# A new method for measuring RGR can uncover the costs of defensive compounds in *Arabidopsis thaliana*.

Cloé Paul-Victor[1], Tobias Züst[1], Mark Rees[2], Daniel J. Kliebenstein[3] and Lindsay A. Turnbull[1].

[1] Institute of Evolutionary Biology and Environmental Studies, Winterthurerstrasse 190, University of Zurich, Zurich, Switzerland, CH-8057; [2] Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN, UK; [3] Department of Plant Sciences, University of California, Davis, California 95616, USA.

Author for correspondence:
Lindsay A. Turnbull
Tel: 0041 44 635 6120
Fax: 0041 44 635 5711
Email: lindsay.turnbull@ieu.uzh.ch

## Summary

• Most plants suffer some degree of herbivore attack and many actively defend themselves against such an event. However, while such defence is generally assumed to be costly, it has sometimes proved difficult to demonstrate the costs of defensive compounds.

• Here, we present a method for analysing growth rates which allows the effects of variation in initial plant size to be properly accounted for and apply it to 30 lines from a recombinant inbred (RIL) population of *Arabidopsis thaliana*. We then relate different measures of relative growth rate (RGR) to damage caused by a specialist lepidopteran insect and to levels of putative defensive compounds measured on the same lines.

• We show that seed size variation within the RIL population is large enough to generate differences in RGR, even when no other physiological differences exist. However, once size-standardised, RGR was positively correlated with herbivore damage (fast-growing lines suffered more damage) and was negatively correlated with the concentration of several glucosinolate compounds.

• We conclude that defensive compounds do have a growth cost and that the production of such compounds results in reduced herbivore damage. However, size standardisation of RGR was essential to uncovering the growth costs of defensive compounds.

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## Introduction

Plants differ in their growth rates and some of this variation is thought to be negatively correlated with traits such as herbivore defences (Bazzaz *et al.*, 1987; Herms & Mattson, 1992), frost resistance (Agrawal *et al.*, 2004; Turnbull *et al.*, 2008) or investment in non-photosynthetic structures such as storage organs (Poorter & Kitajima, 2007). Trade-offs – or negative correlations – between growth rates and investment in defence are predicted by life-history theory because defence is assumed to be costly (Bazzaz *et al.*, 1987; Perrin & Sibly, 1993; Iwasa, 2000). A plant that does not invest in defence can therefore grow more rapidly but it should suffer more damage when herbivores strike; conversely, if a plant invests in defensive compounds it should grow more slowly but suffer less damage. Such trade-offs are usually assumed in theoretical work (Perrin & Sibly, 1993; Iwasa, 2000) but, experimental demonstrations have sometimes proved more difficult (reviewed in Koricheva, 2002), particularly for compounds associated with defence against herbivores (Bergelson & Purrington, 1996; Arendt, 1997; Arendt, 2000); (Almeida-Cortez *et al.*, 1999; Almeida-Cortez & Shipley, 2002; Siemens *et al.*, 2002). Here we explore whether the methods commonly used to calculate growth rates, which fail to account for differences in initial size (Hunt, 1982; Hunt & Cornelissen, 1997), are partly responsible for the difficulties in detecting negative correlations between growth rates and defence, as outlined below.

### The problem with RGR

The most widely-used method to compare growth rates among species or genotypes is relative growth rate (RGR)

$$RGR = \frac{\log(M_2 / M_1)}{t_2 - t_1} \qquad \text{eqn 1}$$

where $M_i$ is the mass of the plant at time $t_i$. Experiments using such calculations are easy to carry out and many

species or genotypes can be compared for relatively little time and effort (Hunt, 1982). The problem with such calculations is that RGR is itself size-dependent and declines as individual plants grow (Hunt, 1982; Hunt & Cornelissen, 1997; Enquist *et al.*, 1999; West *et al.*, 2001); hence larger individuals are expected to have lower RGR than smaller individuals when measured over the same time period. This can confound analyses when the species or genotypes differ in their initial sizes (Turnbull *et al.*, 2008; Rose *et al.*, 2009). To overcome this problem, we need to carry out a size-standardised analysis in which species are compared at a common size. When examining growth/defence trade-offs, a size-standardised analysis should reveal whether each new unit of defended tissue is more costly to make than each new unit of undefended tissue for plants of standardised size.

## Defence in *Arabidopsis thaliana*

*Arabidopsis thaliana* produces a variety of secondary metabolites associated with defence. This chemical arsenal consists of a group of glucosinolates, alongside protease inhibitors, phenolics and terpenoid volatiles (Kliebenstein, 2004). Glucosinolates are amino-acid derived thioglycosides consisting of a conserved core structure and a highly diverse side chain. So far, at least 43 different glucosinolate compounds have been identified in *Arabidopsis* (Reichelt *et al.*, 2002; Kliebenstein *et al.*, 2007), the majority of which have an aliphatic side-chain, while another group of glucosinolates has indolic side-chains (Kliebenstein *et al.*, 2001b). Glucosinolates serve as a major chemical defence mechanism against insect herbivores, bacteria and fungi (Bones & Rossiter, 1996). For example, a number of studies have indicated that high glucosinolate content can delay larval development and reduce the survival of leaf-chewing lepidopteran insects (Kliebenstein *et al.*, 2002; Barth & Jander, 2006; Beekwilder *et al.*, 2008). Different types of herbivores are also affected by different glucosinolate compounds, for example, phloem-feeding aphids are mainly impaired by indolic glucosinolates (Kim & Jander, 2007).

Given their molecular structure, accumulation of glucosinolates by *Arabidopsis* might be expected to incur some metabolic or regulatory cost, leading to reductions in growth rate. However, when looked for, such growth costs have not been detected (e.g. Siemens *et al.*, 2002). Given that *Arabidopsis* lines vary in seed size and emergence time (germination day), comparisons among lines carried out over a fixed time period inevitably compare lines at different sizes. The failure to detect the growth costs of glucosinolates could therefore be due to the lack of size-standardisation when calculating growth rates.

