








Insect egg-killing: a new front on the evolutionary arms-race between brassicaceous plants and pierid butterflies

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Summary

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- Evolutionary arms-races between plants and insect herbivores have long been proposed to generate key innovations such as plant toxins and detoxification mechanisms that can drive diversification of the interacting species. A novel front-line of plant defence is the killing of herbivorous insect eggs.
- We test whether an egg-killing plant trait has an evolutionary basis in such a plant–insect arms-race. Within the crucifer family (Brassicaceae), some species express a hypersensitive response (HR)-like necrosis underneath butterfly eggs (Pieridae) that leads to eggs desiccating or falling off the plant. We studied the phylogenetic distribution of this trait, its egg-killing effect on and elicitation by butterflies, by screening 31 Brassicales species, and nine Pieridae species.
- We show a clade-specific induction of strong, egg-killing HR-like necrosis mainly in species of the Brassiceae tribe including *Brassica* crops and close relatives. The necrosis is strongly elicited by pierid butterflies that are specialists of crucifers. Furthermore, HR-like necrosis is linked to *PR1* defence gene expression, accumulation of reactive oxygen species and cell death, eventually leading to egg-killing.
- Our findings suggest that the plants' egg-killing trait is a new front on the evolutionary arms-race between Brassicaceae and pierid butterflies beyond the well-studied plant toxins that have evolved against their caterpillars.

Introduction

The biodiversity on Earth is shaped by numerous factors including interorganismal interactions that can result in coevolution of adaptive traits. For example, the coevolutionary interactions between plants and insects as described by Ehrlich & Raven (1964) has driven the diversification of plant defensive metabolites (Swain, 1977; Becerra, 2015). In turn, specialist herbivores have evolved detoxification mechanisms, which allow them to feed on their host plants despite these toxic metabolites (Berenbaum, 1983; Despres *et al.*, 2007); for example, caterpillars of the monarch butterfly (*Danaus plexippus*) can feed on cardenolide-containing milkweeds (Cohen, 1985; Malcolm & Brower, 1989), and caterpillars of Pierinae and *Plutella xylostella* in the Plutellidae can feed on glucosinolate-containing Brassicaceae (Wittstock *et al.*, 2004; Wheat *et al.*, 2007; Heidel-Fischer & Vogel, 2015).

The role of plant defences against herbivore eggs has been understudied, especially in a coevolutionary context between

herbivores and plants. The majority of studies on plant–insect interactions have focused on the feeding life stages of herbivorous insects. Yet, in almost half of the ~400 000 known herbivorous insects, especially lepidopteran and sawfly species, eggs may be the first life stage to come into contact with the targeted host plant. Indeed, plants can already perceive and respond physiologically to the presence of herbivore eggs before they hatch (Hilker & Fatouros, 2016). Plant defences against insect eggs may have evolved as an important first line of defence, as every insect egg being detected and killed, is one less herbivorous larva or adult insect feeding on the plant in the near future.

Different types of plant defences against insect eggs have been reported in >30 plant species including gymnosperms and angiosperms (both monocots and eudicots) (Fatouros *et al.*, 2016). In response to insect egg deposition, plants can produce ovicidal substances (Seino *et al.*, 1996), form neoplasms (Doss *et al.*, 2000; Petzold-Maxwell *et al.*, 2011) or express a hypersensitive response (HR)-like necrosis beneath the eggs (Shapiro & DeVay, 1987; Balbyshev & Lorenzen, 1997; Petzold-Maxwell

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et al., 2011; Fatouros *et al.*, 2014). HR-like necrosis is an egg-killing defence leading to eggs desiccating and/or falling off the leaf. It has so far been observed in the plant families Pinaceae (Bittner *et al.*, 2017), Poaceae (Yang *et al.*, 2014), Fabaceae (Garza *et al.*, 2001), Solanaceae (Balbyshev & Lorenzen, 1997; Petzold-Maxwell *et al.*, 2011) and Brassicaceae (Shapiro & DeVay, 1987; Fatouros *et al.*, 2014; Pashalidou *et al.*, 2015a; Griese *et al.*, 2017). To understand whether egg-killing traits have evolved as counter-adaptations to specialist herbivores and their detoxification mechanisms, the phylogenetic occurrence of the HR-like egg-killing trait across these plant families and reciprocal insect pest-clades need to be investigated.

Sequence-based phylogenetic analysis (Al-Shehbaz, 2012; Huang *et al.*, 2015; Guo *et al.*, 2017) has established that the Brassicaceae family is split into a core clade containing 3680 species, which is subdivided into three major lineages, and a smaller sister clade containing only the genus *Aethionema* (61 species) (Beilstein *et al.*, 2006; Beilstein *et al.*, 2008). The model plant *Arabidopsis thaliana* is a representative of Lineage I and the Brassica crop plants are representatives of Lineage II. Lineage III is a smaller group mostly restricted to Asia and lacking a model or crop species. Cleomaceae is the sister family of the Brassicaceae (Hall *et al.*, 2002). Within the Brassicaceae, defences against feeding herbivores and the genetic basis of this defence have been studied intensively (Xue *et al.*, 1992; Graser *et al.*, 2000; Rask *et al.*, 2000; Windsor *et al.*, 2005). Aliphatic glucosinolates evolved as defensive compounds near or at the origin of the Brassicales clade, and became more diverse and complex with plant species radiation. Although these compounds play an important role in defending the plants against herbivory, many feeding insects have specialized and evolved effective glucosinolate detoxification and/or excretion mechanisms (Winde & Wittstock, 2011; Heidel-Fischer & Vogel, 2015; Erb & Robert, 2016; Heidel-Fischer *et al.*, 2019).

The Pieridae butterflies (whites and sulphurs), including approximately 1000 species today (Wahlberg *et al.*, 2014), use host plants belonging to two major plant orders, the Fabales (Fabaceae) and Brassicales (Brassicaceae, Resedaceae, Capparaceae and Cleomaceae), although some species in certain clades also have shifted to Rosales (Rhamnaceae, Rosaceae) or Santalales (Edger *et al.*, 2015). Recent phylogenetic reconstruction of the Pieridae indicates that the ancestral host appears to be fabaceous with multiple independent shifts to other orders. Although the Dismorphiinae and nearly all Coliadiinae are Fabales feeders, the sister to the Coliadiinae, Pierinae, feed primarily on Brassicales (Braby & Trueman, 2006; Wheat *et al.*, 2007). The latter, thus, represent a single origin of feeding on glucosinolate-producing plants.

Shortly after the initial evolution of the order Brassicales, some ancestral Pierinae evolved nitrile-specifier proteins (NSPs) that detoxify glucosinolates. This enabled a host shift from their prior Fabaceae hosts to the Brassicales c. 80 million years (Myr) ago (Edger *et al.*, 2015). Likewise, the evolution of glucosinolate sulfatase in *Plutella xylostella* (Plutellidae) allowed the caterpillars of this moth to feed on Brassicaceae (Wheat *et al.*, 2007; Heidel-Fischer & Vogel, 2015). It has been shown that speciation-rate shifts, as well as genome-duplication events with gene birth–death dynamics

occurred in both Brassicales and Pierinae, usually following a key defence (glucosinolates) or counter-defence (NSPs) invention in one of the coevolutionary partners (Edger *et al.*, 2015). Defence responses targeting eggs might have added a new layer of traits evolved in response to herbivore specialization. To pinpoint the evolution of transitions and innovations of plant defences to insect eggs, it therefore is necessary also to investigate these trait(s) of interest in a proper phylogenetic context.

