Associational anti-herbivore defense in the trichome dimorphism of *Arabidopsis halleri* subsp. *gemmifera* (Brassicaceae)

ハクサンハタザオ（アブラナ科）のトライコームによる被食防御における連合効果

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Summary

A number of plant species are capable of developing defensive traits against herbivores. Associational effects refer to ecological interactions in which the herbivory risk on a focal plant species depends on the neighborhood composition of other plant species. Recent studies have emphasized that such associational effects can be a causal determinant of plant coexistence since they may regulate plant population in a density- or frequency-dependent manner. Despite increasing evidence for associational effects between different plant species, this perspective has rarely been applied to anti-herbivore defense polymorphism within a species. Here, we used a genetic dimorphism of trichome-producing (hairy) and trichomelss (glabrous) plants of *Arabidopsis halleri* subsp. *gemmifera* with the aim of investigating whether and how associational effects cause frequency dependence of plant damage and fitness.

The present thesis provides the following line of evidence on intraspecific associational effects in *A. halleri*. First, it was found that the leaf beetle, *Phaedon brassicae*, preferred glabrous leaves particularly when hairy ones were rare, indicating a potential cause of frequency-dependent damage on the two plant morphs (chapter 2). Second, our laboratory experiment then revealed that associational effects against the leaf beetles mediated a rarity advantage in defense and growth between the two plant morphs (chapter 3). Based on the evidence of previous chapters, we then conducted a multi-year field survey and a semi-field experiment (chapter 4). Both supported the hypothesis that associational effects against the leaf beetle caused a fitness advantage for rare morphs and then promoted the coexistence two morphs within a population. We then extended these studies to interactions with multiple herbivores (chapter 5). We found evidence suggestive of a resistance role of the trichome production against specific types of herbivores such as butterflies; however this resistance effect did not depend on neighboring phenotypes in a plant patch. These findings suggest that the presence/absence of frequency-dependent herbivory under intraspecific associational effects contribute to the maintenance of the trichome dimorphism observed within *A. halleri*.

要約（Summary in Japanese）

植物は自ら動くことができないため、植食者に対して巧みな防御形質を発達させている。植物の個体が受ける食害が自身の形質だけでなく周りの他の植物にも影響される現象は連合効果とよばれ、その例は数多く報告されている。連合効果は、植物の食害や成長に対して少数派有利な状況（負の頻度依存性）をもたらすことで複数の植物種の共存に影響する可能性がある。しかし、植物種間での連合効果はよく研究されてきた一方で、植物種内での連合効果はほとんど着目されてこなかった。そこで本研究では、ハクサンハタザオ(*Arabidopsis halleri* subsp. *gemmifera*)の有毛型と無毛型を対象に、連合効果が有毛・無毛型間で食害や成長に対して頻度依存性をもたらすかを検証した。ハクサンハタザオではトライコームの有無が1遺伝子の変異と対応していることが分かっており、自然集団で見られるハムシやチョウなどの植食性昆虫に対する防御として機能することが予測される。

本学位論文では、植物種内の連合効果について以下4つの研究を行った。まず、第2章では、ダイコンサルハムシ(*Phaedon brassicae*)について、有毛葉が少ない時のみ成虫が無毛葉を選好することを発見した。次に、第3章の室内実験では、ハムシに対する連合効果が有毛・無毛型の食害率と成長に頻度依存性をもたらすことで少数派有利な状況を創出することが明らかとなった。第4章では、連合効果が有毛・無毛型の共存機構となりえるかを検証するために、野外調査と圃場実験を行った。野外では少数派の頻度が時間とともに復帰したこと、圃場実験ではハムシの存在下でのみトライコーム二型に負の頻度依存選択が見られたことから、連合効果が二型の維持機構となる可能性が示唆された。さらに第5章では、複数の植食者が出現する状況で連合効果を検証した。野外調査と圃場実験を行った結果、チョウなど特定の植食者に対してトライコームが防御形質となりえることが示唆されたものの、食害に頻度依存性は見られなかった。以上の結果から、連合効果は食害における頻度依存性の有無を介してトライコーム二型の維持や喪失をもたらすことが示唆された。

Contents

Chapter 1
General introduction

Plant-herbivore interaction plays an essential role in terrestrial ecosystems. It has been estimated that over 15% of annual primary productivity is lost to herbivory in terrestrial plant communities (Cyr and Face 1993). Because this resource loss negatively impacts on plant fitness, plants protect themselves by means of various physical and/or chemical traits (Schoonhoven *et al*. 2005). It is now generally recognized that these defensive traits exhibit a large extent of diversity within a plant species (Hughes 1991; Kennedy and Barbour 1992; Elle *et al*. 1999) as well as between species (Wink 2003; Windsor *et al*. 2005; Agrawal and Fishbein 2006; Beilstein *et al*. 2006).

Although defensive traits provide plants with benefits by discouraging herbivory, they are also costly for plant growth and/or reproduction (Simms and Fritz 1992; Koricheva 2002; Züst *et al*. 2011; Joseph *et al*. 2013). On the basis of the tradeoff between defense and growth, optimal defense theory has long assumed that the amount of herbivory experienced by individual plants depends mainly on their investment in defensive traits (e.g., Rhoads 1979; Simms and Fritz 1992; Stamp 2003). However, there is a growing body of evidence that the herbivory risk depends not only on the plant's own traits but also on the neighborhood composition of other plant species (reviewed by Agrawal *et al*. 2006; Barbosa *et al*. 2009; Underwood *et al*. 2014). These phenomena are called "associational effects" which have been defined as "consumer effects on individuals of one resource organism type, at a given density of that type, are a function of the neighborhood composition of other resource types at particular spatial scales" (Underwood *et al*. 2014). Because of their potential impact on the maintenance of plant species diversity, associational effects have increasingly gained attention in the study of plant-herbivore systems (Callaway *et al*. 2005; Stastny and Agrawal 2014; Kim and Underwood 2015).

Brief introduction to the background of associational effects

Impacts of vegetation heterogeneity on the outcome of plant-herbivore interactions have been studied over decades. This long-standing interest was initiated by observational evidence that a simple plant community, such as may be present in agricultural systems, suffers from outbreaks of herbivorous insects (Elton 1958; Pimentel 1961; Tonhasca and Byrne 1994; Castagneyrol *et al*. 2013, 2014). The focus of studies has now expanded to community levels, where researchers intend to reveal the extent to which plant species diversity predicts the richness of herbivore species (reviewed by Jactel and Brockerhoff 2007; Castagneyrol and Jactel 2012).

The importance of plant species diversity for the likelihood of individual plants being damaged was first proposed by Root and colleagues (Tahvanainen and Root 1972; Root 1973). These studies were among the first to discuss associational effects in connection with plant defense against herbivory. Two major outcomes are expected under associational effects. Palatable plants may gain *associational resistance* when protected from herbivory by nearby unpalatable plants (Tahvanainen and Root 1972; Rausher 1981; Hambäck *et al*. 2000). More recently, an opposite pattern of *associational susceptibility* has also been reported, under which unpalatable plants suffer from severe herbivory when they occur near palatable ones (White and Whitham 2000; Rand 2003). Empirically, the phenomenon of associational effects is now recognized in agricultural (Tahvanainen and Root 1972; Root 1973; Risch 1981), forest (White and Whitham 2000; Castagneyrol *et al*. 2013, 2014), grassland (Courant and Fortin 2010; Wang *et al*. 2010), and even aquatic (e.g., Hay 1986) ecosystems (see Table 1.1 for selected examples).

Recent literature has argued that associational effects may constitute a crucial mechanism of plant coexistence by mediating frequency- or density-dependent damage in multiple plant species (reviewed by Underwood *et al*. 2014). If rare plant species are more likely to be protected from herbivory, associational effects would create a negative feedback that works against the species dwindling to extinction, thus promoting the coexistence of multiple plant species. Several investigations on associational effects have so far documented density- or frequency-dependent herbivory between plant species (Russell and Louda 2004, 2005; Kim and Underwood 2015: Table 1.1), although empirical tests for frequency-dependent damages remain rare.

Associational effects as a potential mechanism for maintaining plant genetic diversity

Despite the fact that associational effects have been studied as a type of interspecific interaction, similar phenomena can be observed within a plant species. In the context of community genetics, for example, a growing number of studies have illustrated that genotype-by-genotype interaction in a plant patch can alter plant-arthropod interactions (Genung *et al*. 2012), plant fitness (Johnson *et al*. 2006; Parker *et al*. 2010) or productivity (Cahill *et al*. 2005; Kotowska *et al*. 2010). This line of evidence suggests that the concept of associational effects (or neighbor effects) is applicable to intraspecific interactions between different plant genotypes.

Prior to the rise of community genetics, several investigations were made into how associational effects protect a dimorphism of defended and undefended plants. Studies from the 1990s connected the concept of associational effects to evolutionary game theory (Sabelis and de Jong 1988; Augner *et al*. 1991; Till-Bottraud and Gouyon 1992; Augner and Bernays 1998; Tuomi *et al*. 1999; see also Rautio *et al*. 2012; McNickle and Dybzinski 2013 for reviews). One major question in this theoretical framework is whether or not rarer types have higher fitness compared to abundant ones (Rautio *et al*. 2012). This criterion of invasibility for rare types helps empirical researchers determine whether the rare genotypes are able to invade into a population dominated by the other genotypes, and whether consequently multiple genotypes can coexist within a population. Empirical tests in this regard are however limited where genetic polymorphism in anti-herbivore defense traits is concerned (but see Wise *et al*. 2009). The present study focused on associational effects as a specific mechanism that led to a rarity fitness advantage between different plant genotypes.

Aim

Negative frequency-dependent selection (or rare-morph advantage) is a theoretically convincing mechanism of maintaining polymorphism within a population (e.g., Ayala and Campbell 1974; Bond and Kamil 1998; Fitzpatrick *et al.* 2007). The purpose of the present thesis was to test whether and how intraspecific associational effects generate the rare-morph advantage and thus can affect the maintenance of anti-herbivore defense polymorphism within a plant species. Three specific questions were addressed in this study: (1) How do associational effects occur between defended and undefended plants within a species? (2) If so, do the associational effects allow rare genotypes to be advantageous in defense and/or growth? (3) Finally, can the rare-type advantage caused by associational effects promote the maintenance of defense polymorphism? We combined laboratory experiments, semi-field experiments, and field surveys to answer the specific questions.

Study system

The present study used a temperate perennial herb, *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz subsp. *gemmifera* (Matsum.) O'Kane & Al-Shehbaz [Brassicaceae/Cruciferae] (syn. *Arabis gemmifera*: referred to as *A. halleri* hereafter; Fig. 1.1). This subspecies is distributed across Japan and the Russian Far East (Hoffmann 2005) and often occurs in isolated habitats such as open gravel sites along streams or in gaps within vegetation (Ihara 1976). The population size ranged from ca. 50 to 7000 in the number of rosette plants (Table 1.2). Plants form rosettes (< 15-cm radius in usual) from summer to winter, and then elongate their flowering stems during spring season (< 1-m height). The flowers are self-incompatible and produce fruits through cross-pollination (Tsuchimatsu *et al.* 2012). The pollinators are small solitary bees and flower flies (Y. Sato, personal observation), and the seeds have no specific structure for long-distance dispersal. Flowering usually starts from late March and last until late May. Fruit set is ca. 80% unless the flowers are consumed by herbivores (Kawagoe and Kudoh 2010). After flowering, plants produce vegetative organs on the main and lateral meristems (Fig. 1.1e), and these rosettes establish as clonal offspring once they are rooted into the ground. Thus, the vegetative growth as well as seed production should contribute to the regeneration and population dynamics of *A. halleri*

To test intraspecific associational effects, we herein focused on the genetic dimorphism of trichome-producing (hairy) and trichomeless (glabrous) plants of *A. halleri* (Fig. 1.1a, b). Several species of the genus *Arabidopsis* have a dimorphism of hairy and glabrous phenotypes (Hauser *et al*. 2001; Kivimäki *et al*. 2007; Steets *et al*. 2010), where the glabrousness has independently evolved because of mutations on a trichome-related gene, *GL1* (Hauser *et al*. 2001; Kivimäki *et al*. 2007). The hairy phenotype is considered genetically dominant, because the association between homozygosity of mutated alleles and glabrous phenotype is perfect (Kawagoe *et al.* 2011) and the heterozygotes exhibit the hairy phenotype. The presence/absence of *A. halleri* trichomes is also associated with allelic variation in *GL1* (Kawagoe *et al*. 2011). Hairy plants produce non-glandular trichomes on their leaf and stem surface, whereas glabrous morphs had no trichomes except for their leaf margin (cf. Fig. 1.1c, d). In a model species *A. thaliana*, *GL1* is involved in the initiation of trichome developments but not in root hair developments (Hülskamp 2004; Grebe 2012). Furthermore, a preceding study of *A. halleri* revealed that (1) the candidate *GL1* locus was unlinked with the two flanking regions as well as several other genes, and that (2) the hairy and glabrous phenotypes were randomly fertilizing each other (Kawagoe *et al*. 2011). The line of genetic evidence indicates that pleiotropy or epistasis is unlikely involved in the *GL-1*-mediated trichome production and root-hair formation. This visible trait with a relatively simple genetic basis helped us concentrate on the discrete phenotypes of hairy and glabrous plants. In addition to the genetic basis, hairy plants produced fewer fruits than glabrous ones in absence of herbivory (Kawagoe *et al*. 2011). This result of a preceding study provides evidence for a fitness cost of the trichome production, supporting the assumption of the existence of defense costs in plants.

The trichome production of *A. halleri* is expected to function as a resistance to herbivory as they are attacked by diverse array of herbivorous insects (see also Yano and Ohsaki 1993). We were able to observe butterflies, beetles, sawflies and aphids feeding on *A. halleri* plants (Fig. 1.2; Table 1.2, 1.3; Appendix 1.1). One major group of herbivorous insects on *A. halleri* is beetles, specifically the flea beetle *Phyllotreta striolata* Fabricius [Coleoptera: Chrysomelidae] and the Brassica leaf beetle *Phaedon brassicae* Baly [Coleoptera: Chrysomelidae]. In natural populations of *A. halleri*, we often found a number of small holes made by flea beetles (Table 1.2), but the leaf area lost to these holes seemed small. In contrast, *P. brassicae* impacts on plant fitness as they directly infest flowering stems of *A. halleri* during spring season (Kawagoe and Kudoh 2010). Another major group is leaf-chewing caterpillars, specifically *Pieris napi* L. [Lepidoptera: Pieridae] and *Athalia infumata* Marlatt [Hymenoptera: Tenthredinidae]. These two species are widely observed in natural populations of *A. halleri*, but their abundance and herbivory intensity are moderate (Table 1.2, 1.3). In particular, *A. halleri* is known to be an unsuitable host for *Pieris* butterflies (Ohsaki and Sato 1999) and may thus be under less feeding pressure from them. Aphids may also be present during the flowering season of *A. halleri*, but were observed only once in a natural population during this study (Fig. 1.2g). These herbivorous insects on *A. halleri* are also known as oligophagous pests on wild and cultivated plants of Brassicaceae: *P. striolata* and *P. napi* are distributed across Europe and Asia, while *P. brassicae* and *A. infumata* are restricted to the Asia region.

Organization of the thesis

This thesis consists of six chapters. Chapter 1 is the general introduction to the background of associational effects. In chapter 2, we examined frequency-dependent foraging by a leaf beetle on the trichome dimorphism as a potential cause of associational effects. In chapter 3, we experimentally tested whether associational effects against *P. brassicae* caused a rarity advantage in defense and/or growth for hairy and glabrous plants of *A. halleri*. In chapter 4, we conducted a multi-year field survey and semi-field experiment to test whether associational effects have driven negative frequency-dependent selection on the trichome dimorphism and consequently promote the coexistence of the two morphs in the field. In chapter 5, we tested associational effects occurring in the presence of multiple herbivores other than the leaf beetle (leaf-chewing caterpillars, flea beetles, and aphids) to extend the previous chapters towards multispecies interaction. In chapter 6, we finally discussed and compared our findings with other examples of associational effects and the maintenance of polymorphism in plants.

Figure 1.1 Pictures of hairy and glabrous plants of *Arabidopsis halleri* subsp. *gemmifera*: (a) rosette of a hairy plant, (b) rosette of a glabrous plant, (c) a flowering meristem of a hairy plant, (d) a vegetative meristem of a glabrous plant, (e) a stem of *A. halleri* producing clonal rosettes (dashed arrow) and inflorescence (including pedicels and undeveloped fruits: solid arrow), (f) a hairy (left) and glabrous (right) plant occurring in a neighborhood, and (g and h) plants severely damaged by herbivory at their growth (g) or flowering (h) stage.

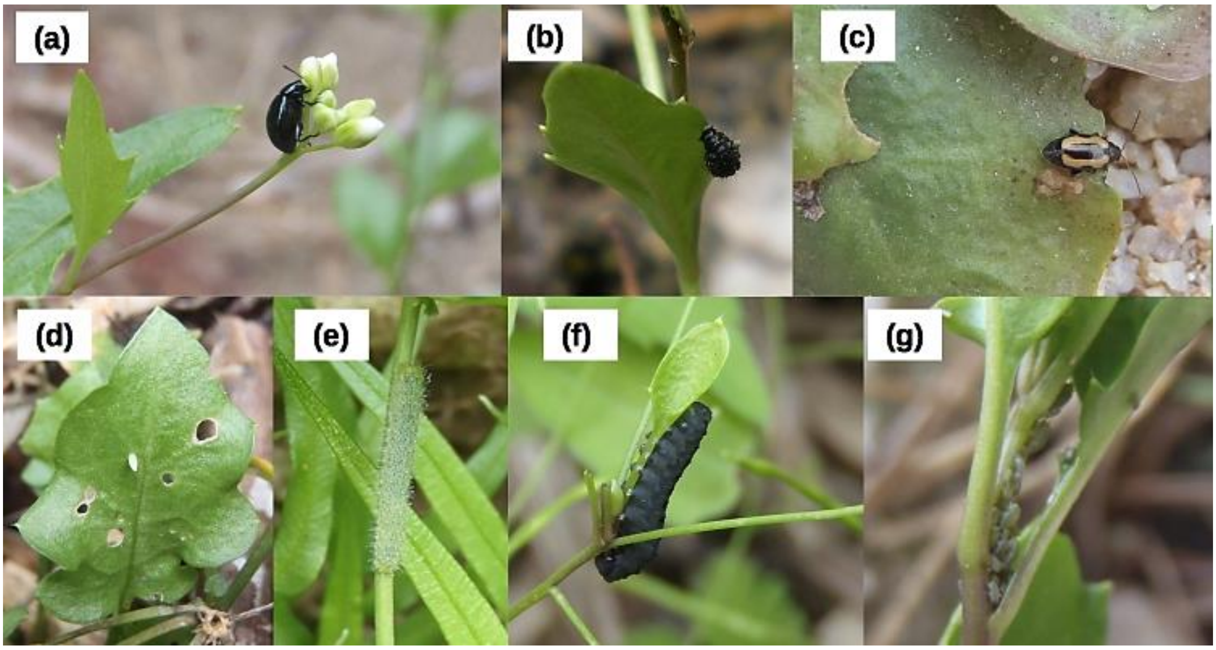


Figure 1.2 Pictures of insect herbivores observed on *Arabidopsis halleri* subsp. *gemmifera* plants: (a and b) adult and larva of *Phaedon brassicae*, (c) the flea beetle, *Phyllotreta striolata*, (d and e) egg and larva of the green-veined white butterfly, *Pieris napi*, (f) larva of the cabbage sawfly, *Athalia infumata*, and (g) crucifer-sucking aphids, *Lipaphis erysimi*.

Table 1.1 Selected examples of plant-herbivore systems in which associational effects have been reported. Focal herbivores and plants (species and traits) are listed. Abbreviation: AR, Associational resistance; AS, Associational susceptibility; NA, Not available.

References	Herbivore	Plant species	Plant traits	Items recorded	Results	Mechanism discussed	Studies discussing similar mechanisms
Tahvanainen & Root (1972)	A specialist flee beetle (*Phyllotreta cruciferae*)	*Brassica oleracea* and tomato or tobacco	Odor (shown by a choice test)	Number of beetles per *Brassicae* plant and plant biomass	AR：The number of beetles per plant was higher, and plant biomass decreased under the monoculture.	Odor cues for herbivores	Root (1973); Risch (1981); Sholes (2008); Plath *et al*. (2012); Zakir *et al*. (2013)
Rausher (1981)	A specialist butterfly (*Battus philenor*)	*Aristolochia reticulata* and others species in nearby vegetation	Unknown (but traits that affected a searching behavior of the butterfly)	Oviposition per *A. reticulata* plant	AR：Isolated plants suffer more oviposition, butterflies spend more time in the isolated patch.	Searching efficiency of herbivores	Hambäck *et al*. (2000)
Risch (1981)	Mono-, oligo-, polyphagous beetles (6 species)	Corn (*Zea mays*), beans (*Phaseolus vulgaris*), and squash (*Cucurbita maxima*)	Unknown	Number of beetles per plant	AR: The beetle abundance reduced under polycultic conditions except for a squash-bean diculture.	Resource concentration (plant density)	Tahvanainen & Root (1972); Root (1973)
Hay (1986)	Generalist fishes	Multiple (>8) species of marine algae	Unknown	Changes in wet mass (%)	AR for palatable algae to generalist fishes.	Physical attachments to unpalatable algae	Hambäck *et al*. (2003)
White & Whitham (2000)	Fall cankerworm (*Alsophila pometaria*).	Cotton (*Populus angustifolia* × *P. fremontii*), and box elder (*Acer negundo*)	Unknown, (but a choice test showed that the cankerworm prefer to the box elder).	Larvae density per shoot of juvenile cotton	AS: Cotton suffered from the higher density near a heterospecific counterpart than near conspecifics or standing alone.	Herbivore dispersal from palatable to unpalatable plants	Agrawal (2004); Plath *et al*. (2012)

Karban & Maron (2002)	Generalist grass-hoppers (incl. multiple species)	Sagebrush (*Artemisia tridentata*) and tobacco (*Nicotiana attenuata*)	Volatile chemical	Number of flowers and capsules per tobacco	AR: tobacco produced more flowers and capsules near damaged sagebrush.	Volatile communication between plants	NA
Hambäck *et al*. (2000)	A specialist leaf beetle (*Galerucella calmariensis*)	An aromatic shrub (*Myrica gale*) and a perrenial herb (*Lythrum salicaria*)	Unknown	Oviposition, leaf damage, seed production per individual *L. salicaria*.	AR: *L. salicaria* incurred less oviposition and damage, and produce more seeds near *M. gale.*	Odor or visual cypsis. Predator abundance did not differ between the two conditions.	Hambäck *et al*. (2003); Jactel *et al*. (2011)
Rand (2003)	A generalist leaf beetle (*Erynephalam aritima*)	Two forbs, *Atriplex patula* var. *hastata* and *Salicorniae uropaea*	Unknown, (but feeding preference was shown by a choice test)	Leaf damage, the number of larvae, and survival (%) of plants.	AS: *Atriplex* suffered more leaf damage and less survival near *Salicornia.*	Diet-mixing foraging by generalist herbivore.	NA
Agrawal (2004)	A specialist beetle (*Tetraopes tetraophthalmus*)	A milkweed (*Asclepias syriaca*) and a grass (*Liriomyza asclepiadis*)	Trichome, Latex, Nitrogen contents	Milkweed biomass, fruit production, fruit mass; and grass biomass	AS: milkweed biomass and fecundity was Control ≒ Grass+ > Beetle+ > Grass&Beetle+.	Dispersal from primary host plants to secondary ones.	White & Whitham (2000)
Callaway *et al*. (2005)	Mammals	Four unpalatable herbs (*Cirsium*, *Veratrum*, *Luzula*, *Alchemilla*) and two palatable herbs (*Anthoxanthum*, *Phleum*)	Unknown except for spines (*Cirsium*) or toxin (*Veratrum*)	Annual growth rates of four plant species.	AR: increased growth rate of two palatable species, no increase of growth rate of unpalatable two species near *Cirsium* and *Veratrum.*	Grazer's avoidance to plant spines and toxin.	González-Teuber & Gianoli (2008)
Russell & Louda (2005)	Florivorous weevil, *Rhinocyllus conicus*	Two congeneric natives, *Cirsium canescens* and *C. undulatum*	Plant phenology	Proportion of weevils and their eggs on *C. undulatum*.	AR for *C. undulatum*: The proportion of weevils and eggs was negatively correlated with the number of *Cirsium canescens.*	Phenological synchronicity of flowering times between two species.	NA

Bergvall *et al*. (2006)	Fallow deer (*Dama dama*)	Unknown. Synthetic food containing different tannin levels	Chemical (Tannin)	Log-consumption per bucket (g).	AS for low-tannin buckets: More consumption on low-tannin buckets in the bad patch than in the good patch. AR for high-tannin buckets: Less consumption on high-tannin buckets in the good patch than in the bad patch.	Within/Among-patch foraging behavior of mammalian herbivores.	Andrew *et al*. (2007); Emerson *et al*. (2012); Wang *et al*. (2010); Courant & Fortin (2010); Miller *et al*. (2009)
Barton & Bowers (2006)	None (only defense phenotypes were evaluated)	Interspecific: two congeners, *Plantago lanceolata* and *P. major*	Chemical (Iridoid glucoside)	Within-patch: % Dry Weight of Iridoid Glycosides	AS for *P. lanceolata*?: *P. lanceolata* surrounded by heterospecific plants produced less defensive chemicals compared to those surrounded by conspecifics.	Plant trait change via direct competition between plants.	Agrawal (2004)
Le Guigo *et al*. (2012)	A specialist aphid (*Brevicoryne brassicae*) and generalist aphid (*Myzus persicae*)	Three *Brassica* species and tomato (*Solanum lycopersicum*)	Chemical (glucosinolate)	Number of aphids on a focal *B. oleracea*, compared between conspecific and interspecific conditions	AS when a herbivore is specialist; AR when a herbivore is generalist.	Chemical attraction or repulsion	Jactel *et al*. (2011); Zakir *et al*. (2013)
Castagneyrol *et al*. (2013)	Leaf-chewers (beetles, caterpillars, and grasshoppers) or Leaf-miners (incl. 5 species)	*Betula pendula*, *Quercus robur*, *Quercus pyrenaica*, *Quercus ilex*, *Pinus pinaster*	Plant height, and leaf area, N and P contents, leaf life span	Leaf area loss (by chewers) and leaf-miner abundance at plot and individual scale.	AR against leaf-miners: no AR against leaf-chewers	Variation in leaf traits, and apparency (plant height)	Sholes (2008); Hughes (2012)
Kim & Underwood (2015)	3 species of specialists (for *S. carolinense*), and 3 species of generalist herbivores	*Solanum carolinense*, *Solidago altissima*	Unknown	Leaf damage (%) of individual plants	AS for *S. carolinense*; damage was positively density-dependent.	Unknown (but several possibilities are discussed)	NA

Table 1.2 The number of hairy and glabrous plants and the observational evidence of herbivore fauna in 26 populations of *Arabidopsis halleri* subsp. *gemmifera* located in the central Japan. See Appendix 1.1 for the procedure of field survey.

ID	Name	Latitude	Longitude	Altitude(m)	#Hairy	#Glabrous	Herbivores observed
1	Sofudani, Gifu	35°19'N	136°27'E	190	0	488	1 larval *Athalia infumata*
2	Midoridani, Gifu	35°37'N	136°36'E	260	0	81	2 adult *Pieris napi*
3	Fujiwara-Mikuni, Mie	35°13'N	136°27'E	240	0	296	2 adult *P. napi*
4	Fujiwara-Ogaito, Mie	35°10'N	136°28'E	120	26	135	2 adult *P. napi*, 3 adult *Phyllotreta striolata*
5	Kiwada, Shiga	35°06'N	136°22'E	310	185	548	1 adult *P. napi*, 3 larval *A. infumata*
6	Ojigahata, Shiga	35°13'N	136°23'E	310	449	602	8 adult *P. napi*, 1 larval *A. infumata*
7	Ibuki, Shiga	35°24'N	136°23'E	350	11	403	1 adult and 1 egg of *P. napi*
8	Gongendani, Shiga	35°15'N	136°22'E	380	134	162	9 adult *P. napi*
9	Asibidani, Shiga	35°13'N	135°51'E	410	0	376	2 adult *P. napi*
10	Umenoki, Shiga	35°16'N	135°52'E	430	0	511	1 adult *P. striolata*
11	Katsuragawa-Sakashita, Shiga	35°11'N	135°51'E	480	6	58	1 adult *P. napi*
12	Kutsuki, Shiga	35°22'N	135°55'E	180	294	20	*Leaf holes observed for many plants*
13	Hanase-Yamasu, Kyoto	35°13'N	135°47'E	410	656	77	7 adult *P. striolata*, 5 larval *A. infumata*, 1 *Eurydema rugosa*
14	Hanase-Bessho, Kyoto	35°11'N	135°47'E	500	742	185	1 adult *P. striolata*, 10 larval *A. infumata*
15	Miyama, Kyoto	35°18'N	135°42'E	350	0	46	*Leaf holes observed but herbivores could not be found*
16	Kurama, Kyoto	35°08'N	135°47'E	420	363	141	5 adult *P. striolata*
17	Ohara, Kyoto	35°10'N	135°51'E	330	0	3686	*Leaf holes observed but herbivores could not be found*
18	Shizuhara, Kyoto	35°07'N	135°48'E	230	0	618	1 larval *A. infumata*

19	Minoh, Osaka	34°51'N	135°28'E	160	1353	982	2 adult *P. napi*, 14 larval *A. infumata*, 6 adult *P. striolata*
20	Myoken, Osaka	34°55'N	135°27'E	230	5292	0	2 adults and 3 eggs of *P. napi*, 7 adult *P. striolata*
21	Tada, Hyogo	34°54'N	135°21'E	140	7335	0	23 adults and 18 eggs of *P. napi*
22	Mikohata, Hyogo	35°15'N	134°43'E	320	120	3	7 adults and 6 eggs of *P. napi*, and 1 adult *P. striolata*
23	Takacho-Tada, Hyogo	35°06'N	134°53'E	190	75	6	2 adults and 4 eggs of *P. napi*, 1 *E. rugosa*
24	Ikuno, Hyogo	35°10'N	134°49'E	360	483	0	3 adults and 13 eggs of *P. napi*, 2 adult *Phaedon brassicae*, 3 adult *P. striolata*
25	Omoide-gawa, Hyogo	35°06'N	134°56'E	200	1051	997	14 adult *P. brassicae*
26	Monzen, Hyogo	35°05'N	134°54'E	160	1441	1699	7 adults and 5 eggs of *P. napi*, 1 larval *A. infumata*, 1 adult *P. striolata*

Table 1.3 Leaf damage of *Arabidopsis halleri* subsp. *gemmifera* plants in 26 natural populations located in the central Japan. Shown are dates when hairy and glabrous plants counted (CountDate), survey dates (DamageDate) and sample sizes (N_h and N_g) for average leaf damage within a population (AvDamage) in 2014 and 2015. See also Appendix 1.1 for the procedure of survey.

ID	Name	CountDate	2014				2015			
			AvDamage	N_h	N_g	DamageDate	AvDamage	N_h	N_g	DamageDate
1	Sofudani, Gifu	4-May-2014	0.040	0	20	12-July-2014	0.073	0	25	7-June-2015
2	Midoridani, Gifu	5-May-2014	0.033	0	20	13-July-2014	0.052	0	20	7-June-2015
3	Fujiwara-Mikuni, Mie	3-May-2014	0.029	0	20	12-July-2014	0.035	0	20	6-June-2015
4	Fujiwara-Ogaito, Mie	31-Dec-2011	0.039	0	20	13-July-2014	0.069	8	20	7-June-2015
5	Kiwada, Shiga	3-May-2014	0.085	15	25	14-July-2014	0.084	20	25	8-June-2015
6	Ojigahata, Shiga	4-May-2014	0.068	10	30	12-July-2014	0.075	30	30	6-June-2015
7	Ibuki, Shiga	14-June-2014	0.111	0	30	14-June-2014	0.045	6	20	28-June-2015
8	Gongendani, Shiga	4-May-2014	0.099	20	20	26-June-2014	0.113	20	20	6-June-2015
9	Asibidani, Shiga	27-Apr-2014	0.020	0	20	28-June-2014	0.038	0	20	23-June-2015
10	Umenoki, Shiga	12-May-2012	0.036	0	20	28-June-2014	0.051	0	20	23-June-2015
11	Katsuragawa-Sakashita, Shiga	27-Apr-2014	0.046	0	20	28-June-2014	0.040	0	20	23-June-2015
12	Kutsuki, Shiga	19-May-2012	0.085	10	20	28-June-2014	0.109	20	20	23-June-2015
13	Hanase-Yamasu, Kyoto	26-Apr-2014	0.142	10	20	21-June-2014	0.128	20	20	20-June-2015
14	Hanase-Bessho, Kyoto	26-Apr-2014	0.115	10	20	21-June-2014	0.100	20	20	20-June-2015
15	Miyama, Kyoto	26-Apr-2014	0.021	0	20	21-June-2014	0.010	0	20	20-June-2015
16	Kurama, Kyoto	26-Apr-2014	0.112	7	20	21-June-2014	0.092	20	20	20-June-2015

17	Ohara, Kyoto	26-Apr-2014	0.051	0	31	21-June-2014	0.040	0	30	20-June-2015
18	Shizuhara, Kyoto	26-Apr-2014	0.040	0	20	21-June-2014	0.051	0	20	20-June-2015
19	Minoh, Osaka	18-Dec-2011	0.095	30	40	18-June-2014	0.118	30	30	16-June-2015
20	Myoken, Osaka	10-Dec-2011	0.102	0	40	30-June-2014	0.134	0	40	12-June-2015
21	Tada, Hyogo	11-May-2014	0.093	0	40	30-June-2014	0.148	0	40	12-June-2015
22	Mikohata, Hyogo	6-July-2014	0.045	20	3	13-June-2014	0.131	20	3	13-June-2015
23	Takacho-Tada, Hyogo	6-July-2014	0.082	20	3	13-June-2014	0.108	20	2	13-June-2015
24	Ikuno, Hyogo	6-July-2014	0.122	0	20	13-June-2014	0.138	0	25	13-June-2015
25	Omoide-gawa, Hyogo	23-Apr-2014	0.250	35	35	3-June-2014	0.154	30	30	9-June-2015
26	Monzen, Hyogo	28-Apr-2014	0.070	15	35	23-June-2014	0.080	30	30	15-June-2015

Appendix 1.1 Methods of the field survey on plant damage and herbivore fauna

We surveyed leaf damage and the number of hairy and glabrous plants for 26 populations located in the Kinki area (Table 1.2, 1.3). The total number of plants within each site was surveyed during 2011–2013 and ranged from 40 to 7400, while herbivory was examined in 2014 and 2015 during early summer (June–mid July). In these populations, the green-veined white butterfly (*Pieris napi*), the flea beetle (*Phyllotreta striolata*), and the cabbage sawfly (*Athalia infumata*) were frequently observed (Table 1.2; Fig. 1.2). We scored the proportion of leaf area lost to herbivory (referred to hereafter as leaf damage) as 0 (< 12.5%), 1 (25 ± 12.5%), 2 (50 ± 12.5%), 3 (75 ± 12.5%), or 4 (> 87.5% and < 100%) for all leaves of an individual plant. We also measured the length of the largest leaf (referred to hereafter as maximum leaf length) as an indication of plant size. These traits were evaluated for 20–40 hairy or glabrous plants randomly chosen for each population, although either hairy or glabrous data were available in completely or mostly (< 10% for rare morphs) monomorphic populations. The plants measured were kept at least 2 m apart from each other to avoid multiple samplings from a single clone. We identified and counted herbivorous insects observed on each site during the survey. All surveys were conducted during 7:30–18:00. Detailed information of survey dates and sample sizes are listed in Table 1.1. We calculated the proportion of leaf area lost for the entire plant by averaging the corresponding area loss of the damage score of leaves (i.e., scores of 0, 1, 2, 3, and 4 = 0.0, 0.25, 0.50, 0.75, and 1.0, respectively). The average leaf damage within a population was calculated as the average of the proportion of leaf area lost for plants sampled in a site.

Chapter 2-1
Frequency-dependent herbivory by a leaf beetle on hairy and glabrous plants

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 behavior 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 behavior 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 behavior 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* Baly [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 behavior 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 behaviors 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, b). 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*. 2007a, b, c). 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 $\mu mol/m^2s$ (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 xylostella* L. However their abundance is much lower than that of *P. brassicae* throughout the year (Kawagoe and Kudoh 2010) and we found only a few *P. napi* and *P. xylostella* during the present study.

Larval growth on hairy and glabrous leaves

First-instar larvae were used within three days after hatching in the laboratory-reared population. Several hundred young radical leaves were harvested from approximately 100 intact hairy and glabrous plants growing in our study site. The hairy and glabrous leaves were kept separately in a plastic case filled with water. A petiole of a single leaf was wrapped with moistened paper and placed in the center of a Petri dish. Nineteen individual larvae were separately released onto the upper surface of either a hairy or a glabrous leaf. The larvae were allowed to infest the leaves for eight days under 20˚C, 12L:12D conditions. The weight of larvae was measured before, four days, and eight days after release. Because adult beetles do not grow in size after emerging from pupae, the weight of larvae in the early developmental stage was used as an indicator of the herbivore performance. Measurements for each larva were performed three times to the nearest 10^{-2} mg (AEL-40SM, Shimadzu, Tokyo, Japan) and the average values were used for analyses. Four days after the first release, the leaves were replaced with fresh leaves that had been kept in a refrigerator.