Here we present a method for calculating size-standardised RGR which requires multiple harvests and apply it to data collected on 30 lines of *Arabidopsis* from a recombinant inbred (RIL) population. We then combine this growth data with published data on the same RIL population to examine the correlations between 1) growth rates and the concentrations of several glucosinolate compounds and 2) growth rates and herbivore damage inflicted by a specialist insect.

# MATERIAL AND METHODS
## Plant material

To demonstrate the potentially confounding effects of seed size on growth rates, we selected a RIL population derived from crosses between two accessions of *Arabidopsis thaliana*: the small-seeded Landsberg *erecta* (L*er*: mean mass of 100 seeds ± 1 SD: 1.93 mg ± 0.10) and the large-seeded Cape Verde Islands (Cvi: mean mass of 100 seeds ± 1 SD: 3.51 mg ± 0.08) (Alonso-Blanco *et al.*, 1998; Alonso-Blanco *et al.*, 1999). For the growth experiment, we selected 30 RILs from the possible set of 162. The 30 lines were selected by dividing the original 162 lines into six equally-spaced seed mass groups and selecting five lines at random from each group. Half of the selected lines carry the *erecta* mutation inherited from the L*er* parent, while the other half carries the wild-type *ERECTA* allele (Table S1). Lines carrying the *erecta* mutation have reduced height and different flower morphologies (Arabidopsis Biological Resource Centre (ABRC)). A summary of published information about the lines is available in Table S1. The seeds were obtained from The Arabidopsis Information Resource (TAIR) and we estimated sown seed mass by weighing one batch of 100 seeds from each of the 30 selected lines.

## Experimental design

Plants were grown in small (20 mm diameter), medium

**Table 1** Schedule of harvest dates showing the average developmental stage observed at each harvest. On average, germination occurred 4.7 days after sowing.

| Harvest | Days after sowing | Average age (Days after germination) | Developmental stage |
| --- | --- | --- | --- |
| 1 | 7 | 2.3 | 2 leaves |
| 2 | 11 | 6.3 | 4 leaves |
| 3 | 15 | 10.3 | 6 leaves |
| 4 | 20 | 15.3 | 8 leaves and bolting |
| 5 | 28 | 23.3 | First flowers seen |
| 6 | 33 | 28.3 | First fruits seen |

(30 mm diameter) and large cylinders (40 mm diameter) inserted into standardized cells (65 mm diameter) within a flat completely filled with a mixture of 50% sand and 50% compost. Each flat contained 35 cells and was 70 mm deep. The cylinders allowed us to randomise pot diameter treatments within flats and ensured that the spacing of individuals in different pot sizes and the surface area available to growing rosettes was exactly the same. However, the three pot sizes provide different degrees of belowground growth restriction (Paul-Victor & Turnbull, 2009). Pots were sown with four seeds and thinned as soon as seedlings emerged to leave one plant per pot (the most central healthy seedling). The plants were grown in a glasshouse with both natural light and additional artificial lighting which came on automatically when the natural light was below 25 kLux and kept under a cycle of 16 h light (22°C) and 8 h dark (20°C). Germination, bolting (initiation of the flowering stem) and flowering (opening of the first flower) were recorded for each plant to the nearest day.

Biomass was collected during six sequential, destructive harvests. We separated the plant parts into roots, rosette leaves and inflorescence (when present) and counted the number of leaves. Plant parts were dried at 80°C for three days and weighed to the nearest microgram. We focussed on the active stages of plant growth by harvesting at relevant points of the plants' development; thus each harvest represents a developmental stage observed in most individuals (Fig. 1 and Table 1). By the last harvest (33 days after sowing) no siliques were observed to have opened and hence no biomass was lost as seeds; however, rosette growth had mostly stopped (evidenced by relatively little change in rosette mass between harvests 4 and 5). At each harvest there were two replicates of each line and pot size combination, giving 1080 plants in total. A few plants are missing due to germination failures in the growth experiment.

## Size-standardised RGR

We modelled total biomass (rosette + roots + inflorescence) as a function of plant age (days since germination) using a three-parameter asymptotic regression model. Plant biomass was log-transformed giving:

$$\log(M_{i,t}) = A_i + (\log(M_{i,0}) - A_i)\exp(-\exp(r_i)t)$$

eqn 2

where $M_{i,0}$ is the starting mass at $t = 0$, $A_i$ is the asymptotic mass as $t \rightarrow \infty$ and $r_i$ is the logarithm of the rate constant (the rate constant is log-transformed to ensure positive growth). The time required to reach a given a reference mass, $M_{ref}$, is given by

$$t(M_{ref}) = \log\left(\frac{\log(M_{i,0}) - A_i}{\log(M_{ref}) - A_i}\right) \times \exp(-r_i)$$

eqn 3

RGR is given by $d(\log(M_t))/dt$, hence we can calculate size-standardised RGR by differentiating eqn 2 and substituting for $t=t(M_{ref})$. This gives

$$RGR_i = \exp(r_i)(A_i - \log(M_{ref}))$$

eqn 4

Thus, size-standardised RGR declines with mass and depends on three parameters, the rate constant ($r_i$) the asymptotic mass ($A_i$) and the reference mass ($M_{ref}$).

To calculate size-standardised RGR for each of the 30 lines we fitted the above model using the function *nlme* in the statistical package R (R Development Core Team, 2008). Lines were treated as a random effect and pot volume and seed mass as fixed effects. Throughout, we followed the model-building approach advocated by the developers of *nlme* (Pinheiro & Bates, 2000) which includes assessment and removal of non-significant terms. The significance of fixed effects (pot volume and seed mass) was assessed using F-tests while the significance of the random effects (lines) was assessed using likelihood ratio tests (Pinheiro & Bates, 2000).