Defence responses induced by cabbage white butterfly eggs have been studied mainly in *A. thaliana* and the black mustard *Brassica nigra* (Little *et al.*, 2007; Fatouros *et al.*, 2014; Pashalidou *et al.*, 2015b; Firtzlaff *et al.*, 2016; Paniagua Voirol *et al.*, 2020; Stahl *et al.*, 2020). In *A. thaliana*, *Pieris brassicae* and *P. rapae* eggs activate a plant immune response, that resembles pattern-triggered immunity (PTI) against pathogens. It includes expression of defence genes (e.g. pathogenesis-related genes (*PR*)), accumulation of reactive oxygen species (ROS) and a local cell death response. However, a visible necrosis is rarely expressed and egg-killing has never been shown (Little *et al.*, 2007; Reymond, 2013; Groux *et al.*, 2020). Egg-killing resulting from a strong necrosis has been shown for *B. nigra* in response to *Pieris* spp. Within *B. nigra*, there is variation frequency and severity of HR-like necrosis between accessions (Fatouros *et al.*, 2014; Pashalidou *et al.*, 2015a; Griese, *et al.*, 2017).

The current study explores whether egg-killing HR-like necrosis evolved as a specific response to pierid egg deposition in a subset of Brassicaceae. Thus far, to the best of the author's knowledge, no effort has been made to map the phylogenetic history of any egg defence trait for any plant family. Doing so would be a first necessary step to show an adaptive response to egg deposition. We investigated the phylogenetic occurrence of HR-like necrosis in the Brassicaceae (mainly lineages I and II) and three species in the Cleomaceae, and also explored the reciprocal phylogenetic co-occurrence in the Pieridae clade. We tested eggs from four *Pieris* butterflies (Pierinae) and five relatives: *Anthocharis cardamines* (Pierinae) feeding on *Cardamine* spp. (Brassicaceae Lineage I), *Aporia crataegi* (Pierinae) feeding on *Prunus* spp. (Rosaceae), *Gonopteryx rhamni* (Coliadiinae) feeding on *Rhamnus* spp. (Rhamnaceae), *Colias* spp. (Coliadiinae) and *Leptidea sinapis* (Dismorphiinae) both feeding on different species of the Fabaceae. As an outgroup, we used the butterfly *Aglais io* (Lepidoptera: Nymphalidae) that feeds on *Urtica* plants (Urticaceae). Additionally, we studied elicitation of the eggs of two moths, *Mamestra brassicae* (Noctuidae) and *Plutella xylostella* (Plutellidae), both feeding on Brassicaceae. Besides screening for HR-like necrosis, we investigated whether important components of plant defences, such as *PR* gene expression, cell death and accumulation of ROS, correlated with the egg-induced necrosis. We tested the effect of HR-like on survival of singly-laid *Pieris* spp. eggs in different plant species under both field and glasshouse conditions. Finally, we hypothesized the evolution of potential counter-adaptations to egg-killing by some pierid butterflies.

Specifically, we addressed the following questions: (1) Is HR-like necrosis induced in a clade-specific manner within the Brassicaceae? (2) Are HR-like necrosis and other defence responses induced by eggs specific to a particular clade of butterfly species

(e.g. genus, subfamily or family) and/or specific to species that co-evolved with the Brassicaceae? And (3) Is the observed necrosis lowering egg survival under glasshouse and field conditions?

Materials and Methods

Plants and insects

For our study, we obtained seeds of thirty-one species in the Brassicales (28 Brassicaceae and three Cleomaceae), from various sources. For each plant species, between one and 11 accessions were grown (Supporting Information Table S1). Per accession, between three and 17 plants were treated with egg wash to assess elicitation of a HR-like response by *Pieris brassicae*. *Brassica nigra* plants were used to assess elicitation of the HR-like necrosis by different butterfly species. Finally, egg-killing was tested for six plant species. In preliminary trials, plant species with unknown developmental times were grown to assess their germination and flowering after sowing. Then, plants were sown in a scheme to ensure that they had reached similar life stages (i.e. vegetative growth) when used for experiments. Therefore, plants were between three and six weeks old when being treated with butterfly eggs or egg wash.

In order to assess the occurrence of HR-like necrosis across the selected Brassicales species, we used a wash of *P. brassicae* eggs (see the Egg wash preparation section below). To assess induction of HR-like necrosis on *B. nigra* plants, we used egg deposition by different butterfly/moth species and populations (for details, see Methods S1 and Table S2).

Egg wash preparation

Not all butterflies and moths used in this study naturally deposit eggs on all plant species that were selected. In order to be able to test those species and screen a large number of plants efficiently, we developed a method to prepare an egg wash that can be used to mimic oviposition as plant treatment. The development and testing of this method will be submitted elsewhere. We showed that there is no difference in the symptoms induced on *B. nigra* leaves between eggs and egg wash of *P. brassicae* (L. Caarls *et al.*, unpublished). For this method, female butterflies of *P. brassicae*, *Pieris rapae* and *P. napi*, and *Mamestra brassicae* moths were persuaded to lay eggs on paper that was pinned to the underside of a leaf. Wash from *Aglais io*, *Anthocharis cardamines*, *Aporia crataegi*, *Colias* spp., *Gonepteryx rhamni* and *Leptidea sinapis* eggs was made by carefully removing eggs from leaves or inflorescences. Eggs of *P. xylostella* were collected on parafilm. Collected eggs were counted and washed overnight in MES buffer, and buffer was applied on plant leaves. Concentrations of the egg washes were adjusted based on the size of the eggs used (for details see Methods S2).

Phenotyping of HR-like necrosis on Brassicales species

Experiments were carried out in a glasshouse compartment (22–27°C, 50–90% RH, 16 h : 8 h, light : dark). For the screening of

31 Brassicales plant species, 5 µl of *P. brassicae* egg wash was pipetted on a fully mature leaf (the third or fourth leaf from the top) of each plant. Another fully matured leaf (the third or fourth from the top) received pure water containing Tween20 as a control. After four days, leaf disks were harvested from the area where egg wash had been applied using a 1-cm cork borer and put in a rectangular Petri dish with wet blue filter paper. Pictures were taken using a Dino-Lite digital microscope (AnMo Electronics Corporation, New Taipei City, Taiwan). These pictures were visually scored for expression of HR-like necrosis (see below).

Elicitation of HR-like necrosis by diverse Pieridae species

Female butterflies of *P. brassicae* (two populations), *P. napi* and *P. rapae* (two populations) were allowed to lay between five and 10 eggs on two different *B. nigra* accessions (SF19 and SF48) (Table S1) (Griese *et al.*, 2017). *Aglais io*, *A. cardamines*, *Colias* sp. and *G. rhamni* egg wash respectively, were tested on both *B. nigra* accessions. *Aporia crataegi*, *L. sinapis*, *P. manni*, *M. brassicae* and *Plutella xylostella* wash, were each tested on *B. nigra* accession SF48. After 4 d, HR-like necrosis was scored using a scoring system described previously by Griese *et al.* (2017).

Pathogenesis-related protein 1 (PR1) gene expression by diverse butterfly and moth species

In order to measure *PR1* gene expression, 10 µl egg wash of *P. rapae*, *P. manni*, *A. crataegi*, *A. cardamines*, *G. rhamni*, *Colias* spp., *M. brassicae* and *P. xylostella* were each pipetted on the abaxial leaf side of 20 *B. nigra* (SF48) plants per butterfly/moth species, except for *P. xylostella* where egg wash for only six plants was available. After 24 h, two 6-mm diameter leaf disks were taken from the egg wash application site and snap frozen in liquid nitrogen. *PR1* transcript levels were measured on five biological replicates composed of four pooled individual plants. RNA isolation according to (Oñate-Sánchez & Vicente-Carbajosa, 2008), real-time quantitative PCR analysis and primers are described in detail in Methods S3 and Table S3.

Histochemical staining

Pieris brassicae females were allowed to lay two egg clutches of 5–20 eggs on a single leaf per plant. From every plant, one clutch was used for histochemical staining (hydrogen peroxide (H₂O₂) or cell death) whereas the other one was used to score the necrotic leaf area. Samples were taken at 48, 72 or 96 h after oviposition by taking a 10-mm diameter leaf disc around the egg clutch (for details, see Methods S4).

