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RESEARCH ARTICLE

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Pleiotropic effect of the *Flowering Locus C* on plant resistance and defence against insect herbivores

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Abstract

1. Plants vary widely in the extent to which they defend themselves against herbivores. Because the resources available to plants are often site-specific, variation among sites dictates investment into defence and may reveal a growth–defence trade-off. Moreover, plants that have evolved different life-history strategies in different environments may situate themselves on this trade-off curve differently. For instance, plants that flower later have a longer vegetative life span and may accordingly defend themselves differently than those that flower earlier.
2. Here, we tested whether late-flowering plants, with a longer vegetative life span, invest more in defence than early-flowering plants, using recombinant genotypes of the annual herb *Cardamine hirsuta* that differ in flowering time as a result of differences in the activity of the major floral repressor *Flowering Locus C* (*FLC*).
3. We found that variation at *FLC* was mainly responsible for regulating flowering time and allocation to reproduction, but this partially depended on where the plants grew. We also found that variation at *FLC* mediated plant allocation to defence, with late-flowering plants producing higher levels of total glucosinolates and stress-related phytohormones. Nonetheless, plant growth and the qualitative values of plant defence and plant resistance against specialist herbivores were mainly independent from *FLC*.
4. *Synthesis*. Our results highlight pleiotropic effects associated with flowering-time genes that might influence plant defence and plant–herbivore interactions.

KEYWORDS
altitudinal gradients, flowering time, glucosinolates, growth–defence trade-off hypothesis, jasmonic acid, *Pieris*, plant–herbivore interaction

1 | INTRODUCTION

Plants have evolved a complex array of barriers to reduce damage imposed by herbivore attack, ranging from the production of low-nutritional quality leaves to the accumulation of toxic molecules in their tissues (Schoonhoven, van Loon, & Dicke, 2005). Such toxins may be constitutively produced throughout life or may be induced following herbivore attack (Karban & Baldwin, 1997). These defence responses are typically mediated by stress-related phytohormones, including jasmonic (JA), salicylic (SA) and abscisic acids (ABA), which tend to increase in concentration after insect or pathogen attack (De Vos et al., 2005; Erb et al., 2009; Farmer, Alméras, & Krishnamurthy, 2003; Schmelz, Alborn, Banchio, & Tumlinson, 2003).

Despite several decades of work, we still lack a full understanding of the ecological and evolutionary factors that contribute to plant defence-trait variation (Benderoth et al., 2006; Futuyma & Agrawal,

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2009). Syndromes of plant defence depend on inherited functional traits, biotic and abiotic conditions, and the geographical and historical contingencies affecting the community (Futuyma & Agrawal, 2009). As a consequence, several theories have been advanced to explain relative plant investment in defence and subsequent herbivore performance in terms of resource availability and trade-offs between defence and other traits, such as growth or development time (Agrawal, Conner, & Rasmann, 2010).

Growth–defence trade-offs ultimately give rise to a negative correlation between the ability to grow fast and the ability to defend well (Herms & Mattson, 1992). Intra- and interspecific comparisons revealed that inherently fast-growing genotypes have lower levels of defence and generally experience higher levels of herbivory than inherently slow-growing ones (e.g. Cates & Orians, 1975; Chapin, Johnson, & McKendrick, 1980; Coley, 1983; Coley, Bryant, & Chapin, 1985; Endara & Coley, 2011; Fine, Mesones, & Coley, 2004). In other words, the environment selects among species or genotypes that grow at a particular rate, within the context of investment trade-offs mediated by responses to herbivore damage (Agrawal et al., 2010; Fine et al., 2004). For annual plants, investment in growth should be strongly associated with short generation times. Environments selecting for shorter generations (i.e. early flowering) might therefore be expected to select for decreased allocation to defence. If so, we might expect pleiotropic effects of genes that govern flowering time on the expression of defence against herbivores. Pleiotropic effects in general have been observed in several well-studied plant systems such as *Arabidopsis thaliana* (Loudet, Chaillou, Krapp, & Daniel-Vedele, 2003; McKay, Richards, & Mitchell-Olds, 2003; Swarup et al., 1999) and *Mimulus guttatus* (Hall, Basten, & Willis, 2006) including evidence for intersection of flowering time and biotic stress pathways (Winter, Garcia, & Holtum, 2011). However, information on pleiotropic effects of flowering-time genes on plant defence against herbivores in a natural setting remains scarce. This is an important lacuna in our knowledge of how trade-offs between diverse, seemingly unrelated traits shape plant phenotypic variation.

We tested for pleiotropic effects of genes influencing flowering time on herbivore resistance/defence traits in *Cardamine hirsuta* (Brassicaceae), an annual plant that occurs throughout Europe and Asia and that shows wide variation across a number of traits, including flowering time. *Cardamine hirsuta* produces a particular class of secondary metabolites, the glucosinolates, that are common in the Brassicaceae. Herbivory causes these molecules to spill from cell vacuoles and come into contact with myrosinases, which transform them into molecules that are poisonous or distasteful to generalist herbivores and, to some extent, even to specialists (Bodenhausen & Reymond, 2007). Because flowering time and defence chemistry are both well known in *C. hirsuta* (see below), and are both likely to affect fitness through trade-offs with one another, the species provides an ideal model to seek novel pleiotropic effects of genes affecting both life history and defence.

Our study involved growing genotypes of *C. hirsuta* with differences in flowering time. In particular, we used near-isogenic lines (see Section 2) that differ at a genomic location harbouring *Flowering Locus C* (*FLC*), a floral integrator with major effect on flowering time (Cartolano et al., 2015; Michaels & Amasino, 1999), and possible pleiotropic effects on other processes including water use efficiency (McKay et al., 2003), circadian leaf movement (Swarup et al., 1999), biotic stress (Winter, 2011), seed size (Alonso-Blanco, Blankenstijn-de Vries, Hanhart, & Koornneef, 1999), seed dormancy (Alonso-Blanco, Bentsink, Hanhart, Blankestijn-deVries, & Koornneef, 2003), germination (Chiang, Barua, Kramer, Amasino, & Donohue, 2009) and nitrate content (Loudet et al., 2003); see Figure S1. We conducted our experiment on plants grown at four contrasting sites that were likely to differ markedly in their growth conditions and interactions with herbivores. We measured flowering time, biomass and resistance to herbivore damage. We subsequently assayed levels of constitutive and induced glucosinolate production as part of a feeding experiment using a sample of plants brought back to a glasshouse. We specifically asked whether late-flowering plants differ in biomass or growth, whether they have increased levels of glucosinolates and defensive phytohormones and whether they are more resistant to herbivory than early-flowering genotypes.