Choice experiments under different leaf frequencies

We conducted choice or no-choice experiments under five leaf frequency conditions (hairy: glabrous = 4:0, 3:1, 2:2, 1:3, 0:4). Adult beetles were used in the 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 S2.1).

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 10×10^{-2} 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.

Statistical analysis

For the data set from the larval growth experiment, the weights of larvae fed on the hairy and the glabrous leaves were compared with a Mann-Whitney *U*-test. The analysis was done separately for the weight before the release, four days, and eight days after the release. For the data set from the choice experiments, we calculated the average leaf loss (cm^2) for each trichome type per dish to analyze herbivory on each leaf type in the choice experiment. A Wilcoxon signed rank test was used to compare the average leaf loss between the hairy and glabrous leaf discs for choice conditions (hairy: glabrous = 3:1, 2:2, 1:3). For no-choice conditions (hairy: glabrous = 4:0, 0:4), the average leaf loss was compared between the hairy and glabrous leaf discs by a Mann-Whitney *U*-test. In all the analyses for the choice conditions, *P*-values were adjusted using sequential Bonferroni correction to control the risk of increased type I error due to multiple testing. To test whether the relative frequency of hairy and glabrous leaves affected the total amount of leaf loss (cm^2) in each arena, we analyzed the effect of the frequency conditions on the total amount of leaf loss in each arena with a Kruskal-Wallis test. Further, to analyze the preference of adult beetles in the choice conditions, Chesson's selectivity index (Chesson 1978) was calculated for each preference arena for the three choice conditions. Chesson's α for diet type i is denoted as $\alpha_i = (r_i / P_i) / \Sigma(r_i / P_i)$, where r indicates the relative frequency of diet i in total consumption by predators and P indicates the relative frequency of diet i in the environment. When there are two types of diets, $\alpha > 1/2$ and $\alpha < 1/2$ mean positive and negative preference for the focal diet, respectively. The parameter r for the hairy and glabrous leaf discs was estimated as the proportion of the hairy or glabrous leaf area consumed relative to the total leaf area consumed in each preference arena. The 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 Bonferroni 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: Bates *et al*. 2012) 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 (Mann-Whitney U-test, $U = 157$, $n_1 = n_2 = 19$, $P = 0.49$: Fig. 2.1). The weight of larvae four days after release also showed no significant difference between the hairy and glabrous leaves ($U = 126$, $n_1 = n_2 = 18$, $P = 0.25$: Fig. 2.1). The weight of larvae eight days after release on the hairy leaves was significantly lower than that on the glabrous leaves ($U = 43$, $n_1 = 11$, $n_2 = 14$, $P < 0.05$: Fig. 2.1). 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 (Wilcoxon signed rank test, $V = 224$, $n = 23$, $P < 0.05$ with sequential Bonferroni correction: Fig. 2.2a) and the hairy: glabrous = 2:2 condition ($V = 163$, $n = 18$, $P < 0.05$: Fig. 2.2a). The average leaf loss did not differ significantly between the hairy and glabrous leaves under the hairy: glabrous = 3:1 condition ($V = 161$, $n = 22$, $P = 0.26$: Fig. 2.2a). Under no-choice conditions, no significant difference in leaf loss was found between the hairy and glabrous leaves (Mann-Whitney *U*-test, $U = 109$, $n_1 = n_2 = 15$, $P = 0.88$: Fig. 2.2a). 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 ($V = 239$, $n = 23$, $P < 0.01$: Fig. 2.2b) and the hairy: glabrous = 2:2 condition ($V = 153$, $n = 18$, $P < 0.01$: Fig. 2.2b). The selectivity index did not differ significantly between the hairy and glabrous leaves under the hairy: glabrous = 3:1 condition ($V = 162$, $n = 22$, $P = 0.26$: Fig. 2.2b). 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 S2.1).

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 S2.2). 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 2.1), 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.

Leaf damage of hairy plants tended to decrease concomitantly as the proportion of glabrous plants increased in a patch in both of these two years (Table 2.2; Fig. 2.3a, c), though the correlation was not significant in 2012 (Table 2.2). Leaf damage of glabrous plants decreased in 2011, while it increased in 2012, as the proportion of glabrous plants increased in a patch (Table 2.2; Fig. 2.3b, d). The leaf damage of glabrous plants increased significantly as the total number of *A. halleri* in a patch increased in 2012 (Table 2.2). The leaf damage of the hairy plants was 0.154 ± 0.009 in 2011 (Mean ± SE, $n = 318$) and 0.136 ± 0.012 in 2012 ($n = 260$), while the leaf damage of the glabrous plants was 0.134 ± 0.009 in 2011 ($n = 232$) and 0.163 ± 0.011 in 2012 ($n = 195$).

Glucosinolate analysis of hairy and glabrous leaves

Five aliphatic glucosinolates were found in the most pairs of hairy and glabrous leaf samples, but the score of LC-MS/MS values of these glucosinolates showed no significant difference between hairy and glabrous leaves (6-Methylsulfinyl-n-hexyl-glucosinolate, $n = 9$ pairs, $V = 38$, $P = 0.37$; 7-Methylsulfinyl-n-heptyl-glucosinolate, $n = 9$ pairs, $V = 23$, $P = 1$;

8-Methylsulfinyl-n-octyl-glucosinolate, $n = 9$ pairs, $V = 21$, $P = 1$;
7-Methylthio-n-heptyl-glucosinolate, $n = 8$ pairs, $V = 21$, $P = 1$;
8-Methylthio-n-octyl-glucosinolate, $n = 8$ pairs, $V = 18$, $P = 1$: Fig. 2.4). The other twelve glucosinolates (including sinigrin and one indolic glucosinolate) were found in some samples (Table S2.3).

Discussion

The choice experiment demonstrated frequency-dependent herbivory by *P. brassicae* with respect to trichome polymorphism of *A. halleri*. We observed less 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 behavior (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 choosing host plants (Ôtake and Funaki 1958). Therefore the results of our choice experiments presumably represent the feeding preference of *P. brassicae* adults and its frequency dependence within a single plant patch.

We found that leaf damage on hairy plants decreased as the proportion of glabrous plants increased within local patches (1 m in diameter) in 2011. A similar pattern was found in

2012, although it was not statistically significant. This tendency is consistent with the frequency-dependent herbivory detected in the choice experiments. We observed a positive correlation between leaf damage of glabrous plants and the frequency of glabrous plants within patches in 2012. This pattern would be expected according to the frequency-dependent preference changes observed in our experiments. However, the negative correlation we observed between leaf damage and frequency of glabrous plants in 2011 was inconsistent with the laboratory evidence of frequency-dependent herbivory. We also observed significant density-dependent herbivory on glabrous plants in 2012 (Table 2.2b). The effect of plant density could not be tested in our choice experiments under the condition of equal leaf density. Overall, our field observations support the existence of frequency-dependent herbivory at least on hairy plants, but it remains unclear whether our experimental evidence can account for the frequency-dependent herbivory on glabrous plants in the field. We need further studies before we can reach a rigorous conclusion about how important the frequency-dependent herbivory by adult beetles is under natural conditions.

Our previous studies revealed that intensive leaf damage is predominantly caused by larvae feeding in our field site (Kawagoe and Kudoh 2010; Kawagoe *et al*. 2011). In the flowering period, adult beetles were found on less than 2% of plants 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. 2.1), 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.

Table 2.1 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).

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

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

Table 2.2 Coefficients and their standard error (SE) for terms of proportion of glabrous plants in a patch and total number of *Arabidopsis halleri* subsp. *gemmifera* in a patch in GLMMs explaining the leaf damage (arcsine-transformed proportion of leaf area lost by herbivory) in 2011 and 2012 in the field. Upper rows (a) present results of models including the proportion of glabrous plants, and lower rows (b) present results of models including both the proportion of glabrous plants and the total number of plants. Bold values indicate significant deviation of coefficients from zero (Wald 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**

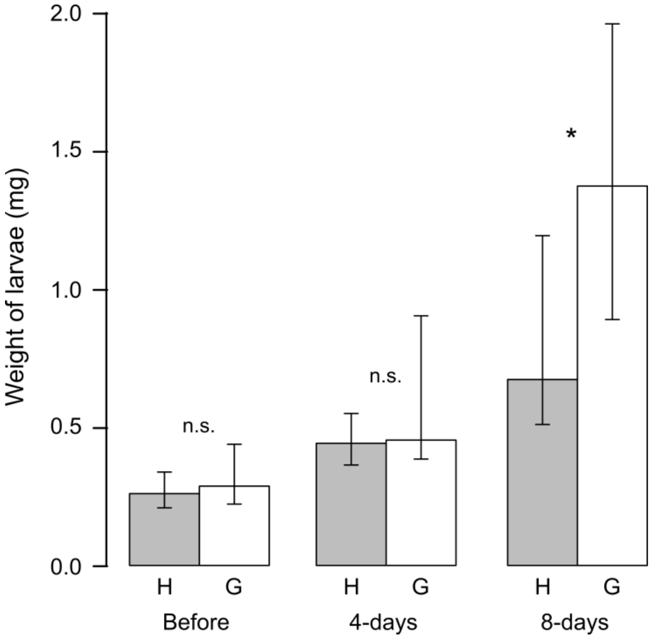


Figure 2.1 Weight of larvae (Median ± 95% CI) fed on hairy (H; filled bars) and glabrous (G; open bars) leaves before release, and four days and eight days after release. Asterisks indicate significant differences with Mann-Whitney *U*-test (n.s., not significant; *, $P < 0.05$).

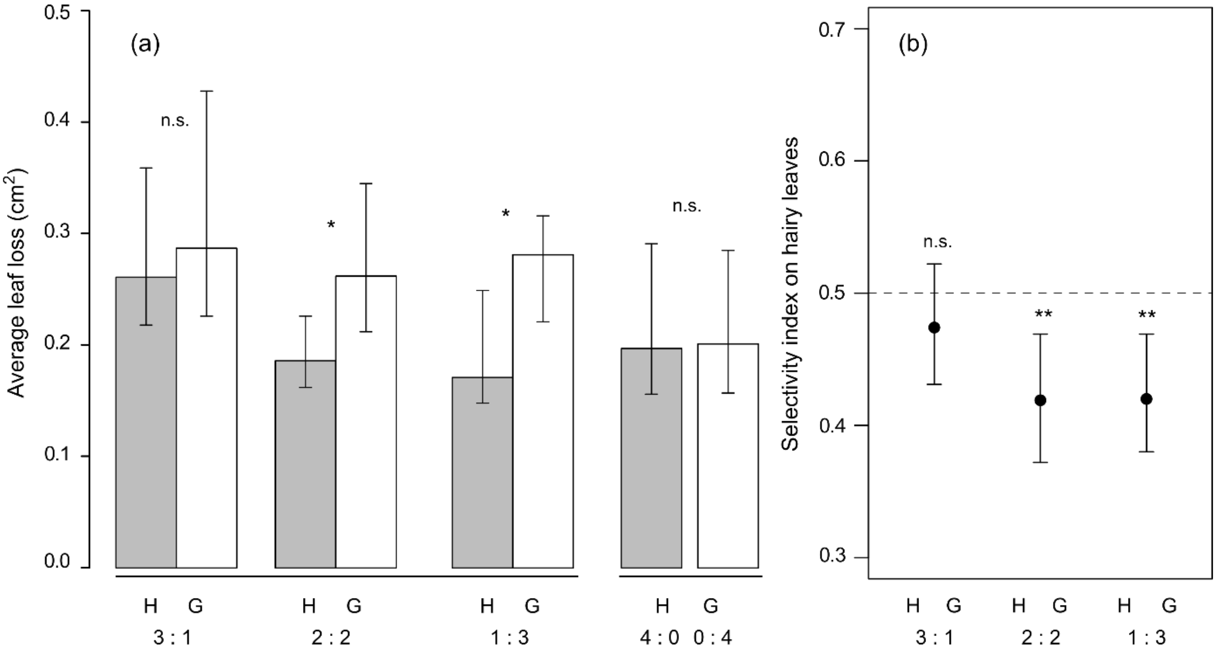


Figure 2.2 Frequency-dependent herbivory by adult beetles on hairy (H) and glabrous (G) leaves in choice experiments. The left panel (a) shows the average leaf loss (Median ± 95% CI) for each trichome type in the choice and no-choice conditions (hairy: glabrous = 4:0, 3:1, 2:2, 1:3, 0:4), where filled and open bars indicate the hairy and glabrous leaf type, respectively. The right panel (b) shows Chesson's selectivity index (Median ± 95% CI) for hairy leaf type under the three choice conditions (hairy: glabrous = 1:3, 2:2, 3:1). Asterisks indicate significant differences with Wilcoxon signed rank test or Mann-Whitney *U*-test (n.s., not significant; *, $P < 0.05$; **, $P < 0.01$; see the Results section for details).

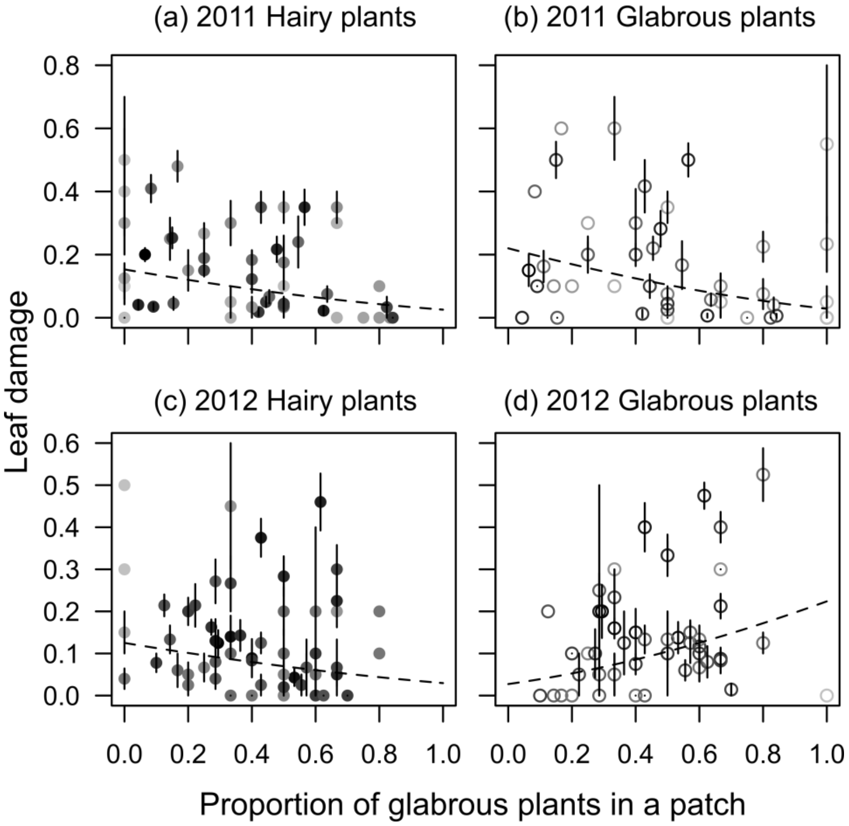


Figure 2.3 Average leaf damage (proportion of leaf area lost by herbivory) plotted against the proportion of glabrous plants growing in a 1-m-diameter patch. The leaf damage of hairy (closed circles) and glabrous (open circles) plants is shown separately for each survey (a-d). A circle represents a single patch and vertical bars indicate SE of average leaf damage within a patch. Darker tones of the circles indicate larger numbers of plants in a patch. Trend lines (dashed lines) were added based on the results of single regressions (see also Table 2.2 for the results of multiple regressions). Data are not transformed in the figures.

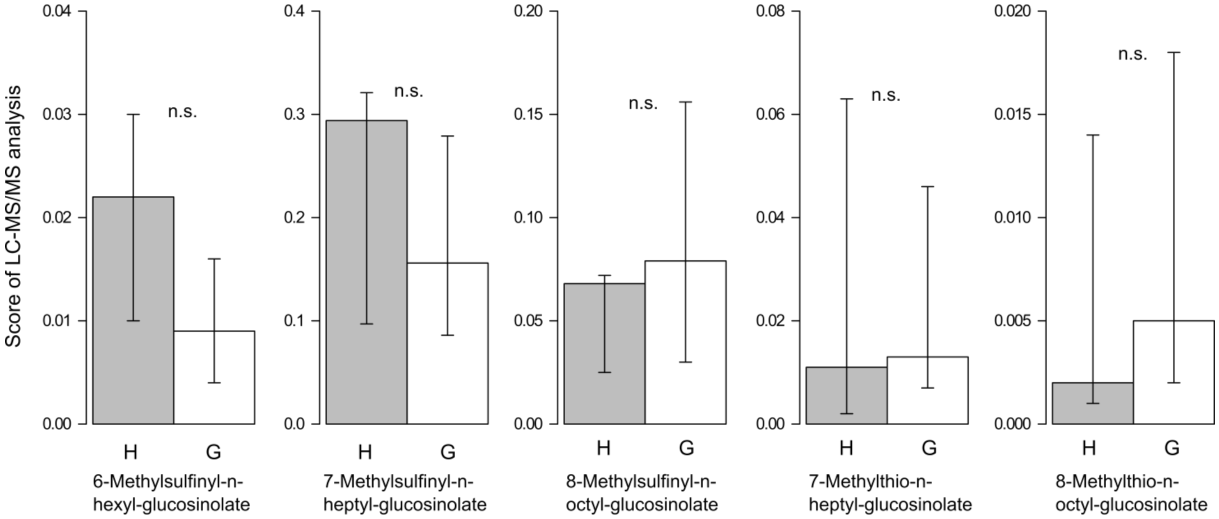


Figure 2.4 Score of LC-MS/MS analysis of five glucosinolates in hairy (H) and glabrous (G) leaves harvested in the field. Median and quartiles are shown for each leaf type (95% CI could not be calculated due to the sample size). n.s. indicates no significant difference between hairy and glabrous leaves with Wilcoxon signed rank test (see the Results section for details).

Supporting information

Table S2.1 Summary table showing the results of choice experiments when replicates with no leaf infestation were included in the analyses (these cases were excluded from the analyses presented in Figure 2.2 in the main text). Median and 95% CI values are listed for average leaf loss and the selectivity index for each leaf type. Bars (---) represent the values that are impossible to define. The sample number (*n*) indicates 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	---	---

Table S2.2 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).

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

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

Table S2.3 Peak area values of twenty glucosinolates analyzed for leaves of hairy (H) and glabrous (G) plants of *Arabidopsis halleri* subsp. *gemmifera* growing in the field.

Name	H_pair1	G_pair1	H_pair2	G_pair2	H_pair3	G_pair3	H_pair4	G_pair4	H_pair5	G_pair5	H_pair6	G_pair6	H_pair7	G_pair7	H_pair8	G_pair8	H_pair9	G_pair9
10-camphorsulfonic acid*	31784.9	45899.0	41755.9	42478.5	47812.7	46866.1	37725.4	47556.4	48788.3	30858.1	40599.7	38237.5	35628.7	38007.2	33699.9	45198.0	42251.0	34572.0
sinigrin	1.0	NA	NA	NA	NA	NA	NA	NA	NA	16.4	NA	NA	NA	NA	NA	NA	NA	NA
3-Methylsulfinyl-n-propyl-glucosinolate	NA	NA	NA	NA	NA	NA	4.6	NA	0.2	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-Methylsulfinyl-n-butyl-glucosinolate	NA	NA	2.6	6.5	2.3	NA	23.1	65.5	4.8	NA	58.9	NA	NA	NA	NA	59.2	0.6	NA
5-Methylsulfinyl-n-pentyl-glucosinolate	NA	NA	NA	3.4	0.2	NA	17.6	21.3	10.8	13.8	73.7	NA	6.6	NA	NA	23.7	2.6	NA
6-Methylsulfinyl-n-hexyl-glucosinolate	951.4	262.6	404.2	675.0	525.8	166.8	1712.8	1318.1	343.8	358.2	2840.4	2495.7	804.8	28.7	194.3	387.9	929.4	21.5
7-Methylsulfinyl-n-heptyl-glucosinolate	10202.4	4696.7	3819.2	14347.0	4434.6	4042.7	12321.0	13263.9	7364.9	5755.9	20915.0	32312.3	10735.1	2366.1	3260.2	7067.7	12427.8	415.1
8-Methylsulfinyl-n-octyl-glucosinolate	1994.5	1255.3	767.4	9806.1	3269.3	7303.4	2550.5	2149.8	1110.4	8573.7	10678.4	4466.0	2581.3	2987.3	839.1	1339.6	10341.1	668.4
3-Methylthio-n-propyl-glucosinolate	NA	NA	NA	NA	NA	0.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-Methylthio-n-butyl-glucosinolate	NA	NA	NA	NA	NA	NA	NA	1.4	3.4	0.7	NA	NA	NA	NA	NA	22.2	NA	NA
5-Methylthio-n-pentyl-glucosinolate	NA	NA	NA	NA	NA	NA	NA	0.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
6-Methylthio-n-hexyl-glucosinolate	314.2	68.9	13.4	NA	NA	NA	NA	40.9	83.8	108.9	NA	13.7	116.1	8.8	150.1	99.7	37.1	NA
7-Methylthio-n-heptyl-glucosinolate	4142.1	2022.6	58.9	45.0	1.2	10.1	117.6	633.3	943.1	2901.6	73.7	336.8	2045.2	450.4	2751.4	2383.8	543.1	NA

8-Methylthio-n-octyl-glucosinolate	688.5	673.5	1.6	68.3	4.8	77.5	29.3	155.9	124.5	4179.1	39.6	75.2	394.9	1052.3	901.4	335.2	453.6	NA
3-Hydroxy-n-propyl-glucosinolate	NA	0.4	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	0.5	NA	NA	NA	NA	NA	NA	NA	0.7	NA	NA	NA	NA
3-Benzoyloxy-n-propyl-glucosinolate	NA	NA	NA	NA	1.1	NA	NA	NA	NA	2.2	NA	NA	NA	NA	NA	19.3	NA	NA
4-Benzoyloxy-n-butyl-glucosinolate	NA	NA	NA	0.6	NA	NA	NA	6.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Indol-3-ylmethyl-glucosinolate	1.2	NA	NA	NA	NA	0.3	NA	NA	NA	6.7	NA	NA	NA	NA	0.9	NA	NA	NA
1-Methoxyindole-glucosinolate	NA	NA	NA	NA	NA	NA	NA	1.6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4-Methoxyindole-glucosinolate	13.5	NA	NA	17.5	355.4	57.1	NA	NA	NA	20.2	NA	NA	5.5	145.9	358.5	2.0	12.1	NA

*, Used as internal standards; NA, not found

Chapter 2-2

Leaf trichomes affect the feeding preference of a leaf beetle *Phaedon brassicae*

Abstract

We examined the effects of leaf trichomes (plant hairs) on the preference and performance of a crucifer-feeding pest, *Phaedon brassicae* Baly (Coleoptera: Chrysomelidae), which is distributed throughout East Asia. In this study, we used two hairless mutants as well as a major ecotype of *Arabidopsis thaliana* (*gl1-1*, *gl1-2*, and Col-0, respectively) to control genetically for trichome production. Our binary choice experiments showed that adult *P. brassicae* fed less on hairy leaves than glabrous ones. These results were consistent between the two independent mutants of the trichome-related gene, providing firm evidence for causality between trichome production and the feeding preference of adult beetles. Adult beetles fed less on hairy leaves than glabrous leaves under no-choice conditions, though this difference was not statistically significant. Additionally, larvae fed on hairy leaves tended to grow less compared to those fed on *gl1-1* or *gl1-2* (glabrous leaves), though this was also not statistically significant. Overall, this study illustrates a resistance function of leaf trichomes to *P. brassicae*, especially regarding the feeding preference of adult beetles.

Introduction

Mechanical structures of plants are a major resistance trait against herbivory (Hanley *et al.* 2007). For example, plant trichomes (epidermal hairs) are considered a physical resistance trait (Levin 1973; Hanley *et al.* 2007) that can alter the performance or preference of herbivores (Yencho and Tingey 1994; Zvereva *et al.* 1998; Handley *et al.* 2005). Field evidence accumulated thus far has indicated that trichome density was negatively correlated with leaf damage in both natural (e.g., Mauricio 1998) and cultivated plants (e.g., Chu *et al.* 2001). However, one issue underlying the correlative evidence is that resistance traits other than trichomes are also correlated with reduced herbivory in some cases. For instance, in natural populations of *Arabidopsis thaliana*, not only trichomes but also glucosinolate contents were genetically correlated with reduced leaf damage (Mauricio 1998). The causality between trichomes and herbivory may be tested by shaving plant hairs (Yencho and Tingey 1994; Zvereva *et al.* 1998), but this approach raises another concern: injured leaf surfaces may emit volatile chemicals that attract herbivores (Visser 1986; Bruce *et al.* 2005). In this respect, mutant accessions of a model plant species provide an excellent opportunity to control genetically for a focal resistance trait (Züst *et al.* 2011).

Several species of the genus *Phaedon* [Coleoptera: Chrysomelidae] are known as major pests that infest cruciferous vegetables (Hamnett 1944; Ôtake and Funaki 1958). Of these species, *P. brassicae* Baly is distributed throughout East Asia and feed on cultivars (Ôtake and Funaki 1958; Wang *et al.* 2007a, b, c) and wild species of Brassicaceae (Yano and Ohsaki 1993). This species usually reaches the adult stage within three weeks after hatching; adults survive for approximately two months under laboratory conditions with various temperature and photoperiod ranges (Wang *et al.* 2007a, c). The longevity and rapid life cycle of *P. brassicae* can lead to severe damage to cruciferous plants. Adults of *P. brassicae* are flightless and access host plants exclusively by walking. Larvae of *P. brassicae* are far less mobile; larvae appear on the host plants on which adults feed (Ôtake and Funaki 1958). Thus, for predicting the loss of plant productivity caused by *P. brassicae*, it is important to elucidate plant traits that affect adult host choice and consequent larval growth.

In this study, we examined the feeding preference and performance of *P. brassicae* for trichome-producing (hairy) and trichomeless (glabrous) leaves of a model plant, *Arabidopsis thaliana* [Brassicaceae], in order to characterize the resistance functions of trichomes. As a source of hairless plants, we focused on two independent mutants of a major trichome-related gene *GL1* (see Grebe 2012 for a review of trichome developments). First, we conducted choice experiments using adults of *P. brassicae* to test their feeding preference for hairy and glabrous leaves. Additionally, we performed no-choice experiments to assess the feeding efficiency of adult beetles with the two types of leaves. Then, larvae of *P. brassicae* were grown on hairy or glabrous leaves to quantify their performance.

Materials and Methods

Insects

We used a laboratory-reared population of *Phaedon brassicae* that had been established in our previous work (Sato *et al*. 2014), derived from adults collected in a natural population of *A. halleri* subsp. *gemmifera* (Taka-cho, Hyogo Prefecture, Japan: 35°06′N, 134°56′E) and maintained on Chinese cabbage under 12L:12D with a 20 °C constant air temperature in an environmental chamber. We added ca. 30 adults from the natural to the laboratory population every year to maintain genetic variation. Because eggs of *P. brassicae* are embedded in plant tissue by their adults, it is difficult to distinguish oviposition from injuries on plants. Thus, in this study we evaluated the preference of *P. brassicae* by adult feeding. Adults and last-instar larvae grow to ca. 4–8 mm in body length. Because adult beetles do not grow in size after emerging from pupae, the weight of larvae in the early developmental stage was used as an indicator of the herbivore performance in this study.

Plants

Seeds of our experimental plants were derived from The Arabidopsis Information Resource (TAIR: http://www.arabidopsis.org/). To control for the trichome phenotype, we used two independent mutants of a trichome-related gene, *GL1* (*gl1-1*, stock no. = CS1644, mutagen = spontaneous; *gl1-2*, CS3126, mutagen = X-rays), and their wild type, Col-0 (CS3176). The *gl1-1* completely lose trichomes while the *gl1-2* produce trichomes sparsely around the leaf margin (Yoshida *et al*. 2009). The number of trichomes was 102 ± 11, zero, and 12 ± 4 per leaf for Col-0, *gl1-1*, and *gl1-2* in our samples, respectively (Mean ± SE, n = 10 counted under a 8× magnified glass). Seeds of three accessions were sown separately on a 6 × 6 block plastic tray (4.5 cm^3 for each block) filled with vermiculite. Water was supplied every three or four days. Liquid fertilizer was 1000-fold diluted and supplied twice during the study (N:P:K = 6:10:5; Hyponex, Hyponex Japan, Osaka, Japan). Seedlings were harvested to keep the density moderate (one plant per block). Plants were grown under 12L:12D with a 20 °C constant air temperature. Photosynthetically active radiations ranged from 40 to 60 μmol/m^2/s at pot surface levels (LI-190 Quantum Sensor, LI-COR, Lincoln, Nebraska, USA), in which the red to far-red ratio was 2.1–2.4.

Feeding preference for hairy and glabrous leaves

We conducted binary choice and no-choice experiments to assess the feeding preference and efficiency, respectively, of adult beetles. Feeding arenas were constructed using Petri dish (6 cm diameter, 1.5 cm depth, Kord-Volmark Co., Brampton, Ontario, Canada) seated with a moisten filter paper (5.5 cm diameter, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). Rosette leaves (1.0–1.5 cm in length of leaf area) were harvested from plants grown for 1.5 months after sowing. For the choice experiments, a pair of hairy (Col-0) and glabrous (either *gl1-1* or *gl1-2*) leaves was set on each arena. For the no-choice experiments, two hairy leaves or two glabrous leaves were set on each arena. Fifteen replicates were made for the both choice and no-choice experiments. Adult beetles were chosen randomly from the laboratory population and starved overnight. Then, one beetle was released on each Petri dish and allowed to feed on the leaves for 12 hours. The experiments were conducted under the same condition as we cultivated the plants. Leaf remains were scanned into a digital image after the experiment. We quantified leaf area loss (cm^2) using a 1 mm^2 grid paper and Image J software (Abramoff *et al*. 2004). For no-choice conditions, the area loss of the two leaves was averaged within each arena. To compare the leaf area loss between hairy and glabrous leaves, Wilcoxon signed-rank tests or Mann-Whitney *U*-tests were used for the choice or no-choice conditions, respectively. *P*-values were adjusted using sequential Bonferroni correction to control for the risk of Type I error. Leaf pairs that had no infestation were excluded from analysis (see Fig. 2.5 for the final sample size). All statistical analyses in this study were performed using R version 3.0.1 (R Core Team 2013).

Larval performance on hairy and glabrous leaves

We examined growth of larvae fed on hairy or glabrous leaves to assess their performance. One leaf of Col, *gl1-1*, or *gl1-2* was set on a Petri dish (6 cm diameter, 1.5 cm depth) seated with a moisten filter paper (5.5 cm diameter). Eighteen replicates were made for each plant accession. One larva (1.0–1.5 mm in body length) was randomly chosen from the laboratory population and released on the leaf. Larvae were allowed to feed on the leaves for a week, with one leaf supplied every two days (i.e., D0, D2, D4, and D6). The weight of each larva was measured three times to the nearest 0.01 mg using an electronic scale (AEL-40SM, Shimadzu, Tokyo, Japan), and averaged among the three measurements. Larvae that died during the experiment were recorded as zero mg. Mann-Whitney *U*-tests and sequential Bonferroni correction were used to compare the weight of larvae between hairy and glabrous leaves.

Results and Discussion

Our choice experiments showed that adult *P. brassicae* preferred to feed on hairy rather than glabrous leaves (Wilcoxon signed-rank tests with sequential Bonferroni correction, Col vs. *gl1-1*, $V = 9$, $n = 11$ pairs, $P < 0.05$; Col vs. *gl1-2*, $V = 10$, $n = 13$ pairs, $P < 0.05$, respectively: Fig. 2.5a). Although *gl1-2* had sparse trichomes (ca. 10 per leaf in our samples), the feeding preference for glabrous leaves was observed consistently in both mutants *gl1-1* and *gl1-2*. These results from two independent mutants thus provide firm evidence for causality between trichome production and the feeding preference of adult beetles.

In contrast, the no-choice experiments did not detect any significant differences in leaf area loss between hairy and glabrous leaves (Mann-Whitney *U*-tests with sequential Bonferroni correction, Col vs. *gl1-1*, $U = 59$, $n_{\mathrm{Col}} = 14$, $n_{\mathrm{gl1\text{-}1}} = 12$, $P = 0.21$; Col vs. *gl1-2*, $U = 65$, $n_{\mathrm{Col}} = 14$, $n_{\mathrm{gl1\text{-}2}} = 15$, $P = 0.17$: Fig. 2.5b). Although the area lost on hairy leaves was less than on glabrous ones (Fig. 2.5b), the damage on hairy leaves was still larger than choice conditions (Fig. 2.5a). Therefore, while trichomes may play a certain role in decreasing feeding efficiency, they likely have a stronger impact on the feeding preference of adult beetles.

As for larval performance, we did not find any significant difference between larvae fed on hairy and glabrous leaves (Col vs. *gl1-1*, $U = 129$, $n_{\mathrm{Col}} = n_{\mathrm{gl1\text{-}1}} = 18$, $P = 0.28$; Col vs. *gl1-2*, $U = 117$, $n_{\mathrm{Col}} = n_{\mathrm{gl1\text{-}2}} = 18$, $P = 0.30$: Fig. 2.5c). However, larvae fed on hairy leaves grew less well than those fed on glabrous ones, and there were more dead larvae on hairy than on glabrous leaves in the experiment (10, 5, and 1 larvae on Col, *gl1-1*, and *gl1-2*, respectively). Thus, it is still possible that trichomes negatively affect the growth or mortality of larvae despite a lack of conclusive evidence for interfering effects of trichomes on larvae performance.

A resistance function of trichomes has been reported for interactions between cruciferous plants and *Phaedon* species. For instance, Kühnle and Müller (2012) found that host acceptance of the mustard leaf beetle, *P. cochleariae*, was inhibited on Brassica cultivars producing dense trichomes. Specifically in *P. brassicae*, our previous study revealed a feeding preference of adult beetles for glabrous leaves of *A. halleri*. In addition, Sato *et al*. (2014) found poorer performance of *P. brassicae* larvae that fed on hairy leaves of *A. halleri*. While the present study was unable to detect any significant reduction in performance of larvae fed on hairy leaves, the results from *A. thaliana* mutants could support a resistance function of trichomes counter to the feeding behavior of *Phaedon* species.

In natural populations of *Arabidopsis thaliana*, there is growing evidence for an anti-herbivore resistance function of trichomes (e.g., Mauricio 1998; Handley *et al*. 2005; Sato and Kudoh 2015a). In this respect, *A. thaliana* mutants are useful for testing for causality between a focal trait and insect resistance (Züst *et al*. 2011). We must note, however, that correlated resistance traits may still exist with trichomes. For example, trichomes are known to accumulate heavy metals (Zhao *et al*. 2000) and synthesize glucosinolates (Frerigmann *et al*. 2012). In a relative, *P. cochleariae*, adult beetles also responded to leaf chemicals such as glucosinolates and flavonoids (Kühnle and Müller 2009). Although there is no evidence yet of chemical response in our studied insects, further studies on these candidate factors are needed to understand fully any causal connection between trichome production and resistance to *P.*

brassicae.

In summary, the present study illustrated a resistance function of trichomes against *P. brassicae*, especially regarding the feeding preference of adult beetles. Moreover, the consistency between results of two independent mutants strongly supports the notion of a pivotal role for leaf trichomes in altering the preference of beetles. Although we did not detect interfering effects on herbivore growth, larval growth tended to be lower on hairy leaves, so trichomes may indeed have a negative impact on both performance and preference of *P. brassicae*. Our results could offer mechanistic insights into *Phaedon*-Brassicaceae interactions in both natural and managed ecosystems.

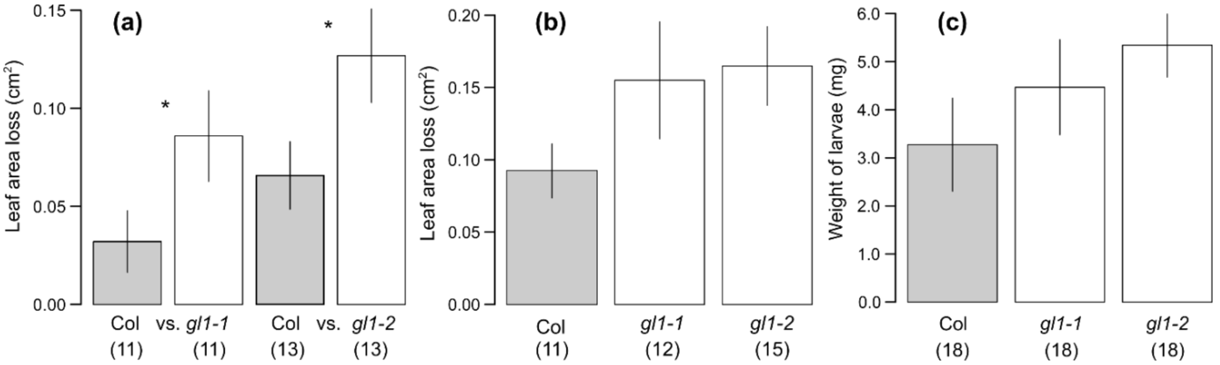


Figure 2.5 Preference and performance of *Phaedon brassicae* on hairy (gray) and glabrous (white) leaves of *Arabidopsis thaliana*. Leaf areas eaten by adults under choice (a) and no-choice (b) conditions and weight of larvae (c) are shown for the wild type (Col) and two hairless mutants (*gl1-1* and *gl1-2*). Bars indicate standard errors of means, while asterisks highlight significant differences at $P < 0.05$; sample sizes are shown within parentheses.

