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Tolerance to apical meristem damage in *Arabidopsis thaliana* (Brassicaceae): a closer look and the broader picture

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Abstract of the dissertation

Tolerance to apical meristem damage in *Arabidopsis thaliana* (Brassicaceae): a closer look and the broader picture

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Tolerance of damage to reproductive tissues is a form of plant defense against herbivory. Although most attention has focused on resistance to herbivory, evidence has been accumulating that tolerance is more important than had been previously assumed. I examined the ecology of tolerance to apical meristem damage (AMD) in natural strains of *Arabidopsis thaliana* originating from three different areas of Europe, using high and low soil nutrient levels, to understand if tolerance is contingent on soil nutrients and if the response is similar species-wide. I found that there was no species-wide relationship between tolerance and nutrients in natural populations of *A. thaliana*, which has important implications for theoretical modeling of tolerance.

Tolerance is not well understood on a fine-scale genetic level. Therefore, I tested for a role of gibberellin hormones in tolerance in *A. thaliana* using five mutants. I next used oligonucleotide microarrays to pinpoint genes involved in the response to AMD in two natural accessions of *A. thaliana*, to identify genes whose expression levels were significantly altered by AMD. I found that gibberellins do not mediate tolerance, although they have major effects on growth and development. I did, however, identify 58 genes involved in the response to AMD that can be targeted in follow-up studies to verify their roles in tolerance.

Finally, I explored the similarities of the tolerance response in *A. thaliana* and several relatives in a broad group of species, to understand how tolerance evolves. I found that tolerance can evolve readily in *A. thaliana* and its relatives, unhindered by developmental constraints imposed by the mating system or life history.

In summary, I suggest that continued studies of tolerance using *A. thaliana* are worthwhile, given its advantages as a model system, the natural genetic variation in tolerance it harbors, and the potential to understand how tolerance evolved in a broad, diverse of group of species.

Table of Contents

List of Figures

List of Tables

Intoduction

Herbivory plays a major role in plant evolution (Painter 1958; Marquis 1992; Abrahamson and Weis 1997; Juenger and Lennartsson 2000; Stowe et al. 2000). There are two ways that plants cope with herbivory: by having a phenotype that decreases the likelihood of being grazed (resistance; this includes cryptic coloration, masting and any other strategies that decrease the average likelihood of herbivory; Weinig et al. 2003a), and by having the ability to recover from tissue loss and damage (tolerance; Rausher 1992). Although there has been extensive research on the evolution of resistance to herbivory, the evolution of tolerance has received comparatively less attention (Stowe et al. 2000; Tiffin and Rausher 1999; Juenger and Lennartsson 2000) because it has generally been assumed to be important only in perennial plants, where below-ground resources may be shunted above ground in response to tissue damage (van der Meijden et al. 1988; Belsky et al. 1993; Stowe et al. 2000), or in monocarpic plants living in grasslands and other areas of exceptionally high, predictable herbivory (Juenger and Lennartsson 2000).

Recent research on the response of monocarpic plants to vertebrate herbivory in non-grassland systems, however, indicates that tolerance is more prevalent and can play a greater role in plant evolution than this conventional wisdom suggests. Several studies have demonstrated that such plants can recover substantially from vertebrate herbivory (for reviews see Stowe et al. 2000; Hawkes and Sullivan 2001; Wise and Abrahamson 2007) even if it inflicts a loss of as much as 95% of their aboveground biomass (Paige and Whitham 1987). Surprisingly, some plants from some species even have higher fitness when damaged by herbivores than when they remain undamaged (Paige and Whitham 1987; Paige 1994; Lennartsson et al. 1997; Lennartsson et al. 1998; Paige 1999; Juenger et al. 2000).

The goal of this dissertation work is to begin to understand a particular form of tolerance (tolerance to apical meristem damage, or AMD) in one species and its close relatives, in order to fill significant gaps in our knowledge of this understudied, potentially very important, aspect of plant defense. Unresolved questions in this field include: What range of ecological conditions allow for high levels of tolerance? Is tolerance appropriately characterized on a species-wide basis, or is it variable within species depending on the genetic background? What genes are involved in modulating the tolerance response? How variable is tolerance among species? How readily does tolerance evolve?

The model species *Arabidopsis thaliana* (Brassicaceae) offers a unique opportunity to address these questions. It has the architecture necessary for AMD (i.e., one main inflorescence that grows from the base of the rosette), and this form of damage has been documented in the field (Weinig et al. 2003a). It is small, grows readily, and has a short life cycle, allowing large experiments under controlled conditions. The underlying mechanisms for tolerance to AMD in *A. thaliana* can be studied because its biology is extremely well known, and the genomic tools available for it are unparalleled for plants

(Pang and Meyerowitz 1987; Anderson and Roberts 1998; Krysan et al. 1999; Mitchell-Olds and Schmitt 2006). Furthermore, extensive phylogenies of *A. thaliana* and its relatives are available (e.g., Koch et al. 2001; Al-Shehbaz 2003, 2005; Dobeš et al. 2006), allowing one to study the interspecific variation in a phenotypic response, and to what extent this variation is associated with patterns of relatedness.

The work here is presented in five chapters. Chapter one examines the ecology of tolerance to apical meristem damage in natural strains of *Arabidopsis thaliana* originating from three different areas of Europe, using high and low soil nutrient levels, to understand if tolerance is contingent on soil nutrients and if it is similar species-wide. The importance of using the best fitness proxy possible for estimating tolerance is also discussed.

Because tolerance is measured solely as the ability to recover fitness after damage, it is uninformative as to the various possible patterns of relationships among traits that may result in the ability to recover fitness; these relationships cannot be inferred from the degree of tolerance alone. Therefore, chapter two employs structural equation modeling to understand how *A. thaliana*'s tissues that are regrown after damage are related to components of fitness. Genetic and environmental variation in these relationships are also examined, and the implications of these results for a species-wide picture of the mechanistic basis of tolerance are discussed.

While the study of tolerance lags behind that of resistance to damage in general, the study of the molecular basis of tolerance is particularly neglected. Virtually nothing is known about the genetic pathways involved in tolerance responses to damage. Chapter three, as published in the journal Heredity, tests the hypothesis that gibberellin hormones help mediate the tolerance response to AMD, via their effects on stem elongation after damage, using five different *A. thaliana* mutants deficient in the production of, or sensitivity to, gibberellins.

Continuing with the goal of chapter three -- identifying molecular processes accounting for tolerance responses to damage -- chapter four uses oligonucleotide microarrays to screen the entire genome of *A. thaliana* for genes involved in the response to AMD. The use of two *A. thaliana* accessions allows for the flagging of genes involved in the response to AMD in different genetic backgrounds. The results of this study can be used in follow-up studies to verify the roles played by different genes in tolerance.

Finally, chapter five explores the similarity of the tolerance response of *A. thaliana* and a broad group of species to explore how tolerance evolves. Inorganic soil nutrient levels are varied to understand the contingency of the tolerance response on this ecological variable and how this contingency differs among species. The difficulties of comparing tolerances across very different species, with different mating systems and life histories, are discussed.

Using *A. thaliana* as a focal species, this dissertation is constructed by combining different levels of analyses, "zooming in" to explore molecular mechanisms of tolerance to AMD, "zooming out" to understand intraspecific variation in tolerance and lability of tolerance to nutrient levels, and then zooming even farther out to investigate the evolution of tolerance at the interspecific level. Taken together, this work goes far in advancing our understanding of tolerance to AMD on multiple levels comprising different subdisciplines of ecology and evolutionary biology, connected by the common thread of a very well understood model species, facilitating rich, productive, and integrated follow-

up investigations in the future.

I. Theoretical models do not account for the effect of nutrient levels on tolerance to apical meristem damage in *Arabidopsis thaliana*

Abstract

Theoretical models come to different conclusions about the effects of inorganic soil nutrient levels on tolerance. We tested two models -- the growth rate model and the continuum of resources model/limiting resources model version IV -- by examining tolerance to apical meristem damage (AMD) in laboratory-reared natural populations of *Arabidopsis thaliana*. We used plants from three different regions of Europe, spanning a wide latitudinal gradient, to test for genetic variation in tolerance and for consistency in the results across the landscape. We found that nutrient levels mediated tolerance to AMD, but this mediation depended on the region a plant was from and on the choice of fitness proxy used to estimate tolerance. None of the models accounted for the regional variation in our results. We suggest that characterizing tolerance in nature will be difficult with simple models, and may require detailed prior information about the populations and environments under consideration. Furthermore, we caution that it is not always prudent to assume fruit production provides a reasonable approximation of fitness in *A. thaliana*, contrary to common practice in the literature.

INTRODUCTION

Herbivory by mammals on monocarpic dicots can involve the consumption of the inflorescence tissue at the bolting stage. This same sort of damage (referred to here as apical meristem damage, AMD) can also be due to abiotic sources of stress, such as frost (Belsky 1986; Paige and Whitham 1987; Juenger et al. 2000; Tiffin 2000). In plants that produce one main inflorescence growing from a vegetative rosette, AMD releases them from apical dominance, and several basal inflorescences can grow from the rosette where only one or a few were hormonally permitted before (Paige and Whitham 1987; Benner 1988; Mopper et al. 1991; Huhta et al. 2000a; Juenger et al. 2000). This can result in different degrees of tolerance, ranging from decreased fitness (undercompensation), to no change in fitness (exact compensation), to an increase in fitness (overcompensation) when damaged as compared to when undamaged, as indicated by the slope of the reaction norm for fitness across apically damaged and undamaged treatments (Juenger et al. 2000; Mauricio 2000; Simms 2000; Stowe et al. 2000).

Theoretical models make different predictions about the effects of inorganic soil nutrient availability on tolerance to AMD. The "continuum of resources" model (CRM, a.k.a the compensatory continuum hypothesis; Maschinski and Whitham 1989) predicts that tolerance of AMD should increase directly with nutrient availability. In other words, the slope of the fitness reaction norm across AMD treatments (Juenger et al. 2000; Mauricio 2000; Simms 2000; Stowe et al. 2000) should be more positive, or less negative, in high nutrients than low nutrients. At low nutrient levels, plants cannot recover the biomass, or fitness, lost to AMD, whereas at higher nutrient levels plants

have sufficient resources to recover and take advantage of the release from apical dominance, producing more fruits and seeds.

The "growth rate" model (GRM; developed by Hilbert et al. 1981 and coined by Hawkes and Sullivan 2001), on the other hand, predicts the opposite. According to it, plants growing slowly in nutrient-poor conditions only have to grow slightly faster to compensate for damage, and this is an easier feat to achieve as compared to plants growing more quickly in nutrient-rich conditions, which are already growing close to their maximum rate when undamaged, and which are therefore unable to grow any faster to compensate for damage.

The more comprehensive "limiting resources" model (LRM; Wise and Abrahamson 2005; Wise and Abrahamson 2007) makes different predictions depending on different assumptions. Its premise is that tolerance depends on whether a "focal resource" (e.g., soil nutrient levels) or an "alternative resource" (the damaged plant tissue itself) limits fitness (see also Rautio et al. 2005, who lay out the same general framework).

We used *A. thaliana* populations to test the effect of nutrient levels on tolerance against the theoretical models. If the CRM is correct, we expected plants to be more tolerant at high nutrient levels. If the GRM is correct, we expected plants to be more tolerant to AMD at low nutrient levels. While the predictions made by the GRM and the CRM are static, the LRM predicts various outcomes. We made the following assumptions to test a specific version of the LRM: when nutrient levels are low nutrients limit fitness, whereas when nutrient levels are high, the number of meristems available from which to build inflorescences and fruits limits fitness (Wise and Abrahamson 2005, Figure 2, prediction IV). Therefore, AMD should have a more stimulating effect on fitness at high nutrient levels than at low nutrient levels. This version of the LRM (which we call LRM-IV) is identical to the CRM, although to our knowledge this has not been pointed out before.

We used populations collected across a wide latitudinal gradient of Western Europe -- northern Spain, the Netherlands, and southern Sweden -- to evaluate whether our findings are geographically robust and whether there is genetic variation for tolerance among natural *A. thaliana* plants. Using natural populations of *A. thaliana*, rather than laboratory strains, will make our results more representative of the species in the wild, and will provide a more meaningful test of the models.

We also evaluated the validity of the assumptions of the CRM/LRM-IV. We examined the reaction norm for fitness across nutrient levels to verify that nutrients were limiting fitness at low nutrient levels. We tested whether fitness limitation switched, at high nutrient levels, to the number of active shoot meristems by regressing basal inflorescence production on fitness (Rautio et al. 2005); we expected to see no relationship between basal inflorescence production and fitness at low nutrient levels, and a positive relationship at high nutrient levels, if the CRM/LRM-IV were correct.

In addition to the standard proxy for estimating fitness for *A. thaliana*, fruit production, we used a more refined one, total viable seed production, which is a composite variable based, in part, on fruit production. While Westerman and Lawrence (1970) found a strong relationship between fruit and seed production in *A. thaliana*, their study used laboratory lines and did not examine the relationship to *viable* seed production. We assessed whether the choice of fitness proxy, fruit production versus total

viable seed production, would alter our conclusions.

MATERIALS AND METHODS

Plant material, handling, and experimental protocol---Maternal seed families of *Arabidopsis thaliana* were collected from populations in three different regions along a broad latitudinal gradient in Western Europe during the spring of 2001 (Figure 1). Although *A. thaliana* is a ruderal species often associated with disturbances (Napp-Zinn 1985), the populations were selected to be sufficiently far from roadsides, railroads, and footpaths so that they could reasonably be considered "natural."

We generally followed the guidelines for germination and growth of *A. thaliana* recommended by the *Arabidopsis* Biological Resource Center (2008). We germinated seeds under laboratory conditions and used selfed-seeds produced by these plants in our experiment to minimize maternal effects. We soaked the seeds in tap water on Whatman™ Grade No. 2 moist filter paper in 16 x 50 mm BD Falcon™ tissue culture dishes and exposed them to a seven-day dark stratification treatment at 4°C to facilitate germination. We then planted them in 3.25 x 3.25 x 5 cm pots on two high-intensity light racks (approximately 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ photon flux) at the University of Tennessee - Knoxville. While the light intensities might have been slightly different in the center versus the periphery of the racks, the experimental design was fully randomized to prevent any spatial confounding. We also rotated the three shelves within each rack weekly to homogenize the light conditions.

Rather than potting soil, we used a 50:50 mixture of river sand and vermiculite to ensure low baseline soil nutrient levels. When the seeds in a pot failed to germinate, we transplanted a seedling of the same maternal seed family into that pot. The seedling came either from another pot with extra germination or from extra seeds left over from planting, which had germinated in the tissue culture plates and had been kept moist at room temperature since then.

We set the photoperiodic regime to that typical of the Netherlands, roughly in the middle of the geographic range from which these populations were collected. During seed germination, we set the photoperiod to September 15th (12 hours and 44 minutes of light), approximately when winter annual ecotypes of *A. thaliana* would be expected to germinate in the field, and used constant room temperature (around 25°C) during daytime and nighttime, which facilitates germination (*Arabidopsis* Biological Resource Center 2008). Twenty-five days later, we placed the plants into a walk-in refrigerator for rosette vernalization, with a daytime/nighttime temperature of 4°C and a photoperiod of January 4th (seven hours and 47 minutes of light). After six weeks, we returned them to room temperature during daytime and nighttime and set the photoperiod to April 30th (14 hours and 52 minutes of light). These conditions are a compromise between the need for as natural a setting as possible and the inevitable logistical limitations of experimental designs.

Our experiment included plants from three regions (Northern Spain, the Netherlands, and Southern Sweden), within which we had sampled four populations from Northern Spain (three molecularly distinct maternal seed families per population; Cruzan et al. unpubished), two populations from the Netherlands (three distinct maternal seed families in one population and two in the other), and three populations from Southern

Sweden (two distinct maternal seed families in two populations and three maternal seed families in the other; Table 1). The number of replicates for each family-treatment combination averaged 6.5 and there were a total of 625 viable plants.

We added nutrients in the form of Scott's™ Osmocote® Classic 14-14-14 time-release nitrogen-phosphorus-potassium prills applied to the sand-vermiculite surface. All plants received one prill 11 days after planting. The high-nutrient plants received another seven prills 23 days after planting, about the time the first true leaves appeared. There was some variation in prill size, which probably contributed somewhat to the residual variance in the analyses. Due to the desiccation caused by the fluorescent lights and the poor water-retention ability of the sand-vermiculite mixture, we sub-irrigated the plants twice daily. Therefore, the prills may have expunged their nutrients faster than the three-to-four month time interval indicated by the manufacturer, and in more of a series of pulses than in a continual disbursement. In spite of this variation, the nutrient levels were still quite high in the high-nutrient treatment, and were sufficient for growth in the low-nutrient treatment, approximately three months into the experiment (average low nutrients - 6.75:18:129 ppm NPK; average high nutrients - 49.5:46.25:180 ppm NPK).

Clipping for AMD was done at the time of bolting, when the inflorescences were in the unopened flower bud stage. The entire inflorescence was clipped off at the base of the rosette with scissors while we were careful not to remove or damage any rosette leaves. All plants that survived germination and/or transplanting bolted.

After the reproductive period, we measured the following traits that were potentially affected by AMD and the interaction of AMD with nutrient levels: 1) number of basal inflorescences, the inflorescences growing out of the rosette; 2) number of lateral branches, the secondary and higher-order branches off of the basal inflorescences; 3) number of fruits, an estimate of lifetime reproductive fitness; 4) number of total viable seeds, an integrated estimate of lifetime reproductive fitness, calculated as fruit production times the average number of seeds per fruit (determined from a sample of 5 fruits per plant) times the proportion of viable seeds (determined from a sample of 20-40 seeds tested for germinability per plant); for instances where a plant did not produce fruits, or produced fruits but no seeds or produced non-viable seeds, we set the number of viable seeds to zero.

Analysis of variance---We analyzed the data with a mixed model analysis of variance (ANOVA) for the following traits: number of basal inflorescences, number of lateral stems, bolting to fruit ripening time, number of fruits, and total viable seed production. We improved normality, homoscedasticity and kurtosis (Sokal and Rohlf 1995) by performing the following data transformations: $\log_{10}$ transformation of number of basal inflorescences, number of lateral branches, fruit ripening time, and total viable seed production. Analyses were performed with JMP IN version 5.1 using the method of moments approach (SAS Institute, Inc. 2003). For each trait, the full model included: region (fixed effect), population nested within region (random effect), maternal seed family nested within population nested within region (random effect), nutrient levels (fixed effect), AMD treatment (damaged vs. undamaged; fixed effect), all possible interaction effects among those factors, light rack (overall effects of one light rack versus the other; fixed effect), and transplant status (non-transplants, pot-to-pot transplants, or Petri dish to-pot transplants; fixed effect). For the model for fruit ripening time we excluded the family x treatment interactions because some plants failed to reach maturity

after bolting, and therefore not all maternal seed families were represented by every nutrient level-AMD treatment combination for this trait.

We did not use Bonferroni correction due its conservativeness (i.e., lack of power). Following Moran (2003), we instead report *p*, the probability of finding a particular number of significant test results using the equation

$$p = \left(\frac{N!}{N-K} K! \right) \alpha^K (1-\alpha)^{N-K}$$

where K refers to the α value (0.05), and N is the number of tests performed under the null hypothesis of no true effect (for examples, see Bossdorf et al. 2004; Muth and Pigliucci 2007).

In order to account for the possibility that our results might not reflect nature and instead derive from growing the plants in non-native photoperiodic regimes, we determined whether rosette size affects the response to AMD. An *A. thaliana* rosette responding to novel vernalization or photoperiodic regimes might grow more, or fewer, rosette leaves than it would in its native environment, leading to a concomitant increase, or decrease, in the number of quiescent shoot meristems able to respond to AMD. By determining whether rosette size affects the multivariate response to AMD, we tested if our findings were sensitive to rosette size, which might be altered by the experimental conditions as compared to native conditions. We re-ran the models for the number of basal inflorescences, fruit production, and total viable seed production in an analysis of covariance (ANCOVA; Sokal and Rohlf 1995) with rosette size. To represent rosette size, we performed principal components analysis on the covariance matrix of standardized values (Dillon and Goldstein 1984, p. 36) of rosette diameter and the number of rosette leaves at bolting, and used the first principal component (Somers 1989), which accounted for 83% of the variation, as a compound measure of size.

