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Hypersensitive-like response in *Brassica* plants is specifically induced by molecules from egg-associated secretions of cabbage white butterflies

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Plants perceive and respond to herbivore insect eggs. Upon egg deposition on leaves, a strong hypersensitive response (HR)-like cell death can be activated leading to egg desiccation and/or dropping. In *Brassica* spp., including many crops, the HR-like mechanism against eggs of cabbage white butterflies (*Pieris* spp.) is poorly understood. Using two *Brassica* species, the crop *B. rapa* and its wild relative *B. nigra*, we studied the cellular and molecular plant response to *Pieris brassicae* eggs and characterized potential insect egg-associated molecular patterns (EAMPs) inducing HR-like cell death. We found that eggs of *P. brassicae* induced typical hallmarks of early immune responses, such as callose deposition, production of reactive oxygen species and cell death in *B. nigra* and *B. rapa* leaf tissue, also in plants that did not express HR-like cell death. However, elevated levels of ethylene production and upregulation of salicylic acid-responsive genes were only detected in a *B. nigra* accession expressing HR-like cell death. Eggs and egg wash from *P. brassicae* contains compounds that induced such responses, but the eggs of the generalist moth *Mamestra brassicae* did not. Furthermore, wash made from hatched *Pieris* eggs, egg glue, and accessory reproductive glands (ARG) that produce this glue, induced HR-like cell death, whereas washes from unfertilized eggs dissected from the ovaries or removal of the glue from eggs resulted in no or a reduced response. This suggests that there is one or multiple egg associated molecular pattern (EAMP) located in the egg glue a that teresponse in *B. nigra* is specific to *Pieris* species. Lastly, our results indicate that the EAMP is neither lipidic nor proteinaceous. Our study expands the knowledge on the mechanism of *Brassica*-*Pieris*-egg interaction and is a step closer toward identification of EAMPs in *Pieris* egg glue and corresponding receptor(s) in *Brassica*.

KEYWORDS

***Pieris*, cabbage, cell death, plant-insect interaction, oviposition-induced response, egg-associated molecular pattern**

1. Introduction

Plants rely on an immune system that regulates the perception of attackers and subsequent activation of inducible defenses (Wilkinson et al., 2019). Perception involves detection of pathogen-derived effector proteins and of molecular patterns, which can derive from different organisms, such as microbes (MAMPs) or herbivores (HAMPs; Gust et al., 2017; Stahl et al., 2018; Van der Burgh and

Joosten, 2019). Perception is followed by an early signaling cascade including rapid ion-flux changes and production of reactive oxygen species (ROS; Couto and Zipfel, 2016). Induced defenses typically include reinforcement of extracellular barriers, for example by callose deposition, the production of antimicrobial or insecticidal metabolites and proteins, and a localized, rapid cell death response, the hypersensitive response (Cui et al., 2015; Couto and Zipfel, 2016; Campos et al., 2018; Balint-Kurti, 2019; Dalio et al., 2020). Most in-depth studies on activation of the plant immune system have been performed on interactions with plant pathogens. So far, 119 HAMPs and/or effectors mostly from oral secretions of piercing sucking and chewing herbivores, have been identified, but pattern recognition receptors for HAMPs are just starting to be discovered (Erb and Reymond, 2019; Steinbrenner et al., 2020).

The detection by plants of herbivore eggs deposited on plant tissue is remarkable, as eggs are immobile and seemingly harmless structures. However, insect eggs will turn into feeding larvae and thus pose a future threat to the plant. Upon detection, plants can mount defenses against eggs that range from plant-mediated desiccation of eggs, egg dropping, egg crushing and the production of ovicidal substances (Hilker and Fatouros, 2015, 2016). Eggs of cabbage white butterflies (*Pieris* spp.) trigger necrotic lesions in leaves of the black mustard, *Brassica nigra* that can result in egg-killing by desiccating and/or dropping off singly laid *Pieris* eggs (Shapiro and DeVay, 1987; Griese et al., 2017). As the egg-induced plant phenotype resembles an HR, it has been referred to as hypersensitive response-like ("HR-like"; Fatouros et al., 2012). Oviposition by *Pieris* butterflies has been shown to induce HR-like cell death in several other plants of the Brassicaceae family, although the severity of the response varies between, and within species (Pashalidou et al., 2015; Griese et al., 2020, 2021; Groux et al., 2021; Bassetti et al., 2022).

Besides HR-like cell death, eggs of different insect species induce immune responses similar to pattern-triggered immunity (PTI), including callose deposition, accumulation of ROS and SA, and transcriptome changes of several defense genes, including *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*) in *A. thaliana* (Little et al., 2007; Bruessow et al., 2010; Gouhier-Darimont et al., 2013; Reymond, 2013; Lortzing et al., 2020). These early signaling defense responses to eggs seem to be conserved across different plant species (Lortzing et al., 2020). Expression of *PR1* was also induced in leaves of *B. nigra* underneath *P. brassicae* and *P. rapae* eggs (Fatouros et al., 2014, 2015). Whether brassicaceous species that are natural hosts of *Pieris* spp., including *B. nigra* and *B. rapa*, respond with a general immune response, including ROS, callose and expression of different defense genes, to insect eggs, and whether there is genetic variation for this response, is largely unknown.

So far, few studies have identified EAMPs that activate defense against insect eggs (Reymond, 2013; Hilker and Fatouros, 2015; Stahl et al., 2018). In some studies, secretions surrounding eggs were sufficient to elicit defense responses in plants and a few elicitors have been isolated from these secretions (Hilker et al., 2005; Tamiru et al., 2011; Salerno et al., 2013). Most EAMPs were found to be organic compounds of low-molecular weight associated with the eggs, while recently a first proteinaceous compound, diprionin, was found as an elicitor of pine defense against sawfly eggs (Hundacker et al., 2021). In *P. brassicae*, egg-enveloping secretions are produced by the females' accessory reproductive gland (ARG) and form a glue-like structure between the eggs and leaves (Beament and Lal, 1957; Fatouros et al., 2012). Treatment of *Brassica* plants with extracts from ARGs has shown to induce HR-like cell death and plant chemical cues attracting egg parasitoids, and to prime plants for future larval attack (Fatouros et al., 2008, 2009, 2015; Paniagua Voirol et al., 2020). Anti-aphrodisiacs transferred from the butterfly male to the female during mating were shown to be present in minute amounts in the ARG secretion and were suggested as potential elicitors (Fatouros et al., 2008, 2009). However, glands from unmated females induced HR-like, and therefore another, female-derived elicitor is likely to play a role (Fatouros et al., 2015). In *A. thaliana*, egg-derived phosphatidylcholines were found to induce production of H_2O_2, SA and result in cell death as shown by trypan blue staining (Stahl et al., 2020). It is still unclear which compounds from eggs of *Pieris* spp. and *Anthocharis cardamines* are detected by *Brassica* spp. and close relatives that result in HR-like activation, and whether these reside in the eggs themselves or in the secretions surrounding the eggs (Griese et al., 2021).

In this study, we developed and implemented a method to characterize the compounds from *Pieris* spp. eggs and egg-enveloping secretions. We specifically addressed: (1) the cellular and molecular response of two *Brassica* species, (2) the specificity of these responses by comparing eggs and egg washes of *Pieris* with the generalist moth *M. brassicae*, (3) the origin of EAMP inducing HR-like cell death, and (4) the chemical nature and stability of the elicitor.