2 | MATERIALS AND METHODS

2.1 | Seed material

The hairy bittercress *C. hirsuta* (Brassicaceae) is an annual plant native to Asia and Europe (Barkoulas, Hay, Kougioumoutzi, & Tsiantis, 2008; Canales, Barkoulas, Galinha, & Tsiantis, 2010; Hay & Tsiantis, 2010; Hay et al., 2014). In the Swiss Alps, where we conducted our study, *C. hirsuta* preferentially grows in lowlands, between about 300 and 700 m above sea level (a.s.l.), but it may also occur at altitudes up to 1,500 m a.s.l. (Rasmann S., personal observations, www.infoflora.ch). In the field, *C. hirsuta* can be heavily damaged by a variety of herbivores including, for instance, caterpillar species in the family Pieridae (Pellissier et al., 2016). To test whether adaptations in flowering time affect plant resistance and defence, we used seeds from the two *C. hirsuta* wild strains Ox and Wa (Oxford and Washington, Hay et al., 2014) that differ in their flowering time (early flowering vs. late flowering, respectively), as well as seeds from two near-isogenic lines (NILs) of *C. hirsuta* (NIL_Ox and NIL_Wa). These two NILs are genetically nearly identical across the genome, but differ in a genomic region of 1.3 Mbp comprising the *FLC*, a major regulator of flowering responses to seasonal environmental factors (Cartolano et al., 2015; Chiang et al., 2009). The NILs were generated from an F1 intercross of Ox and Wa accessions, followed by repeated backcrossing with the Ox accession, with extensive genotyping (Cartolano et al., 2015). The NIL_Ox should be essentially the same as Oxford, while the NIL_Wa has an introgressed allele from Washington at the *FLC* locus.

2.2 | Experimental design

Seeds of the four genotypes of *C. hirsuta* (the two wild strains, and the two corresponding NILs) were cold-stratified for 7 days, sown and germinated in the glasshouse at the University of Lausanne, Switzerland.

On 26 July 2012, that is around 1 week after germination, seedlings started to produce their first pair of true leaves, and they were transplanted into plastic pots (13 cm in diameter), filled with a mixture of potting soil (Orbo-2, Schweizer AG, Lausanne; Switzerland) and vermiculite (3:1). Four days later, they were moved to four common gardens at sites in the Alps that differ in their altitudes (from about 400 m to 1,800 m a.s.l., see Figure S2) and associated growth conditions, especially temperature (Körner, 2007). The sites where chosen both to represent habitats where the study species grows (see above), as well as to investigate phenotypic variation in *C. hirsuta* in response to contrasting environments. A total of 35 replicates of each genotype were placed at each site. Plants were watered ad libitum in order to avoid extreme desiccation in periods of hot weather, and they were allowed to grow for a total of 7 weeks in the field. Flowering time was recorded 14, 20 and 30 days after establishment of the common gardens by scoring all plants of each genotype at each site at the time of bolting (i.e. the production of flowering stems).

To measure natural herbivore damage, we randomly selected and marked 15 plants per genotype at each site at the onset of the experiment and scored herbivore damage after 7 weeks on a percentage scale from 0% to 100%, with 5% increments. Visual estimation is both rapid and cost-effective and provides a precise and accurate method for quantifying herbivory (Johnson, Bertrand, & Turcotte, 2016).

After 4 weeks of growth, on 30 August 2012, 10 plants were haphazardly selected (excluding those that had been damaged by herbivores) at each site from each genotype (i.e. 10 of the initial 35 plants per genotype at each site described above). These plants were brought back to the glasshouse to be assayed for herbivory.

Finally, after 7 weeks of growth outside, when all plants were setting fruits, we harvested the above-ground biomass of 12 plants, haphazardly selected from the remaining 25 plants at each site, to measure their reproductive effort, that is the ratio of reproductive dry mass (i.e. flowering stems + fruits) to vegetative dry mass (i.e. rosette dry mass). Dry mass was obtained by oven-drying at 78°C for 4 days.

2.3 | Herbivory assay

To measure plant resistance and defence induction, we performed an herbivory assay on 10 haphazardly selected plants that were brought back to the glasshouse (on 30 August 2012) from each genotype from all four sites. Plants were brought to the glasshouse after 4 weeks of growth outside, and not later, to avoid losing too many plants to herbivory. Once in the glasshouse (25/18°C, 60% relative humidity and a photoperiod consisting of 14 hr of daylight), we initiated the treatments as follows: seven plants per genotype and site were inoculated with five first-instar larvae of the specialist *Pieris brassicae* (Lepidoptera, Pieridae), whereas the remaining (undamaged) plants were later measured for constitutive levels of secondary metabolites (N = 10 plants × 4 genotypes × 4 altitudes = 160 plants).

After a week of feeding, on 7 September 2012, we assessed plant resistance against caterpillar herbivory by measuring larval weight (i.e. resistance is a measure of insect performance Karban & Baldwin, 1997), after drying the larvae at 70°C for 48 hr. Immediately after larval collection, two leaves per plant were collected in damaged (N = 4 plants × 4 genotypes × 4 altitudes) and undamaged plants (N = 3 plants × 4 genotypes × 4 altitudes), weighed fresh and frozen in liquid nitrogen in two separate tubes, one for the measurement of glucosinolates, and the other for the measurement of phytohormones. Plant biomass was next measured by drying the above-ground biomass in an oven at 70°C for 48 hr. For each plant, we also visually scored damage on a percentage scale as for the field survey and transformed this value into mg of tissue consumed by the caterpillars in terms of (percentage damage × plant biomass)/(100 − percentage damage). For this experiment, we did not measure reproductive effort, as flowering had just commenced in most individuals at the time of the herbivory assay.

2.4 | Leaf chemistry

We measured plant defence in terms of glucosinolate levels in the *C. hirsuta* genotypes following the protocol of Glauser, Schweizer, Turlings, and Reymond (2012), with slight modifications. Briefly, about 15 mg of lyophilized and powder-ground leaf material was extracted in 2.0 ml of ice-cold MeOH:water (70:30, v/v) by incubation at 80°C for 15 min. UHPLC-QTOFMS analyses of 1 µl of extracted solution were performed on an Acquity UPLC™ (Waters), interfaced to a Synapt G2 QTOF (Waters) with electrospray ionization. We found that five glucosinolates (gluconapin, glucobrassicanapin, glucotropaeolin, glucobrassicin and gluconasturtiin) accounted for more than 99% of the total glucosinolate content in all samples of *C. hirsuta*. These five glucosinolates were quantified as gluconapin equivalents using standard curves of gluconapin.

