1 **Trade-off between constitutive and inducible resistance against herbivores is only**

2 **partially explained by gene expression and glucosinolate production**

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34

35   Summary statement

36   The observed partial correlation between herbivore resistance, defensive metabolites

37   accumulation, and gene expression suggests complex network of gene interactions

38   governing the postulated trade-off between constitutive defences and their

39   inducibility.

40

41

42   Abstract

43   The hypothesis that constitutive and inducible plant resistance against

44   herbivores should trade-off because they use the same resources and impose costs to

45   plant fitness has been postulated for a long time. Negative correlations between

46   modes of deployment of resistance and defences have been observed across and

47   within species in common garden experiments. We therefore tested whether that

48   pattern of resistance across genotypes follows a similar variation in patterns of gene

49   expression and chemical defence production. Using the genetically tractable model

50   *Arabidopsis thaliana* and different modes of induction, including the generalist

51   herbivore *Spodoptera littoralis,* the specialist herbivore *Pieris brassicae*, and

52   jasmonate application, we measured constitutive and inducibility of resistance across

53   seven *A. thaliana* accessions that were previously selected based on constitutive

54   levels of defence gene expression. According to theory, we found that modes of

55   resistance traded-off among accessions, particularly against *S. littoralis,* in which,

56   accessions investing in high constitutive resistance did not increase it substantially

57   after attack, and vice-versa. Accordingly, the average expression of eight genes

58   involved in glucosinolate production negatively predicted larval growth across the

59   seven accessions. We next measured glucosinolate production and genes related to

60   defence induction on healthy and herbivore-damaged plants. Surprisingly, we only

61   found a partial correlation between glucosinolate production, gene expression and the

62   herbivore resistance results. These results suggest that the defence outcome of plants

63   against herbivores goes beyond individual molecules or genes but stands on a

64   complex network of interactions.

65

66   Key words: glucosinolates, jasmonic acid, plant defences, plant-herbivore

67   interaction, specificity of resistance, VSP2

68 Introduction

69   Plants, to ward off herbivore attack, have evolved a whole array of defence

70 traits (Schoonhoven *et al.*, 2005), which can be always present or only induced after

71 herbivore feeding (Karban and Baldwin, 1997). The general consensus argues that

72 inducible defences have evolved as a cost-saving strategy (Karban *et al.*, 1997), in

73 which undamaged plants can divert resources from defence to growth and

74 reproduction. Zangerl & Rutledge (1996) postulated that the pattern of constitutive

75 and inducible defences, at the plant or at the organ level, depends on the probability

76 of the attack and the value of the organ. In other words, plants or organs, which are

77 regularly attacked by herbivores, should have high levels of constitutive defences

78 and low levels of induced defences. By extrapolations, in populations where

79 herbivory is low, plants should invest little in constitutive defences and more in

80 inducibility of defence, in which inducibility is the difference between the induced

81 state minus the constitutive state of defence in an organ of the plant. Recent

82 examples have shown that inducibility is dependent on the spatial variation on the

83 plant populations and herbivore pressure (e.g. Moreira *et al.*, 2014; Rasmann *et al.*,

84 2014), suggesting that at the landscape level there are constraints on simultaneously

85 producing both types of defence investment within one species.

86   Indeed, because we know that the expression of redundant traits is costly for

87 the plant (Koricheva *et al.*, 2004), and because we assume that constitutive and

88 induced defences are two traits in competition for the same resources in the plant, we

89 should expect a trade-off (or negative correlation) between them (Agrawal *et al.*,

90 2010). In other words, if both constitutive and inducible resistance traits are adaptive,

91 we should observe a negative correlation between constitutive and induced resistance

92 across populations or species of plants (Agrawal *et al.*, 2010). Several examples have

93 shown trade-offs between constitutive and inducible resistance, both within (e.g.

94 Gianoli, 2002; Rasmann *et al.*, 2014; Rasmann *et al.*, 2011) and across species (e.g.

95 Kempel *et al.*, 2011; Moreira *et al.*, 2014; Rasmann and Agrawal, 2011; Zhang *et al.*,

96 2008). Additionally, Thaler & Karban (1997) mapped constitutive and inducible

97 defences along the phylogeny of *Gossypium* spp., and showed independent and

98 repeated origins and losses of both defence traits, indicating evolutionary lability and

99 independence in the mode of defence investment. In *Acacia*, it was shown that

100 constitutive extrafloral nectar production originated from inducible production in

101 closely related species (Heil *et al.*, 2004). To summarize, past research indicates that

102   constitutive and inducibility of resistance evolve depending on the herbivore pressure

103   and the probability of attack at a particular site. Nevertheless, constrains imposed by

104   resource acquisition force the two mode of defence investment to negatively

105   correlate with each other.

106   With this study we aimed to take a step further in the study of the

107   interactions, and putative trade-off, between inducible and constitutive resistance and

108   investigate the genetic bases explaining the pattern. We specifically asked whether

109   patterns of trade-off between constitutive and inducible resistance (i.e. the effect of

110   the plant's defensive arsenal on the performance of the herbivores, according to

111   Karban & Baldwin (1997)) is correlated to similar patterns of defensive secondary

112   metabolites and gene induction. To address our questions we used a highly

113   genetically-tractable plant, the thale cress *Arabidopsis thaliana* (Brassicaceae); a

114   small annual plant from Eurasia but naturalized across all continents expect

115   Antarctica. Basal genome-wide expression levels have been characterized for over

116   750 Arabidopsis accessions. In addition, major biosynthetic pathways involved in

117   insect resistance, including the jasmonate pathway (Howe and Jander, 2008), are

118   well characterized (Bodenhausen and Reymond, 2007). Furthermore, Arabidopsis,

119   like most species in the Brassicales, contains glucosinolates. When insect herbivores

120   feed on the plant, they damage tissues and bring glucosinolates in contact with an

121   activated enzyme, the myrosinase, which results in the production highly toxic

122   hydrolysis breakdown products such as nitriles, isothiocyanates or thiocyanates

123   (Halkier and Gershenzon, 2006). Moreover, several studies have already shown

124   specificity in inducible resistance against specialists versus generalist herbivores in

125   Arabidopsis (De Vos *et al.*, 2005; Rasmann *et al.*, 2012). Generally, it was shown

126   that the glucosinolates have a negative impact on generalist herbivores fitness, but it

127   has little, none, or positive effect on specialist herbivores (Mueller *et al.*, 2010;

128   Schweizer *et al.*, 2013).

129   Here we hypothesize that, 1) according to classic theory, previously induced

130   plants are more defended against subsequent herbivore attack than undamaged

131   plants; 2) generalist herbivores are more susceptible than specialist herbivores, 3)

132   there is a negative genetic correlation between constitutive and inducibility of

133   resistance, and 4) both glucosinolate production, and gene expression related to

134   defence induction correlate with patterns of induced resistance.