2. Materials and methods

2.1. Plant material and rearing of butterflies

Black mustard (*B. nigra* L.) accessions WUR-01 and WUR-02 used, originated from plants that were collected near the river Rhine in Wageningen, Netherlands (N51.96, E05.68). *Brassica rapa* L. genotypes used in this study (L58, R-o-18, RC-144) were obtained from the Laboratory of Plant Breeding (WUR). Plants were grown in a greenhouse (18 ± 5°C, 50–70% RH, L16, D8) and were used when three to five weeks old.

Pieris brassicae L. (Lepidoptera: Pieridae) was reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus) in a climate room (21 ± 1°C, 50%–70% RH, L16: D8) at the Laboratory of Entomology, Wageningen University. Virgin adult females were obtained by isolating female butterflies immediately after eclosion. Otherwise, twenty females and males could mate in a large cage (60 × 60 × 90 cm) and females were used for oviposition in experiments or oviposition on filter paper (grade 3 hw, Sartorius, Germany) for egg wash production (see section below). The cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) was reared on Brussels sprouts plants in a climate room (21 ± 1°C, 50%–70% RH, L16: D8) at the Laboratory of Entomology, Wageningen University.

2.2. Preparation of egg washes

The protocol for preparation of egg washes has been newly developed to prepare egg wash free from leaf-surface related chemical compounds which enables us to conduct chemical analysis and identify possible EAMPs. Full procedure is described in Supplementary Data (Supplementary Figure S1). *Pieris brassicae* eggs were collected on filter paper pinned underneath a *B. oleracea* leaf in a cage containing 20 mated females. Egg clutches laid on the paper were cut out and submersed in 1 mL 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer per 400 eggs, overnight (16 h) without disturbance. Previously, we have

tested different buffers and found that using Mes buffer resulted in the most consistent plant response (data not shown). The solution (egg wash) was pipetted into a new tube the next morning.

To obtain egg-enveloping secretions for wash of only egg glue, 1-day old *P. brassicae* eggs were collected as above, counted, and then carefully removed from the paper using a brush. The spots of secretions underneath eggs were then cut out and washed overnight (16 h) in 1 mL 20 mM Mes buffer (pH 5.7) per 400 eggs. A wash of the pieces of the same filter paper without secretions was used as control.

To remove egg-enveloping secretions from eggs, eggs on paper were submersed in 1 mL per 400 eggs of either a solution of 1% bleach and 2% Tween-20 or in 250 mM $NaPO_4$ pH 9.0 for 30 min (two different treatments that were shown to remove egg glue; Jacobs et al., 2013). After treatment, eggs were rinsed once with 1 mL MILLI-Q® water (Sigma-Aldrich, MO, United States), hereafter "MQ water," and then 1 mL Mes buffer and then washed overnight (16 h) in 20 mM Mes buffer pH 5.7. Eggs that were used to compare where egg-enveloping secretions were not removed, were submersed in Mes for 30 min, rinsed in MQ and Mes, and then washed.

To study the induction by eggs of different ages, eggs were collected as above on paper, and then kept at room temperature until they were washed at the end of each day, until they hatched (6–7 days after oviposition). After hatching, young caterpillars were carefully removed using a brush. Empty eggshells and associated secretions (on paper) were then washed overnight (16 h).

To collect unfertilized eggs for egg wash, filter paper was pinned underneath a *B. nigra* leaf of a plant placed in a cage with 10 virgin adult females and then prepared in a similar way as fertilized eggs.

Paper sheets with *M. brassicae* eggs were obtained from a rearing population from the Laboratory of Entomology (WUR). To obtain egg wash, eggs were counted, spots with eggs were cut out and washed in 1 mL 20 mM Mes buffer (pH 5.7) per 400 eggs. The wash was pipetted off the next morning and frozen at −20°C until use.

2.3. Plant treatments with egg wash and egg deposition by butterflies

For all experiments testing the effects of treating plants with egg washes, 10 µL egg wash (i.e., an equivalent of 25 eggs) was pipetted on the abaxial side of the fourth or fifth emerged leaf of 3–4 weeks old *B. nigra* plants, unless otherwise specified. Symptoms induced by egg wash were scored 4 days after treatment. To quantify severity, a scoring system from 0 to 4 was adapted from Griese et al. (2017). Severity score 0: no visual response; score 1: brown spots underneath eggs or egg wash spot, only visible at abaxial side leaf; score 2: cell death also visible at adaxial side of leaf, spot smaller than 2 mm diameter, score 3: cell death the size of egg wash spot, and score 4: spreading lesion beyond spot of treatment (Supplementary Figure S1). Score 0 and 1 are classified as "non-HR," score 2–4 are classified as "HR".

For experiments with oviposition on plants, one mated female *P. brassicae* butterfly was placed in a cage with a *B. nigra* or *B. rapa* plant and removed when ~10–25 eggs were laid. HR-like cell death was scored 4 days after oviposition, using the same scoring system as with egg wash. For experiments with *M. brassicae*, five mated female moths were placed together with a *B. nigra* plant in a cage to allow egg deposition overnight (16 h) and removed in the morning. No visible cell death was observed under *M. brassicae* eggs as was also reported in our previous studies (Fatouros et al., 2012; Griese et al., 2021).

2.4. Histochemical staining

For histochemical staining of plant tissue underneath eggs, *P. brassicae* egg-laden plants were sampled 24, 48, and 72 h after oviposition by taking a 10 mm diameter leaf disk of the area surrounding the eggs. Similarly this was done with plants treated with egg wash. Eggs were carefully removed with a brush before proceeding with histochemical staining. Pictures of the leaf disks were taken with a Dino-Lite digital microscope (AnMo Electronics Corporation, Taiwan) before staining (with eggs) and after staining (without eggs). As cell death is generally preceded by production of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide anion (Torres, 2010), we visualized ROS with different histochemical stainings. 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, MO, United States) was used to stain hydrogen peroxide (H_2O_2). Leaf disks (10 mm Ø) were submersed in 1 mg/mL DAB solution and samples were incubated for 30–60 min in the dark. Nitroblue tetrazolium chloride (NBT; Sigma-Aldrich, MO, United States) was used to stain superoxide radical $O_2^{\bullet-}$. For this leaf disks (10 mm Ø) were submersed in 0.2% NBT with 50 mM sodium phosphate buffer (pH 7.5) and samples were incubated 30–60 min in the dark. For visualization of cell death, leaves were submersed in 0.08% trypan blue solution (Sigma-Aldrich, MO, United States), overnight (16 h). For all three stainings, destaining of leaves was performed with 96% ethanol. Pictures of leaf disks before staining (with eggs) and after staining (without eggs) were taken with a Dino-Lite digital microscope (AnMo Electronics Corporation, Taiwan). For staining of callose, leaf disks were first destained and then submersed in 0.01% aniline blue (Sigma-Aldrich, MO, United States) with 150 mM K_2HPO_4 and imaged after at least 2 h of incubation using a DAPI filter on a fluorescence microscope equipped with a DS-5MC camera and NIS elements AR 2.30 software.

2.5. Dissection of butterfly reproductive tract

In female butterflies, eggs are produced in the ovaries, pass through the common oviduct and are fertilized by sperm released from the bursa copulatrix (BC) into the vagina. Before being expelled through the ovipore, the eggs are covered by secretions released from the accessory reproductive gland (ARG), a paired gland that contains egg-enveloping secretions and glue-like material to attach eggs to leaves (Supplementary Figure S2). For dissection of tissues from the female reproductive tract, namely ARGs and BCs or eggs from the ovarian tubules (hereafter termed "ovarian eggs"), mated *P. brassicae* females were obtained by pairing a virgin female and virgin male 1 day after eclosion, which were kept together until mating was observed. Virgin females were obtained by keeping them separately shortly after eclosion. Three to four days after eclosion, mated and virgin *P. brassicae* females were killed by beheading using a scalpel and then dissected. ARGs, BC and ovarian eggs were dissected from females under a stereomicroscope (optical magnification 20×) in 20 mM Mes buffer. Dissected structures were washed overnight (16 h) in 20 mM Mes pH 5.7 using 50 µL per each ARG, 100 µL buffer per each BC or 5 µL buffer per egg. Solution was pipetted off the next morning and frozen until use (modified after Fatouros et al., 2015).