For phytohormone analyses, we focused on measuring the major hormones involved in the expression of defence against biotic attack: abscisic acid (ABA), jasmonic acid (JA), jasmonoyl isoleucine (JA-Ile) and salicylic acid (SA) (Erb & Glauser, 2010). JA and, in part, ABA mainly mediate herbivore attack (Howe & Jander, 2008), whereas SA mainly mediates pathogen attack (Ton et al., 2002), and JA-Ile is directly involved in JA signalling (Katsir, Chung, Koo, & Howe, 2008). Other phytohormones such as ethylene have also been shown to affect resistance against herbivore crosstalk with JA and ABA, but never directly linked to chewing herbivore performance (Pieterse, Leon-Reyes, Van der Ent, & Van Wees, 2009). Phytohormone accumulation in the healthy and damaged plants was monitored according to Glauser, Vallat, and Balmer (2014). The extraction of phytohormones was performed by grinding 200 mg of fresh leaves to a powder under liquid nitrogen and mixing with 990 µl of extraction solvent (ethylacetate/formic acid, 99.5:0.5) and 10 µl of internal standards (ISs; containing isotopically labelled hormones at a concentration of 100 ng/ml for d5-JA, d6-SA, d6-ABA, 13C6-JA-Ile) in a mixer mill at 30 Hz. After centrifugation, re-extraction of the pellet with 500 µl of extraction solvent and evaporation of the combined supernatants, the residue was resuspended in 100 µl 70% MeOH. 5 µl of the solution was injected for UHPLC-MS/MS analysis, following Glauser et al. (2014). The final concentration of the phytohormones was calculated for each sample using calibration curves in which the ISs were present at the same concentrations as in the plant samples.

2.5 | Data analysis

All statistical analyses were performed with R software, version 3.2.2 (R Development Core Team, 2015).

For the field survey, we assessed the effects of site, genotype and their interactions (fixed effects) on flowering time, reproductive effort and percentage natural herbivore damage using two-way permutation ANOVAs (PERMANOVAs), accounting for heteroscedasticity of the residuals using the *aovp* function in the package *lmPerm* (Wheeler, 2010). We examined the mean differences among factors using Tukey's HSD post hoc tests by means of *TukeyHSD* function in R.

For the resistance bioassay, to determine whether herbivore treatment had influenced the composition (i.e. identity and relative abundance) of glucosinolate and phytohormone compounds, we used non-metric multidimensional scaling (NMDS) implemented in the *vegan* package in R (Oksanen et al., 2013). Differences in glucosinolates and phytohormone composition among genotypes, herbivore treatment and their interaction were tested using PERMANOVA, using the *adonis* function in the package *vegan* in R (Oksanen et al., 2013). The Bray-Curtis metric was used to calculate a dissimilarity matrix of all compounds among samples for both the NMDS and PERMANOVA.

The effects of site, genotypes, herbivore treatment and all interactions on the total amount of phytohormones and glucosinolates were assessed with three-way PERMANOVAs, while the effects of site, genotype and their interactions on larval biomass and plant biomass were assessed with two-way PERMANOVAs using the *aovp* function in the package *lmPerm* (Wheeler, 2010). We examined the mean differences among factors using Tukey's HSD.

Finally, we analysed the relationship between herbivore-induced glucosinolates (and phytohormones, separately) and the data from the herbivore bioassay (larval mass, plant mass and tissue consumed) using the environmental fitting analysis (*envfit* function) on the NMDS analysis of the chemical compounds. When applied to NMDS, the environmental fitting analysis can estimate the strength of the correlation of maximal correlation between the NMDS configuration and the dependent variables. This approach can be used to indicate whether one or more variables (larval mass, plant mass and tissue consumed in our case) are associated with differences between samples (genotypes in our case), as represented in the NMDS ordination. Differences in herbivore-induced phytohormones and glucosinolates among genotypes were then visualized using a principal component analysis (PCA), and by including plant biomass and plant tissue consumed as covariates, using the *prcomp* function in R.

3 | RESULTS

3.1 | Flowering time, reproductive effort and natural herbivore damage

Flowering time differed among genotypes in a site-specific manner (see genotype × site interaction, Table 1, Figure 1a). Specifically, while there were no differences between the genotypes at site 1, at sites 2, 3 and 4, the late-flowering genotypes (Wa and NIL_Wa) took an average of 12 days longer to flower than the early-flowering genotypes (Ox and NIL_Ox) (Figure 1a).

TABLE 1 Two-way permutation ANOVA table for flowering time, reproductive effort and percentage natural herbivore damage of the four *Cardamine hirsuta* genotypes (G) including the late-flowering genotypes Wa = Washington genotype and NIL_Wa, a near-isogenic line, in which the Wa *FLC* allele is introgressed into Ox genetic background, and the early-flowering genotypes Ox = Oxford genotype, and NIL_Ox, a near-isogenic sibling line with the Ox *FLC* allele and Ox genetic background. Each genotype was grown at four sites (S) in the Swiss Alps (Figure S2)

Variable	Factor	*df*	Iter	*p*-value
Flowering time	Genotype (G)	3	5,000	**<.0001**
	Site (S)	3	5,000	**<.0001**
	G x S	9	5,000	**<.0001**
	Residuals	617		
Reproductive effort	G	3	5,000	**<.0001**
	S	3	5,000	**<.0001**
	G x S	9	5,000	**<.0001**
	Residuals	185		
Percentage damage	G	3	1,213	.25
	S	3	5,000	**<.0001**
	G x S	9	3,710	.08
	Residuals	231		

Bold *p*-values indicate statistical significance ($p < .05$).

Reproductive effort varied among genotypes and sites (Table 1, Figure 1b). Overall, early-flowering genotypes sharing the Ox *FLC* allele (Ox and NIL_Ox) allocated relatively more to reproduction than late-flowering genotypes sharing the WA *FLC* allele (Wa and NIL_Wa). However, the magnitude of those differences varied among sites (Figure 1b).

We detected no effect of genotype on the extent to which plants were eaten in the field (Table 1). However, herbivory levels differed among sites, with plants grown at lower-altitude sites (1 and 2) showing the highest damage (8% and 13% damage per plant, respectively), while those at sites 3 and 4 experienced 7% damage (Table 1), independently of genotype (Table 1).