135

Material and methods

Plant material

Seeds of all accessions were obtained from The Nottingham Arabidopsis Stock Centre (NASC). For all the experiments (see below), all plants were grown in a growth chamber (short days, 20°C, 55% RH) with a 3:1 mix of commercial potting soil (Orbo-2, Schweizer AG, Lausanne; Switzerland) and perlite. All plants were six weeks old at the time of the experiments.

Microarray data

Constitutive expression data for Arabidopsis accessions were downloaded from the ArrayExpress repository database (http://www.ebi.ac.uk/arrayexpress; experiment E-TABM-18). Data are part of the At GenExpress project (http://arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp) and consist of expression values from 4-day-old seedlings from 34 accessions grown in soil in the same conditions and at the same time (Lempe *et al*, 2005).

Inducible resistance experiment

To measure the specificity of trade-offs between inducible and constitutive resistance in Arabidopsis, we performed an experiment with three different induction treatments: a control treatment (no induction), a jasmonic acid (JA) application, and an herbivore induction. Jasmonic acid has been shown to be the master regulator of plant inducible resistance against chewing herbivores in many plants, including Arabidopsis (Howe, 2004; Howe and Jander, 2008). For the herbivore treatment, we chose the highly generalist herbivore *Spodoptera littoralis* (Lepidoptera, Noctuidae), and the cabbage family specialist herbivore *Pieris brassicae* (Lepidoptera, Pieridae). Eggs of *S. littoralis* were provided by Syngenta (Stein Switzerland) and first-instar larvae were obtained by placing eggs at 30°C during three days. First-instar larvae of *P. brassicae* were obtained from rearing insects on cabbage (*Brassica oleracea*) in controlled greenhouse conditions at the University of Lausanne.

For all treatments, plants were enclosed in hermetic Plexiglas boxes (N = 7 genotypes x 3 treatments x 2 herbivores x 3 plants = 63 plants). Treatments were performed as follow: 1) the control-treated plants were left without further treatment for three days; 2) the JA treatment included plants that were induced by putting three cotton buds in the box, each one spiked with 5 µL of Methyl Jasmonate (MeJA)

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170 (Sigma-Aldrich CAS Nb 39924-52-2). JA treatment lasted 24h after which lids were

171 opened allowing the evaporation of the JA left in the box. Finally, 3) plants were

172 induced by placing 8-10 first-instar *S. littoralis* larvae per pot. Larvae were allowed

173 to feed for three days prior to removal. We used *S. littoralis* for the induction

174 treatment as this herbivore was used to measure the induction of defence genes in

175 selected accessions (see below).

176 After the induction, plants were individually surrounded with 330 ml volume

177 deli plastic cups with the bottom cut off, and 10 *S. littoralis* or 10 *P. brassicae* larvae

178 were added to each plant (N = 30 larvae per herbivore, per genotype and per

179 treatment). Cups were covered with fine-meshed nylon nets to prevent larvae from

180 escaping, and larvae were allowed to feed for 7 days, after which, all surviving larvae

181 were flash frozen in liquid nitrogen, oven-dried for 4 days at 50 °C and weighed.

182

183 *Glucosinolate and gene expression analyses*

184 For glucosinolate and gene analyses we planted 12 plants per genotype and

185 after six weeks, half of the plants were induced with 10 *S. littoralis* caterpillars for

186 three days as described above. At the end the induction treatment, 200 mg of fresh

187 tissue per plant was ground with a homogenizer in 2 ml ice cold MeoH:water (70:30,

188 v/v) with 25 μl of sinalbin 1.56 mmol as the internal standard. Samples were then

189 incubated for 15 min at 80 °C in a block heater (Techne dri-block, Staffordshire,

190 UK), centrifuged at 3500 x g for 10 min, and the supernatant was transferred to an

191 appropriate vial for analysis. Glucosinolate identification and quantification was

192 performed using an Acquity UPLC from Waters (Milford, MA, USA) interfaced to a

193 Synapt G2 QTOF from Waters with electrospray ionization, using the separation and

194 identification method as described in Glauser *et al.* (2012).

195 For gene expression analyses, two leaves were sampled from half of the

196 control and treated plants (n = 3), added together in one Eppendorf tube and flash

197 frozen in liquid nitrogen. We selected three genes known to be induced after

198 caterpillar attack in Col-0 (Reymond *et al.*, 2000), including: 1) *ALLENE OXIDE*

199 *CYCLYSE2* (*AOC2*), a gene that catalyses an essential step in jasmonic acid

200 biosynthesis*; 2) VEGETATIVE STORAGE PROTEIN2* (*VSP2*), a highly inducible

201 gene after herbivory or JA treatment; and 3) *CYTOCHROME P450 79B3*

202 (*CYP79B3*), a gene involved in indole-glucosinolate biosynthesis. RNA extraction

203 and qPCR analyses were done following standard protocols using the reference gene

204   *At2g28390* (Arabidopsis SAND family protein) as described in Hilfiker *et al.* (2014).

205   Primer efficiencies (E) were assessed by a five-step dilution regression. The

206   expression level of a target gene (TG) was normalized to the reference gene (RG)

207   and calculated as Normalized Relative Quantity (NRQ) as follows:

208     $NRQ = E^{Ct_{RG}}/E^{Ct_{TG}}$.

209

210   *Statistical analyses*

211   We analysed the effect of the genotypes, the induction treatment, and the two

212   herbivore species using a full-factorial three-way ANOVA. Secondly, to test for

213   trade-offs between constitutive and inducibility of resistance, we regressed the

214   inducibility (i.e. the difference in mean larval mass values for each genotype between

215   control and induced plants) against the genotype mean of that trait in the control

216   treatment (i.e. the constitutive level). As we regressed a variable against a difference

217   that includes the same variable (i.e. inducibility of resistance = induced plants –

218   control plants), the errors in the two axes are not independent, and thus there is a

219   possibility of obtaining spurious correlations from these analyses (Morris *et al.*,

220   2006). Therefore, to evaluate the significance of these correlations, we employed the

221   Monte Carlo simulation procedure proposed by Morris et al. (2006) using MATLAB

222   (Version 7.5.0.342 – R2007b, MathWorks Inc., USA).

223   Glucosinolate data were analysed with a three-way permutation ANOVA

224   using the package LmPerm in R (Wheeler, 2010), because we could not reach

225   normality of the errors, and included genotype, herbivore treatment, and compound

226   identity as main effects.