**Table 2** Estimates of the fixed effects from the final growth model. Random, i.e. line effects were retained for $r_i$.

|  | Estimate | S.E. | t-value | p-value |
|---|---|---|---|---|
| Asymptotic mass (Asym) | 2.41 | 0.0785 | 30.7 | <.0001 |
| Asym (Pot diameter = 30) | 1.32 | 0.117 | 11.2 | <.0001 |
| Asym (Pot diameter = 40) | 1.84 | 0.128 | 14.4 | <.0001 |
| Rate parameter ($r_i$) | -2.20 | 0.0493 | -44.7 | <.0001 |
| $r_i$ (Pot diameter = 30) | -0.204 | 0.0507 | -4.02 | 0.0001 |
| $r_i$ (Pot diameter = 40) | -0.296 | 0.0509 | -5.82 | <.0001 |
| $M_{i,0}$ (intercept) | -0.097 | 0.660 | -0.147 | 0.883 |
| $M_{i,0}$ (log(sown.seed.mass)) | 0.753 | 0.182 | 4.14 | <.0001 |
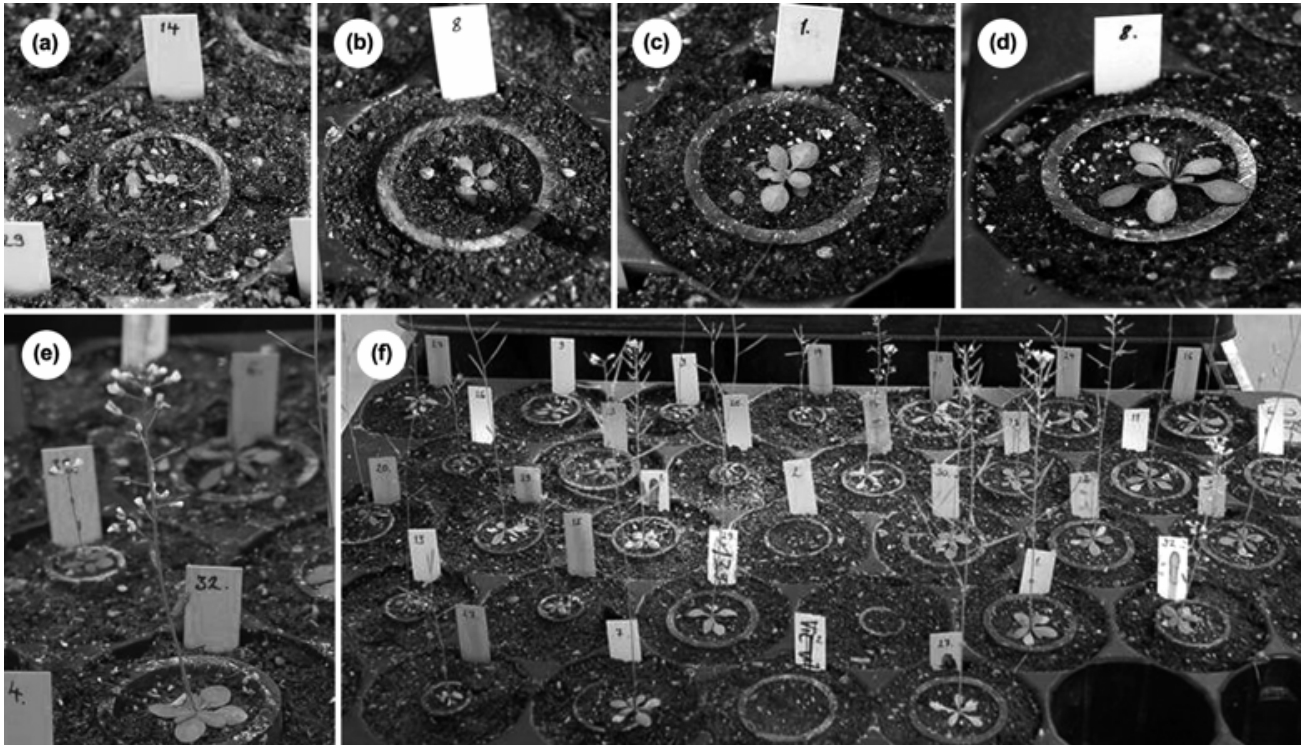
**Figure 1** Picture of the experiment showing the developmental stages of the plants at each of the six harvests (pictures a–f correspond to harvests 1–6; see Table 1). Note that the surface area available to grow rosettes is exactly the same for the three pot size treatments.

## Conventional RGR

Conventional RGR is an average over some specified time period. Average RGR ($RGR_{av}$) is typically measured by making two harvests separated by a short time interval and applying eqn 1. Here we calculate average $RGR_{av}$ over the whole growth interval (harvest 1 – harvest 6). We also calculated early RGR ($RGR_{early}$) using data from the first two harvests (conducted 7 and 11 days after sowing).

## Secondary compounds and herbivory

Estimates of growth rates ($RGR_{av}$, $RGR_{early}$ and size-standardised RGR) in the largest pot size (diameter = 40 mm) were used to test associations between different measures of growth rate with herbivore damage and with glucosinolate concentrations in leaves and seeds. In an earlier experiment, Kliebenstein et al. (2001a) measured glucosinolate concentration in leaves and seeds on the same RILs and recorded levels of damage inflicted by two insects after feeding for a short time interval (Kliebenstein *et al.*, 2002). Although glucosinolate concentrations, damage by herbivores and growth rates were not measured in the same individuals, the genetic stability of a RIL population allows data from different experiments to be compared as long as strong environment x genotype interactions are lacking (West *et al.*, 2006; Keurentjes *et al.*, 2007; Sønderby *et al.*, 2007; Wentzell *et al.*, 2007; Hansen *et al.*, 2008; Keurentjes *et al.*, 2008; Fu *et al.*, 2009; Sulpice *et al.*, 2009).

