Frequency-dependent herbivory by a leaf beetle, *Phaedon brassicae*, on hairy and glabrous plants of *Arabidopsis halleri* subsp. *gemmifera*

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Abstract

Frequency-dependent prey choice by natural enemies may influence the coexistence of multiple prey types, but little is known about whether frequency-dependent foraging choice occurs in herbivory on plants showing resistance polymorphism within a single population. Here we examined frequency-dependent foraging by a crucifer-feeding leaf beetle, *Phaedon brassicae*, on trichome-producing (hairy) and trichomeless (glabrous) plants coexisting within a natural population of the perennial herb *Arabidopsis halleri* subsp. *gemmifera*. Larvae of *P. brassicae* fed on hairy leaves showed slower growth than those fed on glabrous leaves. Although adult beetles consumed similar amounts of leaves when they were fed either hairy or glabrous leaves in no-choice conditions, our choice experiment showed that adult beetles fed at less than the proportionally expected level on hairy leaves compared to glabrous leaves when the hairy leaves were less or equally abundant. Both types of leaves were consumed at the proportionally expected levels when the hairy leaves were more abundant than the glabrous leaves. In a natural population, the leaf damage on the hairy plants was negatively correlated with the local proportion of the glabrous plants in a 1-m diameter patch across two years, while correlations between the leaf damage on the glabrous plants and their proportion differed between the two years. Additionally, we found five glucosinolates in leaves of *A. halleri*, but their accumulation did not differ between hairy and glabrous plants. Our experimental results indicate that hairy plants incur less herbivory by *P. brassicae* when glabrous plants are abundant. The field pattern provides evidence suggestive of frequency-dependent herbivory acting on hairy plants. The present study highlights one of the putative mechanisms of maintaining plant resistance polymorphism.

Introduction

Natural enemies often alter their foraging tactics depending on the relative frequency of multiple prey or host types (Greenwood 1984; Endler 1991; Sherratt and Harvey 1993). Frequency-dependent foraging on various prey types has been reported for predators (Endler 1991; Sherratt and Harvey 1993), parasitoids (Sherratt and Harvey 1993) and herbivores (Cottam 1985; Behmer et al. 2001). The frequency dependence of foraging behaviour may be profitable when predators encounter multiple prey types that are distributed unevenly in their foraging environments. For example, if the cost of searching for a rare prey is large, a predator should increase foraging success by concentrating on major prey types (Greenwood 1984; Endler 1991). In a broad sense, frequency-dependent foraging can be defined as the behaviour by which predators feed on a given prey type at a disproportionately higher or lower rate. Although definitions of frequency-dependent foraging have been discussed in different publications (Greenwood 1984; Behmer et al. 2001; Bergvall and Leimar 2005), here we follow the above broad-sense definition.

Frequency-dependent foraging has long been investigated because of its potential impacts on the coexistence or extinction of multiple prey types (Greenwood 1984; Sherratt and Hervey 1993). If predators feed more on a major prey type than proportionally expected, rare prey types experience less predation risk as the frequency of the major type becomes larger. This may lead to negative frequency-dependent selection on multiple prey types, thereby allowing them to coexist (Greenwood 1984). Conversely, if predators feed less on a major prey type, positive frequency-dependent selection may occur and accordingly promote the extinction of the rare prey types (Greenwood 1984). Empirically, frequency-dependent foraging has

been studied with respect to anti-predator behaviour of prey such as warning coloration or aggregation (reviewed by Endler 1991).

Frequency dependence can also occur regarding herbivory on multiple plant types that share a common herbivore. Some insect and mammalian herbivores are known to forage on multiple plant species (Chandra and Williams 1983; Cottam 1985) or diets containing different nutritional quality (Behmer et al. 2001; Bergvall and Leimar 2005) in a frequency-dependent manner. Within a plant species, natural populations often exhibit genetic polymorphism of chemical and physical resistance traits against herbivores (e.g. Hughes 1991; Elle et al. 1999; Kivimaki et al. 2007). In addition to frequency-dependent host choice, selectivity or host preference of herbivores is also known with respect to anti-herbivore resistance polymorphism (Burgess and Ennos 1987; Sletvold et al. 2010). Few attempts, however, have been made to test a frequency-dependent host choice by a herbivore with respect to the polymorphism within a single plant species (Wise et al. 2009).

The purpose of this study was to examine the existence of frequency-dependent foraging of herbivores with respect to anti-herbivore resistance polymorphism. To test this, we used the leaf beetle *Phaedon brassicae* Baley [Coleoptera: Chrysomelidae] and natural variation in trichome production of *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz subsp. *gemmifera* (Matsum.) O'Kane & Al-Shehbaz [Brassicaceae/ Cruciferae] (referred to as *A. halleri* hereafter). Both adults and larvae of *P. brassicae* forage on trichome-producing and trichomeless plants (hereafter referred to as hairy and glabrous plants, respectively) in a natural population of *A. halleri* (Kawagoe and Kudoh 2010; Kawagoe et al. 2011). This system is suitable for testing frequency-dependent foraging of a herbivore on plants

showing resistance variation because, in our study site, interspecific interactions are specific between *P. brassicae* and *A. halleri*. As to the herbivore fauna, *P. brassicae* is the most influential insect herbivore of *A. halleri*, and other herbivorous insects are much less abundant (Kawagoe and Kudoh 2010). As to the vegetation, other cruciferous plants are absent and hence *P. brassicae* feeds exclusively on *A. halleri*. This simple interspecific interaction helps to exclude confounding effects of other crucifer-feeding herbivores or cruciferous plants.

In addition to the simplicity of species interactions, the plant and beetle characteristics allowed us to interpret and design our study straightforwardly. For *A. halleri* in our study site, trichome polymorphism is strongly associated with allelic variation in a single candidate gene, *GLABROUS1* (*GL1*) (Kawagoe et al. 2011) and therefore we can assume that the visible phenotypes represent genetically determined strategies. For *P. brassicae*, the flightlessness of the beetle made it reasonable to ask whether the local frequency of hairy and glabrous plants affected foraging behaviour of the beetle. Furthermore, it has been reported that host choice by adults is a major determinant of the larvae distribution in *P. brassicae* (Ôtake and Funaki 1958). We have also observed migrations between plants by adults, but fewer by larvae in the field. Although larvae cause the majority of damage to plants during the flowering period in the study site, it can be plausibly assumed that adult behaviours play an important role in determining the distributions of damages among plants.

In this study, we performed three laboratory experiments and a field survey. First, to ascertain whether trichome production acts as a resistance trait against *P. brassicae*, we compared the growth of larvae fed on hairy or glabrous leaves. Second, to test whether the feeding preference of adult *P. brassicae* depended on the relative frequency of hairy and glabrous leaves, we conducted choice experiments

manipulating the relative frequency of hairy and glabrous leaves. Third, the relationship between leaf damage and the proportion of hairy and glabrous plants within small patches was investigated in the field to examine whether frequency-dependent herbivory occurs in the natural habitat. Additionally, to examine whether the trichome phenotype was correlated with chemical resistance traits, we quantified glucosinolates, which are major secondary metabolites of Brassicaceae (Kliebenstein et al. 2001; Clauss et al. 2006), in hairy and glabrous leaves.