227

228   Results

229

230   *Selection of Arabidopsis accessions with contrasting constitutive defences*

231   To investigate genotypic variation in constitutive versus inducible resistance

232   we selected seven accessions of Arabidopsis, based on the expression of 16 genes

233   known to be related to defence against chewing herbivores (Reymond *et al.*, 2004);

234   Supplementary Material Table S1). For each individual gene, 34 accessions for

235   which whole-genome expression data were available (see methods) were ranked

236   based on the constitutive expression of defence genes. The computation of the

237   average constitutive expression across all genes provided a list of seven accessions

238 (Table S2), including HR-5, Kindalville-0 (Kin-0), Niederzenz-1 (Nd-1), Columbia-0

239 (Col-0), Moscow-0 (Ms-0), C-24, and Shahdara (Sha).

240

241 *Induction experiment*

242 In accordance with classic predictions, we found an overall effect of previous

243 induction on resistance (Figure 1, Table 1). Particularly, larvae of both species grew

244 22% and 14% less (for *S. littoralis* and *P. brassicae*, respectively) on plants that

245 were previously induced by *S. littoralis* (Figure 1, Table 1), and to a lesser extent on

246 plants that were induced with JA (17% and 10%, respectively, see no effect of

247 treatment by species interaction in Table 1). Overall, we found strong variation in

248 resistance across accessions (Table 1) and strong specificity in resistance across

249 accessions (see significant genotype by species interaction in Table 1).

250 Across seven accessions of Arabidopsis we found a negative genetic

251 correlation between the constitutive resistance and the inducibility of resistance,

252 particularly for the generalist herbivore *S. littoralis* (Figure 2, for *S. littoralis*, larval

253 induction, $r = -0.94$, $p = 0.02$; and JA induction, $r = -0.94$, $p = 0.01$; and for *P.*

254 *brassicae*, larval induction, $r = -0.82$, $p = 0.09$; and JA induction, $r = -0.08$, $p = 0.74$).

255 For *S. littoralis*, the ranking of inducibility from high-induced susceptibility to high-

256 induced resistance for both the larval and jasmonate induction was: C-24, HR-5,

257 Sha, Col-0, Kin-0, Ms-0, and Nd-1. In other words, Nd-1 showed the largest

258 inducibility of resistance, whereas C-24 had the smallest. Interestingly, we observed

259 in some instances that larvae were larger on induced plants than on uninduced ones

260 (Table S3). This was the case for *S. littoralis* feeding on HR-5 and C-24 after

261 treatment with JA, and for *P. brassicae* feeding on Sha and C-24, after herbivory.

262 We next assessed whether natural variation in gene expression could directly

263 influence resistance. We therefore regressed the average expression values of 8 genes

264 related to glucosinolate production, and 8 genes including JA marker genes and JA

265 biosynthesis in Arabidopsis (Table S2) against the larval weight of the generalist *S.*

266 *littoralis* on each genotype (Table S3). We only used *S. littoralis* data for this

267 analysis since only generalist herbivores should be affected by glucosinolates in

268 plants. Additionally, we only used the control treatment as gene expression was

269 measured on undamaged plants. We found that the constitutive expression of

270 glucosinolate biosynthesis-related genes negatively predicted larval weight gain

271 (Figure 3, $n = 7$, $r = 0.80$, $p = 0.03$). This was not true when regressing the average

272   expression of genes related to JA signalling and production (n = 7, r = 0.07, p =

273   0.87). To test whether or not results for the glucosinolate genes were spurious due to

274   random gene sampling, we performed a permutation analysis using the 10'000

275   averages of 10 randomly selected genes from the whole pool of 22'759 genes present

276   in Arabidopsis. As shown in Figure S1, our data indicate that the glucosinolate result

277   is well below the 0.1, and the 0.05 probabilities when compared to correlations with

278   random genes, indicating that the *S. littoralis* result cannot be obtained from random

279   gene sampling of defence genes.

280

281   *Glucosinolate and gene expression analyses*

282   Because we observed a negative relationship between constitutive and

283   inducible resistance (particularly against *S. littoralis*), we next sought defence

284   mechanisms behind the observed trade-off and measured glucosinolates and gene

285   expression of Col-0, HR-0, Ms-0, Nd-1. Our initial results from the resistance

286   experiment indicated that Col-0 and HR-5 showed little or none induced resistance,

287   Ms-0 showed intermediate levels of induced resistance, and ND-1 showed the

288   highest levels of induced resistance (Figure 2A). We therefore predicted that

289   glucosinolate and gene expression profiles would mimic the larval resistance results,

290   and Nd-1 would show the highest induction of defensive metabolites and genes

291   related to defence induction, and Col-0 and HR-5 the lowest (Figure 4A).

292   Glucosinolate analyses yielded 14 individual glucosinolate compounds, all

293   showing different overall levels (Table S4, see compound effect in Table 2) and

294   different inducibilies after herbivore attack (see treatment by compound effect in

295   Table 2), overall, with herbivore treatment increasing average glucosinolate levels by

296   27% compared to control plants (see treatment effect in Table 2). Accessions showed

297   little variation in total amount of glucosinolates, and only Nd-1 and Col-0 showed

298   variation in glucosinolate induction after herbivore attack (Figure 4B, Table S4, and

299   see treatment by genotype interaction in Table 2). Strikingly, some glucosinolates

300   were almost exclusively found in a single accession (Table S4).

301   Expression analyses of selected insect-inducible genes showed strong

302   induction after *S. littoralis* treatment (Figure 4C-E, and Table 3). *VSP2* had the

303   highest inducibility, with 14-fold induction overall (Figure 4E), compared to 2.6-fold

304   and 1.55-fold for *AOC2* and *CYP79B3* (Figures 4C, and 4D, respectively). We also

305   found strong genotype effect, and genotype by treatment effect for inducibility of

306 genes (Table 3). For *VSP2*, Col-0 and Nd-1 showed the strongest induction, MS-0

307 showed average induction and HR-5 the lowest induction after herbivore attack.

308 However, *AOC2* was strongly induced in Col-0, moderately in both HR-5 and Nd-1,

309 but not in Ms-0. Finally, *CYP79B3* was only induced in Col-0 (Figure 4D). Since this

310 enzyme is involved in the synthesis of indole-glucosinolates (Halkier and

311 Gershenzon, 2006), and its expression correlates with accumulation of glucosinolates

312 in Col-0 (Schweizer *et al.*, 2013), it was interesting to see that levels of the main

313 indole-glucosinolates I3M, and to a lesser extent 1MOI3M, increased in Col-0 after

314 herbivory (Table S4). Additionally, both compounds were also induced in Nd-1 and

315 I3M was higher in Ms-0 without the respective changes in *CYP79B3* expression.

316 Thus, our data show that there is not a consistent correlation between inducibility of

317 resistance, accumulation of glucosinolates and defence gene induction between

318 accessions, as it was predicted by the model in Figure 4A.

