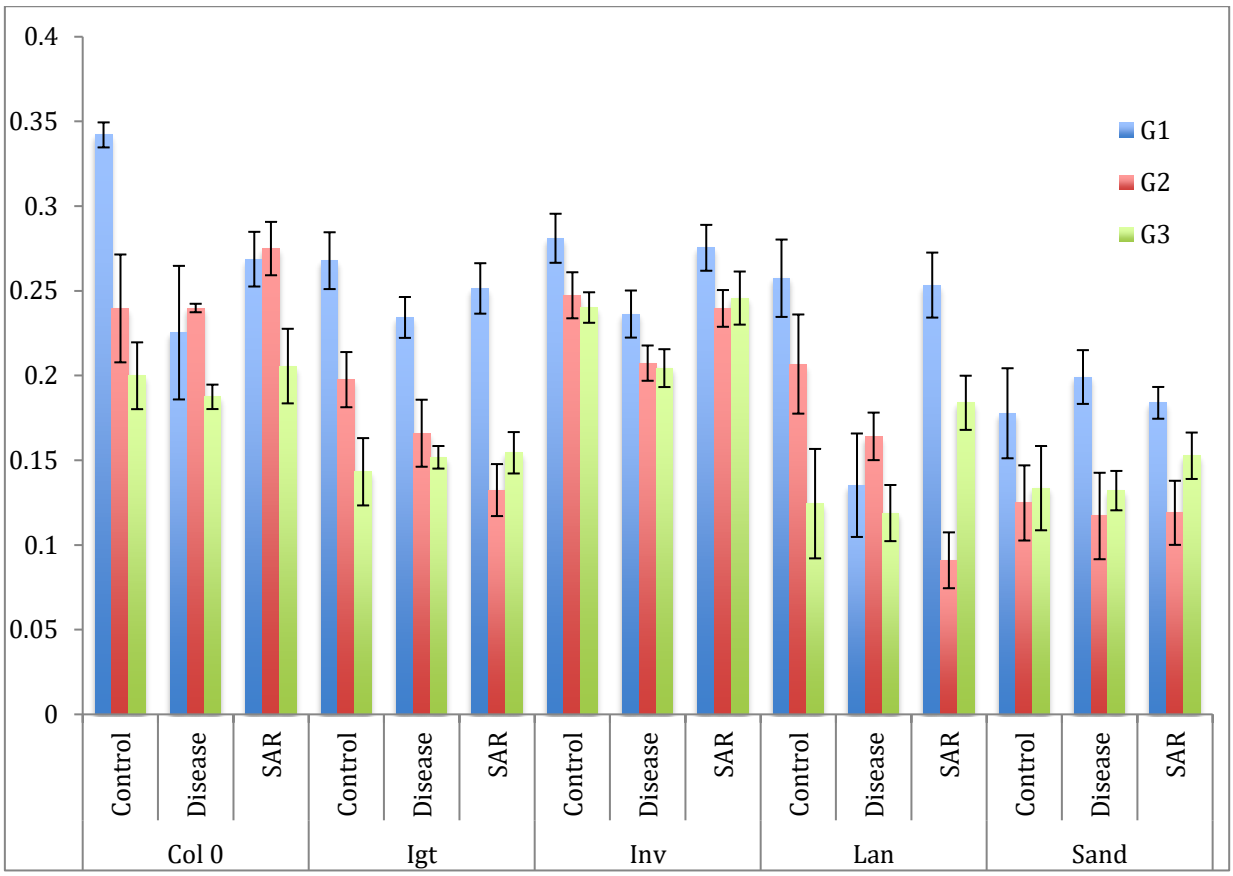

Figure 35 Total reproductive output (seed mass g) of plants in each accession and treatment group. Blue bars- generation one; red bars- generation two, green bars- generation three. Error bars +/- 1SE.

Focusing on those two accessions we can see that the degree of adaptation differed with performance increasing over three generations in the accession from Lanark but not in the accession from Inverness (Figure 36).

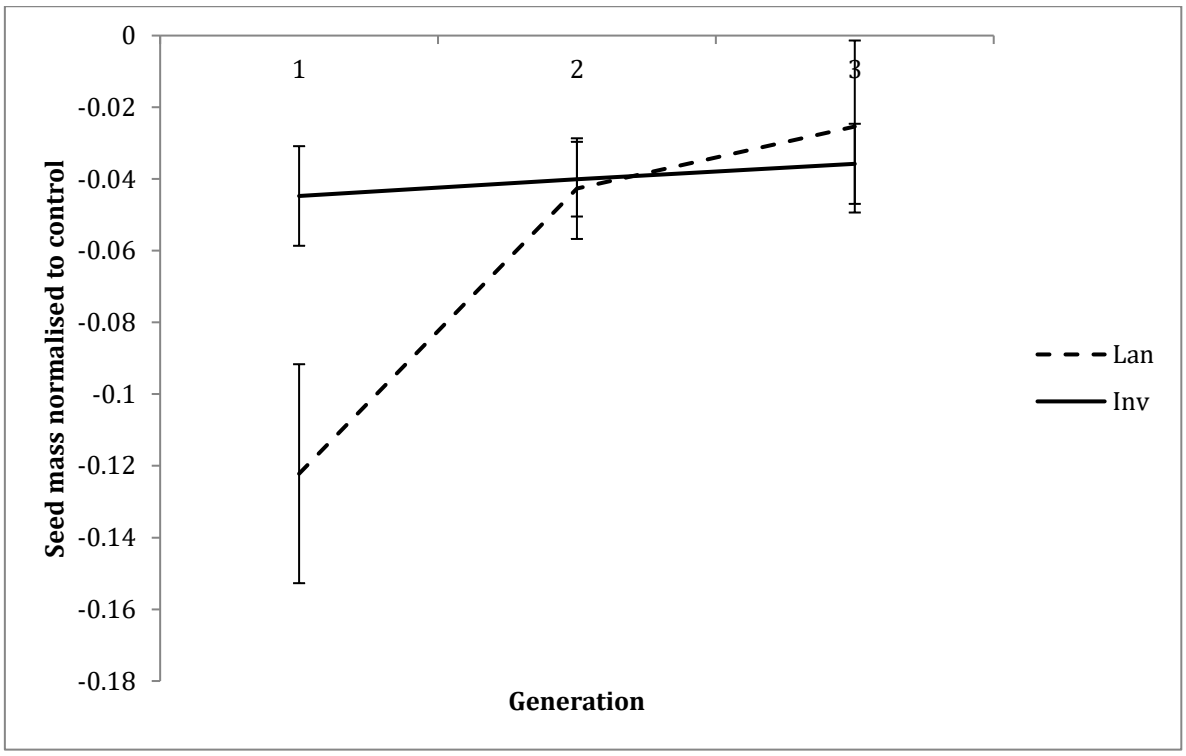

Figure 36 Reproductive output (seed mass in mg) for disease treated plants normalised to control in each generation of the two accessions chosen for further screening (Lanark and Inverness). Error bars represent +/- 1SE.

No significant differences were found in screening *B. cinerea* growth. Bacterial growth assays using DC3000 in both Lanark and Inverness found that treatment group was a significant predictor of bacterial growth (Lanark: ANOVA, F= 6.473, p=<0.001; Inverness: ANOVA, F= 2.888, p=<0.05). For both accessions screened the parental lines permitted the highest levels of bacterial growth suggesting a naïve host is vulnerable to attack. However the lowest levels being found in generation 3 control plants required further investigation (Figure 40).