Materials and Methods

Study system

We conducted field surveys and collected materials in a natural population of *A. halleri* located in Hyogo prefecture in western Honshu, Japan (35°06′N, 134°56′E, ca. 200 m in altitude). The study species is a self-incompatible perennial distributed in Eastern Asia and the Russian Far East (Hoffmann 2005). The plant is a metallophyte and often inhabits soils contaminated by heavy metals (Kubota and Takenaka 2003). In the study site, *A. halleri* occurs near an abandoned mine, along a creek running through open secondary forest. Vegetation is sparse along the creek, probably due to heavy metal contamination of the soil, and no cruciferous species are observed except for *A. halleri*. Approximately half of the plants were hairy and the others were glabrous in this site (Kawagoe et al. 2011). The presence/absence of trichomes has been reported to be associated with the allelic status of a trichome-related gene, *GL1*, but not with its flanking regions and other genes (Kawagoe et al. 2011). Hairy plants produced fewer fruits than glabrous plants in an insect removal experiment (Kawagoe

et al. 2011), indicating that there is a cost of the trichome production. In this study, the glabrous phenotype was defined as the absence of trichomes on leaves and stems. Because this species can reproduce clonally, we designated a plant with no vegetative connection with others as an individual in this study.

Phaedon brassicae is known to be a pest insect of cruciferous vegetables (Wang et al. 2007a). This species usually reaches the adult stage within 3 weeks after hatching, and adults survive for approximately 2 months under laboratory conditions with various ranges of temperature and photoperiod (Wang et al. 2007b). Adults and last-instar larvae are ca. 4-8 mm in body length. In our study site, larvae and adults mainly occur during the flowering period in spring, and severely damage leaves and inflorescences of *A. halleri*, while they also occur from summer to autumn with much lower abundance than in spring (Kawagoe and Kudoh 2010). We collected 31 adults of *P. brassicae* during May-July 2011 and established a laboratory-reared population (> 90 individuals of F1 to F2 generations). The beetles were reared on leaves of Chinese cabbage (*Brassica rapa* var. *glabra*) under 20˚C, 12L:12D conditions with relative humidity of 40-70% in a growth chamber (Biotron NC-220, Nippon Medical & Chemical Instruments, Osaka, Japan). We pre-reared *P. brassicae* on *A. halleri*, Chinese cabbage, cabbage (*Brassica oleracea*) and radish leaves *(Raphanus sativus*). Because *P. brassicae* grew well on the Chinese cabbage and this cultivar had a moderate density of trichomes among the four host plants, Chinese cabbage was chosen to avoid pre-conditioning for hairy or glabrous *A. halleri*. The light intensity of the growth condition was 25.3 ± 2.08 μmol/m^2s (LI-190 Quantum Sensor, LI-COR, Lincoln, NE, USA). The leaf diets were replaced every three or four days.

Other herbivorous insects also feed on *A. halleri* in the study site, including green-veined white butterflies, *Pieris napi* L., and diamondback moths, *Plutella*

185 *xylostella* L. However their abundance is much lower than that of *P. brassicae*
186 throughout the year (Kawagoe and Kudoh 2010) and we found only a few *P. napi* and
187 *P. xylostella* during the present study.
188
189 *Larval growth on hairy and glabrous leaves*
190 First-instar larvae were used within three days after hatching in the laboratory-
191 reared population. Several hundred young radical leaves were harvested from
192 approximately 100 intact hairy and glabrous plants growing in our study site. The
193 hairy and glabrous leaves were kept separately in a plastic case filled with water. A
194 petiole of a single leaf was wrapped with moistened paper and placed in the center of
195 a Petri dish. Nineteen individual larvae were separately released onto the upper
196 surface of either a hairy or a glabrous leaf. The larvae were allowed to infest the
197 leaves for eight days under 20˚C, 12L:12D conditions. The weight of larvae was
198 measured before, four days, and eight days after release. Because adult beetles do not
199 grow in size after emerging from pupae, the weight of larvae in the early
200 developmental stage was used as an indicator of the herbivore performance.
201 Measurements for each larva were performed three times to the nearest 10^{-2} mg (AEL-
202 40SM, Shimadzu, Tokyo, Japan) and the average values were used for analyses. Four
203 days after the first release, the leaves were replaced with fresh leaves that had been
204 kept in a refrigerator.
205
206 *Choice experiments under different leaf frequencies*
207 We conducted choice or no-choice experiments under five leaf frequency
208 conditions (Hairy: glabrous = 4:0, 3:1, 2:2, 1:3, 0:4). Adult beetles were used in the
209 experiment within 1-2 months after emerging from pupae. To stimulate the feeding

motivation of beetles, they were starved for one day. Each beetle was randomly chosen and returned to the colony after experiments. Each trial was performed in a Petri dish (diameter 6 cm, depth 1.5 cm: Kord-Valmark Co., Ontario, Canada) containing a moistened filter paper (diameter 5.5 cm: Toyo Roshi Kaisha, Ltd., Tokyo, Japan). Leaves used for this experiment were harvested as described above and used within 12 h after the harvest. Leaf discs (1 cm^2) were made from the center of each leaf, including a main vain. One disc from hairy plants had 101 ± 32 trichomes (sum of adaxial and abaxial side, Mean ± SD, n = 24: counted using an 8× magnifying glass). Four leaf discs were placed in each Petri dish in a four-way choice manner (Raffa et al. 2002). We examined the five frequency conditions of hairy and glabrous discs (hairy: glabrous = 4:0, 3:1, 2:2, 1:3, 0:4) and the location of hairy and glabrous leaf discs was randomized. Three adult beetles were released into the center of each dish because we often observed an individual plant being infested by multiple adult beetles in the field. They were allowed to infest the leaf discs for 72 h under 20˚C, 12L:12D conditions. The number of arenas analyzed (replicates of trials) was 15, 23, 18, 22 and 15 for hairy: glabrous = 4:0, 3:1, 2:2, 1:3 and 0:4 conditions, respectively. We started 27 replicates per condition and removed arenas in which even one of the four leaf disks showed signs of drying during the 72-h experimental period (22, 26, 23, 27, and 19 cases remained for hairy: glabrous = 4:0, 3:1, 2:2, 1:3 and 0:4 conditions, respectively). We further excluded cases that involved a beetle death (one case) or no leaf-infestation (see also Table S1).

