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Research

Epistasis × environment interactions among *Arabidopsis thaliana* glucosinolate genes impact complex traits and fitness in the field

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Summary

- Despite the growing number of studies showing that genotype × environment and epistatic interactions control fitness, the influences of epistasis × environment interactions on adaptive trait evolution remain largely uncharacterized.
- Across three field trials, we quantified aliphatic glucosinolate (GSL) defense chemistry, leaf damage, and relative fitness using mutant lines of *Arabidopsis thaliana* varying at pairs of causal aliphatic GSL defense genes to test the impact of epistatic and epistasis × environment interactions on adaptive trait variation.
- We found that aliphatic GSL accumulation was primarily influenced by additive and epistatic genetic variation, leaf damage was primarily influenced by environmental variation and relative fitness was primarily influenced by epistasis and epistasis × environment interactions. Epistasis × environment interactions accounted for up to 48% of the relative fitness variation in the field. At a single field site, the impact of epistasis on relative fitness varied significantly over 2 yr, showing that epistasis × environment interactions within a location can be temporally dynamic.
- These results suggest that the environmental dependency of epistasis can profoundly influence the response to selection, shaping the adaptive trajectories of natural populations in complex ways, and deserves further consideration in future evolutionary studies.

Introduction

Understanding the mechanisms controlling adaptive trait evolution is a fundamental objective of evolutionary biology. Accomplishing this broad goal will require elucidating the genotype-phenotype maps within and among populations, including identifying the genes responsible for trait variation and measuring their impact on fitness across environmental conditions. While many studies have successfully mapped ecologically-relevant quantitative trait loci, the underlying genomic regions often harbor tens to hundreds of genes and thus specific causal variants controlling variation remain unidentified (Mitchell-Olds, 1995; Orr, 2001; Maloof, 2003; Koornneef *et al.*, 2004). Furthermore, most adaptive traits have a complex genetic architecture defined by multiple interacting genes (Hayman & Mather, 1955; Weigel & Nordborg, 2005; Mackay, 2014). Epistasis is a fundamental component of the genetic architecture of polygenic traits that occurs when the genes controlling a trait interact such that the phenotypic effect of a gene is nonlinearly dependent on the allelic state of at least one other gene in the genome (i.e. the genetic background). The shapes of evolutionary landscapes depend greatly on the nature of epistatic interactions as well as environmental conditions, which has profound consequences for the evolution of adaptive traits within and across populations (Bateson, 1913; Fisher, 1930; Wright, 1931; Phillips, 2008). Therefore, fully characterizing adaptive trait evolution will require experimental setups that facilitate testing these higher-order interactions across multiple field conditions.

Empirically detecting and characterizing the influence of epistasis within natural populations is often hindered by intrapopulation genetic structure as a result of fixation/loss of alleles and/or linkage disequilibrium among interacting genes. The result is that only a fraction of the potential allelic combinations is actually observable when sampling within a population, obscuring the effects of epistasis on adaptive traits. This problem of population structure can be overcome experimentally by crossing two

divergent parental lines to generate segregating populations of progeny, such as F_2 or recombinant inbred line populations, that introduce additional allelic variation and disrupt linkage disequilibrium through recombination. Utilizing such populations, several empirical studies in recent years have found adaptive traits to be under epistatic control across various taxa (Kim & Rieseberg, 2001; Xing *et al.*, 2002; Kroymann & Mitchell-Olds, 2005; Malmberg *et al.*, 2005; Huang *et al.*, 2012; Taylor & Ehrenreich, 2014; Monnahan & Kelly, 2015). However, the degree to which epistasis accounts for variation in ecologically relevant phenotypic traits is still contested in the literature (Hill *et al.*, 2008; Gerke *et al.*, 2009; da Silva *et al.*, 2010; Huang *et al.*, 2012). This is partly because the conditions under which epistasis evolves in natural systems and its overall importance in adaptive evolution is not well quantified (Fisher, 1930; Wright, 1932; Hansen, 2013; Avila *et al.*, 2014; Maki-Tanila & Hill, 2014).

Furthermore, the expression of adaptive phenotypes often varies greatly across environments. A genotype may be highly fit in one set of environmental conditions but unfit in another. Understanding how intrinsic genetic variation interacts with extrinsic environmental variation to influence trait expression has been the focus of many previous empirical studies (Westerman & Lawrence, 1970; Fry *et al.*, 1998; Juenger *et al.*, 2005). Genotype × environment interactions can impact evolution within populations in complex ways (Links *et al.*, 1973; Holland & Courreges, 1989; Scheiner, 1993). Our understanding of these interactions is primarily limited to the interplay between additive genetic variation and the environment (i.e. G × E). However, as previously stated, numerous studies have shown that nonadditive epistatic interactions are an important contributor to trait variation (Kim & Rieseberg, 2001; Xing *et al.*, 2002; Kroymann & Mitchell-Olds, 2005; Malmberg *et al.*, 2005; Huang *et al.*, 2012; Taylor & Ehrenreich, 2014; Monnahan & Kelly, 2015). While this suggests that interactions between epistasis and the environment (i.e. G × G × E) may play an important role in controlling trait variation, only a handful of empirical studies support this hypothesis (Flynn *et al.*, 2013; Lalic & Elena, 2013; de Vos *et al.*, 2013). In fact, very little is known about how epistasis and the environment interact to influence the evolution of adaptive traits within and across natural populations.

Precisely characterizing how adaptive traits are shaped by additive genetic variation, nonadditive genetic variation, and genetic variation × environment interactions (i.e. G, G × G, G × E and G × G × E) first requires comprehensive knowledge of the identity and regulation of the genes underlying a given trait. An ideal experimental setup would include generating isogenic lines with a common genetic background that harbor different allelic combinations for a defined gene set known to control a trait of interest, growing these lines under various environmental conditions over multiple years in a field setting, and then measuring how the trait varies with genotype and/or environment over time and what impact this has on fitness. Well-described functional molecular pathways are a prerequisite for generating the isogenic lines necessary to study the role of G × G, G × E and G × G × E in adaptive trait evolution. The paucity of systems with cloned and validated genes known to control natural variation in an adaptive trait presents a major hurdle to this endeavor. To date, the few empirical studies investigating the impact of G × G, G × E, and G × G × E interactions between specific genes on the expression of adaptive traits under various environmental conditions have all been conducted in the laboratory (Flynn *et al.*, 2013; Lalic & Elena, 2013; de Vos *et al.*, 2013).

The aliphatic glucosinolate (GSL) defense pathway of *Arabidopsis thaliana* (Arabidopsis) is an excellent model system for exploring the role of epistasis and the environment in adaptive evolution. Across the native range, Arabidopsis populations exhibit high levels of standing variation in their aliphatic GSL profiles, a multivariate phenotype characterized by the presence and relative abundance of the myriad structures within this class of plant-made compounds (Fig. 1) (Mithen & Campos, 1996; Kliebenstein *et al.*, 2001a, 2005; Halkier & Gershenzon, 2006; Sønderby *et al.*, 2007; Chan *et al.*, 2011). Decades of work have led to the identification and validation of the major causal polymorphic genes that control much of the natural aliphatic GSL profile variation observed across Arabidopsis accessions (Halkier & Gershenzon, 2006; Sonderby *et al.*, 2010). In the laboratory, these naturally polymorphic genes have been shown to act both additively and epistatically to produce a diverse array of aliphatic GSL profiles (Kliebenstein *et al.*, 2001b; Li & Quiros, 2003; Hansen *et al.*, 2007, 2008; Hirai *et al.*, 2007; Sønderby *et al.*, 2007, 2010; Li *et al.*, 2008). However, only a fraction of the aliphatic GSL profiles observable in the laboratory have been found among the hundreds of Arabidopsis accessions that have been screened. Certain aliphatic GSL profile types are overrepresented among accessions while others are underrepresented, suggesting that selection has played a role in shaping the phenotypic variation observed in nature (Chan *et al.*, 2010; Brachi *et al.*, 2015; Kerwin *et al.*, 2015). Previous work has linked aliphatic GSL genotype with fitness variation in the field, supporting the idea that these genes are adaptive (Bidart-Bouzat & Kliebenstein, 2008; Kerwin *et al.*, 2015). However, the relative importance of additive vs epistatic effects of specific GSL genes, and the potential interaction with the environment have not previously been assessed in the field.