3.2 | Plant defensive chemistry (glucosinolates and phytohormones)

Across the four *C. hirsuta* genotypes, the five major glucosinolates (gluconapin, glucobrassicanapin, glucotropaeolin, glucobrassicin and gluconasturtiin) represented more than 90% of the total glucosinolates found in this species (Figure S3), a result similar to that found by Pellissier et al. (2016). The PERMANOVA multivariate analysis showed that the identity and abundance of individual glucosinolates differed among genotypes, sites and herbivore treatments (Table 2, Figure 2a, Figure S3). When looking at total glucosinolates, in the absence of herbivory, Wa plants had the greatest constitutive level of glucosinolates (around 38% more than the other genotypes) (Table 3, Figure 3a). However, herbivory induced a 22% increase in the total

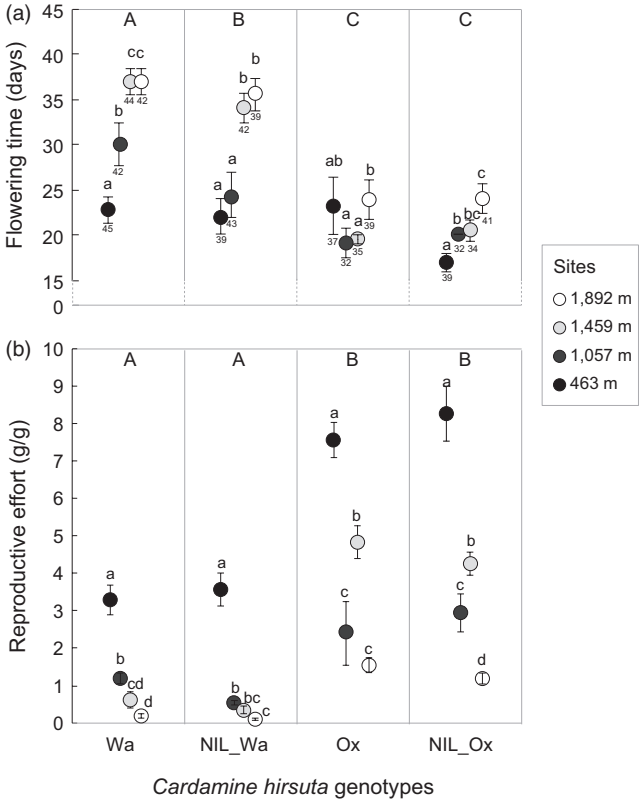

FIGURE 1 Effect of *Flowering Locus C (FLC)* on flowering time and reproductive effort in the field. Shown is (a) the average (±1 *SE*) flowering time of the experiments in the field for four genotypes grown at four different sites, and (b) the reproductive effort, that is the ratio of reproductive dry mass to vegetative dry mass. The four genotypes of *Cardamine hirsuta* include the late-flowering genotypes Wa (Washington), and NIL_Wa, a near-isogenic line in which the Wa *FLC* allele is introgressed into Ox genetic background, and the early-flowering genotypes Ox (Oxford), and NIL_Ox, a near-isogenic sibling line with the Ox *FLC* allele and Ox genetic background growing at different altitudes (m a.s.l.) in the Swiss Alps (see also Figure S2). Different lowercase letters above dots indicate statistically significant differences among sites across all genotypes, and different capital letters indicate significant differences between genotypes (Tukey post hoc test; $p < .05$). Sample sizes are shown under each dot

content of glucosinolates in NIL_Wa, approaching similar levels to those shown by Wa (Table 3 G × T interaction; Figure 3a), and therefore showing an effect of *FLC* on the total amount of glucosinolate production (Table 3). The composition and total content of glucosinolates also varied across sites, depending on the herbivory treatment (see significant herbivory by site interaction, Tables 2 and 3), with the lowest values of total glucosinolates (30% less) found at site 4 for plants not exposed to *Pieris rapae* larva (Figure 3a).

Similar to the glucosinolate analyses, we found a strong effect of genotype, site and herbivore treatment on phytohormonal composition (Table 2, Figure S4, Figure 2b). Overall, the total level of phytohormones differed among genotypes (Table 3), with Wa and NIL_Wa showing almost twice that shown by Ox and NIL_Ox (Figure 3b). We also found an overall phytohormonal induction, particularly mediated by high levels of SA, after herbivore feeding (Table 3, Figure 3b), and the

TABLE 2 Three-way permutation ANOVA table for phytohormones and glucosinolates of the four *Cardamine hirsuta* genotypes (G) including the late-flowering genotypes Wa = Washington genotype and NIL_Wa, a near-isogenic line in which the Wa *FLC* allele is introgressed into Ox genetic background, and the early-flowering genotypes Ox = Oxford genotype, and NIL_Ox, a near-isogenic sibling line with the Ox *FLC* allele and Ox genetic background. Each genotype was grown at four sites (S) in the Swiss Alps (Figure S2)

Variable	Factor	*df*	*F* value	*p*-value
Glucosinolates	Genotype (G)	3	105.67	**<.001**
	Site (S)	3	9.25	**<.001**
	Treatment (T)	1	2.61	.08
	G x S	9	1.14	.31
	G x T	3	1.40	.21
	S x T	3	2.52	**.03**
	G x S x T	9	1.26	.22
	Residuals	124		
Hormones	Genotype (G)	3	5.57	**.002**
	Site (S)	3	10.47	**.001**
	Treatment (T)	1	13.57	**.001**
	G x S	9	1.85	**.02**
	G x T	3	0.47	.87
	S x T	3	1.43	.19
	G x S x T	9	1.64	**.05**
	Residuals	87		

Bold *p*-values indicate statistical significance ($p < .05$).

total levels of phytohormones depended on site (Table 3), with plants at site 2 having around half the phytohormones of those at site 4.

3.3 | Plant growth and plant resistance bioassay

Overall, plant biomass differed among plant genotypes in a way that was similar among sites (Table 3). As expected, plant growth tended to decline with altitude, except that plants growing at site 2 grew least (Figure S5). Site 2 was also the more sun-exposed site, a situation that might have driven plants to experience more severe drought stress than plants growing at the other sites. Differences in size between plant genotypes, however, were only found between two late-flowering strains sharing the WA *FLC* allele: Wa plants were on average 47% larger than NIL_Wa plants (Figure S5). This result and the lack of differences between the early and late genotypes (i.e. Ox vs. Wa, TukeyHSD: $p = .49$, and NIL_Ox vs NIL_Wa, TukeyHSD: $p = .57$) suggest that plant size was largely independent of the *FLC* allelic differences, and rather dependent on the Wa genetic background.

In the glasshouse, we noted a tendency for both the field site locality and plant genotype to affect larval growth, although the result fell short of statistical significance (Table 3). Again, the difference in growth among genotypes was consistent among sites (i.e. no site by genotype interaction, Table 3, Figure 4). More specifically, larvae feeding on plants that grew at site 2 (where the plants were also the smallest) were half the size of those feeding on plants sampled at other sites (Figure 4).