2.6. Treatments of egg wash with heating, proteinase K, freezing, methanol and speedvac extract

To characterize the chemical nature of EAMP in the egg wash we exposed it to different treatments. Untreated egg wash was used as a control. To study the stability of the elicitor, the egg wash was heated and frozen. For heating, egg wash was made as described above, collected in microcentifuge tubes and boiled in a water bath for 30 min at 95°C. For freezing treatment, egg wash was frozen at −20°C and then thawed for 1 h at room temperature, then frozen again. Freezing–thawing cycles were repeated up to four times, after which, all washes were used to treat plants. To study if the elicitor was an intact protein, a proteinase treatment was performed, a stock of 20 mg/mL Proteinase K (Qiagen, Cat No./ID: 19133) was diluted 100× in 100 µL egg wash (10% volume, pH 7.2) and incubated with egg wash for 30 min at 37°C.

2.7. Phospholipid treatments

Phosphatidylcholines (PCs) were recently found to be the main components of *P. brassicae* egg extracts that show a bioactivity in *A. thaliana* Col-0 resulting in induction of SA and *PR1* marker gene as well as ROS accumulation and cell death formation (Stahl et al., 2020). Thus, we tested whether the same PCs used in Stahl et al. (2020) may be associated with development of HR-like cell death by *B. nigra*. Phospholipids were ordered from Avanti Polar Lipids (Alabaster, Alabama, United States). Specifically, PC(16:1/16:1), 1,2-dipalmitoleoyl-sn-glycero-3-phosphocholine, catalog no. 850358 and PC(18:1/18:1), 1,2-dioleoyl-sn-glycero-3-phosphocholine, catalog no. 850357 were used for experiments. Phospholipid stock solutions were made in 100% MeOH. For phospholipid application, the MeOH was evaporated under a nitrogen-flux and the phospholipids were dissolved in MQ water with 1% DMSO, 0.5% glycerol, 0.1% Tween 20 by sonication. Phospholipids were applied on *B. nigra* leaves at different concentrations (1, 5 and 10 µg/µL) that were previously shown to have biological activity in *A. thaliana* (Stahl et al., 2020). An MQ water solution of 1% DMSO, 0.5% glycerol and 0.1% Tween 20 was used as control.

2.8. Production of ethylene

Ethylene production is regularly measured to characterize the induction of early plant immune response by biotic stresses (Fan et al., 2017). Ethylene was measured as previously published (Oome et al., 2014). To measure the plant production of ethylene, in *B. nigra*, ten plants for each accession (with and without HR-like phenotype) were used. For *B. rapa*, three plants of each genotype were used. For each plant, leaf disks (3 mm Ø) were sampled from mature leaves of untreated plants and incubated overnight (16 h) in demineralised water. Subsequently, three leaf disks for each biological replicate (a single plant) were randomly chosen and incubated for 5 h in air tight glass vials with in either 400 µL of 20 mM Mes pH 5.7 or 400 µL egg wash (400 eggs/mL in same Mes buffer). After incubation, 1 mL of the headspace of each sample was taken to measure ethylene concentration on a Focus gas chromatograph (Thermo Electron S.p.A., Milan, Italy) equipped with an FID detector and a RT-QPLOT column, 15 m × 0.53 mm ID (Restek, Bellefonte, PA, United States). The system was calibrated with a certified gas of 1.01 µL L^{-1} (1 ppm) ethylene in synthetic air (Linde Gas Benelux B.V., Schiedam, Netherlands). After sampling leaf disks for the ethylene assay, plants were also treated with *P. brassicae* egg wash to determine their HR-like cell death phenotype.

2.9. Plant treatments for gene expression experiments

To compare gene expression induced by eggs and egg wash, *B. nigra* plants were treated with either 10 µL of egg wash at lower concentration (400 eggs/mL in Mes buffer) or oviposited with an egg clutch of 10 eggs. Six leaf disks (Ø 6 mm) were sampled with a leaf puncher directly next to the eggs or the egg wash-treated spot at 0, 3, 6, 24, and 48 h, as done in previous research (Fatouros et al., 2014; Griese et al., 2021). For each timepoint, four plants were sampled individually and considered biological replicates. *Brassica rapa* plants were treated with three single eggs on a single leaf of each plant. Leaf disks (Ø 6 mm) were then harvested next to the eggs at 0, 3, 6, 24, and 96 h after treatment. For each timepoint, three plants were sampled individually and considered biological replicates.

To compare gene expression between *P. brassicae* and *M. brassicae* egg wash, *B. nigra* plants were treated with 10 µL of either egg wash (400 eggs/mL in Mes buffer) or a negative control (Mes buffer). Six leaf disks (Ø 6 mm) were sampled directly next to the egg wash-treated spot 24 h after treatment. Four plants were used for each treatment as biological replicates.

To compare the gene expression of two *B. nigra* accessions with contrasting ability to develop HR-like cell death, egg wash at higher concentration (~1,000 eggs/mL in demineralised water) was used. Experimental design consisted of two treatments (egg wash, control), two *B. nigra* accessions (WUR-01 without HR, and WUR-02, developing HR), and three time points after treatment (6, 24, and 48 h after treatment). For each treatment combination, egg wash or control were applied with two droplets of 5 µL on the abaxial side of a single leaf. Leaf disks (Ø 6 mm) were harvested at each time point on the treatment spots and disks from the same treatment on a leaf were pooled. For each treatment combination, a total of 15 plants were used and groups of 3 plants with similar treatments were pooled to compose a total of 5 biological replicates. For each gene expression experiment, samples were snap frozen in liquid nitrogen and stored at −80°C until use.

2.10. Expression of genes by real-time qRT-PCR

We investigated to which extent the difference in HR-like phenotype between *B. nigra* accessions WUR-01 and WUR-02 was related to activation of different phytohormones associated with plant immunity. Thus, we quantified expression of genes induced by SA, such as *ICS1*, *PR1*, and *PR2* (Little et al., 2007), and genes regulated by JA, i.e., *MYC2*, *VSP1*, and *VSP2* (Reymond et al., 2004). For experiments on gene expression induced by eggs or egg wash in both *B. nigra* and *B. rapa*, RNA extraction was performed according to Oñate-Sánchez and Vicente-Carbajosa (2008). For experiments on gene expression induced by egg wash in two *B. nigra* accessions, RNA extraction was performed with Direct-zol RNA Miniprep Kit (Zymo Research, CA, United States) following the manufacturer's protocol. For preparation of cDNA of all experiments, 1 µg of RNA was reverse-transcribed using SensiFAST

cDNA synthesis kit (Bioline, United Kingdom) according to the manufacturer's instructions. Real time qRT-PCR reactions were performed using SensiFAST SYBR No-ROX Kit (Bioline, United Kingdom) in 10 µL reaction volumes, containing 3 µL cDNA and 500 nM of primers on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, CA, United States). The following qRT-PCR program was used: 95°C for 2 min followed by 40 cycles of 95°C for 5 s; primer-specific annealing temperature for 5 s and 72°C for 10 s, with data collection at 72°C, followed by a melt curve analysis. Relative gene expression was calculated with the ΔΔCq method, using *GAPDH* as reference gene. Primers sequences are available in Supplementary materials (Supplementary Table S1).