Regression analysis---We tested whether basal inflorescence proliferation limits fitness by regressing basal inflorescence production on our better fitness proxy (total viable seed production; Rautio et al. 2005). We performed this analysis on subsets of the data according to the region of origin of the plants as well as the nutrient level treatments. We used the same data transformations as in the ANOVAs.

RESULTS

Phenotypic variation---Nutrient levels, apical meristem damage (AMD) treatment, light rack, transplant status, and the region of origin x nutrient x AMD interaction all had influence on at least some of the traits, when controlling for the number of simultaneous tests performed (column *p*, Table 1). Analysis of covariance models including rosette size did not differ from analysis of variance (ANOVA) models without it, in terms of model R^2, other significant model factors, interactions, and their visualized trends. Therefore, we only present the results from the ANOVAs.

All ANOVAs were highly statistically significant, and explained between 33% (for basal inflorescence number) and 54% (for fruit ripening time) of the total phenotypic variance in our samples. Effects of light rack and transplant status were significant in several models (Table 1).

Nutrient addition increased the number of basal inflorescences and lateral (i.e., higher order) branches (Figure 2). Removal of the main inflorescence (AMD) also increased the number of basal inflorescences, although it had no effect on the number of lateral branches (Figure 2). There was no significant variation among plants from different regions, populations, maternal seed families, or nutrient level treatments in the effect of AMD on basal inflorescence number or lateral branch number (Table 1).

AMD delayed fruit ripening in all plants (Figure 3), but especially the Dutch plants grown under low nutrients (region x nutrients x AMD $P < 0.05$, Table 1 and Figure 3). Nutrients boosted fruit production in all plants, while AMD reduced fruit production in most (Figure 3, Table 1). The only exception was that added nutrients seemed to allow for greater fruit production in the damaged than in the undamaged Dutch plants (region x nutrients x AMD $P < 0.05$, Table 1 and Figure 3). Nutrients appeared to enhance total viable seed production, but any positive effect was highly dependent on the region of origin of the plants and on whether AMD occurred (region x nutrients x AMD $P < 0.05$, Table 1 and Figure 3). In the Spanish and Swedish plants total viable seed production was, on average, higher, but the Swedish population did not respond at all to nutrients if it experienced AMD (Figure 3). In contrast, the Swedish low nutrient plants showed very little response to AMD, probably because its undamaged total viable seed production was so low to begin with.

Spanish plants showed undercompensation at both low and high nutrient levels (Figure 3g). Dutch plants showed exact compensation at both nutrient levels (Figure 3h), although they appeared to show undercompensation at low nutrient levels and overcompensation at high nutrient levels when the more crude fitness proxy was used (Figure 3e). Plants from Southern Sweden showed exact compensation at low nutrient levels and undercompensation at high nutrient levels (Figure 3i), although they appeared to show exact compensation at both high and low nutrient levels when the more crude fitness proxy was used (Figure 3f).

When comparing the results to the two models of tolerance, Spanish plants did not change in tolerance between low and high nutrient levels whether we used the better or more crude fitness proxy, which was not consistent with either of the models (Figures 3d and 3g). When we used the better fitness proxy, Dutch plants also did not differ in tolerance at low versus high nutrient levels (Figure 3h), which was not consistent with either of the models. When using the more crude fitness proxy, however, we found that Dutch plants were more tolerant at high than at low nutrient levels (Figure 3e). This was consistent with the continuum of resources model-limiting resources model (CRM/LRM-IV). When using the better fitness proxy, we found that Swedish plants were more tolerant at low nutrient levels than at high nutrient levels (Figure 3i), consistent with the growth rate model (GRM). When using the more crude fitness proxy, however, they appeared not to differ in tolerance at low versus high nutrient levels (Figure 3f), which was not consistent with any of the models.

Regression analyses---At high nutrient levels, there was no significant relationship between basal inflorescence number and total viable seed production for plants from any region; at low nutrient levels, however, there was a weak significant positive relationship for Swedish plants ($R^2 = 0.14$, $P < 0.0001$).

DISCUSSION

General patterns---We found that apical meristem damage (AMD) delayed the time to fruit ripening substantially, and Dutch plants at low nutrient levels were even more affected than the others. Considering that *A. thaliana* is an opportunistic ruderal species (Napp-Zinn 1985), and that changes in its timing of germination can have dramatic effects on fitness in the field (Donohue 2002), AMD-induced delay to mature seed set could translate into reduced fitness in natural situations. Delayed flowering and fruiting as a cost of tolerance to damage has been recognized in other studies (Bergelson and Crawley 1992b; Hanley and Fegan 2007; Lennartsson et al. 1998; Huhta et al. 2000b; Juenger and Bergelson 2000; but see Paige and Whitham 1987).

This study demonstrates for the first time the existence of genetic variation in tolerance in natural populations of *Arabidopsis thaliana*, although previously genetic variation had been demonstrated in recombinant inbred lines (Weinig et al. 2003a). This highlights the ecological relevance of studying tolerance to AMD using this model system. The genetic variation in tolerance we observed was only detected among plants from different regions, and not among plants from different populations within regions or among different maternal seed families within the same population. The reason for this spatially coarse-grained differentiation in tolerance is worthy of further investigation; it is probably not due to genetic drift, because Banta et al. (2007) found that neutral molecular differentiation is not associated with either phenotypic differentiation or geographic distances in *A. thaliana* plants collected from these same areas.

Our results show that it is not always safe to assume that fruit production will yield the same results as more refined estimates of fitness, contrary to Westerman and Lawrence (1970) and to common practice in *Arabidopsis* research. We found that "tolerance" sometimes changed appreciably depending on whether it was estimated with the better fitness proxy, total viable seed production, or a more crude one, fruit production. Furthermore, basing our conclusions on the more crude fitness proxy would have altered which models were supported. In particular, an apparent instance of overcompensation, Dutch plants at high nutrients, disappeared when the better fitness proxy was used. This illustrates the need to use more refined estimates of fitness in evolutionary ecology studies (see also Hanley and Fegan 2007). Accordingly, we will only further discuss the results using the better fitness proxy.

Although AMD caused an increase in basal inflorescence number, regardless of the region or population of origin, maternal seed family, or nutrient-level treatments of the plants, this did not translate into an increase in fitness with damage. This could be accounted for by the fact that apically damaged plants had the same total number of lateral branches (i.e., secondary and higher-order branches off of the basal inflorescences) per plant as undamaged plants, meaning that there were actually fewer lateral branches per basal inflorescence on damaged plants, given that damaged plants had more total inflorescences. These same branching patterns were found in Banta and Pigliucci (2005) for different *A. thaliana* accessions. Therefore, it seems that in *A. thaliana* AMD causes a proliferation of the modules produced for growing fruits and seeds (i.e., basal inflorescences), but that these modules also contain less "real estate" for fruits and seeds (i.e., fewer lateral branches) as compared to undamaged plants.

Comparisons to model expectations---We found that neither of the models (i.e. the growth rate model -- GRM, or the continuum of resources model-limiting resources

model version IV -- CRM/LRM-IV) consistently fit our results across the wide area of origination of our samples. Interestingly, this same finding was also made in two other studies comparing these models with other species (Marshall et al; 2008, Suwa and Maherali 2008). The only model to receive any support in our study was the GRM, and only then for Swedish plants. This is consistent with the meta-analysis by Hawkes and Sullivan (2001), which found that dicotyledonous herbs, such as *A. thaliana*, are more likely to show greater tolerance at lower than at higher nutrient levels.

We did not find support for the CRM/LRM-IV (again, discounting the more crude fitness proxy, which is preumably less acurate) because our results show that two of the model's assumptions were not met. First of all, low nutrient levels limited fitness in only the undamaged Swedish plants. This illustrates that the relationship between a resource and fitness should not be generalized across an entire species. Second, we found that the cause of fitness limitation did not switch from nutrient availability at low nutrient levels to the number of shoot meristems at high nutrient levels, contrary to what is predicted by the model. While there was a significant positive relationship between basal inflorescence number and fitness for Swedish plants, this was only true at low nutrient levels. This is opposite of the assumptions of CRM/LRM-IV.

The complete LRM provides a broad paradigm that encompasses contrasting assumptions (e.g., the "focal resource" versus the "alternate resource" limiting plant fitness in various circumstances; Wise and Abrahamson 2005) and therefore makes a wide variety of predictions regarding tolerance in different environments. As a result of this flexibility, it can be fit to most data sets (Wise and Abrahamson 2007). We believe this flexibility results from "the" LRM actually representing a family of models, where every set of paths terminating in a different prediction (Wise and Abrahamson 2005, Figure 2) in effect corresponds to a different model.

Because we chose to only test one model from the LRM family (i.e., the one resulting in prediction IV; Wise and Abrahamson 2005, Figure 2) we cannot test other LRM-family models in an unplanned fashion. These other models, with more appropriate assumptions, will probably be more successful in accounting for tolerance in future studies with these populations. Even considering this possibility, however, it will still be difficult to achieve any species-wide generalization. Selecting different models to test against different datasets without specific ecological information about the particular populations, let alone the species, being studied does not seem useful. We could not have anticipated, for example, which models to test against our data unless we knew, for example, which particular populations were nutrient limited and whether active shoot meristems were limiting for those populations and under what circumstances. This sort of problem is common in biology, due to the contingent evolutionary histories of living systems (Pigliucci 2002), and illustrates that there is probably no one set of assumptions able to account for the tolerance of all plants, even for different populations of a single species (see Hawkes and Sullivan 2001 for a discussion).

An approach to mitigating this problem is to test all of the available models, regardless of their underlying assumptions; in that case, models with different assumptions might make the same prediction, and therefore it would not be possible to claim support for one model to the sufficient exclusion of all the others. Of course, if a shotgun approach to model testing is combined with post-hoc evaluation of the models' assumptions, this would be helpful in discriminating among the models. Wise and

Abrahamson (2007) use just such an approach to validate the LRM family of models against data from various studies. While their descriptive evaluation is heuristically useful, "alternate resource limitation" and "tolerance" were measured using the same variable (the slope of the fitness proxy or biomass reaction norm across damage treatments). The most rigorous post-hoc evaliations of model assumptions from the LRM family require that one measure "alternate resource limitation" independently of "tolerance," as we did by measuring it in terms of shoot meristem limitation of fitness. Aside from our study, to our knowledge this has not been done before to evaluate the LRM-family models (but see Rautio et al. 2005).

We could not evaluate the assumptions of the GRM, namely that the growth rate of the plants is far below the species' maximum at low nutrient levels and that it is at the maximum at high nutrient levels, because our experiment was not designed to do so. This, however, would be interesting, since we found contingent support for the GRM from our data (for Swedish plants). Such test could be carried out by growing plants across a gradient of a much wider set of nutrient levels, ranging from very low to very high and encompassing the limits of the reaction norms of the plants in question.

There are two caveats to interpreting our findings. One is that the significant effects of region in our analyses might be inflated due to non-random sampling within regions. Although we grouped populations together into what we called "regions" to distinguish plants originating from different latitudes, our collections were very localized within those areas. Therefore, there might actually be more phenotypic variation among populations of a given region than evident from this study, perhaps because of habitat variation at finer spatial grain sizes that does not translate into systemic differences at coarser grain sizes. While we cannot rule out this possibility, it does not affect our conclusion that tolerances to AMD, and the effect of nutrient levels on them, are not invariant across the landscape.

Another caveat is that our results might not reflect nature and instead derive from growing the plants in non-native conditions. Because we focused our research effort and statistical power on the effect of inorganic soil nutrient levels on tolerance, we held other environmental conditions constant, even though these naturally do vary, and covary, among populations in the field. However, this sort of problem affects all laboratory experiments using multiple populations, and it simply underscores the complementary roles of studying plant populations *in situ* as well as in the laboratory (see Nuismer and Gandon 2008 for an in-depth discussion). Nonetheless, one possible scenario that we can exclude is that the non-native photoperiodic or temperature regimes enhanced or inhibited growth, and thereby created more, or fewer, basal meristems than the natural phenotype, which in turn affected the tolerance response as mediated by the number of available shoot meristems. If this were the case, then using rosette size, which is presumably related to the number of quiescent meristems, as a covariate in the analyses should have altered the results, as compared to when rosette size was not included; we found, however, that rosette size had no effect on the results.

Concluding Remarks---Our results suggest that tolerance to apical meristem damage, and the effect of nutrient levels on it, is not uniform across the landscape for *Arabidopsis thaliana*, and that the models we tested are inadequate to account for this non-uniformity. The basic problem is that the assumptions of these models do not necessarily apply species-wide. Deploying more models, such as those from the limiting

resources model-family, could help to explain the divergent effects of nutrient levels on tolerance in this and other species, but this would require extensive ecological information about the individual populations of study to ensure that the models, and their underlying assumptions, are appropriate for those populations. It seems that at least sometimes ecology is in fact a rather local affair.

Table 1. Analyses of variance for all traits. We report R^2, degrees of freedom (df), mean squares, and P-values associated with the F-ratio tests. Instead of a multiple-test correction, we report for each factor (row) *p*, the probability of finding by chance the observed number of P-values below $\alpha = 0.05$ given the number of tests performed. Significant factors are highlighted in boldface, but only if, for that factor, there was a significant chance of finding the observed number of P-values below $\alpha = 0.05$ given the number of tests performed (see Moran 2003).

	Basal Inflorescence Number	Lateral Branch Number	Fruit Ripening Time	Fruit Production	Total Viable Seed Production	*p*
R^2	0.33	0.39	0.54	0.52	0.44	
Region; df = 2	0.095 (0.446)	0.072 (0.915)	0.50 (0.059)	223.05 (0.065)	20.35 (0.172)	0.774
Population [Region]; df = 6	0.11 (0.630)	0.83 (0.426)	0.16 (0.040)	55.13 (0.102)	9.31 (0.144)	0.204
Family [Population, Region]; df = 15	0.10 (0.362)	0.63 (0.110)	0.025 (0.0003)	6.68 (0.731)	2.10 (0.635)	0.204
Nutrient Levels (NTL); df = 1	**5.06 (0.00019)**	**32.15 (< 0.0001)**	**0.20 (0.012)**	**948.29 (< 0.0001)**	**46.80 (0.004)**	**< 0.0001**
Apical Meristem Damage (AMD); df = 1	**1.53 (0.0019)**	0.051 (0.624)	**1.97 (< 0.0001)**	**45.86 (0.011)**	**9.60 (0.032)**	**< 0.0001**
NTL x AMD; df = 1	0.22 (0.074)	0.79 (0.106)	0.032 (0.106)	16.48 (0.050)	0.018 (0.881)	0.204
Region x NTL; df = 2	0.085 (0.443)	0.23 (0.568)	0.065 (0.106)	25.85 (0.205)	0.33 (0.886)	0.774
Region x AMD; df = 2	0.17 (0.157)	0.41 (0.184)	0.089 (0.031)	0.108 (0.976)	0.44 (0.737)	0.204
Region x NTL x AMD; df = 2	0.016 (0.736)	0.10 (0.669)	**0.054 (0.026)**	**23.51 (0.013)**	**4.06 (0.036)**	**0.0011**
Population x NTL; df = 6	0.096 (0.287)	0.39 (0.301)	0.028 (0.149)	13.43 (0.191)	2.84 (0.261)	0.774
Population x AMD; df = 6	0.071 (0.499)	0.19 (0.648)	0.021 (0.241)	4.13 (0.560)	1.41 (0.418)	0.774
Population x NTL x AMD; df = 6	0.048 (0.532)	0.24 (0.357)	0.012 (0.365)	2.88 (0.825)	0.76 (0.436)	0.774
Family x NTL; df = 15	0.054 (0.496)	0.13 (0.765)	-	7.82 (0.309)	1.99 (0.029)	0.171
Family x AMD; df = 15	0.078 (0.234)	0.22 (0.394)	-	7.61 (0.327)	1.25 (0.149)	0.815
Family x NTL x AMD; df = 15	0.054 (0.710)	0.20 (0.648)	-	6.01 (0.647)	0.72 (0.668)	0.815
Light Rack; df = 1	**0.62 (0.0029)**	**2.19 (0.0024)**	0.000049 (0.830)	**484.42 (< 0.0001)**	**11.67 (0.0003)**	**< 0.0001**
Transplant Status; df = 2	0.013 (0.834)	0.22 (0.403)	0.0073 (0.934)	**21.88 (0.050)**	**3.54 (0.019)**	**0.021**
Error	0.069; df = 524	0.24; df = 526	0.011; df = 408	7.27; df = 526	0.89; df = 509	

Figure 1. Locations of origin of the *Arabidopsis thaliana* populations used for this study. "SP," "NL," and "SW" signify Spanish, Dutch, and Swedish populations, respectively.

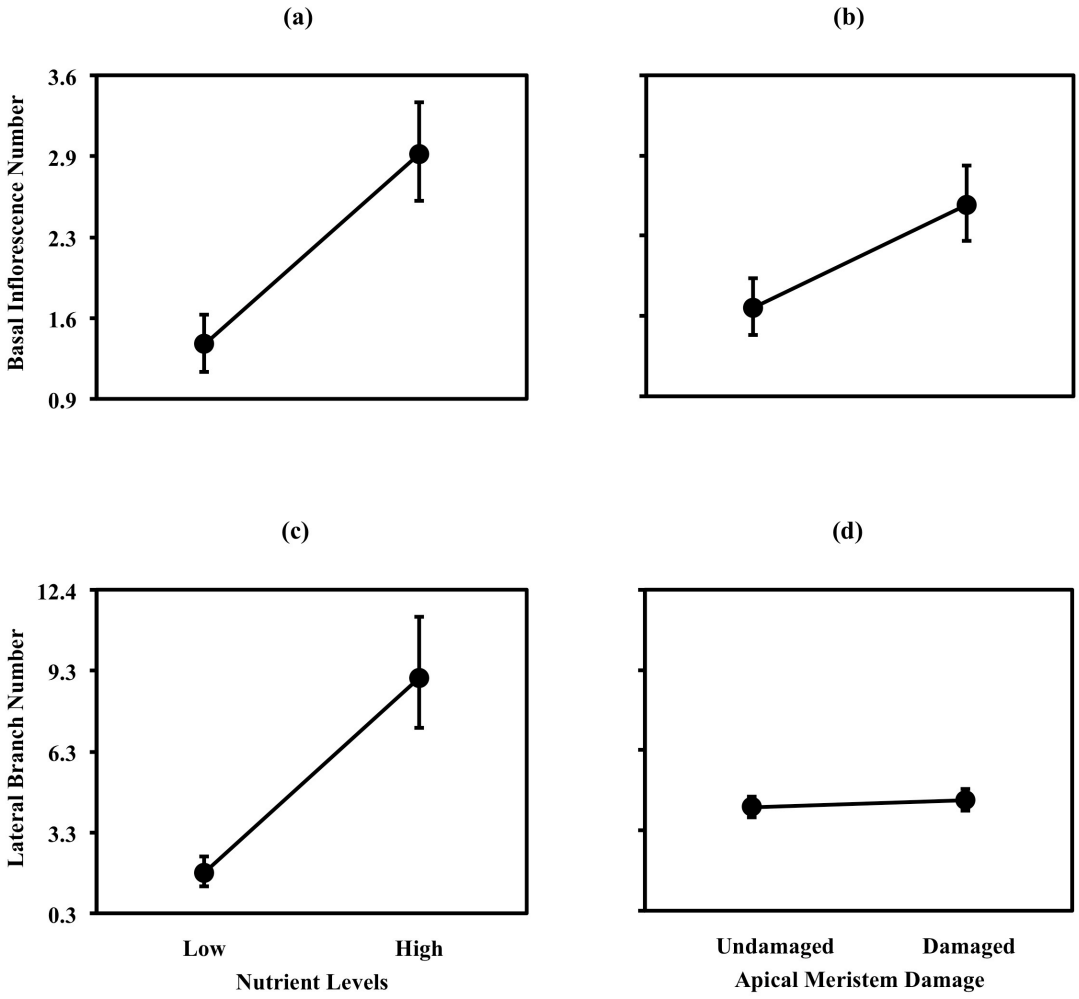


Figure 2. Least squares mean values of basal inflorescence number (top) and lateral branch number (bottom) according to the nutrient levels (left) or apical meristem damage (right) treatment. The error bars are the 95% confidence interval. Apical meristem damage did not have a significant effect on lateral branch number (d).