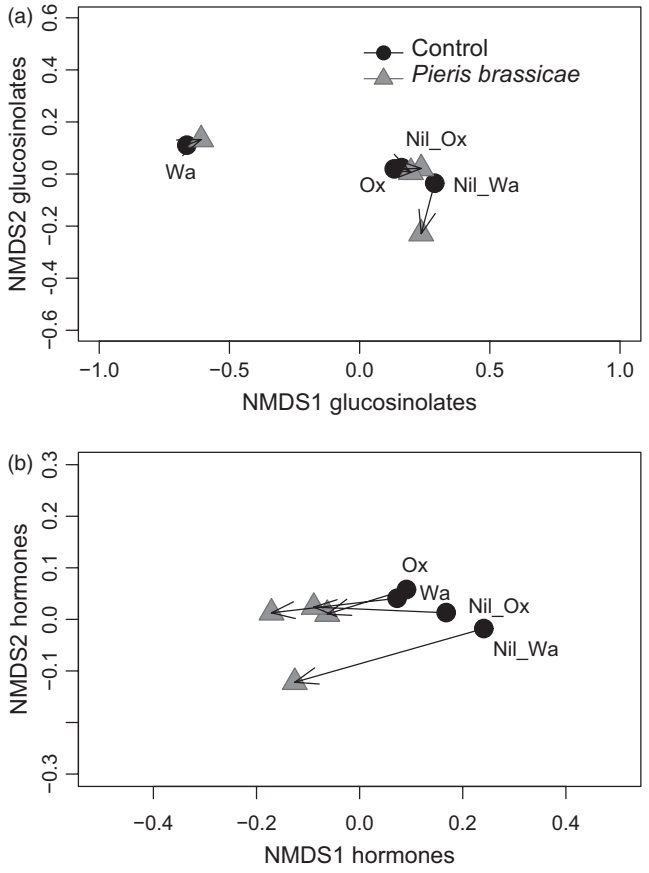

FIGURE 2 Non-metric multidimensional scaling (NMDS) plot illustrating variation in the composition of (a) a foliar glucosinolates, and (b) foliar phytohormones of the four *Cardamine hirsuta* genotypes, and the effects of *Pieris brassicae* herbivory on glucosinolates and phytohormone composition, respectively. Black dots represent the average of control (undamaged) plants, while grey triangles represent response induced by *P. brassicae* attack (Ox = Oxford (n = 25), and NIL_Ox = a near-isogenic sibling line with the Ox *FLC* allele and Ox genetic background (n = 26)), and the late-flowering genotypes (Wa = Washington (n = 26), and NIL_Wa = the *FLC* allele is introgressed into Ox genetic background (n = 26)) of *C. hirsuta*. Arrows represent the distance in the multidimensional space between control undamaged plants (black circle) and the *P. brassicae*-damaged plants (grey triangles)

TABLE 3 Results of the three-way permutation ANOVA for total amount of glucosinolates and phytohormones and the two-way permutation ANOVA for plant biomass and plant resistance (i.e. *Pieris brassicae* larval growth) of the four *Cardamine hirsuta* genotypes (G) including the late-flowering genotypes Wa = Washington genotype and NIL_Wa, a near-isogenic line in which the Wa *FLC* allele is introgressed into Ox genetic background, and the early-flowering genotypes Ox = Oxford genotype, and NIL_Ox, a near-isogenic sibling line with the Ox *FLC* allele and Ox genetic background. Each genotype was grown at four sites (S) in the Swiss Alps (Figure S2)

Variable	Factor	*df*	Iter	*p*-value
Glucosinolates (total)	Genotype (G)	3	5,000	**<.001**
	Site (S)	3	5,000	**<.001**
	Treatment (T)	1	51	.92
	G x S	9	1,309	.43
	G x T	3	2,998	**.04**
	S x T	3	5,000	**.004**
	G x S x T	9	2,823	.25
	Residuals	124		
Phytohormones (total)	Genotype (G)	3	5,000	**.002**
	Site (S)	3	5,000	**<.001**
	Treatment (T)	1	5,000	**<.001**
	G x S	9	1,436	.14
	G x T	3	218	.84
	S x T	3	366	.46
	G x S x T	9	4,789	.19
	Residuals	71		
Plant biomass	Genotype (G)	3	5,000	**<.001**
	Site (S)	3	5,000	**.02**
	G x S	9	604	.45
	Residuals	140		
Larval biomass	G	3	5,000	**<.001**
	S	3	1,878	**.05**
	G x S	9	5,000	.16
	Residuals	90		

Bold p-values indicate statistical significance ($p < .05$).

The environment-fitting analyses showed positive correlations among the defence compounds and the bioassay data. For glucosinolates, both plant biomass and larval growth significantly correlated with variation in compounds across genotypes (R^2 = .41, p = .001, and R^2 = .09, p = .01, respectively), but not plant tissue eaten (R^2 = .04, p = .145). For phytohormones, all three variables of plant biomass, larval growth and tissue eaten were significantly correlated with variation among genotypes (R^2 = .52, p = .001, R^2 = .19, p = .01, and R^2 = .66, p = .001, respectively). The PCA analysis of the glucosinolates and phytohormones corroborates these findings (Figure 5). First, the PCA highlights a clear qualitative difference between Wa (i.e. genetic background Wa) and the other three genotypes. This difference seems to be particularly driven by higher quantities of glucobrassicin (GBC), and gluconapin (GNA) in Wa (Figure 5a). Second, the PCA shows a strong correlation between larval biomass and tissue consumed, and between larval biomass and plant biomass. Finally, the strength of the individual glucosinolates arrows is almost orthogonal to the larval mass, indicating little effect of glucosinolates on plant resistance against *P. brassicae*. The PCA analysis of phytohormones (Figure 5b) highlights a more homogenous production across genotypes, and again an orthogonal effect of almost all phytohormones to larval mass.

4 | DISCUSSION

We measured the effects of *FLC* on flowering time, and its potential pleiotropic effects on plant biomass, plant defence and resistance against herbivores for plants grown at different sites in the Alps.