2.11. Data analysis

All data analysis was carried out in R (R Core Team, 2020). Scoring of HR-like cell death in severity categories were analyzed with a non-parametric method (Kruskal–Wallis test) on HR scores (0, 1, 2, 3, and 4) and different treatments were included as categorical fixed factors. For all other statistical analyses involving comparison of mean values (gene expression, ethylene production), the choice of parametric or non-parametric methods was made after checking the assumptions of normality (Shapiro–Wilk normality test) and homogeneity of variances (Fligner–Killeen test) on the raw data. As parametric methods, Student's *T*-test, Welch *T*-test and ANOVAs followed by Tukey's honestly significant difference test were used. As non-parametric method, Kruskal–Wallis test followed by pairwise Wilcoxon rank sum test was used. Gene expression data from qRT-PCR were calculated with the ΔΔCt method (Livak and Schmittgen, 2001) and were analyzed on log2-transformed data (parametric test) or on raw data (non-parametric test) specifying time points or treatments as factors.

3. Results

3.1. *Pieris* eggs triggered cellular responses in *Brassica* spp.

We first investigated cellular responses against *Pieris brassicae* oviposition in its natural host, the black mustard *Brassica nigra*. Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) accumulated underneath eggs at 24 h (Figure 1A). At the same time point, aniline blue staining in *B. nigra* revealed also callose deposition underneath eggs (Figure 1A). Further, the occurrence of cell death in plant tissue under the eggs was investigated by staining with trypan blue (TB), showing that cell death occurred 72 h after oviposition (Figure 1B). Occasionally, a few TB-stained cells were visible at 48 h (not shown). In *B. nigra*, we regularly observed a strong, macroscopically visible HR-like cell death that spreads beyond the egg site and stops 96 h after oviposition (Figure 1C). Cellular responses against *P. brassicae* oviposition were also investigated in *B. rapa*. ROS accumulation and cell death formation were also detected in *B. rapa* at 24 h and 72 h after oviposition, respectively (Supplementary Figures S2A,B). Although these early cellular responses appeared similar to what was observed in *B. nigra*, the macroscopically visible HR-like cell death developed by *B. rapa* appeared as black necrotic spots that never spread beyond the egg site (Supplementary Figure S2C).

3.2. Egg wash induced a plant response similar to the response to eggs

To isolate potential EAMPs and to quickly screen plants for HR-like cell death, we developed a method to dissolve egg-enveloping secretions from *P. brassicae* eggs (Methods section; Supplementary Figure S1). We then compared visually whether the

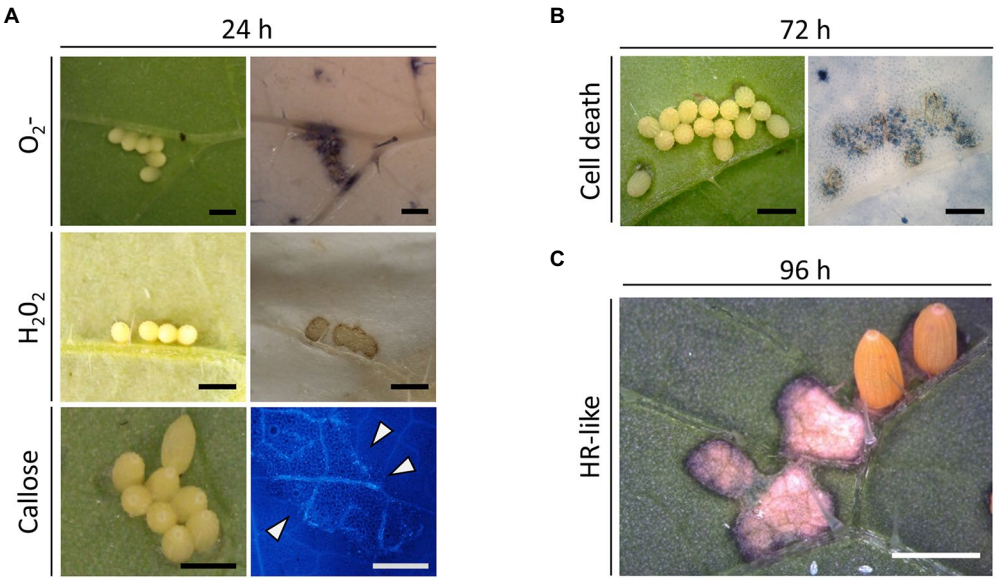

FIGURE 1
Plant immunity responses induced in *Brassica nigra* by *Pieris brassicae* eggs (*Bn*). **(A)** *B. nigra* leaf 24h after oviposition with accumulation of $O_2^{\bullet-}$ (NBT staining), H_2O_2 (DAB staining) and callose deposition (aniline blue staining). **(B)** *B. nigra* leaf 72h after oviposition showing cell death (trypan blue staining). **(C)** Fully developed HR-like cell death that is macroscopically visible at 96h after oviposition. A few eggs were removed from the clutch to show the cell death underneath. Stainings were repeated multiple times with similar results. Scale bars=1mm.