Pieris spp. egg survival on HR-like expressing plants under glasshouse conditions

Experiments were done under long-day glasshouse conditions (21 ± 5°C, 45–70% RH, 16 h : 8 h, light : dark) for *B. nigra*,

B. napus, *B. oleracea*, *B. rapa* and *Crambe hispanica*. *Pieris brassicae* females were manipulated to lay five to 15 separated eggs (i.e. not touching each other) on all lines previously used in the screening of Brassicaceae species. The number of hatching and nonhatching eggs were counted to measure egg survival rates. Previously, *P. brassicae* egg survival was affected only when eggs were laid singly, not touching each other (Griese *et al.*, 2017). The eggs were left on the plant and four days after oviposition HR-like necrosis was scored as present or absent. Egg survival on *Arabidopsis thaliana*, plants were reared under short-day glasshouse conditions ($21 \pm 4^\circ\text{C}$, RH: 70%, 8 h : 16 h, light : dark) to control for fast flowering. Seeds from 36 different Swedish accessions of *A. thaliana* were obtained from the HapMap population (<http://bergelson.uchicago.edu/wp-content/uploads/2015/04/Justins-360-lines.xls>). *Pieris rapae* females were allowed to lay a single egg on one leaf per plant. After five days, survival of eggs was noted by counting the number of hatched caterpillars.

Pieris spp. egg survival assessed by field survey

It has been shown that HR-like necrosis has weaker effects on egg survival under glasshouse than under natural conditions (Fatouros *et al.*, 2014). A survey was conducted to record survival of *P. rapae* and *P. napi* eggs on *B. nigra* plants from a natural population (for details, see Fatouros *et al.*, 2014, and Methods S5).

Phylogenetic trees of Brassicales and Pieridae species

We used a consensus tree based by two recent studies (Huang *et al.*, 2015; Guo *et al.*, 2017) to place our tested Brassicales species accordingly. Both studies analyzed representatives of the three distinct lineages of the core Brassicaceae clade and the first-branching *Aethionema* and the outgroup Cleomaceae (For details see Methods S6 and Table S4).

We mapped the HR-like necrosis induced by the tested butterfly species according to two recent studies: a phylogenomic analysis of Lepidoptera (Kawahara *et al.*, 2019) and phylogenetic analysis of the Papiilionoidea (Wiemers *et al.*, 2020). The first study contained 994 taxa, whereas the second analyzed 496 extant butterfly species in Europe using mitochondrial gene *COI* and ≤ 11 nuclear gene fragments. The European butterflies used were split in 12 subclades. The Pieridae were considered as a single clade and the Nymphalidae divided into seven subclades (Wiemers *et al.*, 2020).

Statistical analysis

Statistical analyses were done using R (R Core Team, 2016). For the screening of plant accessions, contingency tables and χ^2 -tests were used to determine which plant species/genotypes significantly expressed HR-like necrosis after egg wash treatment compared to the control treatment. The contingency tables for the χ^2 -tests were constructed with: the number of egg wash-treated leaves expressing HR-like necrosis; the number of egg wash-treated leaves not expressing HR-like necrosis; the number of

control wash-treated leaves expressing HR-like necrosis; and the number of control wash-treated leaves not expressing HR-like necrosis. With this set-up, all plant accessions within each plant species were tested independently.

Egg survival was analyzed using binomial generalized linear models (GLMs) in which first all variables (plant species, flowering state, HR expression and all interactions between the factors) were used. Based on Akaike information criterions (AICs), unnecessary variables were removed to obtain a more parsimonious model (plant species, HR expression and interaction). Subsequently, R/EMMEANS test or Wilcoxon–Mann–Whitney *U*-test were performed as *post hoc* test.

Differences in induction of HR-like necrosis by different butterflies were tested using binomial GLMs and, to test differences in strength, GLMs with Poisson distribution. Dunn test with Bonferroni–Holm correction was used as *post hoc* test. For differences in HR-severity, Kruskal–Wallis tests followed by *post hoc* Wilcoxon Rank Sum test with Benjamini–Hochberg correction were performed.

Quantification of HR-like necrosis and histochemical staining for each plant species were compared with a Student's *t*-test. Differences in HR-like necrotic area between plant species were analyzed with ANOVA, followed by a Tukey *post hoc* test with Benjamini–Hochberg correction. Gene expression of *PR1* was analyzed using Kruskal–Wallis test followed by a *post hoc* Wilcoxon rank sum test with Benjamini–Hochberg correction.

For all of the statistical analyses involving comparison of mean values (egg survival, histochemical staining, gene expression), the choice of parametric or nonparametric methods was made after checking the assumptions of normality (Shapiro–Wilk normality test) and homogeneity of variances (Fligner–Killeen test) on the raw data.

Results

Origin of HR-like necrosis in the *Aethionema* and core Brassicaceae

Out of 31 Brassicales plant species used this study, five species responded significantly with a HR-like necrosis to *P. brassicae* egg wash. This included species of the tribe Brassiceae and of the genus *Aethionema* (Fig. 1a). In the tribe Brassiceae, egg wash treatment significantly enhanced expression of HR-like necrosis in specific accessions of four species: *B. napus* (in 25–86% of tested plants), *B. nigra* (63–83%), *B. oleracea* (20–40%) and *C. hispanica* (0–86%). HR-like necrosis of *Aethionema arabicum* varied among the tested accessions between 0% and 60% (Table S5). There was no significant induction of HR-like necrosis after egg wash treatment for all other plant species tested compared to control leaves. HR-like necrosis was expressed at low frequency and low severity in several other species of Lineage II. For example, 30% of wild *Lunaria annua* plants showed a weak HR-like necrosis, which was almost significant ($P=0.05$; Table S5). Necrosis was expressed rarely in species in lineage I and III (Fig. 1a): only in single plants of some accessions and once in *Aethionema carneum*.