Chapter 3
Associational effects mediate a minority advantage in defense and growth between hairy and glabrous plants

Abstract

Based on the accumulation of evidence, the risk of herbivory depends not only on the traits of a plant but also on those of neighboring plants. Despite the potential importance of frequency-dependent interactions in the evolutionary stability of anti-herbivore defense, we know little about such associational effects between defended and undefended plants within a species. In this study, we determined whether the intraspecific associational effects against the oligophagous leaf beetle, *Phaedon brassicae*, caused a minority advantage in defense and growth between trichome-producing (hairy) and trichomeless (glabrous) plants of *Arabidopsis halleri* subsp. *gemmifera*. We experimentally demonstrated that the magnitude of herbivory and the number of adult beetles on hairy plants decreased when hairy plants were a minority, whereas the leaf damage and the beetle abundance did not differ between hairy and glabrous plants when glabrous plants were a minority. By contrast, the larvae of *P. brassicae* occurred less when hairy plants were a majority. We also found a reciprocal minority advantage in the biomass production for both hairy and glabrous plants. Additionally, the adults tended to attack glabrous leaves more rapidly than hairy ones, particularly when the beetles were starved or experienced glabrous diets. Furthermore, in the absence of herbivory, the growth of hairy plants tended to be worse than glabrous plants, which indicated a cost for the production of trichomes. Our study suggests that associational effects are a mechanism for the maintenance of trichome dimorphism by contributing to negative frequency-dependent growth.

Introduction

As sessile organisms, plants are exposed to threats of resource loss by herbivory. To mitigate the negative effects of losses from herbivory, many plant species develop defensive traits (e.g., toxins and spines) against herbivores. Based on resource allocation theory of plant defense, the assumption has long been that the magnitude of herbivory on an individual plant depends on the investment of that plant in defensive traits (Rhoades 1979; Simms and Fritz 1992; Stamp 2003). However, the accumulation of evidence now indicates that herbivory risk depends not only on the traits of an individual plant but also on those of neighboring plants (Agrawal *et al*. 2006; Barbosa *et al*. 2009). Some researchers have noted the crucial role of such 'associational effects' in determining the coexistence of multiple plant types because the associational effects may mediate the local frequency or the density dependence of herbivory (Underwood *et al*. 2014; Kim and Underwood 2015). Although several terminologies have been proposed for associational effects (reviewed by Barbosa *et al*. 2009; Rautio *et al*. 2012; Underwood *et al*. 2014), we followed Underwood *et al*.'s (2014) definition which is "Associational effects occur when consumer effects on individuals of one resource organism type, at a given density of that type, are a function of the neighborhood composition of other resource types at particular spatial scales."

Although associational effects are investigated as a type of interspecific interaction (e.g., Root 1973; White and Whitham 2000; Callaway *et al*. 2005), the effects can also be applied to anti-herbivore defense polymorphism within a single species (Tuomi *et al*. 1999; Rautio *et al*. 2012). Based on evolutionary game theory, several authors discuss how defended and undefended plants coexist under associational interactions (Sabelis and de Jong 1988; Till-Bottraud and Gouyon 1992; Tuomi *et al*. 1999). One possible explanation is negative frequency-dependent selection in which associational effects may allow multiple plant types to persist within a population when herbivory decreases plant fitness and rare types can escape from herbivory (Tuomi *et al*. 1999). As a type of associational effect, Bergvall *et al*. (2006) introduced the term 'neighbor contrast defense and susceptibility' to denote, respectively, the minority advantage or disadvantage in herbivory on a given plant type. Additionally, these preceding studies suggest that herbivore perception on contrasting diets is a pivotal mechanism for that type of associational effect (Bergvall *et al*. 2006; Rautio *et al*. 2012). However, empirically, little is known about whether and how associational effects mediate frequency dependence in defense and growth for defended and undefended plants within a species (Wise *et al*. 2009).

From previous research on anti-herbivore defense, defensive traits often incur a cost on the growth and/or reproduction of a plant (Elle *et al*. 1999; Koricheva 2002; Züst *et al*. 2011), and optimal defense theory predicts a single defense strategy balanced between defense and growth (Simms and Fritz 1992; Stamp 2003). In contrast to this prediction, natural plant populations exhibit genetic polymorphisms in both chemical (Hughes 1991; Windsor *et al*. 2005) and physical defenses (Kivimäki *et al*. 2007; Wise *et al*. 2009). Generally, in prey-predator interactions, (1) some fraction of prey abandon defensive traits to avoid the cost, and (2) predation risks for defended and undefended prey sometimes depends on the composition of the neighborhood prey (Brower *et al*. 1970; Speed and Ruxton 2005; Svennungsen and Holen 2007). At least theoretically, a stable dimorphism of defended and undefended prey can evolve under predator-mediated interactions (Svennungsen and Holen 2007). Thus, associational effects may provide insights into why anti-herbivore defense polymorphism is maintained under plant-herbivore interactions.

Trichomes (plant epidermal hairs) are a physical defense trait with distinct phenotypes that are governed by a few primary genes (Kivimäki *et al*. 2007; Grebe 2012). Plant trichomes are a mechanical barrier against feeding by adult and larval insects and against ovipositing by adults (e.g., Zvereva *et al*. 1998; Chu *et al*. 2001; Sletvold *et al*. 2010; Yamawo *et al*. 2012). Trichomes also function in the tolerance to abiotic stresses (Wagner *et al*. 2004). Moreover, trichomes impose a cost on plant growth and/or reproduction (Sletvold *et al*. 2010; Kawagoe *et al*. 2011; Züst *et al*. 2011). To test intraspecific associational effects and their frequency dependence, we focused on trichome production as a representative trait of plant defense that exhibited genetic polymorphism.

Arabidopsis halleri (L.) O'Kane & Al-Shehbaz subsp. *gemmifera* (Matsum.) O'Kane & Al-Shehbaz [Brassicaceae/ Cruciferae: referred to as *A. halleri*, hereafter] possesses a genetic dimorphism of trichome-producing (hairy) and trichomeless (glabrous) morphs (Kawagoe *et al*. 2011). For this subspecies, Kawagoe *et al*. (2011) determined the reproductive cost for the production of trichomes. In an evaluation of the anti-herbivore resistance conferred by trichomes, Sato *et al*. (2014) reports that an oligophagous beetle *Phaedon brassicae* Baly [Coleoptera: Chrysomelidae] preferred to feed on glabrous leaves and avoided hairy leaves only when this leaf type was rare. However, Sato *et al*. (2014) used leaf-discs in the feeding experiment, and whether associational effects can be detected at the whole-plant level is unknown.

The purpose of this study was to test whether the associational effects resulted in a minority advantage in the defense and the growth for the two plant morphs. We experimentally tested intraspecific associational effects in anti-herbivore defense against a leaf beetle on *A. halleri* in which leaf damage, herbivore abundance, and plant biomass were evaluated under contrasting neighborhood conditions of hairy and glabrous plants. We also performed two additional experiments. First, to examine a possible cause of the associational effects, we observed the biting behaviors of the leaf beetles on hairy and glabrous leaves because biting is a useful measure of host preference in *Phaedon* species (Kühnle and Müller 2012). In this additional experiment, we preconditioned adult beetles on the two leaf types with the aim to simulate beetles with experience feeding on hairy-abundant and glabrous-abundant plants. Second, hairy and glabrous plants were cultivated without herbivory to ascertain the growth and/or reproductive cost of trichome production. Because the cost of trichome production can differ depending on plant ontogeny in *Arabidopsis* (Züst *et al*. 2011), we measured the growth and reproduction at multiple points in time.

Materials & Methods

Study system

Arabidopsis halleri is a self-incompatible perennial herb that is distributed across Japan and the Russian Far East. In the lowlands of western Japan, plants begin flowering in late March or early April. After flowering, plants develop leaves and roots on the primary and axillary meristems of the flowering stems, and these new rosettes establish as clonal offspring by rooting into the ground. The presence/absence of trichomes is associated with the allelic status of a trichome-related gene, *GL1*, but not with its flanking regions or other genes (Kawagoe *et al*. 2011). Hairy plants develop nonglandular trichomes on the surfaces of leaves and stems, whereas glabrous plants have no trichomes except for on the leaf margins. From a natural population that was previously studied (located in central Japan, 35°06′ N, 134°56′ E, ca. 200 m in altitude; Kawagoe *et al*. 2011; Sato *et al*. 2014), the seeds of *A. halleri* were collected. The glucosinolate profiles were not associated with the trichome phenotype in the young leaves collected from our study population (Sato *et al*. 2014).

Phaedon brassicae is a pest of cruciferous vegetables (Wang *et al*. 2007a, b). Adults of *P. brassicae* are flightless and access host plants exclusively by walking. The larvae are far less mobile than the adults (Ôtake and Funaki 1958), and therefore, the host plant on which a larva feeds is determined by adult choice. This species of leaf beetle typically reaches the adult stage within 3 weeks after hatching, and adults survive for approximately 2 months under laboratory conditions across various ranges of temperature and photoperiod (Wang *et al*. 2007a, b, c). The adults and the last-instar larvae are ca. 4-8 mm in body length. We used a laboratory-reared population that was previously established and maintained the population at a temperature of 20˚C and a photoperiod of 12:12 h L:D (Sato *et al*. 2014). The sex of the beetles was determined based on the body size (males, 0.5 mm smaller in abdomen height and width than females). To equalize the likelihood of oviposition, we used the sex ratio of 50:50 within each treatment in all of the experiments described below.

Greenhouse test of minority advantage in defense and growth

We conducted a greenhouse experiment that used two contrasting neighborhoods of hairy and glabrous plants to test for the intraspecific associational effects (hairy plants:glabrous plants = 3:1 and 1:3 individuals; referred to hereafter, as hairy-abundant and glabrous-abundant conditions, respectively). These two contrasting neighborhood conditions were adopted because the feeding responses of *P. brassica*e did not differ between the glabrous-abundant condition and the condition with even proportions of the two morphs (i.e., hairy leaves:glabrous leaves = 2:2; Sato *et al*. 2014).

The mature fruits were harvested from 14 maternal plants (seven hairy and seven glabrous plants) in early July 2013 at our study site; the maternal plants were separated by at least 5 m to minimize the likelihood of multiple sampling of a single clone. Hundreds of seeds from each maternal plant were separately sown on Petri dishes (diameter 9 cm, depth 1.5 cm; Kord-Valmark Co., Ontario, Canada) filled with quartz sand. The seeds were germinated under 12:12 h L:D conditions with the respective air temperatures of 25°C and 15°C; in these conditions, > 80% of the seeds germinated successfully. We transplanted three seedlings that each had 2-4 leaves into a plastic pot (diameter 9.0 cm, depth 8.0 cm, Y-pot; SAKATA SEED CO., Yokohama, Japan) filled with seedling-raising compost (Cat. No. 776-274-01; TAKII & CO., Ltd., Kyoto, Japan). The compost contained nutrients (N:P:K = 320:210:300 mg/L, respectively). The plants were grown under 12:12 h L:D with a constant air temperature of 20°C. Water was supplied every 3-4 days during the experiment. The two smallest plants were harvested from each pot after three months of cultivation to avoid competition within the pots. The photosynthetically active radiation ranged from 40 to 60 μmol/m^2/s on the surface of the pots (LI-190 Quantum Sensor; LI-COR, Lincoln, NE, USA) for which the red:far-red ratio was 2.1–2.4.

The experiment began 3.5 months after the seedling transplantations: 120 plants were transferred to 30 nylon nets (23 cm diameter by 33 cm height, supported by a 30-cm long wood pole, with 75% light-transmittance), with four individuals per net. Fifteen replicates were established for each neighborhood condition (4 individuals × 2 neighborhood conditions × 15 replicates = 120 plants in total). The pots were adjoined to allow leaf beetle movement between plants without the necessity of first climbing down to the ground. The four individual plants within each net were arbitrarily chosen from different maternal plants to minimize the frequency of bias of the maternal family within a replicate. The smallest plants (length of largest leaf < 80 mm) were not selected to control for the effects of initial size on the magnitude of herbivory. The adult beetles were allowed to feed on the *A. halleri* individuals under the two neighborhood conditions, with two males and two females randomly chosen from the laboratory-reared population released per net after being starved for a day.

We scored the damage level to each leaf as 0 (intact), 1 (ca. 0% < area loss ≤ 25%), 2 (25% < loss ≤ 50%), 3 (50% < loss ≤ 75%), 4 (75% < loss < 100%), or 5 (100% loss or only a petiole remained) at 1, 2, 3, and 4 weeks after the release of the beetles. The damage levels were evaluated separately for mature and young leaves because these leaf types differed in the density of trichomes (mean ± SE = 2.3 ± 0.5 and 16.0 ± 1.4 trichomes/0.25 cm^2 for mature and young leaves, respectively; Wilcoxon signed rank tests, $V = 0$, $n = 9$ plants, $P < 0.01$) and in the leaf thickness (specific leaf area, mean ± SE = 252 ± 18 and 380 ± 39 cm^2/g for mature and young leaves, respectively; $V = 55$, $n = 16$ plants, $P < 0.01$). For the growth stages, the radial leaves developed first at the core of the rosette toward the vertical axis and then expanded outward toward the horizontal axis. Thus, we used the angle of the leaf petiole as an indicator of leaf age: the mature leaves were classified as leaves with a petiole angle < 45°, whereas the young leaves were those with angles ≥ 45°. The larval and adult leaf beetles on each plant were counted every 3-4 days. Because the eggs of *P. brassicae* are embedded in plant tissue by adults, it was difficult to distinguish oviposition in plants from injuries; thus, the newly hatched larvae were retrieved after each count to evaluate oviposition by adult females in this early period of the greenhouse experiment. For the larvae, we also recorded the leaf type on which the larvae were found (i.e., young or mature leaf) to evaluate which type of leaf was chosen for oviposition by the females. To analyze both the intensity and the duration of the cumulative effects of herbivory, all measurements were repeated until a month after the release of adult beetles.

To examine the effects of trichome phenotype and neighborhood condition on plant growth, we measured the plant biomass (including the aboveground vegetative organs and the primary root) of the experimental plants at the conclusion. All plants were harvested 2 months after the release of the adult beetles. The samples were oven-dried overnight (50˚C, DRK633DB; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). In this later period of the greenhouse experiment, to include the effects of larval feeding on plant growth, we did not remove the larvae after the last measurement of leaf damage that was referred to above. The dried plants were weighed to a precision of 10^{-4} g using an electronic analytical scale (GR-60; A&D Company, Ltd., Tokyo, Japan). We recorded the weight of a dead plant as zero (1 of the 120 plants). The flower production was not evaluated because the plants did not produce flowers at this stage. No plants and only one beetle died during the first month after the release of the beetles.

Additionally, in the greenhouse experiment, we determined whether the trichome density of hairy plants was altered by wounding. We cut 50% of the leaf area from 16 hairy plants that were not used in the aforementioned experiment. A month after the damage, we counted the trichomes on newly expanded leaves under an 8× magnifying glass. However, the trichome density of the hairy plants did not increase (intact plant, mean ± SE = 87.8 ± 14.2 trichomes/cm^2, $n = 8$; damaged plant, 81.5 ± 10.6 trichomes/cm^2, $n = 8$; Mann-Whitney *U*-test, $U = 34$, $P = 1$).

Feeding preference of adult beetles on hairy and glabrous leaves

We observed the feeding preference of adult beetles on hairy and glabrous leaves as a complement to the greenhouse experiment. In this observation, we also tested whether short-term experience with abundant hairy and glabrous diets could enhance/inhibit the host preference by beetles. Young leaves (1.5-2.0 cm in length) were used that were randomly harvested from plants grown under the identical conditions described above. Forty-five male and female adults of *P. brassicae* were randomly selected from the laboratory-reared population. We placed thirty beetles in each of three transparent plastic cases (diameter 8.0 cm, depth 4.5 cm), and these beetles were preconditioned with hairy or glabrous leaves or were starved. Ten young leaves of either hairy or glabrous plants were supplied to the pre-hairy and pre-glabrous conditions, respectively. After the preconditioning, each beetle fed on either a hairy or glabrous leaf within a Petri dish (diameter 6 cm, depth 1.5 cm; Kord-Valmark Co., Ontario, Canada) with a moistened filter paper (diameter 5.5 cm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). We recorded the feeding marks on the leaves every thirty minutes for six hours after the start of the observations. The presence/absence of feeding marks was then checked a day after the beginning of the observations. Twenty-four male and female beetles randomly chosen from each preconditioning treatment were used per observation (8 beetles × 2 leaf types × 3 preconditions). This procedure was repeated three times (i.e., 144 beetles were examined in total). The observations were conducted under the identical conditions used to cultivate the experimental plants, as described above.

Performance of hairy and glabrous plants without herbivory

We cultivated hairy and glabrous plants without herbivory to compare the growth and reproductive potentials. The mature fruits were collected from 14 maternal plants (seven hairy and seven glabrous plants) from our study population in early July 2011. The maternal plants were separated from each other by at least 5 m. We prepared 5 to11 plants from each maternal family in an indoor space without herbivory. Fifty seeds from each maternal family were sown on a Petri dish (diameter 9 cm, depth 1.5 cm) filled with moistened quartz sand on August 15, 2011, to germinate at room temperature (ca. 80% of the seeds germinated). Three seedlings from the identical family were transplanted to the identical pot on November 25, 2011. The plastic pots (diameter 10.5 cm, depth 9 cm) were filled with mixed soil (pumice:leaf mold:peat moss = 1:1:1). All seedlings had 2 to 4 leaves when transplanted. These plants were grown inside a transparent plastic case (75 cm × 45 cm with a depth of 18 cm; 28 pots per case) for four months with natural sunlight (10–12 h in day length) at room temperature (average 10–30°C per day). The locations of the cases were rotated monthly. Liquid fertilizer (N:P:K ratio = 6:10:5, Hyponex; Hyponex, Osaka, Japan) was diluted 1000-fold and supplied monthly. The photosynthetically active radiation was 800-1100 and

100-300 μmol/m^2/s on sunny and cloudy days, respectively. The red:far-red ratio ranged from 1.0 to 1.3 (LI-190 Quantum Sensor; LI-COR, Lincoln, NE, USA).

The largest plant of the three in each pot was identified with a wooden pin 1 month after the transplant, and the marked plants were measured. We recorded the length of the largest leaf and the numbers of leaves and flowers to assess plant growth and reproduction. The number of leaves and the leaf lengths were recorded ca. 1, 4, and 6 months after the transplant (corresponding to December 30, 2011, March 2, 2012, and May 3, 2012, respectively). Of the three plants in each pot, the smallest two (i.e., the unmarked plants) were removed 4 months after the transplant to avoid competition within the pots. The flowers were counted ca. 6 and 7 months after the transplant (corresponding to May 3, and May 26, 2012, respectively). The fruits were not evaluated because the plants did not produce any fruits because of self-incompatibility. All plants except for two individuals survived to the end of the study. The trichome phenotype was identified 6 months after the transplants ($n_{\mathrm{hairy}} = 68$ and $n_{\mathrm{glabrous}} = 42$) because hairs on the stem identified the phenotype more easily. To follow the individual plants without destructive sampling, we estimated plant biomass using plant allometry based on measurements at ca. 4 months after transplant for this growth condition. We used the length of the largest leaf (cm) × ln[no. of leaves] to estimate ln[biomass (mg)] because this metric explained a large proportion of the variation in biomass (linear regression, $Y = 0.13X + 2.48$, $R^2 = 0.80$; see Figure S3.1 in Supporting Information).

Statistical analyses

We used cumulative link mixed models (Agresti 2002) to analyze the effects of the trichome phenotype, the neighborhood condition, and the initial plant size on the leaf damage score. The cumulative model is used to fit ordinal response data (Agresti 2002), and therefore, the model was applied to the ordinal response of the leaf damage score. We treated the trichome phenotype, neighborhood condition, and initial plant size as fixed effects. Additionally, we included an interaction between the trichome phenotype and the neighborhood condition in the model as a fixed effect to test whether the trichome phenotype differentially influenced leaf damage depending on the neighborhood conditions of the two plant types. The length of the largest radial leaf represented the initial plant size, which was an approximation of the radius of the rosette. We included two clustering variables as random effects. First, the maternal plant ID was incorporated to consider potential heritable variation among the maternal families. Second, we considered the individual plant ID among repeated measures as another random effect. The significance of the fixed effects was analyzed using likelihood ratio tests in which we first compared the models with or without an interaction term and then tested each fixed effect using the models without interaction terms. The coefficients of fixed effects were also examined when performing the likelihood ratio tests.

These approaches of cumulative model analyses were performed separately for the young and the mature leaves and for the pooled data on the two leaf types to examine whether the resulting herbivory patterns were specific to the leaf types. Although the damage scores of individual leaves were used for the statistical analyses, we calculated the proportion of leaf area loss of an entire plant by averaging the midpoints of the corresponding area loss of the damage score of the leaves (i.e., damage scores of 0, 1, 2, 3, 4, and 5 = 0.0, 0.125, 0.375, 0.625, 0.875, and 1.0, respectively).

We used generalized linear mixed models to analyze the numbers of adult and larval beetles and linear mixed models (LMMs) to analyze plant biomass. We selected a Poisson error structure for the generalized LMMs to analyze the count response (i.e., the numbers of beetles and larvae). According to these cumulative model analyses, likelihood ratio tests were used to analyze the trichome phenotype, neighborhood condition, interaction between the trichome phenotype and the neighborhood condition, and initial plant size as fixed effects. The cumulative number of beetles was analyzed as the response variable to reflect both the intensity and the duration of herbivory on the plants (Ruppel 1983). For the data on larvae, we separately analyzed the numbers of larvae on the young, mature and all leaves on an individual plant. The maternal plant ID was incorporated as a random effect in these analyses. The biomass data were $\ln(x + 0.1)$-transformed to improve normality. Additionally, for the data of the greenhouse experiment, we compared each pair of the two trichome phenotypes and the neighborhood conditions using pairwise likelihood ratio tests. The sample size of the minority phenotypes was only fifteen in our greenhouse experiment; and therefore, the *P*-values are provided without any correction because of the low statistical power (see also Nakagawa 2004 for an explanation of multiple comparisons).

The time until the initial bite was also analyzed using the cumulative model. The response, i.e., time until the initial bite, was an ordinal variable, and therefore, it was converted to scores of 1-14. The scores of 1-12 corresponded to every < 30-minute time interval up to 6 h, and the scores of 13 and 14 indicated the presence and absence of feeding after a day, respectively. The trichome phenotype, precondition, and interaction between these two factors were analyzed as explanatory variables using likelihood ratio tests. We incorporated the experiment ID as a random effect, but the estimated variance was small ($< 10^{-8}$), and several models did not converge. Thus, the random effect was not included in the cumulative model used to explain the timing of bite initiation.

For the data from the indoor cultivation, we used LMMs for the estimated biomass (4 and 6 months after the transplant) and generalized LMMs with a Poisson error structure for the number of flowers (6 and 7 months after the transplant). The estimated biomass was ln-transformed to improve normality. The trichome phenotype and maternal plant ID were analyzed as fixed and random effects, respectively. To incorporate potential size biases in the initial state, we considered the initial biomass (estimated 1 month after the transplant) as a covariate for the analysis of the estimated biomass. For the number of flowers, to distinguish the direct effects of the trichome phenotype on flower production from those mediated by a growth difference, we also analyzed models with or without a fixed effect of the estimated biomass 4 months after the transplant. We tested the significance of each fixed effect using likelihood ratio tests.

All statistical analyses were performed using the R statistical software package version 3.0.0 (R Core Team 2013). We used the clmm function (in the ordinal package: Christensen 2013) for the cumulative link mixed models, the glmer function (in the lme4 package: Bates *et al*. 2014) for the generalized or ordinal LMMs, and the vglm function (in the VGAM package: Yee 2013) for the cumulative models with no random effects.

Results

Minority advantage in defense and growth for hairy and glabrous plants

The magnitude of leaf damage on hairy plants was lower than that on glabrous plants under the glabrous-abundant condition (LR-$\chi^2_1 = 14.5$, $P < 0.001$), whereas the leaf damage was not significantly different between hairy and glabrous plants under the hairy-abundant condition (LR-$\chi^2_1 = 0.6$, $P = 0.44$: Fig. 3.1a). At the end of the experiment, the less abundant type of plant accumulated a larger biomass than that of the abundant type in the identical neighborhood condition (hairy-abundant, LR-$\chi^2_1 = 5.97$, $P < 0.05$; glabrous-abundant, LR-$\chi^2_1 = 3.89$, $P < 0.05$) or in the identical type in the other neighborhood condition (hairy plants, LR-$\chi^2_1 = 4.77$, $P < 0.05$; glabrous plants, LR-$\chi^2_1 = 7.66$, $P < 0.01$: Fig. 3.1b). The result that less abundant hairy plants had less leaf damage compared with the other three treatments was prominent in young leaves (LR-$\chi^2_1 > 9.0$, $P < 0.01$: Table S3.1; Fig. 3.1c). The differential patterns between the neighborhood conditions were confirmed by a significant trichome × neighborhood interaction for the pooled data of damage on young and mature leaves, damage on young leaves, and plant biomass (Table 3.1a). The leaf damage on mature leaves was far less compared with that on young leaves (Fig. 3.1d) in which no significant effects were detected for the trichome phenotype or the neighborhood conditions on the damage (Table 3.1a). These results for the leaf damage on pooled, young and mature leaf types were detected across all points in time (after 1-4 weeks after the release of the beetles: Figure S3.2). The initial plant size did not differ between the trichome phenotypes or the neighborhood conditions (generalized LMM, $P > 0.2$ for the trichome phenotype, neighborhood condition, and the interaction). The details for the coefficients of the intercepts of the cumulative models are in the supporting information (Table S3.2).

Adults occurred less frequently on hairy plants under the glabrous-abundant condition (LR-χ^2_1 = 19.7, $P < 0.0001$), whereas the cumulative number of adults did not differ significantly (< 1 beetle, on average) between hairy and glabrous plants under the hairy-abundant condition (LR-χ^2_1 = 0.93, $P = 0.33$: Fig. 3.1g). The difference in occurrence of adults on hairy and glabrous plants between the neighborhood conditions was confirmed by a significant trichome × neighborhood interaction (Table 3.1b). More larvae occurred on hairy and glabrous plants under the glabrous-abundant condition than those on the identical plant type under the hairy-abundant condition (hairy plants, LR-χ^2_1 = 4.54, $P < 0.05$; glabrous plants, LR-χ^2_1 = 17.2, $P < 0.0001$: Fig. 3.1h), which was a result that was supported by a significant effect of the neighborhood condition on the abundance of larvae (Table 3.1b). The larvae occurred more frequently on the young leaves of glabrous plants under the glabrous-abundant condition than those under the hairy-abundant condition (LR-χ^2_1 > 6.0, $P <$ 0.05: Table S3.1; Fig. 3.1e), and the larvae occurred less often on hairy leaves than glabrous leaves under the glabrous-abundant condition (although the difference was not significant, LR-χ^2_1 = 0.6, $P = 0.7$: Fig. 3.1e). We observed a larger number of larvae on the mature leaves of hairy plants under the glabrous-abundant condition than those on the other three treatments (LR-χ^2_1 > 9.0, $P < 0.01$: Table S3.1; Fig. 3.1f). For the herbivore abundance, no significant effect of initial plant size was detected (Table 3.1b).

Feeding preference of adult beetles on hairy and glabrous leaves

The glabrous leaves were attacked more rapidly than the hairy ones by adult beetles, and a larger number of hairy leaves remained intact than that of glabrous ones (Fig. 3.2). We also detected a significant effect of the trichome phenotype on the timing of bite initiation of adult beetles (cumulative link models, LR-χ^2_1 = 4.13, $P < 0.05$). Neither the main effect of the precondition nor the interaction between the trichome phenotype and the precondition had a significant effect on the timing of the bite initiation (LR-χ^2_2 = 0.48, $P = 0.79$; LR-χ^2_2 = 3.73, P = 0.15, respectively). However, the timing of the bite initiation suggested no difference between hairy and glabrous leaves for the hairy-preconditioned beetles (upper row, Fig. 3.2). To further examine whether the hairy or glabrous preconditioning affected the bite initiation, we performed the identical analysis but excluded the data set of the starved precondition. In this case, the biting initiation of adult beetles was affected by the interaction of the leaf type and the preconditioning at a marginally significant level (LR-χ^2_1 = 3.2, $P = 0.08$). Detailed results for the coefficients of explanatory variables and intercepts are given in the supporting information (Table S3.3).

Performance of hairy and glabrous plants without herbivory

The estimated biomass of hairy plants was smaller than that of the glabrous ones 4 and 6 months after the transplants (Table 3.2; Figs. 3.3a, b). The estimated biomass was not significantly affected by the initial biomass (*P* > 0.1, both for 4 and 6 months: Table 3.2). The hairy plants produced fewer flowers than those of glabrous plants 6 months after the transplant at a marginally significant level (*P* = 0.07: Table 3.2; Fig. 3.3c), although the hairy plants did not have significantly fewer flowers 7 months after the transplant (*P* = 0.63: Table 3.2; Fig. 3.3d). Trichome production had no significant effect on flower production when the initial biomass (i.e., estimated biomass at 4 months after the transplant) was incorporated as a covariate (*P* > 0.05, both for 6 and 7 months: Table 3.2). The initial biomass was positively correlated to flower production 6 and 7 months after the transplant, at least at a marginally significant level (Table 3.2).

Discussion

We demonstrated experimentally that hairy plants incurred less herbivory by *P. brassicae* when they were surrounded by glabrous plants. This pattern has been reported as a type of associational effect; specifically, our example corresponded with the ‘neighbor contrast defense’ (Bergvall *et al*. 2006; Rautio *et al*. 2012) because we observed less herbivory on the defended phenotype (hairy plants, in our case) when surrounded by undefended individuals. We also found a reciprocal minority advantage in biomass production for the hairy and glabrous plants, which might serve as a potential mechanism to maintain the polymorphism through negative frequency-dependent selection.

Mechanisms responsible for the pattern of associational effects

As a possible mechanism for these associational effects, Bergvall *et al*. (2006) suggest that the contrast of food types perceived by herbivores increases their feeding preference for the more palatable food. In *Phaedon* species, the host preference of adult beetles was less for *Brassica* cultivars that produced dense trichomes (Kühnle and Müller 2012). Our observation that adult beetles were inclined to attack glabrous leaves more rapidly than hairy ones also provided support for the negative influence of trichomes on host preference in *P. brassicae*. Furthermore, when the weak difference in adult abundance between the two plant morphs under the hairy-abundant condition was compared, the feeding preference did not differ considerably between hairy and glabrous leaves when adult *P. brassicae* had been preconditioned with hairy diets. Provided with this circumstantial evidence for feeding preference determined by diet experience, the behavioral response of adult beetles indicated that plant damage was likely dependent on the neighborhood conditions for hairy and glabrous plants. The relevance of this mechanism is also apparent because *P. brassicae* are flightless and access their hosts exclusively by walking (Ôtake and Funaki 1958), which forces the beetles to choose plants within a small-scale area. It has been argued that the spillover of herbivores from undefended to defended plants can alter damage on defended neighbors (spillover effects; White and Whitham 2000); however, in our study, spillover was not likely because the adult beetles actively avoided hairy plants.

Trichome density is often high on young leaves (e.g., Chu *et al*. 2001; Yamawo *et al*. 2012), and consequently, leaf age can influence the effectiveness of trichomes as a defense against herbivory (Chu *et al*. 2001; Puentes and Ågren 2013). In our study, the pattern that less abundant hairy plants suffered less herbivory was the most prominent for the young leaves because the young leaves of hairy plants produced denser trichomes than those of mature leaves. Therefore, trichomes might play an important role in defending the younger leaves that contribute to the future growth of *A. halleri*. Although the pattern of larval abundance between hairy and glabrous plants was not clear, the larvae tended to occur more often on the young leaves of glabrous plants under the glabrous-abundant condition. Because the larvae of *P. brassicae* rarely move between plants, the abundance of newly hatched larvae likely reflected the oviposition patterns of adult females. Thus, the tendency for a larger number of larvae to occur on young glabrous leaves led to the presumption that the adult *P. brassicae* preferred to oviposit on glabrous leaves.

At least at the scale of our experiment, we did not detect significant effects of plant size on the number of *P. brassicae* for either the larvae or the adults; thus, we hypothesized that *P. brassicae* responded to the trichome phenotype rather than the resource abundance. However, it was still possible that density as well as frequency of hairy and glabrous plants might result in the pattern of leaf damage, because our experiment with two neighborhood conditions could not determine the relative importance of density- and frequency-dependent damage on the two plant types (but see Kim and Underwood 2015). We should also note that when herbivores respond nonadditively to plant density (i.e., group effects; Rautio *et al*. 2012) it may lead to similar patterns of damage for an individual plant as those caused by associational effects (see also Hambäck *et al*. 2014 for a theoretical consideration). In future studies, the density and the frequency of the two plant types should be manipulated simultaneously (i.e., response surface design; Damgaard 1998; Inouye 2001) to examine potential interactions between the associational and the resource concentration/dilution effects on plant damage (Underwood *et al*. 2014; Hambäck *et al*. 2014).

Maintenance of polymorphism under associational effects

Classically, a reciprocal minority advantage (or negative frequency-dependent selection) leads to the coexistence of multiple species/genotypes because a reciprocal minority advantage can prevent rare types from going extinct (reviewed by Ayala and Campbell 1974). Our study uncovered a reciprocal minority advantage in plant growth between the hairy and glabrous morphs, which might be a mechanism that maintains the dimorphism within populations. The minority advantage could have a number of possible explanations. First, as assumed in earlier game theories related to associational effects (Augner *et al*. 1991; Tuomi *et al*. 1999), the defended plants might be at a disadvantage in growth because of the cost of defense. In the indoor cultivation study, we also found that the growth and reproduction of glabrous plants were slightly but consistently higher than those of hairy plants in the absence of herbivores. The potential costs of trichomes may even out defense superiority of hairy plants when the defense is weakened under the hairy-abundant condition. However, under the glabrous-abundant condition, the escape from herbivory benefited hairy plants. The associational effects and the costs of defense presumably led to the reciprocal minority advantage in biomass for the two morphs, although these two factors are mutually nonexclusive. Second, the larvae became less abundant when hairy plants were a majority, whereby the less abundant glabrous plants might be protected by the abundant hairy plants (i.e., associational resistance) and therefore grow better than hairy ones under the hairy-abundant condition. To distinguish the associational effects and the plant growth potentials, studies must manipulate the presence/absence of herbivores as well as the neighborhood condition.

Hairy and glabrous plants coexist in a natural population in which *P. brassicae* is the primary consumer of *A. halleri* (Kawagoe *et al*. 2011; Sato *et al*. 2014). In this field site of our previous study, we found that leaf damage on hairy plants decreased as the frequency of neighboring glabrous plants within a 1-m patch scale increased. Although it remains unknown how important biomass production is in the lifetime fitness of *A. halleri* in the field, the growth before flowering could be a major component of fitness because the fecundity of hairy plants depends on their size before flowering (Kawagoe and Kudoh 2010). In the indoor cultivation described in the present study, the biomass in the growth stage was also positively correlated with flower production. In addition to sexual reproduction, *A. halleri* produces clonal rosettes after flowering, and this clonal propagation is expected to depend on biomass at the growth stage. There are no trichomes on the flowers of hairy and glabrous plants, and if associational effects influence pollinator attraction, it will occur indirectly through flower production. If plant neighbor affects host-searching abilities and colonization rates of herbivores between patches in the field, then we must test whether associational effects result in negative frequency-dependent selection in a natural population.

Conclusions and implications

Our study suggests that intraspecific associational effects serve as a mechanism for the maintenance of defense dimorphism by contributing to negative frequency-dependent growth between defended and undefended plants. Notably, associational effects may cause frequency-dependent selection not only in plant-herbivore interactions but also in plant-pollinator and prey-predator interactions (Underwood *et al*. 2014). For example, the learning behaviors of pollinators can maintain flower color polymorphism via negative frequency-dependent selection (Gigord *et al*. 2001). As for plant-pathogen interactions, the evidence is suggestive for frequency-dependent infection between resistant and susceptible plants (e.g., Brunet and Mundt 2000; Siemens and Roy 2005). Thus, the findings of this study position associational anti-herbivore defense in the broad context of mutualistic and antagonistic interactions involving plants. Future studies should incorporate such intraspecific associational effects because these effects might explain how genetic polymorphism is maintained under plant-consumer interactions.

Table 3.1 Effects of trichome phenotype, neighborhood condition, and initial plant size on traits related to plant damage and growth (a) and herbivore abundance (b).