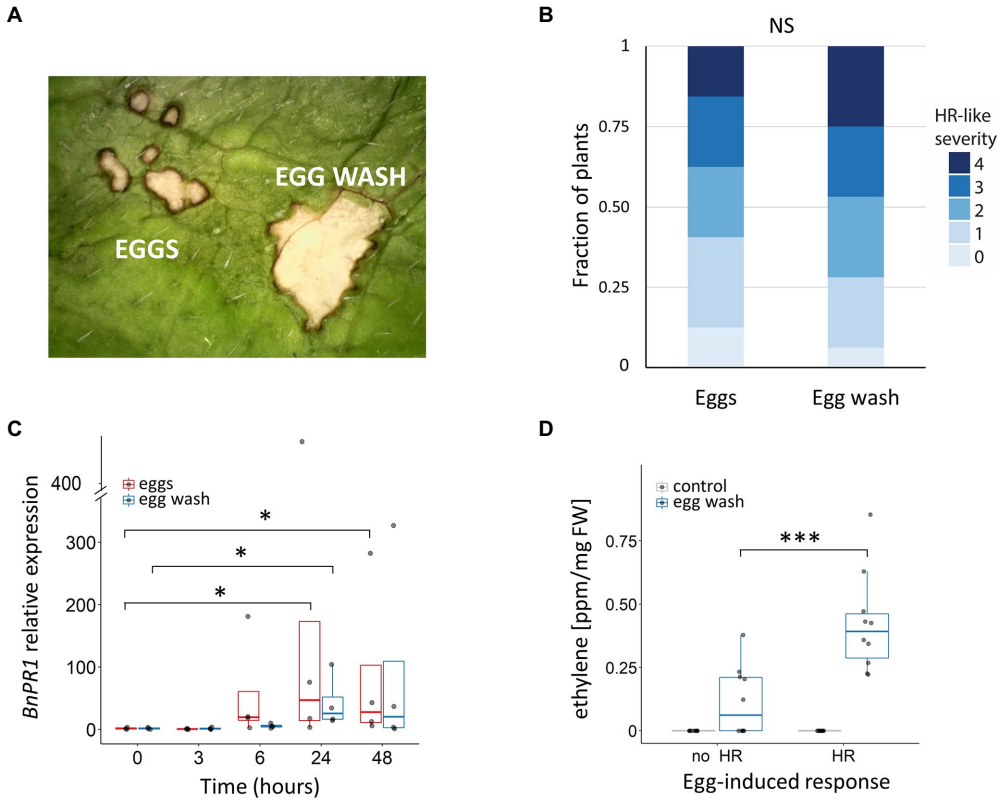

FIGURE 2
Plant responses induced by *Pieris brassicae* eggs and egg wash in *Brassica nigra*. **(A)** Picture of responses induced by eggs and egg wash next to each other on a leaf (microscopic image): both eggs and egg wash induce HR-like necrotic spots. **(B)** Quantification of severity of symptoms induced by egg wash and eggs. Shown is the fraction of plants that were scored in a specific class for each treatment (N=32 plants for both treatments; Kruskal–Wallis, p>0.05). ns=no significant difference. **(C)** *BnPR1* expression in plants after egg deposition (red box plots) or treatment with egg wash (blue box plots). Gene expression was measured by qRT-PCR and normalized to the housekeeping gene BnGADPH. Each treatment consisted of 3–4 biological replicates. Expression at each time point was compared to the 0h timepoint (Kruskal–Wallis, p<0.05 for eggs and p<0.05 for egg wash). **(D)** Ethylene production in parts per million (ppm) by plants treated with egg wash. (Student's T-test, p<0.001). Each treatment consisted of 10 biological replicates. The height of the boxes in C-D 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 data points. Asterisk indicates p<0.05, *** <0.001.

treatment of leaves with egg wash induced a similar HR-like cell death as the butterfly eggs (Figure 2A). As a control, leaves were treated with only the buffer (no eggs were washed). Symptoms induced by eggs or egg wash were scored after 4 days, and HR-like frequency (proportion of plants showing HR-like) and HR-like severity (mean score of induced symptoms) were compared between the two treatments in *B. nigra*. HR-like severity after oviposition by eggs or treatment with egg wash did not differ (Kruskal–Wallis: $\chi^2 = 1.33$, df = 1, $p = 0.24$; Figure 2B; Supplementary Table S2).

We compared expression of SA-marker gene *PR1* in *B. nigra* oviposited on by *P. brassicae* butterflies or treated with egg wash. *PR1* was significantly upregulated both after oviposition and after treatment with egg wash. In *B. nigra*, *PR1* expression increased at 6 h and was significantly upregulated at 24 h and 48 h after egg deposition (Kruskal–Wallis: $\chi^2 = 12.23$, df = 3, $p = 0.015$; Figure 2C). No significant differences in *PR1* expression were found between *B. nigra* plants treated with eggs or egg wash (Figure 2C; Supplementary Table S3). Similarly, *in B. rapa*, *PR1* expression was significantly upregulated by egg deposition at 24 h and showed further increase at 96 h (Kruskal–Wallis: $\chi^2 = 11.18$, df = 4, $p = 0.024$; Supplementary Figure S2D).

Next, we tested whether egg wash induced ethylene. *Brassica nigra* leaves responded with higher ethylene production after incubation with egg wash compared to incubation with control Mes buffer (Figure 2D; Supplementary Table S4). In addition, there was a significant difference in ethylene produced between plants with contrasting HR-like phenotypes. Plants responding with stronger HR-like cell death (score 2 or higher), produced a significantly higher amount of ethylene after incubation with egg wash than plants with no HR-like cell death (Student's T-test = −4.087, df = 18, $p < 0.001$; Figure 2D; Supplementary Table S4). Similarly, *B. rapa* plants developing HR-like cell death, also showed a higher ethylene production upon incubation of leaves with *P. brassicae* egg wash (Student's T-test = −3.876, df = 4, $p = 0.018$; Supplementary Figure S3E). These results suggest that there is an early detection response in plants after contact with egg wash that will ultimately lead to cell death.

3.3. Variation in HR-like cell death severity is associated with SA-related defenses

All SA marker genes were upregulated in *B. nigra* plants upon egg wash treatment compared to control. However, the magnitude of expression of SA-related genes was significantly different between the two *B. nigra* accessions at different time points (Figure 3). In contrast,

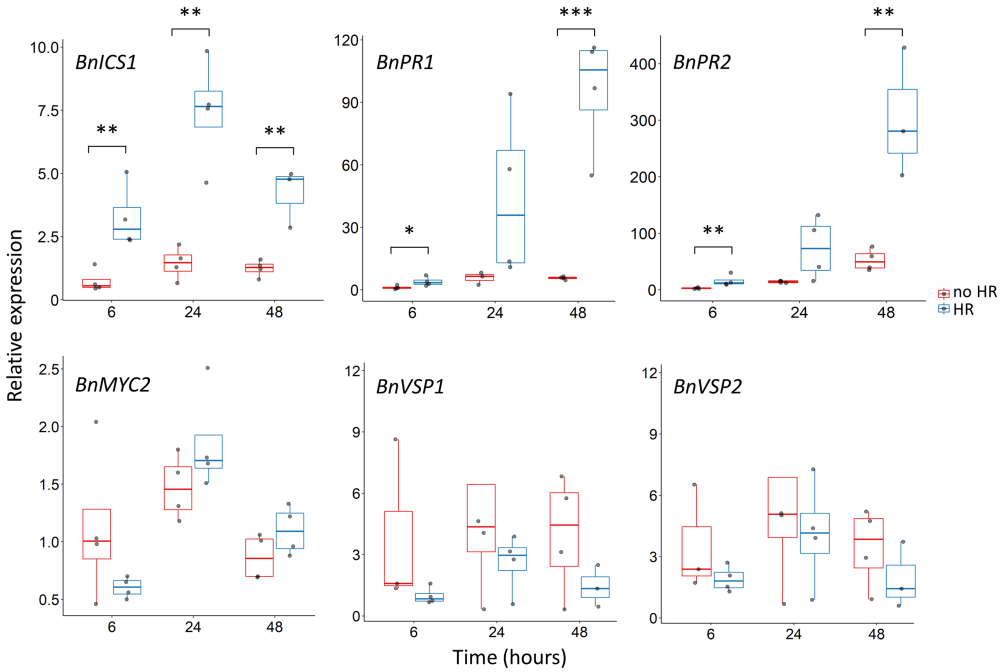

FIGURE 3
Expression of SA- and JA-related defense genes upon *P. brassicae* egg wash treatment in two different *B. nigra* accessions. Gene expression was measured with two treatments (egg wash, control), two accessions (no HR and HR) and at three time points (6, 24, and 48h after treatment). HR phenotype of each plant was scored after sampling for RNA isolation. Gene expression was measured by qPCR-RT and normalized to housekeeping gene *BnGADPH*. Expression levels were calculated at each time point relative to control treatment (water droplets). Each treatment consisted of 4–5 biological replicates and is reported as boxplots. The height of the boxes in 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 data points. At each time point, gene expression between the two accessions was compared (Welch's *T*-test). Asterisks indicate different *p*-values within the same time point: ** <0.01, *** <0.001, no asterisks indicate no significant difference.

relative expression of JA-related genes was generally very low and not significantly different between two accessions. SA-marker *BnICS1* was significantly expressed at higher levels in plants that showed a visible HR already at 6 h after treatment (Welch's T-test = −4.901, df = 5.3, $p = 0.003$). Both, *BnPR1* and *BnPR2* showed increased expression across the time points, although with a different magnitude between the two accessions. Both genes were already expressed at higher level at 6 h in plants that showed a visible HR (Welch's T-test = −2.932, df = 5.3, $p = 0.03$ and Welch's T-test = −4.850, df = 5.8, $p = 0.003$ respectively), and the difference with plants not expressing HR increased up to 5-fold (24 h) and 20-fold (48 h) for *BnPR1* and 4-fold (24 h) and 5-fold (48 h) for *BnPR2*. The expression of JA-related genes showed more stable profiles across time points, with a small peak at 24 h in all treatments, but with no significant differences between the two accessions (MYC2, Welch's T-test = −1.549, df = 5.8 $p > 0.05$; VSP1, Welch's T-test = −0.141, df = 3 $p > 0.05$; VSP2, Welch's T-test = −0.301, df = 3.5 $p > 0.05$).