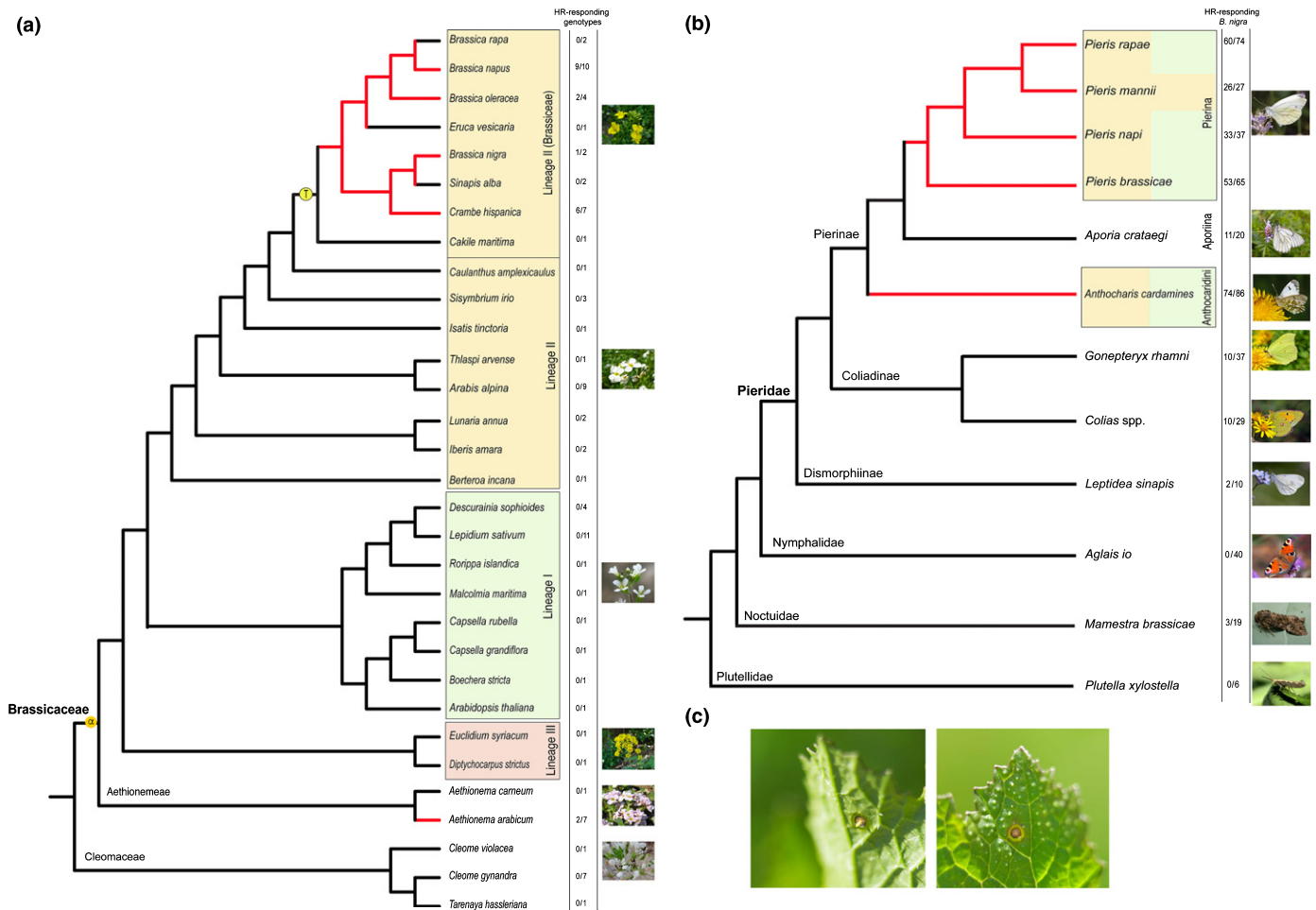


Fig. 1 Presence of hypersensitive response (HR)-like necrosis mapped on phylogenetic trees of Brassicaceae (a) and Pieridae (b). Red lines, at least one genotype of the species expresses HR-like necrosis induced by egg wash significantly more often compared to control; black lines, no genotype expresses HR-like necrosis induced by egg wash significantly more often compared to control; (a) Screening of HR-like necrosis by *Pieris brassicae* egg wash in 28 Brassicaceae species and three Cleomaceae species based on the published phylogenies (Huang *et al.*, 2015; Guo *et al.*, 2017). Different lineages are coloured in orange (Lineage II/tribe Brassiceae), green (Lineage I) and red (Lineage III). The whole genome duplication (WGD) (α) and genome triplication (T) events are marked on the tree. Numbers in the column represent the number of genotypes expressing HR-like (left) from the total number of genotypes tested (right) (b) Elicitation of HR-like necrosis by pierid egg wash or eggs in *Brassica nigra* leaves by different butterfly and moth species shown on phylogenies based on (Kawahara *et al.*, 2019; Wiemers *et al.*, 2020). Responses of the pierid species were compared to the nymphalid *Aglais io*, the noctuid moth *Mamestra brassicae* and the plutellid moth *Plutella xylostella*. Coloured boxes represent species of the Brassicaceae used as main host plants by the butterflies. Brassicaceae and Lepidoptera (sub)families are written on their nodes where they separate from the rest of the clades. Numbers in column represent the number of *B. nigra* plants (SF48) expressing HR-like (left) from the total number of *B. nigra* plants tested (right). Photos of butterflies and moths taken by Zeynel Cebeci, Charles J. Sharp, Juergen Mangelsdorf (all three creative commons license), Jitte Groothuis, Hans M. Smid, Tibor Bukovinszky, and N. E. Fatouros. (c) HR-like necrosis induced by a single *Pieris* sp. egg in *B. nigra* taken from the under and upper side of the leaf (credits N. E. Fatouros).

Elicitation of HR-like necrosis by Pierid species adapted to Brassicaceae

The elicitation of HR-like necrosis in *B. nigra* by egg deposition or egg wash of nine lepidopteran (eight pierid and one nymphalid species) was tested (Fig. 1b). First, we assessed the HR frequency and severity (scored from 0, no symptoms to 3, strong necrosis) in *B. nigra* in response to egg deposition or egg wash of four closely related *Pieris* species, four relatives (*A. cardamines*, *Colias* spp., *G. rhamni*, *L. sinapis*) and *A. io* as outgroup. Eggs or egg wash of *P. brassicae*, *P. napi*, *P. rapae*, *P. manni* and *A. cardamines* induced a high fraction of HR-like necrosis in

B. nigra (0.82 ± 0.06 ; 0.75 ± 0.06 ; 0.86 ± 0.14 , 0.89 ± 0.05 , respectively) and all induced with high severity (Table S6). When several populations were available for butterfly species, all populations elicited HR-like necrosis with similar frequencies (GLM: $\chi^2 = 1.36$, $df = 3$, $P = 0.71$) and severity (GLM: $\chi^2 = 2.60$, $df = 3$, $P = 0.46$). The fraction and severity of HR-like elicited by *G. rhamni*, *Colias* spp. and *L. sinapis* were generally lower than HR-like induced by the eggs of *Pieris* spp. and *A. cardamines* (Tables S6, S7). Moreover, the responses induced in plants by the egg wash of these non-brassicaceous Pieridae in plants appeared to be chlorosis instead of necrosis (Fig. S1). The egg wash of *A. io* induced no symptoms on *B. nigra* (Table S6).

HR-like necrosis severity correlates to *PR1* defence gene expression

We then performed an experiment to compare the response induced by egg wash of Pierinae and their relatives including two moths that can feed on Brassicaceae: *P. xylostella* and *M. brassicae*. As expected, we observed significant differences in HR-like frequency between the Pierinae and other butterfly and moth species (GLM: $\chi^2 = 28.3$, $df = 9$, $P < 0.001$; Table S7) and in HR severity (Kruskal–Wallis: $H = 133.37$, $df = 9$, $P < 0.001$; Fig. 2a). In this experiment, *P. rapae* and *P. mannii* induced HR-like response in all plants tested with high severity (2.88 ± 0.01 and 2.95 ± 0.01 ; Fig. 2a; Table S7). *Anthocharis cardamines* also induced high HR severity on 65% of the tested plants (1.55 ± 0.04). The more distantly related Pieridae *G. rhamni* and *Colias* spp. induced necrosis in only a few plants with low severity (0.31 ± 0.03 and 0.60 ± 0.04 ; Table S7), a similar response to that caused by two moth species and the control treatment (Fig. 2a). HR-like frequency and severity levels induced by *A. crataegi* were between those induced by *Pieris* spp. and *Anthocharis* and the more distantly related species (Fig. 2a; Table S7).

Besides a HR-like necrosis, *Pieris* spp. egg deposition or their crushed eggs also are known to induce other defence responses in *A. thaliana* and *B. nigra*, including *PR1* gene expression following egg deposition or egg wash treatment (Little *et al.*, 2007; Fatouros *et al.*, 2015). We tested if egg washes of other butterfly and moth species also induced a defence response in *B. nigra* by measuring *PR1* gene expression. Interestingly, *PR1* expression was significantly induced by egg wash of all butterfly and moth species tested, except *M. brassicae*. Nevertheless, there were significant differences in *PR1* induction between the different species (Kruskal–Wallis $H = 35.39$, $df = 8$, $P < 0.001$; Fig. 2b). Expression of *PR1* correlated with HR severity and was significantly higher following treatment by egg washes of *P. rapae* and *P. mannii*, and also, although less strongly, by egg wash of *A. cardamines* (Fig. 2b). Plants responding with lower HR severity showed a lower but significant *PR1* expression. Notably, egg wash of the Pierinae *A. crataegi* induced an intermediate *PR1* expression between *A. cardamines* and *G. rhamni*, correlating to HR-like severity. Egg wash of *Colias* spp. induced *PR1* expression that although the highest among the non-Pierinae species, was still about 100-fold lower than that of *P. mannii* (Table S8). Interestingly, *PR1* expression also was induced by *P. xylostella* egg wash, which showed no visual symptoms in these plants (Fig. 2a).