The leaf discs that remained at 72 h were placed on 1-mm-grid paper and converted into a digital image (scanned using MP-460, Cannon, Tokyo, Japan). We used Image J (Abramoff et al. 2004) to estimate the remaining leaf area with the

accuracy of 10^{-3} cm^2. The leaf loss (cm^2) was calculated as [1.1 − the remaining leaf area (cm^2)].

Field survey

Field surveys were conducted for selected *A. halleri* patches along a creek (ca. 200 m in distance) that ran through the center of the study site. We arbitrarily set a circular patch (1 m in diameter) to record the trichome phenotype (hairy or glabrous) and the proportion of leaf area lost to herbivores for all individual plants in each patch. The proportion of leaf area lost by herbivory (referred to as the leaf damage hereafter) was evaluated by eye and recorded as one of 11 successive values, i.e. 0 (no damage), 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 (complete leaf loss). A preliminary survey confirmed that the number of plants within circular patches approached a plateau with increasing patch size: 2.97 ± 0.32, 7.08 ± 0.99, and 8.83 ± 1.25 plants occurred within patches 0.5, 1, and 3 m in diameter, respectively (Mean ± SE, n = 36 patches examined). Therefore, we focused on the local interaction in 1-m-diameter patches. The surveys were conducted twice (on 12 July 2011 and 29 May 2012) after the peak abundance of *P. brassicae* had been observed. The number of hairy and glabrous plants examined was 318 and 232 in 2011; and 260 and 195 in 2012, respectively. At the peak abundance of *P. brassicae*, the number of beetles per plant was 0.18 ± 0.08 on 16 May 2011 and 0.20 ± 0.05 on 8 May 2012 (Mean ± SE, including both adults and larvae: n = 100 plants). We examined 60 patches for each survey while keeping the distance between neighboring patches greater than 3 m.

In addition to the patch-level survey, we collected subset data at the individual level with the following aims. First, to evaluate to what extent our method of

quantifying the leaf damage reflected the intensity of herbivory, we also recorded the number of intact and damaged leaves for 40 plants as an independent estimate of herbivory. This additional measurement confirmed that the leaf damage estimated by our method was highly correlated with the proportion of leaves damaged (Pearson's product moment correlation, both variables were arcsine-transformed, $r = 0.93$, $t_{38} = 15.3$, $P < 0.0001$). Second, to examine whether a correlation between plant size and leaf damage would bias our interpretation of frequency dependence based on trichome phenotype, we measured the length of the longest leaf for the same 40 plants mentioned above. Neither the total number of leaves nor the length of the longest rosette leaf was significantly correlated with the leaf damage ($r = 0.19$, $t_{38} = 1.2$, $P = 0.25$; $r = -0.16$, $t_{38} = -1.0$, $P = 0.32$, respectively, where the leaf damage was arcsine-transformed), indicating that effects of plant size on the leaf damage were negligible.

Glucosinolate analysis of hairy and glabrous leaves

Fully expanded leaves were harvested from flowering stems of intact hairy or glabrous plants on 15 May 2013. Two or three leaves in proximate positions were selected to minimize the within-individual variation of glucosinolate concentration. Furthermore, pairs of a hairy and a glabrous plant (< 1m apart) were sampled to control for micro-environmental variation. Leaves from each individual were separately packed into a plastic bag. The bags were then immediately frozen using 70% ethanol cooled with dry ice at the field site. The leaf samples were stored at −80 ˚C until use. Glucosinolates were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Sawada et al. (2009a, b, 2012) using 4 ± 0.4 mg crushed leaves per individual plant for nine pairs of hairy and glabrous plants.

283 *Statistical analysis*

284 For the data set from the larval growth experiment, the weights of larvae fed
285 on the hairy and the glabrous leaves were compared with a Mann-Whitney *U*-test. The
286 analysis was done separately for the weight before the release, four days, and eight
287 days after the release. For the data set from the choice experiments, we calculated the
288 average leaf loss (cm^2) for each trichome type per dish to analyze herbivory on each
289 leaf type in the choice experiment. A Wilcoxon signed rank test was used to compare
290 the average leaf loss between the hairy and glabrous leaf discs for choice conditions
291 (Hairy: glabrous = 3:1, 2:2, 1:3). For no-choice conditions (Hairy: glabrous = 4:0, 0:4),
292 the average leaf loss was compared between the hairy and glabrous leaf discs by a
293 Mann-Whitney *U*-test. In all the analyses for the choice conditions, *P*-values were
294 adjusted using sequential Bonferroni correction to control the risk of increased type I
295 error due to multiple testing. To test whether the relative frequency of hairy and
296 glabrous leaves affected the total amount of leaf loss (cm^2) in each arena, we analyzed
297 the effect of the frequency conditions on the total amount of leaf loss in each arena
298 with a Kruskal-Wallis test. Further, to analyze the preference of adult beetles in the
299 choice conditions, Chesson's selectivity index (Chesson 1978) was calculated for each
300 preference arena for the three choice conditions. Chesson's α for diet type i is denoted
301 as $\alpha_i = (r_i / P_i) / \Sigma(r_i / P_i)$, where r indicates the relative frequency of diet i in total
302 consumption by predators and P indicates the relative frequency of diet i in the
303 environment. When there are two types of diets, $\alpha > 1/2$ and $\alpha < 1/2$ mean positive
304 and negative preference for the focal diet, respectively. The parameter r for the hairy
305 and glabrous leaf discs was estimated as the proportion of the hairy or glabrous leaf
306 area consumed relative to the total leaf area consumed in each preference arena. The
307 parameter P was the relative frequency of the hairy or glabrous leaf discs in each Petri

dish. A Wilcoxon signed rank test was used to compare Chesson's α between the hairy and glabrous leaf discs.

For the field data, we analyzed the trichome phenotype (hairy or glabrous), the proportion of glabrous plants in a patch (which represents the relative frequency of the two phenotypes), and the total number of *A. halleri* in a patch (which represents the density of *A. halleri*), and the study year as fixed effects explaining the leaf damage. We also analyzed up to three-way interaction terms among the fixed effects to test the dependency of the trichome phenotype on the other factors. However, interaction terms involving the proportion of glabrous plants and the total number of *A. halleri* were not analyzed, because this interaction term corresponded to the number of glabrous plants in a patch and was therefore strongly correlated with the main effect of the proportion of glabrous plants in a patch ($r = 0.67$, $t_{1003} = 28.5$, $P < 0.0001$). The patch ID was incorporated as a random effect in order not to treat multiple plants in a patch as independent samplings. These factors were analyzed using generalized linear mixed models (GLMMs: Bolker et al. 2009) with a normal error structure. The leaf damage (response variable) was arcsine-square-root transformed to improve the normality of residuals. The analysis of field data consisted of three steps. First, we performed a stepwise model selection procedure to search the best-fitted model from a number of possible combinations involving three-way interaction terms among the trichome phenotype, the proportion of glabrous plants in a patch, and the study year; and among the trichome phenotype, the total number of *A. halleri* in a patch, and the study year. We used Akaike's information criteria (AIC) for the model selection criteria. Both forward and backward searches on the fixed effects were allowed in the stepwise model selection. Second, based on interactions between the study year and the other factors in the first analysis, we separately performed model selections for

