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Correlated Induction of Phytohormones and Glucosinolates Shapes Insect Herbivore Resistance of Cardamine Species Along Elevational Gradients

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Abstract

Although the production of phytohormones has been commonly associated with production of plant defence and stress-related traits, few studies have simultaneously investigated this phenomenon across several plant species that grow along large-scale ecological gradients. To address these knowledge gaps, we performed a common garden experiment with six *Cardamine* species, which collectively encompass an elevational gradient of 2000 m. We quantified constitutive and *Pieris brassicae* caterpillars-induced phytohormones and chemical defences in leaves. We found a correlated expression of phytohormone production and the subsequent induction of chemical defences, and this correlated expression reduced herbivore performance. Furthermore, we found that abiotic conditions associated with the optimal elevation range of each species influenced the production of phytohormones and chemical defences, as well as plant growth and productivity. In particular, we found that plant species adapted to milder abiotic conditions at low elevations grew faster, were more productive and produced greater levels of chemical defences. In contrast, plant species adapted to harsher abiotic conditions at high elevations tended to produce greater levels of defence-related oxylipins. Overall, these findings highlight the importance of disentangling the role of phytohormones in mediating plant adaptations to shifting biotic and abiotic conditions.

Keywords Swiss Alps · Elevation gradients · Temperature · Plant-herbivore interaction · Chewing herbivore

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Introduction

Plants are sessile organisms that are constantly confronted to both biotic (*e.g.*, herbivory, disease) and abiotic (*e.g.*, drought, resource limitation) stresses during their lifetime. Plants have thus developed a wide spectrum of mechanisms to cope with and adapt to different types of stress (Gupta et al. 2017; Jeandroz and Lamotte 2017; Nejat and Mantri 2017). In the case of biotic stresses, a crucial step is the rapid and reliable recognition of the biotic aggressor, and the subsequent induction of appropriate plant defences. In response to these attacks, plants perceive aggressor-associated elicitors and rapidly accumulate plant phytohormones, which are small diffusible molecules regulating the production of induced plant defences (Pieterse et al. 2009). The two most important hormonal signalling pathways associated with induced plant defences against biotic attack are the jasmonic acid (JA) and salicylic

acid (SA) pathways (Howe and Jander 2008; Thaler et al. 2012). While the JA-signalling pathway is involved in defence against chewing and mining herbivores, necrotrophic pathogens, bacteria, the SA-signalling pathway is associated with defences against sucking herbivores, biotrophic pathogens and viruses (Pieterse et al. 2012; Zamioudis and Pieterse 2012). More recently, it was discovered that abscisic acid (ABA) and ethylene (ET) are also crucial regulators of herbivore- and pathogen-induced resistance (Erb et al. 2012a; Ton et al. 2009). Although the production of these phytohormones has been commonly associated with the subsequent production of induced defences in single plant species (Bodenhausen and Reymond 2007; Reymond et al. 2004), few studies have investigated this phenomenon across several plant species simultaneously (Rasmann et al. 2015).

Besides their role in regulating induced plant responses against herbivores and pathogens, phytohormones are also central mediators of plant responses and adaptations to the abiotic environment (Peleg and Blumwald 2011). For instance, the role of ABA in drought stress or high salinity has been investigated for decades (Danquah et al. 2014; Mittler and Blumwald 2015; Tuteja and Sopory 2008). Its accumulation in plant tissues controls stomata closure, as well as functional and regulatory protein encoding gene expression (Shinozaki and Yamaguchi-Shinozaki 2007). The role of JA and SA in abiotic-stress integration and adaptation has also recently emerged. JA is involved in plant tolerance to drought (Pedranzani et al. 2007; Suhita et al. 2003) and salinity (Fujita et al. 2006; Moons et al. 1997; Yoon et al. 2009), although the underlying mechanisms and adaptive consequences often remain unclear (Riemann et al. 2015). SA is involved in plant tolerance to extreme temperatures (Horváth et al. 2007; Wahid et al. 2007), drought (Latif et al. 2016; Okuma et al. 2014), salinity (Iqbal et al. 2014; Riemann et al. 2015) and heavy metals (Horváth et al. 2007; Zhang et al. 2015), likely because SA is also involved in the regulation of major physiological processes such as photosynthesis, plant water uptake or nitrogen metabolism (Khan et al. 2015).

Large-scale ecological gradients have enabled a deeper understanding of how biotic and abiotic components of the environment shape natural systems and species interactions (Dobzhansky 1950; Schemske 2009). For instance, elevational gradients and their biotic or abiotic correlates have been shown to be important drivers of concomitant variation in species traits and communities (Rasmann et al. 2014a). Particularly, it is assumed that plants suffer higher herbivory at low elevations (Rasmann et al. 2014b), therefore leading to a higher selective pressure for defences in these plants compared to plants originating from high elevations (Descombes et al. 2017). Additionally, recent studies have demonstrated that abiotic correlates of elevation may concurrently influence levels of herbivory and plant defences. For example, Pellissier et al. (2016) found that both climatic factors (temperature and precipitation) and soil characteristics (fertility) were associated to herbivory and chemical defences across *Cardamine* species growing along an elevational gradient. Nonetheless, how elevation and its climatic correlates influence plant phytohormone production, and in turn affect plant resistance against herbivores and plant growth is still unknown.