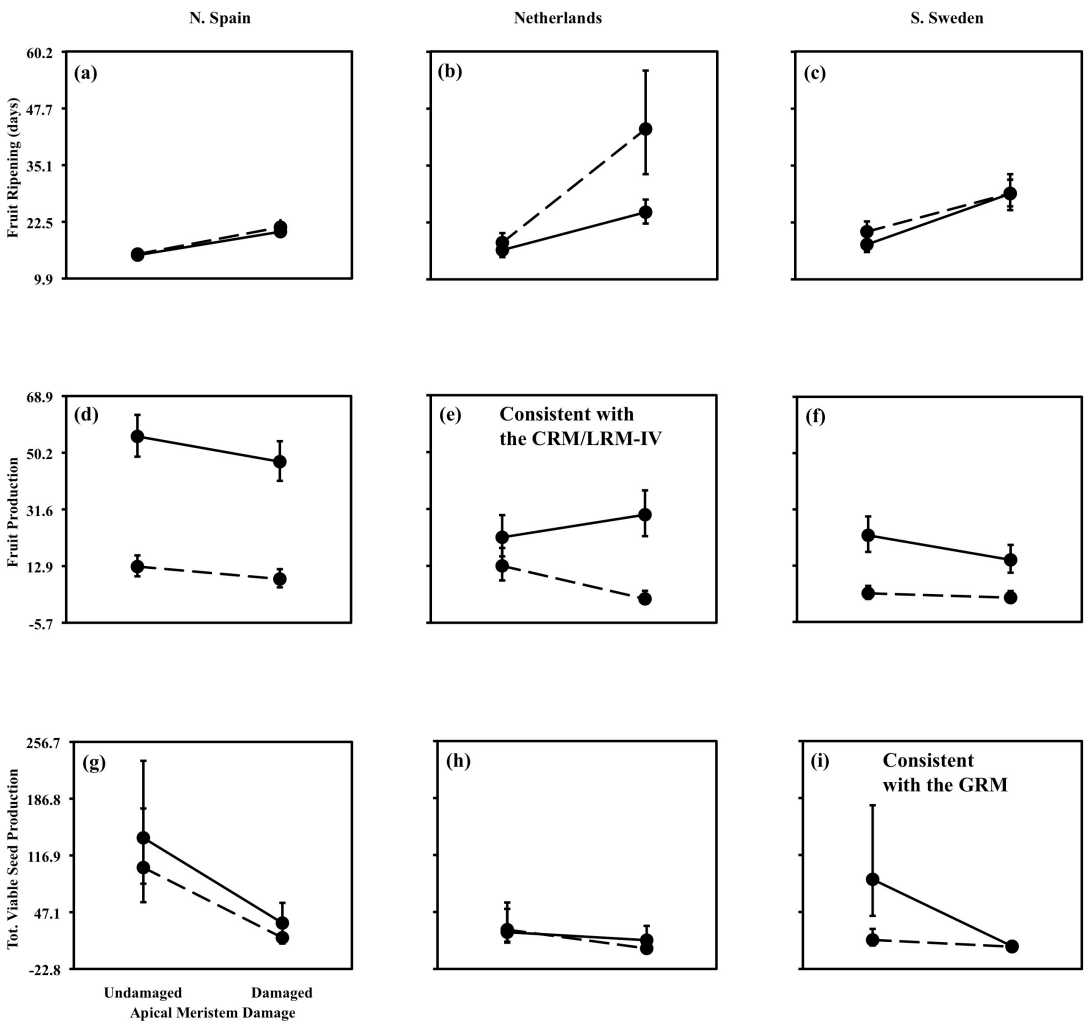


Figure 3. Least squares mean values of fruit ripening time (top row) fruit production (middle row), and total viable seed production (bottom row) for plants from Northern Spain (left column), the Netherlands (middle column), and Southern Sweden (right column). Solid lines are high nutrient levels and dotted lines are low nutrient levels. The error bars are the 95% confidence interval, although they are sometimes too small to be visible.

II. Heterogeneity in the developmental mechanisms of tolerance to apical meristem damage in *Arabidopsis thaliana*

Abstract

To understand the developmental mechanisms that account for tolerance to apical meristem damage (AMD) in *Arabidopsis thaliana*, we employed path analysis to model the relationship among traits related to tolerance. We grew plants at high and low levels of inorganic soil nutrients in order to examine the environmental lability of the developmental mechanisms. Because reliable estimation of tolerance hinges on accurate estimation of fitness, we also examined how well coarser fitness proxies predict more refined proxies closer to true fitness. We found that inflorescence development is altered in apically damaged plants, and that nutrient level variation changes development. We also found that populations qualitatively differ in the relationships among tolerance traits. In terms of the predictive utility of different fitness proxies, we found that whole-plant fruit production predicts seed production with high fidelity; however, we also found that the relationship between fruit production and total viable seed production, and between seed production and total viable seed production, is much weaker. Our study provides an example of how the underlying developmental pathways leading to an ecologically important plastic response can vary depending on the environments and populations under consideration. Furthermore, as in chapter one, we provide more reason for caution when extrapolating from coarse fitness proxies to true fitness.

INTRODUCTION

We examined the developmental pathways leading to tolerance in natural populations of *A. thaliana* using architectural traits directly affected by AMD and downstream components of fitness. We also varied inorganic soil nutrient levels to understand the lability of the developmental processes. We were interested in the following questions: (1) Is inflorescence development the same in undamaged and re-generated inflorescences? (2) Is inflorescence development altered by nutrient level variation? (3) Is inflorescence development the same for plants from different genetic backgrounds?

As mentioned above, we measured multiple fitness components in addition to the usual standard, fruit production (Pigliucci 2003). Westerman and Lawrence (Westerman and Lawrence 1970) found that *A. thaliana* fruit production predicted seed production with great fidelity, but to our knowledge the robustness of this relationship has never been examined across multiple environments, and the ability of seed production to predict whole-plant viable seed production, which is presumably more closely related to true fitness, has never been quantified in this species. Therefore, we also investigated the predictive relationships among these fitness components at different nutrient levels.

MATERIALS AND METHODS

Plant material and handling, and experimental protocol---This study was an analysis of the same data collected in chapter one.

Fitness components relationships---We used a series of linear regression analyses to gage the ability of fruit production to predict seed production and viable seed production, and the ability of seed production to predict viable seed production; analyses were carried out using JMP IN version 5.1 (SAS Institute, Inc. 2003). We performed the regressions on the overall data set, as well as on subsets of the data according to the experimentally imposed nutrient levels and AMD treatments, so as to assess the relationship among fitness components as a function of the experimental treatments experienced by the plants. We used the same trait transformations as in the path analysis.

Path analysis--- We created a path model and performed structural equation modeling (Shipley 2000) using number of basal inflorescences, number of lateral stems, number of fruits, average number of seeds per fruit, and seed germinability. We checked the data for assumptions of normality, homoscedasticity and kurtosis (Sokal and Rohlf 1995), and found that the following transformations were necessary: $\log_{10}$ transformation of number of basal inflorescences and number of lateral branches, square root transformation of fruit production, and arcsine-square root transformation of seed germinability. We standardized the data for each trait to a mean of zero and a standard deviation of one.

Using AMOS version 7.0 (Arbuckle 2003), we employed a phenotypic path model (Figure 1) assuming the following causal sequence: basal inflorescence development precedes development of lateral inflorescences, which precedes development of fruits. After the plants initiate fruit development, they commit to making a certain number of seeds and then provision them with resources (where germinability is a proxy for maternal provisioning).

In two separate multi-group model analyses (Shipley 2000), we modeled (a) each population (across treatments) and (b) every nutrient level-AMD combination regardless of population. We did not perform a multi-group analysis with every possible population-nutrient levels-AMD combination, because this would have decreased statistical power considerably. In developing the best-fit models, each path of the fully constrained model (one assuming equal regression weights across different populations or across different nutrient level-AMD combinations) was sequentially relaxed to assess improvements in model fit (measured as a statistically significant decrease in X^2). Paths were retained as unconstrained when this resulted in a significantly improved model fit. The resulting model performances were evaluated according to the following criteria:

1. *Root mean square error of approximation* (RMSEA). Together with its confidence interval and P-value for the hypothesis of a RMSEA no greater than 0.05 (close fit; Arbuckle 2003), this statistic takes model complexity into account while characterizing its fit to the observed covariance matrix. RMSEA values range from zero to one with models having values approaching zero indicating better fit, or greater parsimony, compared to those with larger values.

2. *Akaike's information criterion* (AIC), a second joint measure of fit and simplicity. The AIC values are given for the reduced model of interest, as well as for saturated and independence models. The saturated model has no degrees of freedom and includes all possible paths, yielding an over-parameterized model with the maximum

possible fit. The independence model assumes independence of all variables and is essentially equivalent to the simple path diagram generated by a multiple regression analysis. Smaller AIC values reflect a better balance of fit and parsimony.

3. Finally, we report the goodness-of-fit using the *comparative fit index* (CFI) in which the covariance of the observed data is compared to that expected assuming that the path model is true. Values close to one indicate good fit, and values greater than 0.9 generally indicate acceptable fit (Bollen 1989).

RESULTS

Fitness component relationships---Fruit production predicted seed production with high accuracy in all nutrient-AMD combinations, with positive relationships and R^2 values ranging from 0.85 – 0.89 (Table 1). However, fruit production predicted total viable seed production less well, with positive relationships and R^2 values ranging from 0.35 – 0.54. Seed production predicted total viable seed production with positive relationships and R^2 values ranging from 0.49 – 0.63. It is interesting to note that, for both the regression of fruit production on total viable seed production and the regression of seed production on total viable seed production, the R^2 values were lowest in the high nutrients-no AMD treatment combination.

Structural equation models---We refer to the model where population of origin is considered regardless of nutrient level-AMD treatment combination as the "across-treatment model," and to the model where treatment combination is considered regardless of the population of origin as the "across-population model." The across-treatment model revealed that populations differed in several path coefficients (Figure 2a). Populations differed in the strength of the direct relationship between lateral branch number and seed germinability (with four populations having a positive relationship and the others having no significant relationship), lateral branch number and seeds per fruit (with three Spanish populations having a negative relationship and the others having none), fruit production and seeds per fruit (with populations differing in the strength of the positive relationship), fruit production and seed germinability (with two populations having a positive relationship, two having a negative relationship and the others having none), and seeds per fruit and seed germinability (with two Swedish populations having a positive relationship and the others having none). The direct relationship between basal inflorescence number and later branch number, basal inflorescence number and seed germinability, and lateral branch number and fruit production was the same for all populations (path coefficients = 0.66, -0.99, and 0.63, respectively). There was no significant direct relationship between basal inflorescence number and seeds per fruit or between basal inflorescence number and fruit production.

Similarly, the across-population model showed that the direct path coefficients changed in several instances depending on the particular treatment (Figure 2, b and c). For both undamaged and damaged plants the direct relationship changed between fruit production and seeds per fruit (where increasing nutrients decreased the positive relationship). For undamaged plants the direct relationship changed between basal inflorescence number and lateral branch number (where increasing nutrients decreased the positive relationship) and between basal inflorescence number and seed germinability (where increasing nutrients turned a negative relationship into no relationship). For

damaged plants the direct relationship changed between seeds per fruit and seed germinability (where increasing nutrients decreased the positive relationship). The direct relationships between lateral branch number and fruit production, lateral branch number and seeds per fruit, and lateral branch number and seed germinability were the same for all plants regardless of the AMD or nutrient levels treatment (path coefficients = 0.52, -0.21, and 0.20, respectively), and there were no direct relationships between basal inflorescence number and fruit production, basal inflorescence number and seeds per fruit, or fruit production and seed germinability.

The best-fit models had root mean square error of approximation (RMSEA) values significantly greater than 0.05. Furthermore, the comparative fit index (CFI) yielded values less than 0.9 (Table 2). This suggests that the covariance structure of the data was not well-accounted for by the multi-group models.

DISCUSSION

The relationship among fitness components---As in chapter one, our results suggest that great caution should be used when researchers extrapolate from fruit production to fitness in this species. An oft-cited justification for this extrapolation is Westerman and Lawrence's (Westerman and Lawrence 1970) finding that fruit production was a very effective proxy for seed production, a more refined fitness component presumed to be closer to true fitness than fruit production. Our study verified this result, but found fruit production to be less correlated with viable seed production, an even more refined fitness component. The ability of seed production to predict viable seed production also did not approach the precision with which fruit production predicted seed production. This suggests that researchers should attempt whenever possible to estimate more iterated measurements of fitness as compared to simple fruit production.

This finding contrasts with the results of Paige and Whitham's (Paige and Whitham 1987) study with *Ipomopsis aggregata* in which the number of seeds per fruit did not differ between damaged and undamaged plants. However, when one considers that *A. thaliana* is a weedy species that produces many more seeds than the environment can accommodate (Napp-Zinn 1985), it is perhaps not surprising that provisioning to seeds in *A. thaliana* can be dramatically affected by changes in environmental conditions. In other words, with little maternal investment in seeds, it seems reasonable that seed development would be unbuffered against environmental stressors like AMD.

The relationship of architectural traits to fitness components---Structural equation modeling suggests that the covariance structure of our data was not well-accounted for by the multi-group models. However, this lack of fit is not surprising, given the group heterogeneity revealed in the two models. When either multi-group model (across-population or across-treatment) was used, the variation attributable to the other grouping scheme (population variation or treatment group variation) was not accounted for, decreasing the fit of the data to the particular model. This can also explain why the direct relationship between fruit production and seed germinability was not significant in the across-population model, whereas it was significant for some populations in the across-treatment model: when variation among populations was not accounted for in the across-population model, the positive relationship between fruit production and seed germinability in two populations was canceled out by the negative

relationship in two other populations.

Basal inflorescence development appears to be altered in apically damaged plants. Although the paths between tolerance traits were the same (with one exception) for undamaged and damaged plants, the strength of the relationships differed for some paths. Specifically, the positive correlations between basal inflorescence production and lateral branch number and between seeds per fruit and seed germinability were stronger for damaged plants. This indicates that the developmental program leading to a particular level of fitness is more flexible in undamaged plants than in damaged plants. In other words, AMD tightens the relationship between plant architectural traits and fitness.

Our study provides evidence that pathways between tolerance traits are altered by variation in nutrient levels in different ways depending on whether or not the plants are damaged. For instance, while nutrient level variation did not have much effect on the relationship between basal inflorescences and lateral branches in damaged plants, it had an appreciable effect on the strength of this association in undamaged plants; the reverse was true for the relationship between seeds per fruit and seed germinability. Interestingly, paths that were sensitive to nutrient levels tended to show a decreased relationship between those traits at high nutrients. Taken together, this suggests that plants are more developmentally flexible at high nutrients, although the points of flexibility are different depending on whether or not the plants are damaged.

Not only does AMD and nutrient level variation alter the relationships among tolerance traits in complex ways, but our study also suggests the relationships are contingent on genetic background. Plants from three of the four Spanish populations, but no others, had a negative relationship between lateral branch number and seeds per fruit, and plants from two of those populations had a negative relationship between fruit production and seed germinability as well. For two other populations, however, one from the Netherlands and one from Sweden, the relationship between fruit production and seeds per fruit was positive rather than negative, and additionally those populations did not have a negative relationship between lateral branch number and seeds per fruit.

Overall, the SEMs indicate that the developmental pathways used to achieve a particular level of tolerance are not easily characterized by a simple developmental model that considers re-grown inflorescences to be developmentally identical to undamaged inflorescences, that assumes development is canalized across nutrient levels, and that treats members of a species as having identical developmental programs, because all of these precepts are violated. Instead, we suggest that tolerance in *A. thaliana* is best understood in the context of specific genetic material and environments, and as such any conclusions are not easily extrapolated to the whole species. This reinforces the same conclusion from chapter one, namely that simple models will be unlikely to characterize tolerance for an entire species. While this might seem antithetical to the goal of explaining patterns in nature using generalizable findings, results such as ours are probably common in ecology due to the inherent complexity and historical nature of living systems (Pigliucci 2002). These results, combined with chapter one, also suggest that tolerance can evolve readily in this species, as evidenced by different relationships between tolerance and the inorganic environment and by the heterogeneity in ontogenetic mechanisms used to re-grow inflorescences after damage.

Table 1. R^2 values of linear regressions of fruit production on seed production, fruit production on viable seed production, and seed production on viable seed production. The regressions are for the overall dataset or for a particular treatment combination subset of the data: low nutrients and no apical meristem damage, low nutrients and apical meristem damage, high nutrients and no apical meristem damage, or high nutrients and apical meristem damage. The following data transformations were used: fruit production$^{0.5}$, seed production$^{0.25}$, and $\log_{10}$(viable seed production).

	Fitness Component	Fruit Production	Seed Production
Overall	Seed Production	0.8755	-
	Viable Seed Production	0.4633	0.5900
Low-No AMD	Seed Production	0.8535	-
	Viable Seed Production	0.5446	0.6304
Low-AMD	Seed Production	0.8776	-
	Viable Seed Production	0.4901	0.6174
High-No AMD	Seed Production	0.8809	-
	Viable Seed Production	0.3522	0.4931
High-AMD	Seed Production	0.8584	-
	Viable Seed Production	0.4118	0.5413

Table 2. Summary statistics for the multi-group structural equation models, either across populations or across nutrient levels-AMD treatment combinations as indicated. The root mean square error of approximation (RMSEA), 90% confidence interval and P-value (where low values indicate significant departure between the observed covariances and those expected from the model structure), and the Akaike information criterion (AIC) both take into account model fit and complexity, penalizing unnecessarily complex models. The comparative fit index (CFI) is a goodness-of-fit index where values close to one indicate very good fit.