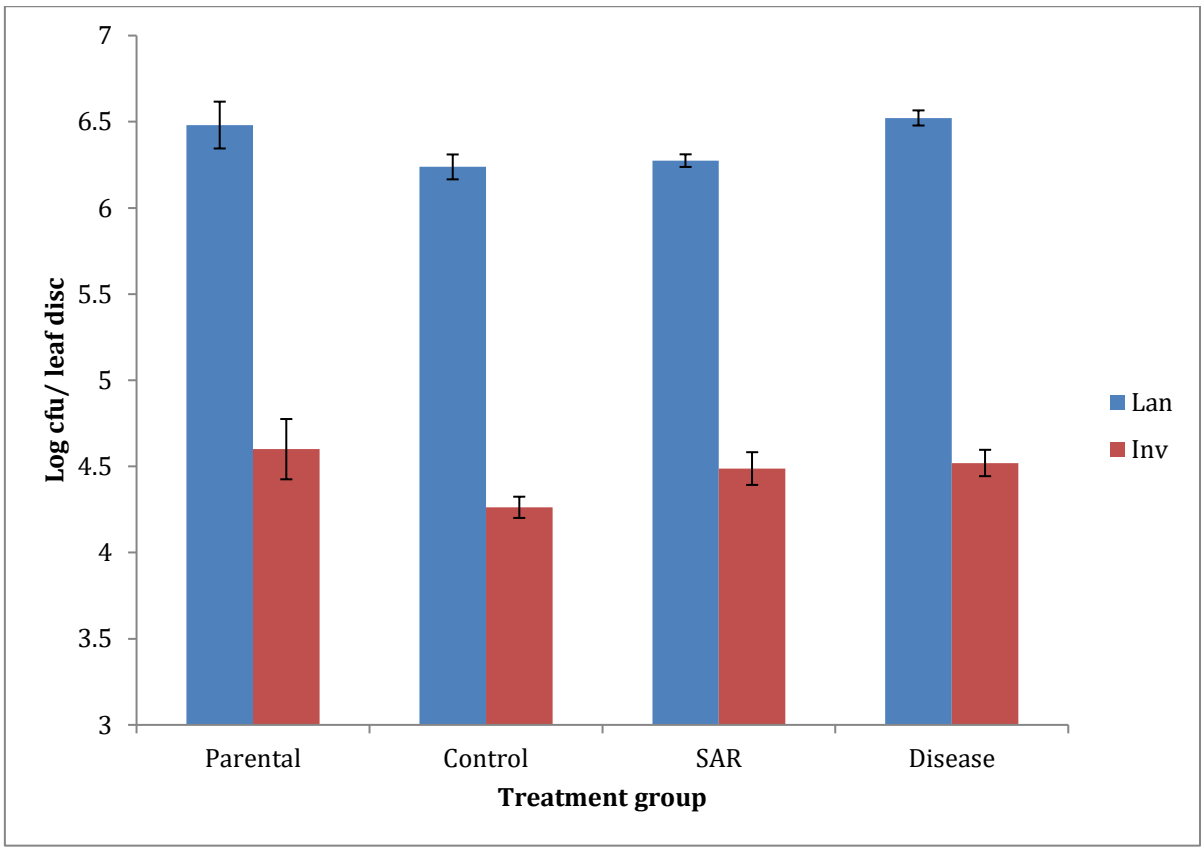

Figure 37 Proliferation of DC3000 (colony forming units per leaf disc) in parental and generation 3 individuals of accessions from Lanark and Inverness at 3 days post infiltration at 0.0002OD. Error bars indicate +/- 1 SE. Parental plants are grown from the original seed stock that were sown in generation 1 and the control plants have been through three generations alongside the experimental plants but without pathogen challenge.

Discussion

Our results suggest that *A. thaliana* can begin to adapt within three generations to an environment that presents a sustained exposure to mixed microbial pathogens. The rapidity of the adaptive response is fairly typical of artificial selection experiments in a wide range of species including guppies (End*ler et al*>, 2001; Kotrschal *et al*., 2013) and fruit flies (Stearns et al., 2000). Among plants, rapid adaptation is characteristic of invasive and colonising species, so it is not surprising to find it in a ruderal like *A. thaliana*, which is likely to encounter a varied set of environments.

The adaptation observed is most plausibly attributed to an epigenetic mechanism because the rapidity of the adaptive response excludes selection of mutations and the lack of recombination in this self-fertilising plant excludes other mechanisms such as meiotic drive. Furthermore, there was no source of artificial selection to drive a change in the gene pool, because all parents were permitted to produce the same number of progeny. The accessions did not behave uniformly and had differing resistance/tolerance of the pathogens at the start of the experiment, thus epigenetic effects are not universal and are to some extent modulated by the underlying genetic scaffolding. Therefore the potential for epigenetic modification will itself be a trait under selection. Individuals living in environments experiencing sustained stress will be more likely to exhibit epigenetic phenomena.

Adaptation by EI could provide an inducible but persistent response to pathogenic challenge for species that encounter large scale patchiness in the prevalence of microbial pathogens coupled with a high cost of constitutive defences. We speculate that founders of a new population of *A. thaliana* need to have an inducible defence that may nevertheless be required for several seasons. Questions remain of the stability of the epigenetic response and its further potential to reduce pathogen burdens. Further experiments could include more generations of challenge to show whether greater resistance/tolerance could be achieved or whether the benefits will plateau from this point. Additionally further generations without challenge could show when the epigenetic mechanisms would revert to the unchallenged state, assuming that there is a metabolic cost to priming in this way.

However we have to express caution in whether our results are evidence of EI as before normalisation fecundity reduced across all accessions and treatments. As the control group was similarly affected we hypothesise that environmental parameters outside of our control could have impacted plant growth. For example building works were being carried out near our growth chamber during the later generations that may have impacted growth of all accessions. Noise pollution (which was extensive during generations 2 and 3) has been shown to impact

plant growth (Woodlief et al., 1969) therefore could in part explain the reduced fecundity exhibited.

Pathogen growth assays (comparing parental to generation 3) showed a significant small increase in resistance to *Pseudomonas syringae* DC3000 in both accessions screened and no significant change in resistance to *Botrytis cinerea*. DC3000 is a highly virulent pathogen with little natural resistance in host populations, in a screening of 1041 accessions only 14 showed resistance to DC3000 (Velásquez et al., 2017), which supports only finding a small reduction in bacterial growth. Where resistance by the hypersensitive response (HR) is not available the speed of general responses could be crucial to reduce pathogen damage. Therefore EI could prime defence responses in progeny, thus reducing the time DC3000 can proliferate *in-planta* before defence is initiated. Curiously, generation 3 control plants also showed a consistent reduction in growth of DC3000 relative to parental plants. This could again be due to epigenetic priming, possibly initiated by volatile signalling between plants, that enables host responses to be more rapidly recruited upon pathogen recognition or for example a stronger MAMP triggered immune response. It is possible that the treatment groups were emitting volatile defence related compounds (Holopainen & Gershenzon, 2010) that would elicit a costly defence response in control plants reducing their fecundity and also invoking some level of epigenetic defence responses.

The increase in fecundity relative to control could be due to treatment groups being primed to optimise seed production despite stressful growth conditions. By challenging 'disease' group plants with multiple pathogens we could have disrupted the epigenetic pathways. Bacterial pathogen defence responses involve salicylic acid (SA) whereas fungal pathogens invoke jasmonic acid (JA). JA and SA are mutually antagonistic pathways, inhibiting signalling of the alternate chemical (Kunkel & Brooks 2002). Therefore it is conceivable that multiple pathogen challenges could have disrupted selection of an optimal defence strategy. The 'SAR' treatment group showed a greater improvement in

fitness than the 'disease' group which could be in part due to the fact that they were consistently challenged with bacteria and experienced no “cost” of disease.

Pseudomonas syringae DC3000 has been shown to invoke a significant adaptive epigenetic effect in *Arabidopsis thaliana (*Luna et al., 2011). We found a small but significant difference in growth of DC3000 however this is not conclusive due to the variation inherent in this assay. Luna et al., (2011) used increasingly high concentration dip inoculation and maintained plants at 100% relative humidity. Whilst this may increase the probability of invoking epigenetic phenomena we chose to keep plants under more natural conditions and inoculate at environmentally relevant concentrations. We found a significant reduction in the fitness cost of challenge (normalised to control) however we cannot say conclusively that this was due to a decrease in disease. Epigenetics is not omnipotent and the pathogens that we had available to screen with were highly virulent. Luna et al., (2012) and Slaughter et al., (2012) screened responses with a weaker pathogen but we did not have a suitable pathogen available in the lab. Therefore we cannot rule out that some level of priming occurred that was not fully revealed by our screening protocol. Looking for molecular evidence of epigenetic marks was outside of the time and financial scope of this work. However it is noted that the studies from Luna et al. and Slaughter et al. originated from the same laboratory and have not yet been validated independently. There is a wealth of conflicting data on SAR responses in the literature depending upon the lab, time of day plants are inoculated, method of inoculation and pathogen strain. Thus it is conceivable that the discrepancies seen in this study and Luna et al. are the result of technical differences. If this is indeed the case then one must question the robustness of EI as an adaptive mechanism.

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Chapter 6: Investigating the effects of genotype and environment on the metabolome of *Arabidopsis thaliana* by untargeted metabolite fingerprinting

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Abstract

Plants are estimated to produce over 100,000 metabolites. The metabolites detected in any individual plant could reveal a huge amount of information about its development and stress levels, as well as information about its underlying genetics. This 'metabolite fingerprint' can therefore be used to investigate the potential for genotype-by-environment (GxE) interactions. We grew 12 genotyped accessions of *Arabidopsis thaliana* in two environments to compare their metabolite and genetic distances. There was significant similarity between genetic and metabolite variation consistent with the genetic basis of metabolic expression. However, the environment had the greatest overall effect on metabolite fingerprint. Potentially, this method of metabolite fingerprinting could therefore be used to distinguish plant responses of genotypes and environments.