(a) Plant damage and growth

Fixed effects	*df*	Damage on pooled leaf types*			End-point biomass		
		Coef. ± SE	LR-χ^2	*P*	Coef. ± SE	LR-χ^2	*P*
Trichome (Hairy)	1	**-0.39 ± 0.11**	**13.09**	**< 0.001**	-0.03 ± 0.08	0.11	0.74
Neighborhood (H > G)	1	0.12 ± 0.10	1.41	0.23	0.032 ± 0.09	0.03	0.86
Initial leaf length	1	0.002 ± 0.003	0.59	0.44	**0.008 ± 0.002**	**12.9**	**< 0.001**
Tri. × Neigh.	1	**0.56 ± 0.20**	**7.67**	**< 0.01**	**-0.55 ± 0.17**	**9.9**	**< 0.01**

Fixed effects	*df*	Damage on young leaves*			Damage on matured leaves*		
		Coef. ± SE	LR-χ^2	*P*	Coef. ± SE	LR-χ^2	*P*
Trichome (Hairy)	1	**-0.52 ± 0.13**	**15.15**	**< 10^{-4}**	0.02 ± 0.32	0.004	0.95
Neighborhood (H > G)	1	0.20 ± 0.13	2.27	0.13	-0.58 ± 0.33	3.15	0.08
Initial leaf length	1	0.003 ± 0.003	0.83	0.36	-0.002 ± 0.008	0.05	0.82
Tri. × Neigh.	1	**0.86 ± 0.24**	**11.93**	**< 0.001**	-0.14 ± 0.65	0.04	0.83

(b) Herbivore abundance

Fixed effects	*df*	Larvae on young leaves			Larvae on matured leaves		
		Coef. ± SE	LR-χ^2	*P*	Coef. ± SE	LR-χ^2	*P*
Trichome (Hairy)	1	0.004 ± 0.123	0.0009	0.98	**0.53 ± 0.18**	**9.07**	**< 0.01**
Neighborhood (H > G)	1	**-0.24 ± 0.12**	**4.43**	**< 0.05**	**-0.95 ± 0.18**	**29.9**	**< 10^{-7}**

Initial leaf length	1	-0.001 ± 0.003	0.16	0.69	-0.001 ± 0.005	0.37	0.85
Tri. × Neigh.	1	0.47 ± 0.24	3.84	0.050	**-1.06 ± 0.34**	**9.31**	**< 0.01**

Fixed effects	*df*	Adults per plant			Larvae per plant		
		Coef. ± SE	LR-χ^2	*P*	Coef. ± SE	LR-χ^2	*P*
Trichome (Hairy)	1	**-0.23 ± 0.11**	**4.00**	**< 0.05**	0.20 ± 0.10	3.72	0.054
Neighborhood (H > G)	1	0.09 ± 0.11	0.59	0.44	**-0.46 ± 0.10**	**22.8**	**< 10^{-5}**
Initial leaf length	1	0.003 ± 0.003	1.25	0.26	-0.002 ± 0.002	0.41	0.52
Tri. × Neigh.	1	**1.05 ± 0.26**	**18.5**	**< 10^{-4}**	-0.10 ± 0.19	0.25	0.62

Notes: Results of mixed models (coefficients and their standard errors) and likelihood ratio (LR-χ^2) are shown for each fixed effect with its degree of freedom (*df*), where bold values indicate significant effects at $P < 0.05$. Coefficients of the neighborhood condition were estimated for the hairy-abundant condition (H > G). We tested the interaction term firstly and then analyzed the main effects of each factor. The initial plant size was represented by the length of the largest leaf. An asterisk (*) indicates response variables analyzed by cumulative link models, whereas no mark indicates those analyzed by generalized or ordinal linear mixed models.

Table 3.2 Effects of trichome phenotype on growth and reproduction of *Arabidopsis halleri* subsp. *gemmifera* cultivated under no herbivory.

Fixed effects	*df*	4 months			6 months						7 months		
		Estimatd biomass			Estimated biomass			No. of flowers			No. of flowers		
		Coef ± SE	LR-χ^2	*P*	Coef ± SE	LR-χ^2	*P*	Coef ± SE	LR-χ^2	*P*	Coef ± SE	LR-χ^2	*P*
Trichome (single)	1	**-0.36 ± 0.09**	**16.3**	**< 0.001**	**-0.25 ± 0.12**	**4.2**	**< 0.05**	-0.13 ± 0.07	3.3	0.07	0.02 ± 0.05	0.23	0.63
Trichome (multiple)	1	**-0.36 ± 0.09**	**16.8**	**< 0.001**	**-0.26 ± 0.12**	**4.4**	**< 0.05**	0.12 ± 0.07	2.7	0.10	0.06 ± 0.05	1.2	0.27
Initial biomass*	1	-0.56 ± 0.56	1.01	0.31	-0.87 ± 0.76	1.32	0.25	**0.71 ± 0.07**	**108**	**$< 10^{-16}$**	0.09 ± 0.05	3.2	0.07

Notes: Results of mixed models (coefficients and their standard errors) and likelihood ratio (LR-χ^2) are shown for each fixed effect with its degree of freedom (*df*). Bold values indicate significant effects at $P < 0.05$ with likelihood ratio tests. The effect of trichome phenotypes was tested with or without the covariate of initial biomass (denoted as single and multiple, respectively). Biomass and flowers were evaluated 4, 6, and 7 months after transplant, where the biomass was estimated on the basis of plant allometry (see Materials and Method for details). Estimated biomass 1 or 4 months after transplant was used as the initial biomass for the analyses of biomass or flowers, respectively.

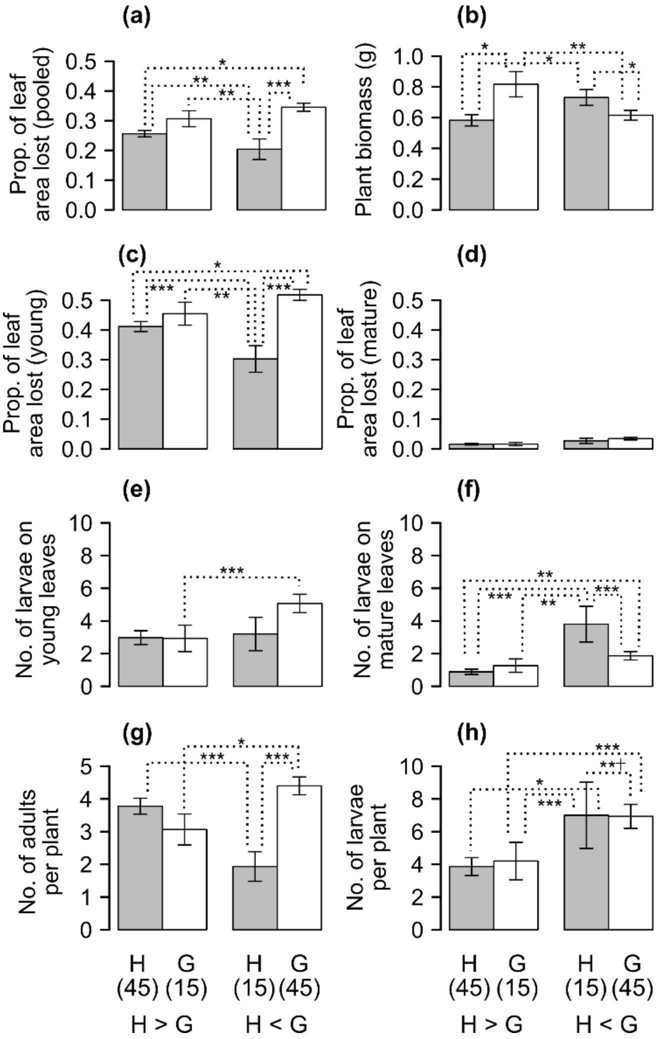


Figure 3.1 Associational effects in defense and growth of hairy (H; grey) and glabrous (G; white) plants under hairy-abundant (H > G) and glabrous-abundant (H < G) conditions. Shown are leaf damage (proportion of leaf area loss) for pooled data of young and mature leaves (a), plant biomass (b), damage for young (c) and mature leaves (d), the cumulative number of larvae on young leaves (e), larvae on mature leaves (f), adult beetles per plant (g), and larvae per plant (h). Leaf damage was presented as values at four weeks after the release of beetles. Error bars indicate SEM of the raw data. Sample sizes are given within parentheses. The number of larvae reflected oviposition by female adults because newly hatched larvae were retrieved after each count. The plant biomass was measured two months after the release of adult beetles. Asterisks indicate significant differences (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) with pairwise likelihood ratio tests by mixed models that incorporated maternal plant ID as a random factor. Dagger mark (†) notifies a significant difference of variance between two groups.

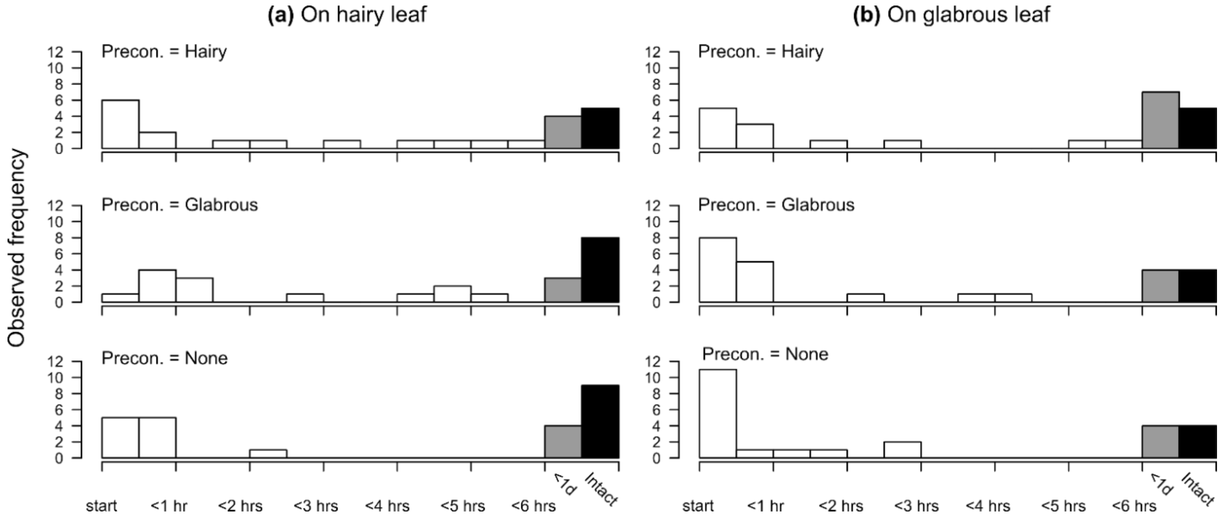


Figure 3.2 Time to initial biting by adult *Phaedon brassicae* on hairy (a) and glabrous (b) leaves. Histograms are shown for overnight preconditioning on hairy (Precon. = Hairy, upper row) and glabrous leaves (Precon. = Glabrous, middle row) and for beetles starved overnight (Precon. = None, lower row). Grey and black bars indicate the presence and absence of feeding marks a day after the release, respectively.

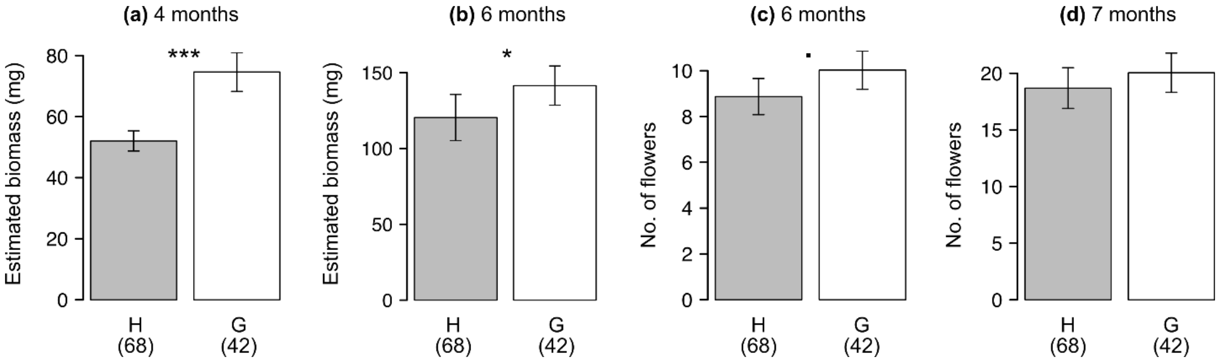


Figure 3.3 Estimated biomass (mg) and the number of flowers of hairy (H; grey) and glabrous (G; white) plants under no herbivory. Figures are shown for plant growth 4 and 6 months after the transplant (a and b) and flowering 6 and 7 months after the transplant (c and d). The biomass was estimated based on plant allometry at ca. 4 months after transplant. Error bars indicate SEM of the raw data. Data are not transformed in these figures. Sample sizes are given within parentheses. Asterisks and dots indicate statistical significances (***, $P < 0.001$; *, $P < 0.05$; $^{\cdot}$, $P < 0.1$).

Supporting information

Table S3.1 Results of pairwise likelihood ratio tests for the greenhouse experiment data including four groups of the trichome phenotypes (H: hairy plants; G: glabrous plants) plants under two neighborhood conditions (H > G: hairy-abundant condition; H < G: glabrous-abundant condition).

Response	Group 1		Group 2		LR-χ^2	*P*
	Tri.	Neigh.	Tri.	Neigh.		
Damage (pooled)	**H**	**H>G**	**H**	**H<G**	**7.69**	**<0.01**
	G	H>G	G	H<G	1.53	0.22
	H	H>G	G	H>G	0.60	0.44
	G	**H<G**	**H**	**H<G**	**14.5**	**<0.001**
	H	**H<G**	**G**	**H>G**	**7.86**	**<0.01**
	G	**H<G**	**H**	**H>G**	**4.86**	**<0.05**
Damage (young)	**H**	**H>G**	**H**	**H<G**	**11.7**	**<0.001**
	G	H>G	G	H<G	2.24	0.13
	H	H>G	G	H>G	0.03	0.86
	G	**H<G**	**H**	**H<G**	**20.9**	**$<10^{-5}$**
	H	**H<G**	**G**	**H>G**	**9.15**	**<0.01**
	G	**H<G**	**H**	**H>G**	**4.52**	**<0.05**
Damage (mature)	H	H>G	H	H<G	1.97	0.16
	G	H>G	G	H<G	1.19	0.28
	H	H>G	G	H>G	0.02	0.90
	G	H<G	H	H<G	0.19	0.66
	H	H<G	G	H>G	1.10	0.29
	G	H<G	H	H>G	2.49	0.11
Plant Biomass	**H**	**H>G**	**H**	**H<G**	**4.77**	**<0.05**
	G	**H>G**	**G**	**H<G**	**7.66**	**<0.01**
	H	**H>G**	**G**	**H>G**	**5.98**	**<0.05**
	G	**H<G**	**H**	**H<G**	**3.89**	**<0.05**
	H	H<G	G	H>G	0.37	0.55
	G	H<G	H	H>G	0.13	0.91

Adult beetles	**H**	**H>G**	**H**	**H<G**	**14.1**	**<0.001**
	G	**H>G**	**G**	**H<G**	**5.42**	**<0.05**
	H	H>G	G	H>G	0.94	0.33
	G	**H<G**	**H**	**H<G**	**19.7**	**$<10^{-5}$**
	H	H<G	G	H>G	3.24	0.07
	G	H<G	H	H>G	1.60	0.21
Larvae per plant	**H**	**H>G**	**H**	**H<G**	**4.54**	**<0.05**
	G	**H>G**	**G**	**H<G**	**17.2**	**$<10^{-4}$**
	H	H>G	G	H>G	<0.001	0.98
	G	**H<G**	**H**	**H<G**	**7.67**	**<0.01**
	H	H<G	G	H>G	3.66	0.56
	G	**H<G**	**H**	**H>G**	**12.4**	**<0.001**
Larvae on young leaves	H	H>G	H	H<G	1.02	0.31
	G	**H>G**	**G**	**H<G**	**14.2**	**<0.001**
	H	H>G	G	H>G	0.62	0.43
	G	H<G	H	H<G	0.10	0.76
	H	H<G	G	H>G	0.04	0.84
	G	**H<G**	**H**	**H>G**	**6.21**	**<0.05**
Larvae on mature leaves	**H**	**H>G**	**H**	**H<G**	**26.0**	**$<10^{-6}$**
	G	H>G	G	H<G	2.25	0.13
	H	H>G	G	H>G	1.49	0.22
	G	**H<G**	**H**	**H<G**	**19.1**	**$<10^{-4}$**
	H	**H<G**	**G**	**H>G**	**9.52**	**<0.01**
	G	**H<G**	**H**	**H>G**	**9.22**	**<0.01**

Notes: Effects of initial plant size and maternal plants were considered as a covariate and random factor in mixed models, respectively (see text for details). Bold values indicate significant differences between groups at *P* < 0.05.

Table S3.2 Intercepts for the leaf damage scores in cumulative link mixed models.

Intercept	(a) Pooled leaf types			(b) Young leaves			(c) Matured leaves		
	Coefficients	SE	*Z*	Coefficients	SE	*Z*	Coefficients	SE	*Z*
0 | 1	0.48	0.29	1.68	-0.23	0.35	-0.65	**2.76**	**0.89**	**3.10**
1 | 2	**1.32**	**0.29**	**4.58**	**0.75**	**0.35**	**2.16**	**6.12**	**0.93**	**6.59**
2 | 3	**1.82**	**0.29**	**6.32**	**1.33**	**0.35**	**3.83**	NA	NA	NA
3 | 4	**2.16**	**0.29**	**7.52**	**1.72**	**0.35**	**4.95**	NA	NA	NA
4 | 5	**2.54**	**0.29**	**8.83**	**2.13**	**0.35**	**6.12**	NA	NA	NA

Notes: Numbers of intercept column indicate the damage score 0 - 5 that correspond to intact leaves - complete area loss, respectively. For example, 0|1 indicates a ratio of probability of being the damage score 0 on that of the score 1. The estimates were calculated using models including all of the fixed effects. Bold values indicate a significant deviation of coefficients from zero at $P < 0.05$ (Wald test). NA indicates no data available.

Table S3.3 Coefficients and intercepts of the time to initial biting by an adult beetle (order 1 - 14) in the cumulative models.

Factors	Coefficient	SE	*Z*
Trichome (Hairy)	**-0.60**	**0.30**	**-2.02**
Precon. (Hairy)	-0.04	0.36	-0.11
Precon. (None)	0.20	0.36	0.56
Tri. × Precon. (Hairy)	1.18	0.73	1.62
Tri. × Precon. (None)	-0.05	0.73	-0.07
Intercept (1|2)	-0.67	0.38	-1.77
Intercept (2|3)	0.01	0.37	0.03
Intercept (3|4)	0.13	0.37	0.35
Intercept (4|5)	0.22	0.37	0.58
Intercept (5|6)	0.30	0.37	0.82
Intercept (6|7)	0.42	0.37	1.12
Intercept (7|8)	0.45	0.37	1.20
Intercept (8|9)	0.48	0.37	1.28
Intercept (9|10)	0.56	0.37	1.51
Intercept (10|11)	0.65	0.38	1.73
Intercept (11|12)	**0.74**	**0.38**	**1.96**
Intercept (12|13)	**0.80**	**0.38**	**2.11**
Intercept (13|14)	**1.64**	**0.40**	**4.15**

Notes: Ordered score 1 - 12 correspond to 30-minute intervals after the beginning of observation, respectively. Score 13 and 14 indicates the presence and absence of biting marks after one day, respectively. For example, 0|1 indicates a ratio of probability of being the damage score 0 on that of the score 1. These intercept estimates were calculated using models including all of the explanatory variables. Bold values indicate a significant deviation of coefficients from zero at $P < 0.05$ (Wald test).

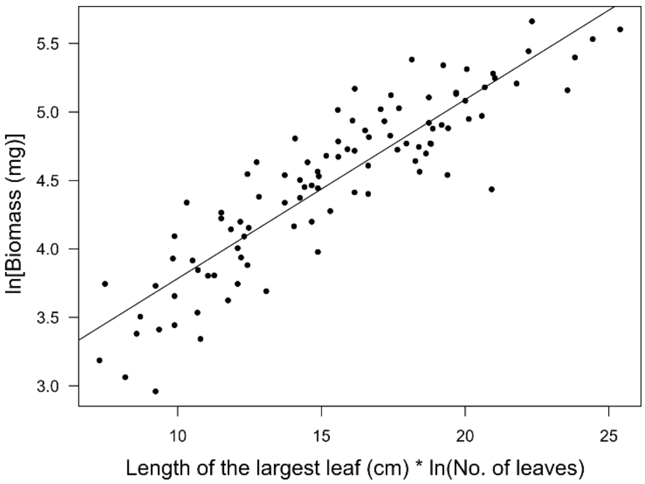


Figure S3.1 Biomass (mg) estimation of *Arabidopsis halleri* subsp. *gemmifera* by the number and length of leaves. The biomass, leaf number, and leaf length were evaluated at ca. 4 months after transplant. Solid line represents the prediction by simple linear regression ($Y = 0.13X + 2.48$, $R^2 = 0.80$). Details of the growth conditions are described in the main text (see Materials & Methods).

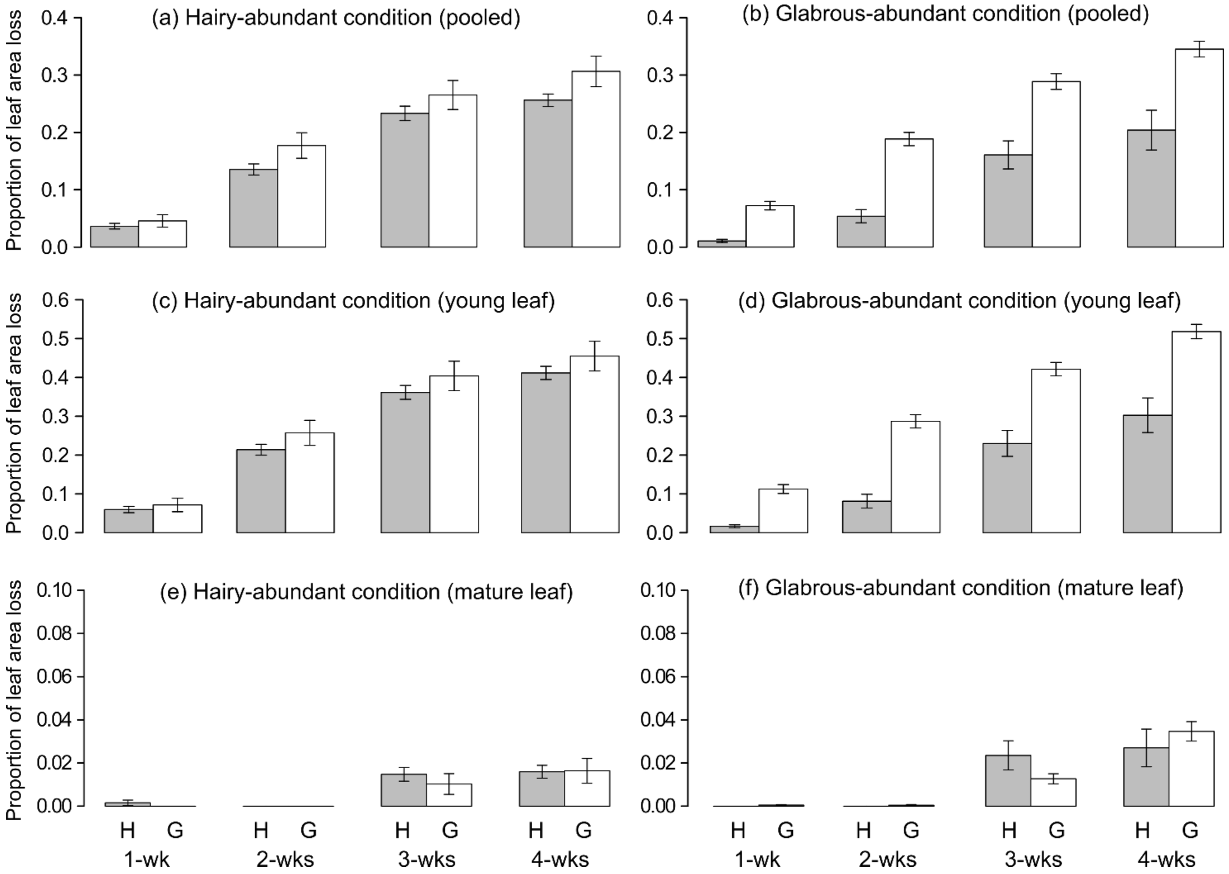


Figure S3.2 Leaf damage data (proportion of leaf area loss) until four weeks after the release of adult beetles. Data are separately presented for pooled dataset of young and mature leaves (a, b), young (c, d), or mature (e, f) leaf type. Mean ± SE values of the raw data are shown for hairy (H; grey) and glabrous (G; white) plants under the hairy-abundant and glabrous-abundant conditions.

Chapter 4

Associational effects promote the local coexistence of trichome dimorphism in a natural plant population

Abstract

Despite a growing body of evidence for herbivore-mediated selection on plant defenses, we still know little about how genetic variation persists in anti-herbivore defense traits. Here, we tested a hypothesis that herbivore-mediated interaction enhances the maintenance of defense polymorphism via negative frequency-dependent selection on the trichome-producing (hairy) and trichomeless (glabrous) plants of *Arabidopsis halleri* subsp. *gemmifera*. First, in a natural population where the specialist beetle *Phaedon brassicae* was prevailing, hairy plants were less damaged with increasing frequency of neighboring glabrous plants, whereas damage on glabrous plants showed no significant relationship with the frequency of neighboring glabrous plants. Furthermore, rarer morphs recovered in frequency in a series of field patches between survey years. Then, our mesocosm experiment demonstrated the rarity advantage in leaf damage for hairy plants and reciprocal fitness advantage between hairy and glabrous plants. This rare-morph fitness advantage was not detected in the absence of *P. brassicae*. Based on the temporal recovery and fitness advantage of rare-morphs, our findings highlight an overlooked but potentially important role of negative frequency-dependent selection in maintaining anti-herbivore defense polymorphism in a contemporary population.

Introduction

Natural plant population often exhibits polymorphism in defense traits against herbivores (Hughes 1991; Elle and Hare 2000; Kivimäki *et al*. 2007). Evidence is now accumulating that herbivores exert selection so strongly as to drive the evolution of plant defense in contemporary time scale (e.g., Zangerl *et al*. 2008; Agrawal *et al*. 2012; Züst *et al*. 2012). As for the evolution of plant defense, resource allocation theory predicts an optimal defense level based on a tradeoff between defense and growth (Simms and Fritz 1992), indicating the loss of genetic variation under strong directional selection. For that reason, it remains unknown why anti-herbivore defense polymorphism persists within a contemporary population (Elle and Hare 2000; Wise *et al*. 2009).

Negative frequency-dependent selection (NFDS) is at least theoretically convincing mechanism that maintains polymorphism, as it creates a negative feedback to prevent rare morphs from going extinct (e.g., Bond and Kamil 1998; Fitzpatrick *et al*. 2007; Takahashi *et al*. 2010). If this type of selection is acting, it predicts a fitness advantage of rare morphs and temporal recovery in frequency of those minorities (Takahashi *et al*. 2010). In plant-animal interactions, for example, learning behaviors of a pollinator species are known to mediate NFDS on a flower color polymorphism in a rewardless orchid (Gigord *et al*. 2001). In antagonistic interactions, several authors examined frequency-dependent pathogen attacks to multiple clonal lineages within a plant species (e.g., Antonovics and Ellstrand 1984; Roy 1998) and a temporal recovery of rare lineages (Siemens and Roy 2005). NFDS has rarely been tested, however, with respect to the anti-herbivore defense polymorphism.

In plant-herbivore interactions, community ecological studies have shown that herbivory risk on an individual plant depends not only on their own traits but on neighborhood composition of other plant species (Agrawal *et al*. 2006; Barbosa *et al*. 2009; Underwood *et al*. 2014). These phenomena of “associational effects” have been recognized as a kind of apparent competition between plant species (see Barbosa *et al*. 2009 for a review). Specifically, recent studies suggest that associational effects may influence the coexistence of multiple plant species by mediating frequency- and/or density-dependent damage (Underwood *et al*. 2014; Kim and Underwood 2015), and these effects may also occur within a plant species (Wise *et al*. 2009; Sato and Kudoh 2015b). Here, in terms of NFDS, we focused on such kind of apparent interaction as a novel mechanism for the maintenance of anti-herbivore defense polymorphism.

Several species of the genus *Arabidopsis* exhibit a genetic dimorphism of trichome-producing (hairy) and trichomeless (glabrous) plants (Hauser *et al*. 2001; Kivimäki *et al*. 2007; Kawagoe *et al*. 2011) which are governed by a few major genes (Grebe 2012). Trichomes of *Arabidopsis* are known to play a major role in physical resistance to biotic and abiotic stresses (Handley *et al*. 2005; Sletvold *et al*. 2010; Sletvold and Ågren 2012).

Likewise relatives, *A. halleri* subsp. *gemmifera* (abbreviated hereafter as *A. halleri*) also have the genetic dimorphism of hairy and glabrous plants (Fig. 1.1). Previously, our study revealed that the specialist beetle *Phaedon brassicae* caused frequency-dependent damage and thus contributed to a minority advantage in growth for hairy and glabrous plants (Sato *et al*. 2014; Sato and Kudoh 2015b). However, whether the herbivore-mediated interaction contributes to the maintenance of trichome dimorphism remains largely unknown.

In this study, we tested a hypothesis that apparent interactions mediated by the specialist leaf beetle can promote the maintenance of hairy and glabrous plants via NFDS. Three specific questions were addressed: (1) Does plant damage depend on the frequency of neighboring plant morphs? (2) Do frequencies of two morphs temporally restore to intermediate levels in the field? (3) Can a herbivore species mediate rare-morph fitness advantage for hairy and glabrous plants? To answer these questions, we first observed leaf damages of two morphs and temporal changes of the morph-frequency in a natural population where *P. brassicae* was a major insect herbivore on *A. halleri*. Then, in a common garden, we manipulated the both morph-frequency and the presence/absence of herbivores to test whether the leaf beetle caused rare-morph advantage in defense and fitness between hairy and glabrous plants.

Materials & Methods

Study organisms

Arabidopsis halleri subsp. *gemmifera* [Brassicaceae] is a self-incompatible perennial herb distributed across Japan and Russian Far East (Hoffmann 2005). Hairy morphs of this subspecies produce non-glandular trichomes on their leaf and stem surface, whereas glabrous morphs had no trichomes except for their leaf margin (Fig. 1.1). The hairy and glabrous phenotypes are associated with allelic variations in a trichome-related gene *GLABROUS1* (*GL1*), but not with other genes including two flanking regions (Kawagoe *et al*. 2011). Wounding did not increase the trichome density of hairy plants (Sato and Kudoh 2015b). Glucosinolate profiles were not associated with the hairy and glabrous phenotype in a population we studied below (Sato *et al*. 2014). In the lowlands of central Japan, plants start flowering from early April and fruit-set was ca. 80% unless the flowers were consumed by herbivores (Kawagoe and Kudoh 2010). After flowering, plants produce new rosettes on the main and lateral meristem, and these rosettes can establish as clonal offspring once they are rooted into the ground (Fig. 1.1e). A plant with no vegetative connection with others was designated as an individual in this study.

Phaedon brassicae [Coleoptera: Chrysomelidae] is an oligophagous pest of Brassicaceae and distributed in Asia (Ôtake and Funaki 1958; Wang *et al*. 2007c). Adults are flightless and access host plants exclusively by walking (Ôtake and Funaki 1958). Newly hatched larvae stayed on a host plant on which adults feed (Ôtake and Funaki 1958), so the damage difference between individual plants is attributable to adult host choice. This species usually reaches the adult stage within 3 weeks after hatching, and adults survive for approximately 2 months across various ranges of temperature and photoperiod (Wang *et al*. 2007c). Adults and last-instar larvae are ca. 4-8 mm in body length. We used a laboratory-reared population previously established and maintained under 20˚C, 12L:12D conditions (Sato *et al*. 2014).

Field survey

We conducted a 3-year survey in a natural population of *A. halleri* located in Taka-cho, Hyogo Prefecture, Japan (35°06′N, 134°56′E, Alt. 200 m). Field surveys were conducted for selected *A. halleri* patches along a creek (ca. 300 m in distance) that ran through the center of the study site. Vegetation is sparse along the creek, and no Brassicaceae plants are observed except for *A. halleri*. No apparent microhabitat differentiation was observed between hairy and glabrous plants (38 cm in median of distance between nearest two morphs, < 1.0% median differences in soil moistures between two morphs). In this site, *P. brassicae* mainly occur during the flowering season (April-May) and heavily infest on *A. halleri* (Kawagoe and Kudoh 2010). Other herbivorous insects (such as *Pieris napi* and *Plutella xylostella*) also occur in the study site, but their abundance is much lower than that of *P. brassicae* in this site (Kawagoe and Kudoh 2010) and they have rarely been observed during the present study.

We randomly established eighty circular patches (diameter 1.0 m, >3 m apart each other) in 2013. The trichome phenotype (hairy or glabrous), the leaf damage, and the number of flowers, and the length of the largest leaf (mm: referred to hereafter as the maximum leaf length) were recorded for all individual plants in each patch. The proportion of leaf area lost to herbivory (referred to as the leaf damage hereafter) was scored as 0 (< 12.5%), 1 (25 ± 12.5%), 2 (50 ± 12.5%), 3 (75 ± 12.5%), or 4 (> 87.5% and < 100%) for all leaves in an individual plant. We focused on local interaction in 1-m-diameter patches, since the number of plants within circular patches approached a plateau with increasing patch size at that scale in this study site (Sato *et al*. 2014). The center of each patch was marked using a steel anchor (15 cm in depth) and checked twice a year to follow a same patch among study years. The surveys were conducted once a year during mid-late May (on 21–23 May 2013, 20–23 May 2014, and 19–22 May 2015) after the peak abundance of *P. brassicae* had been observed (Mean ± SE = 0.23 ± 0.07 beetles including larvae and adults per plant, n = 100 plants on 2013). We newly established 3-10 patches every year to keep the sample size 750–850 individual plants of 80-84 patches per survey.

Mesocosm experiment

We manipulated the neighborhood conditions of hairy and glabrous plants (hairy: glabrous plants = 6:2 or 2:6 individuals, referred to hereafter as the hairy-abundant or glabrous-abundant condition, respectively) and the presence/absence of herbivores. We constructed forty-eight conical plots using gardening poles and wires (diameter 0.75 m and height 0.7 m) to simulate a field patch. These plots were separately enclosed by a nonwoven agricultural net (90% light-transmittance). The experiment was carried out in an agricultural greenhouse (10 m × 3.5 m with a height of 2.0 m) located in the outside open space of Center for Ecological Research, Kyoto University (34°58′N, 135°57′E, Altitude 100 m). We removed all weeds on the ground of experimental area and covered with weed protecting sheets. The greenhouse was covered with an agricultural plastic sheet (thickness 0.1 mm). Air temperature was uncontrolled and ranged from 5 to 40°C during the experiment, which was comparable with that in the field site at a plant height level (8–33°C). Photosynthetically active radiation and the red:far-red ratio inside the greenhouse was 1200–1400 μmol/m^2/s and 1.0–1.2 in a sunny day, respectively (LI-190 Quantum Sensor, LI-COR, Lincoln, NE, USA). Schematic summary of the experiment is available in supporting information (Figure S4.1).

We initially prepared ca. 600 plants in the greenhouse during the winter season of 2014-2015. Matured seeds were collected from ten hairy and glabrous plants in the field site during June-July 2014. The maternal plants were at least 3 m apart each other to avoid multiple samplings on a single clone. Seed were germinated on Petri dish (diameter 9 cm, depth 1.5 cm) filled with moisten quartz sands in an environmental chamber under 12L:12D with 25/15°C respective air temperature, where >80% of seeds were germinated successfully. During late November 2014, we transplanted one or two seedlings that had 2–4 leaves into a plastic pot (diameter 9.0 cm, depth 8.0 cm: Y-pot, SAKATA SEED CO., Yokohama, Japan) filled with seedling-raising compost (TAKII & CO., Ltd., Kyoto, Japan: Cat. No. 776-274-01; fertilizer contents, N: P: K = 320: 210: 300 mg/L, respectively). To prevent herbivory, these potted plants were placed inside transparent plastic cases (75 cm × 45 cm: 28 pots × 22 cases in total). The location of cases was randomized every two weeks. Seven weeks after the transplant, we removed a smaller plant (evaluated by the maximum leaf length) in each pot to prevent competition within the pot. Covers of the plastic cases were removed when plants began to elongate their stems.

Two months after the transplant, we transferred potted plants into the experimental plots where eight plants were included per plot. Eight potted plants were circularly arranged within a plot and two plants of rare morphs were in face-to-face (Figure S4.1). The position of rarer plants was randomized to minimize light condition biases between rare and abundant morphs. In the experimental setup, we excluded small plants (< 40 mm in the maximum leaf length) to control for initial plant size, and the final samples consisted of 2–57 plants from 20 maternal families. Sixteen replicates were established for the beetle-present treatments (8 plants × 2 frequency conditions × 16 plots = 256 plants), while the rest sixteen plots were assigned to the beetle-absent condition (8 plants × 2 frequency conditions × 8 plots = 128 plants). Sexes of adult beetles can be determined on the basis of their body size (0.5 mm smaller in abdomen height and width in adult males); therefore, for all plots of the beetle-present condition we kept the sex ratio 50:50 to equalize the likelihood of oviposition events. Four and twelve plots were set respectively in rows and columns in the greenhouse, where the two frequency conditions were applied in a checkered manner and every three rows were assigned as the beetle-absent condition. Water was supplied every three or four days during the experiment. All plants were not attacked by any herbivores before the experiment.