HR-like severity correlates with increased H_2O_2 and cell death in a subset of Brassicaceae

The screening for HR-like necrosis across different Brassicaceae revealed interspecific variation in HR frequency (Table S5). In addition, we also observed variation in HR severity between plant species that showed high HR frequency. To quantify the differences observed, we measured the area of necrotic tissue induced by *P. brassicae* eggs in three species: *B. nigra*, *B. oleracea* and *C. hispanica*. We found that the necrotic area was largest in

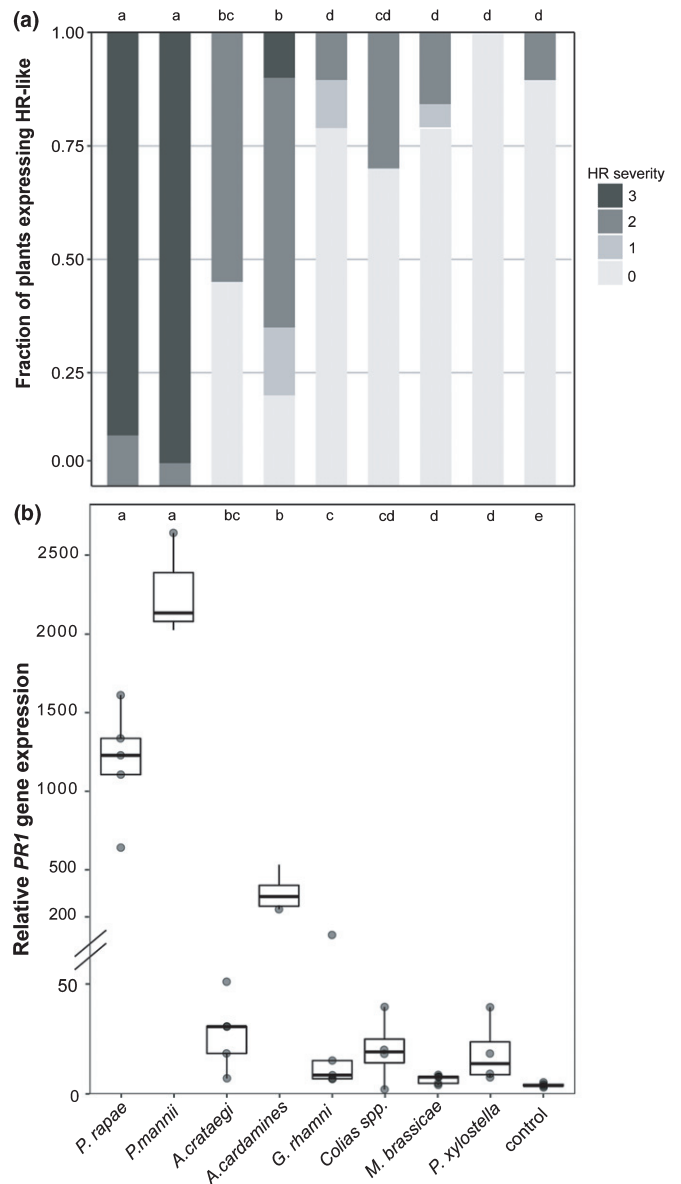


Fig. 2 Hypersensitive response (HR)-like necrosis and pathogenesis-related (*PR1*) gene expression induced by egg wash of different butterfly and moth species in *Brassica nigra* plants. For the experiment, two droplets of 10- μ l egg wash were applied onto one leaf of each plant. (a) Fraction of plants expressing HR-like necrosis at different severities (0, no response; 3, strong necrosis). A total $N = 18$ –20 plants was tested per butterfly species, whereas *Plutella xylostella* egg wash was tested on only six plants. (b) Relative expression of *PR1* gene upon treatment with egg wash of different butterflies/moths or MES buffer as control. Transcript levels were measured by quantitative real-time PCR on four to five biological replicates, each composed of four pooled individual plants. The height of the boxes represents the first to the third quartile of the range; the horizontal line within the box is the median; the whiskers indicate the data minimum and maximum; and dots represent outliers. Letters denote differences in HR severity or mean transcript levels between different treatments (Wilcoxon rank sum test, $P < 0.05$).

B. nigra and significantly smaller in the other two species (ANOVA, $F = 17.028$, $df = 2$, $P < 0.001$; Table S9). Previously, *P. brassicae* eggs on *A. thaliana* were shown to induce

components of plant immunity such as H₂O₂ and cell death despite the absence of a visible HR-like necrosis (Little *et al.*, 2007; Gouhier-Darimont *et al.*, 2013). Therefore, we investigated to what extent the visible stronger or larger HR-like necrosis correlates with induction of H₂O₂ and cell death. Plants that showed a small HR-like necrosis (i.e. *B. oleracea* and *C. hispanica*) exhibited a high accumulation of H₂O₂ and trypan-blue stained cell death compared to the extension of visible necrosis (Fig. S2). *Brassica nigra* showed a strong visible necrosis, > 1 mm² per 10 eggs, exceeding the H₂O₂ and cell death accumulation (Fig. S2). These results suggest that components of the plant immunity are induced regardless of the variation in HR severity and that *B. nigra* induces the visible HR-like response most strongly.

Effect of HR-like necrosis on *Pieris* spp. egg survival on different Brassicaceae species

First, we monitored egg survival on wild *B. nigra* plants of the *Pieris* spp. most abundant under natural field conditions in the Netherlands (*P. napi* and *P. rapae*). Egg survival was 40% lower when eggs induced HR-like necrosis compared to survival of eggs that did not induce a leaf necrosis (GLM: $\chi^2 = 11.02$, df = 1, $P < 0.001$; Fig. 3a), confirming previously reported results (Fatouros *et al.*, 2014). Considering the variation in HR severity and HR frequency between plant species, we investigated the

effect of HR severity in different species on *Pieris* egg survival. We tested egg survival on five plant species from the first screening (Fig. 1a) under glasshouse conditions: three species that showed high HR frequency and contrasting HR severity (*B. napus*, *B. nigra* and *C. hispanica*) and two species with low HR frequency (*B. rapa* and *A. thaliana*). HR-like necrosis significantly lowered the survival of singly laid *P. brassicae* eggs on all three plant species that previously showed high HR frequency (GLM: $\chi^2 = 38.41$, df = 1, $P < 0.001$; Fig. 3b). On *C. hispanica* plants egg survival was significantly lower than on *B. napus* plants (pairwise MWU: $P = 0.006$; Fig. 3b). Conversely, egg survival was not affected by HR-like necrosis for *B. rapa* (GLM: $\chi^2 = 2.61$, df = 1, $P = 0.14$; Fig. 3c) that generally showed low HR severity. On the different *A. thaliana* accessions, no visible HR-like necrosis was observed, and 100% of *P. rapae* eggs survived (Fig. 3d).