For glucosinolate content, five plants per RIL were planted individually in separate pots (diameter = 60 mm), replicated three times. After three weeks, 10 leaves were harvested from each replicate while plants were grown to senescence and seeds collected within each replicate. Both leaf and seed samples were extracted and analysed with previously-described high-throughput methods (Kliebenstein *et al.*, 2001a; Kliebenstein *et al.*, 2001b). Herbivory assays using two lepidopteran species were conducted in another experiment (Kliebenstein *et al.*, 2002). A single 1st instar larva of the specialist *Plutella xylostella* L. or the generalist *Trichoplusia ni* Hübner were placed on 4-week-old plants and the area removed by the herbivores after 48 hours of feeding was measured. Herbivory estimates in *Arabidopsis* based on leaf area removal are highly correlated with herbivory estimates based on larval weight gain (Jander *et al.*, 2001; Barth & Jander, 2006), confirming the reliability of this method. Each RIL was assayed for damage by each lepidopteran species 16 independent times. As *P. xylostella* removed large proportions of the rosettes it was necessary to correct the herbivory scores for rosette size. This was done by fitting a randomized complete blocks ANOVA using the model HERBIVORY = CONSTANT + FLAT + LINE + SIZE, where flat is a blocking term. The predicted herbivory means were then taken as size-standardised herbivory scores. Previous analyses reveal that there is significant among-line variation in both damage inflicted by herbivores and in the concentrations of various glucosinolates compounds (Kliebenstein *et al.*, 2002).

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**Table 3.** Correlations (Pearson's product-moment) between glucosinolate compounds in leaves and seeds and the three different measures of RGR. Significant correlations are highlighted in boldface. The concentrations of some glucosinolate compounds were not measured in the leaves; hence this correlation is not available (NA).

| | | $RGR_{av}$ | | $RGR_{early}$ | | Size-standardised RGR | |
|---|---|---|---|---|---|---|---|
| indolyl-3-methyl | leaves | 0.276 | $p = 0.141$ | 0.217 | $p = 0.268$ | -0.228 | $p = 0.226$ |
| | seed | 0.291 | $p = 0.119$ | -0.350 | $p = 0.068$ | -0.226 | $p = 0.231$ |
| 1-methoxy-indolyl-3-methyl | leaves | NA | NA | NA | NA | NA | NA |
| | seed | 0.211 | $p = 0.263$ | 0.102 | $p = 0.607$ | -0.336 | $p = 0.070$ |
| 4-methoxy-indolyl-3-methyl | leaves | 0.113 | $p = 0.552$ | 0.316 | $p = 0102$ | -0.102 | $p = 0.593$ |
| | seed | NA | NA | NA | NA | NA | NA |
| Total indolic glucosinolates | leaves | 0.314 | $p = 0.091$ | 0.208 | $p = 0.287$ | -0.215 | $p = 0.254$ |
| | seed | 0.320 | $p = 0.084$ | -0.331 | $p = 0.085$ | -0.288 | $p = 0.123$ |
| | | | | | | | |
| 3-hydroxypropyl (3C) | leaves | NA | NA | NA | NA | NA | NA |
| | seed | -0.360 | $p = 0.050$ | -0.116 | $p = 0.555$ | 0.360 | $p = 0.051$ |
| 3-methylthiobutyl (3C) | leaves | NA | NA | NA | NA | NA | NA |
| | seed | -0.034 | $p = 0.857$ | -0.005 | $p = 0.981$ | 0.016 | $p = 0.933$ |
| 4-methylthiobutyl (4C) | leaves | NA | NA | NA | NA | NA | NA |
| | seed | **0.394** | **$p = 0.031$** | 0.025 | $p = 0.899$ | **-0.430** | **$p = 0.018$** |
| 7-methylsulfinylheptyl | leaves | NA | NA | NA | NA | NA | NA |
| | seed | **0.478** | **$p = 0.008$** | 0.177 | $p = 0.365$ | **-0.566** | **$p = 0.001$** |
| 7-methylthioheptyl | leaves | **0.423** | **$p = 0.019$** | -0.012 | $p = 0.953$ | -0.197 | $p = 0.296$ |
| | seed | **0.570** | **$p = 0.001$** | -0.156 | $p = 0.426$ | **-0.557** | **$p = 0.001$** |
| Total 7C aliphatic glucosinolates | leaves | **0.487** | **$p = 0.006$** | 0.002 | $p = 0.991$ | -0.278 | $p = 0.137$ |
| | seed | **0.578** | **$p = 0.0008$** | -0.090 | $p = 0.646$ | **-0.586** | **$p = 0.0006$** |
| 8-methylsulfinyloctyl | leaves | **0.474** | **$p = 0.008$** | -0.078 | $p = 0.694$ | **-0.380** | **$p = 0.039$** |
| | seed | 0.178 | $p = 0.348$ | 0.141 | $p = 0.473$ | -0.292 | $p = 0.117$ |
| 8-methylthiooctyl | leaves | 0.210 | $p = 0.266$ | -0.038 | $p = 0.846$ | 0.012 | $p = 0.949$ |
| | seed | 0.069 | $p = 0.719$ | -0.088 | $p = 0.655$ | -0.117 | $p = 0.539$ |
| Total 8C aliphatic glucosinolates | leaves | 0.416 | $p = 0.022$ | -0.034 | $p = 0.862$ | -0.202 | $p = 0.284$ |
| | seed | 0.115 | $p = 0.544$ | -0.007 | $p = 0.970$ | -0.196 | $p = 0.298$ |
| Total methylsulfinyl glucosinolates | leaves | 0.469 | $p = 0.009$ | -0.017 | $p = 0.930$ | **-0.469** | **$p = 0.009$** |
| | seed | 0.232 | $p = 0.217$ | 0.147 | $p = 0.454$ | -0.353 | $p = 0.056$ |
| Total aliphatic glucosinolates | leaves | 0.159 | $p = 0.401$ | -0.027 | $p = 0.892$ | -0.144 | $p = 0.447$ |
| | seed | 0.184 | $p = 0.330$ | -0.050 | $p = 0.801$ | -0.333 | $p = 0.072$ |
| | | | | | | | |
| Total glucosinolates | leaves | 0.298 | $p = 0.109$ | -0.116 | $p = 0.556$ | -0.186 | $p = 0.326$ |
| | seed | 0.162 | $p = 0.391$ | -0.061 | $p = 0.757$ | -0.341 | $p = 0.065$ |