data collected in 2011 and 2012 to investigate whether the trichome phenotype and the relative frequency of trichome dimorphism had interactive effects on the leaf damage. In the second analysis, the full model included five fixed effects: (1) trichome phenotype × proportion of glabrous plants in a patch, (2) trichome phenotype × total number of *A. halleri* in a patch, (3) trichome phenotype, (4) proportion of glabrous plants in a patch, and (5) total number of *A. halleri* in a patch. Third, based on interactions between the trichome phenotype and the other fixed effects in the second analysis, we estimated coefficients of the independent variables, i.e., "proportion of glabrous plants in a patch" and "total number of *A. halleri* in a patch", to examine the sign and magnitude of the effects of the frequency of hairy and glabrous plants and their density on the leaf damage. Additionally, to add trend lines for figure presentation, we estimated coefficients of the variable "proportion of glabrous plants in a patch" for models including this fixed effect alone.

For the data set from glucosinolate analysis, we analyzed glucosinolates detected in more than eight out of nine sample pairs, in which individual glucosinolates with peak area values of > 1.0 were regarded as detected for each sample. The score of LC-MS/MS analysis was calculated as the peak area value of a certain glucosinolate divided by that of the internal standard (10-camphorsulfonic acid) for each sample. A Wilcoxon signed rank test was used to compare the peak area values of the glucosinolates between hairy and glabrous leaves. In this analysis, proximate hairy and glabrous plants were treated as a pair to control for spatial heterogeneity of environmental conditions among plant patches. To control for the risk of increased type I error due to multiple testing, *P*-values were adjusted with the number of glucosinolates tested using sequential Bonferonni correction.

All statistical analyses were performed using R version 2.15.0 (R Development Core Team 2012). We used the lme function (in the nlme package) and the stepAIC function (in the MASS package) for the stepwise model selection; and the lmer function (in the lme4 package) for GLMM analyses. In all of the GLMM analyses, we used the maximum likelihood method to estimate AICs and coefficients.

Results

Larval growth

The initial weight did not differ significantly between the larvae released on the hairy and glabrous leaves (Fig. 1; $U = 157$, $n_1 = n_2 = 19$, $P = 0.49$). The weight of larvae four days after release also showed no significant difference between the hairy and glabrous leaves (Fig. 1; $U = 126$, $n_1 = n_2 = 18$, $P = 0.25$). The weight of larvae eight days after release on the hairy leaves was significantly lower than that on the glabrous leaves (Fig. 1; $U = 43$, $n_1 = 11$, $n_2 = 14$, $P < 0.05$). The reduction in sample size at later time points was due to mortality of larvae during the experiments.

Choice experiments

The average leaf loss of hairy leaves was significantly smaller than that of glabrous leaves under the hairy: glabrous = 1:3 condition (Fig. 2a; Wilcoxon signed rank test, $V = 224$, $n = 23$, $P < 0.05$ with sequential Bonferroni correction) and the hairy: glabrous = 2:2 condition (Fig. 2a; $V = 163$, $n = 18$, $P < 0.05$). The average leaf loss did not differ significantly between the hairy and glabrous leaves under the hairy: glabrous = 3:1 condition (Fig. 2a; $V = 161$, $n = 22$, $P = 0.26$). Under no-choice

conditions, no significant difference in leaf loss was found between the hairy and glabrous leaves (Fig. 2a; Mann-Whitney *U*-test, $U = 109$, $n_1 = n_2 = 15$, $P = 0.88$). The total leaf loss per dish did not differ significantly among the five frequency conditions (Kruskal-Wallis test, $\chi^2_4 = 5.30$, $P = 0.26$).

The selectivity index of hairy leaves was significantly smaller than that of glabrous leaves under the hairy: glabrous = 1:3 condition (Fig. 2b; $V = 239$, $n = 23$, $P < 0.01$) and the hairy: glabrous = 2:2 condition (Fig. 2b; $V = 153$, $n = 18$, $P < 0.01$). The selectivity index did not differ significantly between the hairy and glabrous leaves under the hairy: glabrous = 3:1 condition (Fig. 2b; $V = 162$, $n = 22$, $P = 0.26$). We also performed the same statistical analyses including cases that involved no leaf-infestation or beetle death, but inclusion of these cases did not affect the conclusions (Table S1).

Field survey

A three-way interaction term among the trichome phenotype, the proportion of glabrous plants, and the study year was included as a result of the stepwise model selection (Table S2). Then, based on this year dependence, we separately analyzed data collected in 2011 and 2012. The interaction term between trichome phenotype of the focal plant and the proportion of glabrous plants was included in the best-fitted model explaining the leaf damage in 2011 and 2012 (Table 1), indicating that the trichome phenotype and the proportion of glabrous plants had interdependent effects on the leaf damage. Therefore, we separately analyzed the data set for each of hairy and glabrous plants for each of these study years, and estimated the coefficients of the terms of the proportion of glabrous plants and total number of plants for each data set.

406 Leaf damage of hairy plants tended to decrease concomitantly as the
407 proportion of glabrous plants increased in a patch in both of these two years (Table 2,
408 Fig. 3a, c), though the correlation was not significant in 2012 (Table 2). Leaf damage
409 of glabrous plants decreased in 2011, while it increased in 2012, as the proportion of
410 glabrous plants increased in a patch (Table 2, Fig. 3b, d). The leaf damage of glabrous
411 plants increased significantly as the total number of *A. halleri* in a patch increased in
412 2012 (Table 2). The leaf damage of the hairy plants was 0.154 ± 0.009 in 2011 (Mean
413 ± SE, n = 318) and 0.136 ± 0.012 in 2012 (n = 260), while the leaf damage of the
414 glabrous plants was 0.134 ± 0.009 in 2011 (n = 232) and 0.163 ± 0.011 in 2012 (n =
415 195).
416
417 *Glucosinolate analysis of hairy and glabrous leaves*
418 The score of LC-MS/MS values of the five glucosinolates showed no
419 significant difference between hairy and glabrous leaves (Fig. 4; 6-Methylsulfinyl-n-
420 hexyl-glucosinolate, n = 9 pairs, V = 38, P = 0.37; 7-Methylsulfinyl-n-heptyl-
421 glucosinolate, n = 9 pairs, V = 23, P = 1; 8-Methylsulfinyl-n-octyl-glucosinolate, n =
422 9 pairs, V = 21, P = 1; 7-Methylthio-n-heptyl-glucosinolate, n = 8 pairs, V = 21, P = 1;
423 8-Methylthio-n-octyl-glucosinolate, n = 8 pairs, V = 18, P = 1). The results for the
424 other fifteen glucosinolates measured are given in supporting information (Table S3).
425
426
427 **Discussion**
428 The choice experiment demonstrated frequency-dependent herbivory by *P.*
429 *brassicae* with respect to trichome polymorphism of *A. halleri*. We observed less
430 herbivory on hairy leaves when they became a minority. Greenwood (1984) defined