Discussion

Pierid butterflies and their brassicaceous host plants are a fascinating model system of co-evolutionary interactions, and research so far has explored the evolutionary and genetic basis of these interactions by focusing on the diversifying selection on plant chemical defences (i.e. glucosinolates) and insect nitrile-specifier protein (NSP) detoxification genes (Edger *et al.*, 2015b; Nallu *et al.*, 2018). Here, we attempt for the first time to map the

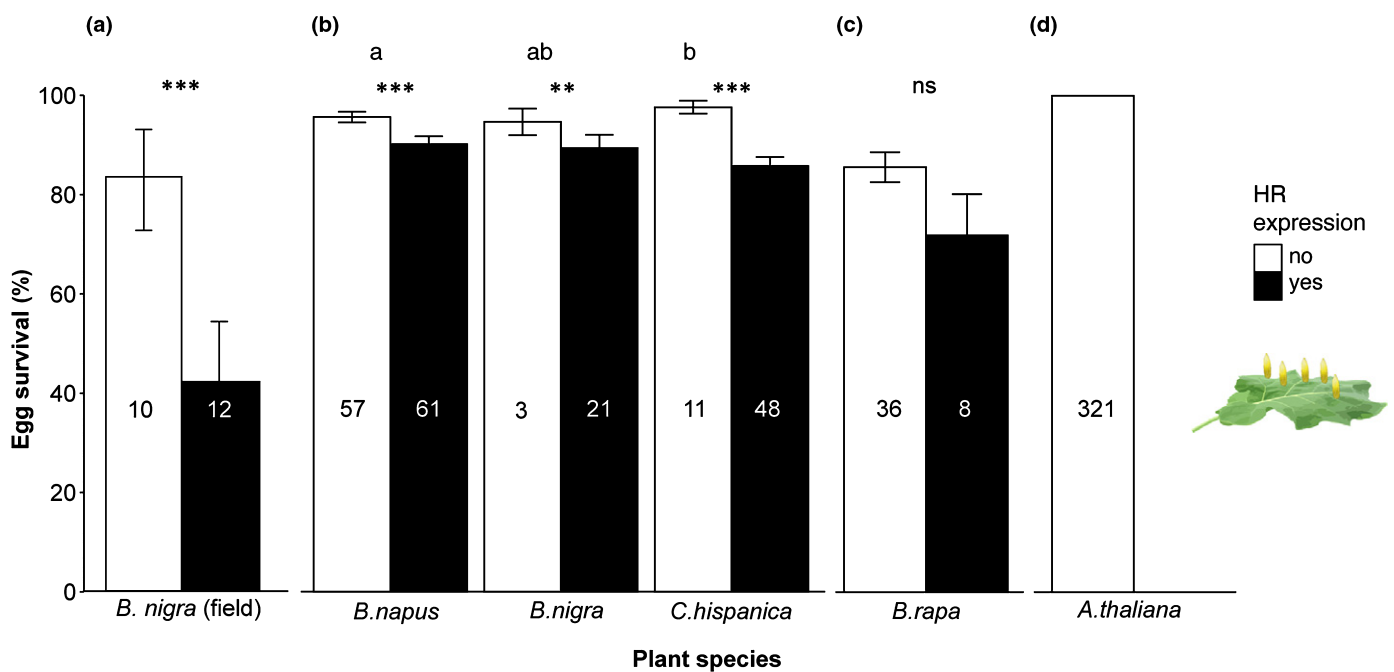


Fig. 3 Effect of hypersensitive response (HR)-like necrosis on survival rates of singly laid *Pieris* eggs on different plant species. (a) Effect of HR-like necrosis on egg survival in field conditions. Survey of *P. napi* and *P. rapae* eggs on *Brassica nigra* plants located near the river Rhine in Wageningen (the Netherlands). One to 13 eggs were sampled per plant. (b–d) Effect of HR-like necrosis on egg survival under glasshouse conditions. Single eggs were separately laid on the leaf without touching each other as shown on the right side. Three experiments were performed with singly laid *P. brassicae* eggs on different accessions of *B. napus*, *B. nigra*, *Campe hispanica* (b) and *B. rapa* (c) as well as *P. rapae* eggs laid on *Arabidopsis thaliana* (d). Ten single eggs were laid on each plant for experiment (b), five single *P. brassicae* eggs on each plant for experiment (c) and a single *P. rapae* egg per plant for (d). If a plant expressed HR-like necrosis under at least one egg it was counted as HR-expressing 'yes'. Numbers in columns represent number of plants tested. Egg survival represents mean \pm SE of hatched eggs for each plant. If a plant expressed HR-like necrosis under at least one egg it was counted as HR-expressing 'yes'. Asterisks indicate significant differences in egg survival between plants with or without HR-like necrosis. Different letters indicate significant differences in egg survival between plant species, without taking HR-like necrosis into account (GLM; ns, not significant; **, $P < 0.01$; ***, $P < 0.001$).

phylogenetic history of egg induction (i.e. hypersensitive response (HR)-like necrosis) as a plant defence trait and its reciprocal co-occurrence in the herbivore clade. We show that a strong HR-like necrosis induced by *Pieris* eggs most frequently occurs in one clade within the Brassicaceae. Half of the tested plant species from the Brassicaceae tribe in Lineage II express HR-like necrosis with high frequency in response to *P. brassicae* egg wash. The visual necrosis was accompanied with increased levels of hydrogen peroxide (H_2O_2) and cell death in three representative HR+ plant species. For the tested *Brassica* and *Crambe* spp. (tribe Brassicaceae), HR-like necrosis lowered egg survival both under natural and glasshouse conditions, except for *B. rapa* that does not express a strong HR-like necrosis as (e.g.) *Crambe hispanica* or *Brassica nigra*. Interestingly, egg survival was generally lower on *B. rapa*, which could hint to plant defences other than HR-like necrosis or nonideal circumstances for *Pieris* eggs. Furthermore, we showed for the first time that only egg wash from species of the subfamily Pierinae that are specialized on the Brassicaceae (i.e. *Pieris* butterflies and *Anthocharis cardamines*) elicit a strong HR-like necrosis and high levels of pathogenesis-related (*PR1*) defence gene on *B. nigra*. Species that are specialized on Fabaceae or Rhamnaceae from the Coliadinae, *Colias* spp. and *Gonopteryx rhamni*, and Dismorphiinae, *Leptidea sinapis*, elicited a weak necrosis or sometimes just a chlorotic response similar to that of *Solanum dulcamara* to *Spodoptera* eggs (Geuss *et al.*, 2017). Our results suggest that the elicitation of strong HR-like necrosis by *Pieris* eggs may have a single origin in the ancestor of the Brassicaceae (Fig. 1) with variation in the frequency and severity of the trait between species and accessions, whereas the trait is most strongly expressed in the Brassicaceae tribe. Moreover, we show that *B. nigra* plants specifically evolved strong HR-like necrosis to the eggs of those pierid species that evolved effective glucosinolate detoxification mechanisms.

Evolution of HR-like necrosis in Brassicaceae and *Aethionema*

Four of eight tested Brassicaceae species showed consistent HR-like necrosis to *Pieris* egg wash in high frequency and severity in at least one of the genotypes tested. In other plant species in the Brassicaceae, we found no consistent induction of HR-like necrosis by *Pieris* egg wash. It is unlikely that the genome triplication event specific to the Brassicaceae clade is the only factor involved in the evolution of HR-like as one *Aethionema* species responded to *Pieris* eggs with a strong necrosis. There may be ecological reasons, such as overlap in spatial distribution between butterflies and plant species, that can explain why HR-like necrosis appears more severe and at higher frequency within the Brassicaceae and *Aethionema*. In fact, besides many of the tested Brassicaceae plants, *Aethionema* are natural host plants for Pierinae species as well. *Pieris ergane*, *Anthocharis gruneri* and *Euchloe ausonia* are specialized on *Aethionema* species in their southeastern European habitat (Tolman & Lewington, 2009). Because of high abundances of Pierinae species occurring on *Aethionema*, it could be that species of this basal clade of the Brassicaceae retained a severe HR-like necrosis as an effective trait against eggs of these butterfly species.