Arabidopsis employs its GSL defense chemistry against numerous plant pests, including generalists that feed on many different plant species and specialists that prefer GSL-containing crucifers (Giamoustaris & Mithen, 1995; Raybould & Moyes, 2001). Differences in amounts of herbivory and plant fitness that are dependent on the genetically controlled GSL profile as well as the environment, such as species of pests present (i.e. G × E interactions), have been observed in both laboratory and field studies (Giamoustaris & Mithen, 1995; Stowe, 1998; Raybould & Moyes, 2001; Bidart-Bouzat & Kliebenstein, 2008; Hansen *et al.*, 2008). Given that herbivory and plant fitness are dependent on G × E interactions, and GSL profile variation is influenced by epistasis (G × G) between aliphatic GSL genes, it follows that there could be measurable interactions between epistatic and environmental factors (i.e. G × G × E) controlling aliphatic GSL profile, amount of herbivory and/or plant fitness in the field. Quantifying the influence of G × G × E interactions on ecologically important traits in the field is necessary to refine

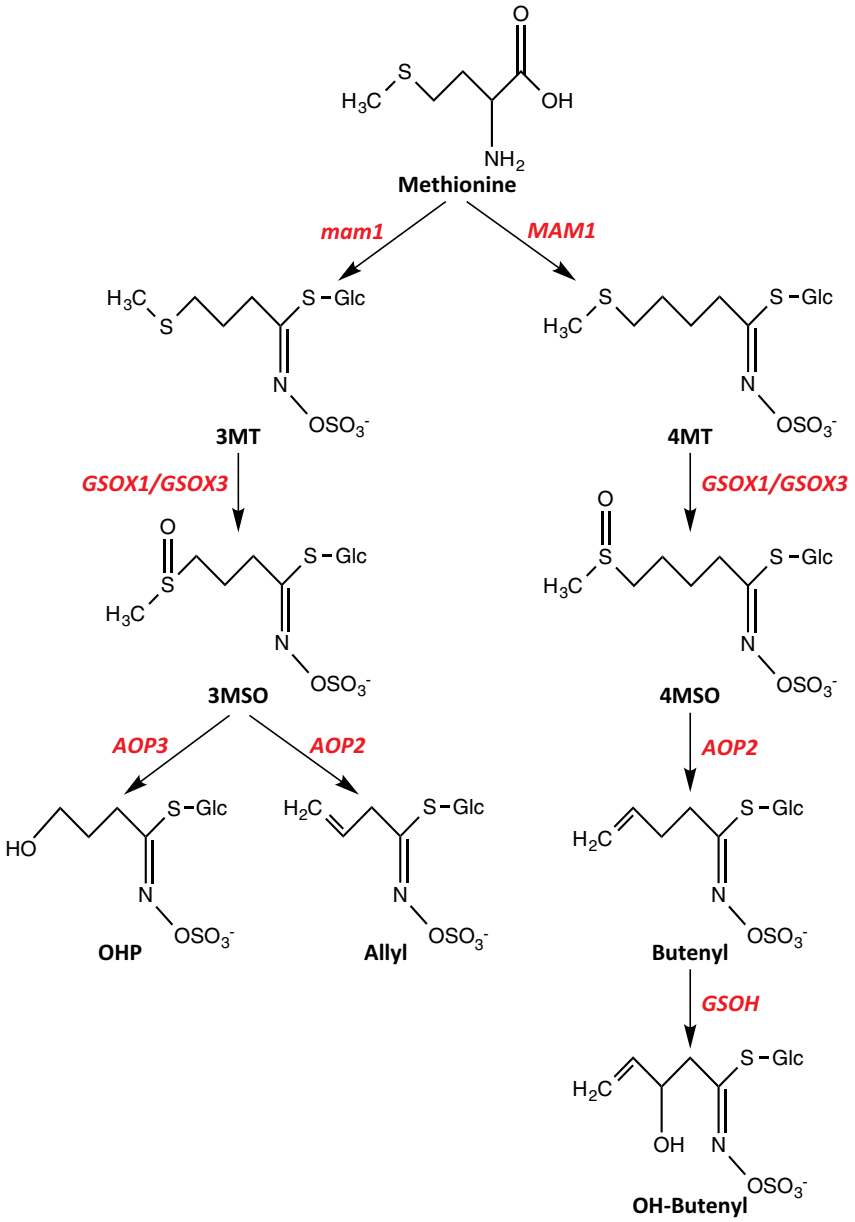

Fig. 1 Overview of aliphatic glucosinolate (GSL) biosynthesis in *Arabidopsis thaliana* (Arabidopsis). Arrows represent enzymatic steps in aliphatic GSL biosynthetic pathway that are genetically variable within Arabidopsis. The names of causal genes underlying enzymatic function are listed next to arrows in red italicized font. Names/acronyms of compounds utilized and/or produced by each reaction are listed below the molecule structures. 3MT, 3-methylthio glucosinolate; 4MT, 4-methylthio glucosinolate; 3MSO, 3-methylsulfinyl glucosinolate; 4MSO, 4-methylsulfinyl glucosinolate; OHP, 3-hydroxy-propyl glucosinolate; Ally, 2-propenyl glucosinolate; butenyl, 3-butenyl glucosinolate; OH-butenyl, 2-hydroxy-but-3-enyl glucosinolate. Aliphatic GSL biosynthesis begins with an amino acid precursor, predominantly methionine in Arabidopsis. During initial chain elongation, carbons are added to methionine through a series of reactions to produce an elongated amino acid. Variation at the Elong locus, which contains *MAM1*, determines the side-chain length of the final GSL molecule. A functional *MAM1* allele at this locus leads to production of primarily four carbon (4C) length aliphatic GSL molecules, whereas a nonfunctional *mam1* allele leads to accumulation of three carbon (3C) length aliphatic GSL molecules. Once elongated, the amino acid precursor is subsequently converted into a GSL via the core pathway (not shown). The GSL molecules produced via the core pathway subsequently undergo a series of side-chain modifications, resulting in a suite of potential GSL compounds. Side-chain modification in Arabidopsis is controlled by variation at *GSOX1*, *GSOX3*, *AOP2*, *AOP3* and *GSOH*. *GSOX1* and *GSOX3* are flavin-monooxygenases that catalyze the *S*-oxygenation of 3MT and 4MT to 3MSO and 4MSO, respectively. *AOP2* and *AOP3* encode 2-oxoglutarate-dependent dioxygenases. *AOP2* converts 3MSO and 4MSO to allyl and butenyl, respectively. *AOP3*, on the other hand, only converts only 3MSO to OHP and does not act on 4MSO. *GSOH* encodes a 2-oxoacid-dependent dioxygenase that converts butenyl to OH-butenyl. A functional *AOP2* is required for *GSOH* functionality because it lies directly upstream of *GSOH* in the biosynthetic pathway and produces its substrate. Therefore, *AOP2* is (functionally) epistatic to *GSOH*. As an example, the wild-type Arabidopsis genotype, Columbia (Col-0), which carries functional alleles at *MAM1*, *GSOX1* and *GSOX3*, nonfunctional alleles at *AOP2* and *AOP3*, and a functional allele at *GSOH*, primarily accumulates 4MSO and does not produce OHP, allyl, butenyl or OH-butenyl GSL. Also not shown are *MYB28* and *MYB29*, two R2R3 MYB transcription factors necessary for aliphatic GSL biosynthesis. A single knockout in either gene leads to a partial reduction in overall aliphatic GSL accumulation, while a double knockout results in nearly complete loss of aliphatic GSL accumulation in the plant.

our models of the relationship between genetic variation (both additive and epistatic) and phenotypic variation across environmental conditions. Empirically elucidating these genotype-phenotype maps under field conditions is critically important to further our understanding of how adaptive traits evolve in nature.

The goal of this study was to measure the effects of epistasis (G × G) and epistasis × environment interactions (G × G × E) on ecologically important traits in a field setting, using the aliphatic GSL defense metabolite pathway in Arabidopsis as a model. To enable this work, we utilized previously collected data from a multi-year field trial conducted on a population of Arabidopsis mutants that vary only at specific GSL genes but are otherwise genetically homogeneous (i.e. share the same genetic background) (Kerwin *et al.*, 2015). In this study, we focused on a subset of 12 aliphatic GSL genotypes from the full Arabidopsis mutant population which were organized into six epistatic interaction groups that each sample all four haplotype combinations for a different pair of aliphatic GSL genes (i.e. AABB, aaBB, AAbb and aabb) (Table 1). We found that additive genetic variation and epistasis (G × G) between pairs of GSL genes had a large impact on aliphatic GSL accumulation, and the environment (E) was largely responsible for variation in leaf damage, while epistasis (G × G) and epistasis × environment interactions (G × G × E) had the largest impact on relative fitness in the field.

Materials and Methods

Epistatic interaction groups

Examples of epistasis (G × G) between genes in the aliphatic GSL pathway controlling GSL chemical profile have been well characterized in laboratory studies of Arabidopsis (Sønderby *et al.*, 2007; Hansen *et al.*, 2008). In this study, our goal was to validate previously identified G × G interactions found to control aliphatic GSL production in the laboratory, and to test for G × G between additional GSL genes in the field. To this end, we focused on a set of 12 genotypes from a laboratory population of synthetic Arabidopsis mutants that vary only at specific causal aliphatic GSL genes and otherwise all share the Columbia (Col-0) accession genetic background. These genotypes were chosen because they fell into one of six pairwise interaction groups that allowed us to test directly the epistatic (G × G) effects of different aliphatic GSL genes on various phenotypes previously measured in the field (Kerwin *et al.*, 2015). Each interaction group consists of four genotypes that sample all homozygous allele combinations for a pair of aliphatic GSL genes (Table 1).

Experimental field setup

Field trials were conducted in two locations, the University of California-Davis (UCD) in Davis, CA, USA and the University of Wyoming (UWY) in Laramie, WY, USA, over three field seasons. The three field trials are referred to as three environments in our statistical models. The first field trial was performed at UWY during summer 2011 (UWY2011), the second at UCD in spring 2012 (UCD2012), and the third at UWY during summer 2012 (UWY2012). Across the three field trials, experimental plants were organized into 120 blocks total. Each block contained a single replicate of all of the genotypes in a randomized position (Supporting Information Fig. S1). Each field trial location contained 40 blocks, half of which were treated with pesticides every 14 d, to decrease foliar herbivory. At UWY, plants were sprayed with the insecticide Sevin (GardenTech, Palatine, IL, USA) to repel flea beetles, the dominant pest in the area. At UCD, plants were treated with Marathon 1% granular (OHP, Mainland, PA, USA) and Lily Miller Slug, Snail & Insect Killer Bait (Lily Miller Brands, Walnut Creek, CA, USA) to repel a variety of pests. As a given block experienced only one insecticide treatment condition and one field trial location, the field trial setup has a split-plot design (see the 'Statistical analyses' section for more details).