3.4. Responses to eggs and egg wash from *Mamestra brassicae* are dissimilar to *Pieris brassicae* in *Brassica nigra*

Staining of leaves showed that plant cells did not die underneath *M. brassicae* eggs as leaves did not stain with trypan blue (Figures 4A,B). Further, *M. brassicae* eggs induced $O_2^{\bullet-}$ production in some plants, but weaker than *Pieris* eggs (Figures 4C,D). While *P. brassicae* egg wash induced the expression of *PR1* after 24 h (ANOVA followed by Tukey, $p < 0.01$), *PR1* expression induced by *M. brassicae* egg wash was not different from the control treatment (Figure 4C; Supplementary Table S5). In addition, incubation with *M. brassicae* egg wash induced lower ethylene production compared to *P. brassicae* egg wash (Kruskal–Wallis: $\chi^2 = 21.36$, df = 2, $p < 0.001$; Figure 4D), comparable to the ethylene levels previously observed in *B. nigra* plants lacking a visible cell death (Figure 2D). Overall, eggs of *M. brassicae* did not induce cell death and only a weak ROS and ethylene response in *B. nigra*.

3.5. EAMP derived from female accessory reproductive glands

There was no significant difference in eliciting activity of eggs of increasing age, from one-day-old, to five-day-old eggs, although HR-like severity decreased slightly. Egg wash was also prepared from eggshells and secretions that remained on the paper after caterpillars hatched, and this still induced HR-like symptoms (Kruskal–Wallis: $\chi^2 = 3.20$, df = 5, $p > 0.1$; Figure 5A; Supplementary Table S6).

A wash of dissected accessory reproductive glands (ARGs) induced HR-like cell death, similar to the positive control *P. brassicae* egg wash (Figure 5B). On the contrary, neither a wash of mature but unfertilized eggs dissected from the ovary ("ovarian eggs") nor a wash of the sperm-containing bursa copulatrix (BC) induced symptoms (Figure 5B). HR-like severity was significantly higher in plants treated with egg wash or a wash of ARG, compared to wash of ovarian eggs or bursa copulatrix (Kruskal–Wallis: $\chi^2 = 19.833$, df = 4, $p < 0.001$; Supplementary Table S6).

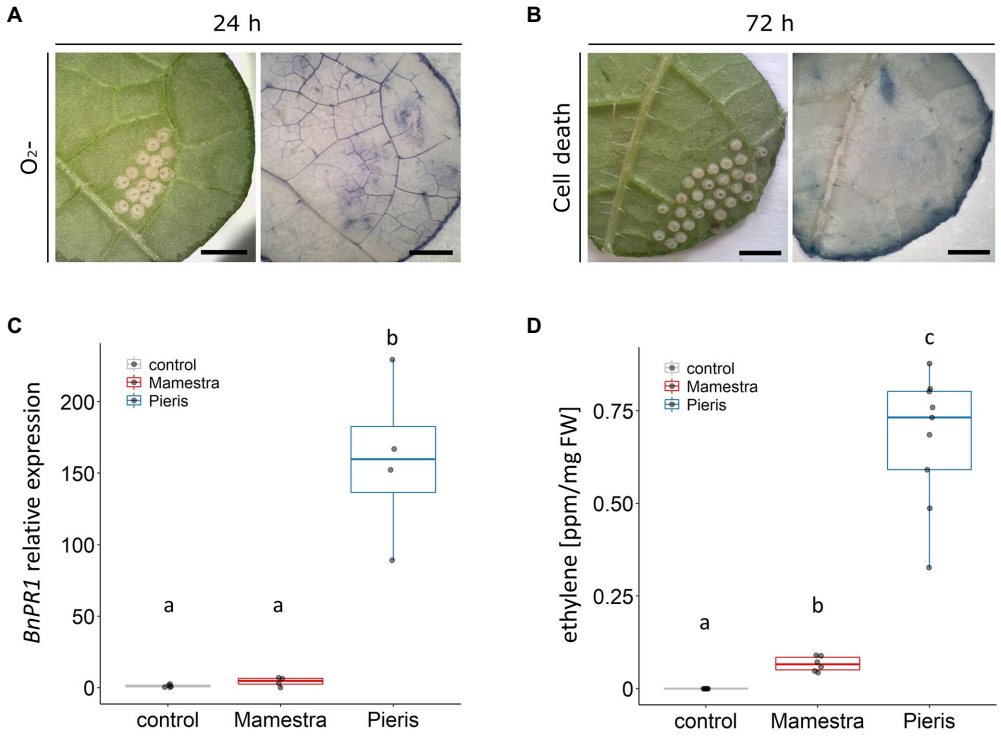

FIGURE 4
Responses to eggs and egg wash of *M. brassicae* in *B. nigra*. **(A)** Leaf of *B. nigra* oviposited on by *M. brassicae* moth by NBT showing light $O2^{\bullet-}$ accumulation underneath *M. brassicae* eggs. **(B)** Leaf stained by trypan blue showing no cell death underneath *M. brassicae* eggs. **(C)** *BnPR1* expression in leaf tissue treated with control solution, *P. brassicae* wash or *M. brassicae* wash. Different letters indicate significant differences in mean *PR1* expression (ANOVA followed by Tukey, $p<0.01$). **(D)** Ethylene production in *B. nigra* leaf in response to egg washes. The height of the boxes in **C,D** 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 data points. Values that have no accompanying letters in common differ significantly in production of ethylene (Kruskal–Wallis followed by pairwise Wilcoxon test, $p<0.001$). Scale bars=1mm.

A wash from unfertilized, deposited eggs (containing secretions from the ARG) from virgin butterflies induced a similar response as wash from fertilized, deposited eggs of mated females (Figure 5C). There was no significant effect of the mating status of the female (mated or virgin) on the frequency of HR-like cell death elicited or on HR severity (Kruskal–Wallis: $\chi^2 = 2.61$, df = 1, $p > 0.1$). In addition, the wash of ARGs from virgin females induced strong symptoms similar to those of mated females (Figure 5B). These results show that egg fertilization is not necessary for the induction of the HR-like cell death in *B. nigra,* and that an EAMP resides in the ARG, and is female-derived.

Finally, both egg wash and wash of glue alone induced a severe HR-like cell death, and HR-like severity was significantly lower when *B. nigra* was treated with a wash of eggs from which the glue was removed (Kruskal–Wallis: H = 26.60, df = 3, $p < 0.001$; Figure 5D; Supplementary Table S6).

3.6. EAMP is neither a protein nor lipid

None of the egg wash treatments conducted, i.e., freezing and thawing, proteinase K, or boiling, had an effect on its bioactivity. That the elicitor does not lose the capability to induce HR in *B. nigra* leaves after these treatments, indicates that it is a small, stable, water-soluble molecule that is likely not a protein (Figure 6; Supplementary Table S7).

Lastly, we tested whether two phosphatidylcholines (PC) could also induce a HR-like responses in *B. nigra*. We did not observe any visible cell death upon treatment of *B. nigra* with neither PC16:1/PC16:1 nor PC18:1/PC18:1 (Figure 7; Supplementary Table S8).