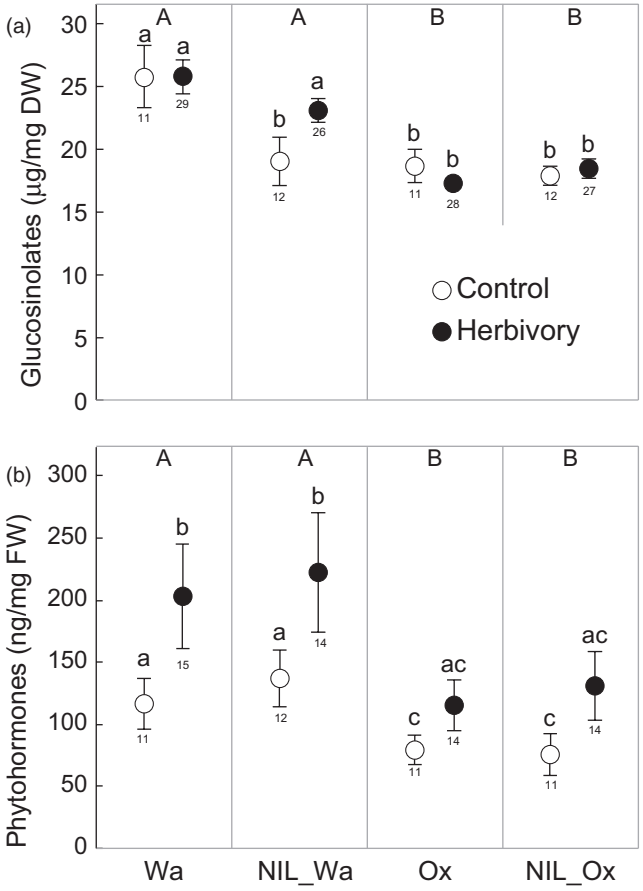

FIGURE 3 *Flowering Locus C (FLC)* effects on *Cardamine hirsuta* defensive chemistry. Shown are *M* ± 1 *SE* of (a) total glucosinolates (i.e. the sum of the five major glucosinolates found in the plant, including gluconapin, glucobrassicanapin, glucotropaeolin, glucobrassicin and gluconasturtiin), and (b) total phytohormones (i.e. the sum of four major phytohormones including salicylic acid, jasmonic acid, jasmonoyl-L-isoleucine and abscisic acid) found in early-flowering genotypes (Ox = Oxford and NIL_Ox = a near-isogenic sibling line with the Ox *FLC* allele and Ox genetic background), and the late-flowering genotypes (Wa = Washington and NIL_Wa = the *FLC* allele is introgressed into Ox genetic background) of *C. hirsuta*. Plants were grown at four different locations and were either left undamaged (Control), or they were induced for 7 days by the larvae of the specialist herbivore *Pieris rapae* (Herbivory). Different lowercase letters above dots indicate statistically significant differences among sites across all genotypes, and different capital letters indicate significant differences between genotypes (Tukey post hoc test; $p < .05$). Sample sizes are shown under each dot

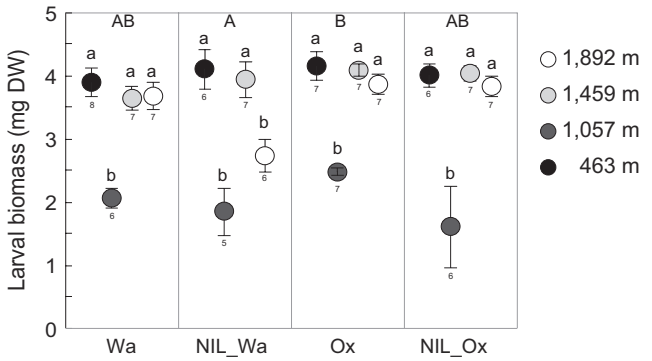

FIGURE 4 *Flowering Locus C (FLC)* effect on *Cardamine hirsuta* resistance against herbivores. Shown are *M* ± 1 *SE* of *Pieris brassicae* larval weight gain when feeding on early-flowering genotypes (Ox = Oxford and NIL_Ox = a near-isogenic sibling line with the Ox *FLC* allele and Ox genetic background), and the late-flowering genotypes (Wa = Washington and NIL_Wa = the *FLC* allele is introgressed into Ox genetic background) of *Cardamine hirsuta*. Plants were grown at four different locations prior to this glasshouse bioassay (Figure S2). Different lowercase letters above dots indicate statistically significant differences among sites across all genotypes, and different capital letters indicate significant differences between genotypes (Tukey post hoc test; $p < .05$). Sample sizes are shown under each dot

Variation at *FLC* was mainly responsible for regulating flowering time and allocation to reproduction (fruits and seeds), but this partially depended on where the plants grew. The flowering locus also indirectly mediated plant allocation to defence, with late-flowering plants producing higher levels of total glucosinolates and stress-related phytohormones. Nonetheless, plant growth and the qualitative values of plant defence and plant resistance against specialist herbivores (i.e. as measured in terms of reduced growth rates by the specialist herbivore, *P. rapae*) were mainly independent of the *FLC* locus (Figure 6). Through its effects on plant growth and secondary metabolism, *FLC* is likely to affect plant resistance against a guild of more generalist herbivores, which are more susceptible to changes in glucosinolate levels.

4.1 | *FLC*, flowering time and G × E effects

As expected, variation at the *FLC* locus affected flowering time (Michaels & Amasino, 1999; Michaels, He, Scortecci, & Amasino, 2003). However, we observed important variation among sites in early- and late-flowering genotypes, highlighting the influence of the environment on gene expression in general (i.e. plasticity) (Kooke & Keurentjes, 2012). In particular, differences in flowering time between the genotypes depended on the site at which they were growing: at site 1, the site at lowest altitude and likely the site offering the best conditions for *C. hirsuta* growth, all genotypes began flowering within the interval of a week, whereas larger differences between late- and early-flowering genotypes were apparent at the remaining sites (lower amount of glucosinolates at site 4). Theory would suggest that the different ontogenetic stages of plant growth at different altitudes might itself modify plant chemistry (Barton & Koricheva, 2010). Accordingly, high altitude-growing plants, due to a decreased, temperature-mediated, development and a growth–defence trade-off, should produce more glucosinolates. However, because we did not find this pattern, and because measurements were taken when most plants had already started bolting, we could rule out a site-mediated ontogenetic effect on plant defences.

Nonetheless, our results suggest that differences among plants brought about by variation at *FLC* become more evident under more stressful conditions (e.g. colder and drier conditions) (Marais, Hernandez, & Juenger, 2013; Mitchell-Olds & Schmitt, 2006). Also,