Plant growth and harvest

Arabidopsis seeds were sown into 50-celled flats containing two inch circular net pots filled with Sunshine Mix #5 (Sungro, Agawam, MA, USA) potting soil formulated with slow-release fertilizer. Seeds were stratified at 4°C for 4 d to facilitate germination synchrony before being transferred to the glasshouse at either UWY or UCD. Germinating in the glasshouse served to minimize variation in the initial seedling conditions. In the UWY

Table 1 Pairwise epistatic interaction groups identified from a synthetic laboratory population of aliphatic glucosinolate (GSL) mutants in *Arabidopsis thaliana*

Alleles		Interaction group					
Gene 1	Gene 2	MYB28 × MYB29	AOP2 × GSOH	MAM1 × MYB28	MAM1 × MYB29	MYB28 × GSOH	MYB28 × AOP2
+	+	Col-0	*AOP2*	Col-0	Col-0	Col-0	*AOP2*
–	+	*myb28*	Col-0	*gsm1*	*gsm1*	*myb28*	*myb28/AOP2*
+	–	*myb29*	*AOP2/gsoh*	*myb28*	*myb29*	*gsoh*	Col-0
–	–	*myb28/myb29*	*gsoh*	*myb28/gsm1*	*myb29/gsm1*	*myb28/gsoh*	*myb28*

Each interaction group is composed of four genotypes representing all homozygous haplotype combinations for a different pair of aliphatic GSL genes in *A.s thaliana* (Arabidopsis) accession Columbia (Col-0). The genotypes in each interaction group contain the allele combinations listed on the left. Gene 1 and gene 2 correspond to the first and second genes in the interaction group name, respectively. '+' indicates a functional allele and '–' indicates a non-functional allele. Double mutants were generated by manually crossing two single mutants of Col-0 each harboring a loss-of-function or gain-of-function allele of one of the aliphatic GSL genes in the interaction group pair.

glasshouse, plants were grown under a 15 : 9 h, light : dark natural photoperiod with temperatures fluctuating diurnally from 10 to 30°C. In the UCD glasshouse, plants were grown under a 14 : 10 h, light: dark natural photoperiod with temperatures fluctuating from 15 to 35°C. Upon germination in the glasshouse, seedlings were thinned to one individual per pot and genotypes were organized into blocks that each contained a single replicate of all genotypes in a randomized position. Two weeks after germination, seedlings were transplanted directly into the field soil in their net pots. The pots were perforated with 4 mm^2 openings to allow roots to grow freely into the field soil, once they outgrew the pots. Transplanted seedlings were grown in the field 4–5 wk before being harvested. Upon harvest, the aerial portion of the plant was collected into a quart-sized freezer bag and transferred to 4°C until all field plants were harvested. After harvest, the UCD2012 field plants were placed into −80°C for long-term storage. The UWY field plants were shipped to UC Davis overnight on dry ice and then placed in −80°C for long-term storage.

GSL extraction, high-performance liquid chromatography (HPLC) separation and GSL structure identification

Glucosinolate content was measured on all field trial plants to assess the environmental effects on aliphatic GSL accumulation across all the genotypes. A single fully expanded, green leaf was collected from 4-wk-old field plants for GSL extraction. To account for variation in size, we measured the area of each leaf collected from the field as follows. First, leaves from 12 plants at a time were harvested onto a white sheet of paper with a grid overlay. Next, a ruler was placed on the sheet of paper and a digital image was captured using a Nikon D3100 digital single-lens reflex (DSLR) camera (Nikon, Tokyo, Japan). Finally, the photographed leaves were transferred into separate wells of 96 deep well plates containing 400 µl 90% methanol and stored in the freezer until extraction. For the UWY field trial, leaves were stored at −20°C for 3–4 wk before being shipped overnight to Davis, CA, on dry ice where they were stored at −20°C until extraction. For the UCD2012 field trial, all plates were stored at −20°C until extraction. After harvest, desulfoglucosinolates were extracted from all samples using a high-throughput protocol (Kliebenstein *et al.*, 2001c). One gram of Sephadex DEAE A-25 (Sigma-Aldrich) was added to each well of a 96-well filter plate using a column loader. To hydrate the Sephadex, 300 µl of H_2O was transferred to each well using a multichannel pipet, and the plate was then incubated at room temperature for 1 h. Excess H_2O was removed from the Sephadex by placing the filter plate on top of a 96 deep well discard plate used to catch the flow through then centrifuged at 175 ***g*** for 2 min. To extract all organic compounds, 96 deep well plates containing a single *Arabidopsis thaliana* leaf, two 2.3 mm ball bearings and 400 µl of 90% methanol in each well were homogenized for 3–5 min in a Harbil 5-Gallon Mixer (Fluid Management Co., Wheeling, IL, USA). Plates were centrifuged at 2750 ***g*** for 20 min. To bind GSL to Sephadex, 150 µl of supernatant from each well (containing the extracted organic compounds) was transferred to the corresponding well of the 96-well filter plate containing hydrated Sephadex and centrifuged at 250 ***g*** for 3 min on top of the 96 deep well discard plate. To wash away all the nonbinding organic compounds from the Sephadex, 150 µl of 90% methanol was added to each well and the plate was centrifuged at 250 ***g*** for 3 min. To remove excess methanol, two wash steps were conducted by adding 150 µl of H_2O to the plate followed by centrifugation at 250 ***g*** for 3 min. To release the GSL compounds from the Sephadex binding agent, 10 µl of Sulfatase (Sigma-Aldrich) and 100 µl of water were added to each well of the 96-well filter plate and then incubated overnight at room temperature in the dark. This reaction cleaves the glucose-sulfate bond, releasing desulfoglucosinolates from the Sephadex. Desulfoglucosinolates were then eluted into a 96-well round bottom plate by centrifugation at 250 ***g*** for 3 min. For each sample, 50 µl of the 110 µl of extract was injected on an Agilent 1100 HPLC (Agilent, Santa Clara, CA, USA) using a Lichrocart 250–4 RP18e column (Hewlett-Packard, Palo Alto, CA, USA). Individual GSL compounds were detected at 229 nm and separated utilizing the following program with an aqueous acetonitrile gradient: a 6 min gradient from 1.5% to 5.0% (v/v) acetonitrile, followed by a 2 min gradient from 5% to 7% (v/v) acetonitrile, a 7 min gradient from 7% to 25% (v/v) acetonitrile, a 2 min gradient from 25% to 92% (v/v) acetonitrile, 6 min at 92% (v/v) acetonitrile, a 1 min gradient from 92% to 1.5% (v/v) acetonitrile, and a final 5 min at 1.5% (v/v) acetonitrile (Kliebenstein *et al.*, 2001c). For each peak, the GSL structure was determined by comparing the retention time and UV absorption spectrum with known standards. The integral under each peak was automatically calculated and this value in milli-absorption units was converted to pmol mm^{-2} tissue using response factor slopes determined from purified standards and area of leaf tissue used per sample as measured by ImageJ (Kliebenstein *et al.*, 2001c; Reichelt *et al.*, 2002).

Leaf damage estimates

Leaf damage was assessed visually in the field on 4-wk-old plants the day before tissue was collected for GSL extraction. A scale from 0 to 10 was used to determine the amount of leaf tissue removed from each plant, with 0 representing no damage and 10 representing complete destruction of the plant (i.e. aerial portion of the plant completely eaten).

Absolute and relative fitness estimates

To determine whether GSL genotype alters plant fitness in the field, we measured the fecundity of each individual grown across all three environments. After harvest, total fruit count (TFC) was estimated for each field plant as the total number of fruits, flowers and buds present. As Arabidopsis reproduces through self-fertilization, fruit set is a measure of both male and female fitness effects. In addition, total number of seeds per plant is highly correlated with total number of fruits (Mauricio *et al.*, 1997).

Absolute fitness was calculated as TFC × survival. Plants that survived to harvest were given a survival score of 1. Plants that germinated and were transplanted into the field but did not survive to harvest were given a score of 0. Individuals that did not germinate or did not survive to transplantation were scored as missing data in statistical analyses. Relative fitness was calculated separately for each interaction group as absolute fitness of an individual divided by average fitness of the interaction group. The transformed data were used in the statistical analyses conducted for this study (see the next section).