4. Discussion

In this study, we show that HR-like inducing EAMPs are in the egg-enveloping secretions that are produced in female ARGs. Our results indicate that the EAMP is unlikely of lipidic or proteinaceous nature but an organic compound of low-molecular weight. When plants are treated with egg wash, HR-like cell death is induced in responsive plant genotypes, together with upregulation of SA-responsive genes and elevated levels of ethylene suggesting that the HR-like cell death is mediated by one or both phytohormones. In addition, deposition of eggs leads to ROS and callose deposition, also in plant genotypes that do not show HR-like cell death. All phenotypes observed were shared between the two *Brassica* species that vary in cell death severity and are specific to eggs and egg wash of the specialist *P. brassicae*.

The increasing knowledge on plant defense mechanisms to eggs of herbivores suggests that plants can specifically recognize and respond to egg deposition, presumably *via* the detection of EAMPs. However, very few EAMPs have been identified, and those that have so far, are organic compounds of low-molecular weight associated with the eggs (Reymond, 2013; Hilker and Fatouros, 2015; Stahl et al., 2018). Only recently a novel type of EAMP, a proteinaceous compound, diprionin, was found as an elicitor of pine defense

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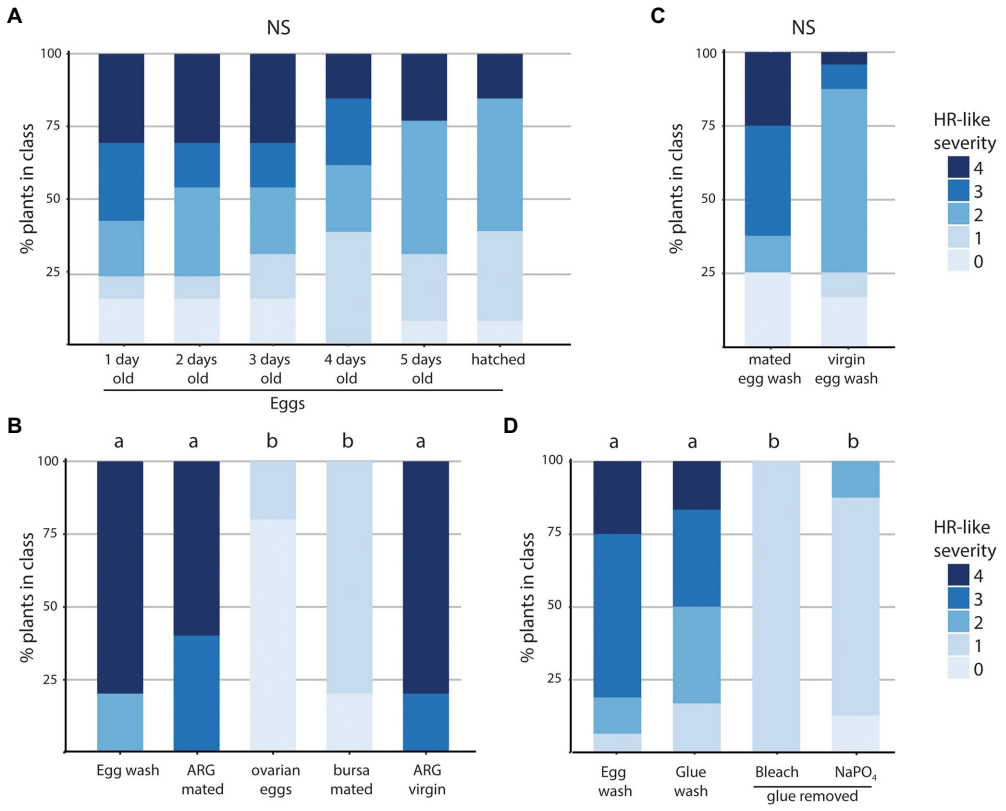

FIGURE 5
Proportion of HR-like cell death induced by washes made of eggs, washes made of tissues of the reproductive tract of *Pieris brassicae,* and washes of eggs with and without glue, in *Brassica nigra*. **(A–D)** **(A)** Egg washes made of eggs of different ages or eggshells and egg glue remaining on filter paper. N plants=13–26 **(B)** Egg wash of dissected structures of reproductive tract. N plants=5. **(C)** Egg wash of eggs of mated females versus virgin females. N plants=8–24 **(D)** Wash of eggs, glue alone (in filter paper) or wash of eggs with egg-enveloping secretions removed. For glue removal, two treatments were used, either a 1% bleach wash or wash with $NaPO_4$. N plants=8–16. Values that have no accompanying letters in common differ significantly (Kruskal–Wallis).

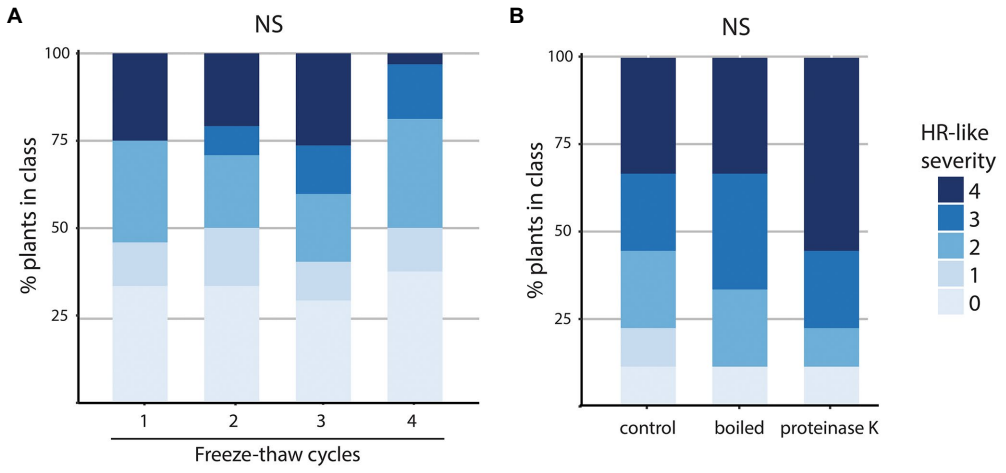

FIGURE 6
Proportion of HR-like cell death induced by differently treated egg washes made of *Pieris brassicae* eggs in *Brassica nigra*. **(A,B)** Severity of HR-like symptoms induced in plants by the different washes. **(A)** Egg washes that were subjected to increasing amount of freeze–thaw cycles. N plants/treatment=24–32. NS **(B)** Egg washes subjected to boiling (95°C for 30 min) or incubated with proteinase K. N plants/treatment=9. NS, not significant, Kruskal–Wallis test.

against sawfly eggs (Hundacker et al., 2021). Diprionin is an annexin-like protein which is released with the egg-associated secretions of the sawfly *Diprion pini* into needles of pines. When pine twigs were treated with diprionin they emitted similar quantities of the sesquiterpene (*E*)-β-farnesene that is known to attract egg parasitoids like when laden with sawfly eggs (Hundacker et al., 2021).

