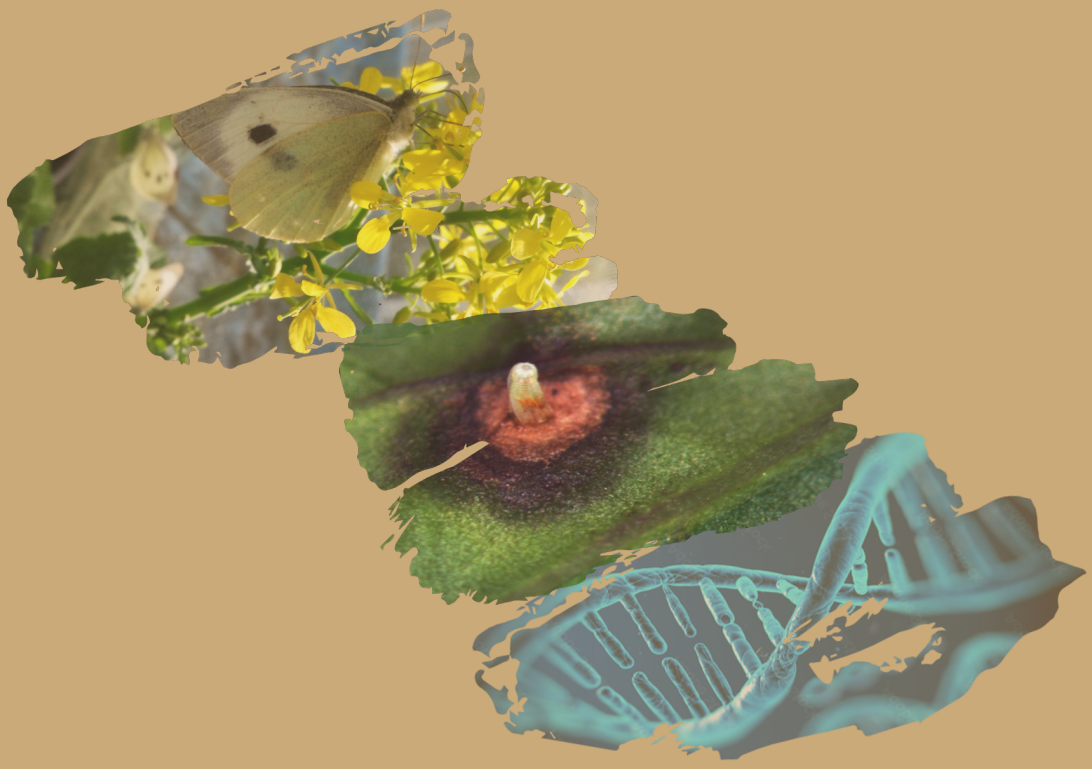
E(gg)xit strategy of plant defense:

Evolution and genetics of a butterfly egg-triggered cell death



Niccolò Bassetti

Propositions

1. To understand the selective pressure of defense traits, life cycle and population dynamics of both a host and its attacker should be considered.
   (this thesis)

2. Although rarely reported in literature, egg-killing traits in crop plants are potential new targets for plant breeding.
   (this thesis)

3. Artificial Intelligence that fails to be transparent and open-source should be regulated.

4. Novel science is overvalued until replicated.

5. Scientific innovations solve global societal challenges only if implemented together with collective thinking.

6. Ecological transition requires a nature-based education in every curriculum.

7. Silence of scientists in public debates gives space to a minority of zealots.

Propositions belonging to the thesis, entitled

"E(gg)xit strategy of plant defense: Evolution and genetics of a butterfly egg-triggered cell death"

Niccolò Bassetti
Wageningen, 3 October 2022

E(gg)xit strategy of plant defense:

Evolution and genetics of a butterfly egg-triggered cell death

Niccolò Bassetti

Thesis Committee

Promotors
Prof. Dr M.E. Schranz
Professor of Biosystematics
Wageningen University & Research

Prof. Dr B.J. Zwaan
Professor of Genetics
Wageningen University & Research

Co-promotors
Dr N.E. Fatouros
Associate Professor, Biosystematics Group
Wageningen University & Research

Dr A.B. Bonnema
Associate Professor, Laboratory of Plant Breeding
Wageningen University & Research

Other members
Prof. Dr J.J.A. van Loon, Wageningen University & Research
Prof. Dr A. Steppuhn, University of Hohenheim, Germany
Dr L. Fokkens, Wageningen University & Research
Dr M.R. Kant, University of Amsterdam, the Netherlands

This research was conducted under the auspices of the Graduate School Experimental Plant Sciences (EPS).

E(gg)xit strategy of plant defense:
Evolution and genetics of a butterfly egg-triggered cell death

Niccolò Bassetti

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 3 October 2022
at 11 a.m. in the Omnia Auditorium.

Niccolò Bassetti
E(gg)xit strategy of plant defense: Evolution and genetics of a butterfly egg-triggered cell death
232 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2022)
With references, with summary in English

ISBN: 978-94-6447-357-5
DOI: https://doi.org/10.18174/575127

To nonno Giotto

for his insatiable curiosity about the world

which inspired me to become who I am

Table of contents

Chapter 1

General introduction

Background

Plants are the primary source of energy for terrestrial life on Earth. They represent the 80% of organic matter of the whole planet and constitute an indispensable part of terrestrial ecosystems (Bar-On *et al.* 2018). Since the appearance of the first land plants over 450 Million years ago (Mya) (Kenrick & Crane 1997), adaptation to ever-changing abiotic and biotic stresses resulted in an incredible diversity, currently estimated of ~350,000 species (Kew 2020). It is thought that this diversity has been partially driven by coevolution with other organisms (Delaux & Schornack 2018). For example, coevolution between plants and insects dates back over 400 Mya and to date, around 250,000 angiosperm species have an interaction with insects (Grimaldi 1999, Grimaldi & Engel 2005). Remarkably, about half of the described one million insect species are estimated to be herbivores (Schoonhoven *et al.* 2005, Stork 2018). The majority of the insect herbivores are considered specialists, that is feeding only on a narrow range of host plants, while the rest are considered generalists, that is feeding on multiple host plants often from different plant families (Ali & Agrawal 2012). Understanding the evolution, the mechanisms and the specificity of the interactions between herbivorous insects and host plants is thus fundamental to comprehend terrestrial biodiversity (Futuyama & Agrawal 2009).

The enormous diversity of insects is thought to be related to diversity of plants (Grimaldi &Engel 2005, Strong *et al.* 1984, Mitter *et al.* 1988). The cospeciation between plants and insects was proposed to follow a so called "escape-and-radiate" model (Thomson 1989). The model was first conceived by Ehrlich and Raven (1964) as a co-evolutionary arms race between plants which develop new chemical defences and insects that consequently evolve detoxification mechanisms as counter-adaptations. In this arms race, successful adaptations allow organisms to escape the selection pressure exerted by the enemy and further diversify. For example, the interaction between the parsley family (Apiaceae) and swallowtail butterflies (Papilionidae) appears to be driven by plants evolving increasingly complex furanocoumarins and by the butterflies counter-adapting with detoxification enzymes (Berenbaum 1983). On the other side, insect diversification may be shaped by insect host plant shifts (Winkler *et al.* 2009), which is still favored by similar phytochemistry across plant taxa. Another notable coevolutionary interaction occurs between Brassicales plants and Pieridae butterflies. In this system, the butterflies' host switch from Fabaceae plants to glucosinolate (GLS)-containing Brassicales plants was possible after the evolution of nitrile-specifier proteins (NSP) that detoxify glucosinolates, leading to radiation of the subfamily Pierinae (Wheat *et al.* 2007). Improvements in phylogenetics and genomics enabled further testing of

the role of putative key innovations on this coevolutionary interaction. For example, increase in Pierinae diversification rate and host shift followed the expansion of plants` GLS biosynthetic pathways and butterflies` NSP gene copies which were driven by gene and genome duplications (Edger *et al.* 2015).

Plant defence and immune system

Plant secondary metabolites are well studied as a defence against insect herbivores but they represent only one aspect within the broader plant immune system (Jones & Dangl 2006, Erb & Reymond 2019). A first layer of plant defences is formed by the so called "constitutive defences", such as trichomes, spines or thick cuticles which physically hamper herbivory and they are always expressed. A second layer of defences are the "induced direct defences" which are only expressed upon attack, such as changes in nutritional quality, production of toxic proteins and metabolites, callose deposition or cell death (Kessler 2015). Finally, plants benefit also from "induced indirect defences" such as herbivore-induced plant volatiles (HIPVs) which are emitted upon herbivore attack to recruit natural enemies of the herbivores (Dicke & Baldwin 2010).

The elicitation of induced defence responses results from the ability of the plants' innate immune system to perceive biotic stresses. It is widely accepted that basal plant immunity follows the recognition of biotic attackers via multiple classes of protein receptors (Dangl & Jones 2006). Generally, membrane pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs), which are conserved molecules that are required for microbial cell integrity, leading to a first layer of immunity, known as pattern-triggered immunity (PTI) (Couto & Zipfel 2016, Gust *et al.* 2017). Remarkably, PRRs are also increasingly associated with defence against insect herbivores (Reymond 2021, Snoeck *et al.* 2022). Insects induce plant immunity through the release of either herbivore-associated molecular patterns (HAMPs) contained in larval oral secretions and frass, or damage-associated molecular patterns (DAMPs), that is molecules derived from damaged plant tissues (Acevedo *et al.* 2015). Overall, this first layer of immunity is sufficient to keep at bay most of non-adapted (generalist) biotic stressors. On the contrary, host-adapted attackers can modulate and/or suppress PTI through the secretion of molecules that are required for virulence, known as effectors (Acevedo *et al.* 2015; Toruño *et al.* 2016). Plants evolved, nonetheless, a second layer of immunity known as effector-triggered immunity (ETI) through cytosolic nucleotide-binding leucin-reach repeat containing receptors (NLRs) which recognize effectors (Cui *et al.* 2015). Genetically, ETI activation is the result of a plant resistance (*R*) gene, often a NLR

protein, which recognizes a specific attacker effector, known as avirulence (*Avr*) gene, thus motivating the concept of "gene-for-gene" resistance model (Flor 1971). The recognition between *R* genes and *Avr* effectors is allelic specific and it results in plant cultivars being resistant or susceptible to specific pathogen "races", or insect "biotypes".

PTI and ETI were once thought to be two sequential layers of plant immunity and they were framed according a so-called "zig-zag" model (Dangl & Jones 2006). Currently, the dichotomy between the two immune responses is considered more blurred (Cook *et al.* 2015) and indeed PTI and ETI seem to share signalling pathways and potentiate each other (Nguo *et al.* 2021, Yuan *et al.* 2021).PTI and ETI largely consist of a similar array of immune responses. In fact, perception of an attacker induces early signalling through modulation of Ca^{2+} fluxes (Thor 2019) and production of a variety of reactive oxygen species (ROS) and nitric oxide (NO) (O'Brien *et al.* 2012, Kulik *et al.* 2015). Early signalling is followed by modulation of phytohormones such as salicylic acid (SA) and/or jasmonic acid (JA) (Pieterse *et al.* 2012) which orchestrate a massive transcriptional reprogramming leading to downstream defences, such as callose deposition, cell death, antimicrobial proteins and/or toxic secondary metabolites (Bürger & Chory 2019). Despite PTI and ETI consist of similar immune responses, they can be distinguished based on the timing and the intensity of the aforementioned responses (Ngou *et al.* 2022).

A commonly observed phenotype of ETI is the hypersensitive response (HR), which is often associated with gene-for-gene resistance as the result of NLR activation (Cui *et al.* 2015). Still not universally definable, HR is a broad term indicating a rapid cell death that is localized in plant cells under attack and limits the spread of a microbial infection or herbivore feeding (Mur *et al.* 2008, Dickman & Fluhr 2013). Physiologically, HR is a unique form of programmed cell death (PCD) that shares features from known PCDs in both plants and animals, such as apoptosis, pyroptosis, autophagy and necrosis (Mur *et al.* 2008, Kunstler *et al.* 2016). Genetically, HR is often a trait underlying monogenic (qualitative) resistance following the gene-for-gene model (Balint-Kurti 2019). However, it is also evident that in some pathosystems, HR can be uncoupled from the actual resistance mechanism (Coll *et al.* 2010, Kunstler *et al.* 2016). Furthermore, HR is certainly less important in polygenic quantitative resistance based on many quantitative trait loci (QTLs) (St Clair 2010). Besides pathosystems, HR involving a gene-for-gene model has been described also in interactions between plants and piercing-sucking insects (Harris *et al.* 2012, Bentur *et al.* 2016, Wang *et al.* 2021b). In many other plant-insect interactions, however, a gene-for-gene relationship has

so far not been demonstrated and the plant defense responses are therefore defined as "HR-like" cell death (Fernandes 2001, Reymond 2013).

Plant responses to insect eggs

Insect eggs often represent the first contact between insect herbivores and plants. Seemingly inert structures, eggs constitute an upcoming danger as they carry future voracious larvae. Accordingly, plants evolved different defence strategies against insect eggs (Hilker & Fatouros 2015). A first line of defence is represented by constitutive defences that deter females from eggs being deposited, for example a high density of leaf trichomes or a waxy leaf surface (Blenn *et al.* 2012, Wagner & Doak 2017). Induced direct defences include different responses such as the formation of neoplasm (Doss *et al.* 2000, Petzold-Maxwell *et al.* 2011), ovicidal chemicals (Seino *et al.* 1996), tissue crushing (Desurmont *et al.* 2011) and HR-like cell death (Shapiro & De Vay 1987, Balbyshev & Lorenzen 1997, Garza *et al.* 2001). Indirect defences such as oviposition-induced plant cues leading to the attraction of egg and larval parasitoids have also been described for several plant-herbivore interactions (Wegener *et al.*2001, Hilker *et al.* 2002, Fatouros *et al.* 2012). Additionally, eggs can prime defences against upcoming herbivory by larvae (Beyaert *et al.* 2011, Pashalidou *et al.* 2013, Austel *et al.* 2016, Bandoly *et al.* 2016, Altmann *et al.* 2018, Valsamakis *et al.* 2020).