The aims of this study were to investigate: (1) whether the production of phytohormones and chemical defences are correlated across species growing along large-scale ecological gradients, (2) whether such correlated expression determines herbivore resistance, and (3) whether abiotic factors associated with elevation determine the production of phytohormones and chemical defences, as well as plant herbivore resistance and plant growth. To address these goals, we performed a common garden greenhouse experiment with six *Cardamine* (Brassicaceae) species that collectively encompass an elevational gradient spanning 2000 m. For each plant, we quantified the concentration of constitutive phytohormones and chemical defences (glucosinolates) in leaves, as well as their inducibility after feeding by *Pieris brassicae* (Lepidoptera: Pieridae), a specialist herbivore feeding on leaves of Brassicaceae species. We also estimated *P. brassicae* growth rate as proxy of herbivore resistance, the abiotic factors (moisture, temperature and solar radiation) associated with the niche of each *Cardamine* species and plant functional traits associated with plant growth and productivity (plant biomass, specific leaf area and photosynthesis) (Díaz et al. 2016). Overall, this study builds towards a better understanding of the ecological and evolutionary drivers of plant defence pathways mediating resistance against herbivores.

Materials and Methods

Plant Material, Greenhouse Experiment and Leaf Sampling

We sampled six species of *Cardamine* (*C. amara, C. flexuosa, C. hirsuta, C. impatiens, C. pratensis,* and *C. resedifolia*) out of 19 (including sub-species) currently growing in Switzerland (Aeschimann et al. 2004). Together, these species encompass an elevational gradient spanning 2000 m, growing from the bottom of Alpine plains (300 m above sea level (a.s.l., *e.g. C. hirsuta*) to more than 3000 m a.s.l. in the Alps (*e.g. C. amara* and *C. resedifolia*) (Defossez et al. 2018; Pellissier et al. 2016), and mostly represent independent speciation events during the radiation of the group (Fig. 1). Seeds of each species were collected on 2–3 wild population on about 10 plants per population across the Alps, and on sites representing the optimal climatic and edaphic niche of each species (see detail of sampling site in Pellissier et al. (2016)). Seeds were germinated in standard potting soil (Orbo-2, Schweizer AG, Lausanne; Switzerland) with perlite (3:1) for one generation to remove potential maternal effects, and the

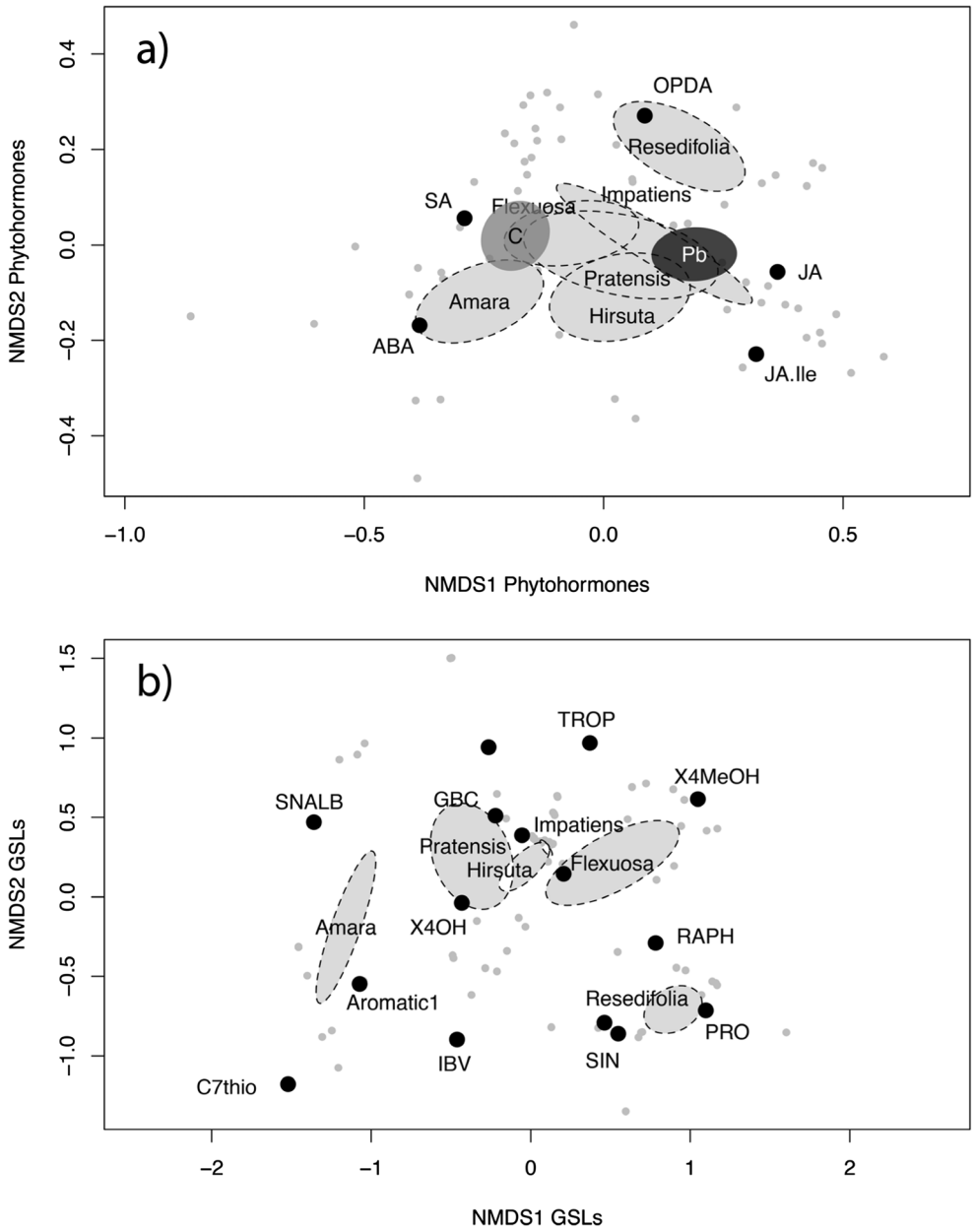

Fig. 1 Non-metric multidimensional scaling (nMDS) plot displaying similarities within herbivore treatment for the six *Cardamine* species. Panel **a** represents similarities in hormonal production across the six species and two herbivore treatment, as either control (C) (light grey circles), or *P. brassicae* herbivore treatment (dark grey circles). Panel **b** represents similarities in glucosinolates (GSLs) production. Because the PERMANOVA analysis found no effect of the herbivore treatment on GSLs, only species differences are depicted. Ellipses represent a 95% confidence bubble around the mean for species and treatments