frequency-dependent predation to describe cases in which feeding preference changes inversely with the frequency of a given prey type (i.e. anti-apostatic or pro-apostatic predation: reviewed by Sherratt and Harvey 1993). When hairy leaves became abundant, we observed a disproportional increase of herbivory on them to levels equal to those found in glabrous leaves. Because we did not observe the inverse change in feeding preference, our results correspond to "potentially frequency-dependent predation" (Greenwood 1984). To our knowledge, the present results are one of a few reported examples of frequency-dependent herbivory with respect to plant resistance polymorphism within a single population. Behmer et al. (2001) documented that a locust, *Locusta migratoria*, consumed more of abundant but sub-optimal artificial foods. Wise et al. (2009) found frequency dependence in associational resistance between the erect-stemmed and candy-cane phenotype of *Solidago altissima* against a gall-fly, but they reported that increased frequency of the resistant phenotype lowered attacks by the herbivore for both phenotypes. Our growth experiment using larvae confirmed that trichome production of *A. halleri* reduced the larval performance, indicating that trichome production functioned as a resistance trait against *P. brassicae*. In our discussion, therefore, we could consider glabrous and hairy leaves as optimal and sub-optimal diets for *P. brassicae*, respectively.

The spatial structure of foraging patches relative to the searching area of predators can alter the consequences for foraging behaviour (Greenwood 1984; Endler 1991; Sherratt and Harvey 1993) and thus determine whether one detects frequency-dependent predation. In host plant choice by herbivores, for example, Janz et al. (2005) showed that frequency-dependent oviposition preference of the polyphagous butterfly *Polygonia c-album* for two host species was detected among plant patches, but not within a patch. In contrast, *Phaedon brassicae* is less mobile with regard to

456 choosing host plants (Ôtake and Funaki 1958). Therefore the results of our choice
457 experiments presumably represent the feeding preference of *P. brassicae* adults and
458 its frequency dependence within a single plant patch.
459 We found that leaf damage on hairy plants decreased as the proportion of
460 glabrous plants increased within local patches (1 m in diameter) in 2011. A similar
461 pattern was found in 2012, although it was not statistically significant. This tendency
462 is consistent with the frequency-dependent herbivory detected in the choice
463 experiments. We observed a positive correlation between leaf damage of glabrous
464 plants and the frequency of glabrous plants within patches in 2012. This pattern would
465 be expected according to the frequency-dependent preference changes observed in our
466 experiments. However, the negative correlation we observed between leaf damage
467 and frequency of glabrous plants in 2011 was inconsistent with the laboratory
468 evidence of frequency-dependent herbivory. We also observed significant density-
469 dependent herbivory on glabrous plants in 2012 (Table 2b). The effect of plant density
470 could not be tested in our choice experiments under the condition of equal leaf density.
471 Overall, our field observations support the existence of frequency-dependent
472 herbivory at least on hairy plants, but it remains unclear whether our experimental
473 evidence can account for the frequency-dependent herbivory on glabrous plants in the
474 field. We need further studies before we can reach a rigorous conclusion about how
475 important the frequency-dependent herbivory by adult beetles is under natural
476 conditions.
477 Our previous studies revealed that intensive leaf damage is predominantly
478 caused by larvae feeding in our field site (Kawagoe and Kudoh 2010, Kawagoe et al.
479 2011). In the flowering period, adult beetles were found on less than 2% of plants
480 censused, while ca. 0.5 larva was observed on a single plant (Kawagoe et al. 2011).

Active host choice by larvae, however, is unlikely to occur, since they feed on the host plant upon which an adult female oviposits, and rarely move between plants. Therefore, we assume that the frequency-dependent leaf damage in the field is attributable to the frequency-dependent foraging and oviposition by adults. Given the slow growth of larvae on hairy leaves (Fig. 1), the leaf damage in the field probably reflected not only the adult choice but also the effects of trichomes on larval feeding activity. Although it was difficult to distinguish whether plant injury was due to feeding choice or oviposition choice in the field, the oviposition preference should next be examined to determine the relative importance of adult host choice and larval feeding in causing the frequency-dependent leaf damage.

One caveat is that other ecological functions or traits correlated with the trichome phenotype may also influence the observed frequency of hairy and glabrous plants. For instance, trichomes have been reported to reduce evapo-transpiration, and to increase UV reflection and tolerance to drought (Wagner et al. 2004, Steets et al. 2010, Sletvold and Ågren 2012). At least within our study site, both hairy and glabrous plants were observed without distinctive segregation throughout a range of microhabitats that may have differed in droughtness and sun exposure. It has been reported that the density of trichomes increases in response to damage in *Arabidopsis thaliana* (Yoshida et al. 2009). Although the polymorphism examined in this study (presence/absence of trichomes) is expected to be determined by a single locus, *GL1* (Kawagoe et al. 2011), further study will be required to evaluate how variation in trichome density among hairy plants is affected by herbivory. In leaves of *A. halleri* we found glucosinolates that have also been found in leaves of related *Arabidopsis* species (e.g. methylthio- and methylsulfinyl-glucosinolates: Kliebenstein et al. 2001 for *A. thaliana*; Clauss et al. 2006 for *A. lyrata*), but little association between

trichome production and glucosinolate contents was observed during the flowering season, when *P. brassicae* infestation was most intensive. It is also known that *A. halleri* accumulates heavy metals in trichomes (Zhao et al. 2000). We do not have any evidence so far that *P. brassicae* discriminates hairy and glabrous plants by any correlated traits.

In summary, this study is one of the first examples to show frequency-dependent herbivory with respect to anti-herbivore resistance polymorphism coexisting within a natural population. Although frequency-dependent food choice by herbivores has been suggested to promote coexistence of multiple plant species at community levels (Chandra and Williams 1983; Cottam 1985), the same process can explain the maintenance of resistance polymorphism within a single species by incorporating a tradeoff between defense and growth (Pacala and Crawley 1992). Previous studies revealed that herbivory by *P. brassicae* greatly reduced fruit production (Kawagoe and Kudoh 2010). Therefore, the frequency-dependent herbivory found in this study could be a candidate mechanism that would result in frequency dependence of plant fitness. Future studies should especially focus on this point, because it may explain why hairy and glabrous plants coexist within a population.

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Table 1 AICs of generalized linear mixed models explaining the leaf damage (arcsine-transformed proportion of leaf area lost by herbivory) on *Arabidopsis halleri* subsp. *gemmifera* in the field. The AICs of models with and without trichome phenotype, frequency, and density terms were compared for each study year. Interaction terms were subtracted sequentially from the full model, and then models with or without each main term were compared. The smallest values of AIC (shown by bold letters) indicate the best-fitted model. The patch ID was incorporated as a random effect in these analyses (see text).