319

320 Discussion

321   We found that overall inducible resistance against herbivores in Arabidopsis

322 is underlined by strong genotypic variation, in which accessions that have high

323 constitutive resistance are weak inducer, whereas accessions that have low

324 constitutive resistance are strong inducers. This pattern generates the predicted trade-

325 off between constitutive and inducible resistance in plants. Interestingly, despite the

326 fact that basal expression of genes related to glucosinolate biosynthesis also predicts

327 the observed resistance to herbivory, we found that constitutive and induced

328 glucosinolate levels and defence gene induction only partially relate to the observed

329 resistance. This suggests that plant defence allocation strategies goes beyond the

330 individual molecules or genes but stands on a complex network of interactions.

331 Below we discuss the possible causes and consequences of the observed results.

332

333 *Specificity of induction of defences and herbivore responses*

334   The seminal book on plant defence induction by Karban and Baldwin (1997)

335 has paved the way to the general wisdom that plants, under herbivore attack, are able

336 to increase their basal levels of defences to a higher level. Whereas the ability to

337 increase resistance only after attack has undoubtedly clear benefits in term of costs

338 (Karban *et al.*, 1997), several drawbacks still impair a full grasp on the phenomenon,

339 including high specificity on the induction/response, and strong genotypic variation

340 in induction.

341   First, as we show here, there is high level of specificity on both sides, in

342 which either the induction agent (an insect or a phytohormone in our case) can result

343 in different inducibilities, and the response of the herbivore is species specific.

344 Indeed, plant induction of defences is driven by the complex chemistry of plant-

345 herbivore interaction (Halitschke *et al.*, 2003; Walling, 2000), which takes into

346 account the counter-response of the herbivore (Felton and Eichenseer, 2000; Karban

347 and Agrawal, 2002), and surely goes beyond simple application of jasmonic acid to

348 the plant (but see e.g. Rasmann *et al.*, 2012). Therefore, only by studying the effect

349 of several inducing agents can we generalize on the existing patterns. Next, we show

350 that specialist herbivores such as *P. brassicae* are less affected by previous plant

351 induction than the generalist herbivore *S. littoralis*, and this seems to be a general

352 rule in plant-insect interaction studies (Ali and Agrawal, 2012). Whether variation in

353 induced resistance and subsequent formation of trade-offs is mainly generated by

354 generalist herbivores is an enticing questions, and to our view merits further studies.

355   Second, this is not the first example of genotypes becoming more susceptible

356 to herbivores after induction. Indeed, induced susceptibility is more common than we

357 might expect (Karban and Baldwin, 1997), and it has been suggested that defence

358 suppression could even benefit the plant rather than the herbivore (Kahl *et al.*, 2000).

359 Although there is generally still little evidence for it, other studies show that plants

360 decrease their defences (Bede *et al.*, 2006; Kahl *et al.*, 2000; Lawrence *et al.*, 2008),

361 and become more susceptible to attacks by herbivores after previous attacks by other

362 species of herbivores (Poelman *et al.*, 2008; Sarmento *et al.*, 2011; Sauge *et al.*,

363 2006). Mechanisms behind induced susceptibility might include trade-offs between

364 defence types against different herbivore species (via so-called antagonistic cross-

365 talk between signalling pathways involved in plant defence (Thaler, 1999), even

366 within the same species (Bruessow *et al.*, 2010). It is therefore possible that the

367 physiological (and evolutionary) constraints generating the trade-offs between

368 constitutive and inducibility of resistance might also be behind patterns of induced

369 susceptibility, and future work with Arabidopsis in this regard might answer this

370 question.

371

372 *Genetic correlations among resistance strategies*

373   By measuring caterpillar growth on undamaged and previously damaged

374   plants, we found a negative genetic correlation between constitutive resistance and

375   inducibility of resistance. Thus, Arabidopsis accessions appear to have a maximal

376   potential for resistance, and this is either allocated constitutively (i.e. always

377   present), following herbivore attack, or in equal balance between the two. Such

378   trade-offs between constitutive and induced responses suggests that the expression of

379   resistance traits in plants is costly or otherwise constrained, or that there is simply no

380   benefit in to additional resistance beyond a particular threshold level (Agrawal *et al.*,

381   2010). Similar patterns in deployment strategies of defence were previously observed

382   within genotypes (Rasmann *et al.*, 2011), or across species of plants (Kempel *et al.*,

383   2011; Moreira *et al.*, 2014). Nevertheless, others have failed to observe trade-offs

384   between constitutive defences and inducibility, at least across species (Rasmann and

385   Agrawal, 2011). Such discrepancies in the experimental observations are difficult to

386   explain as long as we lack a mechanistic understanding of how trade-offs arise,

387   particularly at the gene level (Agrawal *et al.*, 2010). As mentioned above, variable

388   production of defences can be triggered by insect-derived elicitors (Halitschke *et al.*,

389   2003), plant hormones (Harfouche *et al.*, 2006), herbivore-induced volatile organic

390   compounds (Ton *et al.*, 2007), or indeed, differential constitutive levels of gene

391   expression (Ahmad *et al.*, 2011)

392   Additionally, differential investment in plant defence deployment could arise

393   from different herbivore pressures across the effective niche distribution of the

394   species. For instance, we have recently shown that *Vicia sepium* plants at high

395   elevation have lower basal levels of volatile organic compounds production but are

396   more inducible than their conspecifics at lower elevation. This pattern of defence

397   deployment goes hand-in-hand with lower herbivore pressure and lower abundance

398   of predatory ants at high elevation (Rasmann *et al.*, 2014). We thus suggest that the

399   observed pattern in Arabidopsis accessions is generated both by the physiological

400   constrains of the plant (i.e. some genotypes are simply at the maximum level of

401   resistance and thus could not be induced even more as was shown in Córdova-

402   Campos *et al.* (2012)), and the different selection pressures at different locations

403   where the accessions originated.

404

405   *Genotype – phenotype correlations*

13

406   Contrary to our expectations, we did not observe a consistent correlation

407   between the phenotypic response (i.e. herbivore growth), glucosinolate production

408   and defence gene induction. For instance, although the increasing induction of VSP2

409   between HR-5, Ms-0 and Nd-1 was correlated with the inducibility of resistance

410   results (as predicted in Figure 4A), Col-0 displayed the strongest induction of

411   defence genes and it displayed a high constitutive defence. Similarly, accumulation

412   of glucosinolates after *S. littoralis* feeding was not higher in Nd-1 than Col-0, despite

413   their different inducibility of resistance. In addition, the constitutive expression level

414   of glucosinolate biosynthesis genes was negatively correlated with larval weight,

415   although this was not true for glucosinolate levels, implying another level of

416   complexity. In a related study with Arabidopsis, Ahmad *et al.* (2011) showed that a

417   high induction of the defence gene *PR1* was correlated with a reduced bacterial

418   infection in different accessions.