Introduction

Understanding the metabolome can elucidate latent functional differences between individuals. There is increasing interest in its potential to inform ecological and evolutionary investigations (Bundy et al., 2008; Riach et al., 2015) because it can be used to demonstrate the interactions between genotype, phenotype and environment (Keurentjes, 2009). Genotype-by-environment

interactions (GxE) are revealed by the displayed phenotype when phenotypic plasticity means that each genotype encodes a range of possible outcomes depending upon environmental stimuli. Thus, the phenotype summarises the impact of environmental variables on the overall expression of the genome of an organism at a given moment in time. For every gene sequence that defines an organism, an array of chemicals act to deliver the genetic goal. Understanding the dynamic metabolome could be used in plant breeding for crop improvement both in terms of productivity, by targeting environmentally relevant beneficial traits, and enhancements such as nutritional content or flavour (Geleta et al., 2005; Fernie & Schauer, 2009; Schauer & Fernie, 2006).

The expression of metabolites is due to the underlying genetic material, therefore it should be possible to use metabolite fingerprinting to infer the relatedness of organisms by the similarity of their metabolite profile at the same age when grown under the same conditions. However, even under controlled conditions the same genotypes will express metabolite variation. Chan *et al.,* (2010) grew the same accessions of *A. thaliana* in batches at different times in the same growth chamber and about 75% of metabolite features were detected in both batches, but of these 70% were present at different abundances. QTL mapping of recombinant inbred lines has shown that metabolite composition is under genetic control (Keurentjes et al., 2006). However detection of the underlying genetics is confounded by seeming redundancy as high levels of genetic variation in natural accessions are not reflected in high levels of metabolite variation (Chan et al., 2010). Untargeted metabolite fingerprinting has been shown to be valuable in phylogenetic reconstruction across species (Martucci et al., 2014; Wahyuni et al., 2013). It has been less precise at reconstructing within species (Houshyani et al., 2012; Mochida et al., 2009). Houshyani et al., (2012) compared the metabolite fingerprint of 9 *A. thaliana* accessions to 149 SNP data, and found only a weak correlation between genetic and metabolite distances which raises the question of whether higher resolution SNP coverage would aid or further confound the identification of phylogenetic consensus?

The complexity and dynamic nature of the metabolome creates unique challenges. There are estimated to be over 100,000 metabolites present in plants, with species-specific differentiation (Keurentjes et al., 2006). Within and between species, individuals may differ both qualitatively by presence or absence of chemical features and quantitatively in expression levels. Accurate quantification of a given metabolite requires identification of the chemical and running of appropriate standards to correct for methodological biases, such as recovery rates, etc. Metabolites involved in environmental responses include, for example, various plant hormones co-ordinating stress responses and the anti-herbivore or anti-microbial compounds (Wink et al., 1988; Kliebenstein et al., 2005; Wittstock and Gershenzon, 2002). Metabolites are considered as primary if they are involved in growth and development, or as secondary if they are involved in dynamic environmental responses. However these distinctions can breakdown under close inspection, as transcriptional studies have revealed that many primary metabolites are also pivotal in defence responses (Schwachtje & Baldwin, 2008). Thus, metabolites can have multifaceted roles in the dynamic 'metabolic space'. Unravelling the function of metabolites can be problematic because it can involve complex interactions and non-additive effects, as when multiple stresses affect a plant simultaneously. For example, the metabolite proline is thought to be important in plant drought response (Cushman and Bohnert, 2000), but plants under combined drought and heat stress will suppress production of proline while accumulating various sugars (Rizhsky et al., 2004). Illustrating that when stresses are combined, we are still a long way from being able to predict the "optimal" plant enhancement strategies for any given environment.

Furthermore the sensitivity and selective nature of the instrumentation used to characterize metabolome composition adds further challenges. No assay will reveal all metabolites as their detection depends upon the interaction of their unique chemistry with the assay's instrumentation, which is normally the column and ionisation source of liquid chromatography mass spectrometry (LC-MS). LC-MS has been shown to capture a wide variety of plant chemical features and is the method of choice for revealing a comprehensive snapshot of plant

metabolism (Song et al., 2017). LC-MS methodology involves compromises between sensitivity, speed, selectivity and chemical coverage (Berg et al., 2013) no single solution can maximize all of these outcomes. With the huge array of chemicals detected comparison within the same experimental parameters will still reveal a huge array of phenotypic differences. Experimental factors to consider for maximum efficiency in any given system are extraction protocol, analysis methods and instrumentation (Ortmayr et al., 2016; Lindahl et al., 2017). The metabolites detected will include intermediate and end-products of biochemical pathways, which are minimised during data handling. Data analysis methods are seen as the bottleneck in LC-MS studies as they are the key to understanding the complexity of the large datasets produced. As well as being genetically regulated they are highly labile depending upon environmental conditions therefore randomization and rapid freezing of samples is essential.

The differences in metabolite profile between genotypes or treatments can be revealed by mass spectrometry. Targeted analyses aim to quantify the presence of known features, for example screening plant hormones involved in defence response (de Torres-Zabala et al., 2007) or for untargeted analyses aiming to represent, as closely as is technically possible, the entire metabolome, including unidentified chemical features (Keurentjes et al., 2006; Wahyuni et al., 2013). Untargeted metabolic fingerprinting provides a snapshot of the chemical composition of an organism. Metabolite profile analysis is complicated by its ephemeral nature; we are seeking to characterise a moving target (i.e. the complex manifestation of plant metabolic activities) that responds instantaneously to its environment. Not only does the metabolome shift according to environmental exposure whether experimentally intended or not, but also according to natural factors such as intrinsic circadian rhythms and extrinsic plant-to-plant signalling. The metabolite profile's 'fingerprint' also changes due to factors such as age and environmental conditions. However, the changeably responsive nature of the metabolome also means that metabolite fingerprinting in different environments allows us to begin to explore the dynamic interactions between an organism and its environment. In summary, whilst the genome provides the blueprint to build the organism, the metabolome is an expression of

a transitory moment in time for the functioning organism. Comparative metabolomics allows us to capture a huge amount of information about the organism and its environmental responses. What are the relative importance of the environment and genetics in shaping the metabolome? The aims of this study were: (1) to test whether genetically similar lines of *A. thaliana* exhibited detectable similarity in metabolite fingerprint profiles; (2) to assess the sensitivity of metabolite profiles to the impact of environmental variation; and (3) to assess genetic differences in metabolomics environmental responses (GxE)

Methods

Plant material

All accessions used in this study were from laboratory stocks that have previously been genetically characterized and have publicly available 250K SNP data. We selected 12 accessions (9 randomly chosen from to represent the geographic range of the UK and 3 commonly used lab accessions for comparison). Accessions used are shown in Table 15.

Country of Origin	Accessions
USA	Col-0, Col-5
Germany	Nd-1
UK	Asp-1, Edi-1, Hil-1, Igt-1, Laz-1, Rot-1, Sma-1, Ty-0, Unt-1

Table 15 Accessions used

Plants were grown in 1:4 vermiculite and Levington F2 peat free compost (JFC Munro, http://www.jfcmunro.co.uk). Sowings were stratified at 4°C for 3 days before being transferred to the growth chamber or the glasshouse. The controlled growth chamber was maintained under short-day conditions (12hr light, 100-150 µE, 22°C day, 20°C night). In the glasshouse the plants were grown under natural light during the end of March and beginning of April 2014, daylength estimated up to 13hrs light. Individual plants were pricked out after the establishment of their first true leaves.

Preparation of samples for metabolite analysis by LCMS

Plants were grown for approximately five weeks or until they had reached a suitable size for sampling. Five leaves were harvested from each of three plants per accession and immediately frozen in liquid nitrogen keeping each replicate separate. Replicates were systematically sampled between accessions to reduce the impact of circadean effects, replicate one was sampled from each accession then replicate 2 and so on. Samples were freeze-dried for 24 hr and ground to a powder in a tissue lyser for 2 minutes at 25 Hz (Qiagen, http://www.qiagen.com/). As the leaves sampled from different plants varied in size they were weighed for normalisation by mass.

Sample preparation

Metabolite extraction was carried out on ice using 400µl 80% methanol with umbelliferone (7.2 mg/L) as the internal standard. Samples were sonicated and vortexed before removing the supernatant. This process was then repeated this time using 400µl of 50% methanol and the two supernatants combined before filtering through an unsterile 0.22µm syringe filter.

Metabolite profiling

Metabolite profiling was carried out using a QToF 6520 mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. 5 µl of sample extract was run through a Polaris C18 1.8 µm, 2.1 x 250 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). In positive ion mode the mobile phase A was 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. In negative ion mode, mobile phase A was 5% acetonitrile with 1mM ammonium fluoride in water and mobile phase B was 95% acetonitrile.