	RMSEA		*Saturated*	*Model AIC*	*Independence*	*CFI*
Model	***Estimate C.I.***	***P-value***	***AIC***		***AIC***	
Across Nutrient-AMD Treatments	0.089 - 0.101	< 0.0001	360	906.156	1489.362	0.508
Across Populations	0.115 - 0.134	< 0.0001	160	603.21	1179.76	0.558

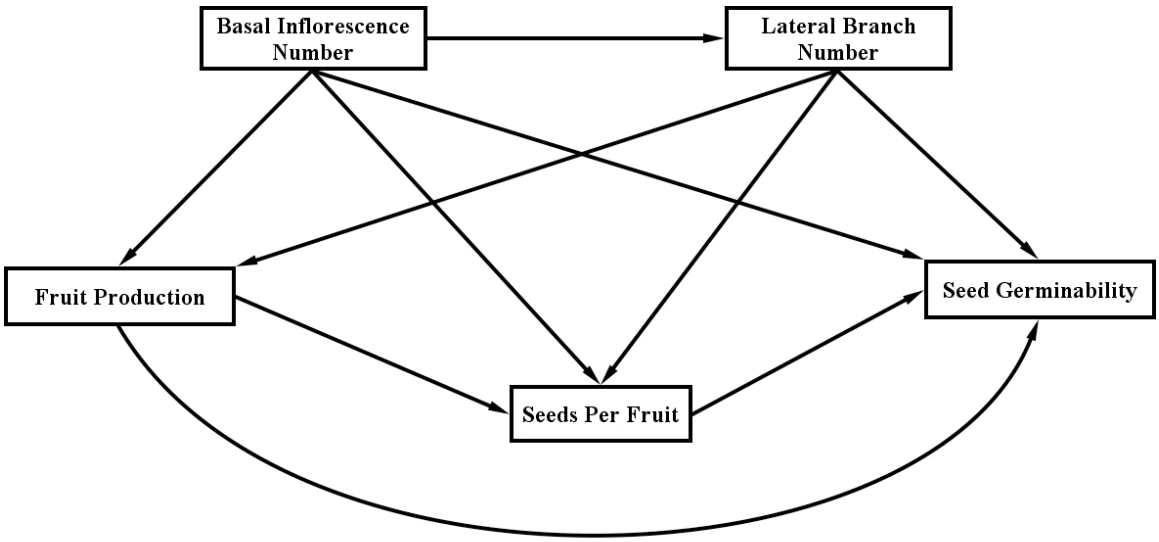
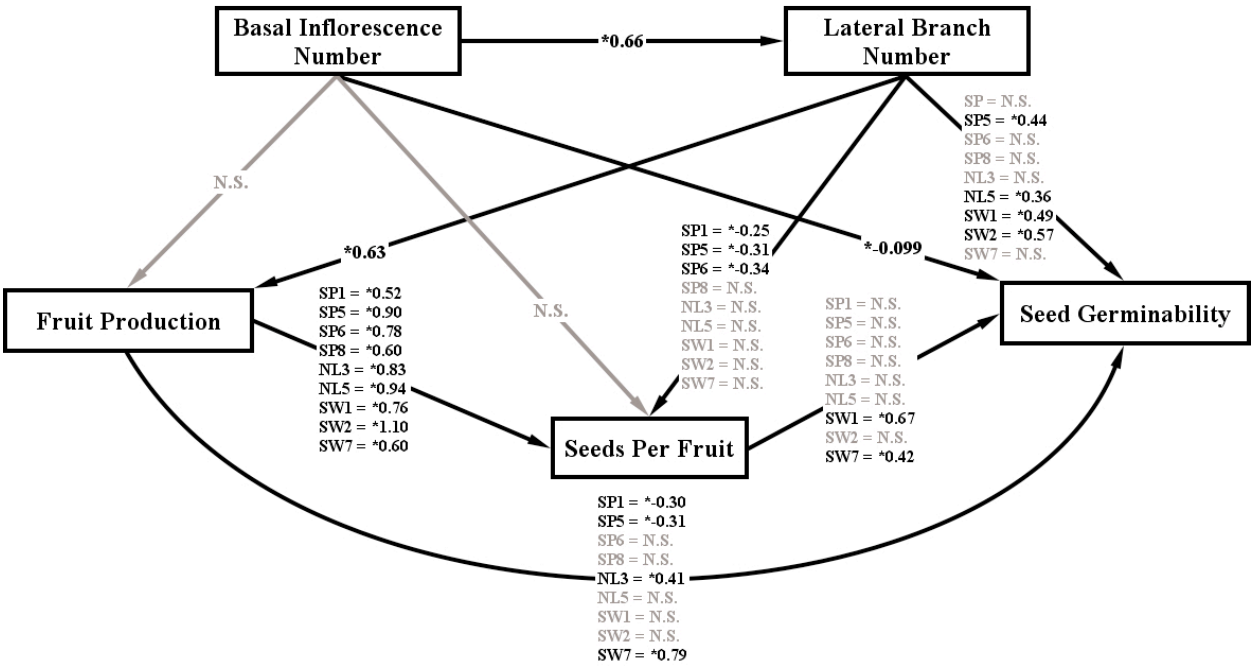
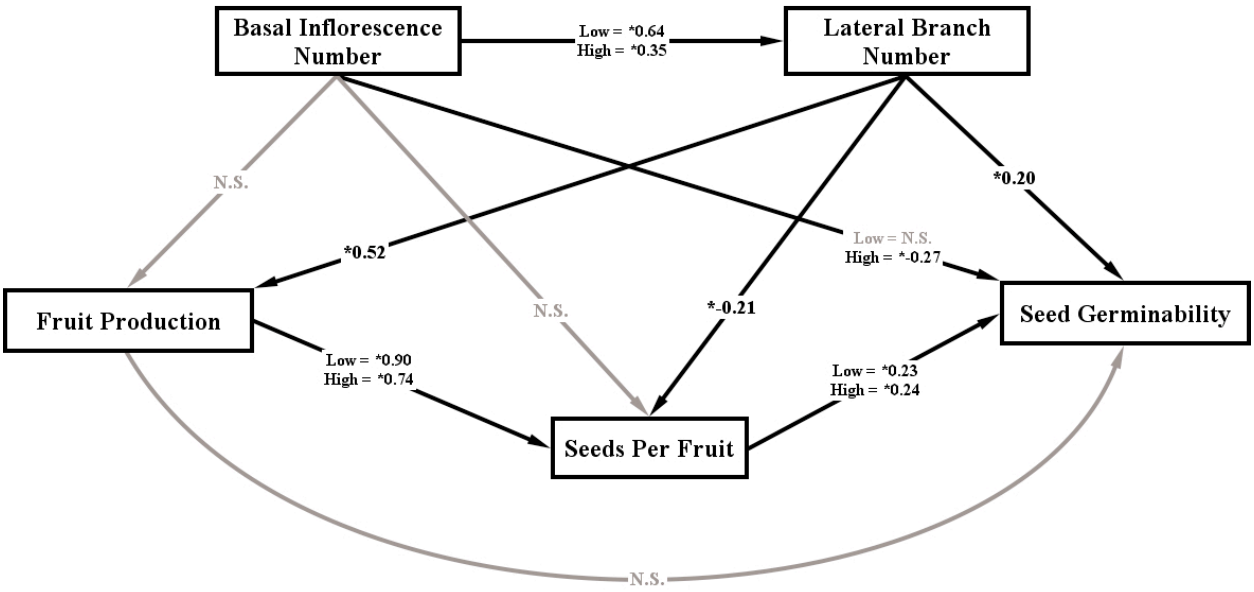


Figure 1. The path analytical phenotypic model used to fit the data for structural equation modeling. The model structure is based on the architecture and development of *Arabidopsis thaliana*.

a


Basal Inflorescence Number
*0.66
Lateral Branch Number
SP = N.S.
SP5 = *0.44
SP6 = N.S.
SP8 = N.S.
NL3 = N.S.
NL5 = *0.36
SW1 = *0.49
SW2 = *0.57
SW7 = N.S.
N.S.
*0.63
SP1 = *-0.25
SP5 = *-0.31
SP6 = *-0.34
SP8 = N.S.
NL3 = N.S.
NL5 = N.S.
SW1 = N.S.
SW2 = N.S.
SW7 = N.S.
*-0.099
Fruit Production
SP1 = *0.52
SP5 = *0.90
SP6 = *0.78
SP8 = *0.60
NL3 = *0.83
NL5 = *0.94
SW1 = *0.76
SW2 = *1.10
SW7 = *0.60
N.S.
Seed Germinability
SP1 = N.S.
SP5 = N.S.
SP6 = N.S.
SP8 = N.S.
NL3 = N.S.
NL5 = N.S.
SW1 = *0.67
SW2 = N.S.
SW7 = *0.42
Seeds Per Fruit
SP1 = *-0.30
SP5 = *-0.31
SP6 = N.S.
SP8 = N.S.
NL3 = *0.41
NL5 = N.S.
SW1 = N.S.
SW2 = N.S.
SW7 = *0.79

b


Basal Inflorescence Number
Low = *0.64
High = *0.35
Lateral Branch Number
N.S.
*0.20
Low = N.S.
High = *-0.27
*0.52
Fruit Production
N.S.
*-0.21
Seed Germinability
Low = *0.90
High = *0.74
Low = *0.23
High = *0.24
Seeds Per Fruit
N.S.

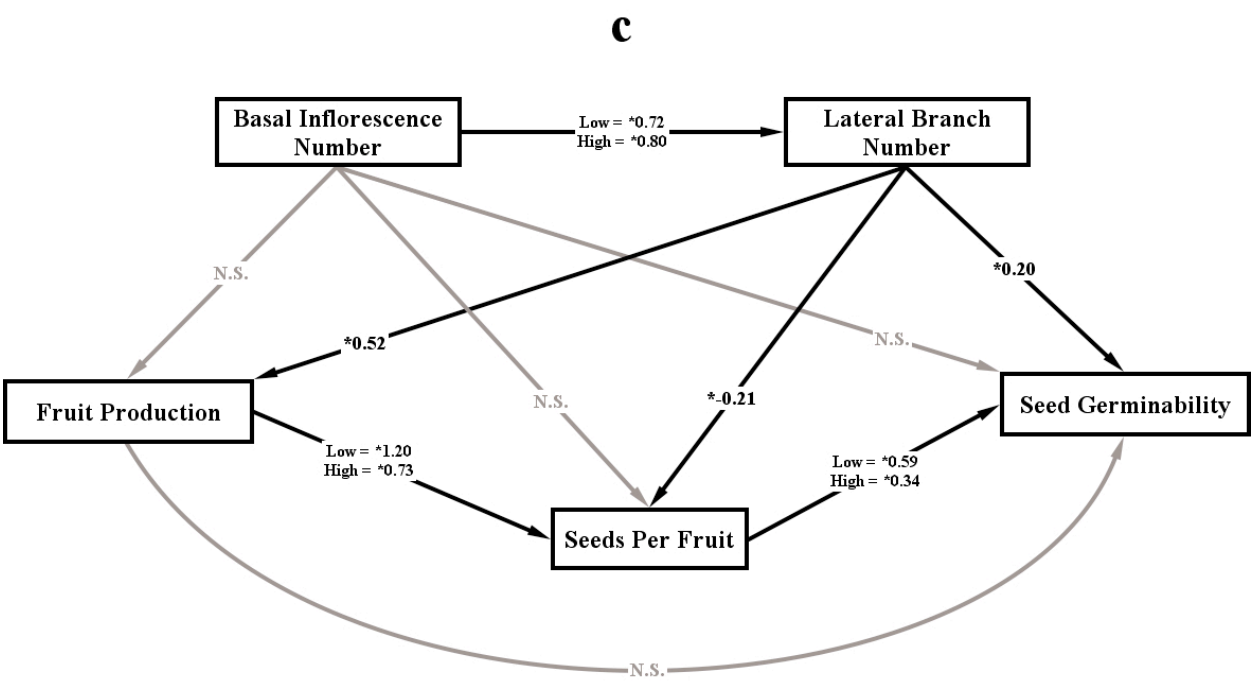


Figure 2. Path models and direct effects of plant architecture traits and fitness components, either (a) across treatments or (b and c) across populations. For part (a), "SP" = Spain, "NL" = Netherlands, and "SW" = Sweden, and the numbers correspond to different population identifiers within regions. Parts (b) and (c) are based on one multi-group model. Part (b) shows path coefficients for undamaged plants at high and low nutrients and part (c) shows damaged plants at high and low nutrients. Path coefficients indicated by an asterisk were statistically different from zero ($P < 0.05$). Multiple coefficients indicate that the relationship between the two traits changes depending on the population or the nutrient levels. “N.S.” and the color gray indicate that a particular path coefficient was not significant.

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Effects of gibberellin mutations on tolerance to apical meristem damage in *Arabidopsis thaliana*

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To examine the role of gibberellin hormones (GAs) in tolerance to apical meristem damage (AMD), we characterized the reaction norms of several GA-deficient and insensitive mutants of *Arabidopsis thaliana* in response to AMD and compared them to those of the wild type, Landsberg, from which they were derived. We included 'natural' genotypes of *A. thaliana* – accessions with shorter lab histories – in order to evaluate how representative Landsberg is of other genotypes. The GA mutations did not alter the level of tolerance to AMD, which was consistent with equal compensation for all genotypes. Generally, the reaction norms to AMD did not differ among the GA mutants themselves, or between the GA mutants and Landsberg. The GA mutations did affect the overall phenotypes of the plants, but these effects were not simply related to whether the mutation was early or late in the biochemical pathways. The GA-insensitive mutant was phenotypically different from the GA-deficient mutants and from Landsberg. The natural populations differed significantly from Landsberg, particularly in attributes related to size and inflorescence production, one more example of the need for researchers to be careful when generalizing the results of studies based upon laboratory strains. Our results indicate that early-flowering genotypes of *A. thaliana* can be remarkably tolerant to AMD, and that GA deficiency/insensitivity does not hinder tolerance to AMD, at least in this genetic background. Moreover, we confirm that mutations at regulatory loci can have noncatastrophic effects on fitness, as recently found by other investigators.
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Introduction

The ecology and evolution of phenotypic plasticity is increasingly being studied from a combined organismal and genetic perspective (Scheiner, 1993; Pigliucci, 1996; Jackson *et al*, 2002). This represents a significant step forward in comparison with the traditional study of plasticity based largely on quantitative genetic methods, which cannot investigate molecular pathways that induce plastic responses (Barton and Turelli, 1989; Pigliucci and Schlichting, 1997). Examples of this new hybrid approach include the examination of the reaction norms of known (and often molecularly characterized) mutants that affect the response to a specific environmental stimulus of interest (Schmitt *et al*, 1995; Pigliucci and Schmitt, 2004). This allows us to see how individual loci that have been previously identified because of their specific phenotypic effects may help modulate a potentially adaptive plastic response.

In this paper, we examine plasticity to apical meristem damage (AMD) in an annual, monocarpic plant, hypothesizing that loci affecting the production of gibberellin (GA) hormones are important in mediating this plasticity. AMD can be caused by vertebrate herbivory among other environmental factors (Belsky, 1986; Paige and Whitham, 1987; Rosenthal and Kotanen, 1994; Juenger *et al*, 2000; Tiffin, 2000). The damage releases apical dominance via a hormonal signal, allowing additional inflorescences to grow from the basal rosette (Paige and Whitham, 1987; Benner, 1988; Mopper *et al*, 1991; Juenger *et al*, 2000). The outcome of this release from apical dominance can be decreased fitness (undercompensation: Bergelson and Crawley, 1992; Huhta *et al*, 2000a, b), little or no change in fitness (compensation: Maschinski and Whitham, 1989; Bergelson *et al*, 1996; Juenger *et al*, 2000), or, somewhat surprisingly, an increase in fitness (overcompensation: Paige and Whitham, 1987; Paige, 1999; Juenger *et al*, 2000, Weinig *et al*, 2003), depending upon how many additional inflorescence branches develop from the rosette after AMD and how many fruits are produced per branch.

In *Arabidopsis thaliana* (Brassicaceae), five GA mutants have been characterized and are available from the *Arabidopsis* seed stock center in Columbus, OH (www.arabidopsis.org). Four of these mutants are deficient in biosynthetic enzymes at different steps in the production of GAs (Koornneef and van der Veen, 1980; Kamiya and Garcia-Martinez, 1999), while the remaining mutant is GA insensitive, meaning that it produces GA but a key receptor is non-functional, so that the signal triggered by the hormones is not transduced (Koornneef *et al*, 1985).

Our aim was to examine the reaction norms of GA mutants under conditions of apical damage or lack thereof in order to investigate if and how GA mediates the effects of AMD. We hypothesized that, in the wild type, GAs are involved in tolerance to AMD by promoting the elongation of proliferating inflorescence branches (Phillips *et al*, 1995, Hedden and Phillips 2000,

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Hay *et al*, 2004) produced after AMD. GA mutants, on the other hand, have a short, bushy phenotype regardless of whether or not they are browsed (Ross, 1994; Ross *et al*, 1997), since they either lack fully functional GAs or are unable to transduce the GA signal. The release from apical dominance that comes with being apically damaged should therefore not be advantageous in GA mutants, since the extra inflorescences produced cannot elongate sufficiently, nor, consequently, produce additional fruits. Therefore, we predicted that AMD would be more detrimental to GA mutants than to the wild type from which they were derived.

Our study included genotypes with either early- or later-acting GA point mutations. The later-stage mutants have less severe GA-deficient phenotypes (Koornneef and van der Veen, 1980; Koornneef *et al*, 1985; Zeevaart and Talon, 1992), and should be more phenotypically similar to the wild type, for example, by more effectively elongating the replacement inflorescences. Therefore, we also predicted that later-acting mutants should show more tolerance to AMD than earlier-acting mutants.

We compared the five GA mutants to the nonmutant (wild type) background genotype from which they were derived, Landsberg *erecta* (Ler-2), which is a laboratory line maintained for decades under controlled conditions. We also included in our experiment three other early-flowering genotypes of *A. thaliana* that have been maintained under laboratory conditions for a much shorter period of time (a few years); these populations are presumably more representative of natural (nonlaboratory) genotypes. We included these accessions in order to evaluate how other *A. thaliana* genotypes respond to AMD. If Ler-2 and these other genotypes react similarly to AMD, then it is plausible to infer that a GA mutation would have similar effects on tolerance in these other genetic backgrounds. On the other hand, it is possible that laboratory lines behave in a highly unusual manner, which should caution evolutionary ecologists from generalizing results based on standard laboratory stocks.

The reason for using only early-flowering genotypes in our study is that early- and late-flowering genotypes of *A. thaliana* are known to be different genetically and ecologically (Zhang and Lechowicz, 1994; Donohue, 2002), and are subject to distinct selection regimes. Therefore, a comparison between early-flowering Landsberg and late-flowering genotypes would not be informative in this context.

Our premises can be summarized in the following questions and expectations: (1) Do mutations in GA signaling alter the tolerance of *A. thaliana* to AMD? We predicted that the GA mutants would be more detrimentally affected by AMD than the wild type. (2) Do the effects of AMD differ for GA mutants deficient at different loci, and are these effects different from those of the GA-insensitive mutant? We expected the later-acting mutants to display a stronger response to AMD than the early-acting or the insensitive mutants (the latter expected to be phenotypically similar to some of the deficient mutants, despite the different genetic basis of its phenotype). (3) Does Ler-2 respond differently to AMD than more natural early-flowering genotypes? Although we did not have strong *a priori* expectations here, it was important to test how much of a good model standard stocks, such as Landsberg, actually are at providing us with generalizations about *A. thaliana*.

Materials and methods

Plant material

A. thaliana is a small, short-lived, highly selfing (Abbott and Gomes, 1989), annual monocarpic plant characteristic of ruderal habitats (Napp-Zinn, 1985). Over the past several years it has been used by an increasing number of evolutionary biologists as a model system (Pigliucci and Schlichting, 1995; Stahl *et al*, 1999; Dorn *et al*, 2000; Jackson *et al*, 2002) because of its logistic advantages as well as the availability of a large knowledge base on its physiology, development, and molecular biology (Pang and Meyerowitz, 1987; Anderson and Roberts, 1998). *Arabidopsis*' annual life history simplifies the measurement of fitness components and how they are affected by genetic variation and environmental factors. In particular, fruit production at the time of senescence is a good estimate of total lifetime fitness (Westerman, 1970), since there is no allocation to vegetative structures for future seasons.

We used nine single-seed descent genotypes of *A. thaliana* from the Arabidopsis Resource Center (Columbus, OH, USA; www.arabidopsis.org): Landsberg *erecta* (Ler-2, Germany); Eilenburg (Eil-0, Germany); Wilna (Wil-2, Russia); Blanes (Bla-12, Spain); *ga1-5* (gibberellin mutant, hereafter referred to as GA); *ga1-6* (GA mutant); *ga4-1* (GA mutant); *ga5-1* (GA mutant); *gai-1* (GA mutant). The three early-flowering genotypes Eil-0, Wil-2, and Bla-12 were picked at random from a set of genotypes previously used by Pigliucci and Marlow (2001). The GA mutants were chosen as part of a broader research program on the reaction norms of hormonal mutants in *Arabidopsis* (Pigliucci and Schmitt, 2004). Ler-2 was included for comparative purposes, since the five GA mutants were originally derived from it. All of our comparisons were either between Ler-2 and the GA mutants, to see how the mutants responded relative to their genetic background, or between Ler-2 and the natural accessions, to see how Ler-2 responded relative to other early-flowering genotypes.

Four of the five mutants examined in this study are GA-deficient, that is, the mutation precludes the formation and accumulation of GAs in the plant. As a consequence, under normal conditions, all these mutants are dwarf or semidwarf, a characteristic of GA-deficient phenotypes consisting of a bushy appearance and a decreased height (Koornneef and van der Veen, 1980; Ross, 1994; Ross *et al*, 1997). Two mutants, *ga1-5* and *ga1-6*, are allelic (Koornneef and van der Veen, 1980; Zeevaart and Talon, 1992), and act very early in the GA production pathways (Ross, 1994; Ross *et al*, 1997). The remaining two gibberellin-deficient lines (*ga4-1*, and *ga5-1*) have a similar general phenotype (Koornneef and van der Veen, 1980), but map to distinct genetic loci (Koornneef and van der Veen, 1980; Zeevaart and Talon, 1992); *ga5* appears to be blocked at several different intermediate steps in the pathways, and *ga4* is interrupted at a still later level as compared to *ga1* (Ross *et al*, 1997). *ga1-5* and *ga1-6* are dwarfs, whereas *ga4-1* and *ga5-1* are classified as semidwarfs (Koornneef and van der Veen, 1980; Ross, 1994; Ross *et al*, 1997). The reason for the differences in the severity of the GA-deficient phenotype between the early-acting (*ga1-5* and *ga1-6*) and the late-acting (*ga4-1* and *ga5-1*) mutants is not clear. All four of these

GA-deficient mutants are suspected to be 'leaky,' that is, they produce some small quantities of active GA, presumably due to redundancy in the GA-production pathways (Koornneef and van der Veen, 1980; Koornneef *et al*, 1985; Zeevaart and Talon, 1992; Phillips *et al*, 1995, Hedden and Phillips, 2000).