Abbreviations: T, Trichome phenotype; P, Proportion of glabrous plants in a patch; N, Total number of *A. halleri* in a patch.

Fixed effects	Terms subtracted	AIC	
		2011	2012
(T × P) + (T × N) + T + P + N	*Full model*	-214.5	-151.6
(T × P) + T + P + N	(T × N)	-216.5	-151.4
(T × N) + T + P + N	(T × P)	-213.2	-135.2
(T × P) + T + P	(T × N) + N	**-217.8**	**-152.2**
(T × N) + T + N	(T × P) + P	-208.6	-137.0
T + P + N	(T × P) + (T × N)	-215.2	-136.0
T + P	(T × P) + (T × N) + N	-216.8	-137.4
T + N	(T × P) + (T × N) + P	-209.9	-137.6
P + N	(T × P) + (T × N) + T	-212.0	-129.9

673 **Table 2** Coefficients and their standard error (SE) for terms of proportion of glabrous
674 plants in a patch and total number of *Arabidopsis halleri* subsp. *gemmifera* in a patch
675 in GLMMs explaining the leaf damage (arcsine-transformed proportion of leaf area
676 lost by herbivory) in 2011 and 2012 in the field. Upper rows (a) present results of
677 models including the proportion of glabrous plants, and lower rows (b) present results
678 of models including both the proportion of glabrous plants and the total number of
679 plants. Bold values indicate significant deviation of coefficients from zero (Wald
680 tests). The patch ID was incorporated as a random effect in these analyses (see text).

Fixed effect	2011		2012	
	Hairy (n = 318)	Glabrous (n = 232)	Hairy (n = 260)	Glabrous (n = 195)
(a) Single regression				
Proportion of glabrous plants	**-0.20 ± 0.10**	**-0.26 ± 0.10**	-0.13 ± 0.09	**0.21 ± 0.09**
(b) Multiple regression				
Proportion of glabrous plants	**-0.20 ± 0.10**	**-0.31 ± 0.11**	-0.15 ± 0.09	**0.25 ± 0.09**
Total number of plants in a patch	-0.06 ± 0.12	-0.12 ± 0.12	0.09 ± 0.10	**0.23 ± 0.10**

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691 **Legends for figures**

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693 **Fig. 1** Weight of larvae (Median ± 95% CI) fed on hairy (H; filled bars) and glabrous
694 (G; open bars) leaves before release, and four days and eight days after release.
695 Asterisks indicate significant differences with Mann-Whitney *U*-test (n.s. not
696 significant, * $P < 0.05$).

697

698 **Fig. 2** Frequency-dependent herbivory by adult beetles on hairy (H) and glabrous (G)
699 leaves in choice experiments. The left panel (a) shows the average leaf loss (Median ±
700 95% CI) for each trichome type in the choice and no-choice conditions (Hairy:
701 glabrous = 4:0, 3:1, 2:2, 1:3, 0:4), where filled and open bars indicate the hairy and
702 glabrous leaf type, respectively. The right panel (b) shows Chesson's selectivity index
703 (Median ± 95% CI) for hairy leaf type under the three choice conditions (hairy:
704 glabrous = 1:3, 2:2, 3:1). Asterisks indicate significant differences with Wilcoxon
705 signed rank test or Mann-Whitney *U*-test (n.s. not significant, * $P < 0.05$, ** $P < 0.01$;
706 see the Results section for details).

707

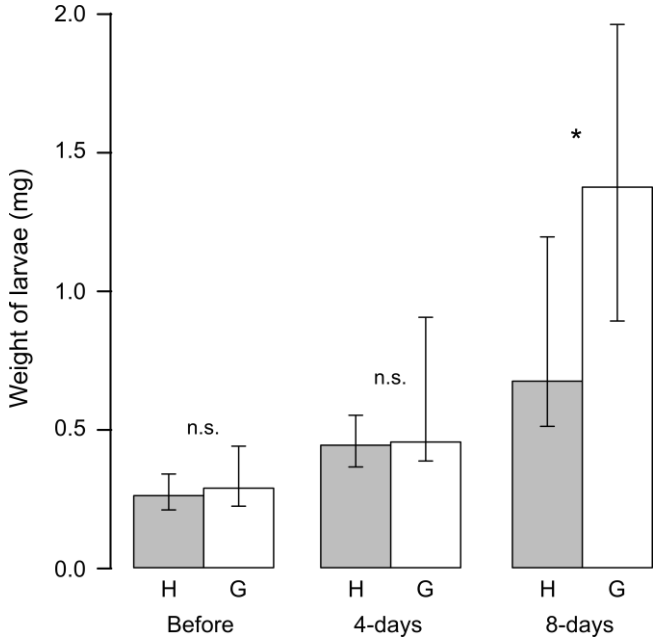
708 **Fig. 3** Average leaf damage (proportion of leaf area lost by herbivory) plotted against
709 the proportion of glabrous plants growing in a 1-m-diameter patch. The leaf damage
710 of hairy (closed circles) and glabrous (open circles) plants is shown separately for
711 each survey (a-d). A circle represents a single patch and vertical bars indicate SE of
712 average leaf damage within a patch. Darker tones of the circles indicate larger
713 numbers of plants in a patch. Trend lines (dashed lines) were added based on the
714 results of single regressions (also see Table 2 for the results of multiple regressions).
715 Data are not transformed in the figures.

716

717 **Fig. 4** Score of LC-MS/MS analysis of five glucosinolates in hairy (H) and glabrous
718 (G) leaves harvested in the field. Median and quartiles are shown for each leaf type
719 (95% CI could not be calculated due to the sample size). n.s. indicates no significant
720 difference between hairy and glabrous leaves with Wilcoxon signed rank test (see the
721 Results section for details).