The gradient applied was 0% B between 0 and 10 min, then 0 to 100% B between 10 and 30 min and 100% B between 30 and 40 min. The flow rate was 0.25 ml

min^{-1} and the column temperature was maintained at 35 °C for the duration. The source conditions for electrospray ionisation were as follows: gas temperature was 325 °C with a drying gas flow rate of 9 l min^{-1} and a nebuliser pressure of 35 psig. The capillary voltage was 3.5 kV in both positive and negative ion mode. The fragmentor voltage was 115 V and skimmer 70 V. Scanning was performed using the autoMS/MS function at 4 scans sec^{-1} for precursor ion surveying and 3 scans sec^{-1} for MS/MS. with a sloped collision energy of 3.5 V/100 Da with an offset of 5V.

Data processing

We used Agilent Masshunter software for feature extraction using parameters as shown in Table 16.

Parameters		Positive Mode	Negative Mode
Adducts :	Consider all Possible Adducts	(+H)	(-H)
		(+Na)	(+Cl)
		(+K)	(+Br)
		(+NH4)	
Charge States	Limit assigned Charge states to a Max of	1	1
	Treat ions with unassigned charge as singly-charged		
Compound Ion Count	Include All		

Table 16 Parameters used for data extraction in MassHunter from Perera, 2011

An in-house data processing algorithm ‘Kernel Feature Alignment’ (Perera, 2011) was used for feature alignment, missing value imputation, data filtering and noise reduction with parameter settings Table 17 . Any feature not present in at least 2 out of 3 replicates was removed.

Parameter	***Value***
RT variance	*0.3*
Mass variance	*10ppm*
Elliptical distribution	*True*
RT range	*3mins- 40 mins*
Ion filtering	*True 2*

Table 17 Alignment parameters for KFA

Distance matrices

We used pairwise distance matrices to infer evolutionary relationships based upon 250K SNP data and untargeted metabolite fingerprinting data. We used distance matrices to avoid any confounding effects of the algorithms behind tree building.

Genetic distance (GD) matrices were calculated by pairwise, accession by accession, comparison following the method of Huff et al., 1993 implemented in R v3.2.1.

Metabolite distance (MD) matrices were calculated by two methods, using binary presence/absence of metabolite features and using abundance data that shows quantities of the metabolite features detected after processing to reduce noise. Using binary presence/absence data the same method could be implemented as for genetic data, whereas Pearson Correlation in R v.3.2.1 was used to calculate a distance matrix for the abundance data.

Col-0 and Col-5 are very closely related being separated only by a single point mutation at gl-1. We therefore calculated the two sets of distance matrices, GD and MD, both with and without the data from Col-5 in order to determine whether this was influencing the results.

Statistical analysis

To determine whether GD and MD matrices were revealing the same evolutionary relationships we performed Mantel tests in R v.3.2.1. based upon 1000 permutations. The null hypothesis of a Mantel test is that no associative relationship exists between the corresponding elements of two matrices.

Core metabolite comparison

To compare the environmental responses of genotypes we compared the relative abundance of common features (the core set) within the two environments. The core set included metabolites present in at least 2 of the 3 biological replicates of all accessions in both environments. The abundance data for the core set was then used to generate a heatmap in Matlab to visualize the differential expression of shared pathways.

Results

Metabolite diversity

Accessions showed extensive variation in chemical composition with the majority of metabolites detected in only a small subset of accessions as shown in Figure 38.

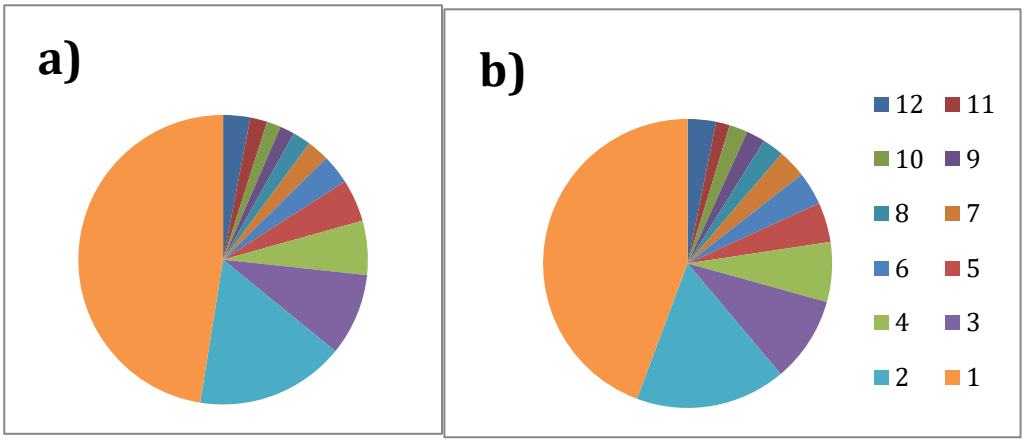

Figure 38 The number of metabolites detected by number of accessions in the Glasshouse (a) and the Growth Room (b).

Association between metabolome distance and phylogenetics

Using all 12 accessions we found significant similarity between GD and MD matrices (Table 18), which implies that metabolite variation had a genetic basis.

Using metabolite abundances we showed a significant relationship between metabolome distance and the underlying genetics in both environments (GH p=0.021*; GR p=0.027*). Whilst using only metabolite presence/absence data the significant relationship was lost in the glasshouse (p=0.053) but enhanced in the more controlled growth room conditions (p=0.005**). The environmental effect was so strong that between the two environments there was no overlap between the metabolite distance matrices for either analysis method.

Matrix 1	Matrix 2	Mantel test results
Genetic Distance	GH-abundance	Mantel statistic r: 0.266 Significance: 0.021
	GR-abundance	Mantel statistic r: 0.331 Significance: 0.027*
	GH-presence/absence	Mantel statistic r: 0.210 Significance: 0.053
	GR-presence/absence	Mantel statistic r: 0.410 Significance: 0.005**
GH-post	GR-post	Mantel statistic r: -0.0001 Significance: 0.490
GH-filter	GR-filter	Mantel statistic r: 0.198 Significance: 0.144

Table 18 Mantel test results comparing all 12 accessions

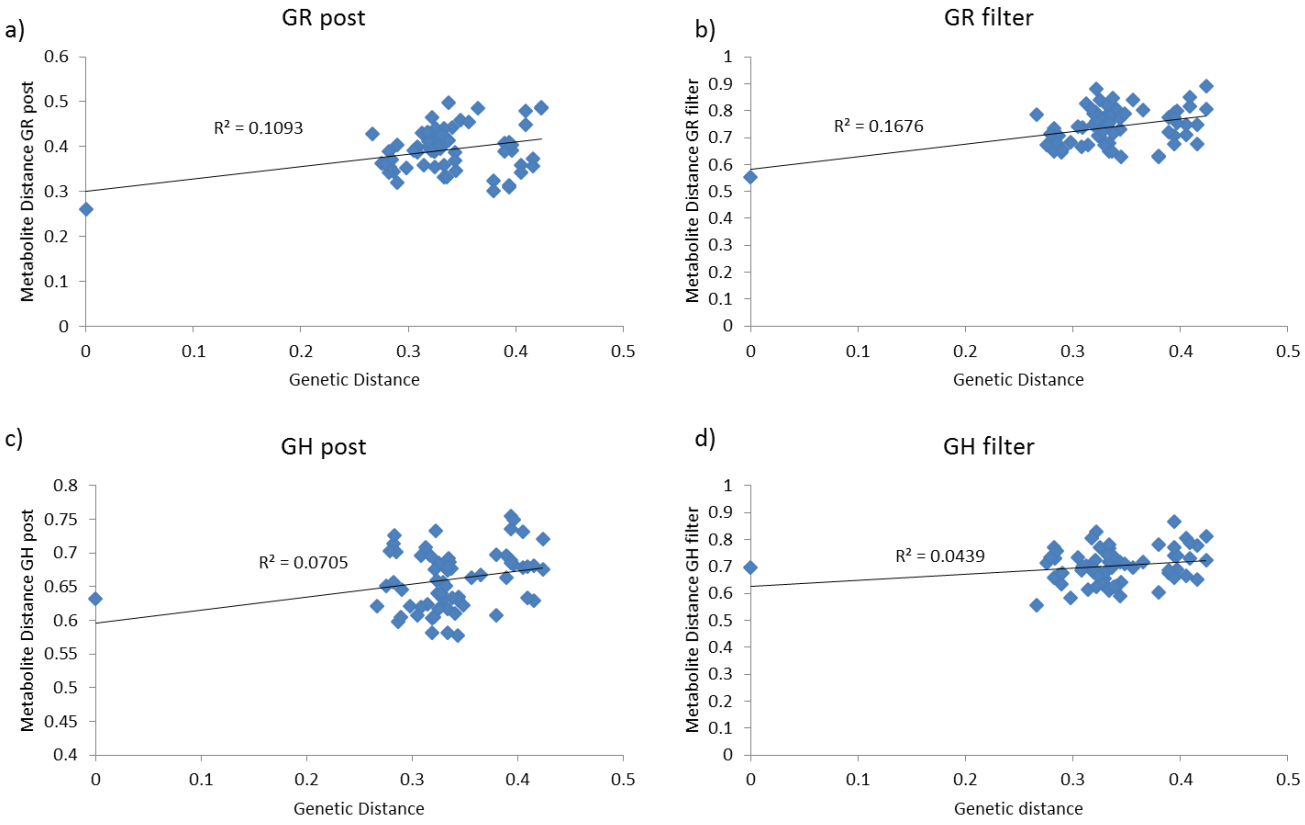

Figure 39 The relationship between genetic and metabolite distance using all 12 accessions. Plots show genetic distance against metabolite distance in: a) growth room using metabolite abundance data, b) growth room using metabolite presence/absence data, c) glasshouse using metabolite abundance data, d) glasshouse using metabolite presence/absence data. Col-0/ Col-5 relationship represented by the plot on 0 genetic distance.