In contrast to all of these, *gai-1* is a GA-insensitive line, although it retains a low level of sensitivity to externally applied GA (Koornneef *et al*, 1985). Bioactive GAs are produced and accumulate in the plant's tissues, but are ineffective, possibly because the mutation damaged a key transcription factor in the transduction pathways (Koornneef *et al*, 1985). This mutant phenotypically resembles GA-deficient genotypes (dwarf appearance), although the genetic and physiological bases of the defect are entirely different (Koornneef *et al*, 1985).

Plant handling and experimental set-up

Seeds of all lines were imbibed with water and exposed to a brief cold treatment (9 days) at 4°C in the dark to facilitate germination. They were then planted in 4 by 4 by 4.5 cm pots with standard promix potting soil that had been autoclaved to avoid pathogen growth. Plants were housed indoors in three-level racks, and the photoperiod was set at 16 h of light per day at room temperature. The 9 days of cold treatment, followed by planting, were carried out on different schedules (because of logistical limitations). We therefore used planting date, which can be thought of as a temporal blocking effect, as a cofactor in the analyses.

The experiment included the nine genotypes exposed to two AMD treatments in a fully factorial design. There were three blocks, originally with 288 plants in each block; however, some of the soil accidentally dried up, leaving 192 plants surviving in block 2. Taking into account the plants lost to desiccation and to random mortality, the number of replicates for each genotype-treatment combination averaged four, and the total number of plants in the experiment was 640.

Nutrients, in the form of Osmocote time reslease pellets, were added 1 week after germination. The plants were either clipped to apically damage the plant's main inflorescence, or were left unclipped. Clipping was carried out at the time of bolting, when the inflorescences were in the unopened flower bud stage. The entire inflorescence was clipped off at the base of the rosette with scissors (although no rosette leaves were damaged or removed). This type of AMD mimics the damage done by mammals such as rabbits and deer, and has been observed in field experiments with *A. thaliana* (Weinig *et al*, 2003) and other monocarpic annuals (Paige and Whitham, 1987; Paige, 1994, 1999, Lennartsson *et al*, 1997).

The following traits, potentially affected by AMD, were measured: (1) Number of basal inflorescences, or inflorescences growing out of the rosette. This is a component of plant architecture during the reproductive phase, and a measure of how much basal inflorescence proliferation occurs when apical dominance is released. (2) Number of lateral stems (or branches), a second measure of plant architecture providing a quantification of the degree of bushiness of the main body of the plant, since lateral stems branch off from the basal inflorescences. (3) Fruit production, an estimate of lifetime reproductive fitness. (4) Inflorescence weight, another estimate of reproductive fitness; we weighed all inflorescences growing on the basal and lateral branches for this measurement. (5) Seed germinability, also a component of fitness. In order to examine this, we gathered between 20and 40 seeds from a subset of plants after senescence, put them in cold treatment for 5 days, and then left them at room temperature in a greenhouse for 10 days. We scored the proportion of seeds that germinated from each plant, and used this as our measure of seed quality.

Data analysis

We analyzed the data with a two-way analysis of variance (ANOVA). We checked for assumptions of normality and heteroscedasticity, and found that the residuals from the models for inflorescence weight and lateral branch number were truncated around the mean in the frequency distribution. We therefore transformed the data by taking the logarithm of the trait values (Sokal and Rohlf, 1981); the residuals of the reanalyzed data were then normally distributed. Analyses were carried out with Jump-In (SAS) version 4.0.4 using a General Linear Model approach (JMPIN, 2001). For each trait, the full model included the following main effects and interactions: genotype (quantifying genetic differences among lines; treated as a fixed effect); AMD (overall plasticity to clipped *vs* unclipped treatments; fixed effect); block (microenvironmental effects; random); planting date (covariate; random); AMD by genotype interaction effect (genetic variation for plasticity to AMD); and error (residual variance). We did not perform sequential Bonferroni or similar corrections for multiple simultaneous tests, following the advice of Moran (2003). Rather, we report the formal *P*-values, effect sizes (in the form of mean sums of squares), and the power of the tests (Cohen, 1992), and discuss the results from a statistically conservative perspective.

For effects that were found to be not statistically significant, we performed power analyses using G-Power (Buchner *et al*, 1997). We followed the conventions of Cohen (1992) and evaluated whether we had the statistical power to detect 'medium' and 'small' effects caused by our treatments. 'Medium' effect size (ES) means that treatment differences are 'visible to the naked eye of a careful observer' (for example, the clipped plants have visibly more fruits than the unclipped plants), whereas 'small' ES means that the treatment differences are 'noticeably smaller than the medium but not so small as to be trivial' (for example, the clipped plants have more fruits than the unclipped plants, but this is not as obvious; Cohen, 1992). This analysis accounts for the possibility that we found an effect to be statistically nonsignificant, not because the effect is actually biologically insignificant, but rather because of a limited sample size. Power values range from 0 to 1, and are calculated for each effect in the model based upon the degrees of freedom and the ES (small, medium, or large) of interest. Power values of 0.8 and higher are considered to be sufficient in order to conclude that there was enough power to detect an effect of the size of interest (Cohen 1992). We will refer to power values for medium ES as 'PW_{med}'and power values for small ES as 'PW_{small}.'

In order to examine the multivariate relationships among traits (ie, taking into account possible intertrait correlations), we performed a principal components analysis (PCA) on all characters (inflorescence weight, lateral branch number, basal inflorescence number, and fruit number), except seed germinability, since we used only a subset of plants to measure that trait. We standardized the data for each character around a mean of zero, and performed a PCA on the covariance matrix (Dillon and Goldstein, 1984, p 36). Plots in principal components (PCs) space allowed us to explore the phenotypic similarity of plants as a function of their genotype and AMD treatment. The PCA was carried out using Jump-In (SAS) version 4.0.4 (JMPIN, 2001).

Results

The main effects of genotype, block, and planting date were significant for all traits (inflorescence weight, number of lateral branches, number of basal inflorescences, fruit production, and seed germinability: Table 1). AMD was not statistically significant for any of the traits except for basal inflorescence number (Table 1), even though we had sufficient power to detect an effect of medium magnitude (all traits, $PW_{med} > 0.99$); we also had relatively good power to detect an AMD effect of small magnitude for all traits except seed germinability ($PW_{small} = 0.71$; for seed germinability, $PW_{small} = 0.61$). Furthermore, for all characters the percentage of variance explained by AMD was in fact very low (less then 1%), indicating little biological relevance regardless of statistical considerations.

In general, mean sums of squares showed that statistically significant main effects accounted for 1–18% of the total variance. There were no significant interaction effects (Table 1), even though we had sufficient power to detect interaction effects of medium magnitude ($PW_{med} > 0.96$ for all traits). We lacked sufficient power to detect interaction effects of small magnitude, as is usually the case for higher-order effects in ANOVAs of large experimental designs. At any rate, mean sums of squares indicate that most interaction effects explained very little of the total phenotypic variance, ranging from 0.5 to 1.8%.

We examined the genotypic means and their 95% confidence intervals for all (across-environment) mean trait values. As an example, we show the plot only for basal branch production (Figure 1), but report the full genotype-by-treatment data in the Supplementary Table 1. In what follows, we focus on comparing the wild-type Ler-2 to its GA mutants, as well as Ler-2 to the other natural populations. We do not stress comparisons of the GA mutants to the non-Landsberg lines, as this would make little biological sense.

As far as the production of basal inflorescences is concerned, the natural genotype Bla-12 had slightly more basal inflorescences than the other natural accessions (Figure 1). The mutants *ga1-6* and *gai-1* had more inflorescences compared to the other mutants and to the wild-type Ler-2. It is worth noting that the only significant AMD main effect was on this trait, with

Table 1 ANOVAs for each trait (columns) and factor (rows) in the experiment

		Inflorescence weight (g)	*Lateral branch number*	*Basal inflorescence number*	*Fruit number*	*Seed germinability*
R^2		0.17	0.25	0.30	0.33	0.23
Genotype (G)	df	**8**	**8**	**8**	**8**	**8**
	MS	**0.016**	**4429.50**	**6.36**	**530157.66**	**2.19**
	MS%	**6.64**	**13.77**	**15.83**	**9.86**	**9.21**
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
AMD (A)	df	1	1	**1**	1	1
	MS	~0	9.32	**0.23**	7492.00	~0
	MS%	0.09	0.03	**0.56**	0.14	0.01
	P	0.4110	0.6270	**0.0312**	0.2587	0.7795
A × G	df	8	8	8	8	8
	MS	0.004	170.63	0.35	27776.76	0.17
	MS%	1.75	0.53	0.88	0.52	0.73
	P	0.1157	0.8259	0.5039	0.7845	0.8251
Block	df	**2**	**2**	**2**	**2**	**2**
	MS	**0.008**	**651.39**	**0.36**	**266331.44**	**1.37**
	MS%	**3.05**	**2.02**	**0.90**	**4.95**	**5.77**
	P	**<0.0001**	**0.0003**	**0.0243**	**<0.0001**	**<0.0001**
Planting date	df	**11**	**11**	**11**	**11**	**11**
	MS	**0.017**	**3017.64**	**3.47**	**986888.22**	**1.06**
	MS%	**6.74**	**9.38**	**8.65**	**18.36**	**4.47**
	P	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**0.0067**
Residual	df	605	606	606	607	471
	MS	0.202	23896.46	29.40	3557457.77	19.01
	MS%	81.72	74.27	73.17	66.17	79.80

For each factor, we report the degrees of freedom (df), mean squares (MS), MS as a percentage of the total, and *P*-values. Statistically significant effects at the $P < 0.05$ α value are highlighted in boldface. R^2 refers to the amount of variance explained by the whole model for a particular trait.

clipped plants having slightly more basal inflorescences than unclipped plants (unclipped 5.78 (confidence interval: 3.27 to 4.35), clipped 6.28 (3.65 to 4.85)), as would be expected with the release from apical dominance by clipping.

Concerning the other traits (plots not shown), the natural genotype Wil-2 had a lower across-environment mean inflorescence weight than the other two natural accessions, Eil-0 and Bla-12, while the GA mutants and Ler-2 had similar inflorescence weights. Bla-12 and Eil-0 produced more lateral branches than Ler-2 or Wil-2, but the lateral branch production of Ler-2 and the GA mutants were similar. With regards to fruit production, the significant genotypic variation in the ANOVA was mostly due to the greater trait mean of *gai-1*, with the other genotypes showing similar levels of fruit production to one another. It is interesting to note that the GA mutants produced as many fruits, or more, than the Ler-2 wild type, and that the latter produced roughly as many fruits as the other natural accessions. Finally, the GA mutants did not differ from Ler-2 in seed germinability (60–70%), and Ler-2 in turn did not differ from the natural genotypes, with the exception of Eil-0, which was characterized by a higher value (about 87%, as compared to 73% for Ler-2).

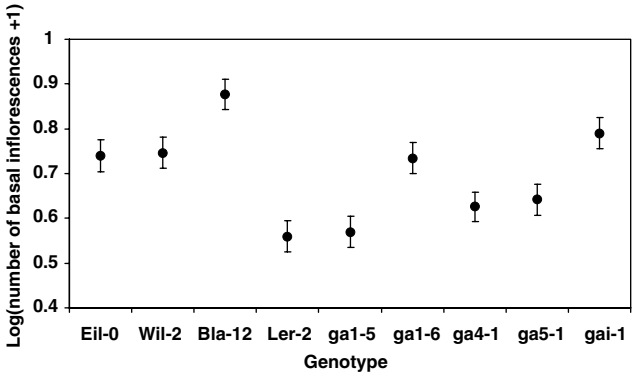

Figure 1 Least-squares (across-environment) means of basal inflorescence production for the different genotypes. The bars represent ± 1 SE.

In our study, no genotypes showed instances of overcompensation or undercompensation, as indicated by the lack of a main AMD effect – or an AMD by genotype interaction effect – for fruit number or seed quality, even though we had the statistical power to detect such effects of medium magnitude or smaller.

In the multivariate analysis, PC 1, which accounted for 66.6% of the variance, was mostly influenced by plant size, since all traits had roughly equal and positive loadings on that component (number of lateral branches $= +0.54$; fruit production $= +0.52$; inflorescence weight $= +0.51$; number of basal branches $= +0.42$). PC 2, which accounted for 16.7% of the variance, was influenced largely by basal branch number, given that this trait had a loading on that component that was much larger than those of the other characters ($+0.87$, as compared to -0.40, which was the next largest value). PC 3 accounted for an additional 8.8% of the total variance, and was influenced by inflorescence weight and fruit production (loadings: inflorescence weight $= +0.70$; fruit production $= -0.70$; next largest value $= 0.10$). It is also interesting to note that the magnitudes of the loadings of inflorescence weight and fruit production on PC 3 were opposite, suggesting a possible weak trade-off between these fitness components.

In the plot of PC 1 *vs* PC 2 (Figure 2), there was generally no difference between the clipped and the unclipped plants of each genotype, with the exception of *ga4-1* and *ga1-6*, both of which produced more basal inflorescences when clipped as compared to when not clipped, and Eil-0, which produced more basal inflorescences when unclipped compared to when clipped. *ga4-1* and Eil-0 were larger when clipped, whereas *ga1-6* was smaller. Ler-2 clustered near some of the GA

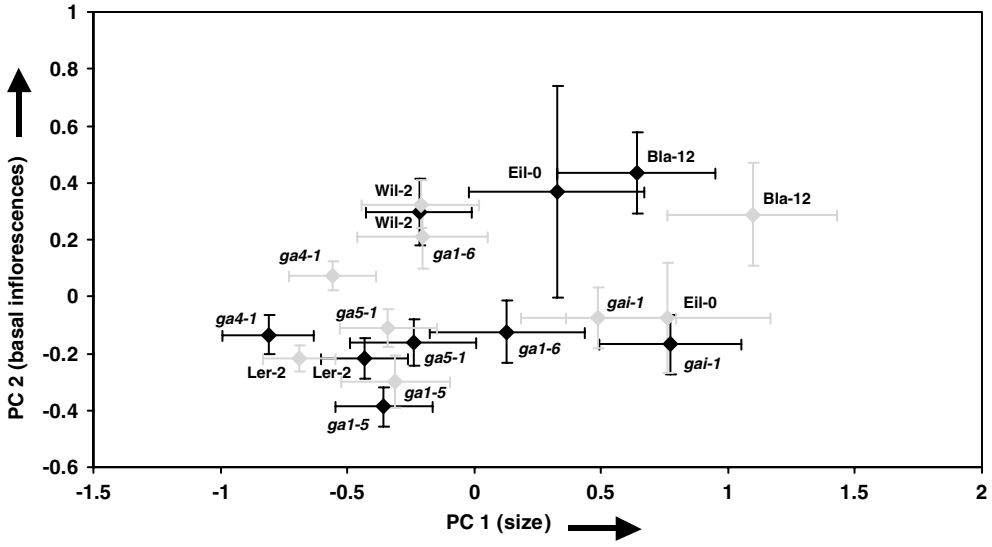

Figure 2 PCA showing how the different genotype-AMD combinations cluster in multivariate space. Fruit production, inflorescence weight, number of basal branches, and number of lateral branches were included in the analysis. The black lines are the unclipped treatment and the gray lines are the clipped treatment. The bars represent ± 1 SE around the centroid means.

mutants (*ga1-5*, *ga4-1* in the unclipped state, and *ga5-1*), but was phenotypically distant from *gai-1*, which was much larger, and *ga1-6*, which was larger than Ler-2 in the unclipped state and had more basal inflorescences than Ler-2 in the clipped state. Ler-2 grouped distinctly from all of the natural accessions, which by comparison were characterized by increased size and proliferation of basal inflorescences (Figure 2).

Discussion

Researchers have insistently been calling for an integration of modern developmental and molecular biology into evolutionary and ecological studies (eg, Schlichting and Smith, 2002). This synthesis is ongoing in the case of phenotypic plasticity (Pigliucci, 2001), particularly concerning the genetic bases of plastic responses (Scheiner, 1993; Pigliucci, 1996; Crews, 2003). Since different molecular mechanisms can underly a given pattern of phenotypic plasticity, it is necessary to directly integrate the molecular level of analysis in our studies to help us discriminate between alternative hypotheses concerning how a particular plastic response is actuated.

Our research combines information from molecular and organismal biology by examining the patterns of an ecologically relevant plastic response in a set of mutant and natural genotypes. Our aim was to determine whether specific mutations of known molecular underpinning, conferring GA deficiency or insensitivity, alter the level of tolerance of *A. thaliana* to AMD. This would suggest the potential for a role of GA in the evolution of tolerance to AMD, although of course not necessarily through the specific mutations examined here.

Effects of GA deficiency and insensitivity on fruit production and tolerance

The GA mutants included in this study did as well as Ler-2 in terms of tolerating AMD, implying that GA deficiency/insensitivity is not a handicap in terms of maintaining reproductive fitness following AMD. The fact that lack of GA did not seem to have an effect on tolerance is interesting, considering that GA is a major plant hormone known to have complex consequences for meristematic growth. In fact, GA deficiency both increases the number of basal meristems that elongate (thereby giving the plant a more 'bushy' phenotype), and reduces the extent of these meristems' growth (yielding a shorter, more compact, appearance) (Hay *et al*, 2004). Our results indicate that both Ler-2 and the GA mutants show little plasticity to simulated herbivory, which implies that GAs have little to do with the slope of the reaction norm for fruit production in responde to AMD.

In addition to not affecting tolerance to AMD, these major regulatory mutations also had little or no apparent detrimental effect on fruit production, regardless of the treatment. In fact, the mutant *gai-1* even had higher fruit production than Ler-2. This surprising outcome may be explained by the double effect of GA deficiency on meristem activity (which is enhanced) and growth (which is diminished) mentioned above: if these two outcomes on average simply balance each other out in the mutants, then the overall fruit production will be similar to that of the wild type. Low fitness was not manifested in lower seed quality, either: the mean seed germinability of the mutants was no different from that of Ler-2. These results are consistent with the work of Pigliucci and Schmitt (2004), who found that the same GA mutants did as well as or better than Ler-2, in terms of reproductive fitness, under greenhouse conditions. Our study provides more evidence (see, for eg, Purugganan and Suddith, 1998) that evolution could proceed by the spread of mutations at major regulatory loci: they are not necessarily hindered by strong deleterious pleiotropic effects; our mutants still flowered and set fruit, producing seeds of as good a quality as the wild type. Of course, how much all of this applies to field conditions is an open question, although mutations at regulatory loci are known to persist in wild populations of *Arabidopsis* (Aukerman *et al*, 1997).

We found no significant main effect of AMD and no AMD by genotype interaction in the statistical model explaining fruit production, even though we had the power to detect such effects. Therefore, we conclude that there were no positive *or* detrimental effects of AMD on reproductive fitness, and that genotypes showed equal compensation at all nutrient levels. This is important, because it implies that all genotypes were able to recover from the removal of the main inflorescence right before flowering, when compared to the situation with an intact apical meristem. Our results are consistent with the findings of Juenger *et al* (2000) in their simulated vertebrate herbivory experiment examining early- and late-flowering populations of *Gentianella campestris*, in which they found that early-flowering populations show equal compensation to simulated herbivory, whereas late-flowering populations display genetic variation for tolerance (although the mechanism accounting for this is currently unknown).

Since the genotypes included in our study all showed compensation, we conclude that the unclipped plants had some potential to produce fruits, which was not realized unless the apical meristems were in fact cut off. One possible explanation for this is that the main inflorescence was acting as an 'herbivory monitor' of when the herbivores leave and when it is opportune to resume the reproductive phase (Van Der Meijden, 1990). Such adaptive explanations, of course, warrant much more empirical investigation before they can be accepted as likely.

The multivariate picture

The principal components analysis clearly separated Ler-2 from the other natural accessions. This is in accordance with what is known about Ler-2's diminutive phenotype relative to other *A. thaliana* genotypes. Although our study involves only one laboratory strain (and does not include other often-used laboratory lines, such as Columbia), these results should at least caution against automatically assuming that long-established laboratory lines are representative of the species of interest. Indeed, results from other studies have sparked quite a debate over the appropriateness of using laboratory-adapted organisms to draw conclusions about the ecology and evolution of model species (eg, Matos *et al*, 2000; Sgrò and Partridge, 2000). The fact that Ler-2 is phenotypically distinct from the natural accessions may imply that the effects of GA mutations in a Ler-2 background are different from their effects in other, more typical, genetic

backgrounds. Therefore, although our study does not indicate a role of GA's in tolerance to AMD, these results should be interpreted with the caveat that the wild type was itself rather atypical; GAs might mediate tolerance in other genetic backgrounds.