Knowledge on the molecular aspects of plant defences to insect eggs is limited and mostly derived from a few systems (Reymond 2013, 2021). Transcriptomic studies showed that eggs induced a massive plant transcriptional response which seems distinct from the response to herbivory (Little *et al.* 2007; Firtzlaff *et al.* 2016; Baruah *et al.* 2017; Bonnet *et al.* 2017; Altmann *et al.* 2018; Drok *et al.* 2018; Geuss *et al.* 2018; Nallu *et al.* 2018). Moreover, a portion of the transcriptional response to eggs appears to be conserved between different plant-insect egg interactions (Lortzing *et al.* 2020). Components of the plant immune response to eggs seem to be shared across unrelated plant species as, for example, the accumulation of ROS (de Puysseleyr *et al.* 2011; Kim *et al.* 2012; Gouhier-Darimont *et al.* 2013; Bittner *et al.* 2017; Geuss *et al.* 2017; Das *et al.* 2021; Oates *et al.* 2021; Ojeda-Martinez *et al.* 2021). However, different phytohormonal pathways may be specifically induced by different interactions. For example, the response of *Arabidopsis thaliana* to *Pieris brassicae* relies on induction of salicylic acid (SA) (Bruessow *et al.* 2010, Gouhier-Darimont *et al.* 2013, Valsamakis *et al.* 2020), while the response of tomato (*Solanum lycopersicum*) to *Helicoverpa zea* induces jasmonic acid (JA) (Kim *et al.* 2012). The response of *A. thaliana* to *P. brassicae* eggs was further shown to be accompanied by callose deposition and cell death (Little *et al.*

2007), but it is unknown whether these responses are also present in other plant-insect egg interactions.

The nature of egg elicitors that trigger the abovementioned immune responses has been revealed in a few cases. Neoplasm formations are induced by eggs of bruchid beetles in pea plants through bruchins, which are lipids originating from egg-associated secretions (Doss *et al.* 2000). Different phospholipids from eggs of the whitebacked planthopper (*Sogatella furcifera*) were instead found to induce the production of benzyl benzoate in rice (*Oryza* spp.), an ovicidal compound (Yang *et al.* 2013). Further, elicitors of oviposition-induced plant cues that attract egg parasitoids were also found, namely in egg secretion of the pine sawfly *Diprion pini* (Hilker *et al.* 2005), or accessory reproductive glands of cabbage white butterflies *Pieris* spp. (Fatouros *et al.* 2008, 2009). Recently, phosphatidylcholines (PCs) were found abundant in eggs of *P. brassicae* and *Spodopera exigua* and they were sufficient to induce ROS, cell death and *PR1* expression in *A. thaliana* (Stahl *et al.* 2020). Despite all this, it is not clear how widespread are these elicitors across insect clades. It is thus unknown whether they represent either a sort of egg-associated molecular patterns (EAMP) conserved across taxa, or rather lineage-specific "egg effectors" that suppresses the initial plant basal immune response.

Similarly, knowledge on plant receptors of egg elicitors is equally scarce. For example, PRRs of receptor-like kinase protein (RLK) type were upregulated in *A. thaliana* upon *P. brassicae* eggs deposition (Little *et al.* 2007) and two L-type LecRKs, LeRK-I.1 and LecRK-I.8, mediated plant response to eggs (Gouhier-Darimont *et al.* 2019, Groux *et al.* 2021b). Beyond these studies, however, there is a lack of knowledge on plant receptors involved in perception of insect eggs. Nevertheless, there is evidence for genetic variation in plant defence traits to eggs in different plant-egg interactions (Yamasaki *et al.* 2003; Tamiru *et al.* 2015; Geuss *et al.* 2017; Groux *et al.* 2021b), which is prerequisite to perform genetic studies.

HR-like cell death induced by *Pieris* eggs

The interaction between eggs of *Pieris* ssp. eggs and Brassicaceae plants represents an amenable system to study molecular aspects of plant responses to eggs. Eggs of *Pieris* spp. induce a HR-like cell death in different Brassicaceae spp. such as *A. thaliana* (Groux *et al.* 2021b), *Brassica* spp., *Sinapis arvensis* and *Moricandia* spp. (Pashalidou *et al.* 2015a, Fatouros *et al.* 2016, Griese *et al.* 2021). This cell death is relatively understudied within the context of plant-butterfly coevolution and its genetic basis is unknown. In *B. nigra* HR-like cell death can potentially act as an adaptive trait. For example, it reduces herbivory pressure as it results in increased egg-killing both in the greenhouse (Griese *et al.* 2017) and in the

field, where it additionally correlates with increased egg parasitism (Fatouros *et al.* 2014). A molecular and physiological characterization of the plant response to *Pieris* eggs was so far only performed in *A. thaliana* (Reymond 2013). Despite the accession Col-0 lacks a macroscopic HR-like cell death, its cellular response to eggs resembles a plant immune response to pathogens (Reymond 2013). Intraspecific variation in HR-like cell death severity and occurrence was observed in *B. nigra* and *A. thaliana* (Griese *et al.* 2017, Groux *et al.* 2021b), suggesting the feasibility of classical forward genetics to investigate its genetic basis. Indeed, natural variation in cell death severity in *A. thaliana* recently resulted in the identification of the first two loci related to plant immunity components (Groux *et al.* 2021b).

Thesis aim and outline

This thesis aims to elucidate evolutionary, physiological and genetic aspects of a HR-like cell death induced by eggs of *Pieris* spp. in *Brassica* spp. The following knowledge gaps and hypotheses guided the design of research objectives (Fig. 1). The *Brassica-Pieris* egg interaction falls under the broader Pieridae-Brassicales system which is a well-described example of plant-insect co-evolution. As the HR-like cell death reduces *Pieris* egg survival, it may represent a plant adaptation in the Pierinae-Brassicales coevolutionary interaction. However, it is not known how widespread this egg-killing trait is across the Brassicales phylogeny and how common is the ability to induce HR across the Lepidoptera, and specifically the Pieridae butterflies. Further, I was interested in characterizing the physiological and molecular processes associated with the egg-induced cell death. Plant immune responses were previously observed under *P. brassicae* eggs in *A. thaliana*. Thus, I hypothesized that similar plant immune responses were also induced in *Brassica* spp. and I expected phenotypic variation for these responses between plant accessions. Finally, I aimed to understand the genetic basis of HR-like cell death in *Brassica* spp. to identify genetic loci which ultimately could link genetic mechanism and evolutionary trajectory of the trait. The research tackling these knowledge gaps was organized in the following Chapters.

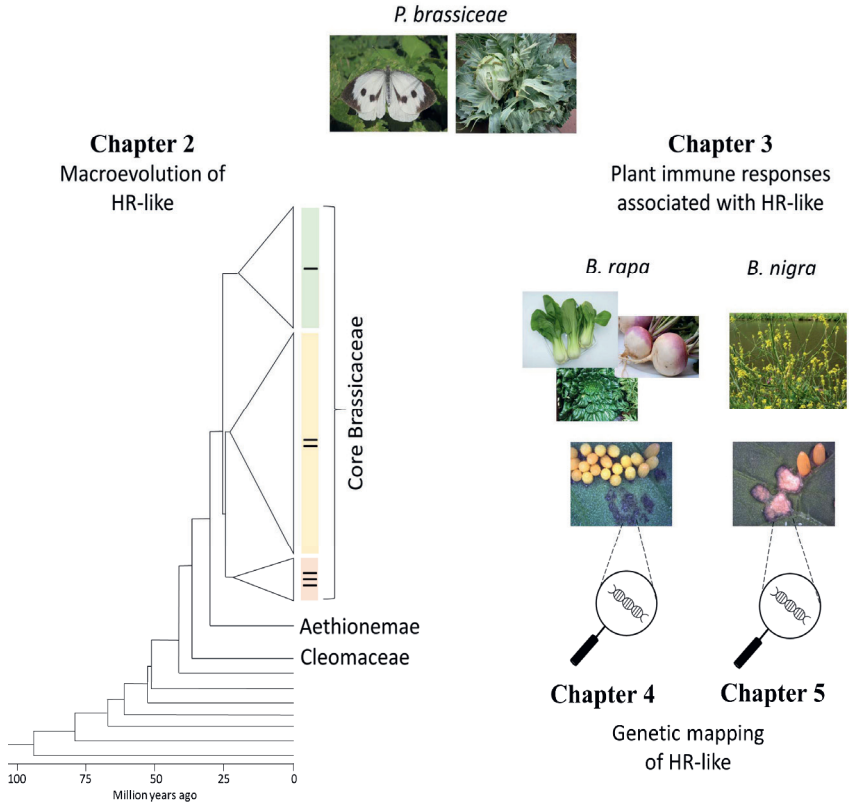


Figure 1. Overview of the approaches used in this thesis. Top panels: *P. brassicae* is a specialist feeder on Brassicaceae (left) and a major pest on *Brassica* crops (right). Pierinae butterflies are adapted to different families of Brassicales while *Pieris* spp. are specialist of Brassicaceae. Left: Chapter 2 explores the macroevolutionary pattern of egg induced HR-like cell death on Brassicaceae (Lineages I, II, III and tribe Aethionemae). Cleomaceae were used as putative outgroup of plants not developing HR-like cell death. Right, top: Chapter 3 focuses on the plant immune responses associated with HR-like cell death by comparing two species, the crop *B. rapa* and the wild host *B. nigra*. Right, bottom: Chapter 4 and Chapter 5 investigate the genetic basis of the HR-like cell death in the two *Brassica* spp. Phylogeny of Brassicales is adjusted from Walden *et al.* (2020). Photos of butterfly and plants are taken from Wikimedia Commons (authors S Sepp, Вальдимар, Karl-Heinz Wellmann, Matt Lavin, baldeaglebluff, thebittenword.com), under Creative Commons BY 3.0 licence. Photos of HR-like cell death are taken by Lotte Caarls.

Chapter 2 explores the overlooked HR-like cell death trait as a potential plant adaptation in the macroevolutionary context of the butterfly-plant arms race. First, I aimed to trace the putative origin of the trait on the plant phylogeny by investigating the occurrence of HR-like cell death in 28 Brassicaceae and 3 Cleomaceae plant species using eggs of *P. brassicae*. Next, I aimed to trace the origin of the putative cell death elicitor on the butterfly phylogeny. Thus, I tested eggs from different butterflies and moths for their ability to induce

in *B. nigra* a cell death and *PR1* gene expression, a plant immunity marker. Finally, few Brassicaceae species were selected to study the effect of cell death severity on eggs survival.

Chapter 3 describes different plant immunity responses associated with HR-like cell death in a crop (*B. rapa*) and a wild relative (*B. nigra*). First, I investigated types and timing of the immune responses developed by the two *Brassica* species. Then, I used these responses to characterize the difference between eggs of *P. brassicae*, a brassicaceaous specialist, with eggs of the generalist moth *Mamestra brassicae*. *B. nigra* accessions with contrasting HR-like phenotypes were tested for their ability to express a canonical HR against pathogens. Further, gene expression of SA- and JA-related defence markers was explored in these *B. nigra* accessions to link variation in egg-induced cell death to different regulation of defense-related phytohormones.

Chapter 4 investigates the strength of HR-like cell death within the crop species *B. rapa.* A subset of 56 *B. rapa* accessions derived from a core collection was screened to explore the diversity and variation in cell death. I developed an image-based phenotyping protocol to quantify cell death size in an accurate and portable manner. This phenotyping protocol was used to identify two accessions with contrasting cell death phenotype and to screen a RIL population to perform QTL mapping. Finally, syntenic relationships between the QTLs that I identified and *A. thaliana* were analysed.

Chapter 5 studies the genetic basis of HR-like cell death in *B. nigra* accessions by crossing plants from a local wild population. First, investigated the inheritance of the trait using different types of crosses. As I expected a simple genetic architecture, I performed genetic mapping using bulk-segregant analysis (BSA) coupled with whole-genome sequencing (BSA-seq). The locus identified with BSA-seq was validated with molecular markers and fine-mapped through recombinant analysis. Next, differential gene expression at the locus region between the two parents was assessed through RNA sequencing. Further, I explored the genetic variation at the locus between the parental accessions and among the available *B. nigra* genomes. Finally, I discussed implications for further fine-mapping of the locus.

Chapter 6 provides a general discussion that places my thesis into a broader context by comparing my results with the literature available. In this final chapter, I speculate how my results could provide a mechanism for perception of *Pieris* eggs by the plant immune system, I cover the implication for plant-insect coevolution and, finally, I discuss perspectives for future research.

Chapter 2

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

Eddie Griese[1*], Lotte Caarls[1,4*], Niccolò Bassetti[1], Setareh Mohammadin[1], Patrick Verbaarschot[1], Gabriella Bukovinszkine'Kiss[1,3], Erik H. Poelman[2], Rieta Gols[2], M. Eric Schranz[1], Nina E. Fatouros[1]

[1] Biosystematics Group, Wageningen University & Research, Wageningen, The Netherlands
[2] Laboratory of Entomology, Wageningen University & Research, Wageningen, The Netherlands
[3] Laboratory of Genetics, Wageningen University & Research, Wageningen, The Netherlands
[4] Laboratory of Plant Breeding, Wageningen University & Research, Wageningen, The Netherlands
[*] These authors contributed equally to this work

New Phytologist, 2021, 230: 341–353 doi: 10.1111/nph.17145

Abstract

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.

Keywords
coevolution, counter adaptation, egg deposition, hypersensitive response, induced plant defences, plant toxins, specialist herbivores.