We released twelve adult beetles per plot for the beetle-present group 4.5 months after the transplant (corresponded to March 2015). The beetles were inoculated at this time point when plants started flowering, because *P. brassicae* become the most abundant and influential on plant fecundity during the flowering season (Kawagoe and Kudoh 2010). We evaluated the herbivory load (leaf damage and herbivore abundance) and plant fecundity (flower production and clones) at the end of reproduction (corresponded to 1.5 month after the release of beetles). The leaf damage was scored in the same procedure as we conducted in the field survey. We counted *P. brassicae* adults and larvae on an individual plant. This herbivore abundance was recorded weekly after larvae had been observed for the first time (2–7 weeks after the release of adult beetles) to evaluate the cumulative herbivory load on plants. We counted flowers as the number of pedicels. Fruit set was not evaluated due to the self-incompatibility of *A. halleri*. The main and lateral meristems of flowering stem of *A. halleri* produce roots and turn into clonal rosettes (Fig. 1.1e); and thus, these meristems were counted to assess clonal reproduction.

Statistical analysis

For the field survey data, we used generalized linear mixed models (GLMMs: Bolker *et al*. 2009) to analyze response variables of the leaf damage score (with a cumulative error structure: Agresti 2002) and the number of flowers (Poisson error structure). We used likelihood ratio tests to examine the fixed effects of the trichome phenotype, the frequency of glabrous plants in a patch (i.e., no. glabrous plants / the total no. plants), the total number of *A. halleri* plants in a patch, and plant size (evaluated by the maximum leaf length). An interaction between the trichome phenotype and the frequency of glabrous plants was also included as a fixed effect to test frequency dependence in the response variables. The plant size was incorporated as a covariate in the cumulative error structure for the leaf damage score; or as an offset term in the Poisson structure for the number of flowers to consider size-dependent damage or fecundity in the statistical model. The study year and patch ID (nested below the study year) was included as random effects. All statistical analyses were performed using the R version 3.2.0 (R Core Team 2015). We used the clmm function with the equidist option (in the ordinal package: Christensen 2013) for the cumulative error structure; and the glmer function (in the lme4 package: Bates *et al*. 2014) for the other error structures in the GLMMs. The proportion of leaf area lost for a whole plant was calculated by averaging the corresponding area loss of the damage score of leaves (0, 1, 2, 3, and 4 = 0.0, 0.25, 0.50, 0.75, and 0.1, respectively).

Additionally, for the field survey data we estimated standardized coefficients of the fixed effects of the frequency of glabrous plants, total number of *A. halleri* plants in a patch, and the maximum leaf length to examine tendencies between these factors and the response variables in GLMMs. Based on the interaction between the trichome phenotype and the frequency of glabrous plants, we conducted the coefficient estimation separately for the hairy or glabrous dataset. Trend lines of the middle category (score 1-2) were added as a representative in cumulative models for the leaf damage. Furthermore, we used ordinal linear regressions to examine temporal changes in the frequency of two morphs between study years. The response variable was the current year's frequency of glabrous plants in a patch, while the explanatory variables were the past frequency of glabrous plants and the past total number of *A. halleri* plants. Since the study site had been attacked by a typhoon in autumn 2014 and sixteen patches were extinct, the sample size was decreased to sixty-four in the analysis of frequency changes from 2014 to 2015. The negative frequency-dependent transition of two morphs was determined by less than one slope coefficients in the linear regression. There was a potential problem for linear trend lines of spanning outside a zero-to-one range; thus, the same analysis was performed using a logistic regression (where we used generalized linear models with a binomial error structure).

The mesocosm experiment data were also analyzed using GLMMs in the same manner as the field survey data. We examined the response variables of the leaf damage score (with a cumulative error structure), and the number of beetles, clonal meristems, and flowers (Poisson error structure). We examined fixed effects of the trichome phenotype, neighborhood condition, and their interaction. The plot ID and maternal plant ID were included as random effects to consider variation among plots and maternal families. The initial plant size (evaluated by the maximum leaf length) was incorporated as a covariate for leaf damage data; or an offset term for the count responses. Significance of each fixed effect was analyzed using likelihood ratio tests. Pairwise likelihood ratio tests were also performed to determine significant differences among the two phenotypes and two neighborhood conditions. *P*-values were adjusted by sequential Bonferroni correction. These analyses were conducted separately for the beetle-present or beetle-absent treatments.

Results

Field survey: frequency-dependent damage and patchy dynamics

Leaf damage on hairy plants decreased as the frequency of glabrous plants became large in a patch (Table 4.1a; Fig. 4.1a), whereas the leaf damage on glabrous plants was not significantly correlated with the frequency of glabrous plants (Table 4.1a; Fig. 4.1b). Differential trends between the leaf damage on two morphs were confirmed by a significant interaction between the trichome phenotype and the frequency of glabrous plants (LR-χ^2 = 67.5, $P < 10^{-16}$: Table S4.1). The leaf damage on hairy plants increased as the total number of plants increased in a patch (Wald test, $Z = 2.09$; $P = 0.04$: Table 4.1a), but no significant relationships were detected between the damage on glabrous plants and the total number of plants ($Z = 1.04$, $P = 0.30$: Table 4.1a). The number of flowers was affected interactively by the trichome phenotype and frequency of glabrous plants in a patch (LR-χ^2 = 87.4, $P < 10^{-16}$: Table S4.1), but there was no significant correlation between the flower production and proportion of glabrous plants neither for the hairy nor glabrous dataset (Wald tests, $P > 0.25$: Figure S4.2a, b). We observed ninety-eight beetles in the total of the three-year survey.

We observed a temporal recovery of frequency of rare morphs between the study years. The frequencies of glabrous plants in a patch increased when they had been small in the past year (Fig. 4.1c, d). By contrast, the frequencies of glabrous plants decreased when they had been large in the past year (Fig. 4.1c, d), indicating that the frequencies of hairy plants (= 1 – frequency of glabrous plants) increased in the local frequency when they were rare. This result was confirmed by the linear prediction whose slope coefficient and 95% confidence intervals (= ± 1.96 × SE) was smaller than one (Table 4.1b). These results were consistent between two yearly intervals of the study years (2013-2014 and 2014-2015: Table 4.1b; Fig. 4.1c, d), and these conclusions did not differ when the trend lines were estimated by logistic regressions (Figure S4.2c, d).

Mesocosm experiment: Rare-morph advantage in plant damage and fecundity

The hairy plants were less damaged than glabrous plants under the glabrous-abundant conditions (LR-χ^2_1 = 129, $P < 10^{-15}$), whereas the leaf damage showed no significant difference between the two morphs under the hairy-abundant condition (LR-χ^2_1 = 0.8, P = 0.74: Table S4.2, S4.3; Fig. 4.2a). Consistent with the pattern of leaf damage, leaf beetles occurred less frequently on hairy than glabrous plants under the hairy-abundant conditions (LR-χ^2_1 = 28.1, $P < 10^{-5}$: Table S4.2, S4.3; Fig. 4.2a). This pattern was observed for both larvae and adults (Figure S4.3), while larvae were more abundant than adults (Mean ± SE = 2.5 ± 0.15 larvae, 0.30 ± 0.03 adults). The neighborhood dependence of leaf damage was still significant (i.e., trichome × neighborhood interaction, LR-$\chi^2_1 = 134$, $P < 10^{-16}$: Table S4.2), though the initial plant size also had a significant effect on the damage (LR-$\chi^2_1 = 89.2$, $P < 10^{-16}$). At the end of experiment, rarer types of plant had a larger number of clonal meristems or flowers than majorities (indicated by a significant trichome × neighborhood interaction, LR-$\chi^2_1 > 119$, $P < 10^{-15}$: Table S4.2; Fig. 4.2a). As to the beetle-absent condition, no rarity advantage was found in the number of flowers and clonal meristems between hairy and glabrous plants (Fig. 4.2b). Glabrous plants produced more flowers and clonal meristems than hairy plants did in the absence of leaf beetles (LR-$\chi^2_1 > 22.8$, $P < 10^{-4}$ for the both hairy- and glabrous-abundant conditions: Table S4.3; Fig. 4.2b).

Discussion

Ecological interactions that generate frequency-dependent selection should be of considerable interests because of their strong potential to maintain genetic variation (e.g., Bond and Kamil 1998; Gigord *et al*. 2001; Fitzpatrick *et al*. 2007). Our field survey and experiment both revealed that hairy plants received less damage when they were rare, whereas leaf damage did not differ between hairy and glabrous morphs when glabrous ones were rare. In the natural population, rarer types of the two plant morphs recovered their frequencies in a series of patches compared to the previous frequencies. Furthermore, we experimentally demonstrated that the presence of *P. brassicae* caused a rare-morph fitness advantage for hairy and glabrous plants. These findings support the hypothesis that the herbivore-mediated interaction promotes the coexistence of hairy and glabrous plants through NFDS.

Herbivore-mediated apparent competition has been suggested as a mechanism for stabilizing the coexistence of different plant species (Louda *et al*. 1990; Pacala and Crawley 1992). Specifically, herbivores can facilitate plant coexistence by selectively feeding on abundant but undefended plants if defended plants are less competitive than undefended ones (Pacala and Crawley 1992). Congruent with the present results, adults of *P. brassicae* are known to prefer to glabrous leaves of *A. halleri* when hairy leaves are rare (Sato *et al*. 2014). This escape from herbivory under the glabrous-abundant condition may result in the rare-morph fitness advantage for hairy plants. Meanwhile, a defense-growth tradeoff likely exists in the trichome dimorphism, since glabrous plants produced more flowers and clones under no herbivory. As hairy plants no longer had the defense profitability when they became abundant, the fitness tradeoff would emerge especially under the hairy-abundant condition. The selective herbivory accompanied with a defense-growth tradeoff is a plausible mechanism of the rare-morph fitness advantage in the presence of *P. brassicae*.

As a kind of antagonistic interaction, several plant-pathogen systems have been investigated to test NFDS on resistant and susceptible plants (e.g., Antonovics and Ellstrand

1984; Brunet and Mundt 2000; Siemens and Roy 2005). Of these studies, some clearly showed rarity fitness advantage in a cultivated plant (Brunet and Munt 2000) but the others found it difficult to detect NFDS due to simultaneous attacks by pathogens and herbivores in natural populations (Roy 1998; Siemens and Roy 2005). In plant-animal interactions, NFDS on floral traits were sometimes offset by positive frequency-dependent selection because flowers can attract not only pollinators (i.e., mutualists) but also seed predators (antagonists) (Toräng *et al*. 2008). Compared with these preceding studies, our study system is relatively simple because the major herbivorous insect, *P. brassicae*, is specialist and no Brassicaceae plants other than *A. halleri* inhabit in our study site. In addition to this simplicity of interspecific interaction, limited dispersal of the flightless leaf beetle enabled us to focus on the local dynamics of plant damage and frequency. To our knowledge, this is thus one of the few examples to simply illustrate NFDS acting in a contemporary population.

Temporal oscillations in morph-frequency are expected to occur under NFDS, and reported as evidence for NFDS in animal species (e.g., Hori 1993; Bond and Kamil 1998; Takahashi *et al*. 2010). Despite several examples of rare-morph fitness advantage in plants (Brunet and Mundt 2000; Gigord *et al*. 2001; Toräng *et al*. 2008), evidence is still limited for morph-frequency changes in the field (Siemens and Roy 2005). Here, the present result that rarer plant morphs increased in frequency within a patch supports the local coexistence of hairy and glabrous plants. In contrast to the local dynamics, the frequency of hairy and glabrous plants has stayed intermediate at least for a decade at the study site (Kawagoe *et al*. 2011; Sato *et al*. 2014). Given also the lack of *P. brassicae* mobility and the patchy structure of an *A. halleri* population, inter-morph interactions and consequent frequency change might be detectable at that local patch scale. The circumstantial evidence led us to hypothesize that the locally frequency-dependent dynamics at the patch level resulted in the intermediate frequency of trichome dimorphism at a whole population level. This notion may also be supported by a theoretical prediction that the morph-frequency oscillation under NFDS becomes weak as the population size increase (Takahashi *et al*. 2010).

While negatively frequency-dependent flower production was detected in the mesocosm experiment, we were unable to observe this pattern in the natural population. This might occur because the initial size of individual plants, which is positively correlated with flower production in *A. halleri* (Kawagoe and Kudoh 2010; Sato and Kudoh 2015b), was uncontrolled and fairly varied in the natural population. Based on the life-history of plants and beetles, however, it was still possible that *P. brassicae* became influential on the population growth of hairy and glabrous plants. Both adults and larvae of *P. brassicae* primarily consume plant leaves and prefer to young leaves (Sato and Kudoh 2015b) which may turn into leaves of clonal offspring. Furthermore, *P. brassicae* mainly occur during spring and heavily damage newly elongated stems of *A. halleri* (Kawagoe and Kudoh 2010). Although it was difficult to identify clonal meristems in the field due to the perennial life-cycle of *A. halleri*, the impact of beetles on clonal spread and its local frequency dependence could be a putative process to be tested for the coexistence of two morphs.

We should note that plant density as well as frequency may affect the strength of herbivore-mediated interactions (Underwood *et al*. 2014; Kim and Underwood 2015). In our field survey, the damage on hairy plants tended to increase as the plant density became large in a patch. This indicates possible relevance of resource concentration (Root 1973; Underwood *et al*. 2014) where denser plant patches could carry more herbivores. Notice also that between-patch movements of herbivores might also contribute to the spatial pattern of plant damage (Hambäck *et al*. 2014). In particular to *P. brassicae*, however, short-term migration is likely limited relative to the within-patch host choice, because adults are flightless and newly hatched larvae tend to stay on a same plant (Ôtake and Funaki 1958). The density- and scale-dependent damage need to be examined to understand how the herbivore-mediated interaction drives ecological dynamics in a longer term.

In summary, the present study provides clear evidence for NFDS acting on anti-herbivore defense polymorphism in a contemporary population. Evidence for the rapid evolution of anti-herbivore defenses has now been accumulated across several plant taxa (Zangerl *et al*. 2008; Agrawal *et al*. 2012; Züst *et al*. 2012; Sakata *et al*. 2014), but this fact raises a further question about how genetic diversity persists in plant defense traits under strong natural selection. Based on the evidence for NFDS on a visible and discrete trait, the present study provides a possible explanation to that question. Future studies should focus on this respect, because it may reveal a mechanism by which even a simple plant-herbivore system leads to genetic diversity in plant defense.

Table 4.1 (a) Effects of the frequency of glabrous plants, total number of plants in a patch, and the maximum leaf length on the leaf damage of hairy and glabrous plants: (b) Effects of the past frequency of glabrous plants and total number of plants on the current frequency of glabrous plants in a patch.

(a) Leaf damage

Factor	Hairy (1341)	Glabrous (1094)
Frequency of glabrous plants	**-0.20 ± 0.07**	0.05 ± 0.08
No. of *A. halleri* plants	**0.20 ± 0.10**	0.12 ± 0.12
Maximum leaf length	0.13 ± 0.02	0.05 ± 0.03

(b) Current frequency of glabrous plants

Factor	2013-2014 (64)	2014-2015 (80)
Past frequency of glabrous plants	**0.57 ± 0.08**	**0.63 ± 0.09**
Past no. of *A. halleri* plants	0.005 ± 0.003	-0.002 ± 0.003

Shown are the estimated coefficients (± SE) of fixed factors in generalized or ordinal linear models. Bold values indicate a significant deviation from coefficients of zero at $P < 0.05$ (Wald tests). The numbers of plants or patches examined are described within parentheses for table (a) or (b), respectively.

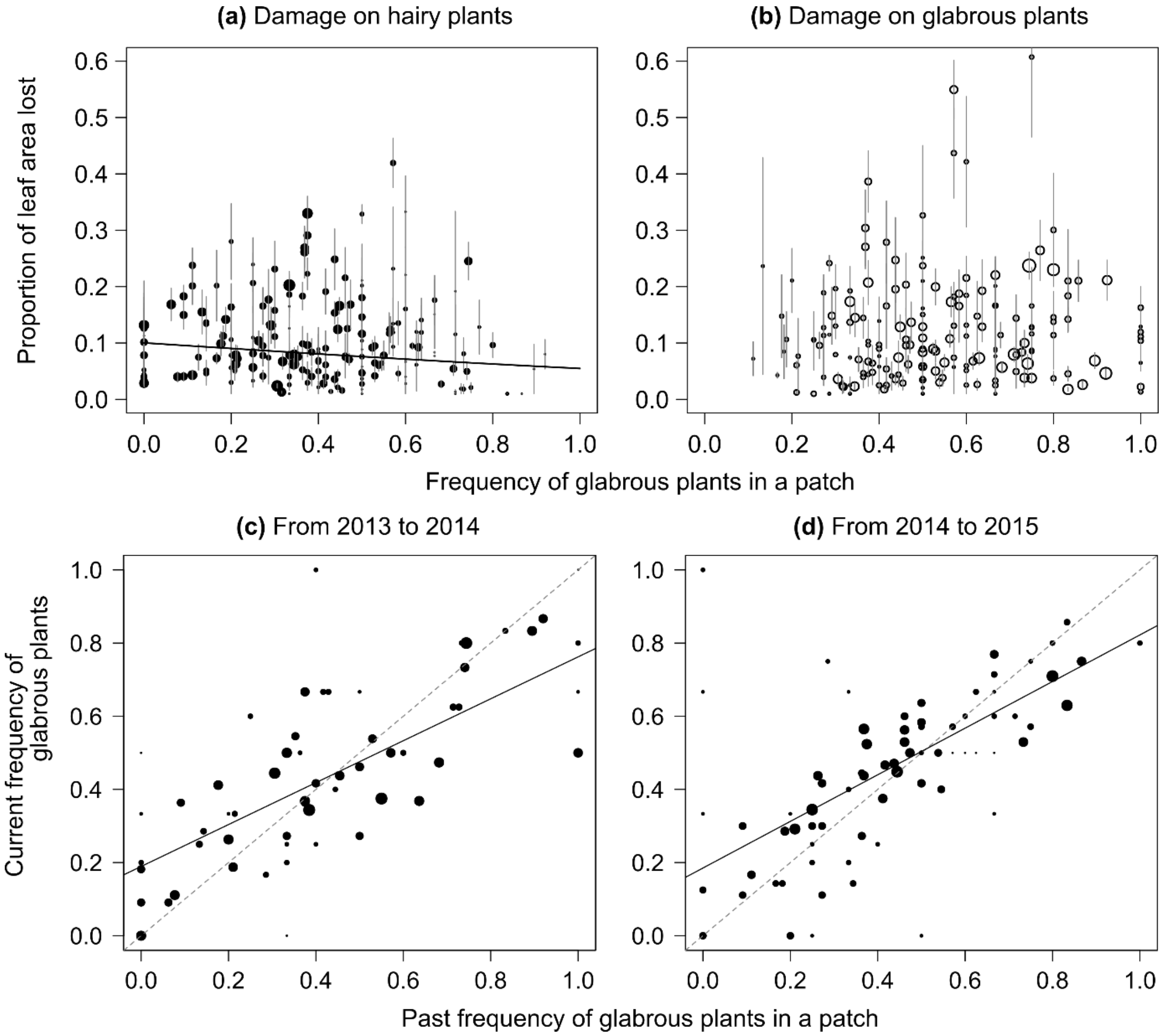


Figure 4.1 Frequency-dependent leaf damages and patchy dynamics of hairy and glabrous plants in the field. A single point represents a patch and its size corresponds to the number of plants in a patch. Upper panels show the proportion of leaf area loss of hairy (a: black circles) or glabrous (b: white circles) plants plotted against the frequency of glabrous plants in a patch. Bars indicate SEM of the raw data of damage. A solid line is a trend line by a generalized linear mixed model. Lower panels show the current years' frequencies of glabrous plants plotted against past frequencies in a patch (c and d). Solid lines indicate linear predictions, while dashed lines indicate no frequency changes between years (y = x).

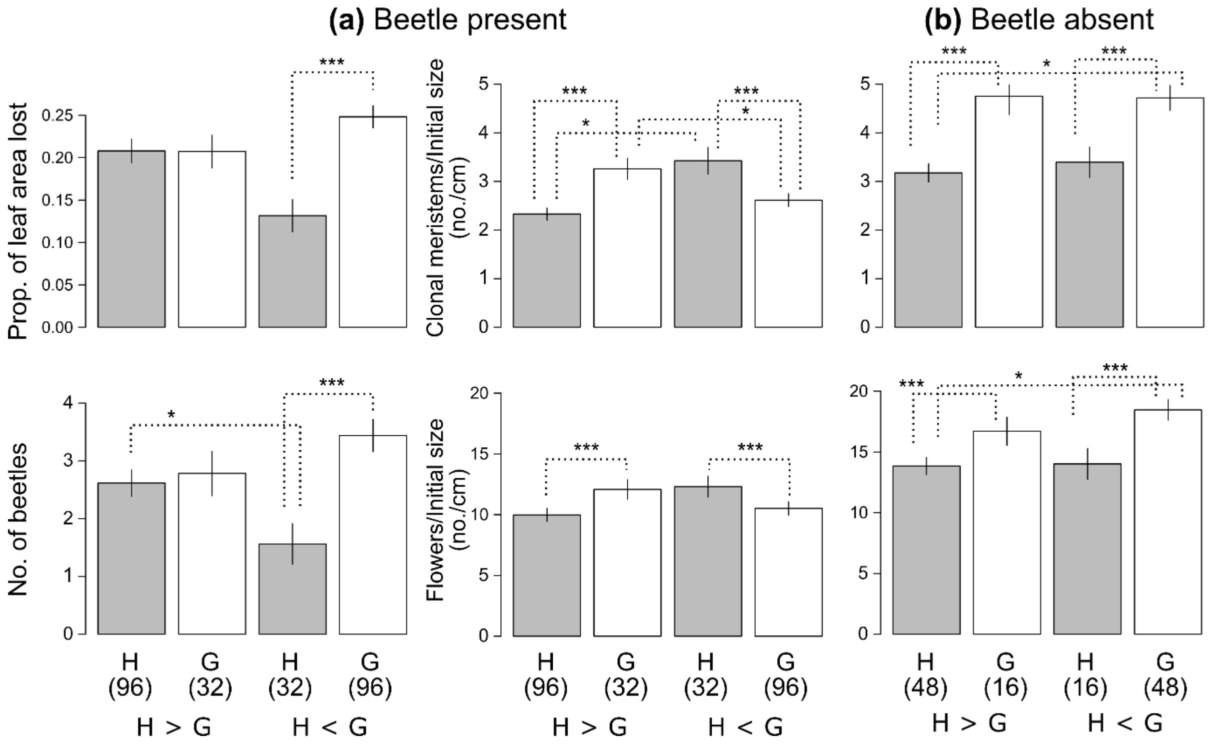


Figure 4.2 Rare-morph advantages in plant damage and fecundity in the mesocosm experiment. Leaf damage (proportion of leaf area lost), beetle abundance (no. of beetles per plant), the number of clonal meristems on flowering stems (which represents a potential of asexual reproduction), and the number of flowers (which represents a potential sexual reproduction) are shown for hairy (H: grey) and glabrous (G: white) plants under the hairy-abundant (H > G) and glabrous-abundant (H < G) condition. The initial plant size was evaluated by the length of the largest radical leaf before flowering. The left and right side present the results under the beetle-present (a) and -absent (b) treatments, respectively. Bars indicate SEM of the raw data. Asterisks show significant differences between the four groups with likelihood ratio tests (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Sample sizes are shown within parentheses.

Supporting information

Table S4.1 Effects of the trichome phenotype, the frequency of glabrous plants, the total number of plants in a patch, and the maximum leaf length on the leaf damage and flower production of *Arabidopsis halleri* subsp. *gemmifera* in the field.

Factor	*df*	Damage		No. of flowers	
		LR-χ^2	*P*	LR-χ^2	*P*
Trichome phenotype	1	**47.2**	**$<10^{-11}$**	**321**	**$<10^{-16}$**
Frequency of glabrous plants	1	2.57	0.11	2.50	0.11
No. of *A. halleri* plants	1	2.73	0.10	0.28	0.60
Maximum leaf length	1	**28.8**	**$<10^{-7}$**	---	---
Trichome × Frequency	1	**67.5**	**$<10^{-16}$**	**87.4**	**$<10^{-16}$**

Likelihood ratio, χ^2, is listed for each factor with its degree of freedom (*df*), where bold values indicate significant effects at $P < 0.05$. Bars (---) represent no information available.

Table S4.2 Effects of the trichome phenotype, neighborhood condition, and their interaction on plant damage and fecundity in the mesocosm experiment.

Factor	*df*	Beetle present								Beetle absent			
		Damage		No. of beetles		No. of flowers		No. of meristems		No. of flowers		No. of meristems	
		LR-χ^2	*P*	LR-χ^2	*P*	LR-χ^2	*P*	LR-χ^2	*P*	LR-χ^2	*P*	LR-χ^2	*P*
Trichome	1	**104**	**$<10^{-16}$**	**15.4**	**$<10^{-4}$**	0.42	0.52	2.91	0.09	**101**	**$<10^{-16}$**	**75.3**	**$<10^{-16}$**
Neighborhood	1	1.64	0.20	0.04	0.84	0.21	0.65	0.64	0.42	0.11	0.74	0.01	0.92
Tri. × Neigh.	1	**134**	**$<10^{-16}$**	**32.9**	**$<10^{-8}$**	**208**	**$<10^{-16}$**	**119**	**$<10^{-16}$**	3.71	0.05	**4.16**	**0.04**

Likelihood ratio, χ^2, is listed for each factor with its degree of freedom (*df*), where bold values indicate significant effects at $P < 0.05$.

The initial plant size was incorporated as a covariate or an offset term in the statistical analyses.

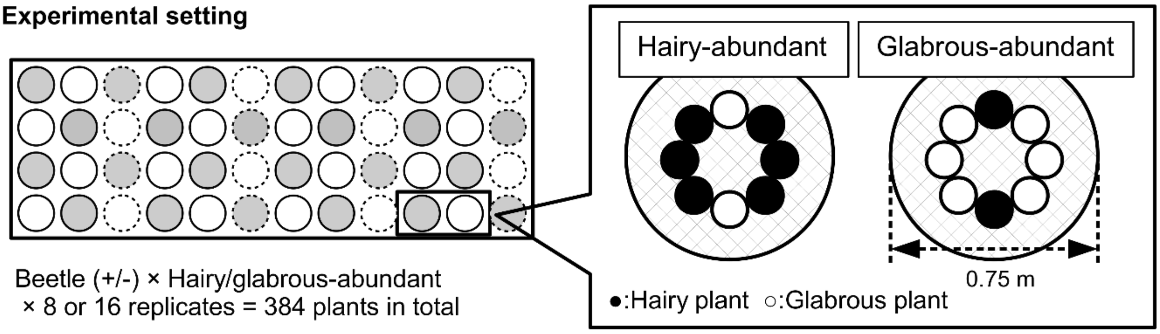
Table S4.3 Results of pairwise likelihood ratio tests for the mesocosm data including four groups of the trichome phenotypes (H: hairy plants; G: glabrous plants) plants under two neighborhood conditions (H > G: hairy-abundant condition; H < G: glabrous-abundant condition) in the presence (+Beetle) or absence (-Beetle) of leaf beetles.

Treatment	Response	Group 1		Group 2		LR-χ^2	P_{adj}
		Tri.	Neigh.	Tri.	Neigh.		
+Beetle	Damage	H	H>G	H	H<G	4.63	0.16
		G	H>G	G	H<G	0.91	0.99
		H	H>G	G	H>G	0.80	0.74
		G	**H<G**	**H**	**H<G**	**129**	**$<10^{-15}$**
		H	H<G	G	H>G	2.60	0.43
		G	H<G	H	H>G	1.07	0.99
	#Beetles	**H**	**H>G**	**H**	**H<G**	**7.28**	**<0.05**
		G	H>G	G	H<G	3.48	0.24
		H	H>G	G	H>G	0.03	0.87
		G	**H<G**	**H**	**H<G**	**28.1**	**$<10^{-5}$**
		H	H<G	G	H>G	5.66	1.1
		G	H<G	H	H>G	2.40	0.24
	#Meristems	**H**	**H>G**	**H**	**H<G**	**11.3**	**<0.01**
		G	**H>G**	**G**	**H<G**	**7.35**	**<0.05**
		H	**H>G**	**G**	**H>G**	**56.7**	**$<10^{-12}$**
		G	**H<G**	**H**	**H<G**	**21.3**	**$<10^{-4}$**
		H	H<G	G	H>G	1.59	0.21
		G	H<G	H	H>G	2.1	0.30
	#Flowers	H	H>G	H	H<G	3.02	0.24
		G	H>G	G	H<G	3.73	0.21
		H	**H>G**	**G**	**H>G**	**51.1**	**$<10^{-10}$**
		G	**H<G**	**H**	**H<G**	**36.0**	**$<10^{-6}$**
		H	H<G	G	H>G	0.52	0.94
		G	H<G	H	H>G	0.08	0.78
-Beetle	#Meristems	H	H>G	H	H<G	0.78	0.76
		G	H>G	G	H<G	0.15	0.69
		H	**H>G**	**G**	**H>G**	**55.2**	**$<10^{-11}$**

	G	**H<G**	**H**	**H<G**	**22.8**	**$<10^{-4}$**
	H	H<G	G	H>G	4.03	0.09
	G	**H<G**	**H**	**H>G**	**7.00**	**<0.05**
#Flowers	H	H>G	H	H<G	0.45	1
	G	H>G	G	H<G	0.01	0.91
	H	**H>G**	**G**	**H>G**	**49.6**	**$<10^{-10}$**
	G	**H<G**	**H**	**H<G**	**44.3**	**$<10^{-8}$**
	H	H<G	G	H>G	0.03	1
	G	**H<G**	**H**	**H>G**	**6.45**	**<0.05**

The maternal plant ID and plot ID were considered as random factors in mixed models (see text for details). Bold values indicate significant differences between groups at $P < 0.05$ with sequential Bonferroni correction.

(a) Experimental setting



(b) Time-line procedure

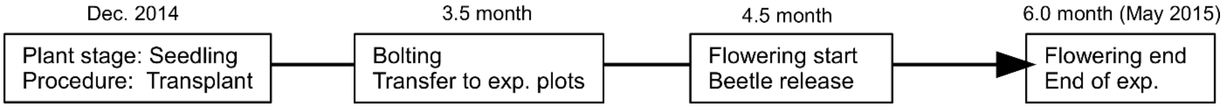
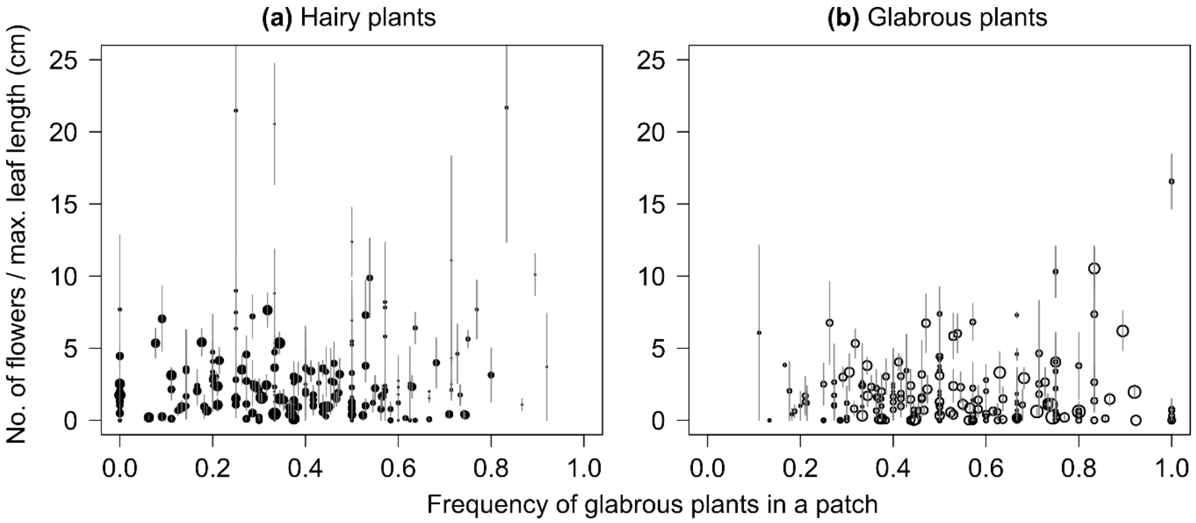


Figure S4.1 Schematic diagram explaining the setting (a) and procedure (b) of the mesocosm experiment. (a) Two neighborhood conditions were set as; hairy: glabrous plants = 6:2 and 2:6 individuals (referred to as hairy-abundant and glabrous-abundant condition, respectively). These two conditions were arranged in a checkered manner (grey: hairy-abundant; white: glabrous abundant), and sixteen or eight replicates were assigned to beetle-present (solid circle) or beetle-absent (dashed circle) treatments, respectively. (b) A horizontal arrow indicates a time series from the start (left, on December 2014) to end (right, on May 2015). Plant stage and experimental procedure are described respectively in the first and second row of a square. Timing of each procedure is shown above each square (*x*-month after the transplant).



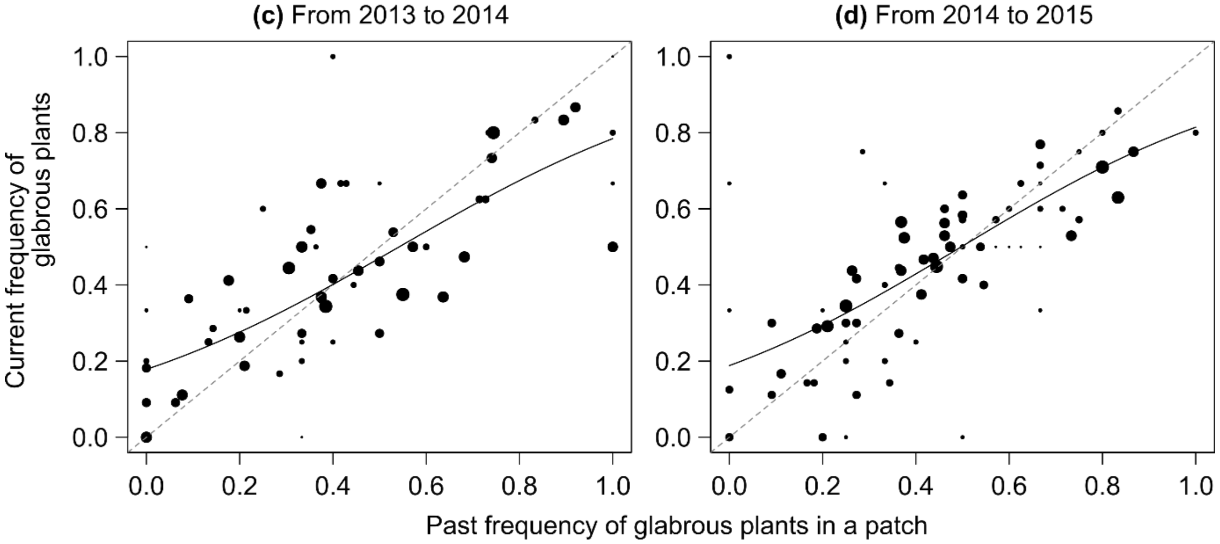


Figure S4.2 Flower production of hairy (a: black circles) and glabrous (b: white circles) plants in relation to the frequency of glabrous plants in a field patch. A single point represents a patch and its size corresponds to the number of plants in a patch. Bars indicate SEM of the number of flowers in a patch. No significant effects were detected in generalized linear mixed models for the frequency of glabrous plants (hairy plants, coefficient ± SE = –0.002 ± 0.054, Z = –0.03, P = 0.97; glabrous plants, –0.12 ± 0.10, Z = –1.12, P = 0.26) and the total number of plants in a patch (hairy plants, –0.11 ± 0.10, Z = –1.12, P = 0.26; glabrous plants, 0.11 ± 0.15, Z = 0.76, P = 0.45). Panel (c) and (d) present the same data with the main figure (Fig. 4.1c, d) but the trend curves were depicted on the basis of logistic regressions.

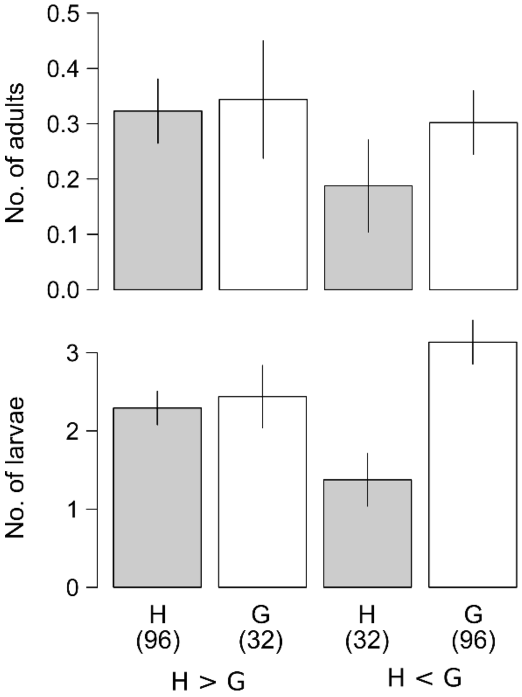


Figure S4.3 The number of larval and adult *Phaedon brassicae* harbored on hairy (H: grey) and glabrous (G: white) plants under the hairy-abundant (H > G) and glabrous-abundant (H < G) condition in the mesocosm experiment. Bars indicate SEM of the raw data. Sample sizes are shown within parentheses.