All three measures of RGR were tested for association with herbivore damage and with the line-specific glucosinolate concentrations in both leaves and seeds using Pearson's product moment correlation. Seed glucosinolate concentrations were also used because they might better reflect the lifetime production of glucosinolates by the plant and because the period of seed production is included in the growth curve. In contrast, leaf concentrations vary according to plant age and size and this can confound analyses (Koricheva, 1999). Concentrations of compounds were tested for normality and transformed where necessary (log or square-root) before correlations were performed.

## Results
### Conventional RGR
$RGR_{early}$ was positively correlated with $RGR_{av}$ ($r = 0.389$, $P = 0.037$, df = 27). As expected, there was a significant negative association between $RGR_{av}$ and seed mass ($F_{1,28} = 6.47$, $P = 0.017$) although $RGR_{early}$ was not significantly associated with seed mass ($F_{1,27} = 1.99$, $P = 0.17$).

### Size-standardised RGR
The asymptotic regression model appeared to provide a good fit to the data (Fig. S1) and model-checking plots revealed no obvious signs of model mis-specification. As judged by comparison of AIC values (a measure of goodness-of-fit; Akaike (1974)), models with pot volume fitted as a factor were better than those in which the

relationship between pot volume and parameters was assumed to be linear or log-linear (although parameters always increased or decreased systematically with pot size). In larger pots the estimated asymptotic mass, $A_i$ was higher ($F_{2,1039}$ = 9.23, $p$ = 0.0001, Table 2) but the rate parameter, $r_i$ was slightly lower ($F_{2,1039}$ = 16.02, $p$ < 0.0001, Table 2). There was a significant effect of sown seed mass on the estimated initial mass, $M_{i,0}$, ($F_{1,987}$ = 17.18, $p$ < 0.0001) which is expected if larger seeds produce larger seedlings. For the random effects, lines varied significantly only in the rate parameter, $r_i$ ($\chi^2$ = 20.9, df = 3, $p$ < 0.0001); the asymptotic mass did not vary among lines ($\chi^2$ = 3.16, df = 3, $p$ = 0.368), nor was there any significant residual variation among lines in the estimated initial mass, $M_{i,0}$ once sown seed mass was fitted ($\chi^2$ = 3.11, df = 1, $p$ = 0.078). The lack of a genotype effect on asymptotic mass probably reflects the pot-grown conditions, in which final size is strongly limited by pot size (Paul-Victor & Turnbull, 2009). Size-standardised RGR was calculated for each line in the largest pot size using parameters taken from the final model and a reference mass, $M_{ref}$ equal to the average mass of the plants half-way through the experiment (eqn 4). However, because only one parameter, $r_i$ varied among lines, the relative ranking of lines with respect to growth rates is independent of the choice of reference mass. The relative ranking of genotypes is also independent of pot size, as there was no RIL × pot size interaction. In addition, this indicates that across-experiment comparisons are unlikely to be influenced by pot size differences. Size-standardised RGR was negatively correlated with RGR$_{av}$ ($r$ = -0.788, P < 0.0001, df = 29) and uncorrelated with RGR$_{early}$ ($r$ = -0.062, P = 0.749, df = 27).

## Understanding relationships between seed size and RGR

To understand the relationships between seed size, conventional RGR and size-standardised RGR we show some simple results for the expected relationship between seed size and RGR assuming plant growth can be adequately modelled by the asymptotic regression equation above (eqn 2). In this case, we first assume that lines differ only in their seed mass ($M_{i,0}$) and that there are no true differences among lines in the two growth parameters (the rate parameter, $r_i$ and the asymptotic mass, $A_i$). We thus assume that each line has an initial mass ($M_{i,0}$) given by its seed size and hence we can calculate the expected mass of each line at harvest 1 and harvest 6 using eqn 4 and average values of $r_i$ and $A_i$ estimated for the largest pot size (Table 2). We can then use these values to calculate RGR$_{av}$ for each line (eqn.1). This reveals that while RGR$_{av}$ is negatively correlated with seed size, size-standardised RGR (eqn 4) is the same for all lines (Fig. 2a and 2b). As other growth parameters are identical among lines, the variation in conventional RGR is entirely due to differences in initial
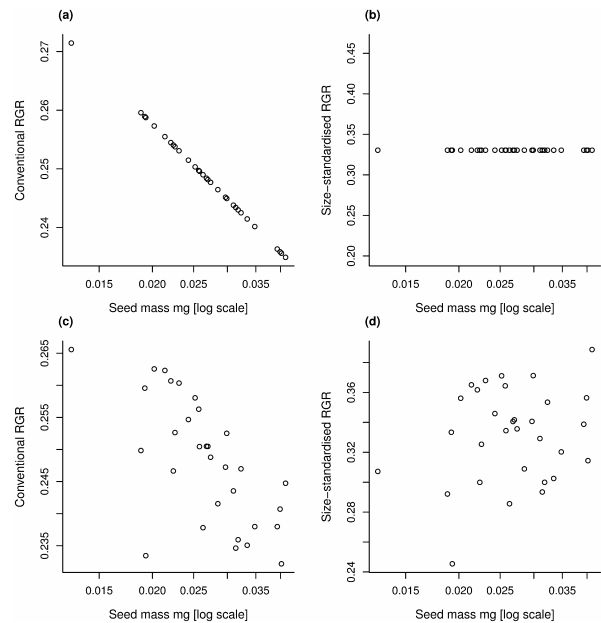