Introduction

The biodiversity on Earth is shaped by numerous factors including inter-organismal 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 *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 Coliadinae are Fabales feeders, the sister to the Coliadinae, 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 (*PR1*)), 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.* 2021b). 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* (Coliadinae) feeding on *Rhamnus* spp. (Rhamnaceae), *Colias* spp. (Coliadinae) and *Leptidea sinapis* (Dismorphinae) 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 *PR1* 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 (Supplementary 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 Supplementary 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* (Caarls *et al.* 2021, Chapter 3). 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) (Supplementary 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. mannii*, *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. mannii*, *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. xyllostella* 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 (Onate-Sanchez & Vicente-Carbajosa 2008), real-time quantitative PCR analysis and primers are described in detail in Supplementary 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 (H_2O_2) 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 non-hatching 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 ± 4 °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 (Li *et al.* 2010). *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 linages of the core Brassicaceae clade and the first-branching Aethionema and the outgroup Cleomaceae (for details see Supplementary 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 Papiolionoidea (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 2021). For the screening of plant accessions, contingency tables and v2-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 v2-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* eggwash. 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% (Supplementary 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 ($P = 0.05$; Supplementary 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*.

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. mannii* 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 (Supplementary Table S6). When several populations were available for butterfly species, all populations elicited HR-like necrosis with similar frequencies (GLM: $v^2 = 1.36$, df = 3, $P = 0.71$) and severity (GLM: $v^2 = 2.60$, 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 (Supplementary Fig. S1). The egg wash of *A. io* induced no symptoms on *B. nigra* (Supplementary Table S6).

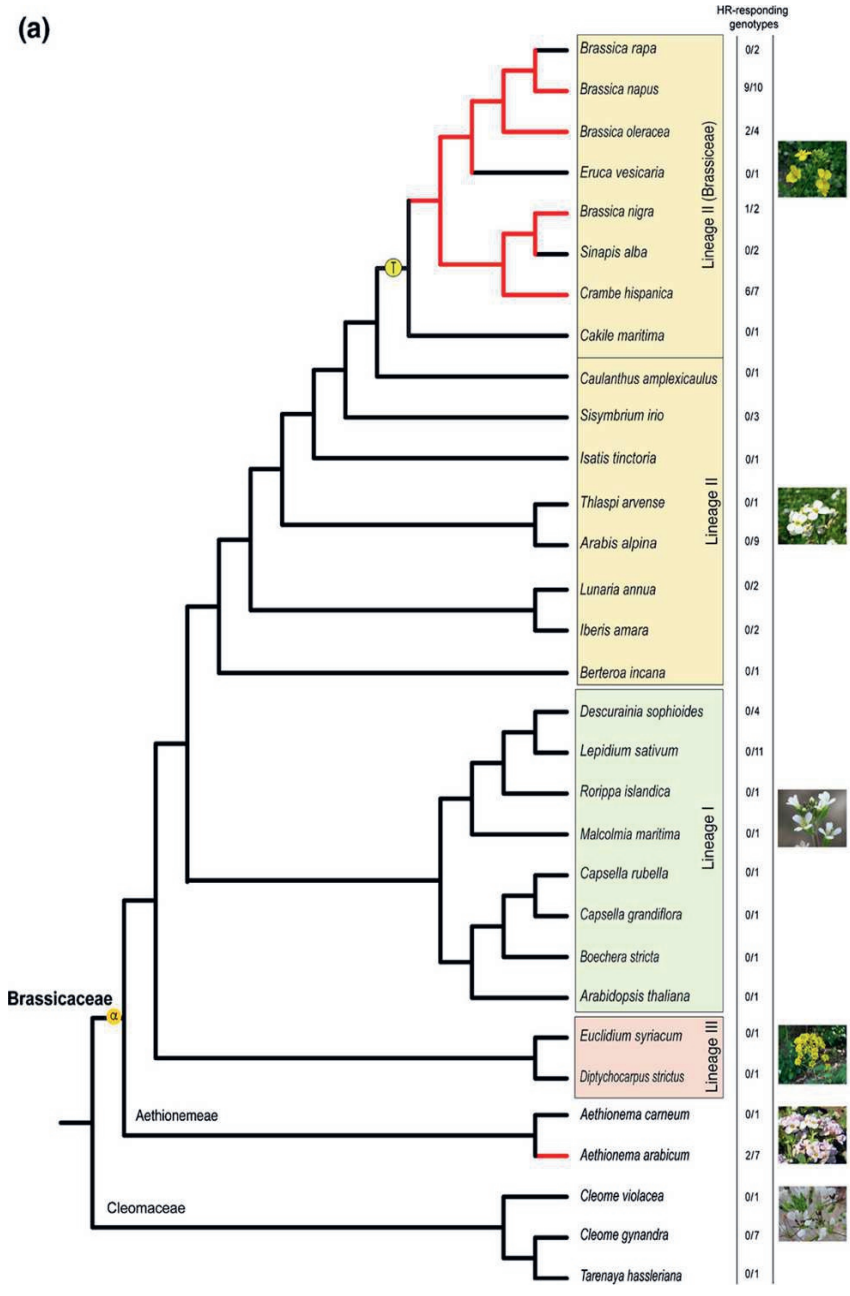


Figure 1. Presence of hypersensitive response (HR)-like necrosis mapped on phylogenetic trees of Brassicaceae (a) and Pieridae (b). Full text caption on page 32.

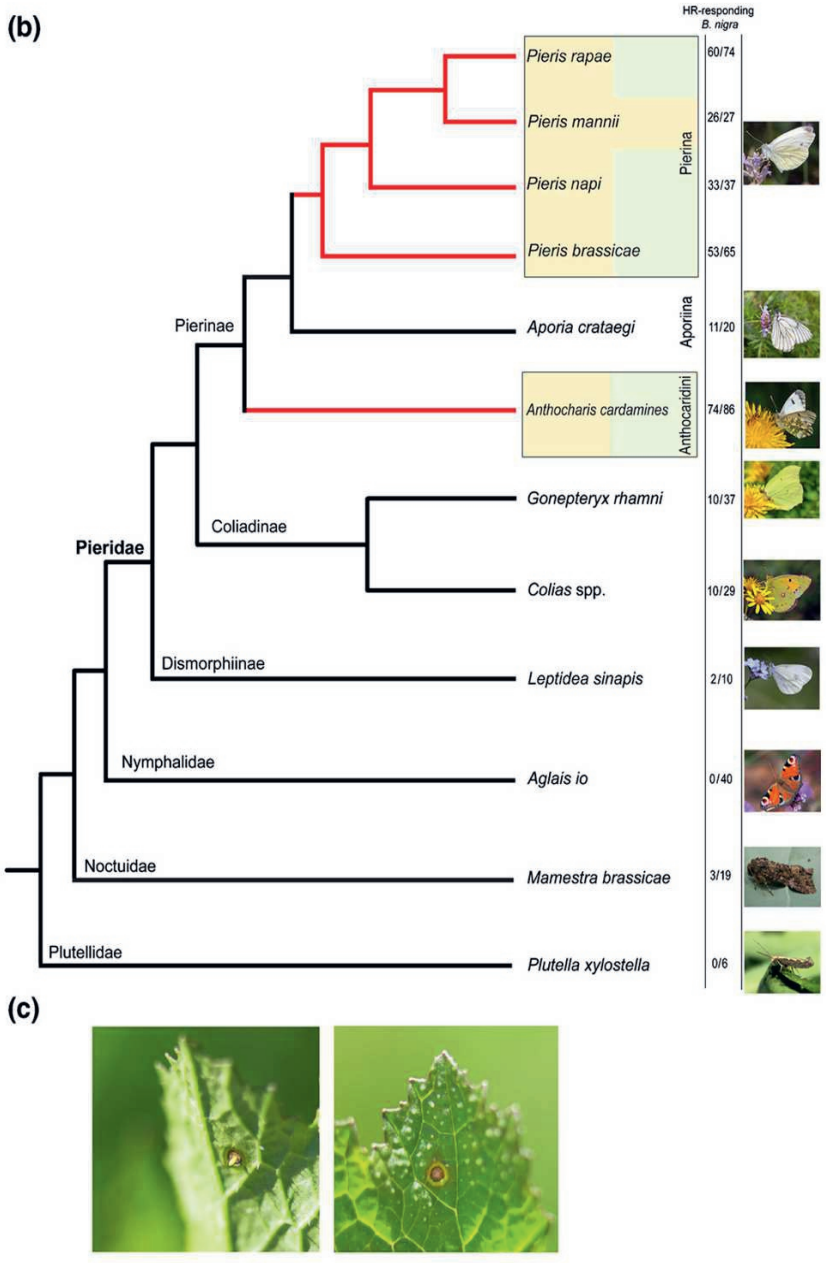


Figure 1. Presence of hypersensitive response (HR)-like necrosis mapped on phylogenetic trees of Brassicaceae (a) and Pieridae (b). Full text caption on page 32.

Figure 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* spp. egg in *B. nigra* taken from the under and upper side of the leaf (credits N. E. Fatouros).

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: $v^2 = 28.3$, df = 9, $P < 0.001$; Supplementary 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; Supplementary 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; Supplementary 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; Supplementary Table S7).

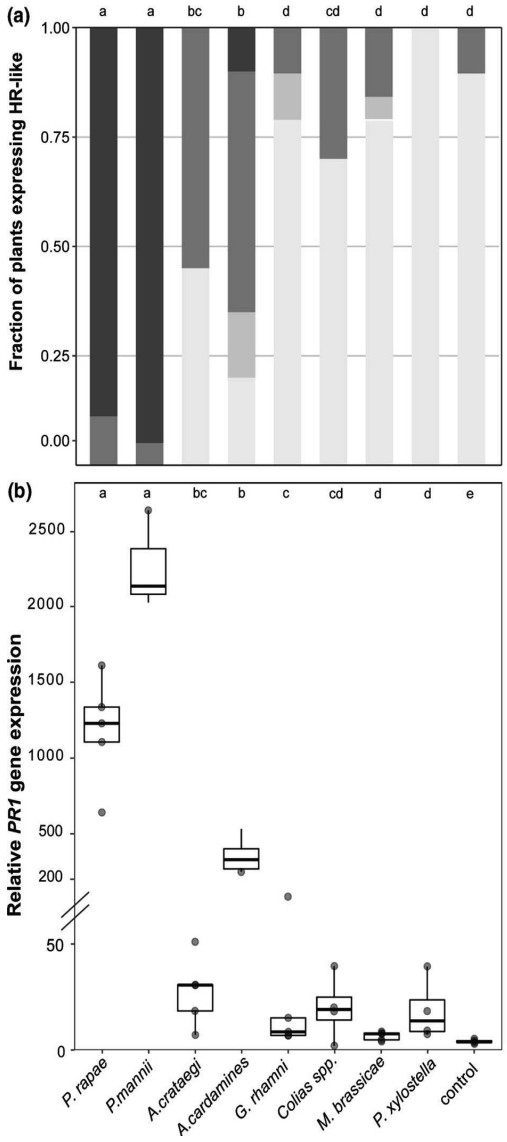


Figure 2. Hypersensitive response (HR)-like necrosis and pathogenesis-related (*PR1*) gene expression induced by egg wash of different butterfly and moth species in *Brassica nigra* plants. For the experiment, two droplets of 10-ll egg wash were applied onto one leaf of each plant. (a) Fraction of plants 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, $P < 0.05$).

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* (Supplementary 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 (Supplementary 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 *B. nigra* and significantly smaller in the other two species (ANOVA, $F = 17.028$, df = 2, $P < 0.001$; Supplementary Table S9). Previously, *P. brassicae* eggs on *A. thaliana* were shown to induce components of plant immunity such as H_2O_2 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 H_2O_2 and cell death. Plants that showed a small HR-like necrosis (i.e. *B. oleracea* and *C. hispanica*) exhibited a high accumulation of H_2O_2 and trypan-blue stained cell death compared to the extension of visible necrosis (Supplementary Fig. S2). *Brassica nigra* showed a strong visible necrosis, > 1 mm^2 per 10 eggs, exceeding the H_2O_2

and cell death accumulation (Supplementary 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: $v^2 = 11.02$, 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: $v^2 = 38.41$, 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: $v^2 = 2.61$, 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).

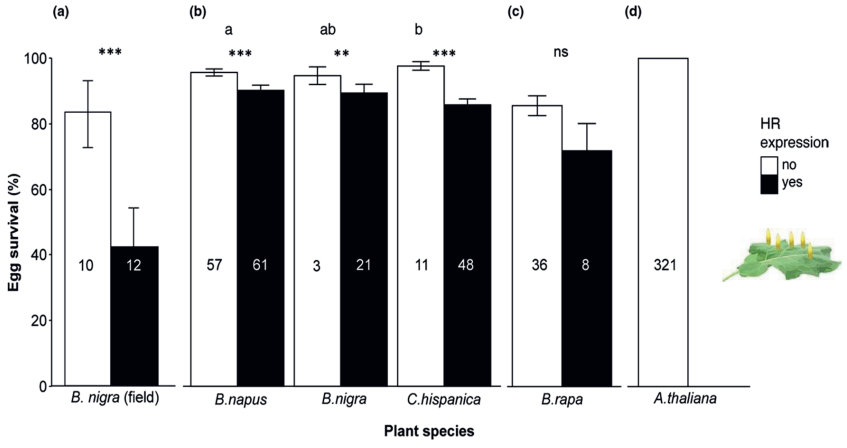


Figure 3. Effect of hypersensitive response (HR)-like necrosis on survival rates of singly laid *Pieris* eggs on different plant species. Full text caption on next page.

Figure 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 Rhine River 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*, *Crambe 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 ± 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; **, $P < 0.01$; ***, $P < 0.001$).

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 phylogenetic history of egg induction (i.e. hypersensitive response (HR)-like necrosis) as a plant defence trait and its reciprocal cooccurrence 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 Brassiceae 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 Brassiceae), 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 Brassiceae 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 Brassiceae and *Aethionema*

Four of eight tested Brassiceae 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 Brassiceae 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 Brassiceae and *Aethionema*. In fact, besides many of the tested Brassiceae 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 Aethionemeae, 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 Brassiceae 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 non responsive 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.* 2021b). For half of the tested species here, only one genotype was tested, increasing the likelihood of selecting only nonresponsive ones (Fig. 1a, Supplementary 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, Supplementary 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 initself 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 Pierinaespecies 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 Brassiceae clade. Only two butterfly species specialized to plant species outside the Brassiceae: *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* o *G. rhamni*) 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.