F1 generation of seeds were germinated and grown for the experiments. After 12 days, seedlings were transplanted into 15-cm diameter plastic pots containing the same soil as for the germination and placed in a greenhouse at 24/18 °C, 55% relative humidity, and a photoperiod consisting of 14 h of daylight (such climatic conditions would reflect low-to-mid elevation climatic conditions in Switzerland). After 6 weeks of growth, plants were infested with four first-instar *P. brassicae* caterpillars (induced treatment, $n = 13$ plants per species) or remained undamaged (control treatment, $n = 7$ plants per species). *Pieris brassicae* larvae were collected near Lausanne (Switzerland) and maintained on cabbage (*Brassica oleracea*) plants in the greenhouse. Four days after inoculation, all caterpillars were collected, frozen, dried at 40 °C for 4 days and weighed. For phytohormone analyses, three damaged leaves per randomly chosen plants ($n = 7$ plants) for the induced treatment, and three undamaged leaves in control plants were collected and immediately flash-frozen in liquid nitrogen. The remaining leaves were oven-dried at 40 °C for 5 days, and weighed. From these tissues, about 50 mg were ground to powder in a Retsch ball mill MM400 (Hann, Germany) for glucosinolates (GSLs) analyses.

Measurements of Plant Functional Traits

Plant functional traits associated with growth and productivity, including plant shoot and root weight, plant height, specific leaf area (light capturing surface of a leaf per unit of dry mass),

leaf toughness and chlorophyll content were measured on the youngest fully-expanded and undamaged leaves of four randomly chosen plants per species. For each plant, height from the ground to the highest leaf was recorded. Specific leaf area was calculated as the ratio of 6 mm diameter leaf disk area to its dry mass (mm^2 mg^{-1}). Toughness was measured as the force (in grams) needed to pierce a 3-mm diameter wide hole into a leaf, and was done by punching three holes in three leaves per plant with a custom-made manual force gauge as in Defossez et al. (2018). Chlorophyll content was measured on the same leaves, three times per leaf, using a SPAD-502 Plus chlorophyll meter (Konica Minolta, Investment Ltd., China). For biomass measures, all plants were cut near the ground, oven-dried at 40 °C for 7 days and weighted. Roots were water-washed, dried as the leaves and weighed.

Phytohormone Analyses

We analysed five phytohormones which are known to be involved in secondary chemistry induction and herbivore performance (Erb et al. 2012b): jasmonic acid (JA), cis-12-oxo-pyhtodienoic acid (cis.OPDA) and jasmonic acid-isoleucine (JA-Ile), salicylic acid (SA) and abscisic acid (ABA). Phytohormones were extracted by adding 1 mL 70% MeOH containing 40 ng of D_6-ABA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), 40 ng of D_5-IAA (Olchemin, Olomouc, Czech Republic), 40 ng of D_4-SA (Sigma-Aldrich), 40 ng of D_6-JA (HPC Standards GmbH, Cunnersdorf, Germany), and 8 ng of JA-[$^{13}C_6$]Ile conjugate as internal standards to 100 mg of ground leaf samples. JA-[$^{13}C_6$]Ile was produced using [$^{13}C_6$]Ile (Sigma-Aldrich) (Kramell et al. 1988). All extracts were vortexed and centrifuged at 16.2 g force for 10 min. The supernatants were collected and used for analysis. Phytohormones separation and quantification were performed using an Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to an API 5000 tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbospray ion source as described in Huang et al. (2017). The hormones were quantified by calculating a calibration equation obtained by linear regression from six calibration points for each compound. Peak areas of the hormones measured in the samples were normalized to the internal standard before applying the calibration equation.

Glucosinolate Analyses

Extraction and analyses of glucosinolates (GSLs) were conducted as previously described (Grosser and van Dam 2017; van Dam et al. 2004). Briefly, between 50 and 100 mg dry leaf material were extracted in 1 ml 70% methanol (MeOH). Samples were immediately heated at 90 °C for 5 min, after which, all the supernatant was added to a DEAE-Sephadex A-25 column (5 × 10 mm) and washed twice with 70% MeOH, once with 1 ml MilliQ water and then twice with 1 ml 20 mM NaOAC buffer (pH 5.5). An on-column aryl sulfatase (20 μl of Type H-1 of *Helix pomatia*; CAS 9016-17-5, Sigma, St. Louis, IL, USA, plus 50 μl NaOAC buffer (pH 5.5)) treatment was performed. The columns were covered with aluminium foil and incubated overnight at room temperature before eluting the desulfoglucosinolates with 2 × 0.75 ml MilliQ water, and freeze-dried. The residue was dissolved in 1.0 ml of MilliQ water and stored at −20 °C until liquid chromatography analysis. The desulfoglucosinolate extract was separated using a reversed-phase C-18 column (Alltima C18 3 μm, 150 mm × 4.6 μm) using a Dionex Ultimate 3000 HPLC with a CH_3CN–H_2O gradient (2–35% acetonitrile from 0 to 30 min; flow 0.75 ml min^{-1}). GSLs were detected with a photodiode-array detector at 229 nm (Dionex, Sunnyvale, CA, USA). Desulfoglucosinolate peaks were identified by comparing the retention times and UV spectra to authentic standards (progoitrin, gluconapin, glucoiberin, glucobrassicanapin, glucotropeaolin, gluconasturtiin, glucoraphanin, glucoerucin, glucobrassicin, sinalbin; Phytoplan, Heidelberg, Germany) or to the in-house retention time and UV-spectra library. The list of all detected compounds can be found in Table 1. For unknown GSLs the different shapes of the UV spectra (between 200 and 300 nm) were compared to known standards to identify the GSL class. GSL concentrations were calculated by using an external sinigrin calibration curve (50–650 μM). Finally, GSLs were categorized as aromatic, indole or aliphatic (Table 1) (Grosser and van Dam 2017).