In other plant species tested, occasionally a single plant showed a light HR-like necrosis. These plants might be able to detect insect eggs and respond with a general immune response, as recently shown for *A. thaliana* (Gouhier-Darimont *et al.*, 2013, 2019; Stahl *et al.*, 2020). Alternatively, it could be a false-positive response due to a contamination or a general stress response, as in rare cases, also control wash induced a weak necrosis. In general, not all tested plant species within the Brassicaceae tribe within Lineage II expressed HR-like necrosis though. Variation for the HR-like necrosis trait between genotypes of one species, as we find here, has been observed before (Pashalidou *et al.*, 2015a; Griese *et al.*, 2017). It is therefore possible that we may have missed HR-like necrosis expressing plants because of the selection of nonresponsive or less sensitive genotypes for some of the plant species or genus. For example, *Sinapis alba* did not show HR-like necrosis (Fig. 1) but previous work on the close relative *S. arvensis* showed that eggs of *P. rapae* and *P. brassicae* strongly induced HR-like necrosis (Griese *et al.*, 2020). For the model species *A. thaliana*, a few accessions other than the ones included in this study did show a chlorosis and/or some necrosis to *P. brassicae* eggs (Reymond, 2013; Groux *et al.*, 2020). For half of the tested species here, only one genotype was tested, increasing the likelihood of selecting only nonresponsive ones (Fig. 1a; Table S1). Some plant species and accessions might have lost the ability to express HR-like necrosis, or only do so rarely. Those plants may be less frequently used as host plants for pierid butterflies, for example because of a phenological mismatch between the plant species and its potential specialist herbivores. This mismatch can be especially true for species belonging to lineages I and III. For example, in central Europe *A. thaliana* usually is not attacked by pierid butterflies, as it is rather small and usually completes its life cycle before pierid caterpillars could develop on the plant (Harvey *et al.*, 2007). Notably, *A. cardamines* was observed to deposit eggs on *A. thaliana* in North Sweden where both life cycles briefly overlap (Wiklund & Friberg, 2009). Yet, *P. rapae* eggs did not induce a leaf necrosis lowering *Pieris* egg survival on Swedish accessions of *A. thaliana* (Fig. 3d), neither did we observe a visible necrosis on the commonly used genotype Col-0 in our experiments when using *P. brassicae* egg wash (Fig. 1; Table S5). The observed variation in HR-like necrosis between genotypes of the same species suggests that expression of this trait might have negative effects on plant fitness and only evolves with high herbivore pressure. Alternatively, variability in a defence trait might in itself be defensive, as postulated by the moving-target strategy to counteract the development of efficient plant defensive responses by herbivores (Adler & Karban, 1994). Phenotypic variation in HR-like necrosis to eggs previously was suggested to be part of such a moving-target game (Hilker & Fatouros, 2015).

Counter-adaptations of brassicaceous-feeding Pierinae species to HR-like necrosis

Previous work has shown that the NSP glucosinolate detoxification gene was a key innovation in the ancestral Pierinae enabling them to shift host plant from Fabaceae to Brassicaceae (Edger

et al., 2015b). We show that strong, egg-killing HR-like necrosis linking to high levels of *PR1* gene expression in *B. nigra* seems specific to species of the two independent lineages, Pierini and Anthocharidini, belonging to the Pierinae subfamily that colonized the Brassicales some 50 Myr ago (Wheat *et al.*, 2007). We suggest that this may be a counter-adaptation of some brassicaceous plants to the nitrile-specifier genes that evolved in the Pierinae (Edger *et al.*, 2015). Because those nitrile-specifier genes detoxify glucosinolates and enabled butterflies of those lineages to conquer the Brassicaceae, a new and separate plant defence mechanism might have evolved. Reciprocally, pierid butterflies also may have found ways to counter-adapt to the egg-killing HR-like necrosis. For example, they could do so by clustering eggs, ovipositing on inflorescences and/or shifting to other host plants (Fig. 4). Clustered eggs of *P. brassicae* were shown to negate the egg-killing effect of the HR-like necrosis (Griese *et al.*, 2017). Although the mechanism underlying this is unknown, it has been shown that desiccation can be slowed down by clustering eggs (Clark & Faeth, 1998; Griese *et al.*, 2017). This might be mitigated by the reduced egg surface area exposed to the environment, compared with single eggs. Besides *P. brassicae*, only the closely-related *P. cheiranthi* feeding on *Crambe* sp. and *A. crataegi* evolved to oviposit eggs in groups within the Pieridae family. In general, most butterflies deposit eggs singly (Stamp, 1980).

Within the Anthocharidini, the majority of species evolved to oviposit on flower buds instead of leaves (Fig. 4) (Tolman, 2001). Inflorescent organs seem unlikely to develop an HR-like. When collecting *A. cardamines* eggs from the inflorescences of *Cardamine* spp. we did not observe any signs of necrosis (N. E. Fatouros, pers. obs.). A few *Euchloe* species of the Anthocharidini colonized non-HR expressing species belonging to Lineage III (*E. penia*) or Resedaceae (*E. charlonia*) (Tolman, 2001), which might have enabled them to oviposit on leaves again (Fig. 4). From the Pierini, only *P. krueperi* seem to have evolved to lay eggs on flower buds. We observed *P. napi* to lay eggs on

inflorescences of flowering *B. nigra* plants (N. E. Fatouros, N. Bassetti, pers. obs.) but other records are not known so far. It would be interesting to further study the evolution of oviposition on inflorescence in the Pierinae, both on a macro- and microevolutionary scale.

After the first shift from Fabaceae to Brassicaceae, some butterfly species have shifted to plants of other families again. The closely related *Pontia* spp., for example, colonized plants from the Resedaceae and Cleomaceae and *A. crataegi* the Rosaceae. Within the *Pieris* spp., many are abundant in nature on species of the Brassicaceae clade. Only two butterfly species specialized to plant species outside the Brassicaceae: *P. ergane* feeds on *Aethionema* spp. and the Southern small white, *P. mannii*, on *Iberis* spp. Egg wash of the latter was shown to induce a strong necrosis in *B. nigra* (Fig. 2). So far, we have not observed that eggs of non-brassicaceous feeding species (e.g. *A. crataegi* or *G. rhamnii*) induce HR-like in their preferred host plants, *Prunus* spp. or *Rhamnus* spp., respectively. However, we cannot exclude that plants in those families have developed ways to defend against Pieridae eggs.

Molecular and cellular responses to insect eggs

When the response to *Pieris* eggs was first described in *B. nigra* some 30 yr ago (Shapiro & De Vay, 1987), the induction of cell death was only known from biotrophic pathogens, whose spread is limited by the death of cells. It is now clear that cell death is a common phenomenon with many different causes, that can be induced by several different biotic interactors, including insects and nematodes (Balint-Kurti, 2019). In our study, we found that HR-like cell death is induced in the Brassicaceae tribe by *P. brassicae* egg wash, and in *B. nigra* by all Pierinae species tested. To understand if the mechanism of this response is shared between these different plant species, and in response to the different butterfly species, detailed knowledge on the molecular responses to eggs, genes that are involved in the detection and