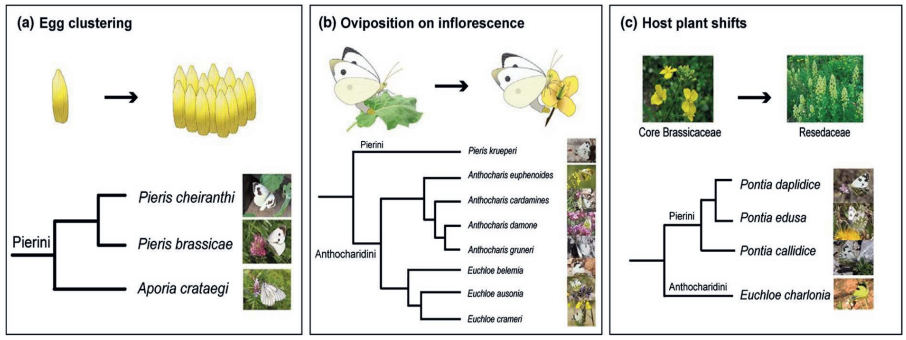


Figure 4. Examples of possible counter-adaptations to egg-killing hypersensitive response (HR)-like necrosis of the Pierinae butterfly clade: egg clustering (a), oviposition on inflorescence (b), and host plant shifts to non-HR expressing species (c). Phylogenetic relationships are according to Wiemers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

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 Brassiceae tribe b *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 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 H_2O_2 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* (Fig. 1-2, Supplementary 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 H_2O_2 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 Brassiceae 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 Brassiceae 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.

Acknowledgements

We thank the employees of Unifarm (Wageningen University and Research) for rearing and caring of the plants used in the experiment. We are thankful to Pieter Rouweler, Andr'e Gidding and Frans van Aggelen for rearing the Dutch *Pieris* butterflies used in the experiment. Guusje Bonnema (WUR, Plant Breeding), and the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (Germany) are thanked for the seeds. Furthermore, we thank Miltos Tsiantis from the Department of Comparative Development and Genetics, Max Planck Institute for Plant Breeding Research for kindly providing *C. hirsuta* seeds, used as host plants for *A. cardamines*, and Niclas Backstrom for collecting and shipping *L. sinapis* eggs and pupae. The authors thank Marcel Dicke and Monika Hilker for reading and commenting on an earlier version of the manuscript.

Availability of data and materials

The datasets supporting the conclusions of this article are temporary available at this link https://figshare.com/s/6cf5e8efe8a20c059de6 (restricted access, to be released upon publication).

Supplementary methods

Supplementary methods are also available online at this https://doi.org/10.1111/nph.17145.

Supplementary Methods S1: Butterflies and moths

Two populations of *P. brassicae* L., *P. napi* L. and *P. rapae* L. were tested (Supplementary Table S2). Furthermore, we tested egg wash from one population of *P. mannii* Mayer, three populations of *Anthocharis cardamines* L., one population of *Aporia crataegi* L., one population of *Leptidea sinapis* L., one population of *Gonepteryx rhamni* L. and *Aglais io* L. (Lepidoptera: Nymphalidae), and two moths species *Plutella xylostella* L. (Lepidoptera: Plutellidae) and *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (Supplementary Table S2). Finally, survival was measured for eggs of *P. brassicae*, *P. napi* and *P. rapae*. *Pieris brassicae*, *M. brassicae* and *P. xylostella* were reared on Brussels sprouts (*B. oleracea* var. *gemmifera* cv. Cyrus) in a climatized room (21 ± 1 °C, 50-70% RH, LD 16:8 h). *Pieris mannii*, *P. napi*, and *P. rapae* were collected outside and reared in a greenhouse (21 ± 4 °C, 60-80% RH, LD 16:8 h), either on flowering *Iberis* spp. plants (*P. mannii*) or Brussels sprouts. One population of *A. cardamines* was obtained from the butterfly farm Farma Motyli Zielona Dolina (Babidół, Poland) as hibernating pupae. Hibernation was broken by storing the pupae at 4 °C in a cold storage room for five months and another month outdoors. After hibernation, the butterflies were kept in a greenhouse compartment (18 ± 2 °C, 50–60% RH, LD 16:8 h) with flowering *Cardamine hirsuta* and *Sisymbrium irio* plants to obtain eggs. *Aglais io* butterflies were kept in cages outside (May to June 2018) with cuttings of *Urtica* spp. plants on which they oviposited. Eggs and/or adults of *A. cardamines*, *Colias* spp. and *G. rhamni* were also collected outdoors (for locations see Supplementary Table S2); adults were released again when sufficient egg depositions were obtained. *Aporia crataegi* eggs were obtained from Worldwide Butterflies, UK (https://www.wwb.co.uk/). *Leptidea sinapis* eggs were obtained from a population collected in Sweden (Supplementary Table S2). *P. brassicae*, *A. crataegi*, *A. io*, *M. brassicae* and *P. xylostella* lay egg clutches, *P. napi* sometimes lays eggs in small groups, while *A. cardamines*, *Colias* spp., *G. rhamni*, *L. sinapis*, *P. mannii* and *P. rapae* lay single eggs.

Supplementary Methods S2: Preparation of egg wash

The concentrations of the egg washes were adjusted based on the size of the eggs used: from 200 eggs per mL for Pieridae to 1000 eggs per ml for *A. io* (compare database on egg size from more than 10.000 insect species: https://shchurch.github.io/dataviz/index.html). To test the number of eggs required, we performed a pilot experiment with *B. nigra* and different concentrations of eggs. With an egg wash of 200 eggs per mL *B. nigra* consistently responded with strong HR-like response. This was the concentration used to test Pieridae eggs. To wash smaller eggs (*P. xylostella*, *A. io*), a concentration of 1000 eggs per mL was used. To screen the Brassicales species, *P. brassicae* wash was made in purified water (Millipore) and Tween20 was added at a 0.01 % concentration to improve distribution of the egg wash drops onto the waxy leaves of some species. To assess HR-elicitation by other butterfly and moth species, eggs were washed in MES buffer. As controls, paper alone, or *Urtica* spp. leaves (*A. io*), a mixture of *C. hirsuta* and *S. irio* inflorescence stems (*A. cardamines*), leaves of *Rhamnus frangula* L. (*G. rhamni*), and inflorescence stems of *Iberis* spp. (*P. mannii*), paper (*M. brassicae*) or parafilm (*P. xylostella*) were washed in the same manner. Eggs and leaves were kept in the solution overnight, after which the supernatant without eggs was pipetted off and stored at 20 °C. As these egg washes were tested on *B. nigra* plants, of which the leaves do not have a wax layer, no Tween20 was added to the washes.

Supplementary Methods S3: RNA isolation and Real Time qPCR(qRT-PCR) analysis of *PR1* genes

1µg of RNA was reverse-transcribed into cDNA using Bioline's SensiFAST cDNA synthesis kit (BIO-65054) in a 20 µl reaction volume according to the manufacturer's instructions and subsequently diluted 8 x in nuclease free water. Real time qPCR reactions were performed using Bioline's SensiFAST SYBR No-ROX Kit (BIO-98050) in 10µl reaction volumes, containing 3µl cDNA and 500nM of each gene-specific primer (Supplementary Table S3) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The following PCR program was used for all PCR reactions: 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s; annealing temperature for 5 s and 72 °C for 10 s, with data collection at 72 °C. To check for unspecific PCR products the reactions were followed by a melt curve analysis. ΔΔCq values were calculated using the Cq values of the untreated plants and normalising using the Cq values of the reference genes *GAPDH* and *ACT-2*.

Supplementary Methods S4: Histochemical staining

H_2O_2 accumulation was measured at 48 h with 3,3′-diaminobenzidine (DAB, Sigma) as previously described by Daudi and O'Brien (2012). Cell death accumulation was measured at 72 h with trypan blue (TB, Sigma) as previously described by Fernández-Bautista *et al.* (2016). Samples taken at 96 h were used to assess the egg-induced necrosis by carefully removing the eggs from the leaf disc. Leaf discs with or without eggs and after histochemical staining were imaged with a Dino-Lite digital microscope (AnMo Electronics, Taiwan). Pictures were analyzed with Fiji (ImageJ 1.52p) using the plugin Trainable WEKA Segmentation v3.2.34 (Arganda-Carreras *et al.* 2017) with a customized script to measure the stained or the necrotic leaf area in mm2 (Bassetti, Caarls, Verbaarschot, Schranz, Fatouros *et al.* in preparation).

Supplementary Methods S5: Pieris spp. egg survival assessed by field survey

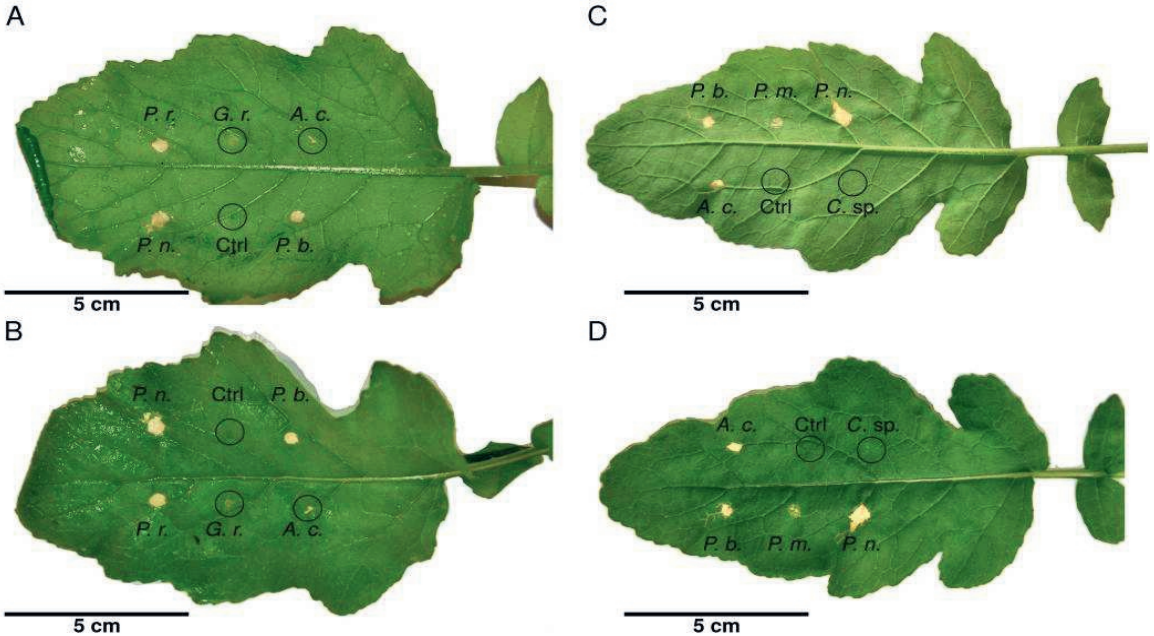
The survey was conducted at a *B. nigra* patch along the River Rhine in Wageningen (Steenfabriek), The Netherlands (coordinates: 51.96°N, 5.68°E) in one season and one butterfly generation (August - September 2017). The total area monitored was approximately 100 m² consisting of ~1000 plants. Plants were monitored for eggs at the edges of a patch or on isolated growing plants so that not all ~1000 plants were monitored. Leaves with *Pieris* eggs were collected and checked for the presence of a HR-like necrotic zone on the leaf. After collection, leaves with eggs were kept in Petri dishes in a climate chamber (25 ± 1 °C, 50–70 % RH, LD 16:8 h) until caterpillars emerged. All hatched and dead eggs were recorded.

Supplementary Methods S6: Phylogenetic tree construction of Brassicales

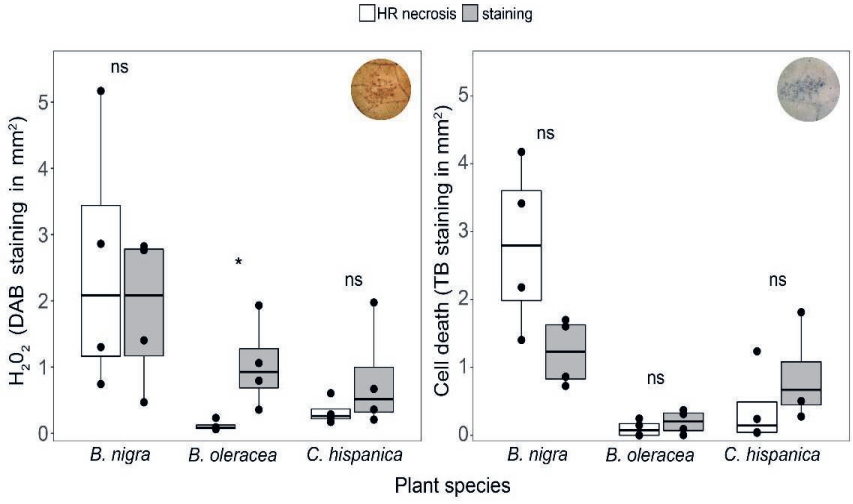
We used the established three-linage classification when planning and conducting our experiments. As some species and genera used in our experiment were not present in neither of the aforementioned studies, we established their relationships with other included species by calculating our own phylogenetic tree using DNA sequences of two chloroplast markers (*rbcL* and *matK*) and one nuclear genome marker (*ITS2*). The sequences were obtained from the BOLD system website (ID numbers see Supplementary Table S4) (Ratnasingham and Hebert 2007). The phylogenetic tree was inferred under maximum likelihood using RaxML v 8.2.4 (GTR+GAMMA, random seed and 1000 bootstrap pseudo-replicates) on the CIPRES science gateway (Miller *et al.* 2010, Stamatakis 2014). The three Cleomaceae species were used as outgroups for the phylogenetic tree.

Supplementary figures

Supplementary figures are also available online at https://doi.org/10.1111/nph.17145.