Carrying out the same analyses without Col-5 found no significant relationships between GD and MD matrices as (Table 19). Therefore the close relationship between Col-0 and Col-5 is influencing the previous positive result. The relationship between Col-0 and Col-5 is a particularly interesting one as there is only a single base pair difference between them, which is not detected by the 250K SNP data giving them a genetic distance of 0. However this base pair difference causes dysfunction in the transcription factor GL1 which affects defence responses and trichome development. Trichomes protect plants from herbivory (Sato and Kudoh, 2017) as they are an active mechanosensory switch detecting the presence of herbivores (Zhou et al., 2017) and triggering toxin synthesis. Trichomes produce an array of glucosinolates which protect against

fungi, bacteria and herbivores (Frerigmann et al., 2012). Consequently the metabolite distance of up to 70% in the glasshouse, where pathogens and insects would be expected to be more prevalent than in the growth room, is unsurprising despite these accessions being genetically similar.

However a relationship was detected between the two environments using metabolite presence/absence data $p<0.05$.

Matrix 1	Matrix 2	Mantel test results
Genetic Distance	GH-Post	Mantel statistic r: 0.016 Significance: 0.389
	GR-Post	Mantel statistic r: 0.227 Significance: 0.171
	GH-Filter	Mantel statistic r: -0.008 Significance: 0.461
	GR-Filter	Mantel statistic r: 0.367 Significance: 0.056
GH-Post	GR-Post	Mantel statistic r: 0.039 Significance: 0.387
GH-Filter	GR-Filter	Mantel statistic r: 0.338 Significance: 0.047*

Table 19 Mantel test results excluding Col-5

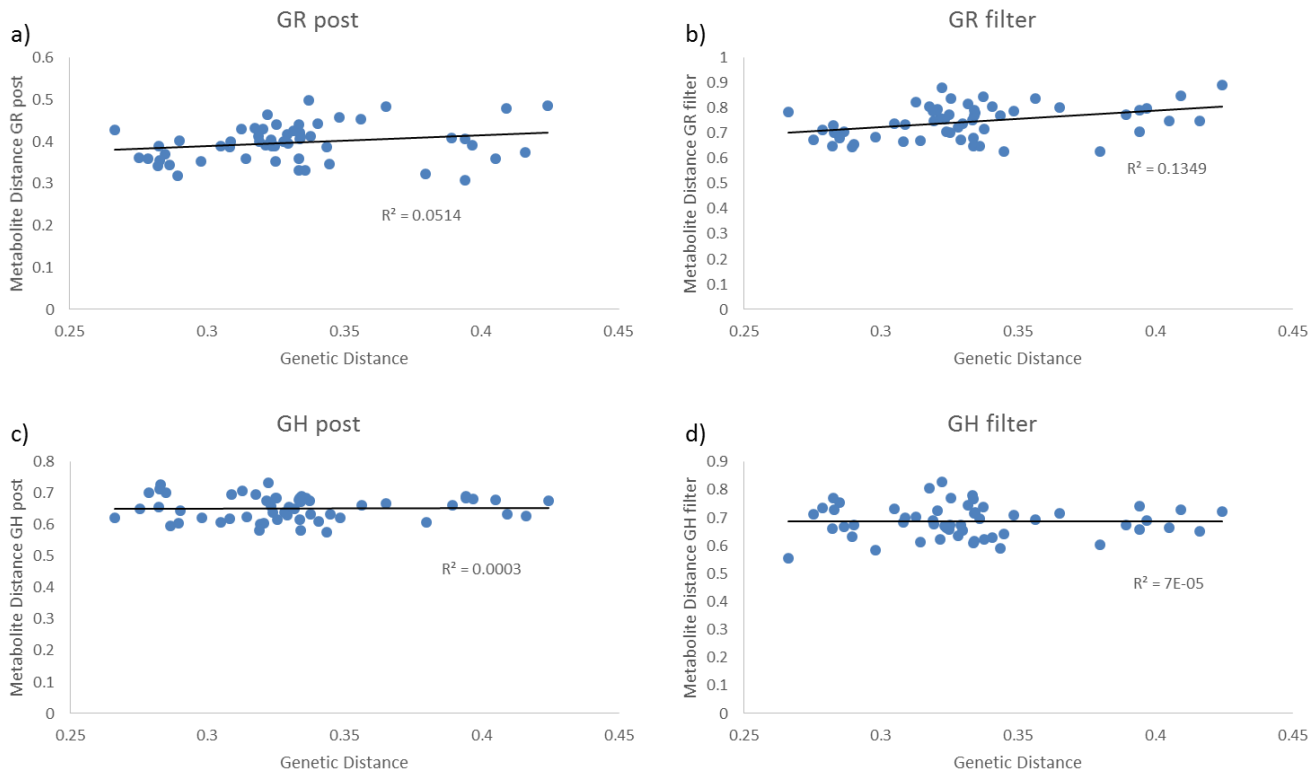

Figure 40 The relationship between genetic and metabolite distance excluding Col-5. Plots show genetic distance against metabolite distance in: a) growth room using metabolite abundance data, b) growth room using metabolite presence/absence data, c) glasshouse using metabolite abundance data, d) glasshouse using metabolite presence/absence data.

The scatterplots of metabolite distance against genetic distance, shown in Figure 39 and Figure 40, are confounded by overrepresentation of the accessions (whilst there are only 12 accessions but multiple datapoints as distance is measured relative to each other accession) however I use them here to simplistically visualise the relationship. When Col-5 is included (Figure 39) there is a weak positive linear trend, where increases in genetic distance produce smaller increases in metabolite distance. When Col-5 is excluded as shown in Figure 40 the trend is lost in the more variable environment of the glasshouse and reduced in the growth room.

Environmental influences on metabolite profile

When the two environments are compared there is only a small core set of metabolites that occur in plants grown both in the glasshouse and the growth room (Figure 41). Unique environmental responses dominate the dataset.

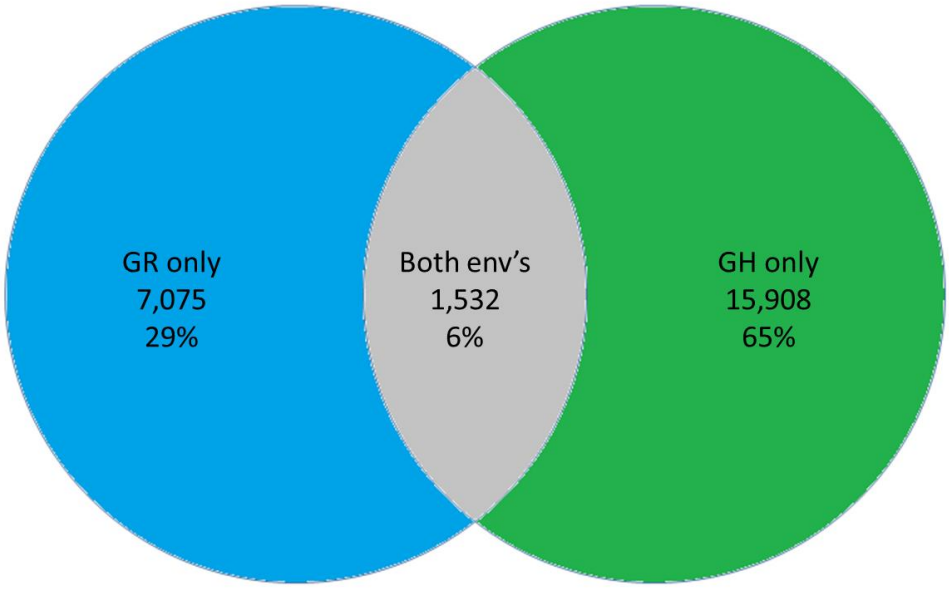

Figure 41 The number of metabolites uniquely detected within or shared between the two environments

The changing abundance of core features shared between environments generated the heatmap in Figure 42. Environment has the greater effect than genetics as the data creates two distinct clusters with the growth room on the left and glasshouse on the right. The clustering of the chemical features down the left hand side of this image is based upon the abundances detected not the underlying chemistries. This clustering is used to infer functional similarity due to response patterns. After the environmental effect accession replicates then cluster together. For ease this is also shown in a dendrogram in Figure 43.