Statistical analyses

To investigate potential epistatic (G × G) and epistasis × environment (G × G × E) interactions between pairs of GSL genes, we conducted linear mixed-model analyses using a restricted maximum likelihood (REML) approach on our field traits, including GSL profile, leaf damage and relative fitness, for each interaction group across the three separate field trials (i.e. environments). The fixed effects in this model were gene 1, gene 2, and environment, and the random factor was block. Gene 1 and gene 2 refer to the binary allelic states of the respective aliphatic GSL genes in a pair defined by epistatic interaction group (Table 1). Environment refers to the field trial site-year and has three levels: UWY2011, UCD2012, and UWY2012. The field trial setup had a split plot design with randomized complete blocks that contain a single replicate of all of the genotypes in a randomized position (Fig. S1). Each block was found in only one environment and therefore block is nested within environment. In a previous study, we showed that insecticide application did not significantly affect any of the measured phenotypes (Kerwin *et al.*, 2015). Further, in this study we were specifically interested in measuring the impact of epistasis (G × G), the environment (E) and the interaction of epistasis and the environment (G × G × E) on our field traits. Therefore, we did not include treatment as a fixed effect in our statistical analyses. We used the following formula (written in its fully expanded form) to fit this model using the LMER() function from the LME4 package in R (Baayen *et al.*, 2008):

```
lmer(Trait ~ Gene 1 + Gene 2 + Environment + Gene 1
  : Gene 2 + Gene 1 : Environment + Gene 2
  : Environment + Gene 1 : Gene 2
  : Environment + (1|Environment:Blk)).
```

The ANOVA() function from the LMERTEST package in R was utilized to generate sums of squares (SS) values and *F* statistic for each fixed effect trait and determine which variables significantly altered the mean of each trait ($P \leq 0.05$), using Satterthwaite's approximation to calculate denominator degrees of freedom (Kuznetsova *et al.*, 2016). The RAND() function from LMERTEST was used to generate the chi-squared (χ^2) statistic and significance terms for the random effect term in the model (Kuznetsova *et al.*, 2016). To account for multiple hypothesis testing, *P*-values were adjusted with the P.ADJUST() function from the R base package using the false discovery rate method (R Development Core Team, 2015). To assess whether the mixed-model normality assumption was violated, a quantile–quantile (Q–Q) plot of the observed residuals vs theoretical residuals, and a histogram of the observed residuals were generated from each model using the QQNORM() and HIST() functions. Based on visual inspection of these plots, we determined that the residuals from each of the field traits showed a skewed or heavily tailed distribution. To address this, the data were transformed, so that the residuals showed a more normal distribution. Leaf damage and total aliphatic GSLs were natural log ($\log_e$)-transformed. TFC was square-root-transformed and the transformed values were used to calculate absolute and relative fitness. To determine whether these transformations caused the homogeneity of variance assumption of mixed-model testing to be violated, we used the transformed data points to generate scatterplots of leaf damage, total aliphatic GSL and relative fitness for each genotype and environment across the six interaction groups. Based on visual inspection of the spread of the data across the genotypes and environments, we determined that the homogeneity of variance assumption was met (i.e. the data were largely homoscedastic). Results from the mixed-model analyses are summarized in Tables 2–4.

Group means estimates

To generate the values used in the interaction plots (Figs 2–4), population means were calculated from the transformed values of each field trait across all genotype × environment groupings using the LSMEANS() function from the DOBY package in R (Højsgaard *et al.*, 2014). These values were then back-transformed to generate figures.

Proportion of variation explained (eta-squared, η^2) estimates

To quantify the proportion of trait variation accounted for by the different factors in our models, we calculated η^2 for each phenotype tested. η^2 was defined as the proportion of the SS variance explained by each factor in the mixed models, including the residual error term. Larger η^2 values indicate that a given factor or source of variation explains a greater proportion of phenotypic variation compared with a factor in the model with a smaller η^2. These values are reported as percentages in the mixed-model summary tables (Tables 2–4).

Results

Aliphatic GSL accumulation is primarily influenced by additive and epistatic (G × G) genetic variation

Aliphatic GSL biosynthetic pathway genes act both additively and epistatically to impact GSL profile in the plant (Sønderby *et al.*, 2007; Hansen *et al.*, 2008) (Fig. 1). A well-studied aspect of GSL pathway regulation involves two transcription factors, *MYB28* and *MYB29*, that together control natural variation in foliar aliphatic GSL accumulation in the plant (Sønderby *et al.*,

Table 2 Control of aliphatic glucosinolate (GSL) accumulation variation in the field

Source of variation	d.f.	SS	*F*/ChiSq	*P*-value		η^2 (%)
MYB28 × MYB29 interaction group						
MYB28	1	504.3	1546.7	0.00	**	57.7
MYB29	1	229.5	703.9	0.00	**	26.3
Environment	2	5.1	7.8	8.9E-04	**	0.6
MYB28:MYB29	1	124.2	380.9	0.00	**	14.2
MYB28:environment	2	6.2	9.5	1.7E-04	**	0.7
MYB29:environment	2	1.3	2.1	0.13		0.2
MYB28:MYB29: environment	2	3.3	5.0	0.01	*	0.4
Environment:blk	1	0.1	19.2	2.3E-05	**	0.0
Residual	345	0.3	na	na		0.0
AOP2 × GSOH interaction group						
AOP2	1	2.1	7.5	0.02	*	22.5
GSOH	1	0.0	0.2	0.68		0.5
Environment	2	3.7	6.7	0.02	*	40.1
AOP2:GSOH	1	0.8	2.9	0.14		8.7
AOP2:environment	2	1.5	2.7	0.14		16.0
GSOH:environment	2	0.5	0.9	0.46		5.5
AOP2:GSOH: environment	2	0.6	1.0	0.46		6.2
Environment:blk	1	0.0	8.4	0.02	*	0.5
Residual	330	0.3	na	na		3.0
MAM1 × MYB28 interaction group						
MAM1	1	29.1	110.9	0.00	**	14.3
MYB28	1	155.4	593.4	0.00	**	76.2
Environment	2	8.4	16.1	1.6E-06	**	4.1
MAM1:MYB28	1	2.4	9.0	3.9E-03	**	1.2
MAM1:environment	2	2.1	4.0	0.02	*	1.0
MYB28:environment	2	4.4	8.4	4.6E-04	**	2.2
MAM1:MYB28: environment	2	1.7	3.3	0.04	*	0.9
Environment:blk	1	0.1	37.0	3.1E-09	**	0.1
Residual	330	0.3	na	na		0.1
MAM1 × MYB29 interaction group						
MAM1	1	1.4	4.9	0.04	*	7.5
MYB29	1	3.1	10.8	3.1E-03	**	16.4
Environment	2	2.0	3.4	0.04	*	10.4
MAM1:MYB29	1	7.0	24.0	1.4E-05	**	36.6
MAM1:environment	2	2.4	4.2	0.03	*	12.7
MYB29:environment	2	0.5	0.9	0.39		2.9
MAM1:MYB29: environment	2	2.5	4.3	0.03	*	13.1
Environment:blk	1	0.1	19.4	4.1E-05	**	0.4
Residual	327	0.3	na	na		1.5
MYB28 × GSOH interaction group						
MYB28	1	56.6	172.0	0.00	**	66.8
GSOH	1	3.6	11.1	2.0E-03	**	4.3
Environment	2	5.1	7.7	2.0E-03	**	6.0
MYB28:GSOH	1	11.1	33.7	7.9E-08	**	13.1
MYB28:environment	2	3.6	5.5	0.01	*	4.3
GSOH:environment	2	3.1	4.8	0.01	*	3.7
MYB28:GSOH: environment	2	1.6	2.4	0.09	.	1.9
Environment:blk	1	0.1	6.6	0.01	*	0.1
Residual	312	0.3	na	na		0.4
MYB28 × AOP2 interaction group						
MYB28	1	61.8	223.6	0.00	**	64.0
AOP2	1	5.5	19.9	2.8E-05	**	5.7
Environment	2	1.6	2.9	0.07	**	1.6
MYB28:AOP2	1	4.6	16.8	9.3E-05	**	4.8
MYB28:environment	2	0.3	0.5	0.61		0.3
AOP2:environment	2	13.3	24.1	1.7E-09	**	13.8
MYB28:AOP2: environment	2	9.3	16.8	3.9E-07	**	9.6
Environment:blk	1	0.1	14.7	1.7E-04	**	0.1
Residual	269	0.3	na	na		0.3

Linear mixed model (MM) results comparing aliphatic GSL accumulation across six epistatic interaction groups composed of different aliphatic GSL genotypes of *Arabidopsis thaliana* (Arabidopsis) from the field. The following generic MM formula was used: aliphatic GSL accumulation ~ gene 1 + gene 2 + environment + gene 1:gene 2 + gene 1:environment + gene 2: environment + gene 1:gene 2:environment + (1|environment:blk). The symbol ':' indicates an interaction term in the model. Gene 1 and gene 2 refer to the aliphatic GSL genes in the epistatic interaction groups listed. See Table 1 for more information on the epistatic interaction groups. Environment refers to the field trial site-year and has three levels: UWY2011, UCD2012, and UWY2012. The environment:blk term refers to the random factor, block, nested within environment. Significance codes: ., $0.1 \geq P > 0.05$; *, $0.05 \geq P > 0.005$; **, $P \leq 0.005$. η^2, percentage variation explained, calculated as the sum of squares (SS) variance for each factor divided by the total SS variance, expressed as a percentage. na, not applicable.

2007, 2010). Individually, these genes control aliphatic GSL accumulation such that loss of function of either transcription factor results in a partial reduction of aliphatic GSL concentrations in the leaves. The double mutant shows a complete loss of foliar aliphatic GSL, an observation not predicted from single mutant analyses, indicating that *MYB28* and *MYB29* also epistatically interact to control aliphatic GSL accumulation. In this study, the impact of GSL genes known to additively and/or epistatically control aliphatic GSL accumulation was tested in the field. Further, the impact of previously untested interactions between GSL genes and the environment on aliphatic GSL accumulation was tested in the field (e.g. G × E, G × G and G × G × E interactions).