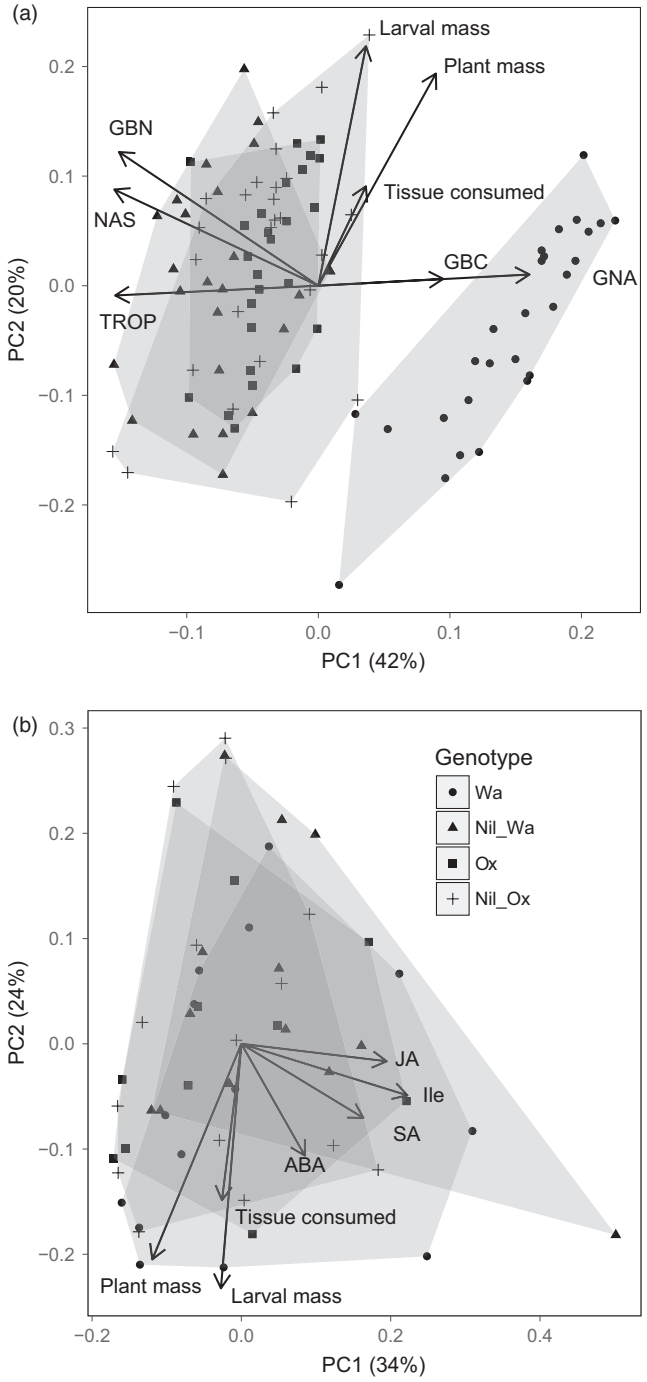

FIGURE 5 Principal component analysis (PCA) of (a) glucosinolates and (b) phytohormones when plotted against plant biomass, larval biomass and tissue consumed. The four different genotypes (the early-flowering genotypes (Ox = Oxford and NIL_Ox = a near-isogenic sibling line with the Ox *FLC* allele and Ox genetic background), and the late-flowering genotypes (Wa = Washington and NIL_Wa = the *FLC* allele is introgressed into Ox genetic background) of *Cardamine hirsuta* are visually separated with shaded polygons. Individual glucosinolates are as follows: GBN = glucobrassicanapin; GNA = gluconapin; NAS = gluconasturtiin; TROP = glucotropaeolin; GBC = glucobrassicin. Individual phytohormones are as follows: JA = jasmonic acid, SA = salicylic acid, Ile = jasmonoyl isoleucine and ABA = abscisic acid

variation expressed among genotypes growing at different sites was mainly attributable to late-flowering genotypes (Wa and NIL_Wa), suggesting that a single-locus introgression may alter the expression of phenotypic plasticity related to flowering time. Finally, it is worth noting that *A. thaliana* plants infested with different strains of pathogens generally reduced their time to flowering (Kazan & Lyons, 2016; Korves & Bergelson, 2003). Therefore, it might be that higher herbivore pressure (at sites 1 and 2) also stimulated a reduction in flowering time, but this hypothesis requires further testing.

Additionally, because our results are based on NILs, where genes located within the introgressed genomic region comprising *FLC* differ between the Ox and Wa strains (see Section 2), it will also be important to validate our conclusions using genome editing approaches to create strains where *FLC* is the only gene mutated in the Ox and Wa backgrounds.

4.2 | Pleiotropic effects of *FLC*

Variation at *FLC* also affected reproduction (fruit production) and total allocation to defence, that is there were clear pleiotropic effects of *FLC*. The greater allocation to reproduction found in the early-flowering genotypes can be related to an earlier flowering time; at high altitude (where differences were also more apparent between genotypes), this may be advantageous, allowing plants to flower and fully mature their fruits before the onset of severe cold compromises their survival.

Variation at the *FLC* locus influenced the total production of glucosinolates, including the induction response after herbivore damage, with a greater content of glucosinolates in the late-flowering genotypes. The *FLC* locus also influenced the phytohormone composition and production, with late-flowering genotypes showing greater levels of phytohormones than early-flowering ones. Although JA is the most important phytohormone linked to plant defence against herbivores, particularly induced by chewing herbivore damage (i.e. caterpillar feeding here), we observed that the greatest differences between the genotypes were brought about by salicylic acid (SA). In addition, SA was predominantly induced after herbivory damage, despite being typically induced in response to piercing and sucking type of insect herbivores. Nonetheless, SA is also an important phytohormone involved in regulation of plant defence against a wide variety of herbivores besides piercing-suckers and has been found to induce several glucosinolates in several species (Kiddle, Doughty, & Wallsgrove, 1994; van Dam et al., 2003). SA induction by specialist herbivores such as *Pieris*, however, merits further exploration, particularly in the light of antagonistic crosstalk between SA and JA (Thaler, Humphrey, & Whiteman, 2012).

Pleiotropy is common for genes involved in the control of flowering time; for example, *FLC* has been found to have an effect on the number of nodes and branches on the inflorescence (Scarcelli, Cheverud, Schaal, & Kover, 2007), on leaf shape and development (Cartolano et al., 2015) and bacterial defence response (Winter, 2011). Evidence also exists for pleiotropic effects of flowering time on the circadian clock period (Swarup et al., 1999), water use efficiency, seed size (Alonso-Blanco et al., 1999), dormany (Alonso-Blanco et al.,