Chapter 5
Tests of associational effects against multiple species of insect herbivores on the trichome dimorphism

Abstract

Trichome-producing (hairy) and trichomeless (glabrous) plants of *Arabidopsis halleri* subsp. *gemmifera* were investigated to test whether plant resistance to herbivory depends on the plants' phenotypes and/or the phenotypes of neighbouring plants (associational effects). We first conducted a common garden experiment in which the relative frequency of hairy and glabrous plants was manipulated. Two species of leaf-chewing insects (larvae of a white butterfly and a cabbage sawfly) were found less often on hairy plants than on glabrous plants. In contrast, the numbers of aphids and flea beetles did not differ significantly between hairy and glabrous plants. For none of these insects did abundance depend on the frequency of the two plant morphs. A field survey was then conducted in two natural populations of *A. halleri*. In the first population, a species of white butterfly was the dominant herbivore, and hairy plants incurred less leaf damage than glabrous plants across two years. In contrast, in the other population, where flea beetles were dominant, there were no consistent differences in leaf damage between the two types of plants. In neither of the two populations did we find evidence for associational effects. This study did not provide any conclusive evidence of associational effects of anti-herbivore resistance, but we did find that trichomes can confer resistance to certain herbivores. Given the results of previous chapter's work on associational effects against a flightless leaf beetle, such associational effects of the trichome dimorphism of *A. halleri* were herbivore-specific.

Introduction

As sessile organisms, plants are consumed by a diverse array of insect herbivores (Strong *et al*. 1984; Lewinsohn *et al*. 2005). Thus, plants developed various resistance traits to protect themselves from herbivory (Schoonhoven *et al*. 2005). For example, trichomes (plant epidermal hairs) are considered a physical resistance trait against herbivory (Levin 1973; Hanley *et al*. 2007). There is increasing evidence that trichomes alter the feeding efficiency and oviposition preferences of various herbivores, including lepidopterans and coleopterans (e.g., Zvereva *et al*. 1998; Reymond *et al*. 2004; Handley *et al*. 2005; Sletvold *et al*. 2010; Yamawo *et al*. 2012). Plant trichomes are also known to show large phenotypic variation that is governed genetically (Ågren and Schemske 1994; Kivimäki *et al*. 2007; Bloomer *et al*. 2012). Although trichomes exhibit quantitative phenotypic variation in their density or number (Ågren and Schemske 1994), the presence/absence of trichomes (i.e., trichome-producing and trichomeless plants) is sometimes regulated by a few major genes (Kivimäki *et al*. 2007; Kawagoe *et al*. 2011; Bloomer *et al*. 2012).

When anti-herbivore resistance traits are sufficiently varied within a plant community or population, the amount of herbivory on an individual plant depends not only on the plant's own phenotype but also on the neighboring plant types (Atsatt and O'Dowd 1976; reviewed by Agrawal *et al*. 2006; Barbosa *et al*. 2009). This phenomenon is referred to as an associational effect and includes any consumer effect mediated by the composition of plant types within a neighborhood on a focal individual plant (Underwood *et al*. 2014). For example, palatable plants benefit from the presence of unpalatable plants via associational resistance if the latter repels herbivores away from the nearby palatable plants (Tahvanainen and Root 1972). In contrast, unpalatable plants suffer from associational susceptibility when herbivores settle on palatable plants and then disperse onto unpalatable ones (White and Whitham 2000). Associational effects are predicted to influence plant diversity, because they may regulate population growth of multiple plant types in a density- or frequency-dependent manner (Hambäck *et al*. 2014).

To date, associational effects have been mostly investigated as a type of interspecific interaction (e.g., Tahvanainen and Root 1972; White and Whitham 2000; Le Guigo *et al*. 2012), but they can also be examined through the lens of intraspecific variation of anti-herbivore resistance traits (Wise *et al*. 2009; Hambäck *et al*. 2009). Several studies of cultivated plants have documented associational effects among resistant and susceptible genotypes within a plant species (Cantero and Sanford 1984; Hambäck *et al*. 2009). In these cases, a focal plant genotype may benefit via associational effects from the increasing proportion of another genotype in close vicinity. These interactions lead to frequency dependence in herbivory, which can promote the coexistence of different genotypes if rare genotypes are protected from herbivory (Wise *et al*. 2009; Underwood *et al*. 2014). Little is known about associational effects and their frequency dependence with respect to natural variation in anti-herbivore resistance traits within a plant species.

Arabidopsis halleri (L.) O'Kane & Al-Shehbaz subsp. *gemmifera* (Matsum.) O'Kane & Al-Shehbaz [Brassicaceae/ Cruciferae] has both trichome-producing (referred to hereafter as hairy) and trichomeless (glabrous) plants. The presence or absence of trichomes is associated with the allelic status of a candidate gene (Kawagoe *et al*. 2011). Our previous study found that leaf damage on *A. halleri* depended on the relative frequency of neighboring hairy and glabrous plants in a natural population where plants were heavily infested by an oligophagous leaf beetle, *Phaedon brassicae* Baly [Coleoptera: Chrysomelidae] (Sato *et al*. 2014). In a choice experiment, we also revealed that when hairy plants were rare, they incurred less herbivory by *P. brassicae* than did glabrous plants, whereas when hairy plants were common, they were not resistant to herbivory (Sato *et al*. 2014). However, white butterflies, flea beetles, moths, and aphids can also be observed in natural populations of *A. halleri* (Y. Sato, personal observation), and the dominant herbivores vary even between closely located populations (Kawagoe and Kudoh 2010). It is thus possible that patterns of associational effects depend on the herbivore species on the trichome dimorphism of *A. halleri*. Here, we examined whether such associational effects exist against herbivores other than the leaf beetle, *P. brassicae*.

In this study, two specific questions were addressed: (1) Does trichome production act as an anti-herbivore resistance trait, and if so, to which herbivores does it provides resistance? (2) Do any of the anti-herbivore effects of trichomes depend on the relative frequencies of hairy and glabrous plants? We performed both an experimental and an observational study to answer these questions. First, we manipulated the relative frequency of hairy and glabrous plants in a common garden to test the resistance role of trichomes and their associational effects against herbivores. Second, we conducted a series of field surveys under different herbivore community structures to examine whether similar patterns of herbivory occurred in natural populations.

Materials and Methods

Study system

Arabidopsis halleri subsp. *gemmifera* is a perennial herb distributed across Japan and the Russian Far East (Al-Shehbaz and O'Kane, 2002). In the lowlands of western Japan, self-incompatible flowers start to bloom in late March or early April, and fruit set is ca. 80% unless the flowers are consumed by herbivores (Kawagoe and Kudoh 2010). After flowering, plants produce new rosettes on the main and axillary meristems of flowering stems, and these rosettes often establish as clonal offspring once they have rooted and attached themselves to the ground. Hairy and glabrous plants co-occur in a natural population in central Japan (Kawagoe *et al*. 2011). The hairy and glabrous phenotypes in this population are associated with allelic variation in a candidate gene, *GLABROUS1* (*GL1*) but are not associated with other genes (Kawagoe *et al*. 2011; see Grebe 2012 for a review of *GL1* in *A. thaliana*). Hairy plants develop trichomes on the surfaces of their leaves and stems, but not on the flowers and fruits, whereas glabrous plants have no trichomes except along the leaf margin. The two morphs have no other apparent morphological differences. Hairy plants produced fewer fruits than glabrous plants under weak herbivory (Kawagoe *et al*. 2011), indicating a trade-off with trichome production. Glucosinolate profiles were not associated with either the hairy or the glabrous phenotype in our seed source population, as described below (Sato *et al*. 2014).

Common garden experiment

Mature fruits of *A. halleri* were collected from a natural population located in Taka-cho, Hyogo Prefecture, central Japan (35°06′N, 134°56′E, ca. 200 m in altitude, Sato *et al*. 2014). The fruits were harvested from 14 maternal plants (including seven hairy and seven glabrous plants) in early July 2011; the maternal plants were spaced at least 5 m apart to minimize the likelihood of multiple samples being taken from a single clone. These fruits provided the seeds from which the plants in our experiment were grown.

For the common garden experiment, we initially prepared ca. 200 plants in an indoor space that contained no herbivores. Fifty seeds from each maternal family were sown on a petri dish (diameter 9 cm, depth 1.5 cm) filled with moistened quartz sand on 15 August 2011 and were allowed to germinate at room temperature. Three seedlings per family were transplanted to a plastic pot (diameter 10.5 cm, depth 9 cm) filled with mixed soil (pumice:leaf mold:peat moss = 1:1:1) on 25 November 2011. All seedlings had two to four leaves when they were transplanted. To prevent herbivory, these plants were placed inside transparent plastic cases (75 cm × 45 cm with a depth of 18 cm, 28 pots per case). The plants grew for four months under natural sunlight (10–12 h in day length) with average daily temperatures of 10–30 °C. The location of the cases was rotated every month. All plants were transplanted individually into plastic pots about a month before the start of the experiment to avoid competition within the cases. The liquid fertilizer Hyponex (Hyponex, N:P:K = 6:10:5, Hyponex Japan, Osaka, Japan) was diluted 1000-fold and supplied monthly until the beginning of the experiment. Photosynthetically active radiation was 800–1100 and 100–300 μmol/m^2/s on a sunny and cloudy day, respectively. The red:far-red ratio ranged from 1.0 to 1.3 (LI-190 Quantum Sensor, LI-COR, Lincoln, NE, USA).

The potted plants were transferred to the experimental garden of the Center for Ecological Research, Kyoto University (34°58′N, 135°57′E, ca. 100 m in altitude) on 28 March 2012. All cultivated plants were sorted in ascending order of leaf length (the length of the longest radical leaf, which ranged from 33 to 71 mm), and the largest 150 plants from the 14 maternal families were used in the experiments, in order to ensure that similarly sized plants were used. We controlled for plant size to avoid confounding effects on herbivore abundance per plant.

Twenty-five individuals were arranged squarely in each of six 1 × 1 m plots filled with fine gravel. We established two treatments for the frequency of hairy and glabrous plants within a plot (Fig. 5.1); three plots consisted of 21 hairy and four glabrous plants (referred to as hairy-abundant plots), and the remaining three consisted of four hairy and 21 glabrous plants (referred to as glabrous-abundant plots). The plots were spaced 1.7 m apart. The hairy or glabrous plants of similar size (< 11 mm difference in the length of the longest radical leaf within a single plot) were placed in the center of the plot, while plants of random size occupied the positions at the edge (Fig. 5.1).

Herbivore abundance and plant performance were monitored weekly on sunny days from April to June 2012 (2, 9, 17, 25, and 30 April; 7, 14, 21, and 28 May; 4, 11, 18, and 25 June). The mustard aphid *Lipaphis erysimi* Kaltenbach [Hemiptera: Aphididae] and green peach aphid *Myzus persicae* Sulzer [Hemiptera: Aphididae] occurred on *A. halleri* at our experimental site (see Supporting information, Figure S5.1). The small cabbage white butterfly *Pieris rapae* L. [Lepidoptera: Pieridae], cabbage sawfly *Athalia infumata* Marlatt [Hymenoptera: Tenthredinidae], and flea beetle *Phyllotreta striolata* Fabricius [Coleoptera: Chrysomelidae] also occurred, mainly after the flowering season (Table 5.1; Figure S5.1). Wingless and winged aphids were counted separately, as the former represented a growing colony while the latter was likely to be associated with dispersal events. We counted *L. erysimi* and *M. persicae* as a single group because the two species were difficult to distinguish accurately in the field due to their similar colors and morphology at the nymph stage. In addition, we recorded the number of mummies (i.e., aphids fed on by parasitoid wasps) after the peak abundance of wingless aphids (14 May 2012). The numbers of intact and damaged leaves were recorded separately in order to distinctly evaluate plant growth and herbivory damage. The numbers of flowers and mature fruits were counted weekly for each plant. We counted flowers as the number of pedicels, including both fruited and non-fruited flowers, to estimate the flower production of each plant throughout the season. The seed production of each plant could not be evaluated because mature seeds spontaneously dropped; therefore, we used the number of mature fruits to estimate seed production. A mature fruit contained 8.3 ± 2.2 seeds under our experimental conditions (mean ± SD, n = 19 fruits). To avoid edge effects in the plots, we analyzed the nine plants in the center of each plot (Fig. 5.1). Three edge plants died from unknown causes (not related to herbivory levels). All measurements were carried out between 8:00 and 17:00.

Field survey

We conducted field surveys in two natural populations of *A. halleri*; Ojigahata, Shiga (35°12′N, 136°23′E, ca. 300 m in altitude) and Minoh, Osaka, Japan (34°50′N, 135°28′E, ca. 50 m in altitude). The two sites were selected because the green-veined white butterflies, *Pieris napi* L. [Lepidoptera: Pieridae], were predominantly observed at one site (Ojigahata) while the flea beetles *Phyllotreta striolata* were predominantly observed at the other site (Minoh). Both sites were located in gravel areas near roads that were covered with sparse vegetation. The population size and proportion of hairy plants were similar between the two sites (no. of plants = 500–1000, percentage of hairy plants = 40–60%). We observed other cruciferous plants (*Cardamine hirsuta*, *C. impatiens*, *C. leucantha*, and *C. scutata*) at these sites, but they occurred only rarely.

Field surveys were conducted twice a year in spring (late May or early June) and autumn (late September or early October), as *P. napi* shows bivoltinism (Fukuda *et al*. 1984). For each population and census, we recorded the trichome phenotype (hairy or glabrous) and the proportion of leaf area lost to herbivory for all individual plants in randomly chosen circular patches (1 m in diameter). Our preliminary survey confirmed that the number of plants within each circular patch began to plateau as the patch size increased. At the Ojigahata site, 2.92 ± 0.25, 5.52 ± 0.70, and 7.40 ± 1.02 plants occurred within 0.5-, 1.0-, and 3.0-m–diameter patches, respectively (mean ± SE, n = 25 patches examined). At the Minoh site, 3.71 ± 0.29, 7.68 ± 0.61, and 10.68 ± 0.95 plants occurred within 0.5-, 1.0-, and 3.0-m–diameter patches, respectively (mean ± SE, n = 28 patches). Therefore, we focused on the local interaction in 1-m–diameter patches. We examined 70–90 patches, including 150–500 hairy and 200–550 glabrous plants, at the Ojigahata site; and we examined 65–85 patches, including 180–350 hairy and 290–380 glabrous plants, at the Minoh site (see also Table 5.2 and 5.3 for detailed survey dates and sample sizes). The proportion of leaf area lost to herbivory (referred to hereafter as the leaf damage) was evaluated visually and recorded as one of 11 successive values: 0 (no damage), 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0 (complete leaf loss). In addition, 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 of 19 plants at the Ojigahata site. This additional measurement confirmed that the leaf damage estimated by our method was highly correlated with the total proportion of leaves damaged (Pearson's product moment correlation; both variables were arcsine-transformed, $r = 0.92$, $t_{17} = 9.4$, $P < 0.0001$). We also identified and counted herbivorous insects within the study area during the survey. All observations and surveys were carried out for 4–6 hours between 9:00 and 16:00.

Statistical analysis

We used the data collected in our experiment to analyze the effects of the trichome phenotype (hairy or glabrous phenotype for each plant) and frequency condition (hairy-abundant or glabrous-abundant for each plot) as fixed effects explaining the number of herbivores, flowers, or leaves per plant (response variables). The interaction term of the trichome phenotype × frequency condition was incorporated as a fixed effect to determine whether the main effects of the trichome phenotype on the herbivore abundance or plant performance depended on the frequency condition (namely, associational effects). To analyze the count response, we used generalized linear mixed models (GLMMs: Bolker *et al*. 2009) with a Poisson error structure and a log link function. The significance levels of the main effects were first analyzed using likelihood ratio tests. Then, the trichome-by-frequency interaction was analyzed to test associational effects. The cumulative number of each herbivore per plant was analyzed as the response variable to reflect both herbivory intensity and duration for plants (i.e., herbivory load: Ruppel 1983; Fournier *et al*. 2005). As an exception, the number of mummified aphids was analyzed using the abundance that was recorded on 28 May because the mummified aphids were attached to plant surfaces and reflected the accumulated number by themselves.

Aphids were observed on leaves throughout the study, whereas white butterflies, cabbage sawflies, and flea beetles were observed only during June (Figure S5.1). Thus, to exclude the direct confounding interactions of the late-coming herbivores, we separately analyzed the cumulative number of wingless and winged aphids counted per plant before 28 May. To examine plant fecundity and growth corresponding to this period, we analyzed the data collected until the end of May regarding the number of flowers and intact leaves. The number of mature fruits was also analyzed; however, the result is not presented in the main text because it followed a similar pattern to that of the number of flowers during the early period of the experiment (Figure S5.2) and no significant effects were detected regarding the number of fruits (see Table 5.1 and Results section below). A small number of leaves (median = 0 leaves) were damaged in the period before 28 May 2012 (Figure S5.2); therefore, the data for this variable during the early period were not analyzed.

For the late-coming herbivores (white butterflies, sawflies, and flea beetles), we analyzed the cumulative number of eggs and larvae of *P. rapae*, larvae of *A. infumata*, and adults of *P. striolata*, in which the cumulative number of each herbivore was calculated throughout the study. Then, to examine the effects of the late-coming herbivores on plant growth, we analyzed the number of damaged leaves at the end of the study (25 June 2012). Many leaves were damaged by the end of the experiment (Figure S5.2); thus, their number was sufficient to estimate the degree of herbivory on the two types of plants. The number of mature fruits during the late season was not analyzed because the impact of these late-coming insects was so severe that a large proportion of the fruits was lost to herbivory.

In some of the GLMM analyses, we included an offset term of $\log_e (x + 1)$ to examine the rates of the count response (Nelder and Frome 1984). This method allows us to handle the effects of sampling effort, or total counts, as a covariate without estimating a slope coefficient in the analysis of the count response. For the analyses of winged and mummified aphids, we included the number of wingless aphids as an offset term to consider the density-dependent dispersal or parasitism on living aphids. The number of flowers was included as an offset term to analyze fruit set (that is, the proportion of mature fruits in the total number of flowers produced) for the number of mature fruits. To analyze the number of damaged leaves, we included the number of intact leaves as an offset term to evaluate the per capita leaf damage.

We incorporated two random effects into all of the GLMMs above. First, to reflect the variance of herbivore abundance and plant performance among the plots in the analyses, we included the plot ID as a random effect. Second, because we designed the experiment to control for plant size, the number of individual plants differed between maternal families (mean ± SD = 4.2 ± 2.8, n = 14 maternal plants). Thus, the seed plant ID was incorporated as another random effect to consider the potential variation in other plant traits related to the maternal families.

We used the field survey data to analyze the effects of the trichome phenotype, the proportion of glabrous plants in a patch (which represents the relative frequency of the two plant types), and the total number of plants (which represents the density of *A. halleri* plants) as fixed effects explaining the leaf damage. Leaf damage was analyzed using linear mixed models, in which the response variable was arcsine-square root transformed to improve the normality of residuals. To test whether the effect of the trichome phenotype on leaf damage depended on the proportion of glabrous plants (i.e., associational effects), we also analyzed the interaction between the trichome phenotype and the proportion of glabrous plants in a patch as another fixed effect. The patch ID was incorporated as a random effect so that multiple individuals in a patch were not treated as independent samples. We used likelihood ratio tests to analyze the significance of each fixed effect, in which the main effects were first tested and then the interaction term was examined. Furthermore, in the linear mixed models, we estimated coefficients of the terms ‘the proportion of glabrous plants in a patch’ and ‘the 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 leaf damage. When the coefficients significantly deviated from zero, we added trend lines to figures using single regression of the leaf damage on the proportion of glabrous plants in a patch. These analyses were separately performed for each survey at the Ojigahata and Minoh sites.

All statistical analyses were performed using R version 2.15.0 (R Development Core Team 2012). We used the glmer and lmer functions (in the lme4 package: Bates *et al.*

2012) for GLMMs and linear mixed model analyses. We chose the maximum likelihood method for the lmer function and the Laplace approximation for the glmer function to estimate likelihoods and coefficients.

Results

Common garden experiment

Aphids emerged in late April, followed by *P. rapae*, *A. infumata*, and *P. striolata* (Figure S5.1). We also observed a few *Thrips palmi* Karny [Thysanoptera: Thripidae] and *Plutella xylostella* L. [Lepidoptera: Yponomeutidae] on *A. halleri* during June, but their abundance was not recorded because they were rare. Three of the 240 *P. rapae* larvae counted in this study were attacked by parasitoid wasps. Although we counted aphids as a single herbivore group, detailed identification under a binocular microscope on 30 April 2012 revealed that 67 specimen samples contained 24 individuals of *L. erysimi* and 43 individuals of *M. persicae*.

During the early period, the number of wingless aphids did not differ between hairy and glabrous plants (Fig. 5.2a; Table 5.1), indicating little resistance role of the trichome production against the aphids. Wingless aphids occurred slightly more often on hairy plants under the glabrous-abundant condition (the trichome × frequency interaction, $P < 0.05$: Table 5.1; Fig. 5.2a). The number of winged aphids was not significantly affected by the trichome phenotype (Table 5.1; Fig. 5.2b). Like the wingless aphids, mummified aphids occurred more often on hairy plants under the glabrous-abundant condition (Table 5.1; Fig. 5.2c). During this early period, glabrous plants showed a higher flower production than hairy plants (the main effect of the trichome phenotype, $P < 0.05$: Table 5.1; Fig. 5.2d). The fruit production was not significantly affected by the trichome phenotype, frequency condition, and their interaction (Table 5.1; Fig. S5.2). The number of intact leaves did not differ between hairy and glabrous plants (the main effect of the trichome phenotype, $P = 0.68$: Table 5.1), but glabrous plants had a larger number of intact leaves than hairy ones under the glabrous-abundant condition (the trichome × frequency interaction, $P < 0.05$: Table 5.1; Fig. 5.2e).

During the later period of the experiment, *P. rapae* eggs, *P. rapae* larvae, and *A. infumata* larvae tended to occur less often on hairy plants within the frequency condition (the main effect of the trichome phenotype, $P < 0.05$, $P = 0.07$, and $P = 0.42$ for *P. rapae* eggs, *P. rapae* larvae, and *A. infumata* larvae, respectively: Table 5.1; Figs. 5.2f, g, h), providing evidence for a resistance role of the trichome production against these herbivores. The number of *P. rapae* and *A. infumata*, was not interactively affected by the trichome phenotype and frequency condition (Table 5.1), indicating no associational effects. Adults of the flea beetle, *P. striolata*, occurred slightly more frequently on hairy plants (the main effect of the trichome phenotype, $P = 0.07$: Fig. 5.2i), but there was no evidence for associational effects (i.e., no significant trichome × frequency interaction: Table 5.1). During this later period, hairy plants tended to have fewer damaged leaves than did glabrous plants (Fig. 5.2j), although the main effect of the trichome phenotype was not significant (Table 5.1).

Field survey

The green-veined white butterfly, *Pieris napi*, was a major insect herbivore in the Ojigahata site, whereas the flea beetle, *Phyllotreta striolata*, was dominant in the Minoh site (Table 5.2a). On 15 September 2012 at the Ojigahata site, we found one egg on a hairy plant and eight eggs on glabrous plants. On 27 May 2013 at the Ojigahata site, two eggs were found on glabrous plants and we observed a larva of *P. napi*. Larvae of *A. infumata* were found occasionally at the Ojigahata and Minoh site (Table 5.2a). We did not observe any parasitoids attacking herbivorous insects in the two populations.

There was less leaf damage on hairy plants than on glabrous plants over the course of two years at the Ojigahata site (the main effect of the trichome phenotype, $P < 0.05$ over the four surveys: Table 5.2; Fig. 5.3a). On the other hand, no consistently significant patterns were observed between the leaf damage on hairy and glabrous plants over the course of two years at the Minoh site (Table 5.2; Fig. 5.3b). In these two populations, we rarely found significant interactions (2 out of 16 cases) between the trichome phenotype and the proportion of glabrous plants in a patch (except for 15 September 2012 and 26 May 2013 at the Ojigahata site, $P < 0.05$: Table 5.2), indicating little associational effects on the leaf damage. The proportion of glabrous plants in a patch was not significantly related to leaf damage in 13 out of 16 cases (except for one case at the Ojigahata site and two cases at the Minoh site: Table 5.3; Fig. 5.4). The total number of *A. halleri* in a patch showed few or negative effects from leaf damage; six out of 16 cases were significantly negative (two cases at the Ojigahata site and four cases at the Minoh site: Table 5.3).

Discussion

In this study, we were unable to detect any conclusive evidence of associational effects; however, we did find evidence to suggest a role for trichomes as an anti-herbivore resistance trait against certain herbivores. In the common garden experiment, we observed fewer eggs and larvae of the small cabbage white, *Pieris rapae*, on hairy plants than on glabrous plants. We also found that larvae of the cabbage sawfly, *Athalia infumata*, tended to occur less often on hairy plants than on glabrous plants. During the later period when these two species of leaf chewers were abundant, hairy plants tended to suffer less leaf damage than glabrous plants. In contrast, we did not observe a reduced abundance of aphids (*Myzus persicae* and *Lipaphis erysimi*) and flea beetles (*Phyllotreta striolata*) on hairy plants in the common garden. In the field surveys, hairy plants incurred slightly less herbivory than glabrous plants in a natural population where the green-veined white butterfly, *Pieris napi*, was dominant. In contrast, no consistent differences in leaf damage were detected between hairy and glabrous plants in the other population where *P. napi* was rarely observed and where the flea beetle was dominant.

Anti-herbivore resistance functions of trichomes have been reported for interactions between other *Arabidopsis* trichomes and leaf-chewing insects. Leaf trichomes are known to prevent damage by the diamondback moth *Plutella xylostella* in *A. lyrata* (Sletvold *et al*. 2010) and to reduce the performance of *P. rapae* larvae in *A. thaliana* (Reymond *et al*. 2004; reviewed by van Poecke 2007). In addition, in *A. halleri*, our previous study revealed that trichome production influenced the feeding preferences of adults and reduced the performance of larvae of the leaf beetle *Phaedon brassicae* (Sato *et al*. 2014). Given the common feeding habit among the white butterflies and sawflies (which tended to occur less often on hairy plants in the common garden) in addition to the leaf beetles, our findings suggest a role for trichomes in resistance against leaf chewers (Table 5.4). In *A. thaliana* and *A. lyrata*, trichomes are also known to repel oviposition by *P. xylostella* (Handley *et al*. 2005; Sletvold *et al*. 2010). The result that fewer eggs of *P. rapae* were observed on hairy plants in our common garden experiment may support such negative effects of trichomes on the oviposition preference of lepidopterans.

Plant trichomes are not always effective as a mechanism of herbivore resistance. Their effects depend on herbivore feeding habits (Andres and Conner 2003), herbivore life stage (Yamawo *et al*. 2012), and plant ontogeny (Puentes and Ågren 2013). For instance, in a community-level study of manzanita (*Arctostaphylos*) species, Andres and Conner (2003) suggested that trichomes were ineffective as a resistance trait against small or sedentary herbivores because such herbivores have less contact with plant hairs during their life cycles. In our study, aphids infrequently contacted *A. halleri* trichomes because the flowering buds of *A. halleri* did not produce trichomes, and many of the aphids were observed on the tops of flowering stems (Y. Sato, personal observation). In addition, young leaves of hairy plants produce ca. 60 trichomes/cm^2, but the trichome density becomes low (ca. 10 trichomes/cm^2) in mature leaves (Chapter 2 of the present thesis). Thus, flea beetle (*Phyllotreta striolata*) adults, which have a body size of a few millimeters, contacted few hairs at such a sparse trichome density. Furthermore, adult flea beetles can move from one leaf to another by jumping, so trichomes are unlikely to interfere with their mobility (Table 5.4). The potential role of trichomes in resistance should be tested with regards to plant ontogeny in order to fully understand factors determining the effectiveness of trichomes against herbivores.

Regarding associational effects in morphological traits, Wise *et al*. (2009) reported that resistance to gall flies did not depend on the frequency of the erect-stemmed and candy-cane phenotypes of *Solidago altissima* in 1.5-m^3 cages. Here, we also found limited evidence of associational effects between white butterflies and the trichome phenotypes of *A. halleri*. Given that adult white butterflies can move among plant patches by flying, we speculate that associational effects are unlikely to cause resistance to oviposition by the butterflies at our 1-m patch scale (Table 5.4). Although this study did not detect associational effects, our previous study found that the magnitude of herbivory on hairy plants depended on the proportion of glabrous plants within 1-m diameter patches in another population where the flightless leaf beetle *Phaedon brassicae* was the predominant herbivore on *A. halleri* (Table 5. 4; Sato *et al*. 2014). Taken together, our data suggest that herbivore mobility affects the occurrence of associational effects between herbivory and trichome phenotype of *A. halleri* (Table 5.4).

As for another ecological function, plant trichomes are known to interfere with the foraging behavior of predators (e.g., Kauffman and Kennedy 1989; reviewed by Dalin *et al*. 2008). We observed a parasitoid, *Aphidius* sp., in the experimental plots, but the number of parasitized aphids was not fewer on hairy plants. Thus this did not support such interfering effects of trichomes on foraging of predators. However, it is still possible that some confounding interactions among herbivores have hindered associational effects. First, size reduction of glabrous plants by two leaf chewers (*Pieris rapae* and *Athalia infumata*) may account for the slightly higher number of *Phyllotreta striolata* on hairy plants in the common garden. Second, the aphid colony contained a specialist, *Lipaphis erysimi*, and a generalist, *Myzus persicae* (Rout and Senapati 1968; Le Guigo *et al*. 2012); therefore, it is possible that anti-herbivore resistance or its associational effects could have been detected if the two species had been distinctly evaluated.

Although the result that abundance of *Pieris rapae* larvae were reduced on hairy plants are congruent with the pattern of leaf damage in a natural population where *Pieris napi* were dominant, one caveat is that *P. rapae* does not utilize *A. halleri* in natural populations (Ohsaki and Sato 1999). Ohsaki and Sato (1999) showed that larvae of *P. rapae* were able to grow on *A. halleri*, but adults did not oviposit on *A. halleri* when they had access to other cruciferous plants, such as *Brassica* and *Raphanus* species. Thus, our results of the common garden experiment should be interpreted carefully about to what extent they are comparable to patterns of leaf damage in a population where a congener, *P. napi*, is the dominant herbivore.

In conclusion, although this study does not provide any conclusive evidence of associational effects of anti-herbivore resistance, we did find that trichomes can act as a resistance trait against certain herbivores in *A. halleri*. However, we should note that conditions under which associational effects can be detected sometimes depend on spatial scales (Janz *et al*. 2005; Hambäck *et al*. 2009). For example, Janz *et al*. (2005) illustrated that the oviposition preference of a polyphagous butterfly, *Polygonia c-album*, did not depend on the frequency of *Urtica dioica* and *Salix caprea* within a patch, but such frequency-dependent oviposition was found among plant patches. In our study, it remains possible that associational effects against flying herbivores (e.g., adults of *Pieris* species) can be detected at a spatial scale greater than 1 m^2. Thus, in future studies, it would be valuable to examine the effects of spatial scales in order to understand when associational effects occur in anti-herbivore resistance.

Table 5.1 Effects of the trichome phenotype and frequency condition on the cumulative number of herbivores and plant performance (the number of flowers or leaves) in the experiment. Early-season herbivores (aphids) and late-season herbivores (*Pieris rapae*, *Athalia infumata*, and *Phyllotreta striolata*) were separately analyzed. Plant performance was also separately analyzed according to the analyses of herbivore abundance. Main effects were tested first and then an interaction term was analyzed. Likelihood ratio tests were performed using GLMMs, where the bold and underlined values indicate significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.1$) effects, respectively.

Fixed effects	#Wingless aphids			#Winged aphids*			#Mummified aphids*			#Flowers			#Matured fruits**			#Intact leaves		
	df	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*
Trichome	1	0.7	0.39	1	0.4	0.52	1	3.6	0.06	**1**	**3.9**	**0.05**	1	0.2	0.65	1	0.17	0.68
Frequency	1	0.7	0.39	1	2.5	0.12	1	0.001	0.98	**1**	**4.7**	**0.03**	1	0.01	0.93	1	2.1	0.15
Tri. × Freq.	**1**	**5.7**	**0.02**	1	2.8	0.10	1	3.7	0.05	1	3.5	0.06	1	0.04	0.84	**1**	**10.7**	**0.001**
Residuals	48	860	---	48	73.6	---	48	80.2	---	48	84.9	---	48	32.4	---	48	242.4	---

*No. of wingless aphids was included as an offset term; **No. of flowers was included as an offset term.

Fixed effects	#*P. rapae* eggs			#*P. rapae* larvae			#*A. infumata* larvae			#*P. striolata* adults			#Damaged leaves***		
	df	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*
Trichome	**1**	**5.4**	**0.02**	1	3.3	0.07	1	0.6	0.42	1	3.2	0.07	1	2.3	0.13
Frequency	1	3.0	0.09	1	0.03	0.87	1	0.2	0.62	1	0.2	0.65	1	0.03	0.87
Tri. × Freq.	1	0.01	0.93	1	0.5	0.47	1	0.002	0.97	1	0.5	0.50	**1**	**138.7**	**< 0.001**
Residuals	48	54.8	---	48	98.4	---	48	67.8	---	48	56.1	---	48	791.4	---

***No. of intact leaves was included as an offset term.

Table 5.2 Insect herbivores counted in the study sites (a) and effects of the trichome phenotype, proportion of glabrous plants in a patch, and total number of *Arabidopsis halleri* plants in a patch on leaf damage (arcsine-transformed proportion of the leaf area loss) (b) at the Ojigahata site and (c) at the Minoh site. Likelihood ratio tests were performed using linear mixed models. Main effects were tested first and then an interaction term was analyzed. The bold and underlined values indicate significant ($P < 0.05$) and marginally significant ($0.05 < P < 0.1$) effects, respectively. The patch ID was incorporated as a random effect in these analyses.

(a) Insect herbivores

Species	2011 Autumn		2012 Spring		2012 Autumn		2013 Spring	
	Ojigahata	Minoh	Ojigahata	Minoh	Ojigahata	Minoh	Ojigahata	Minoh
Pieris napi adults*	8	0	8	0	22(9)	0	8(3)	0
Phyllotreta striolata adults	0	0	15	3	2	0	33	0
Athalia infumata larvae	0	0	1	7	0	0	0	0

*No. of eggs and larvae are shown within parentheses.

(b) Ojigahata

Fixed effects	23 September 2011			27 May 2012			15 September 2012			26 May 2013		
	df	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*
Trichome	**1**	**20.8**	**< 0.001**	**1**	**7.3**	**< 0.01**	**1**	**4.1**	**0.04**	**1**	**36.1**	**< 0.001**
Proportion of glabrous plants	**1**	**5.9**	**0.02**	1	0.4	0.56	1	0.1	0.76	1	0.9	0.34
Total number of plants	1	0.48	0.49	1	0.05	0.83	**1**	**5.0**	**0.03**	1	2.1	0.15
Tri. × Prop. of glabrous plants	1	0.50	0.48	1	0.01	0.91	**1**	**4.8**	**0.03**	**1**	**4.5**	**0.03**
Residuals	1016	-844.4	---	505	-437.3	---	383	-310.9	---	441	-393.1	---

(c) Minoh

Fixed effects	1 October 2011			3 June 2012			22 September 2012			1 June 2013		
	df	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*	*df*	Deviance	*P*
Trichome	1	3.7	0.06	1	0.01	0.93	**1**	**37.2**	**< 0.001**	**1**	**13.6**	**< 0.001**
Proportion of glabrous plants	1	1.5	0.22	1	0.01	0.93	1	0.8	0.37	**1**	**5.0**	**0.025**
Total number of plants	1	1.0	0.31	1	3.8	0.05	**1**	**11.1**	**0.001**	**1**	**7.3**	**< 0.01**
Tri. × Prop. of glabrous plants	1	1.3	0.26	1	2.2	0.13	1	0.3	0.57	1	3.6	0.06
Residuals	691	-687	---	471	-371.9	---	561	-553.1	---	571	-409.6	---

Table 5.3 Effects of the proportion of glabrous plants in a patch and the total number of *Arabidopsis halleri* plants in a patch on leaf damage (arcsine-transformed proportion of the leaf area loss) (a) at the Ojigahata site and (b) at the Minoh site. Standardized coefficients and their standard error (SE) in linear mixed models explaining the leaf damage are shown for the two factors, in which bold values indicate a significant deviation of coefficients from zero at $P < 0.05$ (Wald tests). The patch ID was incorporated as a random effect in these analyses. The number of plant patches examined is shown within the parentheses beside the survey dates, and the number of hairy or glabrous plants examined is presented within the parentheses below each survey date. The observational evidence of herbivore fauna was described in Table 5.2.