419   Clearly, more work is needed to better understand these discrepancies. For

420   example, the apparent absence of correlation between total glucosinolates levels and

421   inducibility of resistance might be explained by the fact that different accessions

422   contain specific glucosinolates. These molecules may have different deterrent

423   properties, and a careful examination of the contribution of each glucosinolate

424   compound to defence will be needed. Furthermore, we restricted our investigation to

425   genes of the jasmonate pathway and to glucosinolates, which are established

426   components of defence against herbivory. Nevertheless, additional factors may

427   contribute to the inducibility of resistance, such as priming (Ahmad *et al.*, 2011; van

428   Hulten *et al.*, 2006), epigenetic modifications (Rasmann *et al.*, 2012), or post-

429   transcriptional effects (Gfeller *et al.*, 2011; Savchenko *et al.*, 2013). A study with a

430   larger number of accessions and defence traits might be needed to explain the

431   mechanistic aspects of the trade-off between constitutive and induced defences.

432

439

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Tables and Figures

Table 1. Three-way ANOVA for assessing the effect of the seven Arabidopsis accessions, the induction treatment (with *S. littoralis* or with methyl jasmonate), on the growth the two herbivore species (*S. littoralis* and *P. brassicae*).

Factor	df	F ratio	P value
Genotypes (G)	6	5.646	<.0001
Treatments (T)	2	3.999	0.022
G*T	12	1.400	0.183
Species (S)	1	261.774	<.0001
G*S	6	3.354	0.005
T*S	2	0.214	0.807
G*T*S	12	1.327	0.220
Residuals	82		

Table 2. Three-way permutation ANOVA table for individual glucosinolate levels across four Arabidopsis accessions. Plants were either left undamaged or induced with *S. littoralis* caterpillars for three days (i.e. treatment effect).

Factor	Df	Iter	P value
Genotype (G)	3	51	1
Treatment (T)	1	3985	0.024
G*T	3	3026	0.032
Compound (C)	13	5000	< 0.0001
G*C	39	5000	<0.0001
T*C	13	5000	0.025
G*T*C	39	5000	0.004
Residuals	560		

Table 3. Three-way permutation ANOVA table for individual gene expression levels across four Arabidopsis accessions. Plants were either left undamaged or induced with *S. littoralis* caterpillars for three days (i.e. treatment effect).

Factor	Df	Iter	P value
Genotype (G)	3	5000	< 0.0001
Treatment (T)	1	5000	< 0.0001
G*T	3	5000	< 0.0001
Genes (Gn)	2	5000	< 0.0001
G*Gn	6	5000	< 0.0001
T*Gn	2	5000	< 0.0001
G*T*Gn	6	5000	< 0.0001
Residuals	48		

Figure legends

Figure 1. Induced resistance against chewing herbivores. Shown are means (± SE) of *P. brassicae* (open bars) and *S. littoralis* (shaded bars) larval mass on Arabidopsis plants that were either left untouched (control), previously induced with *S. littoralis* caterpillar or previously induced with methyl jasmonate (JA). Shown is the average of resistance across seven Arabidopsis accessions. Different letters above bars means difference after post-hoc Tukey test, $p < 0.05$.

Figure 2. Trade-off between constitutive and inducibility of resistance. Shown are means of A) *S. littoralis* and B) *P. brassicae* larval mass when feeding on seven Arabidopsis accessions. Plants were either left undamaged (constitutive) or previously induced by herbivores (open circles, dotted lines), or induced with methyl jasmonate (black dots, solid lines). Inducibility is the average difference of larval weight between induced and constitutive conditions, therefore a negative value means induced resistance, and the lowest values indicate the highest induction of resistance. Lines indicate significant correlation, $p < 0.05$. Legend besides open circles or inside black circles indicate accessions' names: N = Nd-1, M = Ms-0, K = Kin-0, S = Sha, Co = Col-0, H = HR-5, and C = C-24.

Figure 3. Relationship between constitutive gene expression and resistance against chewing herbivores. Shown is the genotypic relationship across seven Arabidopsis accessions of resistance against *S. littoralis* larvae and average gene expression of 8 genes related to glucosinolate production ($p < 0.05$).