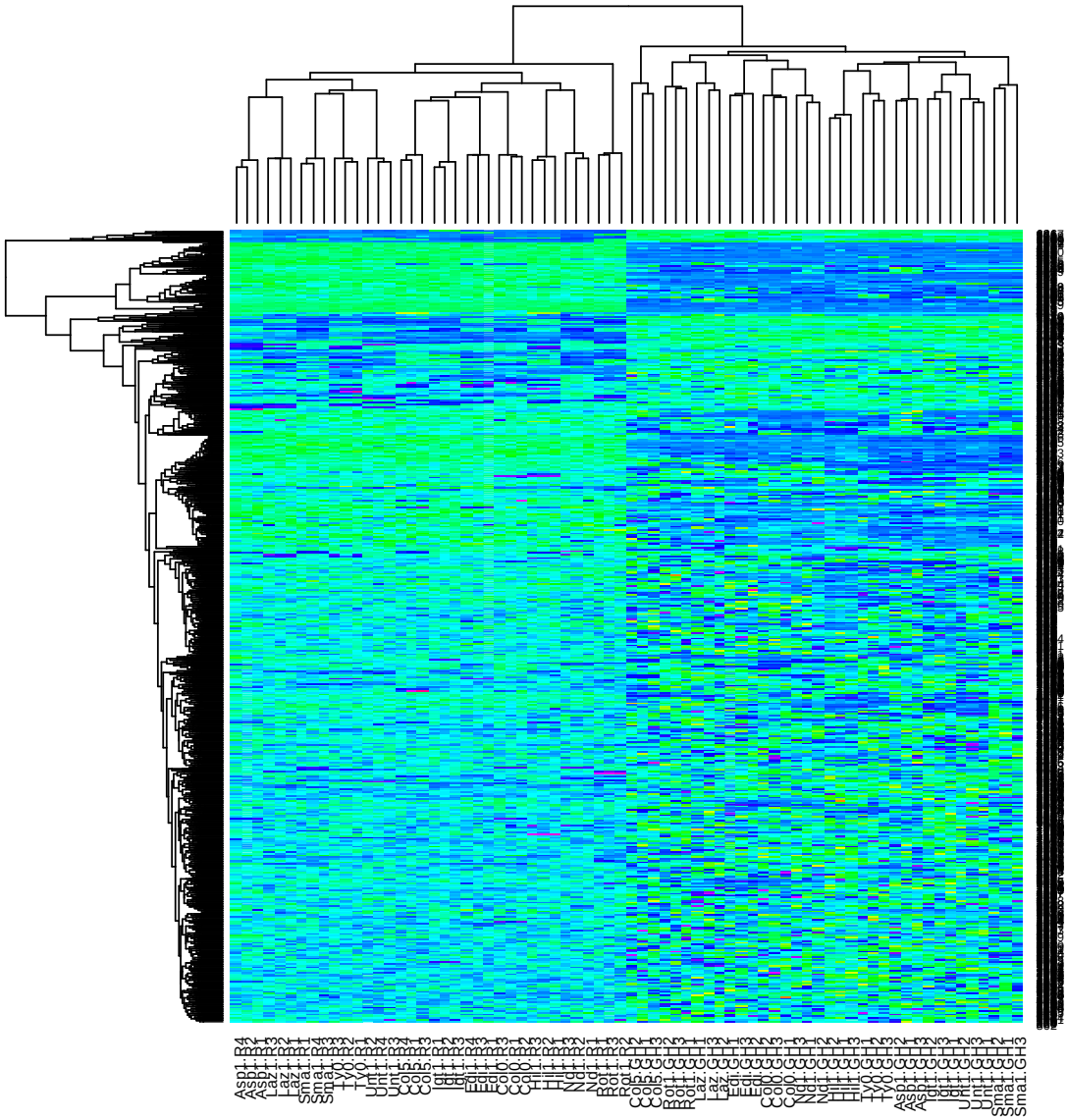

Figure 42 Heatmap of the core features common between the two environments. Accession replicates in columns, metabolite features in rows. Both replicates and metabolite features are clustered by similarity. Produced in R (v.3.2.2). Feature abundance was standardised by dividing by the mean and standard deviation.

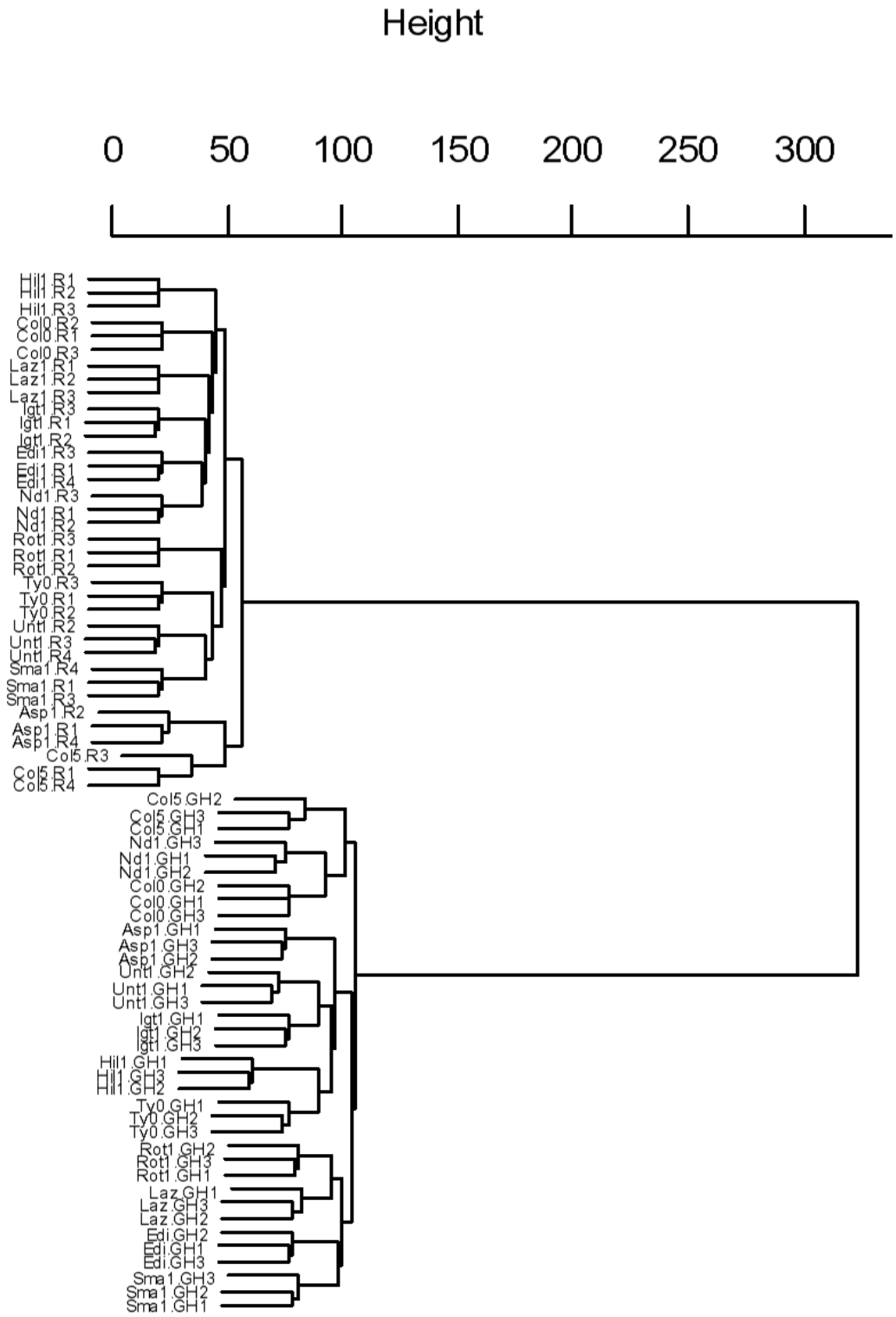

Figure 43 Dendrogram of accession replicates in both environments based upon shared metabolite features. Replicate names ending R denotes Growth Room (top cluster) and GH denotes Glasshouse (lower cluster). Produced in R (v.3.2.2.) Height represents the similarity between branches, the longer the branch the more distant the relationship.