**Figure 2** Expected relationships between seed size and RGR assuming plant growth can be adequately modelled by an asymptotic regression equation. In a and b we assume that there are no differences in the parameters of the growth rate equation other than differences in initial mass, while in c and d we incorporate the estimated among-line differences in the rate parameter, $r_i$.

mass, demonstrating that conventional RGR is sensitive to these differences. In contrast, size-standardised RGR correctly identifies that the growth parameters are identical.

Secondly, we can see the effect of including the line-specific differences in the rate parameter, $r_i$ estimated by the model-fitting process. If we include these differences, the negative relationship between seed mass and conventional RGR persists (Fig. 2c), because RGR is very sensitive to differences in seed mass but relatively insensitive to differences in the growth parameter, $r_i$. Conventional RGR and size-standardised RGR are negatively correlated with each other because conventional RGR is negatively correlated with seed mass but there is a positive correlation between seed mass and size-standardised RGR. Thus lines with heavy seeds have low conventional RGR and high size-standardised RGR, while those with lighter seeds have high conventional RGR and low size-standardised RGR (Fig. 2c vs. 2d).

## Secondary metabolites and herbivory

Among lines, damage by the specialist herbivore *P. xylostella* was positively correlated with size-standardised RGR, meaning that fast-growing lines suffered the most damage (Fig. 3a). In contrast, RGR$_{av}$ was negatively correlated with damage suffered (fast-growing lines suffered the least damage; Fig. 3b) and RGR$_{early}$ showed no correlation with herbivore damage (Fig. 3c). Correlations of damage by *T. ni* with RGR had the same direction as for *P. xylostella*, but were non-significant (not shown). Ten single glucosinolate compounds were assayed in the leaves,

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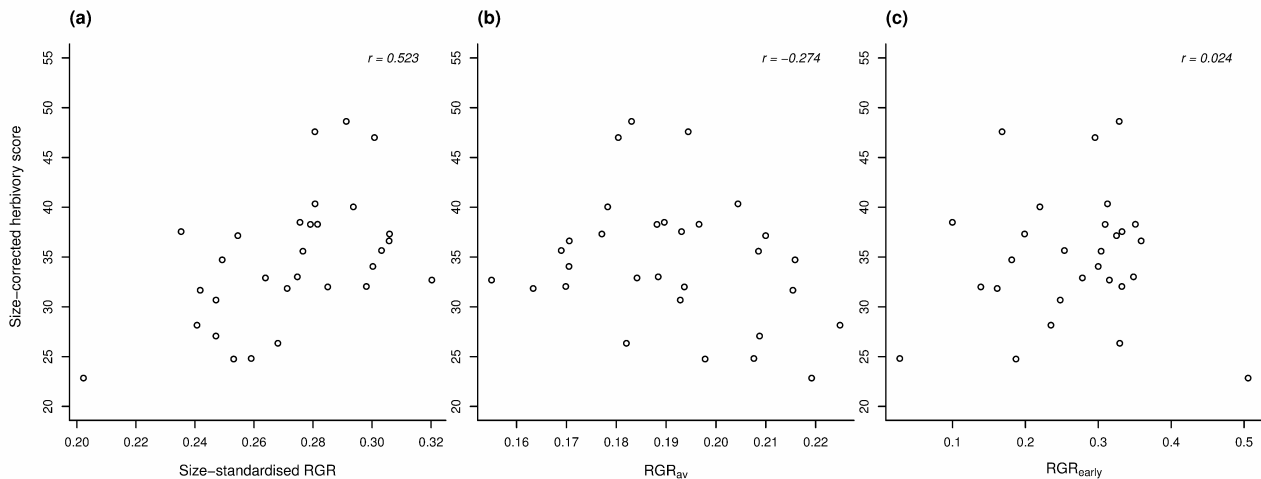
**Figure 3** Residual herbivory means for *P. xylostella* plotted against the three measures of RGR. Values of $r_P$ represent Pearson's product moment correlation. Only the correlation between herbivore damage and size-standardised RGR is significant.

the seeds or both (Table 3). As expected if defensive compounds have a growth cost, correlations between size-standardised RGR and ten glucosinolate compounds were predominantly negative in sign (Table 3). In fact, of the 26 correlations carried out in total, 23 were negative in sign. In contrast, correlations of chemical compounds with $RGR_{av}$ were positive for all but one compound, while no correlation between chemical compounds and $RGR_{early}$ was found (Table 3). Damage by the specialist herbivore was also negatively correlated with most glucosinolate compounds. Again out of 26 possible correlations, 23 were negative in sign although individual correlations were only significant in the case of the indolic glucosinolates (Table S2).

In general, correlations between size-standardised RGR and glucosinolate concentrations were stronger for seeds than for leaves, although not all compounds were measured in leaves, reducing the potential for significant correlations.