722

723 **Fig. 1**



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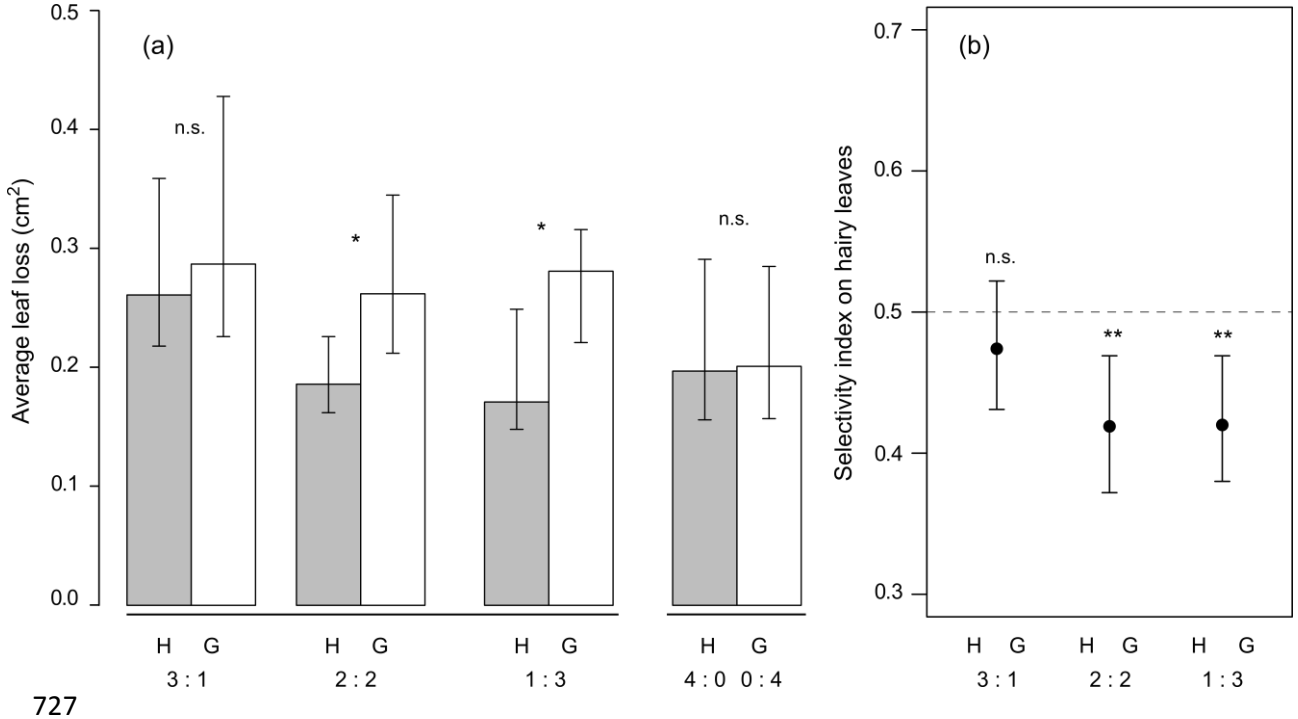
726 **Fig. 2**



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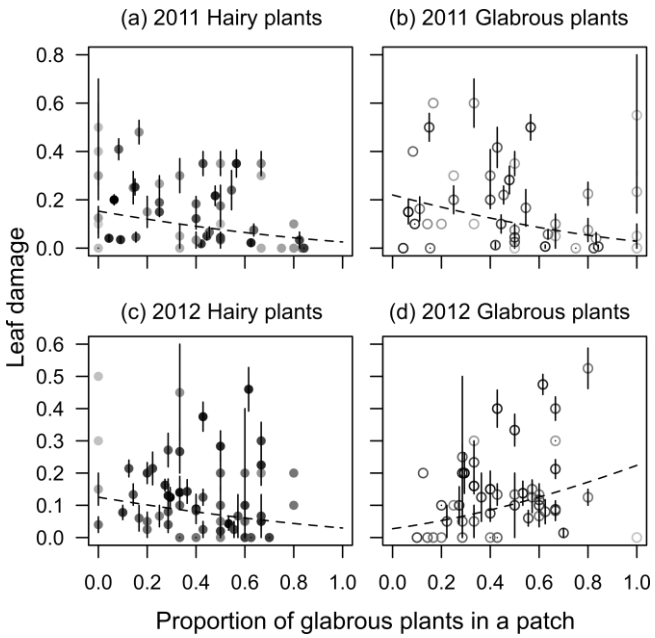
730 **Fig. 3**



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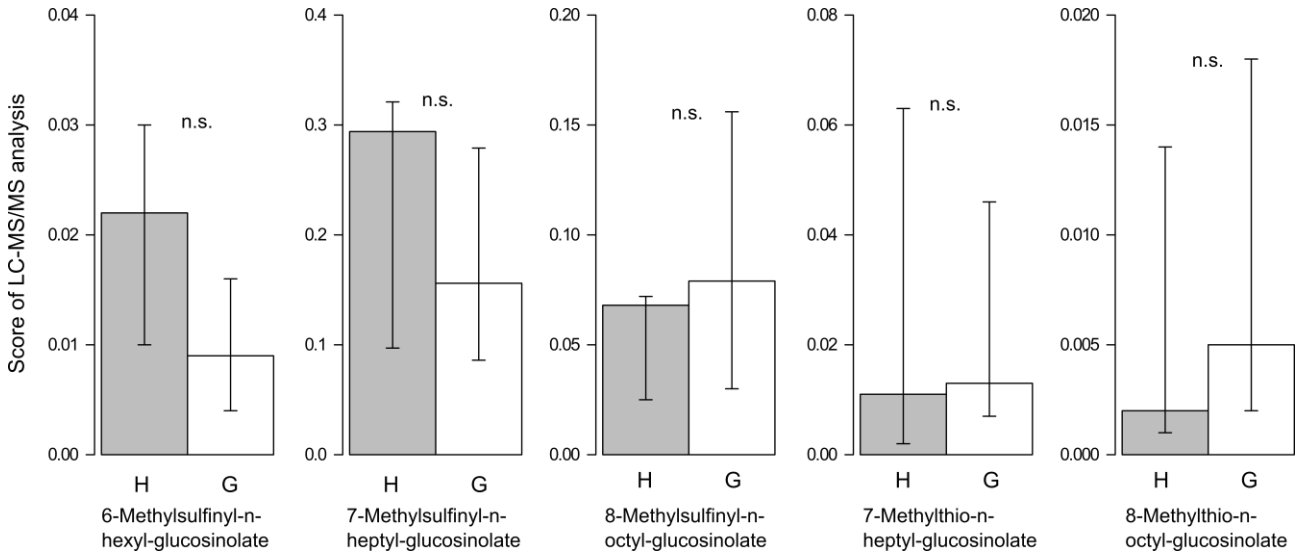
734 **Fig. 4**



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738 **Supplemental Materials**

739 **TableS1** Summary table showing the results of choice experiments when replicates
740 with no leaf infestation were included in the analyses (these cases were excluded from
741 the analyses presented in Figure 2 in the main text). Median and 95% CI values are
742 listed for average leaf loss and the selectivity index for each leaf type. Bars (---)
743 represent the values that are impossible to define. The sample number (*n*) indicates
744 the total number of replicates analyzed.