Genetic basis of metabolite profile in *A. thaliana*

Variation in similarity of metabolite profiles of *A. thaliana* is correlated with genetic similarity; essentially, similar genotypes show similar metabolomes, depending upon the methods applied and the accessions chosen. Previously within species, the reconstruction of evolutionary history using untargeted metabolite fingerprinting has not always been successful (Mochida et al., 2009). For *A. thaliana,* no correlation was found to genetic distances based upon low resolution 149 SNP data (Houshyani et al., 2012). Using high resolution 250K SNP data to infer evolutionary relationships for our accessions, we show that the detection of the underlying evolutionary relationships is aided by the inclusion of extremes. Only by including the closely related Col-0 and Col-5 could we detect genetic relatedness using untargeted metabolite profiling. Whilst the metabolomes of our 12 accessions showed extensive variability with almost 50% of features in both environments being detected in only one accession, this was not reflected in the evolutionary relationships, as the Mantel tests without Col-5 found no significant overlap between GD and MD matrices. However unlike Chan et al., (2010) who found no relationship between increasing genetic and metabolite distances, we found some positive correlations between the two. Whilst this could not be linked to precise phylogenetic correspondence, it supports the existence of a link between genetics and the metabolome.

Environmental effects on the metabolome

We found substantial environmental effects on metabolite profiles in *A. thaliana*. Further, the effect of environment exceeded the genetic effects. In our results, this was manifested by the clustering of replicates by environment before accession. Further work could use this dataset to putatively identify the dominant discriminating features between environments (top section of the heatmap Figure 42). The features within the core set are shared between environments across all accessions, the features that cluster at the top of this image are largely unaffected by genetics (as they are expressed at similar levels by all accessions in that

environment) therefore these are core housekeeping features in the environmental response. Further planned experiments include linking plant chemistry to environmental fitness to determine the metabolite features associated with increased yield.

In essence, environmental effects on the phenotype displayed swamped the evolutionary signal. The genetic signal is also likely to become obscured because many of the chemical features detected will be parts of the same pathway. The expression of these features will therefore be influenced by various feedbacks and regulatory mechanisms in the cellular machinery that are environmentally responsive and not governed precisely by variation in a particular genetic sequence. In summary, while plant chemistry is under genetic control, the metabolites expressed and their levels will reflect the environmental exposure of the individual plant, or plasticity, similar to comparing the transcriptome with the genome. The shifts in expression profile reveal the phenotypic plasticity of the organism as it attempts to optimise itself to environmental conditions. Thus, the differences in metabolite profile among accessions will include a substantial element of 'noise' arising from each individual plant's sensitivity to environmental difference, which may even respond to microsite/microclimate variation in the growth chambers.

Almost half the metabolite features detected in either environment were found in only a single accession, which even allowing for noise suggests some level of convergent evolution in metabolite functionality, as there appear to be many alternate chemical responses to similar environmental conditions. Inconsistency in feature detection between biological replicates shows both technical and biological variation. Technical variation exists due to the sensitive nature of the technology. Biological variation encapsulates differences in the growth and development of individual replicates.

In our experiment, genetic variation is static and the metabolite fingerprint is dynamically shifting between replicates and environments. Between environments, the features detected show an overlap of only 6%, these features

will include those involved in fundamental processes of growth and development, although they still exhibit considerable environmental responsiveness, which also suggests adaptive environmental roles. The features uniquely detected in one environment are inferred to be adaptive to the specific biotic and abiotic conditions encountered there. Significantly more features were detected in the glasshouse (17,440) than the growth room (8,607) in keeping with the fact that the glasshouse is a less controlled environment with higher levels of fluctuations in both biotic and abiotic stresses. The difference in metabolite fingerprint between the two environments captures the dynamic action of phenotypic plasticity.

Phenotypic plasticity allows a plant to shape itself to its environment. For a ruderal species such as *A. thaliana* this ability to optimize phenotypic expression based on biotic and abiotic exposure is fundamentally important in the successful colonization of new environments as well as surviving spatial and temporal heterogeneity. The extent of dynamic environmental effects on phenotype that we detected are what makes *A. thaliana* such a successful colonizing species however further work is required to be able to detect the underlying genetics.

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Chapter 7: Final Discussion

The research presented in this thesis explores the interactions between genotype, phenotype and environment. Utilising natural accessions and populations of the model plant species *Arabidopsis thaliana* I have begun to explain how and why genetic and phenotypic diversity are maintained in natural landscapes.

The species that I have studied, *Arabidopsis thaliana*, is the best genetically characterised plant species on the planet (Weigel and Mott, 2009; Alonso-Blanco et al., 2016) and the extensive laboratory investigations of molecular functionality have provided a powerful resource for plant science. However, my research shows that we need to consider the natural context of genotypes to better understand their evolution and function. My experiments have demonstrated that the full range of observable variation may be obscured by the contingencies of any one site because of non-additive effects and ecological interactions. For example, using the resistance locus *Rpm1* as a marker of genetic diversity, I have revealed extensive differentiation within and between wild UK populations of *A. thaliana* (Chapter 2). Screening diversity across the genome would reveal the evolutionary history of these accessions however other studies have shown that *Arabidopsis thaliana* in the UK exhibits the genetic signature of an ancient and gradual colonization (Alonso-Blanco et al., 2016) supporting our findings.

If my results are generalizable and, therefore, wild plant populations contain huge amounts of genetic and phenotypic diversity, then there are important ecological implications. Impending climate change and concurrent increases in extreme weather events will impose disrupting stresses on populations, creating further threats to global food security and conservation efforts. Some scientists consider that we are already experiencing a mass extinction event. Currently, conservationists typically take a somewhat pessimistic view of the future of ecological systems in the face of anthropogenic climate change. In contrast, my work suggests that potentially wild plant populations contain a rich source of

genetic variation that could enable species to adapt, similarly agriculture will need to embrace the genetic diversity present in crop species to determine the optimal varieties across the globe as the climate changes (Wolkovich et al., 2017). To predict how and when climate impacts will be manifested in wild plant communities across the globe, scientists should therefore consider adaptation. Instead of the rigid 'climate envelopes' that are used to model range shifts, we may in future need to model them instead as elastic shapes; the extent of this elasticity will need to be characterised by further studies like mine.

If genotypes are adaptive to a particular and relatively narrow range of environmental conditions, then it should be possible to identify those aspects of their 'home site' conditions that give them a selective advantage. In Chapter 3, I showed that the pattern of diversity across the UK is explained in part by adaptation, which was demonstrated by linking a genotype's performance in a common garden to the climatic conditions of its home-site. This finding challenges the belief that local adaptation and the effects of selection are swamped by genetic drift and founder effects in a highly vagile weed like *A. thaliana*. The implication is that local adaptation can be expected in even ruderals. Further, finding evidence of local adaptation in *A. thaliana* suggests that the species is valuable not only as a model laboratory plant, but also as a model species in plant evolutionary ecology.

My investigations have also begun to provide examples of how genetic diversity is maintained in the UK's gene pool of *A. thaliana*. As explained in Chapter 1, GxE interactions can be a mechanism for maintaining genetic variation by reversing the fitness hierarchy among genotypes in either time or space. For *A. thaliana*, I have demonstrated reversals in fitness hierarchies in both space (Chapter 2), and also over time (Chapter 3). Besides the accumulation of genetic variation in a persistent seed bank, it appears that genetic diversity in *A. thaliana* is likely to sustained for the foreseeable future by habitat and climatic heterogeneity. If extreme weather events increase by climate change, it is possible that these may have a role in sustaining diversity in the species that I

have studied.

In Chapter 2, I also showed that GxE interactions are a possible driver maintaining genetic diversity for pathogen resistance in *A. thaliana*. Resistance to *Pseudomonas syringae* carrying either *avr-Rpm1* or *avr-B* depends upon the presence of the *RPM1* gene in the plant (Grant et al., 1995). Expression of functional resistance proteins has previously been demonstrated to exact a fitness cost (Tian et al., 2003b), which suggests that GxE could be acting at this locus because the functional allele would be disadvantaged in habitats where the pathogen was absent. In Chapter 2, I show, using natural UK accessions in a group-wise study that the longstanding alleles of *RPM1* and *rpm1-null* are selectively neutral in our experiment, which could be attributed to the evolution of ameliorating modifiers that have eroded the fitness cost (Karasov et al., 2017) and so maintained a stable polymorphism for 9.8 million years (Stahl et al., 1999). However, this conclusion is tentative because I cannot rule out the possibility that fitness differences would have been revealed if I had included a wider range of environmental conditions in my experiments (Roux & Bergelson, 2016).