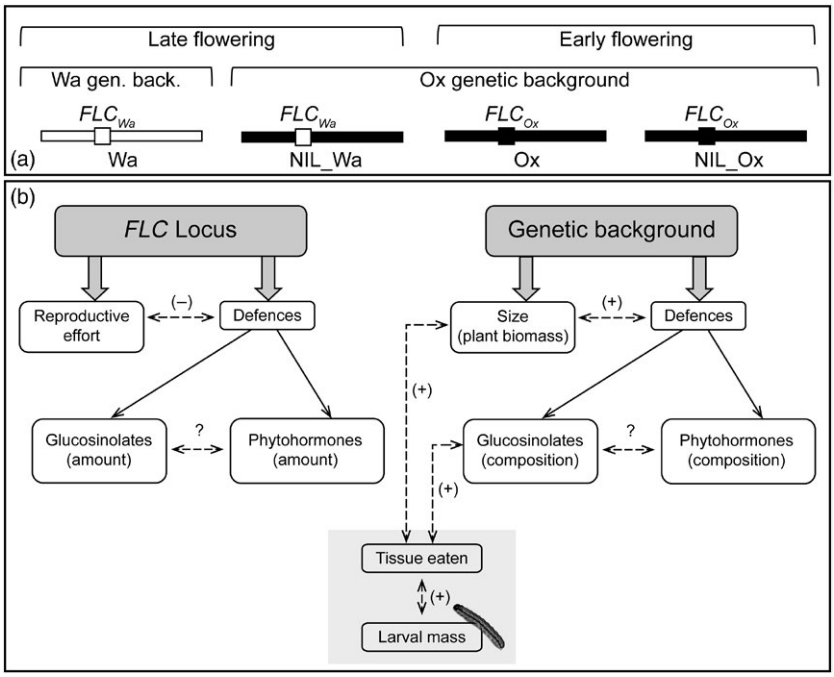

FIGURE 6 Overview of how *Flowering Locus C (FLC)* affects growth reproduction and defences. (a) Schematic representation of the genetic background (long boxes) and *FLC* allele (squares) of the late-(Wa and NIL-Wa) and early-flowering genotypes (Ox and NIL_Ox) used in this experiment; same-colour long boxes represent same genetic background (Wa = white, Ox = black), and same-colour squares represent same *FLC* allele (FLC_{Wa} = white, FLC_{Ox} = dark grey). (b) Overview of the different effects of *FLC* and genetic background of the *Cardamine hirsuta* genotypes that were used in the experiments on reproduction-, growth-, defence- and resistance-related traits. Non-filled boxes represent the different traits measured; note that "defences" are subdivided between glucosinolates and phytohormones. Boxes on a hatched area relate to resistance-related traits. Double-headed arrows (or dashed lines) represent positive correlations (+) or potential trade-offs (−); where the nature of the relationship is unknown, this is indicated as "?". See text for more details

2003), germination (Chiang et al., 2009) and nitrate content (Loudet et al., 2003; McKay et al., 2003). However, to our knowledge, this is the first report of pleiotropic effects of a flowering-time locus on herbivore defence-related traits such as glucosinolate and phytohormone production. Pleiotropic effects are thought to reflect functional and developmental relationships among traits (Cheverud, 2000). In this regard, the greater level of constitutive defence may be related to a physiological trade-off (Agrawal et al., 2010): plants that flower early allocate resources not only to growth but also to reproduction, compromising allocation to defence. On the other hand, late-flowering genotypes need longer to complete their life cycle, and we may therefore expect a greater level of constitutive defence to increase their fitness in an environment with longer herbivore risk of attack.

4.3 | Effects of genetic background on growth and chemical defence

We found no difference in biomass between the late and early genotypes at the time of harvest, suggesting the absence of any clear size threshold that might influence flowering time. However, it has been suggested that differences in leaf development (not investigated here) might influence resource allocation to seeds; early-flowering plants have leaves progressing to adult shapes faster than late-flowering plants, with more leaflets and potentially a higher capacity to produce and transfer photosynthetic metabolites to flowers and fruits (Cartolano et al., 2015). We did find differences in plant size between the two late-flowering genotypes, with the wild-type line (Wa) being greater than the near-isogenic line (NIL-Wa), pointing to a likely role for other genes in controlling plant growth in addition to *FLC* (Cartolano et al., 2015; Hay & Tsiantis, 2010). Interestingly, the wild-type (Wa) also had the greatest levels of constitutive glucosinolates so that the genetic background at loci other than *FLC* was clearly important for some of the variation we observed.

4.4 | Plant growth and resistance

It is widely supposed that variation in defence traits is strongly governed by trade-offs between growth and resistance (Herms & Mattson, 1992; Huot, Yao, Montgomery, & He, 2014). In our experiment, larger plants also had a greater level of constitutive defences (see above for Wa). In addition, the level of defences increased with plant size, even though larger plants were also more susceptible to attack by *P. brassicae*. This result, which is strongly driven by the slower growth of plants at site 2, which were also the most resistant against *P. brassicae*, has two plausible implications. First, it is possible that specialist herbivores might be less sensitive to the outcome of a growth–defence trade-off than generalist herbivores. Indeed, glucosinolates likely defend plants against generalist herbivores, but they may not harm, or might even benefit, specialized herbivores such as *P. brassicae* used in this study (Ali & Agrawal, 2012). In addition, we found

that *P. brassicae* larvae feeding on plant that grew at site 2 gained significantly less weight compared to the other sites. This result points to the interactive effects between plant responses to abiotic stress (warm conditions in this case) and biotic resistance (i.e. leaves of highly stressed plants became more unpalatable) (Rasmann, Alvarez, & Pellissier, 2014). Second, it is possible that biomass alone might not be a good predictor for measuring the postulated plant growth–defence trade-off (Cipollini, Purrington, & Bergelson, 2003; Paul-Victor, Zuest, Rees, Kliebenstein, & Turnbull, 2010). For instance, latex production in milkweeds was also positively correlated with plant growth, while the cost of cardenolide production was observed only when plant growth was dissected into different components, such as relative growth rate and net assimilation rate (Züst, Rasmann, & Agrawal, 2015). In our experiment, we observed that late-flowering genotypes of *C. hirsuta* had higher overall levels of defence. Thus, if resources are shifted towards the production of flowers, fruits and seeds, we might expect to see a trade-off between reproduction and defence, which would have a negative impact on both growth and allocation to defence.

Another way to view trade-offs between resistance and allocation to growth is to consider their impact on herbivore avoidance. Plants with early maturation may, for example, avoid herbivores that only arrive late in the season (Chew & Courtney, 1991). Similarly, when plants delay seed set in favour of vegetative growth, divestment from immediate reproduction may decrease seed predator loads (Janzen, 1971). In the present case, a strategy of delayed flowering may avoid early-fruit herbivore attack, because *P. brassicae* caterpillars tend to move between leaves and flowers and fruits throughout their life while feeding (Mauricio & Bowers, 1990). Interestingly, it was observed that individuals of tarweed plants (*Madia elegans*, Asteraceae), which display natural variation in their phenology, have two distinct phenotypes, a late-season phenotype that also possesses glandular trichomes as indirect defence against herbivores, and an early-season phenotype without trichomes (Krimmel & Pearse, 2014), suggesting that investment in defence traits is costly and may evolve as an alternative to a temporal escape strategy. Along the same lines, late-flowering *Oenothera biennis* plants reduce seed predation by *Mompha brevivittella* moths (Agrawal, Johnson, Hastings, & Maron, 2013), and late-flowering *Lobelia siphilitica* plants suffer decreased herbivory (Parachnowitsch & Caruso, 2008). The effect of delayed flowering on resistance in *C. hirsuta* may thus not only be a mere pleiotropic effect of a complex gene expression network, but also a potentially adaptive strategy into which escape and resistance are incorporated as part of the defence syndrome. Specifically, plants might evolve either to defend against herbivores directly or to avoid them altogether (Agrawal & Fishbein, 2006).

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AUTHORS' CONTRIBUTIONS

S.R., J.S.V. and J.R.P. conceived and designed the experiment; S.R. and J.S.V. collected, analysed the data and led the writing of the manuscript. G.G. carried out the analyses of phytohormones and glucosinolates. M.T. and his collaborators generated the genetic material used. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.d7t8c (Rasmann et al., 2017)

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SUPPORTING INFORMATION

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