We also found that most of the GA-deficient mutants grouped near Ler-2 in multivariate phenotypic space, showing that a clear effect of their common genetic background was still evident in the overall phenotype of the mutants. The phenotype of *gai-1*, however, was appreciably different from Ler-2 and the other mutants, particularly in terms of overall plant size. It is not clear why the only GA-insensitive mutant in our sample should have a markedly distinct phenotype, given that we expected the genetic defect to produce phenotypic effects analogous to those of GA-damaged pathways. However, even among the GA-deficient mutants there were detectable phenotypic differences, although these did not seem to be generally related to whether the mutations were affecting the GA pathways early or late. Indeed, even *ga1-5* and *ga1-6*, which are at the same locus, mapped in different areas of principal components space, possibly due to different degrees of 'leakiness' of their respective allelic mutations.

Concluding remarks

Overall, our results do not demonstrate a role of GA in mediating tolerance to AMD, although GA deficiency and insensitivity do seem to have an effect on other aspects of the phenotype. A role for GA in tolerance to AMD, however, might exist in other genetic backgrounds, especially late-flowering genotypes of *A. thaliana*, which differ dramatically both ecologically and genetically from earlier-flowering accessions (Zhang and Lechowicz, 1994; Juenger *et al*, 2000; Donohue, 2002; Weinig *et al*, 2003). As mentioned earlier, in *G. campestris*, later-flowering populations do show more genetic variation for tolerance than earlier-flowering accessions (Juenger *et al*, 2000), although the mechanistic reasons for this are largely unexplored. Furthermore, in *A. thaliana*, Zhang and Lechowicz (1994) found that there was more phenotypic plasticity in many morphological and physiological traits in later-flowering genotypes than in earlier-flowering ones. Therefore, it is possible that GA becomes more important in mediating responses to AMD in later-flowering populations characterized by a longer life span and different ecological settings.

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Supplementary Information accompanies the paper on Heredity website (http://www.nature.com/hdy)

IV. Phenotypic and molecular genetic responses to apical meristem damage in *Arabidopsis thaliana*

Abstract

Due to the increasing availability of genomics tools, is now possible to study the response to tissue damage in plants on a genome-wide molecular level and thereby to understand some of the genetic pathways and molecular processes involved the phenotypic response. Using oligonucleotide microarrays, we analyzed changes in mRNA transcript levels due to apical meristem damage in a wild-collected accession of *Arabidopsis thaliana*. We also analyzed its phenotypic response to AMD as well as in four others originating from the same area, to evaluate if the molecular responses make sense in light of the phenotypic responses. We found that 83% of the genes with differential expression were up-regulated in response to AMD, and that the majority of them were associated with growth and metabolic processes. This suggests that vigorous re-growth (tolerance) responses were induced by AMD, more so than stress pathways underlying resistance to damage. We found that many of the biological processes associated with the annotated genes could be explained in the context of the phenotypic responses to AMD we observed. We discuss particularly interesting genes with transcript differences in response to AMD and hypothesize how these genes might mediate the response to AMD. Overall, our results show that a functional genomics approach can aid in finding loci involved in the responses to AMD and can guide follow-up studies to better understand the genetic networks involved in the responses.

INTRODUCTION

There has been growing interest in recent years in connecting the wealth of information about plant-herbivore interactions at the phenotypic level to fine-scale molecular genetic mechanisms, facilitated by the proliferation and increasing affordability and dissemination of molecular and genomic techniques (Andrew et al. 2005; Núñez-Farfán et al. 2007; Ouborg and Vriezen 2007; Snoeren et al. 2007; Chen 2008). One set of plant responses to herbivores that has not received much attention at a molecular level is the responses to apical meristem damage (AMD) in monocarpic plants.

Arabidopsis thaliana offers the opportunity study the responses to AMD at both the phenotypic and molecular genetic level -- to understand some of the genetic pathways and molecular processes involved in the phenotypic response. As a close relative of economically important crops, such as broccoli and canola, potential agricultural applications may arise from understanding the molecular genetic responses to AMD in this species.

The few previous attempts to investigate the genetic and molecular basis of the phenotypic responses to AMD have not identified candidate loci or specific molecular processes. Weinig et al. (2003b) found no significant QTLs associated with the ability to

recover from AMD by rabbits in their field study with *A. thaliana*. They interpreted this to mean that many loci of small effect underlie the phenotypic responses to AMD, which suggests that very large sample sizes would be required to find significant QTLs. Similarly, Banta and Pigliucci (2005) found no evidence for a role of gibberellins, an important hormone involved in stem elongation, in the ability to recover from AMD. Taken together, these studies illustrate that the responses to AMD are likely complex processes at the molecular genetic level, and therefore very fine-scaled techniques will be necessary to pinpoint specific genes and molecular pathways involved.

The use of oligonicleotide microarrays is one such approach that promises to facilitate identification of heretofore-elusive genes involved in responses to AMD, and thereby to also get a sense of the molecular processes involved. We used this approach to screen for genes that were up- or down-regulated in response to AMD in *A. thaliana*. Using the Affymetrix™ ATHI GeneChip® array, which covers virtually all of the genes in the *A. thaliana* genome, we were able to screen for genes involved in responses to AMD regardless of prior knowledge about those genes and/or *a priori* hypotheses concerning their involvement. We used an accessions of *A. thaliana*, collected relatively recently from the wild, to make our findings more relevant to natural populations.

In order to make putative connections between the gene lists generated by microarray analysis and phenotypic responses to AMD, we grew a larger number of individuals and scored their phenotypic responses to AMD. We also scored the phenotypic responses to AMD in four other accessions collected from the same area in order to gage how typical the focal accessions' responses to AMD were.

We were specifically interested in the following questions: (1) What are the phenotypic responses to AMD of the focal accessions used in our study? (2) Are the responses to AMD in the focal accessions representative of the larger set of accessions collected from the same area? (3) Does oligonucleotide microarray analysis reveal genes that are differentially expressed in damaged versus undamaged plants, and do these molecular responses make sense in light of the phenotypic responses?

MATERIALS AND METHODS

Plant material, handling, and experimental protocol---We used five *Arabidopsis thaliana* accessions collected from the Waginengen area in the Netherlands during the spring of 2001 (see Banta et al. 2007 for more information). We generally followed the guidelines for germination and growth of *A. thaliana* recommended by the *Arabidopsis* Biological Resource Center (2008). We grew the plants under laboratory conditions for several generations and used the descendant seeds in our experiment to minimize maternal effects. We soaked the seeds in water on VWR™ brand 7.5 cm moist filter paper in 16 x 50 mm BD Falcon™ tissue culture dishes and exposed them to a seven-day dark stratification treatment at 4°C to facilitate germination. We then planted the seeds in a greenhouse in 3.25 x 3.25 x 5 cm pots within watering trays, and used sub-irrigation to water the plants when necessary. Rather than potting soil, we used a 50:50 mixture of sand and vermiculite and added Scott's™ Osmocote® Plus nutrient prills to the soil surface to create lower, more ecologically realistic soil nutrient conditions.

During seed germination, we set the photoperiod to 13 hours and the temperature to 25°C. Twenty-eight days later, we simulated overwintering as a rosette, with a

temperature of 3°C and a photoperiod of eight hours. After six weeks, we simulated spring and summer using a photoperiod of at least 15 hours and a temperature of at least 21°C.

We either clipped the plants, to simulate herbivory by apically damaging the plant's main inflorescence, or left them unclipped as a control. Clipping was done five days after the formation of unopened flower buds, when the plants had bolted, and the entire inflorescence was clipped off at the base of the rosette with scissors while being careful not to remove or damage any rosette leaves. To determine whether there was variation among accessions in the amount of inflorescence tissue removed by damage, we dried and weighed the inflorescence tissue removed.

Tissue collection and RNA extraction---We randomly selected four damaged and four undamaged plants from one accession for microarray analysis. Five days after the formation of flower buds, when the plants had bolted, we harvested the shoot apex and associated flower buds from the main inflorescence of undamaged plants. Five days after the re-formation of flower buds, when the damaged plants had re-bolted, we harvested the same tissue from one of the axillary inflorescences of damaged plants. Harvested tissue was flash-frozen in liquid nitrogen and stored at -80°C, and total RNA was extracted using a Qiagen™ RNeasy® kit (www.qiagen.com).

Phenotypic data collection and analysis---After the reproductive period, we measured the following traits that were potentially affected by AMD: 1) Number of basal inflorescences, the inflorescences growing out of the rosette; 2) Number of lateral stems, the secondary and higher-order branches off of the basal inflorescences; 3) Time from first flower budding to fruit ripening, a measure of how long it took to complete the reproductive period; 4) Number of fruits, a crude estimate of total lifetime fitness; 4) Number of total viable seeds, an integrated estimate of total lifetime fitness, calculated as fruit production times the average number of seeds per fruit (determined from a sample of 5 fruits per plant) times the proportion of viable seeds (determined from a sample of 20-40 seeds tested for germinability per plant).

Analysis of variance---We performed analyses of variance (ANOVAs) on the following traits: number of basal inflorescences, number of lateral stems, first budding to fruit ripening time, number of fruits, and total viable seed production. No data transformations were necessary to comply with statistical assumptions (Sokal and Rohlf 1995). Using JMP version 7.0.1 (SAS Institute Inc. 2007), we modeled each trait as a function of: accession, AMD treatment (undamaged or damaged), accession-by-AMD interaction, watering tray (one tray or the other), transplant status (either the plant grew from a transplanted seedling or it germinated and grew *in situ*), and tissue collection status nested within accession (for plants from the accession chosen for tissue collection, either tissue was either harvested or not). All effects were fixed. Following Moran (Moran 2003), we did not apply Bonferroni or similar corrections for multiple tests because of their tendency to over-correct, thereby inflating type-II error.

To determine if there was variation among accessions in the amount of inflorescence tissue removed by damage, we performed an ANOVA on the dry-weight of the excised inflorescence tissue as a function of the accession. We square-root-transformed inflorescence dry-weight to improve compliance with statistical assumptions.

Oligonucleotide microarrays and analysis---For microarray processing, total

RNA samples were sent to the Genomics Center at the New York University Medical Center Cancer Institute. To achieve sufficient quantities of RNA, one round of amplification was necessary using the Ovation™ RNA amplification system V2 on all samples (www.ovation.com). Then the samples were reverse transcribed, labeled, hybridized and scanned according to the protocols in the Eukaryotic Samples and Array Processing section of Affymetrix's™ GeneChip® Expression Analysis Technical Manual (Affymetrix Inc. 2008). Subsequently, the raw data was sent to the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign for further data processing. After passing Affymetrix's™ recommended quality control diagnostics, the raw data from the arrays were pre-processed using the GCRMA algorithm (Irizarry et al. 2003) as part of the affy (Gautier et al. 2004) and gcrma (Irizarry et al. 2003) packages from the Bioconductor project (Gentleman et al. 2004).

Before statistical analysis, probe sets were discarded if they were not called "present" on at least one of the 18 arrays or "marginal" on two of the arrays, based on Affymetrix's™ call detection algorithm (Affymetrix_Inc. 2008); of the 22,810 probe sets on the array, 18,476 probe sets passed the filter and were retained for analysis. The data that was fit for both of the AMD treatment groups using a cell-means model (Smyth 2005). Differential expression of probe sets was assessed using the limma package (Smyth 2004) from the Bioconductor project, which uses an empirical Bayes "shrinkage" method on the standard errors to improve power. Multiple test correction of the P-values was done using the false discovery rate (FDR) method (Benjamini and Hochberg 1995). Molecular functions of, and biological processes associated with, differentially expressed genes were assigned based on annotations from the GOstats package for Bioconductor (Falcon and Gentleman 2007) and a literature review.

RESULTS

Phenotypic variation---There was a significant effect of apical meristem damage (AMD) treatment on the number of basal inflorescences and fruit ripening time; a marginally significant effect of AMD treatment on the number of lateral branches and total viable seeds; marginally significant variation among accessions in the effect of AMD on fruit ripening time; and a significant effect of tissue collection on fruit ripening time (Table 1). AMD increased the number of basal inflorescences, marginally significantly decreased the number of lateral branches, increased the time to fruit ripening, and marginally significantly decreased total viable seed production (Figure 1). All accessions responded similarly to AMD, since there was no significant variation among accessions in the effect of AMD on the traits. There was no significant variation among accessions in the amount of inflorescence tissue removed by damage ($P = 0.2565$). In other words, all accessions paid a similar cost, in terms of tissue lost, when damaged.

Microarray analysis---Using a false discovery rate-adjusted α of 0.1, 35 genes in the focal accession (Table 2) showed significant differential expression when plants were apically damaged as compared to when they were undamaged. Of these, 29 were up-regulated (22 named and/or of known molecular function, 25 with known associations to biological processes) and six were down-regulated (all named and/or of known function,

four with known associations to biological processes).

These differentially expressed genes have diverse roles. Table 2 shows that most of the genes with known associations to biological processes are involved in either primary metabolism (i.e., carbohydrate metabolism/transport, fatty acid/lipid metabolism/transport, and protein metabolism: 33% of genes with known associations to biological processes), or growth (i.e., photosynthesis, development, and organelle biogenesis: 22%). Furthermore, three genes are involved in responses to stress (i.e., glucosinilate production, toxin catabolism, and defense response to bacteria: 11%) and two are involved in auxin activity (i.e., auxin production and transport; 7%). Eight of the nine genes involved in metabolism, all of the genes involved in growth, two of the three genes involved in the response to stress, and both of the genes involved in auxin activity were up-regulated in response to AMD.

DISCUSSION

Microarray data---We observed significant changes in transcript levels for a number of *Arabidopsis thaliana* genes in response to apical meristem damage (AMD). A striking pattern emerged where 83% of the genes with differential expression were up-regulated, as opposed to down-regulated, in response to AMD. Of these, half of the annotated ones were associated with either metabolism or growth. Taken together, this suggests increased growth and associated metabolic processes in the developing flower buds in response to damage. Therefore, it seems the molecular processes involved in the *re*generation of damaged floral tissues do not occur at the same pace as those used to *generate* undamaged tissues; rather, some of these processes are accelerated.

It is also interesting that there were far more genes associated with growth and metabolism revealed by the microarray analysis than there were genes associated with stress responses. Stress responses in this context could indicate the induction of chemical pathways that increase resistance to future damage (Yang and Hoffman 1984; Murphy et al. 1999; Stotz et al. 2002; Devoto and Turner 2005; Lorenzo and Solano 2005; van Loon et al. 2006; Maffei et al. 2007). This suggests AMD elicits responses that increase tolerance to damage (i.e., re-growth) more so than it induces resistance to future damage. This is perhaps not surprising, given that AMD directly affects fitness, as opposed to damage to other tissues, where the connection to fitness is more indirect and an array of induced defenses is possible (for a review, see Koricheva et al. 2004). In other words, apically damaged annual plants that lose a significant amount of their inflorescence tissue must replace the tissue to achieve reproductive success; becoming better defended against future bouts of damage, or performing any other biological processes for that matter, becomes less important.

Two of the three genes involved in stress responses also are involved in auxin activity. This is significant, because -- based on their known physiological roles -- auxins should be involved in tolerance to (i.e., re-growth following) AMD (Paige and Whitham 1987; Benner 1988; Mopper et al. 1991; Huhta et al. 2000a; Juenger et al. 2000). *SUR1* converts a shared intermediate of both the indole-acetic acid (IAA; an auxin)- and indole glucosinilate- (a nitrogenous anti-herbivore secondary metabolite)-producing pathways into indole glucosinilates rather than IAA (Mikkelsen et al. 2004; Grubb and Abel 2006). Thus the up-regulation of *SUR1* in response to damage should have the dual effect of

increasing tolerance *and* resistance to AMD: increased *SUR1* production increases glucosinilate production and decreases IAA production, thereby making the plants less palatable to herbivores *and* releasing IAA-suppressed shoot meristems in the rosette to re-grow (and proliferate) inflorescences. Genes such as this may help to explain why resistance and tolerance to herbivore damage are often synergistic, rather than competing, plant defense strategies (Leimu and Koricheva 2006; Núñez-Farfán et al. 2007), even though several authors have argued on theoretical grounds that natural selection should favor either tolerance or resistance, but not both (van der Meijden et al. 1988; Herms and Mattson 1992; Belsky et al. 1993; Mauricio 2000). This result also serves to stress a more general point: optimality models can fall short in biological systems because biological processes are often non-independent due to overlaps in the underlying genetic pathways (Taylor and Raes 2004).

The gene *ATGTSU20*, which also showed differential expression, makes a glutathione S-transferase protein (GST) that binds to IAA (Marrs 1996). GSTs have been implicated in facilitating IAA transport, although the exact purpose of GST-binding to IAA is not well understood (Marrs 1996). On its face it is counter-intuitive that this gene would be up-regulated in response to AMD, which would seemingly increase IAA-mediated suppression of shoot meristems and therefore hinder inflorescence re-growth. A possible explanation is that, in some circumstances, GSTs might be used to sequester IAA rather than transport it (Jones 1994; Bilang and Sturm 1995). In such a case, increased *ATGTSU20* activity in response to AMD may increase tolerance by decreasing the IAA-mediated suppression of shoot meristems via a reduction, rather than an increase, in IAA signal transport, thereby facilitating re-growth, and proliferation, of inflorescences. To understand the relationship between tolerance to AMD and *ATGTSU20*, research is needed to clarify whether *ATGTSU20* facilitates or hinders IAA transport in response to AMD.

The defense response gene *TGA4* was down-regulated in response to AMD in the focal accession. *TGA4* encodes a leucine zipper transcription factor, a family of proteins that bind to the promoters of pathogen-response genes inducted by the salicylic acid pathway (Jakoby et al. 2002). *TGA4* down-regulates this pathway (Foley and Singh 2004), and so down-regulation of *TGA4* should up-regulate these pathogen-response genes. Thus, down-regulation of *TGA4* by AMD appears to be a defensive/stress response that de-suppresses pathogen-response genes.

Phenotypic responses to AMD---The phenotypic effects of AMD on the focal accession were typical as compared to other accessions from the same locale, giving us no reason to believe that the molecular responses to AMD we observed were atypical; it is possible that the phenotypic responses to AMD in the non-focal accessions are also achieved by the same or similar genetic pathways as in the focal accession, but of course this would require empirical verification.

It was possible to hypothesize several connections between the gene expression and the phenotypic data in our study. AMD delayed ontology to ripe fruit set. Considering that *A. thaliana* is an opportunistic ruderal species (Napp-Zinn 1985), and that changes in its timing of germination can have dramatic effects on fitness in the field (Donohue 2002), AMD-induced delay to mature seed set could translate into reduced fitness in natural situations. Delayed flowering and fruiting as a cost of tolerance to damage has been recognized in other studies (Bergelson and Crawley 1992b; Lennartsson

et al. 1998; Huhta et al. 2000b; Juenger and Bergelson 2000; but see Paige and Whitham 1987). Therefore, it is perhaps adaptive that the focal accession ramped up genes associated with growth in response to AMD, since this could help mollify the phenological delay incurred by damage.

AMD increased the number of basal inflorescences in both accessions, as in previous studies on *A. thaliana* (Weinig et al. 2003b; Banta and Pigliucci 2005). This is consistent with our finding of up-regulation of *SUR1*, which should thereby shunt an IAA intermediate to glucosinilate production rather than IAA production, and with our finding of up-regulation of *ATGTSU20*, which should (if its speculated purpose is correct) thereby sequester auxins, ameliorating auxin-mediated suppression of basal shoot meristems and allowing for their proliferation. Furthermore, this suggests that re-growth after AMD in *A. thaliana* is mediated, at least in part, by auxins, which help determine the architecture for re-growth.

Fruit production and total viable seed production did not increase, and in fact trended towards decreasing, in response to AMD, despite an increase in the number of basal inflorescences when damaged. This can be explained by the effect of AMD on lateral branch production: fruit number is more closely tied to lateral branch number than it is to basal inflorescence number in this species (Banta et al. in review), and lateral branch production decreased, rather than increased with AMD. This shows that the plants were hindered by AMD, despite re-growth and proliferation of inflorescences after damage; re-grown inflorescences contained less "real estate," in the form of lateral branches, for building fruits and seeds.