Supplementary Figure S1. Leaves from B. nigra treated with egg wash of different butterfly species and controls inducing or not a HR-like necrosis. *Pieris brassicae* (*P. b.*), *P. mannii*, (*P. m.*), *P. napi* (*P. n.*), and *P. rapae* (*P. r.*) and *A. cardamines* (*A. c.*) induce a strong HR-like necrosis. Egg wash of *G. rhamni* (*G. r.*) and *Colias* sp. (*C.* sp.) induces a very faint response resembling a chlorosis that does not fit into the established scoring system. The control (buffer without eggs) does not elicit a HR-like necrosis. All egg washes had the same concentration (200 eggs per ml) and amount applied onto the leaf (5µl). Two leaves were needed as not all egg washes were available at the same time. A) and C) Abaxial side of the leaf where the egg washes were applied onto. B) and D) Adaxial side of the leaf showing how strong the HR-like response is on the side which was not treated with egg wash.



Supplementary Figure S2. Quantification of HR necrosis and histochemical stainingof reactive oxygen species and cell death in *B. nigra*, *B. oleracea* and *C. hispanica* leaves upon oviposition of *P. brassicae* egg clutches. A) H_2O_2 by DAB staining, b) cell death by TB staining. A representative picture of the staining is presented on the top right corner of each graph. Boxplots depicts 1st, 3th quantile and median, each dot represents a plant treated with an egg clutch (10 eggs). For each plant species *N* = 4 plants were used for both experiments. Asterisks denote statistically significant difference between HR necrosis and histochemical staining (Student`s t-test, ns: not significant, * P<0.05).

Supplementary tables

Supplementary tables are available online at https://doi.org/10.1111/nph.17145.

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

Family	Plant species	Genotypes (*N*)	Genotypes	Source(s)	Life history
Brassicaceae	*Aethionema arabicum*	7	84-56-1; 84-56-2; 84-58; Cyp; KE3873; KE3926OT-01-2; Tur	Mohammadin *et al.* (2015)	annual, long day
	Aethionema carneum	1	KM220087	Mohammadin *et al.* (2018)	annual, long day
	Arabidopsis thaliana	1	Col-0		annual, long day
	Arabis alpina	9	ARA; ARAB1; ARAB2; ARAB3; DOR; PAJ; STY; TOT;WCA	Albani *et al.* (2012)	perennial, short day/vernalization
	Berteroa incana	1	Wild	Erik Poelman Laboratory of Entomology, WUR	annual

Supplementary Table S1. Continued.

Brassicaceae	*Boechra stricta*	1	LTM2	Michael Eric Schranz, Biosystematics Group, WUR	perennial, day neutral/vernalization
	Brassica napus	10	4113; 4115; BRA1679; CR185; CR3195; CR3197; CR578; CR671; CR807; CR993	IPK Gatersleben Genebank	annual, long day
	Brassica nigra	2	SF19; SF48	Erik Poelman Laboratory of Entomology, WUR	annual, long day
	Brassica oleracea	4	K34-3; OH30-3; 14027; W53-4	Busto-Segura *et al.* (2017)	perennial
	Brassica rapa	3	BRO-030; RC144; R-o-18	Guusje Bonnema, WUR Plant Breeding	annual, short day
	Cakile maritima	1	KM05-0093-10	Michael Eric Schranz, Department of Biosystematics, WUR	annual, long day

Supplementary Table S1. Continued.

Brassicaceae	*Capsella grandiflora*	1	CAPS3	IPK Gatersleben Genebank	annual, differential
	Capsella rubella	1	CAPS2	IPK Gatersleben Genebank	annual, differential
	Caulanthus amplexicaulus	1	CAB	Michael Eric Schranz, Biosystematics Group, WUR	annual
	Crambe hispanica	7	CR2569; CR2571; CR2572; CR2573; CR2574; CR2578; USDA388853	IPK Gatersleben Genebank	annual, long day
	Descurainia sophiades	5	BGH-01; DES1; DES2; DES3; tetraploid	Michael Eric Schranz, Biosystematics Group, WUR; IPK Gatersleben Genebank	annual
	Diptychocarpus strictus	1	05-0397-10-00	Michael Eric Schranz, Biosystematics Group, WUR	annual, long day?
	Eruca vesicaria	1	USDA-PI6333218	Michael Eric Schranz, Biosystematics Group, WUR	annual, long day

Supplementary Table S1. Continued.

Brassicaceae	*Euclidium syriacum*	1	GC-0587-68	Michael Eric Schranz, Biosystematics Group, WUR	annual, long day
	Iberis amara	2	IBE1; MK2005- 238	IPK Gatersleben Genebank; Michael Eric Schranz, Biosystematics Group, WUR	annual, long day
	Isatis tinctoria	1		Erik Poelman Laboratory of Entomology, WUR	annual, short day/vernalization
	Lepidium sativum	11	LEP40; LEP46; LEP51; LEP54; LEP56; LEP57; LEP61; LEP62; LEP63; LEP64; MK2003-169	IPK Gatersleben Genebank, Michael Eric Schranz, Biosystematics Group, WUR	annual, long day
	Lunaria annua	2	MK2006-705; Wild	Michael Eric Schranz, Biosystematics Group, WUR; Erik Poelman Laboratory of Entomology, WUR	annual, day neutral/vernalization
	Malcomia maritima	1	MK2005-111	Michael Eric Schranz, Biosystematics Group, WUR	annual, short day

Supplementary Table S1. Continued.

Brassicaceae	*Rorippa islandica*	1	GC2006-89	Michael Eric Schranz, Biosystematics Group, WUR	annual/perennial
	Sinapis alba	2	GC0560-79; Wild		annual, long day
	Sisymbrium irio	3	KM88-34-00-20-14; SIS16; SIS4	Michael Eric Schranz, Biosystematics Group, WUR, IPK Gatersleben Genebank	annual, short day
	Thlaspi arvense	1	GC1211-67	Michael Eric Schranz, Biosystematics Group, WUR	annual, long day
Cleomaceae	*Cleoma gynandra*	7	gyn100.1; GYN2; INC-06-015; ODS-15-002; TOT-8889; TOT8892; TOT8917	Biosystematics group WUR, Laboratory of Genetics WUR, Horticulture and Seed Science University of Abomey-Calavi (Benin), German Genebank, World Vegetable Center, Taiwan	annual, long day
	Cleoma violacea	1	JH-HB813	Michael Eric Schranz, Biosystematics Group, WUR	annual, long day
	Tarenaya hasslerian	1	1100	Michael Eric Schranz, Biosystematics Group, WUR	perennial, long day

Supplementary Table S2. Origin of natural and reared butterfly populations usedin the study.

Butterfly species	Population	Origin	GPS
Aglais io	PL	Butterfly farm in Babidół	
Anthocharis cardamines	FR	Ban-de-Laveline, Voges	48.2453°N, 7.0661°E
	FR	Gorges du Segre	42.4408°N, 2.0803°E
	PL	Butterfly farm in Babidół	
Aporia crataegi	UK	Worldwide Butterflies	
Colias sp.	FR	Montgenevre, Alpes	44.5533°N, 6.4130°E
Gonepteryx rhamni	NL	North-East Wageningen	51.9864°N, 5.6797°E
Leptidea sinapis	SE	Fiby	59.5408°N, 17.2131°E
Mamestra brassicae	NL	Laboratory of Entomology, Wageningen University	
Pieris brassicae	NL	Laboratory of Entomology, Wageningen University	
	PL	Butterfly farm in Babidół	
Pieris napi	NL	River Rhine, Wageningen	51.9607°N, 5.6799°E
	FR	Estagel, Pyrenees-Oriental	42.7724°N, 2.6996°E
Pieris rapae	NL	River Rhine, Wageningen	51.9607°N, 5.6799°E
	FR	Estagel, Pyrenees-Oriental	42.7724°N, 2.6996°E
Pieris mannii	NL	Wageningen	51.9705°N, 5.6766°E
Plutella xylostella	NL	Laboratory of Entomology, Wageningen University	

Supplementary Table S3. Primer sequences and annealing temperature used in *PR1* real-time qPCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')	Annealing Temperature (°C)
PR-1	CGCCGACGGACTAAGAGGCG	ACACCTCGCTTTGCCACATCCA	60
GAPDH	GGAGCTGCCAAGGCTGTCGG	CCTTCAGATTCCTCCTTGATAGCC	64
ACT-2	ACATTGTGCTCAGTGGTGGA	TCTGCTGGAATGTGCTGAGG	62

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

Species	Record number			Database
	ITS2	matK	rbcL	
Arabidopsis thaliana	SDH667-14	GBVE3079-11	GBVE3091-11	BOLDSystem
Arabis alpina	MKPCH717-10	GBVP2280-14	GBVP2280-14	BOLDSystem
Berteroa incana	WAT067-12	GBVT316-13	WAT067-12	BOLDSystem
Boechera stricta	BBYUK647-12	GBVE3157-11	BBYUK647-12	BOLDSystem
Brassica napus	SDH677-14	GBVR4446-13	GBVR4446-13	BOLDSystem
Brassica nigra	WAT161-12	GBVE3189-11	GBVE3188-11	BOLDSystem
Brassica oleracea	MKTRT2795-14	GBVE3190-11	GBVE3196-11	BOLDSystem
Brassica rapa	SDH679-14	GBVE3203-11	GBVX6649-15	BOLDSystem
Cakile maritima	ITSAP430-14	GBVT3310-13	GBVE3226-11	BOLDSystem
Capsella rubella	ITSAJ204-14	GBVE3245-11	GBVE3244-11	BOLDSystem
Capsella grandiflora		GBVE3236-11		BOLDSystem
Caulanthus amplexicaulus	ITSAP3272-14			BOLDSystem
Cleome gynandra	ITSAK4345-14	GBVE2267-11	UHURU294- 14	BOLDSystem
Cleome violacea	ITSAK4316-14	GBVS2993-13		BOLDSystem
Crambe hispanica		GBVT3315-13		BOLDSystem
Descurainia sophioides	BBYUK686-12	FCA174-09	BBYUK686-12	BOLDSystem
Diptychocarpus strictus	ITSAJ2329-14	GBVT283-13		BOLDSystem
Eruca vesicaria	PCCM726-14	GBVT3325-13	PCCM726-14	BOLDSystem
Euclidium syriacum	ITSAF1252-14			BOLDSystem

Supplementary Table S4. Continued.

Iberis amara	PCCM703-14	GBVE3336- 11	GBVE3335-11	BOLDSystem
Isatis tinctoria	PCCM740-14	GBVE3341- 11	GBVT4085-13	BOLDSystem
Lepidium sativum	HIMS329-12		GBVY2605- 14	BOLDSystem
Lunaria annua	MKTRT855-13	GBVS1366-13	GBVS934-13	BOLDSystem
Malcolmia maritima	AM905723.1			NCBI
Rorippa islandica		GBVE3455-11	FCA2965-11	BOLDSystem
Sinapis alba	SDH747-14	GBVE3462-11	GBVE3463-11	BOLDSystem
Sisymbrium irio	SDH750-14	GBVE3466-11	GBVE3467-11	BOLDSystem
Tarenaya hassleriana	SDH940-14	GBVP5560-15	SDH940-14	BOLDSystem
Thlaspi arvense		GBVE3505-11	GBVU4434-13	BOLDSystem
Aethionema arabicum				BOLDSystem
Aethionema carneum				BOLDSystem

Supplementary Table S5. Summary of HR-like necrosis induced by 13 P. brassicae egg wash in different Brassicaceae and Cleomaceae species. *N* indicates the number of treated plants for each 15 species; HR frequency indicates the portion of treated plants showing HR-like necrosis. The 16 order is alphabetical and does not reflect species relationships. $\chi^2$and P-values were 17 generated by Chi-square tests (P < 0.05 in bold).

Plant species	Genotype	*N* treated	HR fraction	χ^2	*P*
Aethionema arabicum	84-56-1	6	0.33	0.6	0.44
	84-56-2	10	0.5	4.27	**0.04**
	84-58	15	0.6	10.16	**0.001**
	Cyp	13	0.31	2.66	0.1
	KE3873	5	0	0	
	KE3926OT-01	6	0.33	0.6	0.44
	Tur	6	0.17	0	
Aethionema carneum		3	1	2.67	0.1
Arabidopsis thaliana	Col-0	16	0	0	
Arabis alpina	ARA	9	0.11	0	
	ARAB1	14	0.14	0.54	0.46
	ARAB2	15	0.07	0	
	ARAB3	13	0.08	0	
	DOR	19	0	0	
	PAJ	20	0	0	
	STY	16	0.1	0.002	0.96
	TOT	16	0	0	
	WCA	20	0	0	
Bertero incana	--	16	0.07	0	
Boechra stricta	LTM2	10	0	0	
Brassica napus	4113	7	0.86	8.14	**0.004**
	4115	14	0.43	5.3	**0.02**
	BRA1679	17	0.76	17.93	**<0.001**
	CR185	12	0.25	1.52	0.22

Supplementary Table S5. Continued.

Brassica napus	CR3195	15	0.86	18.54	**<0.001**
	CR3197	16	0.44	6.58	**0.01**
	CR578	15	0.87	19.55	**<0.001**
	CR671	13	0.54	7.04	**0.008**
	CR807	9	0.56	4.43	**0.035**
	CR993	11	0.42	4.04	**0.04**
Brassica nigra	SF19	8	0.63	2.4	0.12
	SF48	6	0.83	5.49	**0.02**
Brassica oleracea	K34-3	14	0.38	4.31	**0.04**
	OH30-3	16	0.375	5.13	**0.02**
	14027	10	0.4	2.81	0.09
	W53-4	15	0.2	1.48	0.22
Brassica rapa	RC144	6	0.17	0	
	RO18	13	0.15	0.54	0.46
Cakile maritima	KM05-0093-10	3	0	0	
Capsella grandiflora	CAPS3	18	0	0	
Capsella rubella	CAPS2	11	0.09	0	
Caulanthus amplexicaulus	CAB	3	0.33	0	
Cleome gynandra	gyn100.1	12	0.25	1.52	0.22
	GYN2	9	0.11	0	
	INC-06-01	4	0.5	0.67	0.41
	ODS-15-002	11	0	0	
	TOT-8889	6	0	0	
	TOT-8892	14	0.08	0.001	0.97
	TOT-8917	10	0	0	
Cleome violaceae	JH-HB813	13	0.07	0.001	0.97
Crambe hispanica	CR2569	13	0.85	15.76	**<0.001**
	CR2571	7	0.86	7.29	**0.007**
	CR2572	2	0	0	
	CR2573	14	0.57	8.58	**0.003**
	CR2574	15	0.87	20.46	**<0.001**
	CR2578	14	0.77	13.16	**<0.001**

Supplementary Table S5. Continued.