Condition	Trichome	*n*	Average leaf loss for each leaf type		Chesson's selectivity index	
			Median	95% CI	Median	95% CI
H:G = 4:0	Hairy	20	0.161	0.138-0.245	---	---
H:G = 3:1	Hairy	26	0.206	0.185-0.328	0.495	0.447-0.557
	Glabrous		0.239	0.195-0.374	0.506	0.443-0.553
H:G = 2:2	Hairy	22	0.179	0.151-0.229	0.448	0.400-0.499
	Glabrous		0.240	0.192-0.323	0.552	0.500-0.600
H:G = 1:3	Hairy	27	0.168	0.143-0.223	0.457	0.401-0.490
	Glabrous		0.221	0.193-0.281	0.543	0.510-0.599
H:G = 0:4	Glabrous	19	0.185	0.141-0.229	---	---

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TableS2 Results of the stepwise model selection for the full model that included three-way interaction terms, i.e., the trichome production, the proportion of glabrous plants in a patch, the total number of *A. halleri* subsp. *gemmifera* in a patch, and the study year. Backward and forward stepwise searches were allowed to minimize AICs. The model selection was performed using the stepAIC function implemented in R. The patch ID was incorporated as a random effect in these analyses (see text).

Abbreviations: T, Trichome phenotype; P, Proportion of glabrous plants in a patch; N, Total number of *A. halleri* in a patch; Y, Study year.

Step	Fixed effects	Term subtracted	AIC
0	(T × P × Y) + (T × N × Y) + (T × P) + (T × Y) + (P × Y) + (T × N) + (N × Y) + T + P + N + Y	*Full model*	-368.1
1	(T × P × Y) + (T × P) + (T × Y) + (P × Y) + (T × N) + (N × Y) + T + P + N + Y	(T × N × Y)	-368.6
2	(T × P × Y) + (T × P) + (T × Y) + (P × Y) + (N × Y) + T + P + N + Y	(T × N × Y) + (T × N)	-370.0
3	(T × P × Y) + (T × P) + (T × Y) + (P × Y) + T + P + N + Y	(T × N × Y) + (T × N) + (N × Y)	-370.4
4	(T × P × Y) + (T × P) + (T × Y) + (P × Y) + T + P + Y	(T × N × Y) + (T × N) + (N × Y) + N	-372.3

Table S3. Peak area values of glucosinolates found in leaves of hairy and glabrous plants of *Arabidopsis halleri* subsp. *gemmifera* growing in the field.
Search results of Kyoto Encyclopedia of Genes and Genomes (KEGG) are also presented.

Name	KEGG LIGAND	KEGG Name	Hairy_pair1	Glabrous_pair1	Hairy_pair2	Glabrous_pair2	Hairy_pair3	Glabrous_pair3	Hairy_pair4	Glabrous_pair4	Hairy_pair5	Glabrous_pair5	Hairy_pair6	Glabrous_pair6	Hairy_pair7	Glabrous_pair7	Hairy_pair8	Glabrous_pair8	Hairy_pair9	Glabrous_pair9
10-camphorsulfonic acid*			31784.947	45899.02	41755.852	42478.516	47812.703	46866.063	37725.406	47556.383	48788.285	30858.113	40599.691	38237.469	35628.664	38007.168	33699.859	45198.031	42251.031	34571.953
sinigrin	C08427	Sinigrin; 2-Propenyl glucosinolate	1.011	NA	NA	NA	NA	NA	NA	NA	NA	16.353	NA	NA	NA	NA	NA	NA	NA	NA
3-Methylsulfinyl-n-propyl-glucosinolate	C08411	Glucoiberin; 3-Methylsulfinylpropyl	NA	NA	NA	NA	NA	NA	4.646	NA	0.208	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-Methylsulfinyl-n-butyl-glucosinolate	C08419	Glucoraphanin; 4-Methylsulfinylbutyl	NA	NA	2.621	6.548	2.303	NA	23.08	65.522	4.818	NA	58.931	NA	NA	NA	NA	59.223	0.646	NA
5-Methylsulfinyl-n-pentyl-glucosinolate			NA	NA	NA	3.359	0.24	NA	17.576	21.325	10.766	13.84	73.724	NA	6.566	NA	NA	23.691	2.614	NA
6-Methylsulfinyl-n-hexyl-glucosinolate			951.416	262.567	404.176	674.981	525.78	166.771	1712.755	1318.077	343.759	358.203	2840.367	2495.737	804.752	28.731	194.348	387.873	929.404	21.519
7-Methylsulfinyl-n-heptyl-glucosinolate			10202.403	4696.734	3819.19	14347.034	4434.618	4042.692	12320.968	13263.879	7364.864	5755.896	20914.969	32312.32	10735.055	2366.148	3260.225	7067.686	12427.809	415.088
8-Methylsulfinyl-n-octyl-glucosinolate			1994.533	1255.32	767.366	9806.058	3269.329	7303.358	2550.52	2149.775	1110.42	8573.693	10678.393	4466.003	2581.281	2987.265	839.083	1339.617	10341.102	668.383
3-Methylthio-n-propyl-glucosinolate			NA	NA	NA	NA	NA	0.727	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-Methylthio-n-butyl-glucosinolate	C08409	Glucoerucin; 4-Methylthiobutyl glucosinolate	NA	NA	NA	NA	NA	NA	NA	1.357	3.381	0.7	NA	NA	NA	NA	NA	22.206	NA	NA
5-Methylthio-n-pentyl-glucosinolate			NA	NA	NA	NA	NA	NA	NA	0.396	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
6-Methylthio-n-hexyl-glucosinolate			314.176	68.931	13.435	NA	NA	NA	NA	40.942	83.796	108.931	NA	13.72	116.136	8.792	150.083	99.712	37.101	NA
7-Methylthio-n-heptyl-glucosinolate			4142.145	2022.624	58.887	45.018	1.174	10.145	117.556	633.326	943.112	2901.59	73.67	336.761	2045.183	450.354	2751.356	2383.806	543.08	NA
8-Methylthio-n-octyl-glucosinolate			688.521	673.525	1.638	68.305	4.791	77.499	29.27	155.916	124.531	4179.127	39.561	75.235	394.857	1052.281	901.358	335.211	453.614	NA
3-Hydroxy-n-propyl-glucosinolate			NA	0.435	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-Hydroxy-n-butyl-glucosinolate			NA	NA	NA	NA	NA	0.496	NA	NA	NA	NA	NA	NA	NA	0.705	NA	NA	NA	NA
3-Benzoyloxy-n-propyl-glucosinolate			NA	NA	NA	NA	1.062	NA	NA	NA	NA	2.243	NA	NA	NA	NA	NA	19.281	NA	NA
4-Benzoyloxy-n-butyl-glucosinolate			NA	NA	NA	0.565	NA	NA	NA	6.715	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Indol-3-ylmethyl-glucosinolate			1.162	NA	NA	NA	NA	0.342	NA	NA	NA	6.738	NA	NA	NA	NA	0.911	NA	NA	NA
1-Methoxyindole-glucosinolate			NA	NA	NA	NA	NA	NA	NA	1.55	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-Methoxyindole-glucosinolate			13.514	NA	NA	17.51	355.352	57.122	NA	NA	NA	20.228	NA	NA	5.539	145.943	358.535	2.009	12.123	NA

*, Used as internal standards; NA, not found