Conclusion---We have identified several genes whose expression is affected by AMD, many of which are involved in either growth or stress processes, and some of which are involved in the control of apical dominance, showing that a functional genomics approach can aid in finding loci involved in the responses to AMD. Future studies can follow up on these patterns to verify and further investigate the roles of these genes in tolerance, induced resistance, and other responses to AMD using various molecular approaches such as, for example: T-DNA insertion mutants (Krysan et al. 1999), near-isogenic lines (Keurentjes et al. 2007), and Ecotilling (a method of probing for nucleotide polymorphisms in natural populations; Comai et al. 2004). Particularly interesting for follow-up studies would be an investigation into the role of the *SUR1* gene in mediating both tolerance and induced resistance to AMD, which is potentially part of the molecular-genetic explanation for the lack of trade-offs between tolerance and resistance often observed at the phenotypic level. It would also be helpful to understand the effect of *ATGSTU20*, since its up-regulation in response to AMD hints at a previously hypothesized, but unverified, role for this gene (sequestration/inactivation of auxins). Future studies could also compare the functional genomics of *A. thaliana*'s responses to AMD to those of its relatives such as *Arabidopsis lyrata* and *Capsella rubella* to look for generalities in the molecular underpinning of these responses across different species.

Table 1. Analyses of variance. For each trait (columns) we report R^2, degrees of freedom (df), mean squares, and P-values (in parentheses) associated with the F-ratio tests. Significant factors at $\alpha = 0.05$ are emphasized in boldface.

	Basal Inflorescences $R^2 = 0.28$	Lateral Branches $R^2 = 0.13$	Fruit Ripening Time $R^2 = 0.62$	Fruits $R^2 = 0.10$	Total Viable Seeds $R^2 = 0.18$
Accession (df = 4)	0.14 (0.7880)	6.18 (0.2669)	**267.54 (< 0.0001)**	222.17 (0.2397)	281229.25 (0.0757)
Apical Meristem Damage (AMD) (df = 1)	**8.20 (< 0.0001)**	15.06 (0.0758)	**1710.33 (< 0.0001)**	201.56 (0.2623)	362459.35 (0.0951)
Accession x AMD (df = 4)	0.093 (0.8942)	3.57 (0.5509)	64.00 (0.0758)	43.43 (0.8937)	93440.35 (0.5692)
Watering Tray (df = 1)	0.0025 (0.9315)	2.12 (0.5016)	0.016 (0.9813)	37.88 (0.6259)	13558.62 (0.7444)
Transplant Status (df = 1)	0.034 (0.7648)	0.010 (0.9628)	65.15 (0.1376)	57.76 (0.5474)	40947.58 (0.5714)
Tissue Collection Status (df = 1)	0.88 (0.1103)	1.01 (0.6425)	**185.13 (0.0135)**	163.24 (0.3127)	22830.14 (0.6724)
Error	0.34 (df = 85)	4.66 (df = 84)	28.89 (df = 72)	158.28 (df = 85)	126571 (df = 68)

Table 2. List of genes with expression changes in response to apical meristem damage. For each gene (rows), we present the Genbank ID, the fold change in gene expression of damaged tissue relative to undamaged tissue, the false discovery rate-corrected P-value ($\alpha = 0.1$), the name of the gene (if named) and its molecular function, and the biological process associated with the gene's activity. Genes shaded grey were down-regulated, and un-shaded genes were up-regulated. Molecular functions and biological processes were assigned based on annotations from the GOstats package for Bioconductor (Falcon and Gentleman 2007) unless otherwise indicated.

Genbank ID	FoJd Change	P-Value	*Name*/Function	Biological Process
AT2G20610	3.19	0.0621	*SUR1*/transaminase activity	indoleacetic acid biosynthesis, adventitious root development; also glucosinilate biosynthesis (Mikkelsen et al. 2004)
AT3G14650	2.72	0.0672	*CYP72A11*/ oxygen binding	electron transport
AT3G56090	2.69	0.0610	ferric iron binding	cellular iron ion homeostasis
AT2G22230	2.52	0.0650	3-hydroxyacyl-[acyl-carrier-protein] dehydratase activity	fatty acid biosynthesis
AT1G62790	2.35	0.0610	lipid binding	lipid transport
AT2G43950	2.33	0.0672	ion channel activity	cation transport
AT1G78370	2.26	0.0621	*ATGSTU20*/ glutathione transferase activity	toxin catabolism, auxin transport
AT3G10260	2.09	0.0610	unknown	unknown
AT5G67560	2.02	0.0672	GTP binding	small GTPase mediated signal transduction
AT4G39880	2.02	0.0610	unknown	ribosome biogenesis and assembly
AT5G09850	1.94	0.0729	unknown	regulation of transcription
AT1G64510	1.90	0.0621	unknown	ribosome biogenesis and assembly
AT5G17840	1.87	0.0621	unknown	unknown
AT1G53520	1.81	0.0610	unknown	unknown
AT1G07020	1.81	0.0621	unknown	unknown
AT3G11945	1.80	0.0621	prenyltransferase activity	unknown
AT4G05180	1.74	0.0894	calcium ion binding	photosynthesis
AT1G12410	1.70	0.0621	*CLPR2*/ endopeptidase Clp activity	ATP-dependent proteolysis
AT1G66430	1.69	0.0672	kinase activity	D-ribose metabolism, sucrose biosynthesis, sucrose catabolism using beta-fructofuranosidase, acetate fermentation
AT2G33450	1.69	0.0621	unknown	translation
AT5G46800	1.66	0.0880	*BOU*/binding	mitochondrial transport
AT1G11860	1.61	0.0880	aminomethyltransferase activity	glycine decarboxylation via glycine cleavage system
AT4G26500	1.60	0.0282	enzyme activator activity, transcription regulator activity	iron-sulfur cluster assembly, embryonic development ending in seed dormancy
AT1G64620	1.56	0.0621	transcription factor activity, DNA binding	regulation of transcription
AT5G19370	1.53	0.0663	isomerase activity	unknown
AT5G58330	1.47	0.0621	malate dehydrogenase activity	malate metabolism
AT5G49940	1.39	0.0880	*NFU2*/ structural molecule activity	chloroplast organization and biogenesis, iron-sulfur cluster assembly
AT4G38970	1.36	0.0929	fructose-bisphosphate aldolase activity	pentose-phosphate shunt
AT3G22110	1.34	0.0621	*PAC1*/ peptidase activity	ubiquitin-dependent protein catabolism
AT5G10030	-3.86	0.0929	*TGA4*/DNA binding, calmodulin binding, transcription factor activity	response to cold, defense response to bacterium
AT3G49600	-1.82	0.0282	*UBP26*/ ubiquitin-specific protease activity	ubiquitin-dependent protein catabolism
AT4G20400	-1.51	0.0610	transcription factor activity	regulation of transcription
AT1G21570	-1.50	0.0610	nucleic acid binding	unknown
AT1G50620	-1.30	0.0621	DNA binding	DNA-dependent regulation of trascription
AT4G13750	-1.26	0.0880	ATP binding	unknown

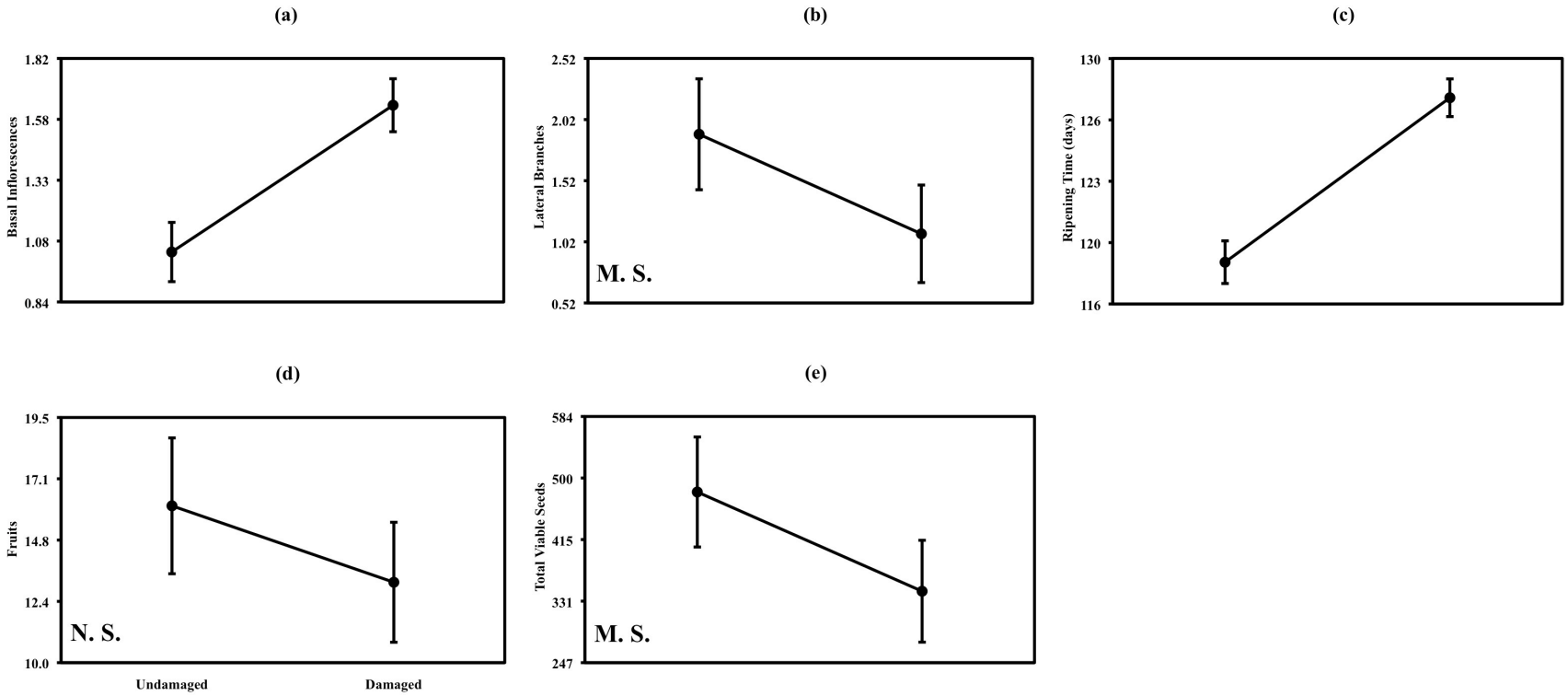

Figure 1. Least squared mean values of (a) basal inflorescence number, (b) lateral branch number, (c) fruit ripening time, (d) fruit production, and (e) total viable seed production, according to the apical meristem damage treatment (AMD). "N. S." indicates that the effect of AMD on a particular trait was not significant at $\alpha = 0.05$, and "M. S." indicates marginal significance ($0.05 < P < 0.10$). The error bars represent ± 1 SE.

V. Tolerance to apical meristem damage in *Arabidopsis thaliana* and other Brassicaceae: a comparative approach

Abstract

Despite calls for an understanding of the evolution of plastic responses, phenotypic plasticity is almost never studied in a broad interspecific context. Therefore, we examined an ecologically important plastic response, tolerance to apical meristem damage, in the model species *Arabidopsis thaliana* and seven of its relatives from multiple genera. We also manipulated inorganic soil nutrient levels to gage the environmental lability of tolerance and how this lability differs among species. We found that tolerance differs among species. Furthermore, the overall picture of tolerance similarities is complicated by nutrient levels variation so as to make it difficult to generalize the tolerance response across species. Differences in tolerance were not accounted for by differences in mating system or life history, and they were only partially accounted for by the degree of relatedness. We also found that the choice fitness proxy is important when evaluating tolerance differences in this group of species. Overall, our study shows that tolerance can evolve readily in this group, unhindered by developmental constraints imposed by the mating system or life history. We suggest that continued studies of tolerance using *A. thaliana* are worthwhile, given its advantages as a model system.

INTRODUCTION

Model species are usually initially selected for study because they are fast-growing and easy to work with in a laboratory setting, among other reasons. However, these properties may make them ecologically and genetically unusual. *Arabidopsis thaliana* (Brassicaceae) illustrates this point. As an annual, highly-selfing weedy species characteristic of ruderal habitats (Napp-Zinn 1985), *A. thaliana*'s ecological and life-history characteristics contrast with those of many species of conservation or agricultural interest (i.e., highly competitive invasive species or obligately outcrossing crop plants). On a genetic level *A. thaliana* is also atypical, with derived characteristics such as a reduced chromosome number ($n = 5$ from an $n = 8$ ancestral state found in close relatives) and multiple chromosomal rearrangements (Schranz et al. 2007b).

Although some researchers have managed to connect findings from *A. thaliana* to distantly related organisms such as *Populus* spp. (aspen; Weigel and Nilsson 1995), it is unclear to what extent aspects of *A. thaliana*'s phenotype are analogous, if not homologous, to those of other species, given its unusual characteristics. As a result, interest has been growing over the last few years in developing other close relatives of *A. thaliana* as alternative model species (Mitchell-Olds 2001; Koch 2003; Koch and Kiefer 2005; Schranz et al. 2007a; Vogel et al. 2007), the idea being that existing genomic techniques could easily be bridged to these species, which are more ecologically interesting and more representative of the "local genetic neighborhood" than *A. thaliana*.

While marking a significant improvement, the development of these new model species leaves open the question: how easily can one extrapolate the findings from a given species, either to close relatives or to ecologically similar species? Furthermore, while there has been a strong tradition of studying phenotypic plasticity in *A. thaliana* (e.g., Westerman and Lawrence 1970; Pigliucci 1995; Callahan and Pigliucci 2002; Pigliucci 2005; Tonsor and Scheiner 2007), most of the work with alternative model species has so far focused on molecular genetics or phenotypes within single environments (but see Pigliucci et al. 1999). Knowledge about phenotypic plasticity is not being migrated into these newly emerging model species at the same rate as other aspects of *A. thaliana* biology.

We studied tolerance to apical meristem damage (tolerance to AMD) in *A. thaliana* and seven of its relatives to understand the generalizability of an ecologically important plastic response and to help bridge its study into new model species. AMD involves the removal of inflorescence tissue and typically occurs right before flowering, due to either herbivores such as deer or rabbits (Paige and Whitham 1987; Weinig et al. 2003a) or to abiotic sources of stress such as frost (Juenger et al. 2000; Tiffin 2000). Plants can recover substantially from AMD (Paige and Whitham 1987; Juenger et al. 2000; Weinig et al. 2003a) even if it inflicts a loss of as much as 95% of their aboveground biomass (Paige and Whitham 1987). Tolerance to AMD is a form of phenotypic plasticity because it is defined as the slope of the reaction norm for fitness across herbivory treatments (Simms 2000). The slopes of the reaction norms for fitness can be used to compare the tolerance of different groups of organisms (e.g., different genotypes within a population, different populations within a species, different species, or even different taxa; Pigliucci 2001). "Undercompensation" refers to a negative slope of the ordered fitness reaction norm across (increasing) damage levels, "exact tolerance" refers to a flat slope, and "overcompensation" refers to a positive slope.

We manipulated inorganic soil nutrient levels to test for interspecific variation in its effect on tolerance. Several studies have documented that tolerance within a species changes depending on the levels of soil nutrients (e.g., chapter one of this dissertation; for reviews, see Hawkes and Sullivan 2001; Wise and Abrahamson 2007). If tolerance were affected similarly by soil nutrients across species then this would add to the generalizability of tolerance studies conducted on a single species. Conversely, if tolerance were differentially affected by soil nutrients in different species then this would complicate efforts to extrapolate the results of tolerance studies to other species, since the "rules" of extrapolation change in different environments.

In summary, we specifically asked the following questions: (1) How does tolerance vary among species? (2) Are more closely related species more similar to one another in their tolerance than they are to more distantly related species? (3) Are there other correlates of tolerance besides the degree of relatedness, i.e., mating system and life history, and to what degree are they decoupled from relatedness? (4) How does the interspecific picture of tolerance change when nutrient levels variation is taken into account?

We also used multiple fitness proxies -- above-ground weight, flower production, and fruit production -- which allowed us to examine to what extent the choice of fitness proxy affects the estimation of tolerance. The accuracy of the estimation of tolerance, the slope of the fitness reaction norm, should depend on how well the fitness proxy stands in

for "true" fitness.

MATERIALS AND METHODS

Plant material and experimental design---We obtained seeds of eight species from the family Brassicaceae (Table 1). Koch et al. (2001) report that *Arabis alpina* and *Arabis blepharophylla* are self-compatible perennials, *Capsella rubella*, *Arabidopsis thaliana*, *Boechera stricta*, and *Olimarabidopsis pumila* are self-compatible annuals, *Arabidopsis lyrata* is a self-incompatible annual, and *Pseudoturritis turrita* is a self-compatible biennial or perennial. Contrary to Koch (2001), our samples of *A. lyrata* were self-compatible (and highly selfing), and our samples of *A. alpina* and *P. turrita* were largely self-incompatible.

Our samples had the following intraspecific structure: *A. lyrata*, two populations with 11 maternal families in one population and five in the other; *A. thaliana*, four populations with two families per population; *C. rubella*, one family; *O. pumila*, four families from unknown populations. We do not know the sample structure of the other species.

We imbibed the seeds with water and exposed them to a seven-day dark stratification treatment at 4°C to facilitate germination. We then planted them in 9 x 9 x 9 cm pots in a greenhouse. Rather than potting soil, we used a 50:50 mixture of sand and vermiculite to ensure low baseline soil nutrient levels. During seed germination, we set the photoperiod to 13 hours and the temperature to 25°C. Nineteen days later we simulated overwintering as a rosette, with a temperature of 4°C and a photoperiod of eight hours. After six weeks, we simulated spring and summer using a photoperiod of at least 15 hours and a temperature of at least 25°C. Ten months later, we simulated a second overwintering for the long-lived species that had not yet flowered, using the same photoperiod and temperature as the previous overwintering. Ten weeks afterward we returned the plants to spring and summer conditions.

We added nutrients, in the form of Osmocote 15-9-12 8-9-month time-release nitrogen-phosphorus-potassium pellets applied to the soil surface. All plants received two pellets four days after planting, eight months after planting, and ten months after planting. The high nutrient plants received an additional eight pellets 19 days after planting, about the time the first true leaves appeared.

We either clipped the plants, to apically damage one of the plant's inflorescences, or left them unclipped as a control. We clipped the plants right before flowering, when unopened flower buds were visible. The clipping protocol differed depending on the architecture of the species. For species that produced a dominant inflorescence and that had a clearly differentiated rosette – *A. blepharophylla*, *A. lyrata*, *A. thaliana*, *C. rubella*, and *O. pumila* – we clipped off the dominant inflorescence at the base of the rosette with scissors. For species that produced a dominant inflorescence but did not have a clearly differentiated rosette – *B. stricta* and *P. turrita* – we clipped off the dominant inflorescence about one cm from the soil surface. *A. alpina* did not produce a dominant inflorescence but produced multiple ramets; therefore, we counted the entire genet in a pot as an individual plant and clipped off the largest ramet at the soil surface. For all species, any plants that did not flower at all were not included in subsequent analyses.

We harvested plants: (1) if they were assigned to the unclipped treatment and they

had finished one bout of flowering (allowing time for ripe fruit production in the highly selfing species), (2) if they were clipped and had finished an additional bout of flowering after re-growing clipped tissue, or (3) if they were clipped and failed to re-flower 17 months after the beginning of the experiment. After harvesting, we measured the total above-ground dry-weight, the number of flowers produced (where fruits are counted as part of the total), and the number of fruits produced.

Analysis of variance---We analyzed above-ground weight, flower production and fruit production with two-way analysis of variance (ANOVA), using $\log_{10}$-transformed values to improve normality (Sokal and Rohlf 1995). We used JMP IN version 5.1 with the method of moments approach (SAS Institute, Inc. 2003). Some species did not survive in the low nutrients treatment. Therefore, we ran two separate sets of ANOVAs: one for plants that experienced high and low nutrients (to examine the effect of nutrient levels, AMD, and their interaction on a restricted number of species), and one for plants that experienced only high nutrients (to examine the effect of AMD, but not nutrient levels, on a larger number of species).