Descurainia sophiades	BGH-01	15	0	0	
	DES1	16	0	0	
	DES2	6	0	0	
	DES3	16	0.06	0	
Descurainia tetraploid	--	16	0	0	
Diptychocarpus strictus	05-0397-10-00	8	0	0	
Eruca vesicaria	USDA-PI6333218	4	0.25	0	
Euclidium syriacum	GC-0587-68	15	0	0	
Iberis amara	IBE1	13	0	0	
	IBE11	8	0	0	
Isatis tinctoria	--	15	0.07	0	
Lepidium sativum	LEP40	15	0	0	
	LEP46	13	0	0	
	LEP51	16	0.06	0	
	LEP54	13	0	0	
	LEP56	15	0	0	
	LEP57	15	0	0	
	LEP61	14	0.06	0	
	LEP62	14	0	0	
	LEP63	15	0	0	
	LEP64	16	0	0	
	MK2003-169	14	0	0	
Lunaria annua	Wild	16	0.29	3.75	0.05
	MK2006-705	5	0.4	0.63	0.43
Malcomia maritima	MK2005-111	14	0.07	0	
Rorippa islandica	GC2006-89	16	0.1	0	
Sinapis alba	Wild	16	0	0	
	GC0560-79	13	0	0	
Sisymbrium irio	KM88-34-00-20-14	8	0	0	
	SIS16	11	0.09	0	
	SIS4	8	0	0	
Tarenaya hassleriana	1100	4	0	0	
Thlaspi arvense	*GC1211-67*	16	0.13	0.53	0.47

Supplementary Table S6. HR- like necrosis (score ranging from 0 to 3) expressed by *B. nigra* plants elicited by different butterfly species. Plants in which the eggs or egg wash tested did induce a HR-like necrosis (Plants HR+) and plants in which they did not (Plants HR-) are counted Different letters indicate significant differences (different when $P < 0.025$) between butterfly species, Dunn-test, Bonferroni Holm corrected.

Butterfly species	HR severity [mean (SE)]	Plants HR+	Plants HR-	HR frequency mean (SE)
Aglais io	0 (0) b	0	40	0 (0) b
Anthocharis cardamines	1.63 (0.10) a	61	5	0.92 (0.03) a
Colias spp.	0.67 (0.10) ab	4	5	0.56 (0.18) a
Gonepteryx rhamni	1.11 (0.33) a	8	10	0.44 (0.12) c
Pieris brassicae	1.69 (0.13) a	53	12	0.82 (0.05) a
Pieris mannii	2.14 (0.40) ac	6	1	0.86 (0.14) ac
Pieris napi	2.46 (0.16) c	33	4	0.89 (0.05) a
Pieris rapae	1.64 (0.15) a	42	14	0.75 (0.06) ac

Supplementary Table S7. HR- like necrosis (score ranging from 0 to 3) expressed by *B. nigra* plants elicited by different butterfly and moth species. Shown are mean score and standard error. Letters denote differences in HR severity (Wilcoxon, $p<0.05$). Number of plants on which the eggs or egg wash was induced a HR-like necrosis (Plants HR+) and plants in which they did not (Plants HR-) were counted and HR frequency calculated. Different letters indicate significant differences between species (EMMEANS test).

Species	HR severity [mean (SE)]	Plants HR+	Plants HR-	HR frequency
Pieris rapae	2.88 (0.01) a	18	0	1.00 a
Pieris mannii	2.95 (0.01) a	20	0	1.00 a
Aporia crataegi	1.10 (0.05) bc	11	9	0.55 ab
Anthocaris cardamines	1.55 (0.04) b	13	7	0.65 ab
Gonepteryx rhamni	0.31 (0.03) d	2	17	0.11 b
Colias spp.	0.60 (0.04) cd	6	14	0.30 b
Leptidea sinapis	0.70 (0.10) bcd	2	8	0.20 b
Mamestra brassicae	0.36 (0.04) d	3	16	0.16 b
Plutella xylostella	0.00 (0.00) d	0	6	0.00 b
Control (MES buffer)	0.21 (0.03) d	2	19	0.10 b

Supplementary Table S8. Relative expression of *PR1* gene 34 upon treatment with egg wash of different 35 butterflies/moths and MES buffer as control. *N* indicates biological replicates (average of 4 pooled 36 plants), PR1 relative expression is represented by median + SE. Letters denote differences in mean 37 transcript levels between different treatments (Kruskal-Wallis rank sum test followed by Dunn`s 38 multiple comparison test, $P<0.05$, performed on log-transformed data).

Species	*N*	*PR1* relative expression mean ± SE
P. rapae	5	1229.3 ± 159.5 (a)
P. mannii	4	2134.2 ± 239.1 (a)
A. crataegi	5	27.9 ± 9.3 (bc)
A. cardamines	5	328.9 ± 51.6 (b)
G. rhamni	5	5.86 ± 12.6 (c)
Colias spp.	4	19.44 ± 7.6 (cd)
M. brassicae	5	5.04 ± 0.9 (d)
P. xylostella	4	15.6 ± 7.3 (d)
Control (MES buffer)	5	1.1 ± 0.39 (e)

Supplementary 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).

Experiment	Species	N	HR-like necrotic area in mm^2 [mean ± (SE)]
ROS (Figure S2a)	*B. nigra*	4	2.52 ± 0.99 (a)
	B. oleracea	4	0.11 ± 0.08 (b)
	C. hispanica	4	0.32 ± 0.10 (b)
Cell death (Figure S2b)	*B. nigra*	4	2.79 ± 0.62 (a)
	B. oleracea	4	0.10 ± 0.06 (b)
	C. hispanica	4	0.39 ± 0.29 (b)

Chapter 3

Hypersensitive response of *Brassica* plants against cabbage white butterfly eggs is specifically induced by egg-associated secretions

Niccolò Bassetti1[*], Lotte Caarls[1,2*], Femke van Doesburg[1], Martijn Flipsen[1], Ewan van Eijden[1], Patrick Verbaarschot[1], Klaas Bouwmeester[1,3], Nina E. Fatouros[1]

[1] Biosystematics Group, Wageningen University & Research, Wageningen, The Netherlands
[2] Laboratory of Plant Breeding, Wageningen University & Research, Wageningen, The Netherlands
[3] Laboratory of Entomology, Wageningen University & Research, Wageningen, The Netherlands
[*] These authors contributed equally to this work

Abstract

Our understanding of cell death in plant immunity remains incomplete. This is especially true for cell death developed in response to herbivorous insects and in particular to their eggs. Here, we characterize at cellular and molecular level a few plant immunity responses associated with a hypersensitive response (HR)-like cell death induced by butterfly eggs in Brassicaceae plants. Specifically, we compared two lepidopteran species, *Pieris brassicae* and *Mamestra brassicae* and two host plants, *Brassica nigra* and *B. rapa*. We show that the macroscopic HR-like cell death is preceded by an accumulation of ROS, callose deposition, cell death, ethylene induction and *PR1* gene expression in both plant species. These plant immunity responses are specifically induced in response to eggs of *P. brassicae*, which are specialist herbivores on Brassicaceae, while they are absent under eggs of *M. brassicae*, a generalist moth. We show that secretions surrounding *Pieris* eggs are sufficient to induce plant immune responses and cell death, unlike the previously described egg elicitor phosphatidylcholines (PCs). Finally, we investigated the specificity of plant intraspecific variation in egg-induced cell death. We show that plant genetic variation in egg-induced cell death is independent from canonical HR as a *B. nigra* accession not displaying a HR-like cell death is still able to develop HR when challenged with pathogenic bacteria and fungi. At molecular level, a *B. nigra* accession developing a macroscopic cell death show an early and more sustained induction of SA-related defense genes compared to the accession lacking cell death, while ROS and cell death are instead equally present regardless of plant intraspecific variation in macroscopic HR-like cell death. Our study is the first one to decipher an insect egg-induced cell death at the cellular and molecular level in the butterflies' natural hosts. A further identification of genetic and molecular components of plant immunity is needed to understand to which extent plants make use of the plant immune system to recognize eggs and anticipate insect attack.

Keywords
Plant-insect interactions, hypersensitive response, egg-associated molecular patterns, oviposition-induced plant responses, *Pieris*, Brassicaceae, plant immune system

Introduction

Plants survival relies on an innate immune system that regulates the perception of attackers and the subsequent activation of inducible defenses (Wilkinson *et al.* 2019). Perception of attackers is mediated by pattern recognition receptors (PRRs) on the surface of plants cells, and/or intracellular nucleotide-binding leucine-rich-repeat (NLRs) receptors (Couto & Zipfel 2016, van der Burgh and Joosten 2019). Generally, PRRs detect molecular patterns (elicitors) that are conserved across different organisms, such as of microbes (i.e. MAMPs) or of herbivores (i.e. HAMPs) (Cook, Mesarich and Thomma 2015, Gust, Pruitt and Nürnberger 2017, Stahl, Hilfiker and Reymond 2018, Steinbrenner *et al.* 2020), while NLRs detect effectors that are specific for a pathogen or insect (Jones & Dangl 2016). Activation of the immune receptors determines an early signalling cascade including rapid ion-flux changes and/or production of reactive oxygen species (ROS) (Boller & Felix 2009, Couto & Zipfel 2016). Subsequently, a transcriptional reprogramming and accumulation of the phytohormones jasmonic acid (JA), salicylic acid (SA) and ethylene link early signalling to plant defense activation (Pieterse *et al.* 2012, Erb & Reymond 2019). Induced plant defenses include reinforcement of extracellular barriers, for example by callose deposition, production of antimicrobial or insecticidal proteins and/or metabolites, or a localized cell death response, in some cases in the form of a hypersensitive response (HR) (Cui *et al.* 2015, Couto & Zipfel 2016, Mukhtar *et al.* 2016, Campos *et al.* 2018, Stahl *et al.* 2018, Balint-Kurti 2019). Besides the HR described in pathosystems, cell death responses to insect eggs are also known but their mechanism remains largely unsolved so far (Shapiro & DeVay 1987, Balbyshev & Lorenzen 1997).

A visible macroscopic cell death was first observed underneath eggs of cabbage white butterflies (*Pieris* spp.) deposited on leaves of the black mustard, *Brassica nigra* L. (Shapiro & DeVay 1987). Depending on the strength and type of egg deposition, it can result in egg-killing by desiccating or dropping off the eggs (Griese *et al.* 2017, Fatouros *et al.* 2014). Insect eggs oviposited on leaves represent a direct threat for plants as voracious larvae hatch from eggs and therefore plants evolved sophisticated defense mechanisms directly targeting eggs (Hilker & Fatouros 2015, 2016). As this egg-induced cell death resembles a pathogen-induced cell death, it was then called a “HR-like” cell death (Fatouros *et al.* 2012). So far, it is not resolved whether HR-like cell death is also accompanied by the hallmarks of the HR that often characterizes plant-pathogens interactions.

From pioneering work on *A. thaliana,* we know that a general immune response is triggered by eggs and considerably differs at transcriptional level from the response against feeding larvae (Nallu *et al.* 2018, Little *et al.* 2007). The response against eggs seem to rather share features of a pathogen-triggered immunity (PTI), such as callose deposition, accumulation of ROS, cell death, accumulation of hormone SA, and transcriptome changes of many defense-related genes (Little *et al.* 2007, Bruessow *et al.* 2010, Gouhier-Darimont *et al.* 2013, Reymond 2013, Lortzing *et al.* 2019). The signalling of *Pieris* egg-induced plant defences is mainly dependent on SA accumulation in *A. thaliana* (Gouhier-Darimont *et al.* 2013, Valsamakis *et al.* 2020). It has also been shown that *P. brassicae* eggs can induce a systemic acquired resistance (SAR) against the foliar pathogen *Pseudomonas syringae* (Hilfiker *et al.* 2014, Orlovskis & Reymond 2020). All these studies were performed, however, using the *A. thaliana* Col-0, which shows a weak macroscopic HR-like cell death (Little *et al.* 2007, Gouhier-Darimont *et al.* 2013). In *B. nigra*, so far only the expression of *PR1* and accumulation of SA were measured (Fatouros *et al.* 2014, 2015, Bonnet *et al.* 2017), but no other plant immunity responses have been studied so far. Thus, it is not known the extent and the timing with which plant immune responses are accompanying the development of HR-like cell death in brassicaceous species that are natural hosts of *Pieris* spp., such as *B. nigra* and *B. rapa*.

Intraspecific variation in frequency (proportion of plants) and severity of the HR-like cell death has been observed in all *Brassica* spp. that were studied until now (Pashalidou, *et al.* 2015a, Fatouros *et al.* 2014, Griese *et al.* 2017, 2021, Bassetti *et al.* 2021) and *A. thaliana* (Groux *et al.* 2021b). Further, we also found interspecific variation HR-like cell death in several other species of the Brassicaceae family (Chapter 2, Griese *et al.* 2021). Overall, there is natural variation in the cell death induced by *P. brassicae* eggs between different accessions (Griese *et al.* 2021, Chapter 2), but a link between visible macroscopic cell death and molecular plant defence responses is still missing. Whether the variation in cell death is also accompanied by intraspecific variation in PTI responses such as accumulation of ROS cell death, gene expression still needs to be explored.