Environmental Variables

In order to assess habitat-driven natural variation in phytohormones and defences, we acquired occurrence data for each species from the National Data Center and Information on the Flora of Switzerland (www.infoflora.ch). For each species, we extracted environmental data, including elevation, degree-days (hereafter referred as temperature), solar radiation, and potential evapotranspiration (hereafter referred as moisture), which represent the most symptomatic niche values of elevation clines (Körner 2007) (Fig. 1), from associated environmental layers (Pellissier et al. 2016). We calculated values for temperature (degree-days) and moisture (potential evapotranspiration) from meteorological stations using a Digital Elevation Model (DEM) at 100 m resolution, and interpolated following Zimmermann and Kienast (1999). We estimated solar radiation values using the tool implemented in ArcGIS 10.

Gluconapin Toxicity Bioassay

Through random forest analyses, we observed that the GSL gluconapin (GNA) was predominantly negatively associated with *P. brassicae* larval growth (*i.e.* insect resistance) across species (see Results section below). We thus performed a

Table 1 Two-way Anova table for testing the effect of six different *Cardamine* species, a *Pieris brassicae* herbivore treatment against a control, and their interaction on plant phytohormones and glucosinolates. Significant values are indicated in bold. Significance: "*" = $p < 0.05$; "**"; $p < 0.01$; "***" = $p < 0.001$

Class	Compound	Factor	Df	F	*p* value
Phytohormones	OPDA	Species	5	2.77	**0.024****
		Herbivore	1	47.51	**<0.001*****
		S * H	5	0.59	0.707
		Residuals	67		
	JA	Species	5	2.61	**0.032****
		Herbivore	1	126.85	**<0.001*****
		S * H	5	2.31	0.053*
		Residuals	67		
	JA.Ile	Species	5	1.97	**0.093°**
		Herbivore	1	84.06	**<0.001*****
		S * H	5	2.08	0.08*
		Residuals	67		
	SA	Species	5	2.91	**0.019****
		Herbivore	1	8.82	**0.004****
		S * H	5	0.51	0.767
		Residuals	67		
	ABA	Species	5	17.13	**<0.001*****
		Herbivore	1	22.45	**<0.001*****
		S * H	5	1.09	0.37
		Residuals	67		
Glucosinolates	PRO	Species	5	100.32	**<0.001*****
		Herbivore	1	0.004	0.95
		S * H	5	3.12	**0.014****
		Residuals	67		
	SNALB	Species	5	7.37	**<0.001*****
		Herbivore	1	0.11	0.745
		S * H	5	0.05	0.999
		Residuals	67		
	GNA	Species	5	42.56	**<0.001*****
		Herbivore	1	1.88	0.175
		S * H	5	0.66	0.650
		Residuals	67		
	GBN	Species	5	21.74	**<0.001*****
		Herbivore	1	0.18	0.67
		S * H	5	0.39	0.85
		Residuals	67		
	GBN	Species	5	21.74	**<0.001*****
		Herbivore	1	0.18	0.67
		S * H	5	0.39	0.85
		Residuals	67		
	TROP	Species	5	6.64	**<0.001*****
		Herbivore	1	0.03	0.85
		S * H	5	3.35	**0.009****
		Residuals	67		
	GBC	Species	5	23.24	**<0.001*****
		Herbivore	1	0.03	0.704
		S * H	5	2.30	**0.054**
		Residuals	67		
	GBN	Species	5	21.75	**<0.001*****
		Herbivore	1	0.18	0.672
		S * H	5	0.39	0.849
		Residuals	67		

P. brassicae resistance bioassay in controlled conditions (25/18 °C day/night temperature, 55% relative humidity; 16/8 h day/night daylight) using a variety of Chinese cabbage (*Brassica rapa pekinensis* var. Nagaoka, obtained commercially, UFA-Samen, Switzerland) that does not produce GNA (Müller et al. 2015). After 4 weeks of growth, two one-centimeter diameter leaf disks per plant were collected and placed individually in petri dishes lined with humid filter paper. One randomly chosen leaf disk per pair was then added with 50 μmol/g of plant dry weight GNA dissolved in distilled water, while the control was added with the same volume of distilled water. One 2-day-old *P. brassicae* larva was added to each petri dish, at the centre of the arena, near each leaf disk, and left to forage freely for 3 days. Larvae were weighted at the beginning and at the end of the experiment to calculate weight gain as ln(final – initial weight).