The ANOVAs for plants that experienced high and low nutrients contained the following effects: clade (one clade contained *A. alpina* and *A. blepharophylla*, while the other contained *A. thaliana*, *C. rubella*, and *O. pumila*; fixed effect), species nested within clade (fixed effect), nutrient levels (fixed effect), AMD (damaged vs. undamaged; fixed effect), all possible interaction effects among those factors, greenhouse bench (random effect), and tray nested within growth chamber (random effect). The ANOVAs for plants that experienced only high nutrients contained more species within clades (one clade contained *A. alpina*, *A. blepharophylla*, and *P. turrita*, while the other contained *A. lyrata*, *A. thaliana*, *B. stricta*, *C. rubella*, and *O. pumila*) and did not include the main and interaction effects of nutrient levels. We could not include the main or interaction effects of clade for the fruit production models because *A. alpina* and *P. turrita* produced virtually no ripe fruits and were therefore excluded from those models. To reduce the likelihood of disregarding the results of truly significant tests (Type II error), we have chosen not to use the sequential Bonferroni or similar corrections for multiple tests, following Moran's (2003) criticism of this method. The reader has all the necessary information from our tables to implement corrections if a diminished Type I error, and concomitantly increased Type II error, is preferred.

RESULTS

As mentioned in the Materials and Methods section, the species comprising the two clades differed depending on whether the models included nutrient levels or were restricted to high nutrients. For simplicity, we refer to one clade as the *Arabis* clade and the other as the *Arabidopsis* clade. When nutrient levels were included, the *Arabis* clade contained *Arabis alpina* and *Arabis blepharophylla*, and the *Arabidopsis* clade contained *Arabidopsis thaliana*, *Capsella rubella*, and *Olimarabidopsis pumila*. When only the high nutrients plants were included, the *Arabis* clade contained *A. alpina*, *A. blepharophylla*, and *Pseudoturritis turrita*, and the *Arabidopsis* clade contained *A. lyrata*, *A. thaliana*, *Boechera stricta*, *C. rubella*, and *O. pumila*. We did not include the main or interaction effects of clade when modeling fruit production because *A. alpina* and *P. turrita* did not produce fruits and were excluded from those models.

Plants from the *Arabis* clade were larger (in terms of above-ground weight), but less fecund (in terms of flower production), than plants from the *Arabidopsis* clade (not shown). Increasing nutrients were associated with increased size and fecundity (in terms of either flower production or fruit production) for all species. However, the effect of increasing nutrients on size was greater in the *Arabis* clade than in the *Arabidopsis* clade (not shown).

According to the model that included nutrient levels, species differed in the effect of AMD on above-ground weight (Table 2). This same finding was not replicated in the model restricted to high nutrients, probably due to a lack of power. Also according to the model that included nutrient levels, clades differed in the effect of AMD on flower production, with the *Arabis* clade showing undercompensation and the *Arabidopsis* clade showing overcompensation in response to AMD (not shown). In contrast, the models restricted to the high nutrients plants, which had a larger number of species within clades, did not register a significant effect of clade on the response to AMD.

With flower production as the fitness proxy, plants from the *Arabis* clade showed roughly exact tolerance to AMD, whereas plants from the *Arabidopsis* clade differed in their tolerance to AMD depending on the species, ranging from undercompensation to overcompensation (Figure 1, first column). *A. thaliana* and *C. rubella* were similar in their tolerance, both showing overcompensation. The patterns were the same at high nutrients when fruit production was the fitness proxy (Figure 1, second column). At low nutrients, however, *A. thaliana* and *C. rubella* became less tolerant, with *C. rubella* switching in magnitude from overcompensation to undercompensation (Figure 1, third column).

These findings are all supported by the highly statistically significant ANOVA models, which explained between 54% and 82% of the total phenotypic variation (Table 2). For the models that included nutrient levels, there were significant effects of clade, species, nutrients, greenhouse bench, and tray, and nutrients-by-apical meristem damage (nutrients-by-AMD), clade-by-nutrients, clade-by-AMD, species-by-nutrients, species-by-AMD, and species-by-nutrient-by-AMD interaction. For the models restricted to high nutrients, there were significant effects of clade, species, greenhouse bench, tray, and species-by-AMD interaction.

DISCUSSION

Despite calls for the study of plasticity in a broad, interspecific context (Doughty 1995; Pigliucci et al. 1999), such work is still uncommon, presumably due in part to the complications of raising distantly related organisms in a common environment necessary for controlled experimentation. While several studies have examined phenotypic plasticity in multiple related species (e.g., Cook 1968; Lee et al. 1986; Schlichting and Levin 1986; Blouin 1992; Semlitsch and Reyer 1992; Wimberger 1992; Breto et al. 1994; Smith 1995), they have always focused on only two or three species, or a set of species within the same genus, and thus are not informative about the plastic responses in a wide group of organisms. Our study, the most extensive we are aware of to examine plasticity using multiple species, and the only such one to consider two different environmental variables simultaneously, demonstrates that tolerance to apical meristem damage (tolerance to AMD) can evolve rapidly in this group of species including *A. thaliana,* and

that nutrient levels variation contributes to variation in tolerance both within and among species.

We found that, within the *Arabidopsis* clade, tolerances to AMD are different among species regardless of whether flower or fruit production is used as a fitness proxy. Furthermore, these differences do not track the degree of relatedness of species. For example, *Arabidopsis lyrata* is not most similar in tolerance to its congener, *A. thaliana*, but rather to its far more distant relative *Boechera stricta* and to the even more distant relatives of the *Arabis* clade. On the other hand, tolerance is similar for the three species the *Arabis* clade, showing exact tolerance to AMD regardless of the fitness component. Determining whether tolerance in either clade evolved neutrally, due indirectly to selection on other characters, or due directly to selection on tolerance per se will require detailed research into the ecology of these species (Gould and Lewontin 1979; Gould and Vrba 1982), about which, for most of them, little is currently known. Whether by drift or selection, however, our data suggests that tolerance can evolve readily, at least in the *Arabidopsis* clade where tolerance has no is highly variable among species.

It is interesting to note that tolerance does not track the two pieces of "ecological" information available for all species: mating system and life history (Koch et al. 2001). At high nutrients, for example, *A. thaliana* was most similar in tolerance to another self-compatible annual, *Capsella rubella*, but was very different from the other self-compatible annuals, *A. lyrata* (which, for the populations used in our study, was self-compatible), *Olimarabidopsis pumila*, and *Boechera stricta*. This suggests that, at least within the *Arabidopsis* clade, tolerance is not developmentally constrained by mating system or life history.

Our study shows that the choice of fitness proxy can be important when estimating tolerance. We found that, when using above-ground weight as a fitness proxy, interspecific differences in tolerance are not registered, in contrast to when flower and fruit production, fitness proxies presumably closer to "true" fitness, are used. It is important to note that we were unable to measure fruit production for several species that were self-incompatible, and therefore we do not know how many other species' estimates of tolerance are sensitive to the choice of flower or fruit production as a fitness proxy. We suggest caution when assuming that coarse fitness proxies, especially above-ground weight, are a reasonable stand-in for true fitness. When studying tolerance in species that are not highly self-compatible, such as some in this study, we suggest that it is probably more appropriate to use natural populations *in situ*, rather than in the laboratory or greenhouse, in order to allow for natural pollination and therefore accurate enumeration of fruit production (see Gronemeyer et al. 1997 and Steets et al. 2007 for examples). Furthermore, this would allow for estimation of seed production, and perhaps viable seed production, which as we suggested in chapter one are preferable fitness estimates that should not be taken for granted. Of course, as with all field studies, such an approach has the disadvantage of not allowing for the manipulation of certain environmental factors while holding others constant, making the disentangling of important factors from covarying ones difficult (see Banta et al. 2007 for a discussion).

Tolerance among species becomes even more heterogeneous when nutrient levels variation is considered, but only if fruit production is used, highlighting again the importance of using the best fitness proxy possible. While it might seem that *A. thaliana* and *C. rubella* are very similar in tolerance when only high nutrient levels are considered,

when under low nutrients the opposite conclusion is reached. This demonstrates that nutrient levels variation affects tolerance differently in different species, even those with similar mating systems and life histories such as *A. thaliana* and *C. rubella*. Also importantly, this result adds a caveat when extrapolating tolerance findings from one species to others: while the tolerances of two species might be similar in one environment (high nutrients), they can become dramatically different in another environment (low nutrients). The species, therefore, to which one can extrapolate tolerance findings depends on a careful consideration of the environments under which tolerance is occurring. This could actually be an intractable problem, depending on the species-specificity of the lability of tolerance to different environmental conditions. Before the scope of this problem is understood, more research is of course needed to determine how many different environmental axes are important to the tolerance response and to what extent they differ interspecifically in their effect on tolerance.

Concluding remarks---We found no compelling reasons to abandon *Arabidopsis thaliana* for the purpose of studying tolerance to apical meristem damage (tolerance to AMD). Of the broad group of species we considered, there appears to be no species with a more "typical" response to AMD than *A. thaliana*, since the responses are so interspecifically diverse. There are, in fact, some compelling positive reasons to continue to use *A. thaliana* to study tolerance: as a highly selfing species, the estimation of tolerance using accurate fitness proxies is easily attainable under controlled conditions; as an annual, reproductive output after one growing season equals the total lifetime reproductive output; as a fast-growing species readily grown under controlled conditions, maximal information can be gleaned from a given amount of research effort; as a long-established model system, genomic techniques for fine-scale investigation are already available at the most economical prices and do not need to be bridged from other species.

Given the highly divergent responses of *Capsella rubella* to AMD at high versus low nutrients it would be interesting to perform studies on tolerance in this species to understand how, molecularly, and why, ecologically and evolutionarily, tolerance is so sensitive to nutrients in this species, and if this result holds up when more refined fitness estimates, like viable seed production, are used.

Table 1. Information about the plant samples used in this study: the species, the locations of the samples' origin in the wild, and the sources from where seeds were obtained.

Species	***Location(s) of Origin***	***Source(s)***
Arabis alpina	Undocumented	B and T World Seeds (catalogue # 14389)
Arabis blepharophylla	Undocumented	B and T World Seeds (catalogue #s 25412, 77777)
Boechera stricta	Undocumented	University of Copenhagen Botanical Garden, Denmark (specimen # E4854-0038)
Arabidopsis lyrata	Alaska	D. Wolf, University of Alaska at Fairbanks
Arabidopsis thaliana	(1) Netherlands (2) Sweden	M. Pigliucci (see Banta et al. 2007)
Capsella rubella	Undocumented	*Arabidopsis* Seed Stock Center (CS22561)
Olimarabidopsis pumila	Undocumented	*Arabidopsi*s Seed Stock Center (CS3700, CS4651, CS22463, CS22562)
Pseudoturritis turrita	(1) Undocumented (2) Turkey	(1) B and T World Seeds (catalogue # 14399) (2) U.S. Department of Agriculture National Plant Germplasm System (accession # PI 312842)

Table 2. Analyses of variance, with model effects in rows and traits in columns. We report model R^2, degrees of freedom (df), mean squares (MS), and P-values associated with the F-ratio tests. Significant effects are emphasized in boldface. The models that include nutrient levels are presented ("High and Low Nutrients"), as well as the models restricted to plants that experienced only high nutrients ("High Nutrients Only").

		Above-Ground Weight R^2 = 0.61 (top); 0.45 (bottom)		Flower Production R^2 = 0.78 (top); 0.81 (bottom)		Fruit Production R^2 = 0.74 (top); 0.82 (bottom)	
		df	MS (P-Value)	df	MS (P-Value)	df	MS (P-Value)
High and Low Nutrients	Clade	1	**0.74 (< 0.0001)**	1	**61.52 (< 0.0001)**	-	-
	Species	5	**0.028 (< 0.0001)**	3	**0.81 (0.0003)**	3	**12.00 (< 0.0001)**
	Nutrient Levels (NTL)	1	**0.88 (< 0.0001)**	1	**4.27 (< 0.0001)**	1	**7.60 (< 0.0001)**
	Apical Meristem Damage (AMD)	1	0.0014 (0.5992)	1	0.17 (0.2524)	1	0.023 (0.6586)
	NTL x AMD	1	0.0026 (0.4810)	1	0.015 (0.7308)	1	**0.48 (0.0420)**
	Clade x NTL	1	**0.050 (0.0019)**	1	0.086 (0.4089)	-	-
	Clade x AMD	1	0.00067 (0.7190)	1	**0.81 (0.0115)**	-	-
	Clade x NTL x AMD	1	0.0083 (0.2038)	1	0.0059 (0.8291)	-	-
	Species x NTL	5	0.0064 (0.2826)	3	0.17 (0.2493)	3	0.060 (0.6730)
	Species x AMD	5	0.0075 (0.2012)	3	0.28 (0.0864)	3	0.31 (0.0501)
	Species x NTL x AMD	5	0.0031 (0.6896)	3	0.20 (0.1858)	3	**0.38 (0.0213)**
	Greenhouse Bench	1	**0.12 (< 0.0001)**	1	**6.73 (< 0.0001)**	1	**7.04 (< 0.0001)**
	Tray	78	**0.0068 (0.0399)**	78	0.17 (0.0544)	78	**0.17 (0.0111)**
	Error	512	0.0051	316	0.13	283	0.12
High Nutrients Only	Clade	1	**0.64 (< 0.0001)**	1	**28.76 (< 0.0001)**	-	-
	Species	7	**0.042 (<0.0001)**	6	**5.96 (< 0.0001)**	5	**19.28 (< 0.0001)**
	Apical Meristem Damage (AMD)	1	0.0035 (0.5198)	1	0.47 (0.0995)	1	0.56 (0.0552)
	Clade x AMD	1	0.00040 (0.8083)	1	0.015 (0.7713)	-	-
	Species x AMD	7	0.013 (0.1291)	6	**0.46 (0.0162)**	5	**0.48 (0.0085)**
	Greenhouse Bench	1	**0.045 (0.0273)**	1	0.34 (0.2266)	1	**0.76 (0.0404)**
	Tray	77	0.0092 (0.2717)	77	**0.24 (0.0298)**	76	0.18 (0.1327)
	Error	362	0.0083	248	0.17	205	0.15

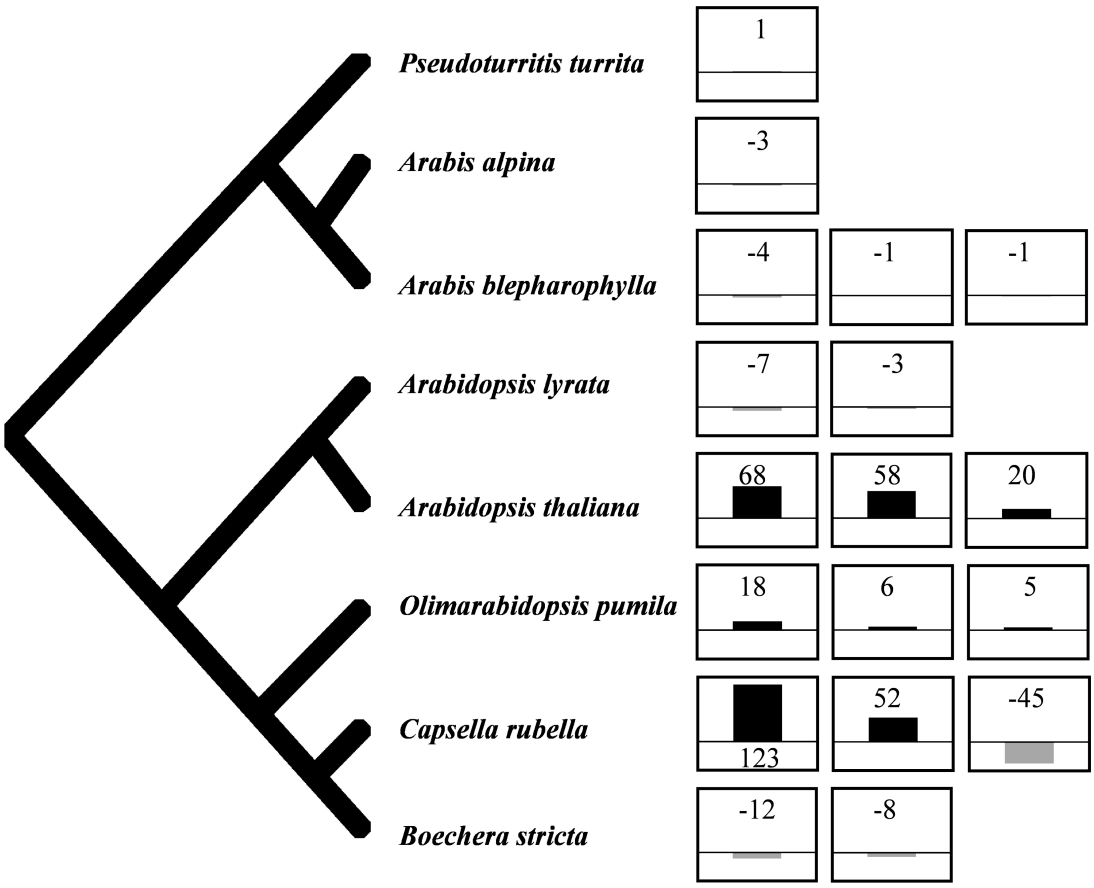

Figure 1. Bar graphs of tolerance superimposed on a phylogeny, based on Koch et al. (2001), Al-Shehbaz (2003, 2005), and Dobeš et al. (2006). Flower production (first column) or fruit production (second and third columns) is used as a fitness proxy. The first and second columns are for high nutrients, and the third column is for low nutrients. Tolerance is measured as the mean fitness of damaged plants minus the mean fitness of undamaged plants. The lines bisecting the graphs represent exact tolerance (zero). Values above zero represent overcompensation and values below zero represent undercompensation. The same scale is used throughout.

VI. Summary

This dissertation focused on tolerance to apical meristem damage (AMD), using a variety of approaches to begin to fill in gaps in our knowledge of this potentially very important, yet understudied, plant defense strategy. In chapter one, I used natural strains of *Arabidopsis thaliana* originating from three different areas of Europe, exposed to high and low soil nutrient levels, to understand whether tolerance to AMD is contingent on soil nutrients and if so, whether the patterns of tolerance are similar species-wide. I found that there was no species-wide relationship between tolerance and nutrients in natural populations of *A. thaliana*, which suggests that extensive ecological information about the individual populations of study is necessary to accurately predict the response to AMD in this species.

In chapter two, I employed structural equation modeling to understand how *A. thaliana*'s inflorescences and branches are related to components of fitness. I found that the relationship between these architectural traits and fitness components is altered in apically damaged plants, suggesting that tolerance is achieved by a modified ontogenetic program, not a reiteration of the normal one used in undamaged plants that has simply been reset by damage. I also found genetic variation, and plasticity to soil nutrient levels, in the ontogenetic program. Given these results, I suggest that tolerance in *A. thaliana* is best understood in the context of specific genetic backgrounds and environments, and as such any conclusions are not easily extrapolated to the whole species.

In chapter three, I used five *A. thaliana* mutants to test the hypothesis that gibberellin hormones help mediate the tolerance response to AMD. I found no evidence that gibberellins were involved in tolerance, at least not in the genetic background and environments studied. This is interesting, considering that gibberellins are a major plant hormone involved in stem elongation, and confirms that mutations at major regulatory loci can have non-catostrophic effects on fitness, probably due to the redundancy of complex gene regulatory networks. In contrast to the aforementioned one-at-a-time approach, in chapter four I used a shotgun approach to pinpoint genes involved in the response to AMD that can be followed upon in future studies. I identified 35 genes in one accession, and 23 in another, that are up- or down-regulated in response to AMD, which can be targeted in follow-up studies to verify their roles in tolerance.

In chapter five, I explored the similarities of the tolerance response in *A. thaliana* and several relatives with a broad group of species, to understand how tolerance evolves. I found that tolerance evolves readily in this group, unhindered by developmental constraints imposed by the mating system or life history, and that there was no universal relationship between tolerance and soil nutrient levels.

In summary, I suggest that continued studies of tolerance using *A. thaliana* are worthwhile, given its advantages as a model system, the natural genetic variation in tolerance it harbors, and the potential to understand how tolerance evolved in a broad, diverse group of species. The goal of finding simple, universal (or even species-wide) models to predict tolerance, however, seems unattainable, given the heterogeneous levels of tolerance, the heterogeneous ontogenetic programs whereby specific tolerance

responses are achieved, and the heterogeneous plasticities of tolerance to nutrient levels within and among species.

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