Figure 4. Defence induction across accessions. A) shows the predicted defence induction of four Arabidopsis accessions based on the resistance bioassay in Figure 2A, in which Nd-1 should have the highest inducibility, HR-5 and Col-0 should have the lowest inducibility, and Ms-0 should have intermediate levels of inducibility. B) show the mean (± SE) levels of constitutive (open bars) and induced (black bars) production of glucosinolates, and C) – E) show the relative expression of *AOC2*, *CYP79B3*, and *VSP2*, respectively. Induction was performed with *S. littoralis* caterpillars. Values (± SE) are the average of three technical replicates.
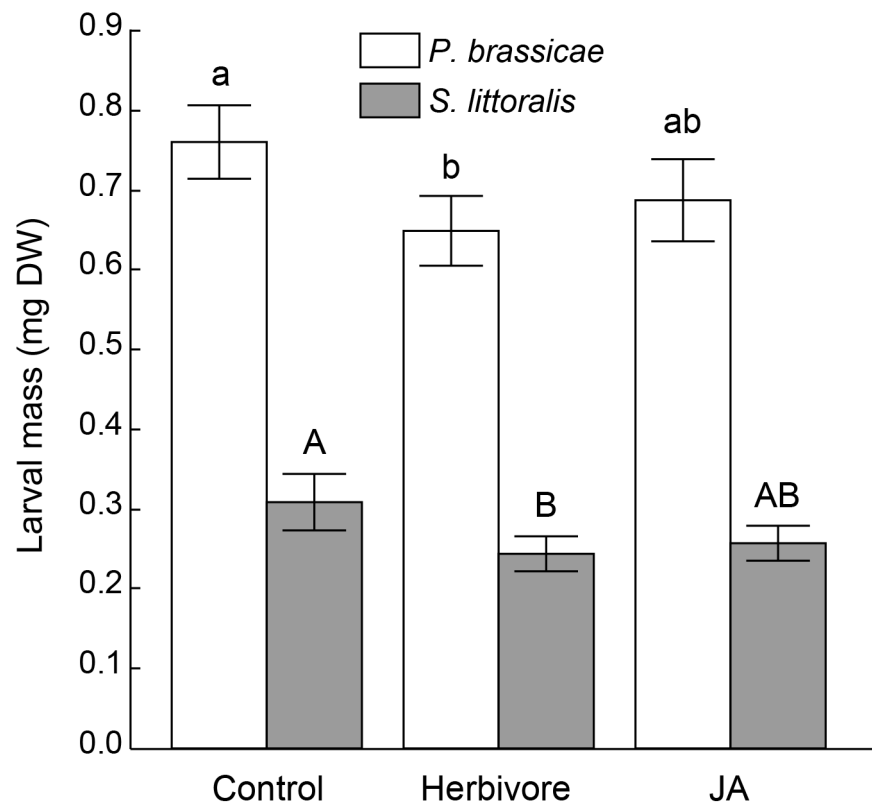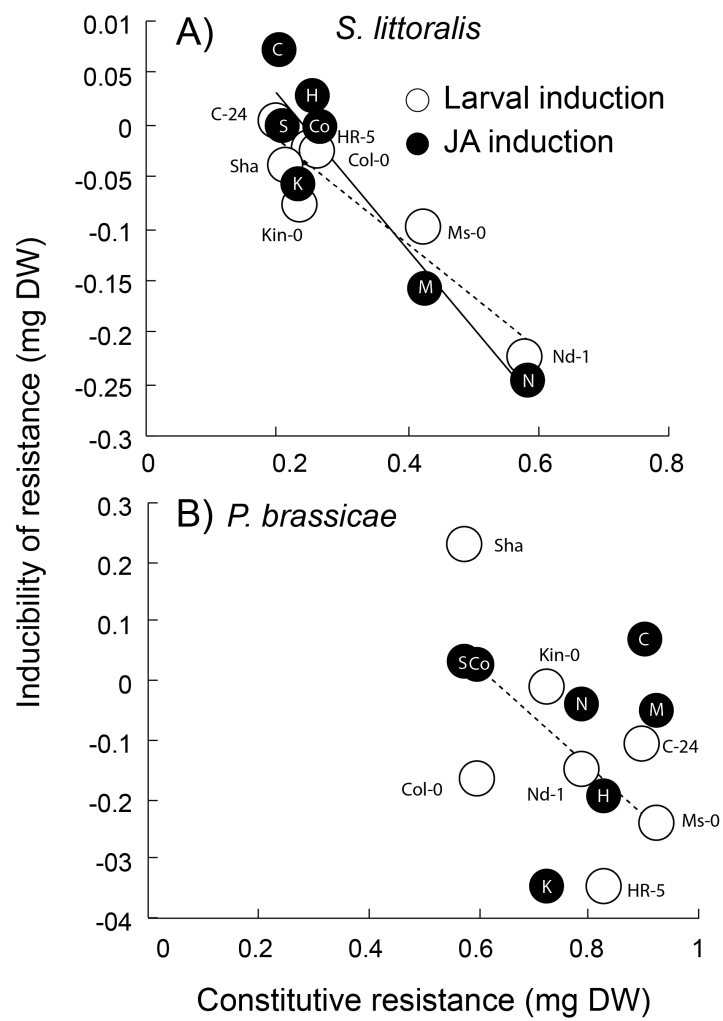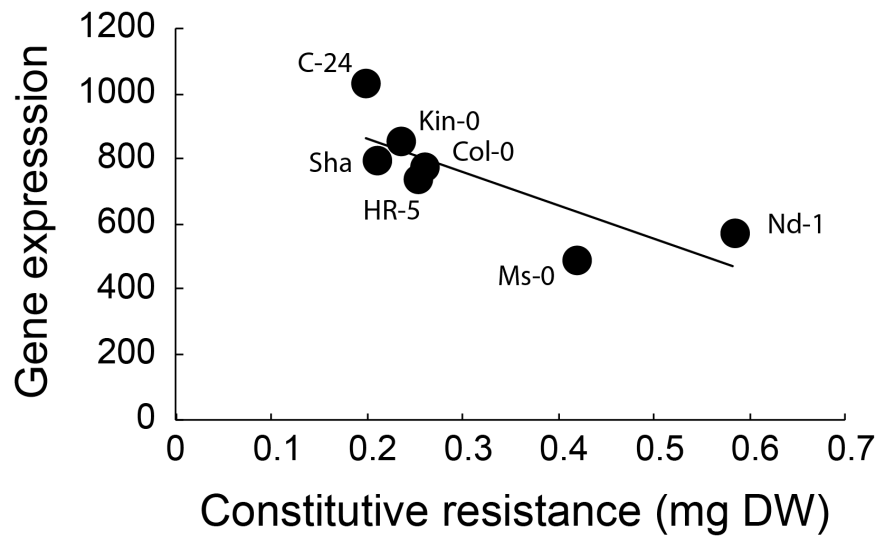
Figure 1.
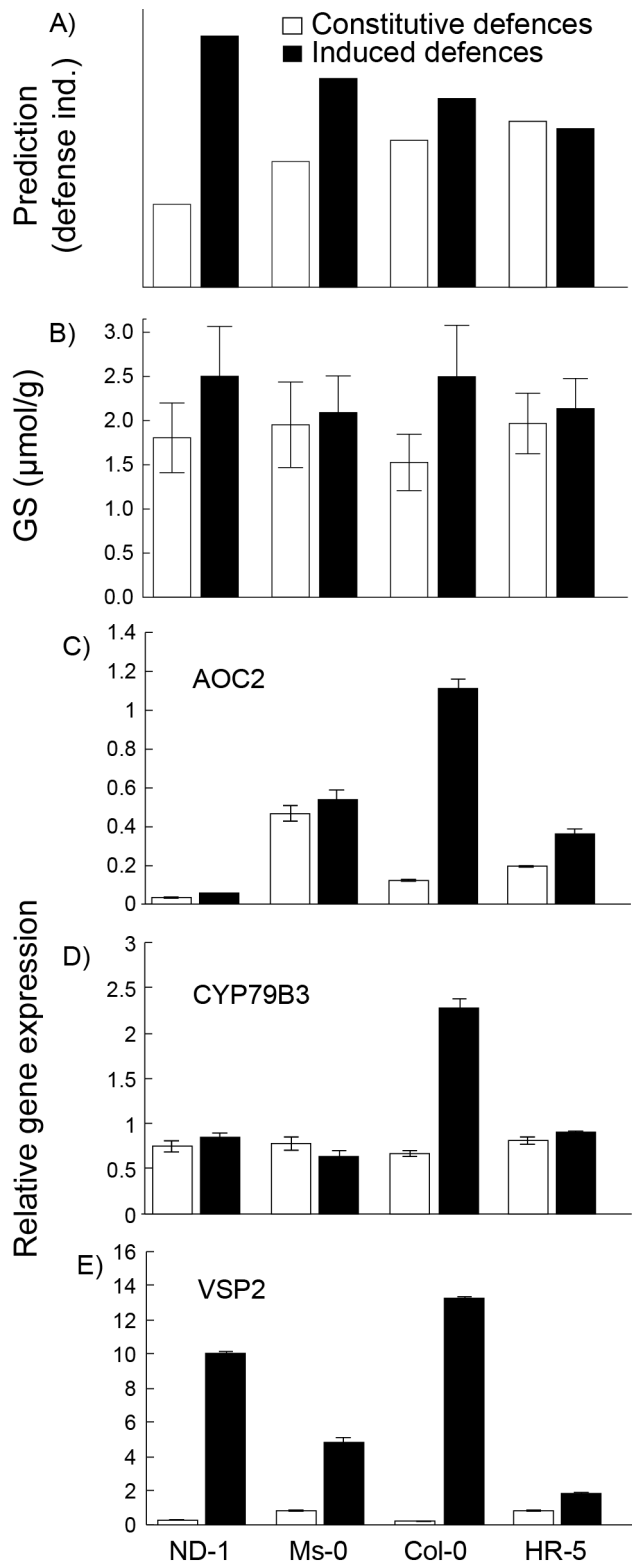
Figure 2.