## Discussion

### The importance of size standardisation

RGR has for many years been accepted as a standardised way of measuring and comparing the growth rates of different species or genotypes. This is despite the fact that several authors have highlighted the problems with such comparisons when the species or genotypes vary in size (Poorter & Remkes, 1990; Reich *et al.*, 1998). Instantaneous RGR is expected to decline with size for both physiological reasons (large plants generally have to allocate more carbon to non-photosynthetic support tissue (Enquist *et al.*, 1999; West *et al.*, 2001)) and for reasons of resource restriction (large plants are increasingly unable to extract sufficient resources to maintain former growth rates). However, because the seed size variation observed in *Arabidopsis thaliana* is only 2–3 fold, it might reasonably be asked whether this variation is large enough to cause a problem. Here we have demonstrated that the seed mass

variation in the L*er* x Cvi population is sufficient to generate a spurious negative correlation between conventional RGR and seed size, even when there is no true underlying variation in physiological growth rates. Thus, the method could be more widely used to disentangle the effects of size from other physiological differences among lines, not just those differences associated with defence (Coleman & McConnaughay, 1995; McConnaughay & Coleman, 1999; Bernacchi *et al.*, 2000). It should also be noted that non-destructive methods of measuring plant biomass (or leaf area) are increasingly available, perhaps removing the need for destructive harvests and hence avoiding some of the additional work associated with this method (e.g. see Durham Brooks *et al.*, 2009 for a new method of measuring root growth continuously).

### The costs of defence

Traditionally, the costs of enhanced investment in defence have been assessed by comparisons of final seed set, as this is more directly correlated with fitness (Bazzaz *et al.*, 1987; Purrington & Bergelson, 1997; Mauricio, 1998). However, in the *Arabidopsis* lines analysed here, genotypes differed in the rate at which the asymptote was approached, and not in the asymptotic mass. Hence, it could be argued that a reduction in early growth rate does not represent a true fitness cost. However, a reduction in early growth rate could translate into a substantial fitness cost when plants are growing in competition rather than alone in individual pots. Rapid early growth allows resource pre-emption and therefore might be a good surrogate for competitive ability in short-lived annual plants (Grime, 2002). Loss of competitive status as a result of allocation to defence instead of early growth is a mechanism sometimes described as an 'opportunity cost' (Coley *et al.*, 1985), which is more easily detected when plants are growing in competition (reviewed in Koricheva, 2002).

7

Using the new RGR methodology, we were able to show that size-standardised growth rates were negatively correlated with a variety of glucosinolate compounds but positively correlated with herbivore damage. This supports the basic assumptions of plant defence theory which assumes that the optimal level of defence represents a balance between the costs of defence and the likelihood and severity of the expected attack (Herms & Mattson, 1992). In contrast, the direction of these correlations is reversed when using conventional RGR. Thus, if conventional RGR is to be believed, we would conclude that faster-growing lines produce more secondary metabolites and suffer less damage from herbivores, in common with some other studies using conventional RGR (Almeida-Cortez et al., 1999; Almeida-Cortez & Shipley, 2002). It thus seems that the lack of negative correlations between growth rates and defensive compounds in some published studies could at least partly be due to the lack of size-standardisation when calculating growth rates.

The negative correlations between size-standardised growth rate and glucosinolate concentration were stronger for some individual compounds than for total glucosinolates (Table 3). Similarly, glucosinolate compounds cannot be treated as a single defence mechanism as there is structural specificity to their effectiveness against various insects, as demonstrated in both lab and field studies (Bidart-Bouzat & Kliebenstein, 2008; Hansen et al., 2008). However, glucosinolates are products of complex metabolic pathways (e.g. Halkier & Gershenzon, 2006) and it might be that cellular processes independent of defence and growth influence their concentration and thus mask the trade-off pattern. Correlations between growth rates and glucosinolate concentration for all compounds were usually stronger in the seeds than in the leaves. The concentration in the seeds might better reflect the life-time metabolic potential of the maternal plant as of all plant organs, seeds have the highest proportion of glucosinolates per unit dry weight; as such they might incur a particularly high cost to the maternal plant (Brown et al., 2003). Additionally some seed glucosinolates are derived from leaf-glucosinolates (Kliebenstein et al., 2007; Nour-Eldin & Halkier, 2009). As a consequence, seed glucosinolates may correlate better with the growth rate calculated here, as this measure of growth is calculated over the whole lifespan of the plants, including the period of seed production.

## Methodological considerations

The use of a homozygous RIL population, in which genetic variation is stable, allowed us to combine data from the present experiment with data on herbivory and glucosinolates collected in other experiments on the same RIL population. This is a well-established concept that has allowed for cross-comparison across numerous experimental conditions for a given RIL population, e.g. identifying causal links between transcriptome and metabolome variation even though the experiments were separated by several years (West et al., 2006; Keurentjes et al., 2007; Sønderby et al., 2007; Wentzell et al., 2007; Hansen et al., 2008; Keurentjes et al., 2008; Fu et al., 2009; Sulpice et al., 2009). It has also been established that the main QTLs controlling glucosinolate structure and concentration within this population do not show extensive genotype × environment interactions within the rosette (Kliebenstein et al., 2001a; Kliebenstein et al., 2002). Thus, although the measurements were made at three different points in time, this simply decreases our statistical power to find significant effects and should not introduce potential bias. The strength of the negative trade-off between growth and defence could therefore be under-estimated.

Correlative analyses such as those presented in this paper are not causal and are most effective for generating new hypotheses. Our analysis also raises the possibility that some of the costs of secondary metabolites are masked by cellular processes not directly associated with growth and defence. In *Arabidopsis*, such hypotheses require more rigorous testing with additional, larger RIL populations or by exploiting mutational variation. We hope, however, that the methods presented in the paper will better allow future studies to better estimate the costs of defence.

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## Supplementary information.

**Table S1:** Information about the 32 recombinant inbred lines selected for the study.

**Table S2:** Correlations between glucosinolate compounds in leaves and seeds and herbivore damage by the specialist insect *Plutella xylostella*.

**Figure S1.** Destructive harvest data for the thirty RILs (the L*er* parent is not shown) grown in one pot size (pot diameter = 30 mm) with fitted curves from the final model.