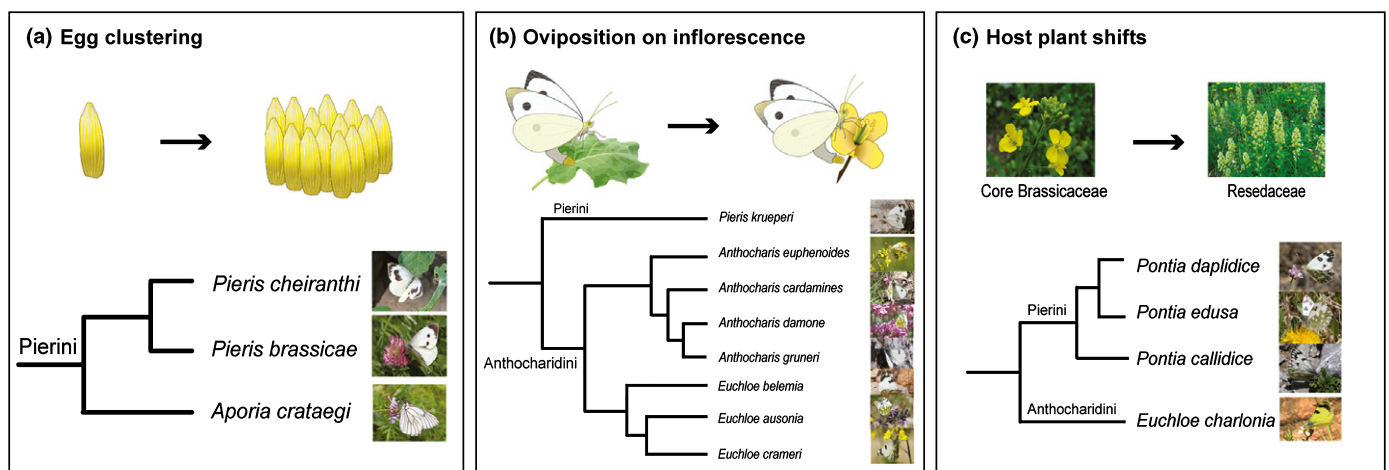


Fig. 4 Examples of possible counter-adaptations to egg-killing hypersensitive response (HR)-like necrosis of the Pierinae butterfly clade: egg clustering (a), oviposition on inflorescence (b), and host plant shifts to non-HR expressing species (c). Phylogenetic relationships are according to Wiemers *et al.* (2020), and oviposition traits and host plants were retrieved from Tolman (Tolman, 2001). Photo credits: Matt Rowlings and creative common license.

recognition, and elicitors of the response are required. An in-depth molecular characterization of the Pierinae egg-induced HR-like compared to other microbial-induced HR goes beyond the aim of this study. Nevertheless, we have attempted to start with a description of the molecular response to insect eggs by studying trypan blue-stained cell death and accumulation of reactive oxygen species (ROS) in three plant species, and *PR1* expression in *B. nigra* towards nine insect species. In this study, ROS accumulation and cell death were induced in all plant species tested, whereas the strong HR-like necrosis and high *PR1* expression was specific to *B. nigra* and to Pierinae insect species. It is possible that also in other species and accessions in the Brassicaceae that we have not investigated closely, a general immune response lacking a strong cell death is activated by *Pieris* eggs, as was shown for *A. thaliana* (Col-0) (Gouhier-Darimont *et al.*, 2013, 2019; Stahl *et al.*, 2020; Valsamakis *et al.* 2020). Our data suggest that the strong HR-like necrosis is always accompanied by ROS and high *PR1* expression. However, because our histochemical stainings involved only three plant species (*B. nigra*, *B. oleracea*, *C. hispanica*), our observations may have been confounded with possible plant interspecific variation in the H₂O₂ and cell death-inducing pathways. To understand whether the different species in which *P. brassicae* egg wash induce cell death share the same or similar mechanisms, requires the identification of genes involved in egg detection and downstream defence response activation in the responsive plant species identified in this study. At the moment, we are undertaking genetic studies to identify putative plant receptors required for perception of *Pieris* eggs in different *Brassica* spp.

Elicitor of HR-like specific to Pierinae eggs

Although induction of strong HR-like necrosis and high levels of *PR1* gene expression in *B. nigra* was specific to *Pieris* and *Anthocharis* species, neither the non-Pierinae butterflies nor the moth species tested induced a strong HR-like necrosis on *B. nigra* (Figs 1, 2; Table S6). This suggests that the elicitor for HR-like necrosis is one or several molecules found only in Pierinae eggs, rather than a general molecule present in (all) butterfly eggs. The differences in the severity of HR-like necrosis elicitation between different Pierinae species could either be caused by quantitative differences of these elicitor(s), or by changes in their chemical composition. In *A. thaliana*, eggs from distantly related insect species were recently shown to release phosphatidylcholines (PCs) that induce a general immune response (i.e. pattern-triggered immunity) involving salicylic acid and H₂O₂ accumulation (Little *et al.*, 2007; Gouhier-Darimont *et al.*, 2013, 2019; Stahl *et al.*, 2020). A lectin receptor kinase, LecRK-I.8, might be involved in early perception of eggs from two widely divergent species, *P. brassicae* and *Spodoptera littoralis* (Gouhier-Darimont *et al.*, 2019). Interestingly, low *PR1* expression was induced by egg wash of Coliadinae butterflies and *P. xylostella* in *B. nigra* also in our experiments. These results support a model where a general egg molecule (PCs) is detected by many plants (including *A. thaliana* and *B. nigra*) and a Pierinae-specific egg-associated molecular pattern (EAMP) may be detected specifically by the

Brassicaceae tribe. This would be similar to the detection of microbe-associated molecular patterns (MAMPs) by the plant immune system (van der Burgh & Joosten, 2019). An exciting next step would be the identification of the Pierinae-specific elicitor(s). Currently, we are analyzing the chemical composition of egg wash from different butterfly species to identify the compounds inducing HR-like necrosis.

In conclusion, we provide a first attempt to disentangle the evolution of HR-like in the Brassicales and show that various Brassicaceae plants can mount an HR-like induced by *P. brassicae* eggs and that this trait might be under similar selective pressures as plant defences against feeding insects. A coevolutionary arms-race between eggs from species of the Pierinae and plant species within the Brassicaceae clade is likely to have occurred. Plants within this clade make use of necrotic lesions to lower egg survival and in this way might have evolved a new mechanism, possibly co-opted from pre-existing plant immunity mechanisms, to combat eggs of specialist herbivores adapted to their host plants' toxins.






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Author contributions

EG, LC, NB, SM, PV, MES and NEF planned and designed the research; ED, LC, NB, SM, PV, GB and NEF performed the experiments and/or analyzed the data; EG, LC, NB, SM, PV, EHP, RG, MES and NEF executed data interpretation; EG, LC, NB and NEF wrote the manuscript with contributions by SM, PV, EHP, RG and MES; and all authors read and drafted the final version. EG and LC contributed equally to this work.

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Data availability

Data available in the Supporting Information.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Leaves from *B. nigra* treated with egg wash of different butterfly species and controls inducing or not a HR-like necrosis.

Fig. S2 Quantification of HR necrosis and histochemical staining of reactive oxygen species and cell death in *B. nigra*, *B. oleracea* and *C. hispanica* leaves upon oviposition of *P. brassicae* egg clutches.

Methods S1 Butterflies and moths.

Methods S2 Preparation of egg wash.

Methods S3 RNA isolation and real-time qPCR analysis of PR1 genes.

Methods S4 Histochemical staining.

Methods S5 *Pieris* spp. egg survival assessed by field survey.

Methods S6 Phylogenetic tree construction of Brassicales.

Table S1 Used plant species in screening of HR-like necrosis by *P. brassicae* eggs or egg wash.

Table S2 Origin of natural and reared butterfly populations used in the study.

Table S3 Primer sequences and annealing temperature used in *PR-1* real-time qPCR.

Table S4 Reference numbers of genes used for generating the phylogenetic tree of Brassicales plant species used in this study.

Table S5 Summary of HR-like necrosis induced by *P. brassicae* egg wash in different Brassicaceae and Cleomaceae species

Table S6 HR-like necrosis (score 0–3) expressed by *B. nigra* plants elicited by different butterfly species.

Table S7 HR-like necrosis (score 0–3) expressed by *B. nigra* plants elicited by different butterfly and moth species.

Table S8 Relative expression of *PR1* gene upon treatment with egg wash of different butterflies/moths and MES buffer as control.

Table S9 Quantification of HR-like necrosis in *B. nigra*, *B. oleracea* and *C. hispanica* leaves upon oviposition of *P. brassicae* egg clutches (10 eggs).

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