Figure 3.

Figure 4.

Supplementary information

Table S1. Genes known to be inducible after chewing herbivore attack in Arabidopsis. Those genes were used for the classification of 34 Arabidopsis accessions based on constitutive gene expression levels, and the selection of the seven accessions used for the experiments.

Function	AGI	Short Description (TAIR10)
Glucosinolates	At4g39950	CYP79B2 (cytochrome P450)
	At1g74100	SOT16 (sulfotransferase)
	At5g60890	MYB34 (transcription factor)
	At3g16390	NSP3 (nitrile specifier protein 3)
	At1g52030	MBP2 (myrosinase-binding protein)
	At2g39330	Jacalin lectin family protein, myrosinase-associated protein
	At3g16470	Jacalin lectin family protein, myrosinase-associated protein
	At1g54000	GDSL-like lipase, myrosinase-associated protein
JA synthesis and signalling	At1g17420	LOX3 (lipoxygenase)
	At1g72520	LOX4 (lipoxygenase)
	At5g07010	ST2A (hydroxyjasmonate sulfotransferase)
	At5g13220	JAZ10 (jasmonate-ZIM-domain protein)
JA marker	At5g44420	PDF1.2 (low-molecular-weight cysteine-rich 77)
	At1g12240	Vacuolar invertase betaFruct4
	At2g24850	TAT3 (tyrosine aminotransferase 3); transaminase
	At5g24420	Glucosamine/galactosamine-6-phosphate isomerase-related

Table S2. Constitutive expression of genes involved in glucosinolate biosynthesis and regulation, and in jasmonate biosynthesis, signaling, and response, for seven Arabidopsis accessions.

Type	AGI ID	C-24	Kin-0	SH	HR-5	Col-0	ND-1	Ms-0
Glucosinolates	At4g39950	254.77	635.1	524.33	371.7	285.2	242.6	201.5
	At1g74100	704.87	706.67	1017.97	632.4	450.37	681.07	492
	At5g60890	212.67	169.9	203.87	264.6	226.37	143.7	120.7
	At3g16390	2087.93	1667.5	1149.27	747.6	1601.1	1325.47	970.5
	At1g52030	12.33	28.83	309.37	24.3	28.63	40.3	49.6
	At2g39330	15.23	20.57	15.37	7.2	16.43	10.83	20.9
	At3g16470	808.47	582.3	819.9	1048.4	632.03	725.13	358.3
	At1g54000	2079.9	1536.83	1370.87	1780.7	1459.83	1096.13	860.1
JA synthesis	At1g17420	55.43	19.1	36.73	23.9	42.43	45.87	29.5
	At1g72520	10.73	8.97	29.23	13.3	14.03	13.43	6.2
	At5g07010	12.3	12.5	12.83	11.1	6.07	9.93	22
	At5g13220	69.63	86.2	58.57	91.1	54.2	64	50.6
JA marker	At5g44420	9.07	3.17	15.17	28.5	15	8.23	2
	At1g12240	659.97	944.67	659.53	931.9	811.13	684.23	444.3
	At2g24850	28.4	20.43	18.57	27.8	24.6	20.43	25.6
	At5g24420	94.47	104.1	222.87	153.4	141.03	323.43	196.1
Average		541	483	447	406	435	397	283

Table S3. Constitutive (control treatment) and induced (*S. littoralis* and MeJA treatment) resistance of seven *A. thaliana* accessions against the specialist caterpillar *P. brassicae*, and the generalist caterpillar *S. littoralis*. Data represent averages (± SE) caterpillar dry weight.

Induction treatment	Accession	*P. brassicae* (mg)	*S. littoralis* (mg)
Control	C-24	0.899 +/- 0.024	0.199 +/- 0.026
	Col-0	0.594 +/- 0.029	0.26 +/- 0.013
	HR-5	0.826 +/- 0.086	0.254 +/- 0.01
	Kin-0	0.725 +/- 0.03	0.235 +/- 0.01
	Moscow-0	0.922 +/- 0.026	0.42 +/- 0.017
	ND-1	0.784 +/- 0.024	0.582 +/- 0.057
	SH	0.573 +/- 0.065	0.211 +/- 0.006
S. littoralis	C-24	0.794 +/- 0.045	0.201 +/- 0.023
	Col-0	0.427 +/- 0.034	0.234 +/- 0.011
	HR-5	0.482 +/- 0.053	0.233 +/- 0.005
	Kin-0	0.714 +/- 0.05	0.157 +/- 0.014
	Moscow-0	0.681 +/- 0.034	0.322 +/- 0.028
	ND-1	0.636 +/- 0.022	0.359 +/- 0.014
	SH	0.808 +/- 0.037	0.175 +/- 0.015
MeJA	C-24	0.971 +/- 0.016	0.272 +/- 0.03
	Col-0	0.619 +/- 0.066	0.257 +/- 0.013
	HR-5	0.628 +/- 0.021	0.284 +/- 0.041
	Kin-0	0.376 +/- 0.061	0.178 +/- 0.003
	Moscow-0	0.869 +/- 0.043	0.265 +/- 0.023
	ND-1	0.744 +/- 0.034	0.335 +/- 0.03
	SH	0.605 +/- 0.01	0.209 +/- 0.006