In a recent study, we suggest that this arms-race has led to the evolution of the HR-like cell death in Brassicaceae family, as an egg-killing trait specifically targeting eggs from brassicaceous-specialist *Pieris* and *Anthocharis* butterfly species (Chapter 2, Griese *et al.* 2021). Eggs from non brassicaceous-feeding Pierinae butterflies and brassicaceous-feeding moths *Mamestra brassicae* and *Plutella xylostella* moths (Lepidoptera: Pieridae) hardly induce *PR1* expression and no macroscopic cell death (Chapter 2, Griese *et al.* 2021). On the other

hand, egg extracts of the generalist herbivores *Spodoptera littoralis* and *Drosophila melanogaster* were shown to induce the expression of PTI marker genes in *A. thaliana* (Bruessow *et al.* 2010). Hence, it is intriguing whether moth eggs are also accompanied by PTI responses despite not inducing a macroscopic cell death in *Brassica* spp.

Given the wide array of plant inducible defenses targeting eggs (Reymond 2013), a plant perception of egg-associated molecular patterns (EAMPs) has been postulated to initiate plant-egg molecular interactions (Erb *et al.* 2012). Eggs of *Pieris* spp. are enveloped by secretions originating from the female's accessory reproductive glands (ARGs) that form a glue-like structure between the eggs and the leaves of *Brassica* spp. host plants (Beament and Lal 1957, Fatouros *et al.* 2012). Treatment of *Brassica* plants with an extract of ARGs induced cell death, the release of oviposition-induced plant volatiles (OIPVs) attracting *Trichogramma* spp. egg parasitoid wasps, and primed plants for future larval feeding (Fatouros *et al.* 2008, 2009, 2015, Paniagua Voirol *et al.* 2020). In *A. thaliana*, the lipid fraction of *P. brassicae* eggs extract induced accumulation of ROS, cell death and PR1 expression (Gouhier-Darimont *et al.* 2013). The lipidic elicitor of these responses was recently identified as phosphatidylcholines (PCs), a major component of cell membranes thus acting as EAMP (Stahl *et al.* 2020). Although *Pieris* egg extract can induce a macroscopically visible cell death in some *A. thaliana* accessions (Groux *et al.* 2021b), direct application of PCs has not been associated yet with this cell death. Thus, it is still unclear whether PCs that are the egg elicitors responsible for the HR-like cell death.

In this study, we assessed the histological and molecular responses that characterize HR-like cell death induced by eggs of the specialist *P. brassicae* by comparing two different *Brassica* spp. and different accessions within these plant species. We hypothesized that the HR-like cell death induced by eggs is specifically induced by *Pieris* eggs. More specifically, we studied (1) which plant immune responses were associated with HR-like cell death in both *B. nigra* and *B. rapa*, (2) whether the responses were part of a conserved response to insect eggs by comparing eggs of the specialist *P. brassicae* to eggs of the generalist *M. brassicae,* (3) whether PCs induce cell death similar to egg-associated secretions, (4) whether variation in HR-like cell death was specific to *Pieris* eggs irrespective of the ability to induce canonical HR against pathogens and (5) whether this variation was correlated with ROS, cell death and induction of SA- and JA-related defence genes.

Material and methods

Plant material and rearing of butterflies

Brassica nigra L. accessions SF48-O1 and DG1-S1 originated from plants that were collected from a local population in the floodplain of the Rhine River next to Wageningen (51°57'38.6"N 5°40'45.3"E), The Netherlands. *Brassica rapa* L. genotypes L58, R-o-18, and RC-144 were obtained from Dr. Guusje Bonnema, Laboratory of Plant Breeding (WUR). Plants were grown in a greenhouse (18 ± 5 °C, RH 50–70%, LD 16:8 h) and were used when four 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, RH 50–70 %, LD 16:8 h). Upon eclosion of the adults, twenty females and males could mate in a large cage (60 x 60 x 90 cm) and females were used for oviposition in experiments or oviposition on paper or leaves for egg wash production. The cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) was reared on Brussels sprouts plants in a climate room (21 ± 1 °C, RH 50–70%, LD 16:8 h) while adults oviposited on paper sheets.

Preparation of egg washes

Pieris brassicae egg wash used for the characterization of plant immunity responses was prepared from one day old eggs oviposited on a filter paper pinned underneath a *B. oleracea* cv. Cyrus leaf (Caarls *et al.* 2021). Pieces of filter paper containing egg clutches were incubated overnight at room temperature in 20 mM MES buffer (pH 5.7) at a concentration of 400 eggs/ml. *Mamestra brassicae* egg wash was prepared with the same procedure using eggs laid on paper sheets. Filter paper without eggs was treated in a similar manner and used as control. *Pieris brassicae* egg wash used for the screening of *B. nigra* accessions and for the comparison with pathogen extracts was prepared with one day old *P. brassicae* egg clutches oviposited directly on *B. oleracea* cv. Cyrus leaves. Eggs were carefully removed with a stainless-steel lab spatula and then incubated overnight at room temperature in an Eppendorf tube with 1 ml demineralised water every 0.02 g of eggs (~1000-1200 eggs/ml). A leaf without eggs was treated in a similar manner and used as control. After overnight incubation, the liquid phase of egg washes was pipetted off and stored at -20 °C until use.

Preparation of fungal and bacterial extracts

The fungal pathogens *Rhizoctonia solani* and *Alternaria brassicicola* isolate MUCL2097 (*Ab*), were cultured on Potato Dextrose agar (PDA) plates at 25 °C. To prepare fungal extracts, as a mycelium covered a whole Petri dish (Ø 100 mm), it was cut into mycelial plugs (1 cm^2), inoculated into 100 ml Potato Dextrose medium and incubated at 25 °C and 200 rpm. Mycelium was harvested from liquid cultures after 1.5 weeks, dried from excess medium and placed into a 50 ml tube containing 5 ml demineralised water. All tubes were kept on ice and ultrasonicated 6 times for 1 minute at maximum amplitude, using an ultrasonication probe. After a centrifugation step of 5 min at 4000 rpm, the supernatant was aliquoted in Eppendorf tubes and stored at -20 °C until use.

The bacterial pathogen *Xanthomonas campestris* pv. *campestris* race 4 (*Xcc*) was maintained on yeast extract-dextrose-carbonate agar (YDCA) plates at 28 °C. To prepare bacterial extract, *Xcc* was grown overnight in 15 ml liquid LB medium at 28 °C and 200 rpm. Cultures of *Xcc* at OD_{600} of 0.6 - 0.7 were centrifuged for 10 min at 3000 rpm, resuspended in a minimal amount of demineralised water and ultrasonicated as described above. Tubes were then centrifuged again for 10 min at 3000 rpm and the supernatant was aliquoted in Eppendorf tubes and stored at -20 °C until use.

Plant treatments with eggs, egg wash and pathogen extracts

For oviposition experiments, each *B. nigra* or *B. rapa* plant was placed in a cage with one *P. brassicae* butterfly to receive one egg clutch and then was substituted by a new plant. HR-like cell death was scored four days after oviposition using a scoring system with discrete categories of severity symptoms from 0 to 4 (Supplementary Fig. S1). For oviposition with *M. brassicae*, female moths were placed together with a *B. nigra* plant in a cage to allow egg deposition overnight and the removed in the following morning. For all experiments with an egg wash treatment, 5-10 µl of egg wash was pipetted on the abaxial side of the fourth or fifth mature leaf of 3-5 weeks old plants. Symptoms induced by egg wash were scored four days after treatment with the scoring system described above. Pathogen extracts were applied by infiltration using a 1 ml syringe. Scoring of the damage induced by extracts was done on a scale from 0 to 4. For histochemical staining, *B. nigra* and *B. rapa* plants were oviposited by *P. brassicae* or *M. brassicae* and samples were taken 24, 48 and 72 h after oviposition by taking a leaf disc (Ø 10 mm) of the area surrounding the eggs or egg wash.

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 by an egg clutch of 10 eggs. Six leaf discs (Ø 6 mm) were sampled directly next to the eggs or the egg wash-treated spot at 0, 3, 6, 24 and 48 hours. For each timepoint, four plants were sampled individually and considered biological replicates. *B. rapa* plants were treated with three single eggs on a single leaf of each plant. Leaf discs (Ø 6 mm) were then harvested next to the eggs at 0, 3, 6, 24 and 96 hours 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 discs (Ø 6 mm) were sample directly next to the egg wash-treated spot 24 hours after treatment. Four plants were used for each treatment as biological replicates.

To compare the gene expression of two *B. nigra* accessions, egg wash at higher concentration (~1000 eggs/ml water) was used. Experimental design consisted of two treatments (egg wash, control), two *B. nigra* accessions (SF48-O1 and DG1-S1), and three time points after treatment (6, 24 and 48 hours 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 discs (Ø 6 mm) were harvested at each time point on the treatment spots and discs 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 quickly frozen in liquid nitrogen and stored at -80 °C until use.

Histochemical stainings

Accumulation of hydrogen peroxide (H_2O_2) was stained with 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, Mo, USA). Leaf discs (10 mm Ø) were submersed in 1 mg/ml DAB solution and samples were incubated for 30 to 60 minutes in the dark. Accumulation of superoxide radicals ($O_2^{\bullet-}$) was stained with nitroblue tetrazolium chloride (NBT, Sigma-Aldrich, Mo, USA). Leaf discs (10 mm Ø) were submersed in 2 ml 0.2% NBT and 50 mM sodium phosphate buffer (pH 7.5) and samples were incubated 30 to 60 minutes in the dark. To visualize cell death, leaves were stained overnight with 0.1% trypan blue (Sigma-Aldrich,

Mo, USA) in lactophenol at room temperature. Destaining of leaves was performed with 96% ethanol. For callose staining, leaf discs (10 mm Ø) were destained with 96% ethanol and then stained with 0.01% aniline blue (Sigma-Aldrich, Mo, USA) in 150 mM K_2HPO_4 for two hours. Callose deposits were visualized using a Nikon 90i epifluorescence microscope using a DAPI filter and a digital DS-5MC camera. Pictures of leaf discs before staining (with eggs) and after staining (without eggs) were taken with a Dino-Lite digital microscope (AnMo Electronics Corporation, Taiwan).

Ethylene assay

Ethylene production was measured as previously described by Oome *et al.* (2014). Leaf discs (3 mm Ø) were sampled from fully developed leaves of untreated plants and incubated overnight in demineralised water. Subsequently, three leaf discs of each plant were incubated for five hours in either 400 µl of 20 mM MES pH 5.7 or 400 µl of the egg wash (400 eggs/ml in MES buffer). After incubation, 1 ml of the headspace of each sample was taken to measure ethylene on a Focus gas chromatograph (Thermo Electron, Italy) equipped with an FID detector and a RT-QPLOT column, 15 m × 0.53 mm ID (Restek, PA, USA). The system was calibrated with a certified gas of 1.01 µl/l (1 ppm) ethylene in synthetic air (Linde Gas Benelux B.V, The Netherlands). After sampling leaf discs for ethylene assay, plants were also treated with by *P. brassicae* egg wash to determine their HR-like cell death phenotype.

Gene expression by qRT-PCR

For experiments with plants and eggs, RNA extraction was performed according to Oñate-Sánchez and Vicente-Carbajosa (2008). For experiments comparing egg wash and pathogen extracts, RNA extraction was performed with Direct-zol RNA Miniprep Kit (Zymo Research, CA, USA) following manufacturer`s protocol. For preparation of cDNA of all experiments, 1µg 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 (Supplementary Table S1) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA). 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.

Statistical analysis

All data analysis was carried out in R (R Core Team 2021). The occurrence of HR-like cell death was analyzed with a generalized linear model (GLM) with a binomial error distribution. The response of plants to each treatment was considered as a binomial response: either non-HR (score 0 or 1) or HR (score 2, 3, and 4) and different treatments were included as categorical fixed factors. When overall differences were found, pairwise differences between factors were tested. HR severity was considered as the score of symptoms induced, and differences in mean HR severity were tested using Kruskal-Wallis test, followed by Wilcoxon Rank Sum test with Benjamini-Hochberg correction. For gene transcription data, ΔΔCq values were used for statistical analysis. To compare eggs and egg wash, data were analyzed with one-way ANOVA for the two treatments (eggs or egg wash) independently followed by Dunnett's test to compare all timepoints to the 0h timepoint. The differences between treatments were tested for each timepoint with Student's t-tests. To compare gene transcription data between plants treated with either *P. brassicae* or *M. brassicae*, ΔΔCq values were used for statistical analysis with one-way ANOVA, followed by Tukey post-hoc tests. Differences in mean ethylene produced (ppm) after treatments or between plants were tested using the Kruskal-Wallis test, followed by the Wilcoxon Rank Sum test with Benjamini-Hochberg correction.

Results

***Pieris* eggs trigger similar immune responses at cellular level in two *Brassica* species**

We assessed whether plant immunity responses may explain the difference in HR-like cell death severity between two Brassicaceae plant species, *B. nigra* and *B. rapa.* Overall, we observed in *B. nigra* SF48-O1 that development of a macroscopically visible HR-like cell death spreads beyond the egg site and it stops 96 h after oviposition (Fig. 1). In *B. rapa* accession L58, however, HR-like cell death appears as black necrotic spots but never spread beyond the egg site (Fig. 2). As cell death is generally preceded by production of reactive oxygen species (ROS) (Torres *et al.* 2010), we visualized ROS with different histochemical stainings. Superoxide anion ($O2^{\bullet-}$), a precursor of other ROS molecules stained by NBT staining, accumulated underneath eggs at 24 h in both *B. nigra* (Fig. 1A) and *B. rapa* (Fig. 2A). At the same time point, we also detected hydrogen peroxide (H_2O_2), a more stable ROS stained with DAB staining, under eggs deposited on leaves of both plant species. Next, we investigated the deposition of callose under eggs, which was shown to be associated with lesions following

pathogen invasion or autoimmune responses (Koga *et al.* 1988, Dietrich *et al.* 1994). Aniline blue staining in *B. nigra* revealed callose deposition underneath eggs at 24 h after oviposition (Fig. 1A). However, callose deposition was not investigated on *B. rapa*. Finally, the occurrence of cell death under the egg site was investigated by staining with trypan blue (TB), showing that cell death is already present 72 h after oviposition in both *B. nigra* (Fig. 1B) and *B. rapa* (Fig. 2B), thus preceding the macroscopic HR-like cell death. Occasionally, few TB-stained cells were visible at 48 h in both plant species (not shown). We further investigated the induction of *PR1* which is a marker gene of the SA-dependent defense pathway (Lefevere *et al.* 2020). In *B. nigra, PR1* expression increased at 6 h and was significantly upregulated at 24 h and 48 h after egg deposition (Kruskall-Wallis: $\chi^2_4 = 12.23$, P = 0.015) (Fig. 1D). Similarly, *PR1* expression was also significantly upregulated at 24 h in *B. rapa* (Kruskall-Wallis: $\chi^2_4 = 11.18$, P = 0.024) and showed further increase at 96 h (Fig. 2D).