# SUPPLEMENTARY INFORMATION

**Table S1**: Information about the 32 lines selected for the study. The two accessions L*er* and Cvi are the parents. The 30 remaining recombinant inbred lines are derived from reciprocal crosses between the two parents.

| NASC | RIL Koornneef | Published Seed Mass (*) [mg] | Sown Seed mass (**) [mg] | *ERECTA* mutation |
|---|---|---|---|---|
| N8581 | L*er* | 0.0193 | 0.0202 | 1 |
| N8580 | Cvi | 0.0351 | 0.0348 | 0 |
| N22002 | CVL3 | 0.0162 | 0.0129 | 1 |
| N22014 | CVL15 | 0.0145 | 0.0193 | 0 |
| N22018 | CVL19 | 0.0251 | 0.0263 | 1 |
| N22026 | CVL27 | 0.0275 | 0.0270 | 1 |
| N22030 | CVL31 | 0.0295 | 0.0334 | 0 |
| N22033 | CVL34 | 0.0236 | 0.0297 | 0 |
| N22036 | CVL37 | 0.0325 | 0.0399 | 0 |
| N22037 | CVL38 | 0.0150 | 0.0188 | 0 |
| N22038 | CVL39 | 0.0202 | 0.0258 | 0 |
| N22043 | CVL44 | 0.0242 | 0.0285 | 0 |
| N22051 | CVL53 | 0.0327 | 0.0310 | 1 |
| N22057 | CVL60 | 0.0286 | 0.0393 | 1 |
| N22059 | CVL62 | 0.0190 | 0.0224 | 0 |
| N22094 | CVL124 | 0.0274 | 0.0252 | 1 |
| N22095 | CVL125 | 0.0200 | 0.0214 | 0 |
| N22098 | CVL128 | 0.0273 | 0.0274 | 0 |
| N22099 | CVL129 | 0.0243 | 0.0268 | 0 |
| N22105 | CVL135 | 0.0327 | 0.0348 | 1 |
| N22107 | CVL137 | 0.0302 | 0.0314 | 0 |
| N22109 | CVL139 | 0.0217 | 0.0231 | 0 |
| N22112 | CVL142 | 0.0315 | 0.0318 | 1 |
| N22124 | CVL154 | 0.0317 | 0.0323 | 0 |
| N22128 | CVL158 | 0.0373 | 0.0411 | 1 |
| N22130 | CVL160 | 0.0361 | 0.0402 | 1 |
| N22132 | CVL162 | 0.0256 | 0.0221 | 1 |
| N22138 | CVL168 | 0.0334 | 0.0299 | 0 |
| N22148 | CVL178 | 0.0207 | 0.0226 | 1 |
| N22149 | CVL179 | 0.0223 | 0.0243 | 1 |
| N22156 | CVL187 | 0.0183 | 0.0192 | 1 |
| N22160 | CVL191 | 0.0280 | 0.0257 | 1 |

(*) Source: Alonso-Blanco et al., 1999.

(**) Source: Arabidopsis center (TAIR).

**Table S2**: Coefficients of Pearson's product moment correlation describing the relation of individual glucosinolates in leaves and seeds with herbivore damage of the specialist *Plutella xylostella* (size-corrected). Concentrations of compounds were log- or squareroot-transformed where necessary to meet the assumptions of normality.

| | | Herbivore damage *P. xylostella* | |
|---|---|---|---|
| indolyl-3-methyl | leaves | -0.216 | $p = 0.253$ |
| | seed | -0.323 | $p = 0.082$ |
| 1-methoxy-indolyl-3-methyl | leaves | NA | NA |
| | seed | -0.130 | $p = 0.493$ |
| 4-methoxy-indolyl-3-methyl | leaves | -0.135 | $p = 0.479$ |
| | seed | NA | NA |
| Total indolic glucosinolates | leaves | -0.144 | $p = 0.447$ |
| | seed | **-0.393** | **$p = 0.032$** |
| | | | |
| 3-hydroxypropyl (3C) | leaves | NA | NA |
| | seed | 0.284 | $p = 0.128$ |
| 3-methylthiobutyl (3C) | leaves | NA | NA |
| | seed | -0.103 | $p = 0.590$ |
| 4-methylthiobutyl (4C) | leaves | NA | NA |
| | seed | -0.211 | $p = 0.263$ |
| 7-methylsulfinylheptyl | leaves | NA | NA |
| | seed | -0.320 | $p = 0.085$ |
| 7-methylthioheptyl | leaves | -0.081 | $p = 0.670$ |
| | seed | -0.306 | $p = 0.100$ |
| Total 7C aliphatic glucosinolates | leaves | -0.145 | $p = 0.443$ |
| | seed | -0.323 | $p = 0.081$ |
| 8-methylsulfinyloctyl | leaves | -0.319 | $p = 0.086$ |
| | seed | -0.147 | $p = 0.439$ |
| 8-methylthiooctyl | leaves | -0.048 | $p = 0.800$ |
| | seed | 0.041 | $p = 0.831$ |
| Total 8C aliphatic glucosinolates | leaves | -0.179 | $p = 0.343$ |
| | seed | -0.031 | $p = 0.873$ |
| Total methylsulfinyl glucosinolates | leaves | -0.197 | $p = 0.297$ |
| | seed | -0.217 | $p = 0.250$ |
| Total aliphatic glucosinolates | leaves | -0.299 | $p = 0.109$ |
| | seed | -0.102 | $p = 0.590$ |
| | | | |
| Total glucosinolates | leaves | -0.215 | $p = 0.254$ |
| | seed | -0.143 | $p = 0.451$ |

**Figure S1.** Destructive harvest data for the thirty RILs (the L*er* parent is not shown) grown in one pot size (pot diameter = 30 mm) with fitted curves from the final model.