As expected for a highly heritable trait, we found that foliar aliphatic GSL accumulation was not greatly influenced by environmental conditions in the field (Table 2). Instead, this trait was largely controlled by additive genetic variation in aliphatic GSL genes observed across the interaction groups (Table 2). For example, *MYB28* and *MYB29* individually control *c.* 60–80% and 15–25% of the variation in aliphatic GSL accumulation, respectively, across interaction groups, in accordance with previous laboratory work (Sønderby *et al.*, 2007; Beekwilder *et al.*, 2008). Furthermore, *MAM1* and *AOP2* accounted for over 15% and 20% of the variation in this trait, respectively. These results are also consistent with previous findings, illustrating that aliphatic GSL accumulation is a highly heritable trait and experimental work from the laboratory translates into the field (Table 2).

For some pairs of GSL genes, epistasis (G × G) accounted for a significant proportion of aliphatic GSL variation in the field, up to 37% (Table 2). To a lesser extent, epistasis × environment (G × G × E) interactions across the six interaction groups accounted for phenotypic variation in this trait, up to 13%

Table 3 Control of leaf damage variation in the field

Source of variation	d.f.	SS	*F*/ChiSq	*P*-value		η^2 (%)
MYB28 × MYB29 interaction group						
MYB28	1	0.1	0.4	0.72		0.8
MYB29	1	0.0	0.0	0.99		0.0
Environment	2	6.4	17.7	9.2E-07	**	75.7
MYB28:MYB29	1	0.4	2.1	0.30		4.4
MYB28:Environment	2	0.0	0.1	0.99		0.4
MYB29:Environment	2	1.3	3.5	0.08	.	15.0
MYB28:MYB29: environment	2	0.3	0.7	0.72		3.0
Environment:blk	1	0.1	27.0	9.2E-07	**	0.8
Residual	343	0.2	na	na		2.1
AOP2 × GSOH interaction group						
AOP2	1	0.0	0.2	0.75		0.6
GSOH	1	0.1	0.3	0.75		1.0
Environment	2	4.2	11.5	1.2E-04	**	72.3
AOP2:GSOH	1	0.1	0.5	0.74		1.7
AOP2:environment	2	0.6	1.5	0.43		9.7
GSOH:environment	2	0.0	0.1	0.93		0.5
AOP2:GSOH: environment	2	0.8	2.1	0.34		13.1
Environment:blk	1	0.1	22.2	2.0E-05	**	1.1
Residual	310	0.2	na	na		3.1
MAM1 × MYB28 interaction group						
MAM1	1	0.6	4.5	0.07	.	7.7
MYB28	1	0.2	1.4	0.28		2.4
Environment	2	5.4	18.8	4.1E-07	**	64.8
MAM1:MYB28	1	0.8	5.7	0.05	*	9.8
MAM1:environment	2	0.5	1.7	0.24		6.0
MYB28:environment	2	0.1	0.5	0.60		1.8
MAM1:MYB28: environment	2	0.5	1.9	0.24		6.4
Blk	1	0.1	55.7	6.7E-13	**	1.1
Residual	329	0.1	na	na		1.7
MAM1 × MYB29 interaction group						
MAM1	1	0.2	1.2	0.36		2.9
MYB29	1	0.0	0.2	0.65		0.5
Environment	2	5.0	14.9	8.2E-06	**	70.5
MAM1:MYB29	1	0.3	1.8	0.32		4.2
MAM1:environment	2	0.2	0.6	0.60		3.0
MYB29:environment	2	0.5	1.6	0.32		7.7
MAM1:MYB29: environment	2	0.7	2.1	0.32		10.1
Environment:blk	1	0.1	33.7	5.2E-08	**	1.1
Residual	326	0.2	na	na		2.4
MYB28 × GSOH interaction group						
MYB28	1	0.2	1.0	0.52		2.4
GSOH	1	0.0	0.0	0.88		0.1
Environment	2	5.7	14.3	2.7E-05	**	72.1
MYB28:GSOH	1	0.0	0.0	0.88		0.1
MYB28:environment	2	0.6	1.5	0.44		7.7
GSOH:environment	2	1.0	2.6	0.21		12.9
MYB28:GSOH: environment	2	0.3	0.8	0.62		3.9
Environment:blk	1	0.1	19.6	3.9E-05	**	0.9
Residual	307	0.2	na	na		2.5
MYB28 × AOP2 interaction group						
MYB28	1	0.0	0.1	0.82		0.2
AOP2	1	0.4	2.5	0.23		4.9
Environment	2	4.8	15.7	4.5E-06	**	61.4
MYB28:AOP2	1	0.1	0.4	0.72		0.7
MYB28:environment	2	0.4	1.2	0.46		4.9
AOP2:environment	2	0.0	0.2	0.86		0.6
MYB28:AOP2: environment	2	2.0	6.7	4.1E-03	**	26.1
Environment:blk	1	0.1	29.2	5.3E-07	**	1.2
Residual	254	0.2	na	na		2.0

Linear mixed model (MM) results comparing leaf damage across six epistatic interaction groups composed of different aliphatic glucosinolate (GSL) genotypes of *Arabidopsis thaliana* (Arabidopsis) from the field. The following generic MM formula was used: leaf damage ~ gene 1 + gene 2 + environment + gene 1:gene 2 + gene 1:environment + gene 2:environment + gene 1:gene 2:environment + (1|environment:blk). The symbol ':' indicates an interaction term in the model. Gene 1 and gene 2 refer to the aliphatic GSL genes in the epistatic interaction groups listed. See Table 1 for more information on the epistatic interaction groups. Environment refers to the field trial site-year and has three levels: UWY2011, UCD2012, and UWY2012. The environment:blk term refers to the random factor, block, nested within environment. Significance codes: ., $0.1 \geq P > 0.05$; *, $0.05 \geq P > 0.005$; **, $P \leq 0.005$. η^2, percentage variation explained, calculated as the sum of squares (SS) variance for each factor divided by the total SS variance, expressed as a percentage. na, not applicable.

(Table 2). Interestingly, the influence of G × G consistently outweighed the influence of G × G × E on aliphatic GSL variation across five of the six interaction groups (Table 2).

Together these results indicate that genetic variation exerted more control over aliphatic GSL variation in the field than did the environment alone. However, we also observed that the influence of epistatic variation was partially dependent on environmental conditions as illustrated by the G × G × E, suggesting that a complex relationship between intrinsic and extrinsic factors influences aliphatic GSL accumulation in the field.

Leaf damage variation is primarily influenced by the environment

Glucosinolates are defensive compounds known to deter leaf herbivory by various insect species. The degree of protection provided by these metabolites is dependent on both the insect species present and GSL profile of the plant. Previous field work has shown that Arabidopsis leaf damage variation is largely controlled by the environment as well as GSL genotype × environment interactions (Kerwin *et al.*, 2015). To extend these analyses and include the impact of pairs of specific aliphatic GSL genes, we proceeded to test the role of the environment, epistasis (G × G), and epistasis × environment interactions (G × G × E) in altering foliar herbivory in the field across the six interaction groups.

As expected, we observed that the environment significantly impacted leaf damage in the field, accounting for 60–75% of the variation in this highly plastic trait (Table 3). In contrast to what was observed for aliphatic GSL accumulation, additive genetic variation did not account for much of the variation in leaf damage across the interaction groups (Tables 2, 3). Depending on the interaction group, G × E, G × G or G × G × E interactions accounted for the majority of the remaining phenotypic variation