Statistical Analyses

Effect of Plant Species and Herbivore Treatment on Phytohormone and Glucosinolate Profiles To assess if plant species and herbivore treatment influenced the composition (*i.e.*, identity and relative abundance) of phytohormones and GSLs in *Cardamine* species, we used non-metric multidimensional scaling (NMDS) implemented in the vegan package (Oksanen et al. 2013) in R version 3.5.1 (R Development Core Team 2017). Differences in phytohormonal and GSL composition among species, herbivore treatment, and their interaction were tested using a permutational multivariate ANOVA (PERMANOVA), using the *adonis* function in the vegan package (Oksanen et al. 2013). The euclidean metric was used to calculate dissimilarity among samples for both the NMDS and PERMANOVA, although results were robust to other distance metrics. Phytohormonal and GSL data were also visualized with principal component analyses (PCAs; *ade4* package (Dray and Dufour 2007)). Following the PERMANOVA, we also measured the effect of species and herbivore treatment on all individual hormones and GSL traits using two-ways ANOVAs, and followed by Tukey's HSD post-hoc tests. Chemical data were $\log_{10}$-transformed prior to analyses. The effect of plant species on *P. brassicae* resistance (larval weight gain till the end of the bioassay) was tested using one-way ANOVA, and followed by Tukey's HSD post-hoc tests.

Correlated Expression of Phytohormones and Glucosinolates Across Species and Its Role on Herbivore Resistance To test if the production of phytohormones and chemical defences were correlated, we tested for a shared structure between the phytohormonal and GSL matrices, which would represent a coupled hormonal-secondary metabolite syndrome, using a coinertia analysis (Defossez et al. 2018; Pellissier et al. 2016). In other words, here we tested whether the matrices of hormonal and GSL production (including abundance and diversity of compounds) are correlated across species. If this were the case, we would conclude that species' production of specific phytohormones correlates with

the production of specific GSLs. We first assessed the correspondence between constitutive phytohormones and GSLs, and then between induced phytohormones and GSLs. The coinertia analyses were performed using the *ade4* package in the R environment (Dray et al. 2003) and the significance of the shared variance was assessed using a Monte Carlo test as implemented in the package *ade4* (Dray and Dufour 2007). Finally, to address if the correlated expression of phytohormones and chemical defences influence herbivore resistance, we correlated the first axis of the coinertia analysis against the average weight gain of *P. brassicae* larvae.

Relationship Between Climatic Niche, Plant Functional Traits, Phytohormones, Chemical Defences and Herbivore Resistance To address if abiotic factors associated with elevation determined phytohormonal and chemical defence production and herbivore resistance, we performed a structural equation modelling to test direct and indirect effects of climate, plant functional traits (as proxy of plant productivity) and phytohormonal concentration on defence compound production and herbivore resistance. The *lavaan* R package (Rosseel 2012) was used for the structural equation modelling. We developed an *a priori* model based on theoretical knowledge of plant defences, in which chemical defences and subsequent plant resistance against herbivores, were potentially indirectly driven by both environmental abiotic conditions (climate; (Coley 1998; Rasmann et al. 2014a) and plant productivity (reflected in variation of plant functional traits (Wright et al. 2004), and mediated by phytohormonal production (Erb et al. 2012b) (Fig. 2). In our structural equation modelling, *climate* corresponded to the first axis of a PCA that included temperature, precipitation and radiation (Fig. 1; positive values indicate warmer and drier conditions). Plant productivity corresponded to the inverse of the first axes of a PCA including species height, dry weight, specific leaf area, photosynthesis, and toughness (Fig. 3; positive values indicated higher and faster biomass production). Because of their multivariate nature, the most influential phytohormones (JA, Fig. 4) and chemical defences (GNA, Fig. 4) were selected using random forest analyses (to estimate the variable importance). For the structural equation modelling, all data were rescaled to correct for large differences in variances. Non-significant relationships were deleted with a *step*-by-*step* approach to select the best-fitted model according to χ^2, root mean square error of approximation (RMSEA), comparative fit index (CFI), and standardized root mean square residual (SRMR).

GNA Toxicity Bioassay The impact of GNA in the diet on the growth of *P. brassica* larvae was tested following a paired design (see above). The relative growth rate of *P. brassicae* was thus analysed using a paired t-test.

Results

Effect of Plant Species and Herbivore Treatment on Phytohormone and Glucosinolate Profiles The six *Cardamine* species significantly varied in their expression of phytohormones (Fig. 1a, PERMANOVA, plant species effect, $F_{5,78} = 3.41$, $p < 0.001$), and how phytohormones were induced (herbivore treatment effect, $F_{1,78} = 46.05$, $p < 0.001$; plant species by herbivore treatment interaction, $F_{5,78} = 2.23$, $p = 0.01$). The production of JA, JA-Ile and OPDA were tightly correlated (along first axis of PCA in Fig. 5), while SA and ABA were orthogonal to jasmonates along the second axis of the PCA (Fig. 5). For all phytohormones, besides strong species-specificity at the individual level (Table 1, Fig. 2), we observed *P. brassicae*-mediated induction (Fig. 2), with JA having the highest induction (1.9 times) and ABA and SA the lowest (1.3 times).

Across the six *Cardamine* species we detected 15 individual GSL compounds (Table 1, Fig. 6), of which, gluconapin (GNA), glucobrassicanapin (GBN), progoitrin (PRO), sinalbin (SNALB), glucotropaeolin (TROP), glucobrassicin (GBC) and an unidentified aromatic GSL were the most abundant. The induction of GSL differed between plant species (Fig. 1b, PERMANOVA, species effect, $F_{5,78} = 17.38$, $p < 0.001$), but it was not affected by herbivory ($F_{1,78} = 1.10$, $p = 0.33$), nor by their interaction ($F_{5,78} = 0.99$, $p = 0.48$).