Table S4. Glucosinolate levels in four Arabidopsis accessions

Glucosinolate	Col-0		HR-5		Ms-0		Nd-1	
	Control	Induced	Control	Induced	Control	Induced	Control	Induced
2-propenyl	0 +/- 0	0 +/- 0	0.017 +/- 0.006	0.024 +/- 0.004	0.839 +/- 0.244	0.743 +/- 0.140	0.005 +/- 0.001	0.005 +/- 0.003
3-hydroxypropyl	0.001 +/- 0.001	0 +/- 0	0.275 +/- 0.172	0.088 +/- 0.083	0 +/- 0	0.001 +/- 0.001	0.871 +/- 0.113	1.191 +/- 0.209
7-methylthioheptyl (7MTH)	0.026 +/- 0.002	0.027 +/- 0.001	0.050 +/- 0.011	0.059 +/- 0.012	0.025 +/- 0.003	0.033 +/- 0.010	0.020 +/- 0.002	0.023 +/- 0.002
8-methylthiooctyl (8MTO)	0.064 +/- 0.005	0.051 +/- 0.004	0.177 +/- 0.017	0.188 +/- 0.037	0.216 +/- 0.042	0.194 +/- 0.019	0.130 +/- 0.016	0.150 +/- 0.018
glucobrassicanapin	0 +/- 0	0 +/- 0	0.033 +/- 0.011	0.041 +/- 0.008	0 +/- 0	0.006 +/- 0.006	0 +/- 0	0 +/- 0
glucobrassicin (I3M)	0.181 +/- 0.027	0.586 +/- 0.163	0.154 +/- 0.017	0.203 +/- 0.015	0.117 +/- 0.025	0.226 +/- 0.030	0.228 +/- 0.027	0.424 +/- 0.080
glucoerucin (4MTB)	0.190 +/- 0.026	0.132 +/- 0.019	0.003 +/- 0.002	0.004 +/- 0.002	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0
glucohirsutin (8MSOO)	0.116 +/- 0.017	0.106 +/- 0.010	0.334 +/- 0.040	0.344 +/- 0.055	0.534 +/- 0.128	0.397 +/- 0.041	0.272 +/- 0.058	0.311 +/- 0.070
glucoiberin (3MSOP)	0.103 +/- 0.014	0.161 +/- 0.022	0.023 +/- 0.014	0.028 +/- 0.028	0.034 +/- 0.008	0.032 +/- 0.006	0.122 +/- 0.017	0.187 +/- 0.032
gluconapin	0.001 +/- 0.001	0 +/- 0	0.344 +/- 0.117	0.449 +/- 0.099	0.029 +/- 0.006	0.094 +/- 0.068	0 +/- 0	0 +/- 0
glucoraphanin (4MSOB)	0.715 +/- 0.1	1.200 +/- 0.172	0.008 +/- 0.004	0.008 +/- 0.002	0 +/- 0	0.001 +/- 0.001	0.020 +/- 0.002	0.025 +/- 0.004
glucotropeolin	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0	0 +/- 0
methoxyglucobrassicin (4MOI3M)	0.019 +/- 0.001	0.025 +/- 0.003	0.015 +/- 0.001	0.016 +/- 0.001	0.020 +/- 0.002	0.020 +/- 0.001	0.012 +/- 0	0.015 +/- 0.001
neoglucobrassicin (1MOI3M)	0.003 +/- 0.002	0.039 +/- 0.010	0 +/- 0	0 +/- 0	0.004 +/- 0.003	0.011 +/- 0.002	0 +/- 0	0 +/- 0
progoitrin isomer	0 +/- 0	0 +/- 0	0.097 +/- 0.032	0.130 +/- 0.028	0 +/- 0	0.045 +/- 0.045	0 +/- 0	0 +/- 0
progoitrin	0.001 +/- 0.001	0 +/- 0	0.302 +/- 0.101	0.405 +/- 0.088	0 +/- 0	0.143 +/- 0.143	0 +/- 0	0 +/- 0

TOTAL	1.420 +/- 0.197	2.327 +/- 0.404	1.832 +/- 0.545	1.987 +/- 0.462	1.818 +/- 0.461	1.946 +/- 0.513	1.680 +/- 0.236	2.331 +/- 0.419

Values (μmol/g FW) are the mean (±SE) of 6 measurements

Table S5. List of primers used in this study

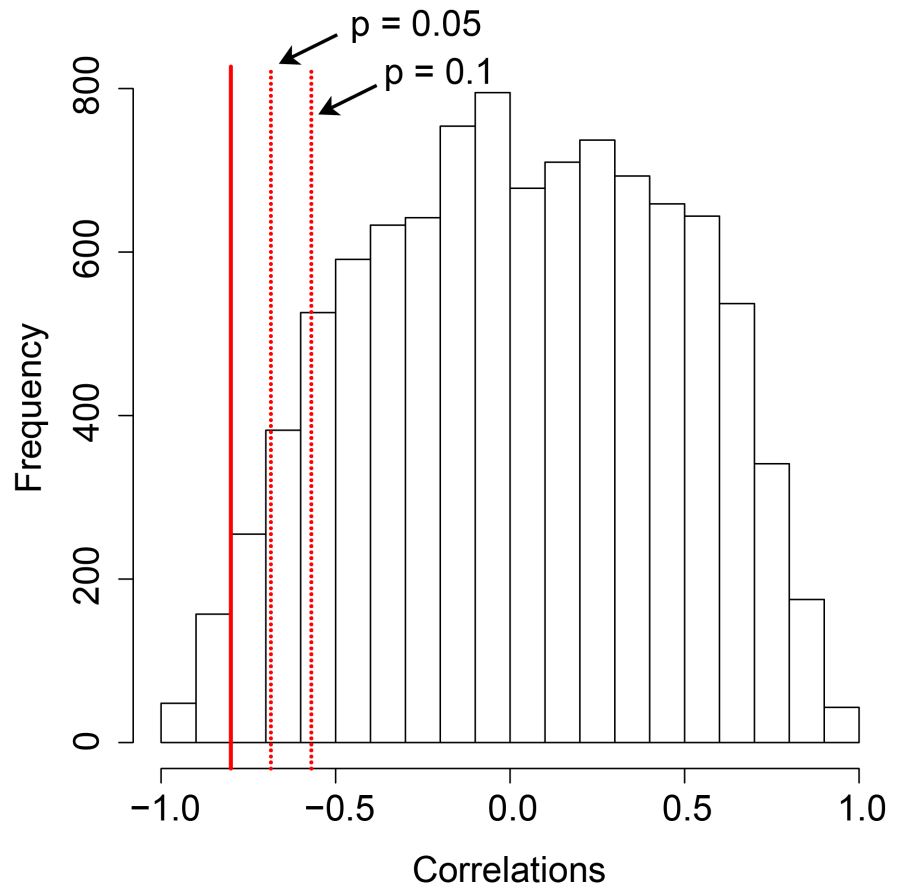| AOC2 (At3g25770)	Fwd	5'-CACGTCCCAGAGAAGAAAGG-3'
	Rev	3'-CGAGGAACGAATCCTCGTAA-3'
CYP79B3 (At2g22330)	Fwd	5'-CTTTGCTTACCGCTGATGAA-3'
	Rev	5'-GCGTTTGA TGGGTTGTCTG-3'
VSP2 (At5g24770)	Fwd	5'-GGTGCCCGCAAATTGCAAAGACTA-3'
	Rev	5'-GGTTGATGCTCCGGTCCCTAACCA-3'
SAND (At2g28390)	Fwd	5'-AACTCTATGCAGCATTTGATCCACT-3'
	Rev	5'-TGATTGCATATCTTTATCGCCATC-3'

Figure S1. Selection of random genes. Shown is the histogram of correlations
between the average of 10 randomly selected genes and the constitutive resis
agaisnt *S. littoralis* across seven Arabidopsis accessions. Solid line indicate t
correlation coefficient for the 8 genes related to glucosinolate producion. Dot
represent the 10% and 5% quantile for the 10000 correlations using random g
selection.