Table 4 Control of relative fitness variation in the field

Source of variation	d.f.	SS	*F*/ChiSq	*P*-value		η^2 (%)
MYB28 × MYB29 interaction group						
MYB28	1	0.32	3.06	0.16		12.3
MYB29	1	0.04	0.35	0.89		1.4
Environment	2	0.00	0.02	0.98		0.1
MYB28:MYB29	1	0.82	7.93	0.01	*	31.9
MYB28:environment	2	0.03	0.14	0.98		1.2
MYB29:environment	2	0.08	0.40	0.89		3.3
MYB28:MYB29: environment	2	1.24	5.96	0.01	*	47.9
Environment:blk	1	0.05	44.74	1.8E-10	**	2.0
Residual	345	0.10	na	na		4.0
AOP2 × GSOH interaction group						
AOP2	1	0.14	1.37	0.39		5.9
GSOH	1	0.40	3.80	0.14		16.4
Environment	2	0.01	0.06	0.94		0.5
AOP2:GSOH	1	0.06	0.53	0.54		2.3
AOP2:environment	2	0.52	2.50	0.17		21.5
GSOH:environment	2	0.21	0.99	0.50		8.5
AOP2:GSOH: environment	2	1.02	4.88	0.03	*	42.1
Environment:blk	1	0.07	48.60	2.5E-11	**	2.7
Residual	317	0.10	na	na		4.3
MAM1 × MYB28 interaction group						
MAM1	1	0.23	2.32	0.21		11.7
MYB28	1	0.00	0.00	1.00		0.0
Environment	2	0.00	0.00	1.00		0.0
MAM1:MYB28	1	0.20	2.00	0.21		10.1
MAM1:environment	2	0.50	2.50	0.20		25.2
MYB28:environment	2	0.54	2.69	0.20		27.1
MAM1:MYB28: environment	2	0.46	2.30	0.20		23.2
Environment:blk	1	0.05	49.36	1.7E-11	**	2.7
Residual	330	0.10	na	na		5.0
MAM1 × MYB29 interaction group						
MAM1	1	0.06	0.50	0.64		3.5
MYB29	1	0.03	0.21	0.74		1.5
Environment	2	0.01	0.05	0.95		0.7
MAM1:MYB29	1	0.29	2.41	0.32		16.8
MAM1:environment	2	0.26	1.07	0.55		14.9
MYB29:environment	2	0.36	1.51	0.45		21.0
MAM1:MYB29: environment	2	0.64	2.66	0.29		37.0
Environment:blk	1	0.08	55.33	8.1E-13	**	4.6
Residual	327	0.12	na	na		7.0
MYB28 × GSOH interaction group						
MYB28	1	0.34	3.03	0.17		11.1
GSOH	1	0.02	0.18	0.88		0.7
Environment	2	0.01	0.02	0.98		0.2
MYB28:GSOH	1	1.18	10.45	0.01	*	38.4
MYB28:environment	2	0.10	0.42	0.88		3.1
GSOH:environment	2	0.06	0.26	0.88		1.9
MYB28:GSOH: environment	2	1.31	5.81	0.01	*	42.7
Environment:blk	1	0.06	36.65	1.1E-08	**	1.9
Residual	312	0.11	na	na		3.7
MYB28 × AOP2 interaction group						
MYB28	1	0.62	5.89	0.05	*	18.6
AOP2	1	0.27	2.58	0.13		8.2
Environment	2	0.01	0.03	0.97		0.2
MYB28:AOP2	1	0.33	3.11	0.12		9.8
MYB28:environment	2	0.86	4.11	0.05	*	26.0
AOP2:environment	2	0.68	3.24	0.08	.	20.5
MYB28:AOP2: environment	2	0.51	2.41	0.12		15.2
Environment:blk	1	0.05	23.51	1.0E-05	**	1.5
Residual	255	0.10	na	na		3.2

Linear mixed model (MM) results comparing relative fitness across six epistatic interaction groups composed of different aliphatic glucosinolate (GSL) genotypes of *Arabidopsis thaliana* (Arabidopsis) from the field. The following generic MM formula was used: relative fitness ~ gene 1 + gene 2 + environment + gene 1:gene 2 + gene 1:environment + gene 2:environment + gene 1:gene 2:environment + (1|environment:blk). The symbol ':' indicates an interaction term in the model. Gene 1 and gene 2 refer to the aliphatic GSL genes in the epistatic interaction groups listed. See Table 1 for more information on the epistatic interaction groups. Environment refers to the field trial site-year and has three levels: UWY2011, UCD2012, and UWY2012. The environment:blk term refers to the random factor, block, nested within environment. Significance codes: ., $0.1 \geq P > 0.05$; *, $0.05 \geq P > 0.005$; **, $P \leq 0.005$. η^2, percentage variation explained, calculated as the sum of squares (SS) variance for each factor divided by the total SS variance, expressed as a percentage. na, not applicable.

in leaf damage. Across the interaction groups, epistasis (G × G) accounted for < 10% of the variation in leaf damage in the field, G × E interactions accounted for up to 15% and epistasis × environment (G × G × E) interactions accounted for up to 26% (Table 3). These results show that extrinsic environmental factors had more influence on the amount of leaf damage observed in our field trials than did intrinsic genetic variation alone.

Relative fitness variation is controlled by epistasis (G × G) and G × G × E

The analyses in the previous section suggest that aliphatic GSL accumulation and leaf damage in the field are largely controlled by different factors. Ultimately, the goal of this study was to explore the roles of epistasis and the environment in shaping adaptive evolution, using the aliphatic GSL defense pathway in Arabidopsis as a model. To understand how epistatic interactions between pairs of aliphatic GSL genes might influence the adaptive potential of a GSL genotype across environments, we calculated relative fitness separately for each of the six interaction groups across the three field trials. Relative fitness, defined as the fitness of a genotype divided by the average fitness of the group, was chosen because it serves as a proxy for the selective advantage of a given genotype within a population under a given set of environmental conditions.

We found that relative fitness in the field was influenced by individual aliphatic GSL genes, although the observed effect size was much smaller for this trait than for aliphatic GSL accumulation, < 20% across the six interaction groups (Tables 2, 4). Epistasis (G × G) significantly impacted relative fitness across some of the interaction groups, accounting for up to 38% of the variation in this trait (Table 4; Fig. 5). Interestingly, interactions between epistasis and the environment (G × G × E) had the greatest influence on relative fitness in the field, accounting for up to 48% of trait

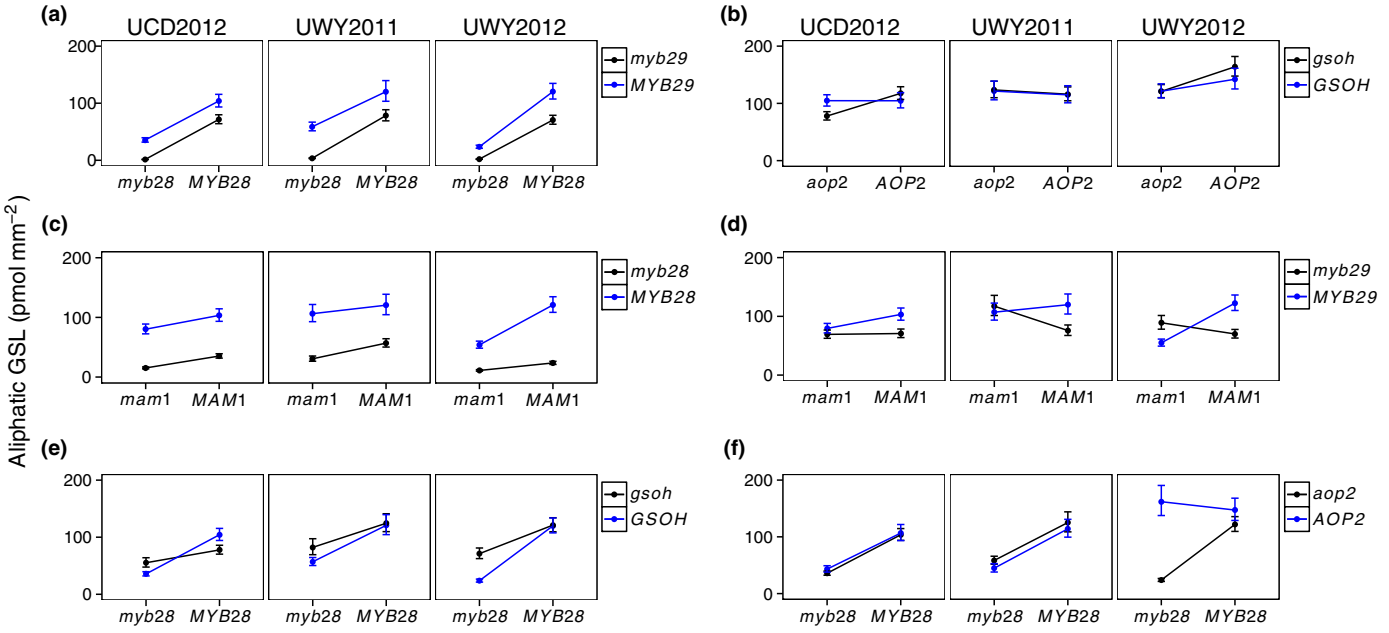

Fig. 2 Genotype × genotype × environment (G × G × E) interaction plots illustrating aliphatic glucosinolate (GSL) accumulation within six epistatic interaction groups, each composed of distinct combinations of *Arabidopsis thaliana* (Arabidopsis) aliphatic GSL genotypes measured across three field trials. Letters correspond to the different interaction groups as follows: (a) MYB28 × MYB29, (b) AOP2 × GSOH, (c) MAM1 × MYB28, (d) MAM1 × MYB29, (e) MYB28 × GSOH, and (f) MYB28 × AOP2. See Table 1 for more information on epistatic interaction groups. UCD2012, University of California at Davis 2012; UWY2011, University of Wyoming 2011; UWY2012, University of Wyoming 2012. Points and error bars show back-transformed population means ± SE. See Table 2 for GSL accumulation mixed model results. 3MT, 3-methylthio glucosinolate; 4MT, 4-methylthio glucosinolate; 3MSO, 3-methylsulfinyl glucosinolate; 4MSO, 4-methylsulfinyl glucosinolate; OHP, 3-hydroxy-propyl glucosinolate; Ally, 2-propenyl glucosinolate; butenyl, 3-butenyl glucosinolate; OH-butenyl, 2-hydroxy-but-3-enyl glucosinolate.