(a) Ojigahata

Fixed effects	23 September 2011 (80)		27 May 2012 (72)		15 September 2012 (90)		26 May 2013 (70)	
	Hairy (498)	Glabrous (523)	Hairy (180)	Glabrous (330)	Hairy (178)	Glabrous (210)	Hairy (156)	Glabrous (290)
Proportion of glabrous plants	-0.07 ± 0.06	**-0.12 ± 0.06**	0.08 ± 0.08	0.01 ± 0.06	-0.12 ± 0.07	0.10 ± 0.09	0.09 ± 0.08	-0.08 ± 0.07
Total number of plants	-0.02 ± 0.08	-0.02 ± 0.07	0.07 ± 0.08	0.002 ± 0.066	**-0.16 ± 0.07**	-0.15 ± 0.10	**-0.23 ± 0.08**	-0.02 ± 0.08

(b) Minoh

Fixed effects	1 October 2011 (67)		3 June 2012 (66)		22 September 2012 (85)		1 June 2013 (70)	
	Hairy (340)	Glabrous (356)	Hairy (186)	Glabrous (290)	Hairy (249)	Glabrous (317)	Hairy (197)	Glabrous (379)
Proportion of glabrous plants	-0.04 ± 0.09	**-0.23 ± 0.07**	-0.07 ± 0.10	0.07 ± 0.08	-0.03 ± 0.09	-0.08 ± 0.07	**0.35 ± 0.08**	0.03 ± 0.07
Total number of plants	-0.06 ± 0.11	-0.12 ± 0.09	**-0.19 ± 0.10**	-0.11 ± 0.08	**-0.31 ± 0.13**	**-0.26 ± 0.09**	-0.08 ± 0.09	**-0.27 ± 0.10**

Table 5.4 Patterns of anti-herbivore resistance and associational effects between different herbivores and hairy and glabrous plants of *Arabidopsis halleri.* Feeding habit, dispersal mode, and body length of these herbivores (less than the maximum size throughout their life cycle) are also listed. Circles and cross marks indicate that associational effects were supported and not supported, respectively. Associational effects cannot be discussed regarding herbivores in which trichomes do not serve as a resistance trait (indicated by 'NA').

Herbivore	Species	Feeding habit	Dispersal	Body length	Resistance effect of trichomes	Associational effects between trichome phenotypes	References
Leaf beetle	*Phaedon brassicae*	Chewer	Walking	< 1.0 cm	○	○	Sato *et al*., 2014
White butterflies	*Pieris rapae*, *Pieris napi*	Chewer	Flying (adult)	< 4.0 cm	○	×	This study
Cabbage sawfly	*Athalia infumata*	Chewer	Flying (adult)	< 2.0 cm	○	×	This study
Flea beetle	*Phyllotreta striolata*	Chewer	Jumping	< 0.3 cm	×	NA	This study
Aphids	*Myzus persicae*, *Lipaphis erysimi*	Sucker	Walking + Flying	< 0.3 cm	×	NA	This study

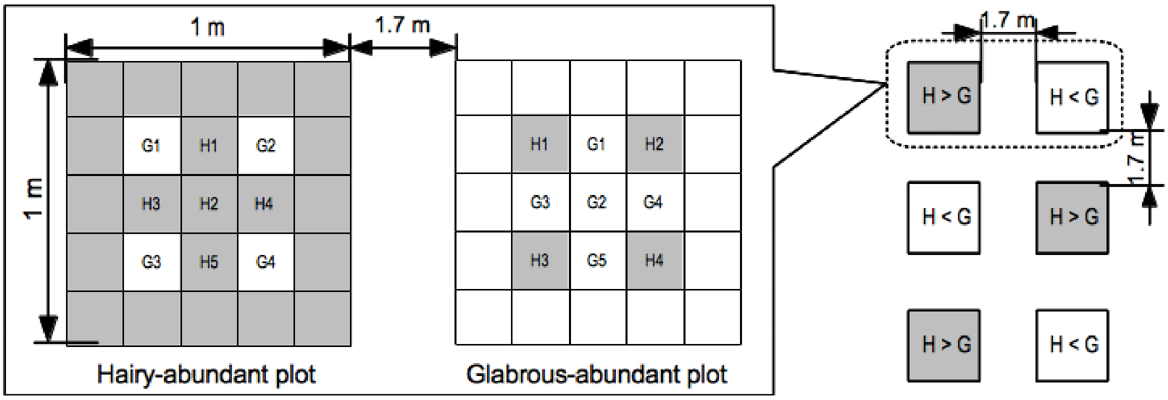


Figure 5.1 A schematic diagram showing the experimental design of the experiment in which the relative frequency of hairy and glabrous plants was manipulated. The hairy-abundant (H > G) plot and glabrous-abundant plot (H < G) consisted of 84% and 16% hairy plants, respectively. Twenty-five plants were planted in a grid, and three replications were set for each plot type. In the left two panels, a filled or open square indicates a hairy (H) or glabrous (G) plant, respectively. Analyzed plants (nine individuals in the center of each plot) were sorted by rosette size, and the number after the 'H' or 'G' indicates the ascending order of sorting. The rest of the hairy or glabrous plants were placed on the edge of the plots with randomization of plant size. Plants were maintained at intervals of 20 cm.

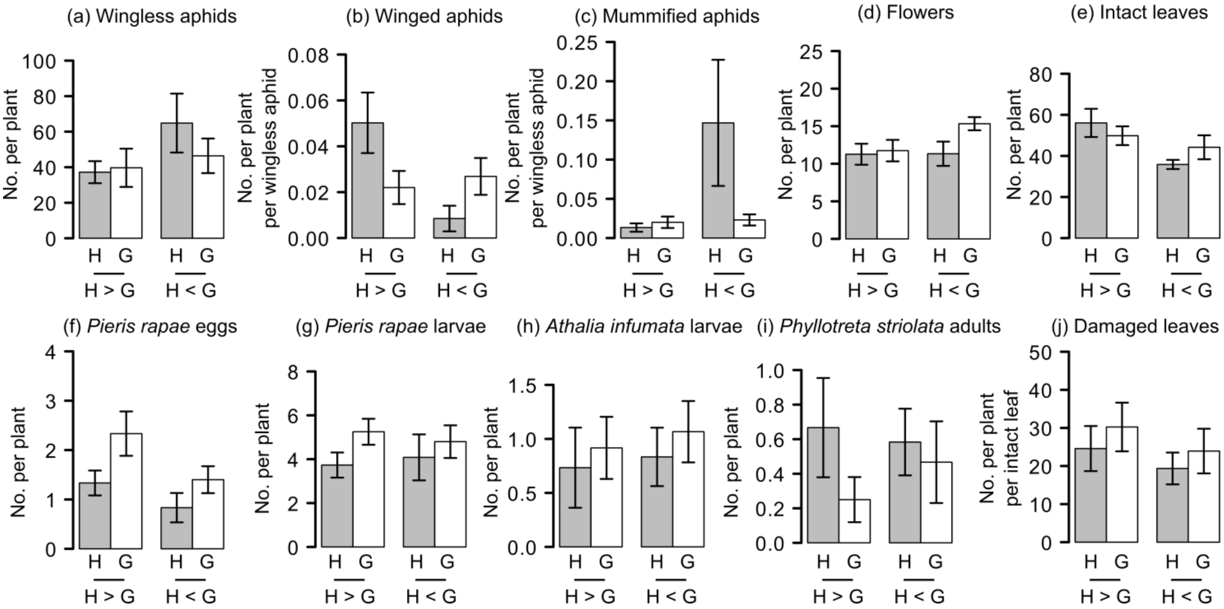


Figure 5.2 The cumulative number (mean ± SE) of herbivorous insects (a–c, f–i) and the number of flowers (d) and leaves (e, j) of the hairy (H; grey) and glabrous (G; white) plants in the hairy-abundant (H > G) and glabrous-abundant (H < G) plots. Data are separately analyzed for the early- and late-season surveys to distinguish the effects of herbivory between the two periods (see also text and Figures S5.1, S5.2).

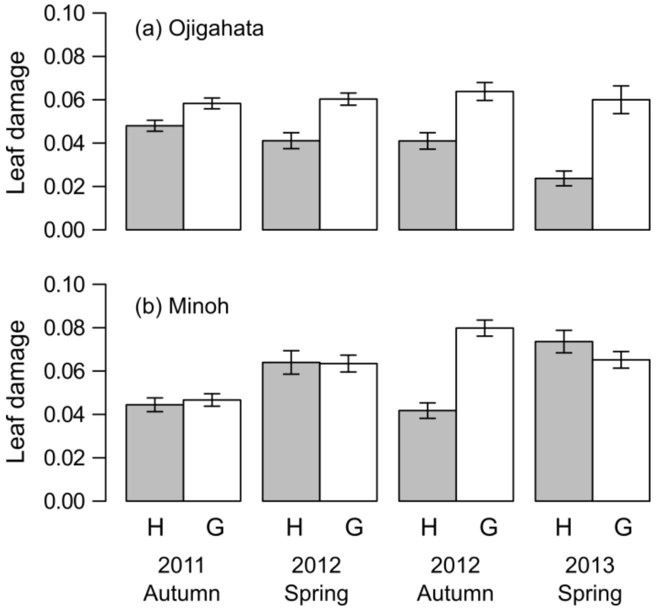
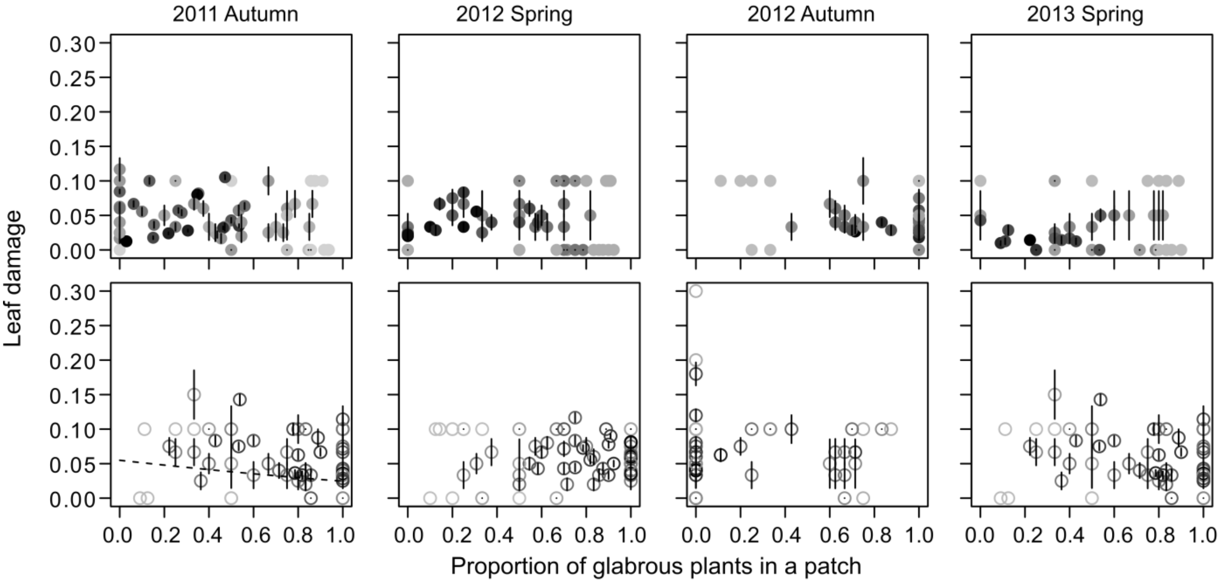


Figure 5.3 Leaf damage (proportion of the leaf area loss: mean ± SE) of hairy (H; grey) and glabrous (G; white) plants (a) at the Ojigahata site and (b) at the Minoh site. Data are not transformed in the figure. Detailed survey dates and observational evidence of herbivore fauna are provided in Table 5.2.

(a) Ojigahata



(b) Minoh

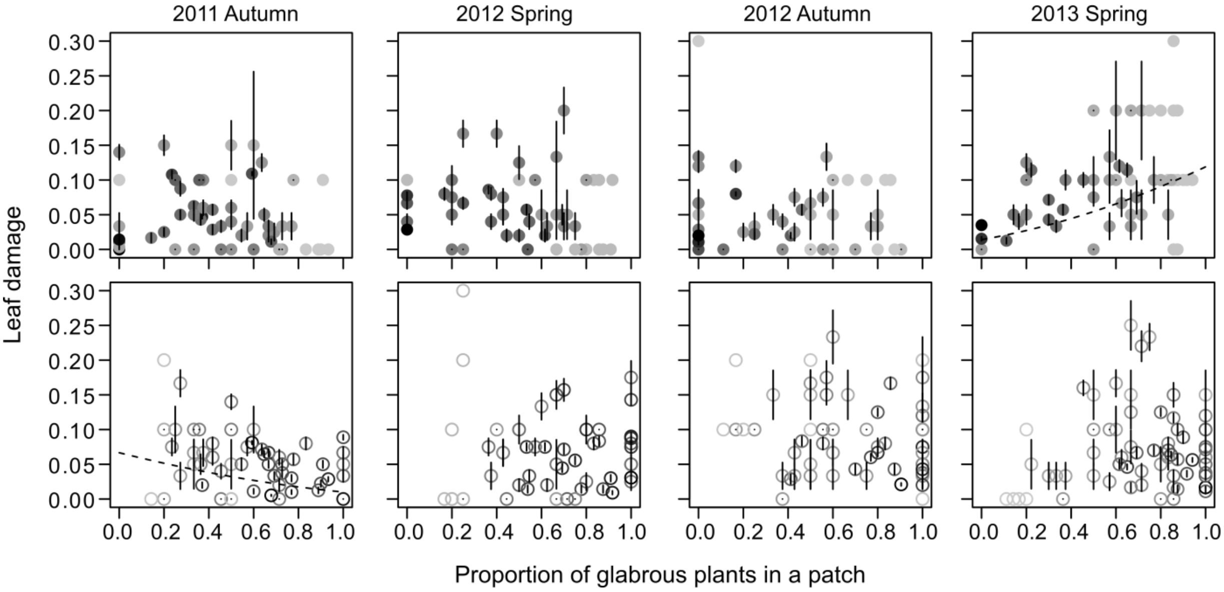


Figure 5.4 Leaf damage (proportion of the leaf area loss) on hairy (black circles) or glabrous (white circles) plants in relation to the increasing proportion of neighboring glabrous plants (a) at the Ojigahata site and (b) at the Minoh site. Circles and vertical bars respectively indicate the mean and SE of leaf damage on individual plants within a 1-m diameter patch. Note that many points do not have error bars because all plants within a patch received the same damage score. Trend lines (dashed lines) were added on the basis of single regressions when the slope coefficients were significant. Darker circles indicate a larger number of plants in a patch. Data are not transformed in the figure. Detailed survey dates are described in Table 5.2 and 5.3.

Supporting information

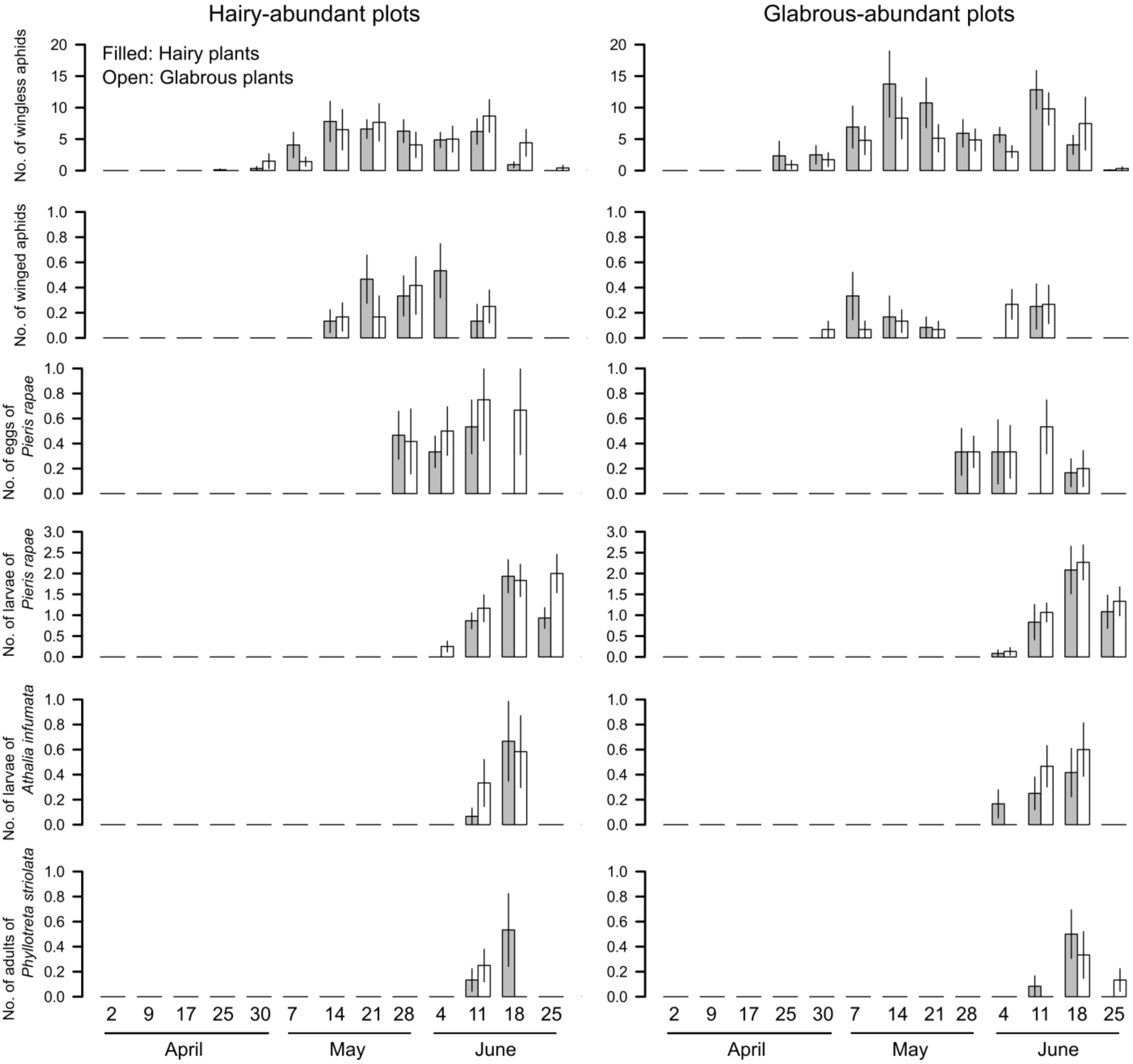


Figure S5.1 Temporal patterns in the number of each herbivore on the hairy (filled) and glabrous (open) plants in the hairy-abundant (left) and glabrous-abundant (right) plots throughout the study. Mean ± SE values are shown.

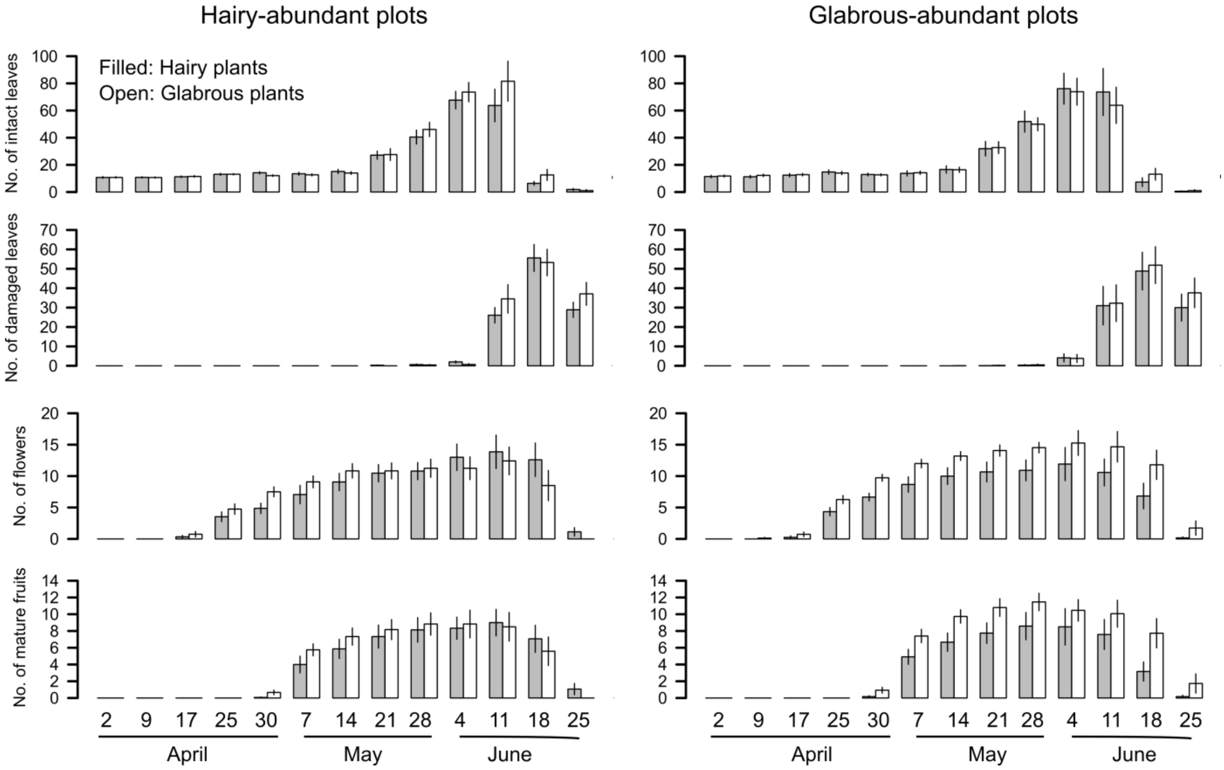


Figure S5.2 Temporal patterns in the performance of hairy (filled) and glabrous (open) plants in the hairy-abundant (left) and glabrous-abundant (right) plots throughout the study. Mean ± SE values are shown. Flowers were counted as the total number of pedicels per plant, including both fruited and non-fruited flowers.

Chapter 6
General discussion

The major findings of our investigation are as follows: The oligophagous leaf beetle, *Phaedon brassicae*, avoided trichome-producing (hairy) leaves in favor of trichomeless (glabrous) leaves when hairy leaves became a minority (chapter 2). Intraspecific associational effects against the leaf beetle caused a rare-morph advantage for hairy plants in defenses and consequently led to a reciprocal rare-morph advantage in growth for hairy versus glabrous morphs (chapter 3). Associational effects against *P. brassicae* mediated negative frequency-dependent selection that could enhance the maintenance of trichome dimorphism within a population (chapter 4). These associational effects specifically occurred against the leaf beetles, while the trichome production could act as a resistance trait to leaf-chewing caterpillars (chapter 5). These results suggest that intraspecific associational effects are influential on the maintenance of trichome dimorphism through the presence/absence of frequency-dependent herbivory.

In this chapter, we first discuss evolutionary implications of frequency-dependent damage under associational effects on the maintenance of defense polymorphism, and then compare our findings with other examples of the maintenance of polymorphism in plants. Finally, we discuss future directions for investigations into intraspecific associational effects and the maintenance of defense polymorphism.

Plant coexistence under associational effects

As noted in the general introduction, the phenomenon of associational effects is widespread in nature (Table 1.1). However, an understanding of their ecological and evolutionary importance has only recently developed. Several authors have pointed out problems with experimental designs for understanding the roles of associational effects (Rautio *et al*. 2012; Hambäck *et al*. 2014; Underwood *et al*. 2014). A summary of study designs in selected publications is shown in Table 6.1 (corresponding to Table 1.1). These studies do not present any consensus regarding the phenotype of a focal plant type (i.e., defended or undefended) and manipulations of the composition of neighboring plants (presence/absence, relative abundance, or total density of multiple plant types) (see Rautio *et al*. 2012 and Underwood *et al*. 2014 for reviews). For example, several studies investigated the magnitude of herbivory on one of two plant species in the presence or absence of another species (Hambäck *et al*. 2000; White and Whitham 2000). A few studies used the contrasting approach of collecting reciprocal data on two species in the presence or absence of their counterparts (Agrawal 2004; Callaway *et al*. 2005), or of manipulating the relative abundance of two plant types to examine the relative magnitude of herbivory between multiple plant types (Bergvall *et al*. 2006). There is also a long-term observational study that reported associational effects on the basis of density-dependent herbivory on two species (Russell and Louda 2004, 2005).

Because density- and frequency-dependent regulation differ in their long-term consequences on competition between two species (Inouye 2001), one solution to understanding the ecological role of associational effects is to manipulate the density and frequency of two plant types independently (response surface design) (Inouye 2001; Underwood *et al*. 2014; see also Discussion in chapter 3). Kim and Underwood (2015) used this approach to show that damage on one species (*Solanum carolinense*) were both density- and frequency-dependent (see Table 1.1 and Table 6.1 for details), providing a key to understanding ecological dynamics under associational effects. However, two questions remain about interspecific associational effects in an evolutionary context. First, little is known about which species are more defensive and whether the defense levels are heritable. Second, the relative advantage of a given fitness component is difficult to ascertain unless damages are compared between two plant species. Information on both areas is needed to first define defended and undefended strategies and then understand the evolutionary trajectory of defense strategies based on their relative fitness.

The evolutionary role of associational effects has been investigated by using two neighborhood conditions of rare and abundant plant types to compare damages on defended and undefended plants (Rautio *et al*. 2012). Bergvall *et al*. (2006) and the present thesis used this design to assess invasibility of rare types. However, this approach may be questionable in an ecological sense, because it remains unclear whether the invasibility is independent of population size. Density-dependent damage is particularly important for understanding whether a polymorphism can persist without extinction of a plant population through time. We thus conclude that there is still insufficient knowledge to enable us to sufficiently understand both ecological and evolutionary roles of associational effects in a single system.

As noted above, evidence has been accumulated to suggest that plant genetic variation creates feedback selection through interactions with arthropod species or communities (e.g., Johnson *et al*. 2006; Genung *et al*. 2010; Agrawal *et al*. 2012). In this context, dissecting density- and frequency-dependent herbivory is critical for understanding intraspecific associational effects, as the former is responsible for ecological (i.e., abundance) changes and the latter for evolutionary (genetic composition) alterations of a plant population. As discussed above, this issue may be addressed by studying reciprocal fitness data derived from two defense strategies under the independent manipulation of density and frequency.

Intraspecific associational effects in plant-animal interactions

The present thesis used a polymorphic trait to address how intraspecific associational effects mediate frequency-dependent damage in defended and undefended plants. In addition to Wise *et al*. (2009) which we have already discussed in chapter 2 and 5, the study of Bergvall *et al*. (2006) is partly comparable with our experimental setting and results (Table 1.1 and 6.1). Notably, Bergvall *et al*. (2006) reported similar patterns of associational effects (i.e., "neighbor contract defense", discussed in chapter 3) despite the fact that the focal herbivore (deer) and plant defense trait (tannin chemicals) were quite different from our study on the insect vs. trichome system. This supports the view that intraspecific associational effects may be potentially ubiquitous in vertebrate and invertebrate herbivores interacting with physical and chemical plant defenses. Bergvall *et al*. (2006) also examined the between-patch response of an herbivore to palatable and unpalatable diets (Table 1.1). Although our study was unable to either assess (due to the limited dispersal ability of *P. brassicae*; chapter 2-4) or find (because of non-significant main effects of frequency; chapter 5) this effect, it is still possible that spatial scale affects patterns of associational effects against mobile herbivores, such as mammals and flying insects (see also Discussion in chapter 5).

Associational effects can also be observed in plant-pollinator systems. For instance, the phenomenon that pollinator visits to a given flower are increased by neighboring attractive flowers has been reported as the "magnet plant" effect (e.g., Johnson *et al*. 2003; Peter and Johnson 2008). Gigord *et al*. (2001) discussed an intraspecific variation example, showing that the learning behaviors of bumblebees create negative frequency-dependent selection on flower color polymorphism in a rewardless orchid, *Dactylorhiza sambucina*. The neighborhood frequency of color morphs was able to alter plant fitness, because the flower color acts as a reward signaling for bumblebees which tend to avoid major but nectarless flower colors (Gigord *et al*. 2001). This frequency-dependent foraging on different flower colors was also reported in a generalized plant-pollinator system, although patterns were sometimes obfuscated by simultaneous visits of different pollinator species (Eckhart *et al*. 2006).

A more complex case in which mutualists and antagonists mediate contrasting types of frequency-dependent selection on floral traits was presented by Törang *et al*. (2008). This study found that pollinators caused negative frequency-dependent selection, but seed predators led to positive frequency-dependent selection on short- and long-scape morphs of *Primula farinose*. This conflicting direction of selection occurs when a floral display can attract seed predators (which negatively affect plant fecundity) as well as pollinators (which positively affect the fecundity). On the basis of this result, Törang *et al*. (2008) suggest that net effects of the two types of frequency-dependent selection in space and time determine the maintenance of scape-length polymorphism. The present study did not evaluate such multispecific interactions, but it was discovered that the presence/absence of frequency-dependent herbivory on hairy and glabrous *A. halleri* differed among populations where different types of insect herbivores occurred. Future studies should therefore address whether this differential occurrence of frequency-dependent damage accounts for spatial variation in the trichome production of *A. halleri*.

Evidence for the maintenance of polymorphism in plants

It is a long-standing hypothesis that counter-adaptation of parasites to abundant host genotype/species exerts frequency-dependent selection on resistant and susceptible plants (Antonovics and Ellstrand 1984; reviewed by Little 2002). Whether these coevolutionary interactions can be considered as associational effects remains unexplored, but the infection risk for a given plant genotype often depends on the genetic composition of its neighbors within a plant patch (Antonovics and Ellstrand 1984; Brunet and Mundt 2000). Some experimental studies have demonstrated a lowered infection rate in rare genotypes (Brunet and Mundt 2000), and other field studies have presented evidence suggestive of a temporal recovery of rare genotypes (Siemens and Roy 2005). These studies are in favor of the pathogen-mediated maintenance of plant genetic polymorphism, though this remains controversial in the field (Little 2002).

In contrast to antagonistic interaction with pathogens, some groups of fungi are known to confer plant tolerance to environmental stresses and thereby alter the competitive abilities of host plants (Cheplick and Faeth 2009). A study of Miller and Rudgers (2014) exploring this mutualistic interaction illustrated experimentally that the grass endophyte, *Epichloë amarillans*, has driven niche differentiation between endophyte-infected and endophyte-free genotypes of the winter betgrass, *Agrostis hyemalis*. More importantly, this study showed that endophyte-mediated niche differentiation intensified intragenotypic competitions, thus creating a condition for the coexistence of two genotypes (Miller and Rudgers 2014). This type of mutualistic interaction with microbes has the potential to maintain polymorphism in a plant species.

Although explicit tests for frequency-dependent selection on anti-herbivore defense polymorphism are limited, it is possible that this type of natural selection may act not only on physical but also chemical defenses. In particular, geographic patterns reported by Berenbaum and Zangerl (1998) imply that frequency-dependent selection can act on chemical defenses showing distinct clusters. Ecological studies have traditionally assumed a quantitative genetic basis for plant defense traits (e.g., Ågren and Schemske 1994; Mauricio 1998; Agrawal *et al*. 2002; Clauss *et al*. 2006). In contrast, recent genetic evidence suggests that even a little variation in chemical structures critically affects plant resistance to herbivores, and that a few major loci are responsible for such chemical variation (Kliebenstein *et al*. 2002; Kerwin *et al*. 2015; see also Windsor *et al*. 2005 and Kliebenstein 2009 for reviews). Given qualitative inheritance in the major types of plant defense (chemical and physical traits), frequency-dependent selection may be more likely in plant defenses than previously thought.

Future direction

This thesis focused on pairwise plant-herbivore interactions with a polymorphic trait. However, such a simple system is just one component of the diverse interactions involving plants and herbivores, as it is commonly observed that many plant species have evolved multiple defense traits against an array of herbivores (Lewinsohn *et al*. 2005; Schoonhoven *et al*. 2005). As discussed above in a case of floral traits (Törang *et al*. 2008), when a plant species interacts with multiple species simultaneously, the strength and direction of selection on plant defenses may differ from those in a pairwise interaction (i.e., diffuse selection; Iwao and Rausher 1997; Strauss *et al*. 2005). In addition to the community contexts, a single plant species often adopts combinations of multiple defenses (Agrawal and Fishbein 2006), and genetic correlation (Leimu and Koricheva 2006) or plastic changes (Yamawo *et al*. 2014) of these defenses may complicate plant-herbivore interactions. To incorporate the community and trait complexity, it would be valuable to use a plant species that employs multiple defenses against multiple species of herbivores.

Another caveat is that current ecological interactions are a snapshot of the evolution of plant defense, and hence the present ecological approach may not reveal the origin of variation in defense traits. Several specific questions remain in our study system. First, the glabrous type of *A. halleri* has very recently evolved from the hairy type (Kawagoe *et al*. 2011). The molecular evidence raises the question of how long the dimorphism can be maintained in a natural population. Second, it is still unknown whether the anti-herbivore defense has been a primary function of the trichome production. For instance, trichomes can function as part of a tolerance mechanism against drought in a related species (Løe *et al*. 2007; Sletvold and Ågren 2012), and the frequency of trichome dimorphism was found to be divergent between different geographic regions (Løe *et al*. 2007). Although our studied subspecies do not occur in sandy habitats, a variety of *A. halleri* produced dense trichomes in several alpine populations (described previously as *Arabis gemmifera* var. *alpicola*: Kubota *et al*. 2015). This indicates a possibility that abiotic stresses involved in the altitudinal gradient (e.g., frost damage or UV reflection) may be important for the primal evolution of trichome dimorphism in *A. halleri*. The growing number of molecular techniques that have become available will enable us to infer past events during the evolution of trichome dimorphism in *A. halleri*.

Conclusion

The present thesis highlights an overlooked but potentially important role of intraspecific associational effects in the maintenance of plant defense polymorphism via frequency-dependent selection. The discovery of intraspecific associational effects implies that a major assumption of plant defense (i.e., plant damage is independent of neighbors' strategies) might not always hold true. Further studies incorporating this point of view are required for understanding how intraspecific associational effects alter outcomes of plant-herbivore interaction in more complex environments.

Table 6.1 Summary of the experimental design of selected publications (listed in Table 1.1) to detect associational effects in plant-herbivore systems. Columns list methods of manipulation of neighborhood conditions, patch-scale, and whether reciprocal data of interacting species are available (NA: no information available).

References	Neighborhood conditions	Scale	Reciprocal data or not
Tahvanainen & Root (1972)	Monoculture of *Brassica* and polyculture of three species. Tomato and tobacco were added to an equal density of *Brassica* in the polyculture.	Within-patch	NA for tomato and tobacco
Rausher (1981)	0.5 m-radius vegetation surrounding an *Aristolochia reticulata* individual was removed. Non-removed patches were assigned to the control.	Within-patch	NA for counterparts
Risch (1981)	Intercropping one crop into another; monoculture, diculture, and triculture for 3 species (= 7 conditions).	Within-patch	Reciprocal
Hay (1986)	Solitary or paired culture in a closed cage.	Within-patch	Reciprocal
White & Whitham (2000)	Transplanting juvenile cotton to three conditions: nearby box elder, nearby matured cotton, stand alone.	Within-patch	NA for the box elder
Karban & Maron (2002)	Tobacco was transferred next to nearby damaged or intact sagebrush.	Within-patch	NA for Sagebrush
Hambäck *et al*. (2000)	Stand-alone or within-*Myrica gale* patch condition for *Lythrum salicaria*.	Within-patch	NA for *M. gale*

Rand (2003)	Presence/absence of herbivores for 3 conditions: *Atriplex* alone with low density, *Atriplex* alone with high density, *Atriplex* with *Salicornia*.	Within-patch	NA for *Salicornia*
Agrawal (2004)	Grasses or beetles were added to a potted milkweed: 4 treatments of Control, Grass+, Beetle+, and Grass&Beetle+; in addition to Grass alone, Grass&Milk&Beetle, Grass&Milk.	Within-patch	Grass biomass data: Grass alone = Grass&Milk&Beetle > Grass&Milk
Callaway *et al*. (2005)	Four focal species × within/outside grazing fence × exclusion of *Cirsium* or *Veratrum* = 24 treatments.	Within-patch	NA for *Cirsium* and *Veratrum*
Russell & Louda (2005)	> 10-yrs field census of 13 sites where the two plant species co-occur.	Within-site	NA for *Cirsium canescens*
Bergvall *et al*. (2006)	Bad-patch and good-patch contain 7:1 and 1:7 of high:low tannin buckets, respectively. Single- and group-foraging of deer was also tested.	Within/Between patch	Reciprocal
Barton & Bowers (2006)	2 species of *Plantago* × (conspecific, heterospecific, no neighbor) × 2 harvesting time points = 12 conditions.	Within-patch	Reciprocal
Le Guigo *et al*. (2012)	A *Brassicae oleracea* individual was surrounded by 6 conspecific or heterospecific individuals (= 7 treatments in total).	Within-patch	Reciprocal
Castagneyrol *et al*. (2013)	31 possible mono- and poly-culture combinations of 1–5 plant species.	Within/Between patch	Relative values of two species (for plant height data)
Kim & Underwood (2015)	Response surface design (1, 6, 12, 18 total plants/m^2 with 4 frequencies of *Solidago altissima*).	Within-patch	NA for *Solidago altissima* in the main text (but available in Appendix)

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References

Abramoff, M.D., Magalhaes, P.J., and Ram, S.J. (2004) Image processing with Image J. *Biophotonics International* 11:36–42.

Agrawal, A.A. (2004) Resistance and susceptibility of milkweed: competition, root herbivory, and plant genetic variation. *Ecology* 85:2118–2133.

Agrawal, A.A., Conner, J.K., Johnson, M.T.J., and Wallsgrove, R. (2002) Ecological genetics of an induced plant defense against herbivores: additive genetic variance and costs of phenotypic plasticity. *Evolution* 56:2206–2213.