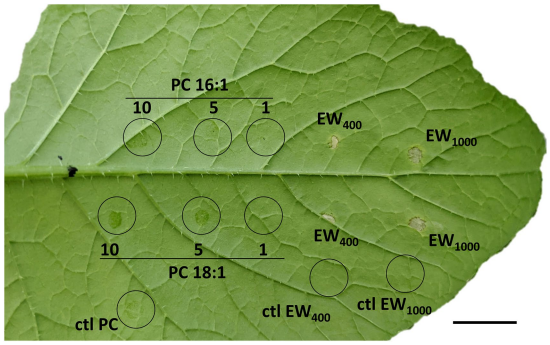

FIGURE 7
Phosphatidylcholines (PC) does not induce HR-like cell death in *B. nigra* plants unlike *P. brassicae* egg wash. *Brassica nigra* (SF48-O1) plants were treated with phosphatidylcholines PC(16:1/16:1) and PC(18:1/18:1) or with egg wash (EW). PCs were solubilized in 1% DMSO, 0.5% Glycerol and 0.1% Tween and were applied at different concentrations (1, 5, and 10 µg/µL). A solution of 1% DMSO, 0.5% Glycerol and 0.1% Tween was used as control (ctl). Egg wash$_{400}$ was prepared with 400 eggs/mL, egg wash$_{1000}$ was prepared with 1,000–1,200 eggs/1 mL. All treatments were applied on the same leaf, with two leaves per plant, on 15 biological replicates. Scale bar=1.

Our study reveals that the EAMP associated with *P. brassicae* eggs likely belongs to the first category of non-proteinaceous molecules. Neither boiling nor treatment of the egg wash with proteinase K had an effect on elicitation of HR-like. Recently, phosphatidylcholines (PCs) were identified as EAMPs of the *P. brassicae* egg-induced response in *A. thaliana*. Active PCs were mainly containing C16- and C18-fatty acyl chains and treatment with the latter resulted in SA, H_2O_2, induction of *PR1* and trypan blue staining (Stahl et al., 2020). PCs are components of cell membranes. Earlier, phospholipids of *Sogatella furcifera* Horváth were also found to induce an ovicidal response in rice (Yang J. O. et al., 2014). Our results suggest that the EAMPs in *P. brassicae* eggs that induce HR-like cell death in *B. nigra* are other compounds than PCs. First, the *B. nigra* response is specific to *Pieris* eggs and egg wash and is absent in response to other lepidopteran eggs. Second, given our method used of washing eggs in a water-like buffer, lipids are not expected to be present in (high amounts in) the wash. Finally, as PCs are present in membranes, ovarian eggs should also induce the response. Indeed, ovarian eggs alone induced *PR1* expression in *A. thaliana* (Little et al., 2007) while we showed that ovarian eggs did not induce HR-like cell death in *B. nigra*.

When washing eggs, mainly compounds from outside of the eggs and from egg-enveloping secretions are dissolved. We present evidence that at least one *Pieris*-specific EAMP is in these secretions that envelop the eggs: (i) wash of glue on filter paper is sufficient to induce the HR-like cell death, (ii) when the secretions are removed from eggs, and eggs are then washed, HR-like cell death in plants is diminished, and (iii) a wash of ARGs, the organs where secretions are produced, is also sufficient to induce HR-like cell death. As the egg surface and egg-exterior associated secretions are in direct contact with the leaves, it could be expected that plants evolve to detect elicitors in egg-enveloping secretions. It resembles the natural situation of leaf-egg interaction, more so than, for example, crushing of eggs (Little et al., 2007; Bruessow et al., 2010) or crushing of adults (Doss et al., 2000; Yang Y. et al., 2014). Previously, a male-derived anti-aphrodisiac compound transferred during mating to the female ARG, benzyl cyanide, was suggested as potential elicitor (Fatouros et al., 2008). We find no evidence for a male-derived elicitor: eggs and ARGs of virgin *P. brassicae* butterflies induced HR-like cell death in an equal manner as mated butterflies. We thus hypothesize that at least one HR-inducing EAMP is present in the egg-enveloping secretions and ARGs and is female-derived. Chemical analysis of egg wash and glands is currently carried out to identify this EAMP.

Plant responses to insect eggs are similar to responses to (microbial) pathogens, and include SA and ROS accumulation, callose deposition, defense gene expression and cell death (Reymond, 2013). For example, callose deposition was shown to be associated with lesions following pathogen invasion or autoimmune responses (Koga et al., 1988; Dietrich et al., 1994). We observed the deposition of callose in *B. nigra* associated with lesions induced by *P. brassicae* eggs. Natural variation in strength of egg-induced cell death was found in several brassicaceous species, including *B. nigra* (Pashalidou et al., 2015; Griese et al., 2021; Groux et al., 2021). Here, we show that plants that do not express a strong HR-like cell death, still responded with ROS accumulation and cell death as showed by trypan blue staining. Similarly, in *S. dulcamara*, variation exists for egg-induced chlorosis, and a genotype that did not respond with chlorosis and on which egg hatching rate of *S. exigua* was not reduced, still accumulated SA after oviposition (Geuss et al., 2017). We thus hypothesize that *Pieris* eggs generally induce an immune response in all plants of *B. rapa* and *B. nigra*, that is only in some plants accompanied by a stronger cell death response. In pathogen-induced HR, cell death can often be uncoupled from (preceding) biochemical and molecular changes and the two processes can be genetically dissected (Künstler et al., 2016). In that case, cell death is dispensable for resistance. However, in egg-induced HR, previous studies show that the stronger the HR-like cell death, the higher egg mortality (Fatouros et al., 2014; Griese et al., 2017, 2020, 2021).

In *A. thaliana*, *PR1* expression was also found in response to crushed egg extracts of different insects (Bruessow et al., 2010; Stahl et al., 2020). In *B. nigra*, there was no cell death underneath *M. brassicae* eggs, and neither induction of *PR1* nor ethylene production in response to *M. brassicae* egg wash. Our results suggest that cell death, ethylene production and gene expression, at least in *B. nigra*, are specific to *P. brassicae* eggs, and we expect the response to be activated after detection of a Pierinae-specific elicitor. This hypothesis is strengthened by our previous work which showed Pierinae-specific induction of *PR1* in *B. nigra* (Griese et al., 2021). It is possible that a mild cellular defense-like response is activated against a general insect-derived EAMP, for example PCs, while the strong HR-like is activated only by EAMPs specifically derived from *Pieris* spp. in those plants that can detect these. The production of ethylene after incubation with egg wash only in plants that show a strong HR-like cell death (and not in non-HR plants), points to this effect. Mapping efforts in *B. nigra* plants, can reveal whether the genetic variation in plants is for the HR-like cell death and/or for the detection of a *Pieris*-specific elicitor.

Molecular patterns that are detected by plants are thought to be structurally conserved molecules (Van der Burgh and Joosten, 2019). The presence of EAMPs in the glue suggests that their function could be a structural component of the glue, or a compound with an essential function to the fertilized eggs. Many proteins are found in glue of insect eggs (Li et al., 2008), and egg glue of *P. brassicae* was described to consist of proteins and unsaturated lipoids (Beament and Lal, 1957). In addition, the ARG secretions could contain molecules that are produced by the parents and/or microbial symbionts to protect the vulnerable egg,

for example compounds with antimicrobial activity (Flórez et al., 2018). Further identification of EAMPs can lead to new research in this direction.

In summary, we present a method to obtain EAMPs from insect eggs, and using this method, show that the EAMP inducing the HR-like cell death response is in the egg glue, derived from the female ARG. We furthermore assess the chemical nature and specificity of these elicitors and the molecular response of *Brassica* plants to *Pieris* and other eggs. The obtained knowledge paves the way for future studies on identification of EAMPs in *Pieris* egg glue, and the corresponding receptor genes in *Brassica* plants.

Data availability statement

The datasets presented in this study can be found in the data repository Zenodo at doi.org/10.5281/zenodo.7194716.

Author contributions

LC, NB, JL, RM, and NF planned and designed the research. LC, NB, and PV performed the experiments and/or analyzed the data. LC, NB, and NF wrote the manuscript with contributions by PV, RM, JL, and MS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found at: https://www.frontiersin.org/articles/10.3389/fevo.2022.1070859/full#Supplementary-material

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