In contrast, genotypes that carried the more recently evolved *rpm1* alleles (Rose et al., 2012), which are expressed but functionally neutral, exhibited significantly lower fitness than *RPM1* genotypes. Whilst *rpm1-null* is a total deletion of the resistant sequence, by contrast the *rpm1* alleles contain a non-functional sequence, which is probably the basis for the fitness differences between these susceptible alleles. By quantitative RT-PCR, I showed that accessions carrying *rpm1* expressed non-functional resistance protein in the presence of the pathogen, which could incur fitness costs by diverting limiting resources from other functions. The novel *rpm1* alleles occur at the highest frequency (52%) in the UK. Taken together, these findings predict that the interacting avirulence genes (*avr-Rpm1* and *avr-B*) should occur at low frequency among pathogens in the UK. My screening of natural fauna in South-West populations supports this hypothesis because only 0.1% of the samples contained *avr-Rpm1* and *avr-B* was not detected at all. It is also possible that these alleles could be being co-

opted for some novel exapted function which we did not consider and this may impact their environmental fitness regardless of pathogen presence (Huibers, 2008). Further work to characterise the natural fauna present on *Arabidopsis thaliana* is needed to determine whether the supposed co-evolutionary battle is actually a drifting relic of ancient history or active in current day warfare. Overall, these findings to date support an adaptive explanation for gene frequencies in both hosts and pathogens.

My observations show that the accessions from UK-wide sampling exhibit a wide range of functional differences in environmentally relevant traits. For example, variation in leaf shape was readily evident as well as variation in other traits such as herbivory, pathogen and drought resistance. In my research, I explored many aspects of the phenotype using both classical ecological methods such as common gardens (Chapters 2 and 3) and laboratory-based response screenings to study other traits such as pathogen resistance (Chapters 4, 5 & 6). I studied environmental effects on phenotypic plasticity by growing the same accessions in two distinct environments, capturing phenotypic differences in fecundity to show G x E in common gardens (Chapter 2) and differences in the metabolic feature space by metabolite fingerprinting (Chapter 6).

Metabolite fingerprinting captures a huge dataset capable of revealing the growth, health and exposure of an organism, as well as aspects of its underlying genetics. Metabolite profiling is used across research fields from the identification of medical disease biomarkers (González-Domínguez, 2017; Carrizo et al., 2017a), and pollutant exposure (Carrizo et al., 2017b) to plant defence responses (Eloh et al., 2016). Understanding the layers of information contained within the metabolite fingerprint can reveal the interaction of genotypes and phenotypes with their environment. Further work to identify discriminating metabolites within and between environments could be linked to fitness and function in field studies, which could ultimately enhance agricultural knowledge and practices.

Arabidopsis thaliana in the UK contains a wealth of genetic and phenotypic

diversity, in line with the evolutionary history revealed by genetic analysis (Alonso-Blanco et al., 2016). The evidence I found for climate adaptation in the UK (Chapter 3) suggests that these accessions would be interesting candidates for genetic analysis to look for the genes responsible (for example by GWA).

Whereas the differences between the experimental environments in Chapter 6 were predominantly in abiotic characteristic, in this thesis I also considered biotic interactions, which are characterised by evolutionary feedback between the co-evolving species. For example, co-evolving host-pathogen interactions are typically considered as an arms race or trench warfare, where co-evolving species vie for supremacy. Range expansions of microbes due to anthropogenic causes (from facilitated dispersal by stock movement or by natural dispersal in response to climate change) can cause devastating plant diseases in naive hosts. To investigate the relative resilience of a host to a novel pathogen vs. an habitual pathogen, I explored both the long-standing natural host-pathogen interaction between *A. thaliana* and *Pseudomonas syringae*, and novel interactions with Xanthomonad xenopathogens (Chapter 4). In effect, studying a plant species response to a xenopathogen allows us to study the host-pathogen 'arms race' from the firing of the starting pistol. My results showed that the diversity in both qualitative and quantitative screenings suggests that *A. thaliana* harbours great potential for rapid evolution in the face of novel threats. I have interpreted these results by invoking the concepts of 'exaptation', or the co-opting of traits to a new or extended function, and cross-resistance – both can help to provide rapid protection for species under novel threats. As before, my results provide a basis for optimism as wild plants are confronted by some of the consequences of climate change. Further work could include improving the large scale screening of phenotypic responses to a more accurate quantification of resistance, for example by thermal imaging or chlorophyll florescence (Rousseau et al., 2013; Mahlein, 2016) due to inaccuracies inherent in visual phenotyping this could reveal a greater range of resistance and tolerance strategies. Further study should also include analysis of the basis of enhanced resistance seen in God-1.

While exaptation provides a means of rapid evolution through pre-adaptation, in Chapter 5 I considered another mechanism for rapid evolution in response to an environmental stress, namely epigenetic inheritance. We are taught that evolution is a slow, stepwise process ultimately dependent upon the accumulation of random mutations. However we now know that an organism's heritable phenotype is not defined only by their genetics. In a single generation, phenotypic plasticity buffers organisms from environmental changes and allows them to optimise their growth to the prevalent conditions. Scientists are increasingly discovering that phenotypic alterations can in some instances be passed to subsequent generations by epigenetic inheritance, allowing rapid evolution. To contribute to this growing awareness, I show (Chapter 5) that epigenetic inheritance adaptively reduces the relative cost of environmental stress (pathogen exposure) across subsequent generations. Accessional variation in the strength of epigenetic rescue from environmental stress suggests that the underlying genetics are still ultimately important in the capacity for this phenotypic response. Given more time this study would be enhanced by additional generations of pathogen exposure to determine if the resistance possible via epigenetic mechanisms is limited and will plateau after only a few generations, or whether sustained exposure would increase resistance. Additionally the offspring could also be grown in a pathogen free environment to see how quickly they revert to parental levels of susceptibility. Molecular analysis to screen for cytosine methylation using bisulfite sequencing could reveal the basis of the epigenetic changes detected (Verhulst et al., 2016; Verhoeven et al., 2016). Ultimately screening wild plants for the presence of similar epigenetic modifications could show the frequency and prevalence of epigenetic responses in the natural setting.

Conclusion

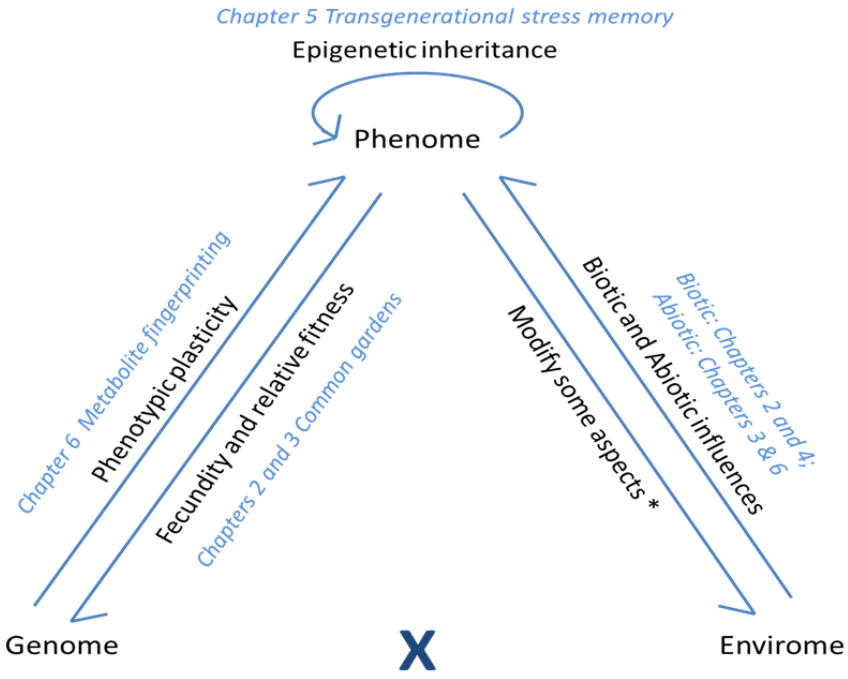

Figure 44 The interactions between genome, phenome and envirome studied in this thesis

The fundamental message of Figure 44 is that there are many sources of phenotypic variation, which can contribute to adaption. My research has made contributions in all sections of the interactions triangle (Figure 44) and has revealed previously undescribed variation and capacity for adaptation in a small ruderal, *A. thaliana*.

Both natural and agricultural systems will have to evolve in order to survive anthropogenic changes, including climate change. My research shows that there are grounds for optimism in expecting resilience to these challenges among wild plants. Indeed, my work begins to show how natural genetic variation could enable persistence of plants in the face of various stressors. In future, GM technology based on this natural variation may help to protect crop species from devastating pathogens. Meanwhile, my work strongly reinforces the principle of conservation of wild species in as many environments as possible in order that

standing stocks of natural genetic diversity can be protected for future prospecting. In future, global food security may depend in part on our guardianship, characterisation and utilisation of the stocks of natural genetic diversity.

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