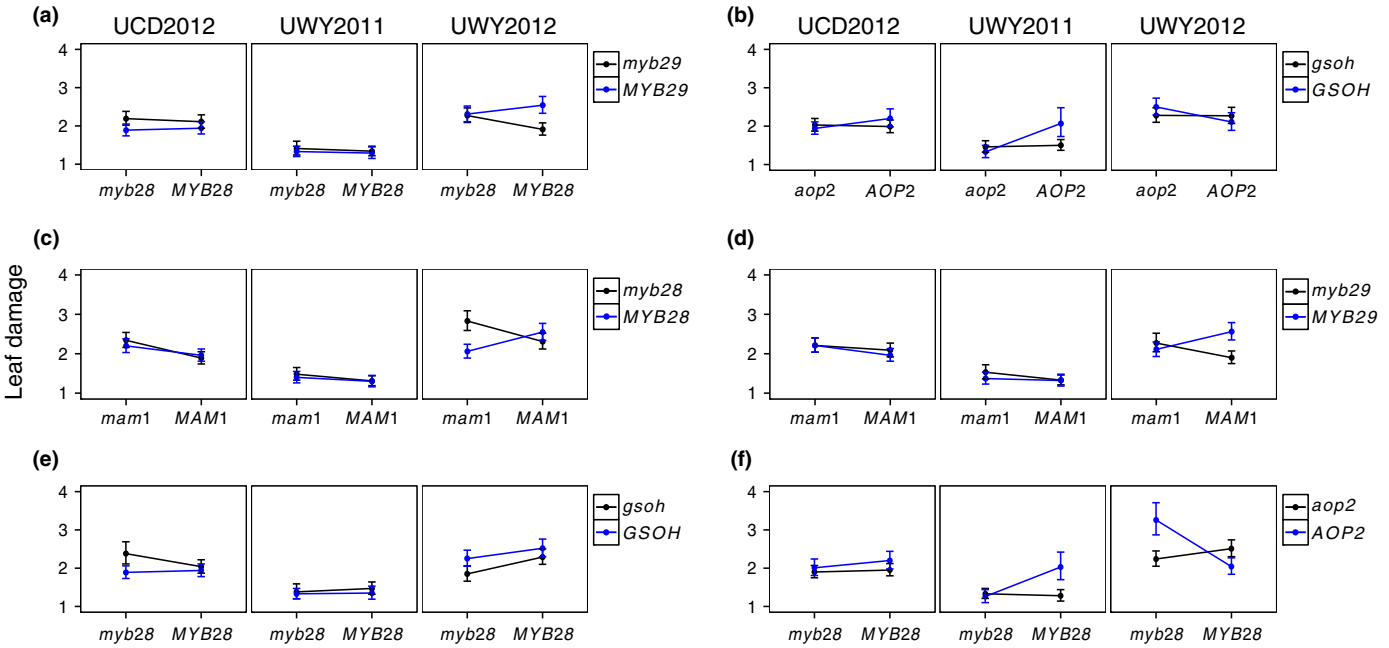

Fig. 3 Genotype × genotype × environment (G × G × E) interaction plots illustrating leaf damage within six epistatic interaction groups, each composed of distinct combinations of *Arabidopsis thaliana* (Arabidopsis) aliphatic glucosinolate (GSL) genotypes measured across three field trials. Letters correspond to the different interaction groups as follows: (a) MYB28 × MYB29, (b) AOP2 × GSOH, (c) MAM1 × MYB28, (d) MAM1 × MYB29, (e) MYB28 × GSOH, and (f) MYB28 × AOP2. See Table 1 for more information on epistatic interaction groups. UCD2012, University of California at Davis 2012; UWY2011, University of Wyoming 2011; UWY2012, University of Wyoming 2012. Points and error bars show back-transformed population means ± SE. See Table 3 for leaf damage mixed-model results.

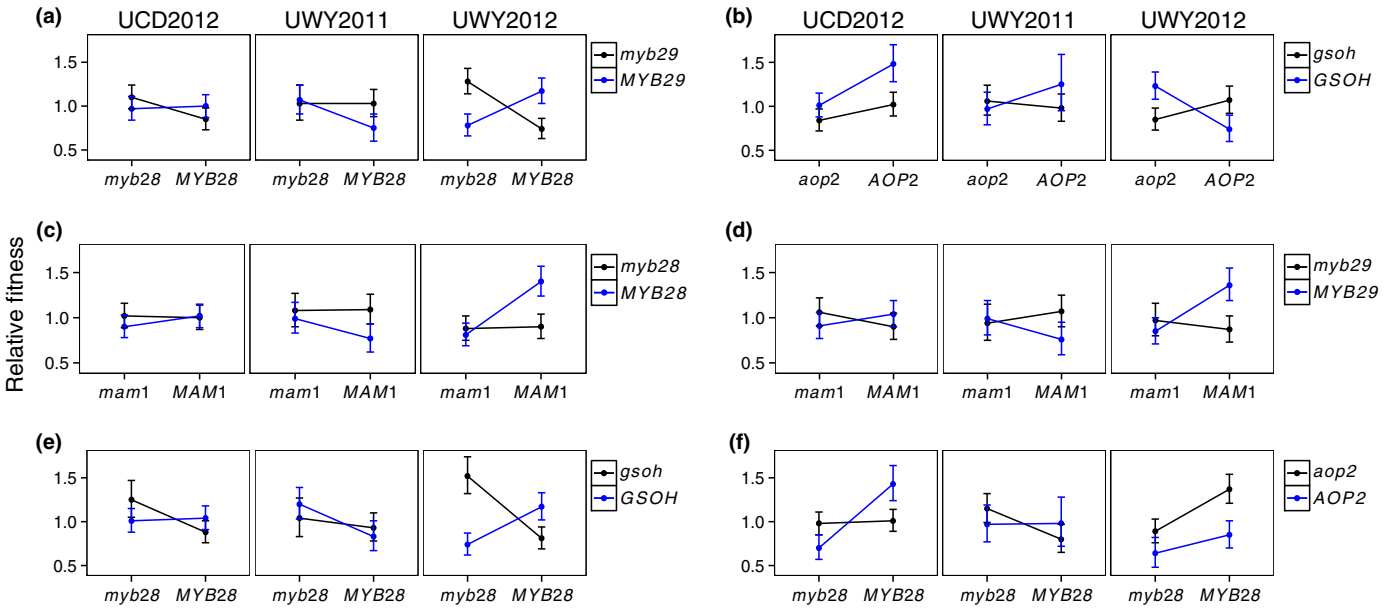

Fig. 4 Genotype × genotype × environment (G × G × E) interaction plots illustrating relative fitness within six epistatic interaction groups, each composed of distinct combinations of *Arabidopsis thaliana* (Arabidopsis) aliphatic glucosinolate (GSL) genotypes measured across three field trials. Letters correspond to the different interaction groups as follows: (a) MYB28 × MYB29, (b) AOP2 × GSOH, (c) MAM1 × MYB28, (d) MAM1 × MYB29, (e) MYB28 × GSOH, and (f) MYB28 × AOP2. See Table 1 for more information on epistatic interaction groups. UCD2012, University of California at Davis 2012; UWY2011, University of Wyoming 2011; UWY2012, University of Wyoming 2012. Points and error bars show back-transformed population means ± SE. See Table 4 for relative fitness mixed model results.

variation (Table 4; Figs 4, 5). These results suggest that extrinsic environmental variation can greatly modify the effect that intrinsic aliphatic GSL gene interactions have on fitness in the field. This environmental dependency of epistasis could profoundly impact the ability of Arabidopsis to respond to natural selection acting on GSL variation, shaping the adaptive trajectories of natural populations in complex ways (Table 4; Fig. 4).

Discussion

Epistasis for fitness can theoretically constrain or otherwise influence evolution within populations, generating a rugged adaptive landscape characterized by the presence of many local optima rather than a single fitness peak towards which all genotypes evolve (Wright, 1932; Whitlock *et al.*, 1995). Further, the topography of adaptive landscapes can vary across environmental conditions, indicating that the evolutionary fate of an allele or mutation is dependent on its genetic background as well as the environment in which it occurs (Kirkpatrick, 1982; Whitlock, 1995). The goal in this study was to empirically test these theoretical predictions regarding the influence of epistasis (G × G) and epistasis × environment (G × G × E) interactions on the adaptive landscape. In a previous study, we investigated genotype (G) and genotype × environment (G × E) interactions in the field using an Arabidopsis mutant population with 17 aliphatic GSL genotypes (Fig. S1) (Kerwin *et al.*, 2015). In the current study, to explicitly test the impact of G × G and G × G × E interactions on the field data collected in Kerwin *et al.* (2015), we focused our analyses on groups of genotypes that sampled all four haplotype combinations for discrete pairs of aliphatic GSL genes (i.e. AABB, aaBB, AAbb and aabb) (Table 1). In total, we analyzed 12 aliphatic GSL genotypes from the full set of 17, which were organized into six epistatic interaction groups (Table 1). This allowed us to explicitly test the effect of G × G and G × G × E interactions for pairs of genes known to control natural variation in Arabidopsis aliphatic GSL defense chemistry. To address the potential influence of these interactions on the adaptive landscape, we measured their impact on relative fitness across three field trials. These analyses showed that nonadditive G × G interactions between aliphatic GSL genes greatly impacted relative fitness in the field. Across the six interaction groups, G × G accounted for 10–38% of the variation in this trait (Table 4; Fig. 5). Strikingly, epistasis interacted strongly with the environment, such that G × G × E interactions controlled 15–48% of the variance in relative fitness in the field (Table 4; Fig. 5). Together, our results show that G × G and G × G × E interactions are probably key components of how genetic variation in the aliphatic GSL pathway influences relative fitness in the field across Arabidopsis populations.