Correlated Expression of Hormones and Glucosinolates Across Species and Its Role on Herbivore Resistance The coinertia analysis showed a non-significant correlated expression of constitutive phytohormones and GSLs ($r = 0.06$, $p = 0.91$), but when induced, plants displayed a shared structure of the hormonal and the GSL matrices ($r = 0.31$, $p = 0.002$). Specifically, after herbivore feeding, the six species aligned along a first axis that moves from SA, ABA to JA and JA-Ile and corresponding GSL production. Plant species also varied greatly in their resistance against the specialist herbivore *P. brassicae* (Fig. 3), and the first axis of the coinertia was negatively associated with *P. brassicae* larval biomass (Fig. 4, $F_{1,34} = 5.66$, $p = 0.02$).

Relationship Between Climatic Niche, Plant Functional Traits, Phytohormones, Chemical Defences and Herbivore Resistance

Based on random forest analyses, we selected the phytohormone JA and the GSL gluconapin (GNA) for building the structural equation modelling (Fig. 5). Overall, we found a positive relationship between climate and productivity where plant species growing under warmer and drier conditions at low elevations were more productive (Fig. 5). We also found a negative relationship between climate and JA (but not GNA) where

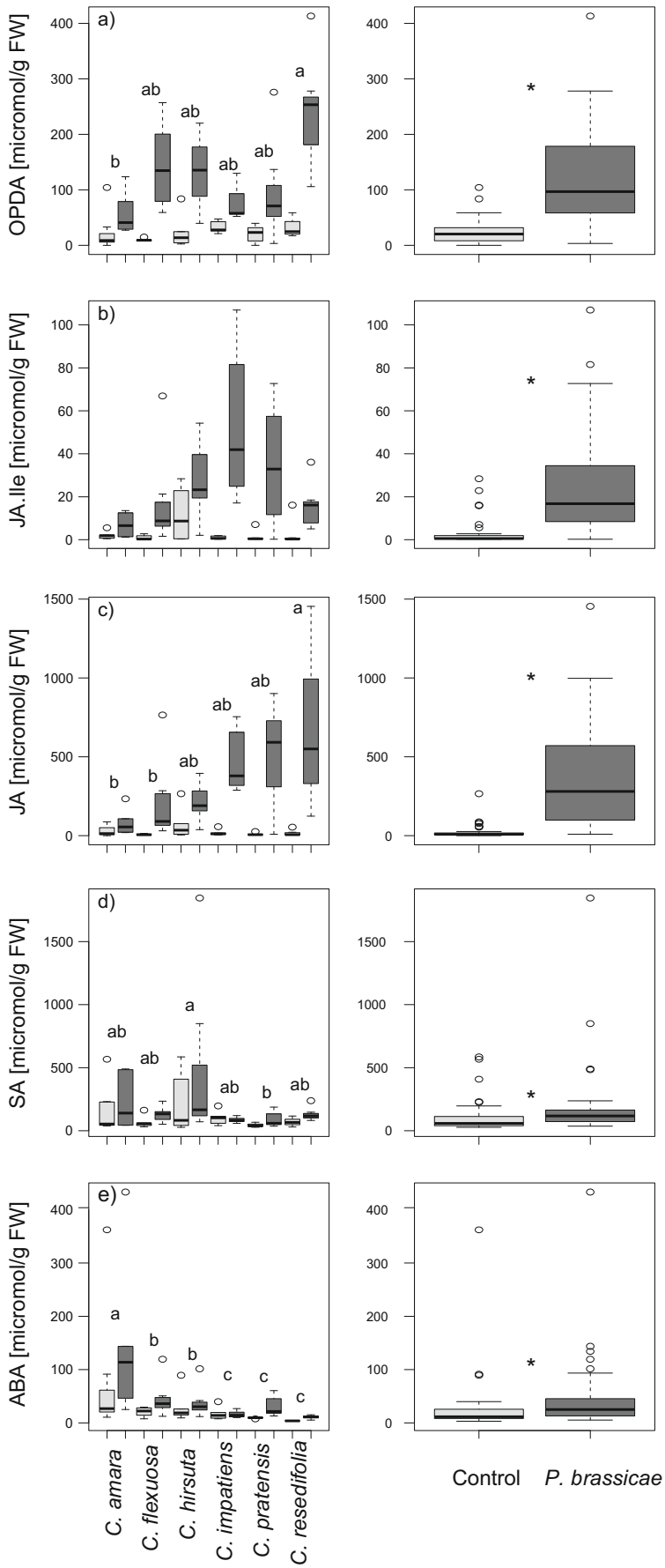

Fig. 2 Hormone production across six *Cardamine* species. Boxplots represent species differences across healthy (control, C, light grey) plants, and herbivore (*P. brassicae,* dark grey) treatments for **a** cis-12-oxo-pyhtodienoic acid (cis.OPDA), **b** jasmonic acid-isoleucine (JA-Ile), **c** jasmonic acid (JA), **d** salicylic acid (SA), and abscisic acid (ABA). Species are aligned from left to right according to increasing averaged elevational distribution. Panels on the left represent species means for both treatments. Dots above or below the boxplots indicate outliers. Asterisks, and letters above boxplots indicate significant difference after TukeyHSD post-hoc tests ($p < 0.05$)