Agrawal, A.A., and Fishbein, M. (2006) Plant defense syndromes. *Ecology* 87:S132–S149.

Agrawal, A.A., Lau, J.A., and Hambäck, P.A. (2006) Community heterogeneity and the evolution of interactions between plants and insect herbivores. *Quarterly Review of Biology* 81:349–376.

Agrawal, A.A., Hastings, A.P., Johnson, M.T.J., Maron, J.L., and Salminen, J.P. (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. *Science* 338:113–116.

Ågren, J., and Schemske, D.W. (1994) Evolution of trichome number in a naturalized population of *Brassica rapa*. *American Naturalist* 143:1–13.

Agresti, A. (2002) *Categorical Data Analysis* (2nd edition). John Wiley & Sons, Hoboken, NJ.

Al-Shehbaz, I.A., and O'Kane, S.L. (2002) Taxonomy and phylogeny of *Arabidopsis* (Brassicaceae). *The Arabidopsis Book* 1:e0001.

Antonovics, J., and Ellstrand, N.C. (1984) Experimental studies of the evolutionary significance of sexual reproduction. I. A test of the frequency-dependent selection hypothesis. *Evolution* 38:103–115.

Andres, M.R., and Connor, E.F. (2003) The community-wide and guild-specific effects of pubescence on the folivorous insects of manzanitas *Arctostaphylos* spp. *Ecological Entomology* 28:383–396.

Andrew, R.L., Peakall, R., Wallis, I.R., and Foley, W.J. (2007) Spatial distribution of defense chemicals and markers and the maintenance of chemical variation. *Ecology* 88:716–728.

Atsatt, P.R., and O'Dowd, D.J. (1976) Plant defense guilds. *Science* 193:24–29.

Augner, M., Fagerström, T., and Tuomi, J. (1991) Competition, defense and games between plants. *Behavioral Ecology and Sociobiology* 29:231–234

Augner, M., and Bernays, E.A. (1998) Plant defence signals and Batesian mimicry. *Evolutionary Ecology* 12:667–679.

Ayala, F.J., and Campbell, C.A. (1974) Frequency-dependent selection. *Annual Review of Ecology and Systematics* 5:115–138.

Barbosa, P., Hines, J., Kaplan, I., Martinson, H., Szczepaniec, A., and Szendrei, Z. (2009) Associational resistance and associational susceptibility: having right or wrong neighbors. *Annual Review of Ecology, Evolution, and Systematics* 40:1–20.

Barton, K.E., and Bowers, M.D. (2006) Neighbor species differentially alter resistance phenotypes in *Plantago*. *Oecologia* 150:442–452.

Bates, D., Maechler, M., and Bolker, B. (2012) lme4: linear mixed-effects models using S4 classes. R package version 0.999999-0. http://CRAN.R-project.org/package=lme4

Bates, D., Maechler, M., Bolker, B., and Walker, S. (2014) lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-6. http://CRAN.R-project.org/package=lme4

Beilstein, M.A., Al-Shehbaz, I.A., and Kellogg, E.A. (2006) Brassicaceae phylogeny and trichome evolution. *American Journal of Botany* 93:607–619.

Behmer, S.T., Raubenheimer, D., and Simpson, S.J. (2001) Frequency-dependent food selection in locusts: a geometric analysis of the role of nutrient balancing. *Animal Behaviour* 61:995–1005.

Berenbaum, M.R., and Zangerl, A.R. (1998) Chemical phenotype matching between a plant and its insect herbivore. *Proceedings of the National Academy of Sciences* 95:13743–13748.

Bergvall, U.A., and Leimar, O. (2005) Plant secondary compounds and the frequency of food types affect food choice by mammalian herbivores. *Ecology* 86:2450–2460.

Bergvall, U.A., Rautio, P., Kesti, K., Tuomi, J., and Leimar, O. (2006) Associational effects of plant defences in relation to within- and between-patch food choice by a mammalian herbivore: neighbour contrast susceptibility and defence. *Oecologia* 147:253–260.

Bloomer, R.H., Juenger, T.E., and Symonds, V.V. (2012) Natural variation in *GL1* and its effects on trichome density in *Arabidopsis thaliana*. *Molecular Ecology* 21:3501–3515.

Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, M.H.H., and White, J.S.S. (2009) Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology & Evolution* 24:127–135.

Bond, A.B., and Kamil, A.C. (1998) Apostatic selection by blue jays produces balanced polymorphism in virtual prey. *Nature* 395:594–596.

Brower, L.P., Pough, F.H., and Meck, H.R. (1970) Theoretical investigations of automimicry, I. single trial learning. *Proceedings of the National Academy of Sciences* 66:1059–1066.

Bruce, T.J.A., Wadhams, L.J., and Woodcock, C.M. (2005) Insect host location: a volatile situation. *Trends in Plant Science* 10:269–274.

Brunet, J., and Mundt, C.C. (2000) Disease, frequency-dependent selection, and genetic polymorphisms: experiments with stripe rust and wheat. *Evolution* 54:406–415.

Burgess, R.S.L., and Ennos, R.A. (1987) Selective grazing of acyanogenic white clover: variation in behaviour among populations of the slug *Deroceras reticulatum*. *Oecologia* 73:432–435.

Cahill, Jr., J.F., Kembel, S.W., and Gustafson, D.J. (2005) Differential genetic influences on competitive effect and response in *Arabidopsis thaliana*. *Journal of Ecology* 93:958–967.

Callaway, R.M., Kikodze, D., Chiboshvili, M., and Khetsuriani, L. (2005) Unpalatable plants protect neighbors from grazing and increase plant community diversity. *Ecology* 86:1856–1862.

Cantero, W.W., and Sanford, L.L. (1984) Insect population response to mixed and uniform plantings of resistant and susceptible plant material. *Environmental Entomology* 13:1443–1445.

Castagneyrol, B., Giffard, B., Péré, C., and Jactel, H. (2013) Plant apparency, an overlooked driver of associational resistance to insect herbivory. *Journal of Ecology* 101:418–429.

Castagneyrol, B., and Jactel, H. (2012) Unraveling plant-animal diversity relationships: a meta-regression analysis. *Ecology* 93:2115–2124.

Castagneyrol, B., Jactel, H., Vacher, C., Brockerhoff, E.G., and Koricheva, J. (2014) Effects of plant phylogenetic diversity on herbivory depend on herbivore specialization. *Journal of Applied Ecology* 51:134–141.

Chandra, S., and Williams, G. (1983) Frequency-dependent selection in the grazing behaviour of the desert locust *Schistocerca Gregaria*. *Ecological Entomology* 8:13–21.

Cheplick, G.P., and Faeth, S.H. (2009) *Ecology and Evolution of the Grass-endophyte Symbiosis*. Oxford University Press, Oxford.

Chesson, J. (1978) Measuring preference in selective predation. *Ecology* 59:211–215.

Christensen, R.H.B. (2013) ordinal – regression models for ordinal data. R package version 2013.9-30 http://www.cran.r-project.org/package=ordinal/.

Chu, C.C., Freeman, T.P., Buckner, J.S., Henneberry, T.J., Nelson, D.R., and Natwick, E.T. (2001) Susceptibility of upland cotton cultivars to *Bemisia tabaci* biotype B (Homoptera: Aleyrodidae) in relation to leaf age and trichome density. *Annals of the Entomological Society of America* 94:743–749.

Clauss, M.J., Dietel, S., Schubert, G., and Mitchell-Olds, T. (2006) Glucosinolate and trichome defenses in a natural *Arabidopsis lyrata* population. *Journal of Chemical Ecology* 32:2351–2373.

Courant, S., and Fortin, D. (2010) Foraging decisions of bison for rapid energy gains can explain the relative risk to neighboring plants in complex swards. *Ecology* 91:1841–1849.

Cottam, D.A. (1985) Frequency-dependent grazing by slugs and grasshoppers. *Journal of Ecology* 73:925–933.

Cyr, H., and Face, M.L. (1993) Magnitude and patterns of herbivory in aquatic and terrestrial ecosystems. *Nature* 361:148–150.

Damgaard, C. (1998) Plant competition experiments: testing hypotheses and estimating the probability of coexistence. *Ecology* 79:1760–1767.

Dalin, P., Ågren J., Björkman, C., Huttunen, P., and Kärkkäinen, K. (2008) Leaf trichome formation and plant resistance to herbivory. In: Schaller, A. (ed.) *Induced Plant Resistance to Herbivory*. Springer, Netherlands, pp. 89–105.

Eckhart, V.M., Rushing, N.S., Hart, G.M., and Hansen, J.D. (2006) Frequency-dependent pollinator foraging in polymorphic *Clarkia xantiana* ssp. *xantiana* populations: implications for flower colour evolution and pollinator interactions. *Oikos* 112:412–421.

Elle, E., van Dam, N.M., and Hare, J.D. (1999) Cost of glandular trichomes, a "resistance" character in *Datura wrightii* Regel (Solanaceae). *Evolution* 53:22–35.

Elle, E., and Hare, J.D. (2000) No benefit of glandular trichome production in natural populations of *Datura wrightii*? *Oecologia* 123:57–65.

Elton, C.S. (1958) *The Ecology of Invasions by Plants and Animals*. Methuen, London, 18.

Emerson, S.E., Brown, J.S., Whelan, C.J., and Schmidt, K.A. (2012) Scale-dependent neighborhood effects: shared doom and associational refuge. *Oecologia* 168:659–670.

Endler, J.A. (1991) Interactions between predators and prey. In: Krebs, J.R., and Davis, N.B. (eds.) *Behavioral Ecology: An Evolutionary Approach*. Blackwell, London, pp. 169–196.

Frerigmann, H., Böttcher, C., Baatout, D., and Gigolashvili, T. (2012) Glucosinolates are produced in trichomes of *Arabidopsis thaliana*. *Plant Metabolism and Chemodiversity* 3:242.

Fitzpatrick, M.J., Feder, E., Rowe, L., and Sokolowski, M.B. (2007) Maintaining a behaviour polymorphism by frequency-dependent selection on a single gene. *Nature* 447:210–212.

Fritz, R.S., and Simms, E.L. (eds.). (1992) *Plant Resistance to Herbivores and Pathogens: Ecology, Evolution, and Genetics*. University of Chicago Press, Chicago, Illinois.

Fournier, V., Rosenheim, J.A., Brodeur, J., Diez, J.M., and Johnson, M.W. (2006) Multiple plant exploiters on a shared host: testing for nonadditive effects on plant performance. *Ecological Applications* 16:2382–2398.

Fukuda, H., Hama, E., Kuzuya, T., Takahashi, A., Takahashi, M., Tanaka, B., Wakabayashi, M., and Watanabe, Y. (1984) *The Life Histories of Butterflies in Japan*, Vol. I. Hoikusha, Osaka. [in Japanese]

Genung, M.A., Bailey, J.K., and Schweitzer, J.A. (2012) Welcome to the neighbourhood: interspecific genotype by genotype interactions in *Solidago* influence above- and belowground biomass and associated communities. *Ecology Letters* 15:65–73.

Gigord, L.D.B., Macnair M.R., and Smithson, A. (2001) Negative frequency-dependent selection maintains a dramatic flower color polymorphism in the rewardless orchid *Dactylorhiza sambucina* (L.) Soò. *Proceedings of the National Academy of Sciences* 98:6253–6255.

González-Teuber, M., and Gianoli, E. (2008) Damage and shade enhance climbing and promote associational resistance in a climbing plant. *Journal of Ecology* 96:122–126.

Grebe, M. (2012) The patterning of epidermal hairs in *Arabidopsis* - updated. *Current Opinion in Plant Biology* 15:31–37.

Greenwood, J.J.D. (1984) The functional basis of frequency-dependent food selection. *Biological Journal of Linnaean Society* 23:177–199.

Handley, R., Ekbom, B., and Ågren, J. (2005) Variation in trichome density and resistance against a specialist insect herbivore in natural populations of *Arabidopsis thaliana*. *Ecological Entomology* 30:284–292.

Hanley, M.E., Lamont, B.B., Fairbanks, M.M., and Rafferty, C.M. (2007) Plant structural traits and their role in anti-herbivore defence. *Perspectives in Plant Ecology, Evolution and Systematics* 8:157–178.

Hambäck, P.A., Ågren, J., and Ericson, L. (2000) Associational resistance: insect damage to purple loosestrife reduced in thickets of sweet gale. *Ecology* 81:1784–1794.

Hambäck, P.A., Pettersson, J., and Ericson, L. (2003) Are associational refuges species-specific? *Functional Ecology* 17:87–93.

Hambäck, P.A., Björkman, M., Rämert, B., and Hopkins, R.J. (2009) Scale-dependent responses in cabbage herbivores affect attack rates in spatially heterogeneous systems. *Basic and Applied Ecology* 10:228–236.

Hambäck, P.A., Inouye, B.D., Andersson, A., and Underwood, N. (2014) Effects of plant neighborhoods on plant-herbivore interactions: resource dilution and associational effects. *Ecology* 95:1370–1383.

Hamnett, G.G. (1944) An investigation into the life-history and morphology of the mustard beetle, *Phaedon cochleariae* F., on watercress. *Proceedings of the Zoological Society of London* 114:368–381.

Hauser, M.T., Harr, B., and Schlötterer, C. (2001) Trichome distribution in *Arabidopsis thaliana* and its close relative *Arabidopsis lyrata*: molecular analysis of the candidate gene *GLABROUS1*. *Molecular Biology and Evolution* 18:1754–1763.

Hay, M.E. (1986) Associational plant defenses and the maintenance of species diversity: turning competitors into accomplices. *American Naturalist* 128:617–641.

Hoffmann, M.H. (2005) Evolution of the realized climatic niche in the genus: *Arabidopsis* (Brassicaceae). *Evolution* 59:1425–1436.

Hori, M. (1993) Frequency-dependent natural selection in the handedness of scale eating cichlid fish. *Science* 260:216–219.

Hughes, M.A. (1991) The cyanogenic polymorphism in *Trifolium repens* L. (white clover). *Heredity* 66:105–115.

Hughes, A.R. (2012) A neighboring plant species creates associational refuge for consumer and host. *Ecology* 93:1411–1420.

Hülskamp, M. (2004) Plant trichomes: a model for cell differentiation. *Nature Reviews Molecular Cell Biology* 5:471–480.

Ihara, K. (1976) Mode of local differentiation in *Arabis lyrata* and *A. gemmifera* (Cruciferae) in Japan. *Journal of the Faculty of Science, the University of Tokyo, Section 3 Botany* 12:1–36.

Inouye, B.D. (2001) Response surface experimental designs for investigating interspecific competition. *Ecology* 82:2696–2706.

Iwao, K., and Rausher, M.D. (1997) Evolution of plant resistance to multiple herbivores: quantifying diffuse coevolution. *American Naturalist* 149:316–335.

Jactel, H., and Brockerhoff, E.G. (2007) Tree diversity reduces herbivory by forest insects. *Ecology Letters* 10:835–848.

Jactel, H., Birgersson, G., Andersson, S., and Schlyter, F. (2011) Non-host volatiles mediate associational resistance to the pine processionary moth. *Oecologia* 166:703–711.

Janz, N., Bergström, A., and Johansson, J. (2005) Frequency dependence of host plant choice within and between patches: a large cage experiment. *Evolutionary Ecology* 19:289–302.

Johnson, S.D., Peter, C.I., Nilsson, L.A., and Ågren, J. (2003) Pollination success in a deceptive orchid is enhanced by co-occurring rewarding magnet plants. *Ecology* 84:2919–2927.

Johnson, M.T.J., Lajeunesse, M.J., and Agrawal, A.A. (2006) Additive and interactive effects of plant genotypic diversity on arthropod communities and plant fitness. *Ecology Letters* 9:24–34.

Joseph, B., Corwin, J.A., Züst, T., Li, B., Iravani, M., Schaepman-Strub, G., Turnbull, L.A., and Kliebenstein, D.J. (2013) Hierarchical nuclear and cytoplasmic genetic architectures for plant growth and defense within *Arabidopsis*. *Plant Cell* 25:1929–1945.

Karban, R., and Maron, J. (2002) The fitness consequences of interspecific eavesdropping between plants. *Ecology* 83:1209–1213.

Kauffman, W.C., and Kennedy, G.G. (1989) Relationship between trichome density in tomato and parasitism of *Heliothis* spp. (Lepidoptera, Noctuidae) eggs by *Trichogramma* spp. (Hymenoptera, Trichogrammatidae). *Environmental Entomology* 18:698–704.

Kawagoe, T., and Kudoh, H. (2010) Escape from floral herbivory by early flowering in *Arabidopsis halleri* subsp. *gemmifera*. *Oecologia* 164:713–720.

Kawagoe, T., Shimizu, K.K., Kakutani, T., and Kudoh, H. (2011) Coexistence of trichome variation in a natural plant population: a combined study using ecological and candidate gene approaches. *PLoS ONE* 6:e22184.

Kennedy, G.G., and Barbour, J.D. (1992) Resistance variation in natural and managed systems. In: Fritz, R.S., and Simms, E.L. (eds.) *Plant Resistance to Herbivores and Pathogens: Ecology, Evolution, and Genetics.* University of Chicago Press, Illinois, pp. 13–41.

Kerwin, R., Feusier, J., Corwin, J., Rubin, M., Lin, C., Muok, A., Larson, B., Li, B., Joseph, B., Francisco, M., Copeland, D., Weinig, C., and Kliebenstein, D.J. (2015) Natural genetic variation in *Arabidopsis thaliana* defense metabolism genes modulates field fitness. *eLife* 4:e05604.

Kim, T.N., and Underwood, N. (2015) Plant neighborhood effects on herbivory: damage is both density and frequency dependent. *Ecology* 96:1431–1437.

Kivimäki, M., Kärkkäinen, K., Gaudeul, M., Løe, G., and Ågren, J. (2007) Gene, phenotype and function: *GLABROUS1* and resistance to herbivory in natural populations of *Arabidopsis lyrata*. *Molecular Ecology* 16:453–462.

Kliebenstein, D.J., Kroymann, J., Brown, P., Figuth, A., Pedersen, D., Gershenzon, J., and Mitchell-Olds, T. (2001) Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. *Plant Physiology* 126:811–825.

Kliebenstein, D., Pedersen, D., Barker, B., and Mitchell-Olds, T. (2002) Comparative analysis of quantitative trait loci controlling glucosinolates, myrosinase and insect resistance in *Arabidopsis thaliana*. *Genetics* 161:325–332.

Kliebenstein, D.J. (2009) A quantitative genetics and ecological model system: understanding the aliphatic glucosinolate biosynthetic network via QTLs. *Phytochemistry Reviews* 8:243–254.

Koricheva, J. (2002) Meta-analysis of sources of variation in fitness costs of plant antiherbivore defenses. *Ecology* 83:176–190.

Kotowska, A.M., Cahill Jr, J.F., and Keddie, B.A. (2010) Plant genetic diversity yields increased plant productivity and herbivore performance. *Journal of Ecology* 98:237–245.

Kubota, H., and Takenaka, C. (2003) *Arabis gemmifera* is a Hyperaccumulator of Cd and Zn. *International Journal of Phytoremediation* 5:197–201.

Kubota, S., Iwasaki, T., Hanada, K., Nagano, A.J., Fujiyama, A., Toyoda, A., Sugano, S., Suzuki, Y., Hikosaka, K., Ito, M., and Morinaga, S.I. (2015) A genome scan for genes underlying microgeographic-scale local adaptation in a wild *Arabidopsis* species. *PLoS Genetics* 11:e1005361.

Kühnle, A., and Müller, C. (2009) Differing acceptance of familiar and unfamiliar plant species by an oligophagous beetle. *Entomologia Experimentalis et Applicata* 131:189–199.

Kühnle, A., and Müller, C. (2012) Prefeeding and acceptance behavior of an oligophagous beetle is dependent on plant suitability and rearing history. *Journal of Insect Behavior* 25:155–165.

Le Guigo, P., Rolier, A., and Le Corff, J. (2012) Plant neighborhood influences colonization of Brassicaceae by specialist and generalist aphids. *Oecologia* 169:753–761.

Leimu, R., and Koricheva, J. (2006) A meta-analysis of tradeoffs between plant tolerance and resistance to herbivores: combining the evidence from ecological and agricultural studies. *Oikos* 112:1–9.

Levin, D.A. (1973) The role of trichomes in plant defense. *Quarterly Review of Biology* 48:3–15.

Lewinsohn, T.M., Novotny, V., and Basset, Y. (2005) Insects on plants: diversity of herbivore assemblages revisited. *Annual Review of Ecology, Evolution, and Systematics* 36:597–620.

Little, T.J. (2002) The evolutionary significance of parasitism: do parasite-driven genetic dynamics occur ex silico? *Journal of Evolutionary Biology* 15:1–9.

Løe, G., Toräng, P., Gaudeul, M., and Ågren, J. (2007) Trichome production and spatiotemporal variation in herbivory in the perennial herb *Arabidopsis lyrata*. *Oikos* 116:134–142.

Louda, S.M., Keeler, K.H., and Holt, R.D. (1990) Herbivore influences on plant performance and competitive interactions. In: Grace, J.B., and Tilman, D. (eds.) *Perspectives on Plant Competition*. Academic Press, New York, pp. 414–444.

Mauricio, R. (1998) Costs of resistance to natural enemies in field populations of the annual plant *Arabidopsis thaliana*. *American Naturalist* 151:20–28.

McNickle, G.G., and Dybzinski, R. (2013) Game theory and plant ecology. *Ecology Letters* 16:545–555.

Miller, T.E.X., and Rudgers, J.A. (2014) Niche differentiation in the dynamics of host-symbiont interactions: symbiont prevalence as a coexistence problem. *American Naturalist* 183:506–518.

Miller, A.M., McArthur, C., and Smethurst, P.J. (2009) Spatial scale and opportunities for choice influence browsing and associational refuges of focal plants. *Journal of Animal Ecology* 78:1134–1142.

Nakagawa, S. (2004) A farewell to Bonferroni: the problems of low statistical power and publication bias. *Behavioral Ecology* 15:1044–1045.

Nelder, J.A., and Frome, E.L. (1984) Models for rates with Poisson errors. *Biometrics* 40:1159–1162.

Ohsaki, N., and Sato, Y. (1999) The role of parasitoids in evolution of habitat and larval food plant preference by three *Pieris* butterflies. *Researches on Population Ecology* 41:107–119.

Ôtake, A., and Funaki, S. (1958) The distribution of *Phaedon brassicae* Baly, with special reference to influences of the dispersal of the adults upon the distribution of their next generation. *Bulletin of Shimane Agricultural University* 6:107–116. [in Japanese with English summary]

Pacala, S.W., and Crawley, M.J. (1992) Herbivores and plant diversity. *American Naturalist* 140:243–260.

Parker, J.D., Salminen, J.P., and Agrawal, A.A. (2010) Herbivory enhances positive effects of plant genotypic diversity. *Ecology Letters* 13:553–563.

Peter, C.I., and Johnson, S.D. (2008) Mimics and magnets: the importance of color and ecological facilitation in floral deception. *Ecology* 89:1583–1595.

Pimentel, D. (1961) Species diversity and insect population outbreaks. *Annals of the Entomological Society of America* 54:76–86.

Plath, M., Dorn, S., Riedel, J., Barrios, H., and Mody, K. (2012) Associational resistance and associational susceptibility: specialist herbivores show contrasting responses to tree stand diversification. *Oecologia* 169:477–487.

Puentes, A., and Ågren, J. (2013) Trichome production and variation in young plant resistance to the specialist insect herbivore *Plutella xylostella* among natural populations of *Arabidopsis lyrata*. *Entomologia Experimentalis et Applicata* 149:166–176.

R Development Core Team (2012) R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria.

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

R Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Raffa, K., Havill, N., and Nordheim, E. (2002) How many choices can your test animal compare effectively? Evaluating a critical assumption of behavioral preference tests. *Oecologia* 133:422–429.

Rand, T.A. (2003) Herbivore-mediated apparent competition between two salt marsh forbs. *Ecology* 84:1517–1526.

Rausher, M.D. (1981) The effect of native vegetation on the susceptibility of *Aristolochia reticulata* (Aristolochiaceae) to herbivore attack. *Ecology* 62:1187–1195.

Rautio, P., Bergvall, U.A., Tuomi, J., Kesti, K., and Leimar, O. (2012) Food selection by herbivores and neighbourhood effects in the evolution of plant defences. *Annales Zoologici Fennici* 49:45–57.

Reymond, P., Bodenhausen, N., van Poecke, R.M.P., Krishnamurthy, V., Dicke, M., and Farmer, E.E. (2004) A conserved transcript pattern in response to a specialist and a generalist herbivore. *Plant Cell* 16:3132–3147.

Rhoades, D.F. (1979) Evolution of plant chemical defense against herbivores. In: Rosenthal, G.A., and Janzen, D.H. (eds.) *Herbivores: Their Interaction with Secondary Plant Metabolites*. Academic Press, New York, pp. 2–54.

Risch, S.J. (1981) Insect herbivore abundance in tropical monocultures and polycultures: an experimental test of two hypotheses. *Ecology* 62:1325–1340.

Root, R.B. (1973) Organization of a plant-arthropod association in simple and diverse habitats: the fauna of collards (*Brassica oleracea*). *Ecological Monographs* 43:95–124.

Rout, G., and Senapati, B. (1968) Biology of the mustard aphid, *Lipaphis erysimi,* in India. *Annals of the Entomological Society of America* 61:259–261.

Roy, B.A. (1998) Differentiating the effects of origin and frequency in reciprocal transplant experiments used to test negative frequency-dependent selection hypotheses. *Oecologia* 115:73–83.

Ruppel, R.F. (1983) Cumulative insect-days as an index of crop protection. *Journal of Economic Entomology* 76:375–377.

Russell, F.L., and Louda, S.M. (2004) Phenological synchrony affects interaction strength of an exotic weevil with Platte thistle, a native host plant. *Oecologia* 139:525–534.

Russell, F.L., and Louda, S.M. (2005) Indirect interaction between two native thistles mediated by an invasive exotic floral herbivore. *Oecologia* 146:373–384.

Sabelis, M.W., and de Jong, M.C. (1988) Should all plants recruit bodyguards? Conditions for a polymorphic ESS of synomone production in plants. *Oikos* 53:247–252.

Sakata, Y., Yamasaki, M., Isagi, Y., and Ohgushi, T. (2014) An exotic herbivorous insect drives the evolution of resistance in the exotic perennial herb *Solidago altissima*. *Ecology* 95:2569–2578.

Sato, Y., Kawagoe, T., Sawada, Y., Hirai, M.Y., and Kudoh, H. (2014) Frequency-dependent herbivory by a leaf beetle, *Phaedon brassicae*, on hairy and glabrous plants of *Arabidopsis halleri* subsp. *gemmifera*. *Evolutionary Ecology* 28:545–559.

Sato, Y., and Kudoh, H. (2015a) Tests of associational defence provided by hairy plants for glabrous plants of *Arabidopsis halleri* subsp. *gemmifera* against insect herbivores. *Ecological Entomology* 40:269–279.

Sato, Y., and Kudoh, H. (2015b) Associational effects against a leaf beetle mediate a minority advantage in defense and growth between hairy and glabrous plants. *Evolutionary Ecology* doi:10.1007/s10682-015-9809-0

Sawada, Y., Akiyama, K., Sakata, A., Kuwahara, A., Otsuki, H., Sakurai, T., Saito, K., and Hirai, M.Y. (2009a) Widely targeted metabolomics based on large-scale MS/MS data for elucidating metabolite accumulation patterns in plants. *Plant & Cell Physiology* 50:37–47.

Sawada, Y., Toyooka, K., Kuwahara, A., Sakata, A., Nagano, M., Saito, K., and Hirai, M.Y. (2009b) *Arabidopsis* bile acid: sodium symporter family protein 5 is involved in methionine-derived glucosinolate biosynthesis. *Plant & Cell Physiology* 50:1579–1586.

Sawada, Y., Nakabayashi, R., Yamada, Y., Suzuki, M., Sato, M., Sakata, A., Akiyama, K., Sakurai, T., Matsuda, F., Aoki, T., Hirai, M.Y., and Saito, K. (2012) RIKEN tandem mass spectral database (ReSpect) for phytochemicals: a plant-specific MS/MS-based data resource and database. *Phytochemistry* 82:38–45.

Schoonhoven, L.M., van Loon, J.J., and Dicke, M. (2005). *Insect-plant Biology* (2nd ed.). Oxford University Press. Oxford.

Sherratt, T.N., and Harvey, I.F. (1993) Frequency-dependent food selection by arthropods: a review. *Biological Journal of Linnaean Society* 48:167–186.

Sholes, O.D.V. (2008) Effects of associational resistance and host density on woodland insect herbivores. *Journal of Animal Ecology* 77:16–23.

Siemens, D.H., and Roy, B.A. (2005) Tests for parasite-mediated frequency-dependent selection in natural populations of an asexual plant species. *Evolutionary Ecology* 19:321–338.

Simms, E.L., and Fritz, R.S. (1992) Costs of plant resistance to herbivory. In: Fritz, R.S., and Simms, E.L. (eds.) *Plant Resistance to Herbivores and Pathogens: Ecology, Evolution, and Genetics*. University of Chicago Press, Chicago, pp. 392–425.

Sletvold, N., and Ågren, J. (2012) Variation in tolerance to drought among Scandinavian populations of *Arabidopsis lyrata*. *Evolutionary Ecology* 26:559–577.

Sletvold, N., Huttunen, P., Handley, R., Kärkkäinen, K., and Ågren, J. (2010) Cost of trichome production and resistance to a specialist insect herbivore in *Arabidopsis* *lyrata*. *Evolutionary Ecology* 24:1307–1319.

Speed, M.P., and Ruxton, G.D. (2005) Warning displays in spiny animals: one (more) evolutionary route to aposematism. *Evolution* 59:2499–2508.

Stamp, N. (2003) Out of the quagmire of plant defense hypotheses. *Quarterly Review of Biology* 78:23–55.

Stastny, M., and Agrawal, A.A. (2014) Love thy neighbor? Reciprocal impacts between plant community structure and insect herbivory in co-occurring Asteraceae. *Ecology* 95:2904–2914.

Steets, J.A., Takebayashi, N., Byrnes, J.M., and Wolf, D.E. (2010) Heterogeneous selection on trichome production in Alaskan *Arabidopsis kamchatica* (Brassicaceae). *American Journal of Botany* 97:1098–1108.

Svennungsen, T.O., and Holen, Ø.H. (2007) The evolutionary stability of automimicry. *Proceedings of the Royal Society B: Biological Sciences* 274:2055–2063.

Strauss, S.Y., Sahli, H., and Conner, J.K. (2005) Toward a more trait-centered approach to diffuse (co)evolution. *New Phytologist* 165:81–90.

Strong, D.R., Lawton, J.H., and Southwood, S.R. (1984) *Insects on Plants: Community Patterns and Mechanisms*. Blackwell, Oxford.

Tahvanainen, J.O., and Root, R.B. (1972) The influence of vegetational diversity on the population ecology of a specialized herbivore, *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae). *Oecologia* 10:321–346.

Takahashi, Y., Yoshimura, J., Morita, S., and Watanabe, M. (2010) Negative frequency-dependent selection in female color polymorphism of a damselfly. *Evolution* 64:3620–3628.

Till-Bottraud, I., and Gouyon, P.H. (1992) Intra-versus interplant Batesian mimicry? a model on cyanogenesis and herbivory in clonal plants. *American Naturalist* 139:509–520.

Tonhasca, A., and Byrne, D.N. (1994) The effects of crop diversification on herbivorous insects: a meta-analysis approach. *Ecological Entomology* 19:239–244.

Toräng, P., Ehrlén, J., and Ågren, J. (2008) Mutualists and antagonists mediate frequency-dependent selection on floral display. *Ecology* 89:1564–1572.

Tsuchimatsu, T., Kaiser, P., Yew, C.-L., Bachelier, J.B., and Shimizu, K.K. (2012) Recent loss of self-incompatibility by degradation of the male component in allotetraploid *Arabidopsis kamchatica*. *PLoS Genetics* 8:e1002838.

Tuomi, J., Augner, M., and Leimar, O. (1999) Fitness interactions among plants: optimal defence and evolutionary game theory. In: Vuorisalo, T.O., and Mutikaine, P.K. (eds.) *Life-history Evolution in Plants*. Kluwer Academic Publishers, Dordrecht, pp. 63–84.

Underwood, N., Inouye, B.D., and Hambäck, P.A. (2014) A conceptual framework for associational effects: when do neighbors matter and how would we know? *Quarterly Review of Biology* 89:1–19.

van Poecke, R.M.P. (2007) *Arabidopsis*-insect interactions. *The Arabidopsis Book* 5:e0107.

Visser, J.H. (1986) Host odor perception in phytophagous insects. *Annual Review of Entomology* 31:121–144.

Wagner, G.J., Wang, E., and Shepherd, R.W. (2004) New approaches for studying and exploiting an old protuberance, the plant trichome. *Annals of Botany* 93:3–11.

Wang, X.P., Zhou, X.M., and Lei, C.L. (2007a) Development, survival and reproduction of the Brassica leaf beetle, *Phaedon brassicae* Baly (Coleoptera: Chrysomelidae) under different thermal conditions. *Pan-Pacific Entomology* 83:143–151.

Wang, X.P., Zhou, X.M., Wang, Y.Y., and Lei, C.L. (2007b) Internal reproductive system and diapausing morphology of the Brassica leaf beetle *Phaedon brassicae* Baly (Coleoptera: Chrysomelidae: Chrysomelinae). *The Coleopterists Bulletin* 61:457–462.

Wang, X.P., Xue, F.S., Tan, Y.Q., and Lei, C.L. (2007c) The role of temperature and photoperiod in diapause induction in the Brassica leaf beetle, *Phaedon brassicae* (Coleoptera: Chrysomelidae). *European Journal of Entomology* 104:693–697.

Wang, L., Wang, D., Bai, Y., Huang, Y., Fan, M., Liu, J., and Li, Y. (2010) Spatially complex neighboring relationships among grassland plant species as an effective mechanism of defense against herbivory. *Oecologia* 164:193–200.

White, J.A., and Whitham, T.G. (2000) Associational susceptibility of cottonwood to a box elder herbivore. *Ecology* 81:1795–1803.

Windsor, A.J., Reichelt, M., Figuth, A., Svatoš, A., Kroymann, J., Kliebenstein, D.J., Gershenzon, J., and Mitchell-Olds, T. (2005) Geographic and evolutionary diversification of glucosinolates among near relatives of *Arabidopsis thaliana* (Brassicaceae). *Phytochemistry* 66:1321–1333.

Wink, M. (2003) Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* 64:3–19.

Wise, M.J., Yi, C.G., and Abrahamson, W.G. (2009) Associational resistance, gall-fly preferences, and a stem dimorphism in *Solidago altissima*. *Acta Oecologica* 35:471–476.

Yamawo, A., Suzuki, N., Tagawa, J., and Hada, Y. (2012) Leaf ageing promotes the shift in defence tactics in *Mallotus japonicus* from direct to indirect defence. *Journal of Ecology* 100:802–809.

Yamawo, A., Tagawa, J., Hada, Y., and Suzuki, N. (2014) Different combinations of multiple defence traits in an extrafloral nectary-bearing plant growing under various habitat conditions. *Journal of Ecology* 102:238–247.

Yano, S., and Ohsaki, N. (1993) The phenology and intrinsic quality of wild crucifers that determine the community structure of their herbivorous insects. *Researches on Population Ecology* 35:151–170.

Yoshida, Y., Sano, R., Wada, T., Takabayashi, J., and Okada, K. (2009) Jasmonic acid control of *GLABRA3* links inducible defense and trichome patterning in *Arabidopsis*. *Development* 136:1039–1048.

Yencho, G.C., and Tingey, W.M. (1994) Glandular trichomes of *Solanum berthaultii* alter host preference of the Colorado potato Beetle, *Leptinotarsa decemlineata*. *Entomologia Experimentalis et Applicata* 70:217–225.

Yee, T.W. (2013) VGAM: Vector Generalized Linear and Additive Models. R package version 0.9-3. URL http://CRAN.R-project.org/package=VGAM

Zakir, A., Sadek, M.M., Bengtsson, M., Hansson, B.S., Witzgall, P., and Anderson, P. (2013) Herbivore-induced plant volatiles provide associational resistance against an ovipositing herbivore. *Journal of Ecology* 101:410–417.

Zangerl, A.R., Stanley, M.C., and Berenbaum, M.R. (2008) Selection for chemical trait remixing in an invasive weed after reassociation with a coevolved specialist. *Proceedings of the National Academy of Sciences* 105:4547–4552.

Zhao, F.J., Lombi, E., Breedon, T., and McGrath, S.P. (2000) Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. *Plant Cell & Environment* 23:507–514.

Züst, T., Joseph, B., Shimizu, K.K., Kliebenstein, D.J., and Turnbull, L.A. (2011) Using knockout mutants to reveal the growth costs of defensive traits. *Proceedings of the Royal Society B: Biological Sciences* 278:2598–2603.

Züst, T., Heichinger, C., Grossniklaus, U., Harrington, R., Kliebenstein, D.J., and Turnbull, L.A. (2012) Natural enemies drive geographic variation in plant defenses. *Science* 338:116–119.

Zvereva, E.L., Kozlov, M.V., and Niemelä, P. (1998) Effects of leaf pubescence in *Salix borealis* on host-plant choice and feeding behaviour of the leaf beetle, *Melasoma lapponica*. *Entomologia Experimentalis et Applicata* 89:297–303.