G × E, G × G and G × G × E for relative fitness impact evolution and the response to selection

Genotype × environment (G × E) interactions can influence fitness variation within populations in one of two ways. One possibility is that the relative fitness ranking of genotypes shifts depending on environmental conditions, so that no single genotype is consistently the most fit. In this first scenario, as environmental conditions fluctuate across seasons or years, genetic variation is maintained within a population (Gillespie & Turelli, 1989). An alternative possibility is that the relative fitness ranking of the genotypes remains the same across environmental

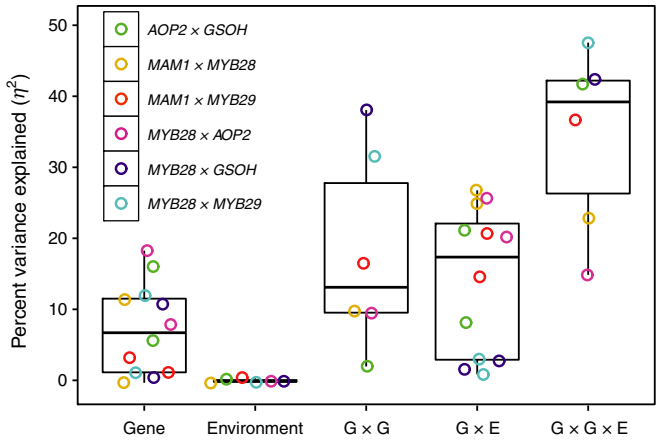

Fig. 5 Boxplot illustrating variation in relative fitness across six epistatic interaction groups composed of different aliphatic glucosinolate (GSL) genotypes of *Arabidopsis thaliana* (Arabidopsis) explained by each fixed factor in the following linear mixed model (MM): relative fitness ~ gene 1 + gene 2 + environment + gene 1:gene 2 + gene 1:environment + gene 2:environment + gene 1:gene 2:environment + (1|environment:blk). Gene 1 and gene 2 refer to the binary allelic states of the respective aliphatic GSL genes in a pair defined by epistatic interaction group. See Table 1 for more information on the epistatic interaction groups. Environment refers to the field trial site-year and has three levels: UWY2011, UCD2012, and UWY2012. Environment:blk refers to the random factor, block, nested within environment. The symbol ':' indicates an interaction term in the model. Percentage variation explained, η^2, was calculated as the sum of squares variance for a given factor or source of variation (SS_S) divided by the total sum of squares variance (SS_T). Larger η^2 values indicate that a given factor controls a greater proportion of phenotypic variation compared with a factor in the model with a smaller η^2 value. Colors refer to the different interaction groups. The *x*-axis labels refer to the different fixed factors in the MM. Gene, gene 1 and gene 2, respectively (note the 12 circles). G × G = gene 1:gene 2. G × E = gene 1:environment and gene 2:environment, respectively. G × G × E = gene 1:gene 2:environment. See Table 4 for relative fitness MM results.

conditions, but the magnitude of difference in relative fitness between genotypes fluctuates with the environment. Here, the same genotype is always the best performer, but the difference in relative fitness between the best and worst genotypes may be negligible or substantial, depending on the season or year. In this second scenario, G × E interactions would not explicitly maintain genetic variation, but may alter the response to selection within a population.

Similarly, epistatic (G × G) interactions can influence fitness variation in two ways. The fitness effect of a gene may be dependent on the allelic state of one or more interacting genes in the genome. Two types of epistasis, magnitude and sign epistasis, can affect fitness variation within populations differently. With sign epistasis, the direction of the fitness effect of a gene depends on the allelic state of the interacting gene(s), leading to a shift in relative fitness ranking across genetic backgrounds (Weinreich, 2005; Poelwijk *et al.*, 2007). Alternatively, under magnitude epistasis, the magnitude of the fitness effect of a gene is dependent on the allelic state of the interacting gene(s), while the relative fitness ranking is unaffected (Weinreich, 2005; Poelwijk *et al.*, 2007). According to adaptive landscape theory, sign epistasis, but not magnitude epistasis, can generate a rugged landscape. When the rank performance of a gene depends on the allelic state of other genes in the genome, selection pressures shift across allelic combinations, creating fitness peaks separated by adaptive valleys. These adaptive valleys prevent deterministic evolution from one adaptive peak to another (Weinreich, 2005; Poelwijk *et al.*, 2011). Therefore, sign epistasis can explicitly maintain genetic variation, even under a single set of environmental conditions.

If both G × E and G × G interactions act on fitness, then evolution can be constrained in complex ways. In this study, we observed both G × E and G × G interactions influencing relative fitness in the field (Table 4; Fig. 5). Additionally, we observed that G × G × E interactions explained much of variation in relative fitness (Table 4; Fig. 5). For example, at the UWY field site, the direction of effect of epistasis on relative fitness reversed between 2011 and 2012, such that allelic combinations that conferred higher relative fitness in UWY2011 resulted in lower relative fitness in UWY2012, and vice versa (Fig. 4). This striking result suggests that temporally fluctuating selection pressures could maintain genetic variation within a single population.

The UWY2012 field trial displayed the highest average leaf damage and the lowest average absolute fitness, suggesting that it represents the harshest environment in this study (Fig. 3). In addition, we observed a greater degree of sign and magnitude epistasis in UWY2012 compared with the other field trials (Figs 2–4). This is true for all three of our field traits but is most striking for relative fitness. Across the pairs of GSL genes, the impact of one allele was highly dependent on the allelic state at the second gene in the pair. In the UWY2011 and UCD2012 field trials, the G × G effect was diminished on average, demonstrating that environmental variation greatly influences the expression of epistasis in this study. These results suggest the intriguing possibility that epistasis may act to maintain genetic variation to a greater degree in harsher environments than in milder environments. Testing this further would require additional field trials over multiple years in more locations to assess if the observed pattern of prevalent epistasis in harsh environments is maintained. These results illustrate how the genetics underlying the phenotypic expression of adaptive traits can be complex and environmentally dependent, which may constrain or otherwise impact evolution in unexpected and complex ways.

Epistasis and G × G × E vary across trait hierarchies

We often think of phenotypes in a hierarchical framework in which some traits, because of their simplicity (i.e. gene expression from a promoter) or temporal precedence (i.e. seedling traits), can deterministically influence the expression of other more complex or temporally antecedent traits (Weinig & Delph, 2001; Joseph *et al.*, 2013). In this study, the proposed hierarchy would be as follows: aliphatic GSL accumulation directly influences leaf damage as a result of herbivory, which, in turn, alters relative fitness. However, the pattern of effect that intrinsic genetic and extrinsic environmental factors had on our field trial traits argues

that this causal hierarchical view of phenotypes may not accurately reflect the complex relationship between the underlying genes and the environment.

For example, across the interaction groups, G × G exerted greater influence on the lowest and highest hierarchical traits, aliphatic GSL accumulation and relative fitness, than it did on the intermediate hierarchical trait, leaf herbivory. A similar pattern was observed for the effect of additive genetic variation on our field traits. Interestingly, a general trend of increasing G × G × E effects across the trait hierarchy can be observed across the interaction groups.

One possible explanation for this pattern is that the damage in the leaves of the plant may not fully reflect the influence of aliphatic GSL variation on fitness variation in our field trials. Potential damage inflicted by nonleaf-chewing pests (e.g. nematode, aphids) and/or pathogens is not captured by the leaf damage measurements taken in the field. Given that aliphatic GSL profile has been shown to influence resistance to these other biotic pests, 'hidden' damage could account for the disparity between the observed genetic architecture of leaf herbivory damage and relative fitness results (Manici *et al.*, 1997). Similarly, it is possible that aliphatic GSL may influence other unmeasured traits, such as flowering time, that ultimately influence fitness variation (Kerwin *et al.*, 2011).

Lastly, Arabidopsis produces other defensive secondary metabolites that may impact leaf damage and/or plant fitness through aliphatic GSL-independent pathways (Kliebenstein, 2004). This suggests that *a priori* assignment of trait hierarchies, while enticing, may not reflect trait relationships in natural systems and illustrates the complexity of mapping genotypes to phenotypes when studying adaptive trait evolution.

Conclusions

To our knowledge, this is one of the first studies to explore the impact of epistasis between specific causal genes, particularly defense-related genes, on fitness across multiple environments in a field setting. Together, our results provide evidence that epistasis among the naturally variable aliphatic GSL genes in Arabidopsis is common and has the potential to significantly affect adaptation to fluctuating local environments. The relationship between epistasis and the environment is complex such that information obtained from one environment in one year is insufficient to fully characterize the relationship between genetic architecture and fitness in nature, underscoring the need to extend laboratory work into the field to truly understand how organisms evolve in nature. The dependency of epistasis on the environment is probably a common phenomenon impacting adaptive evolution in many species that persist in heterogeneous environments, not just Arabidopsis, and deserves greater consideration in future evolutionary studies.

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Author contributions

R.E.K. and D.J.K. planned and designed the research. M.J.R. assisted with the field trial setup. R.E.K., A.M., B. Larson, D.C., J.A.C., M.J.R., M.F., B. Li and B.J. conducted fieldwork. R.E.K., J.F., A.M., C.L. and B. Larson conducted laboratory work. R.E.K. analyzed the data. J.A.C. and D.J.K. assisted with data analysis. R.E.K., D.J.K. and C.W. wrote and edited the manuscript.

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Supporting Information

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Fig. S1 Schematic of the field trial setup in a single environment.

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