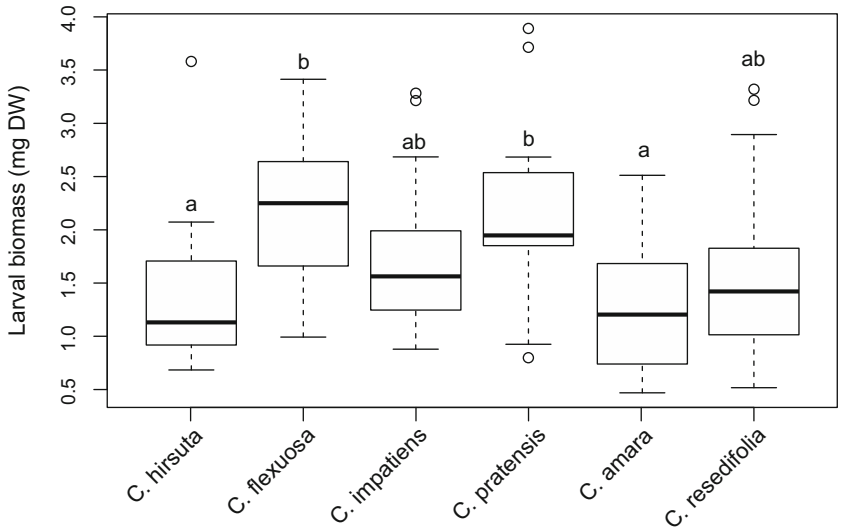

Fig. 3 Insect performance on the six *Cardamine* species. Boxplots represent the average *Pieris brassicae* larval growth rate on six species of *Cardamine*. Species are aligned from left to right according to increasing averaged elevational distribution. Different letters above boxes represent differences across species (TukeyHSD, $p < 0.05$)

plant species growing under colder and wetter conditions at high elevations produced higher levels of JA (Fig. 5). GNA concentration was positively affected by both plant productivity and JA concentration (Fig. 5). As mild environmental conditions affected positively plant productivity our model suggested an indirect positive effect of climate on GNA concentration at low elevations (Fig. 5). However, in light of the direct negative effect of mild environmental conditions on JA concentration, an antagonistic indirect effect emerged at high elevations (Fig. 5). Finally, we found a marginal negative effect of GNA on the herbivores` weight (Fig. 5). GNA significantly reduced the performance of *P. brassicae* larvae when feeding on complemented leaves: the larvae grew 1.6 times more on control leaf disks compared with leaf disks treated with GNA (Fig. 6, paired t-test, $t = 3.23$, $df = 10$, $p = 0.01$).

Discussion

We measured the concomitant constitutive and induced expression of phytohormones and toxic secondary metabolites (glucosinolates, GSLs) across six species of wild-growing plants in the genus *Cardamine*, which span an elevation gradient spanning 2000 m. We found a correlated expression of phytohormones and chemical defences (GSLs) in plants induced by the specialist herbivore *P. brassicae* and this correlated expression increased plant resistance against this herbivore in a bioassay. In addition, we found that abiotic conditions associated with elevation influenced the production of phytohormones and chemical defences, as well as plant growth and productivity. In particular, plant species adapted to milder abiotic conditions at low elevations grew faster, were more productive and produced more toxic (GNA) glucosinolates. In contrast, plant species adapted to harsher abiotic conditions at high elevations tended to produce greater levels of jasmonic acid (JA). These findings highlight the importance of disentangling the role of phytohormones in mediating adaptations to shifting biotic and abiotic conditions, and will sharpen the theoretical framework of plant-herbivore interactions for basic and applied research.

Correlated Expression of Phytohormones and GSLs Across Species

Our results showed that the production of phytohormones and chemical defences (GSLs) across *Cardamine* species was

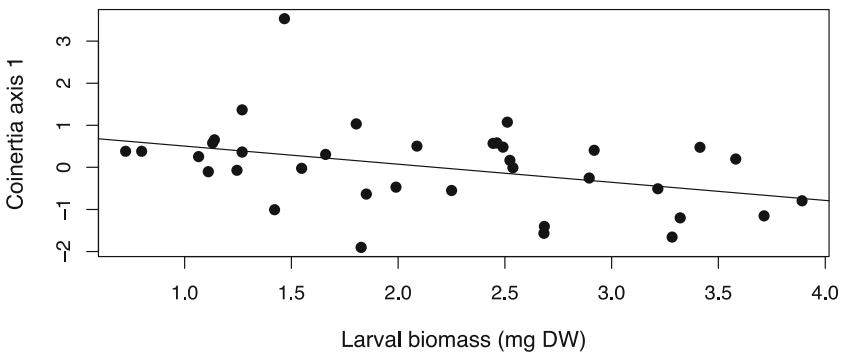

Fig. 4 Correlation between *Pieris brassicae* larval growth and first axis of coinertia analysis. Positive values of the coinertia axis indicate increased production of phytohormones and major glucosinolates (GSLs), while negative values indicate low production of phytohormones and production of minor GSLs

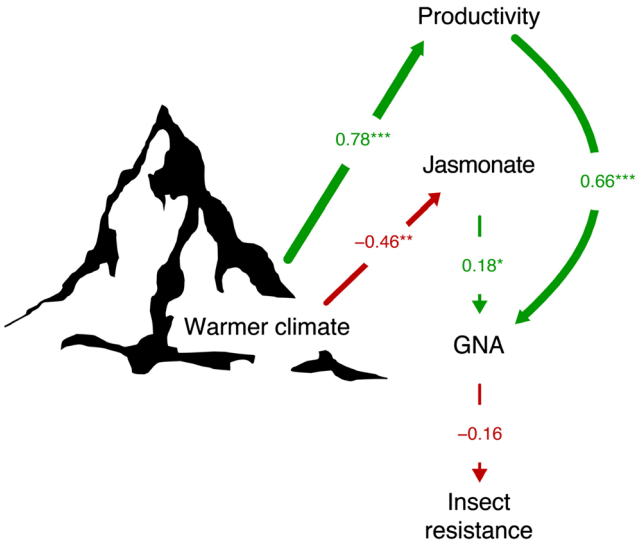

Fig. 5 Selected structural equation model of direct and indirect effect of climate, plant productivity and phytohormonal concentration on plant defence production and herbivore resistance. The weight of the arrows indicates the strength interaction and value of the path coefficient. Positive correlations are represented in green and negatives in red. $\chi^2 = 4.76$, df = 5, $P = 0.452$; CFI = 1.000; TFI = 1.000, RMSEA <0.05; SRMR = 0.07. Significance of regressions: "*" = $p < 0.05$; "**"; $p < 0.01$; "***" = $p < 0.001$

significantly correlated, and such correlated expression increased plant resistance to insect herbivory. Plants activate defence responses to protect themselves from pathogens and herbivores. These induced defences are activated by hormone-mediated signalling cascades (*e.g.*, JA, SA, ABA) in the plant. Previous research has extensively reported that phytohormonal levels in plants positively correlate with the subsequent production of induced chemical defences (Bodenhausen and Reymond 2007; Farmer et al. 2003; Howe and Jander 2008; Kessler and Baldwin 2002). For instance, Schmidt et al. (2011) found increased accumulation of JA and JA-isoleucine in Norway spruce (*Picea abies*) trees in relation to extensive formation of induced resin canals (a physical and chemical barrier

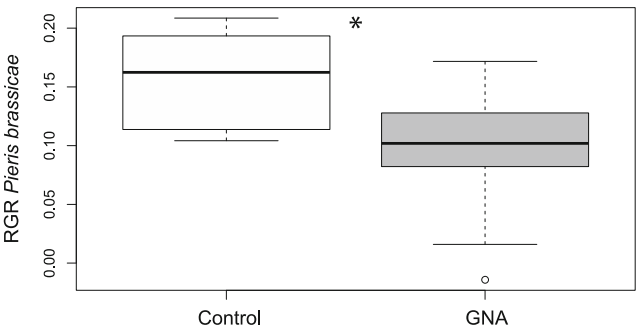

Fig. 6 Gluconapin (GNA) decreases *Pieris brassicae* larval growth rate. *P. brassicae* larvae were grown on leaf disks added with GNA (grey boxes) or added with water (open boxes). The asterisks indicate a significant difference using a paired t-test, t = 3.23, df = 10, $p = 0.01$

in conifers). Similarly, War et al. (2011) found that levels of SA in chickpea (*Cicer arietinum* L.) plants were positively correlated with subsequent induction of phenolics and defensive proteins. Nonetheless, genome-wide search for hormonal-gene orthologues in several species showed that the metabolism and function of plant hormones are generally more sophisticated and diversified in more derived plant species (Jiang and Guo 2010). This would indicate escalation for a more robust phytohormone-chemical defence syndrome, which would promote higher resistance in more derived species. Our limited sample size ($n = 6$ plant species) does not allow teasing this apart statistically. However, although being closely related, *C. amara* and *C. flexuousa* (Pellissier et al. 2016), did not exhibit similar responses (see Fig. 4), suggesting weak phylogenetic inertia in the hormone-defence coupling. Our analyses did not measure glucose conjugates of SA and ABA, which may exhibit different patterns. To summarize, despite increased evidence of the importance of the correlated expression of phytohormones and chemical defences in plant-herbivore interactions, less is known about this phenomenon across several plant species. Future research should address this gap using a wider sampling of species within a same family or genus.

Effects of Abiotic Conditions on Plant Functional Traits, Plant Chemistry and Herbivore Resistance

Our results showed that *Cardamine* species growing under milder abiotic conditions associated with low elevations grew faster and were more productive, which is consistent with previous studies (Defossez et al. 2018; Körner 2003). These authors proposed that the mechanism underlying such effects is slower growth rates at higher elevations, which allows plants to use resources more efficiently in severe climatic environments. Our results also showed that *Cardamine* species growing under milder abiotic conditions associated with low elevations produced greater levels of GNA in their leaves and this in turn increased herbivore resistance in a bioassay. These results are in line with the long-standing hypothesis that increased herbivory in warmer and more stable climates, characteristic of lower elevations, leads to greater investment in plant defences at low relative to high elevations (Rasmann et al. 2014a). For instance, previous research in this system found that *Cardamine* species growing at low elevations suffered greater levels of leaf herbivory and invested more in constitutive GSLs (Pellissier et al. 2016). Similarly, Pellissier et al. (2012) found that, across 16 pairs of congeneric plant species in the Swiss Alps, low-elevation plants supported greater abundance and richness of herbivores, and they were more resistant to herbivory than their high elevation conspecifics.

Our results showed that *Cardamine* species growing under harsher abiotic conditions associated with high elevations produced greater levels of JA in their leaves during herbivore feeding. In addition to their role in plant-herbivore

interactions, jasmonates, including OPDA, JA and JA-Ile, are also involved in plant development (Creelman and Mullet 1995) and in the regulation of plant responses against abiotic stresses (reviewed by Ahmad et al. 2016). For example, Demkura et al. (2010) observed that increased levels of ultraviolet radiation amplified the response of jasmonate-inducible genes such as trypsin proteinase inhibitor in *Nicotiana attenuata* plants. Similarly, De Ollas et al. (2018) found that tomato plants growing under drought conditions accumulated greater levels of JA and JA-Ile in their leaves in comparison with well-watered plants. Finally, Ismail et al. (2012) observed increased levels of JA-responsive genes in a grapevine cell line with salt tolerance. Therefore, the multifaceted effect of hormones in plants is structured according to plant adaptation to specific local biotic and abiotic conditions and their relative contribution to the whole species niche. In other words, higher levels of specific hormones (such as JA at high elevation) does not seem to follow the trend of lower herbivory, but could mainly be a consequence of other abiotic constraints. To finally tease apart if different hormonal networks are under the selective pressure of different axes of the niche, future experiments should include an even higher number of plant species, reciprocal transplants of genotypes adapted to different elevations, and manipulate the activation of several phytohormonal signalling pathways.

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