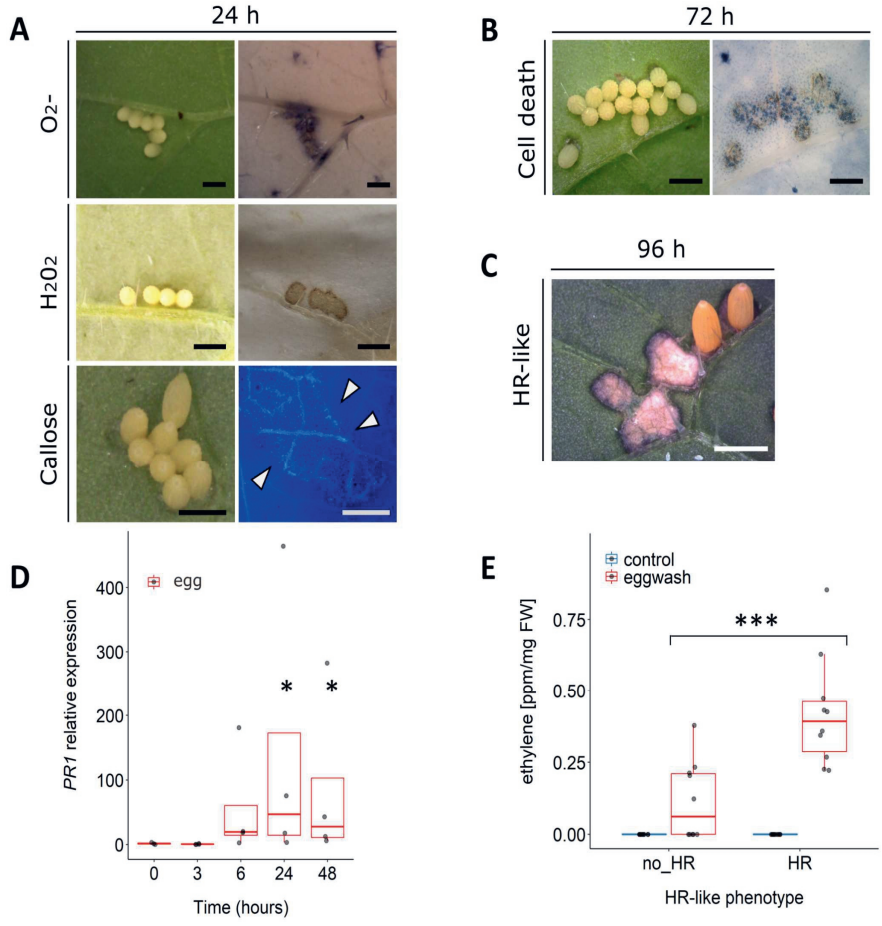


Figure 1. Plant immunity responses induced by *Pieris brassicae* eggs in *Brassica nigra* (*Bn*). A) *B. nigra* leaf 24 h after oviposition with visualized accumulation of O2•– (NBT staining), H_2O_2 (DAB staining) and callose deposition (aniline blue). B) *B. nigra* leaf 72 h after oviposition shows cell death (trypan blue staining). C) Fully developed HR-like cell death that is macroscopically visible at 96 h after oviposition. Few eggs were removed from the clutch to show the cell death underneath. Magnification bars = 1 mm. D) *BnPR1* relative gene expression underneath eggs. Each treatment consisted of four biological replicates, each including six leaf discs. Gene expression was measured by qRT-PCR and normalized to the housekeeping gene *BnGADPH*. Oviposited plants were compared to untreated plants at 0 h time point. E) Ethylene production in parts per million (ppm) in *B. nigra* plants. Ethylene of plants showing HR-like was compared to plants without HR-like. Height of the boxes represents the range between first and third quartile; the horizontal line within the box is the median; the whiskers indicate the data minimum and maximum; and dots represent outliers. Asterisks indicate differences in P-values: * <0.05, ** <0.01, *** <0.01, Student T-test.

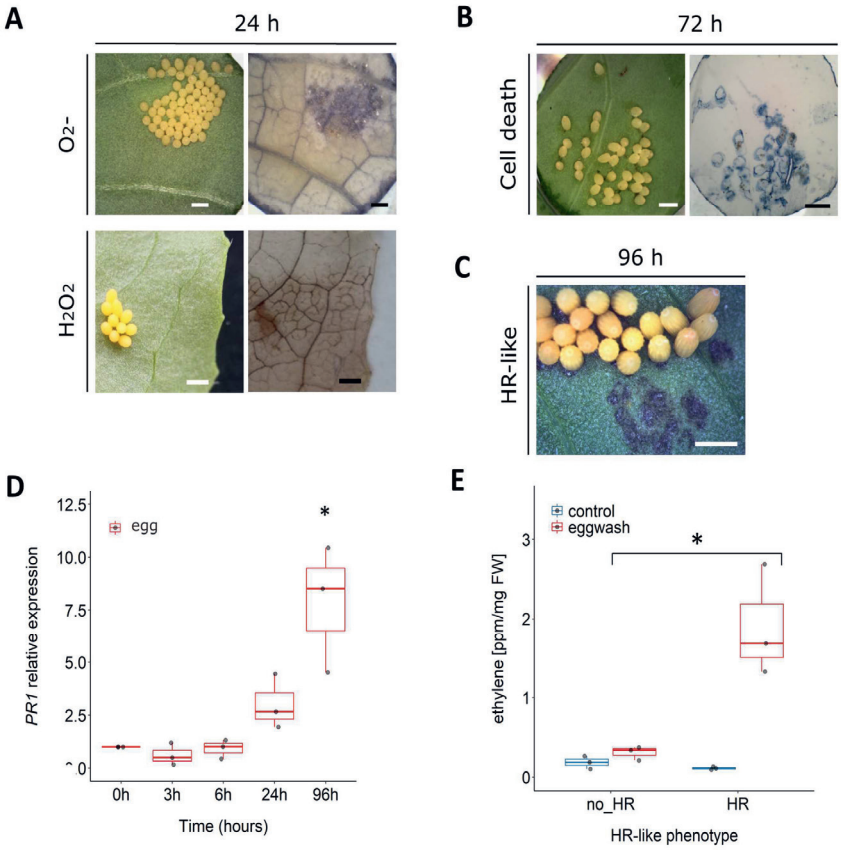


Figure 2. Cellular plant immunity responses induced by *Pieris brassicae* eggs in *Brassica rapa* (*Br*). A) *B. rapa* leaf 24 h after oviposition with accumulation of O2•− (NBT staining) and H2O2 (DAB staining). B) *B. rapa* leaf 72h after oviposition shows cell death (trypan blue staining). C) Fully developed HR-like cell death at 96 h after oviposition that is macroscopically visible. Magnification bars = 1 mm. D) *BrPR1* relative gene expression underneath single *P. brassicae* eggs. Each treatment consisted of three biological replicates, each including three leaf discs. Gene expression was measured by qRT-PCR and normalized on housekeeping gene *BrGADPH*. Oviposited plants were compared to untreated plants at 0 h time point. E) Ethylene production in parts per million (ppm) by *B. rapa* plants treated with egg wash. Height of the boxes represents the range between first and third quartile; the horizontal line within the box is the median; the whiskers indicate the data minimum and maximum; and dots represent outliers. Asterisks indicate significant differences, P-values: * <0.05, Student T-test.

Egg wash induced a plant immune response similar to eggs

Previously, we found that an "egg wash", that is a water-based suspension of the secretions surrounding freshly oviposited eggs, was sufficient to induce HR-like cell death (Chapter 2, Caarls *et al.* 2021, Griese *et al.* 2021). When applied to the abaxial leaf side of *B. nigra*, *P. brassicae* egg wash induced a HR-like cell death visually undistinguishable from the HR-like cell death induced by butterfly's eggs (Supplementary Fig. S1A). HR-like cell death

frequency, indicating the proportion of plants showing cell death, appeared slightly increased after egg wash treatment compared to eggs (GLM: $\chi^2 = 0.24$, df = 1, P = 0.62) (Supplementary Fig. S1B, Supplementary Table S2). Egg wash induced a slightly more severe cell death than eggs (Supplementary Fig. S2B, Supplementary Table S2), but the overall difference was not statistically relevant (Kruskal-Wallis: $H_1 = 3.44$, P = 0.06). We then measured the impact of egg wash at gene expression level. Similarly, to what observed with eggs, *PR1* expression was significantly upregulated at 24 h after egg wash treatment (Kruskall-Wallis: $\chi^2_4 = 10.01$, P = 0.041) (Fig. 3C, Supplementary Table S3). *PR1* upregulation was maintained at 48 h after treatment, although not significantly different than the initial time point. Overall, egg wash proved to be an effective egg-mimicking treatment as it induced similar visual and molecular responses.

Further, we tested whether egg wash could induce ethylene, a common assay used to characterize the induction of early plant immune response by biotic stresses (Fan *et al.* 2017). *Brassica nigra* leaves responded with ethylene production after incubation with egg wash for 5 h compared to incubation with control MES buffer (Fig. 1E, Supplementary Table S4). After the ethylene assay, plants were assayed for their HR-like cell death phenotype. We found that plants responding with a strong macroscopic HR-like cell death, produced a significantly higher amount of ethylene after incubation with egg wash than plants with no visible cell death (Student`s $T\text{-test}_{18} = -4.087$, P < 0.001). A similar effect was also observed in *B. rapa*, as treatment with egg wash of accession L58, which shows HR-like cell death, resulted in higher ethylene production compared to accession R-o-18 which does not show visible cell death (Student`s $T\text{-test}_4 = -3.876$, P = 0.018) (Fig. 2E).

HR-like cell death is induced by *P. brassicae* egg wash but not by phosphatidylcholines

As *Pieris* egg wash appeared sufficient to induce plant immune responses and HR-like cell death, we hypothesized that the elicitor of those responses may be a water-soluble compound. On the other side, phosphatidylcholines (PCs) derived from the egg lipidic fraction were recently identified as the elicitors of plant immune responses in *A. thaliana* (Stahl *et al.* 2020). The active PCs were mainly containing C16- and C18-fatty acyl chains (PC16:1/PC16:1 and PC18:1/PC18:1). Thus, we tested whether also phosphatidylcholines (PC) could induce similar 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 (Fig. 3).

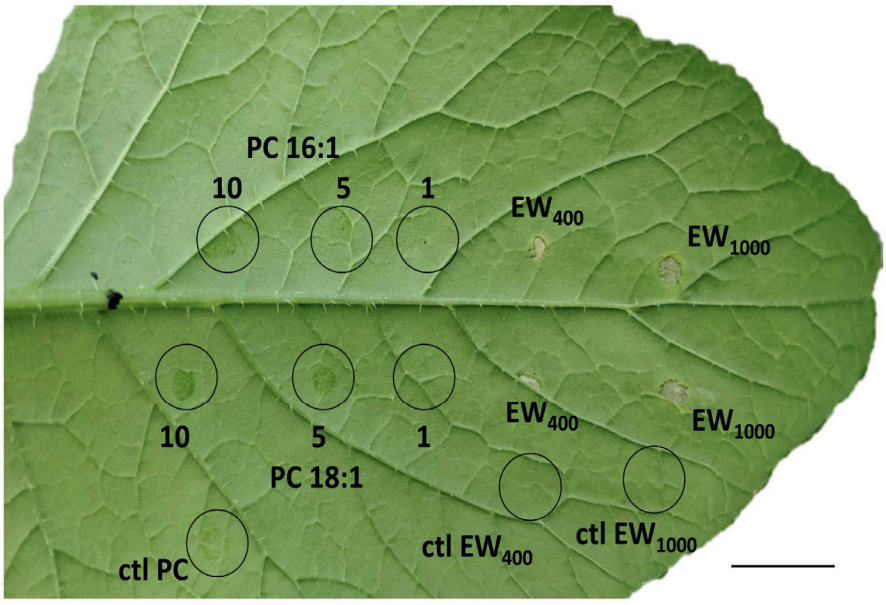


Figure 3. Induction of HR-like cell death by phosphatidylcholines (PC) compared to *P. brassicae* egg wash in *B. nigra* plants. *B. nigra* (SF48-O1) plants (N=15) were treated with phosphatidylcholines PC(16:1/16:1) and PC(18:1/18:1) or with egg wash. PCs were solubilized in 1% DMSO, 0.5% Glycerol and 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. Egg wash$_{400}$ was prepared with 400 eggs/ml, egg wash$_{1000}$ was prepared with 1000-1200 eggs/1 ml. All treatments were applied on the same leaf, with two leaves per plant. Magnification bar = 1 cm.

Moth eggs and egg wash only induce a weak plant immune response on *B. nigra*

We then investigated whether cellular and molecular responses observed in *B. nigra* are specific to eggs and egg wash of the specialist *P. brassicae* compared to eggs and egg wash of the generalist *M. brassicae*. Eggs of *M. brassicae* induced low O_2•– production, given a weak NBT staining on one single plant (Fig. 4A). Similarly, cell death visualized by TB staining was hardly visible (Fig. 4B). While *P. brassicae* egg wash induced the upregulation of *PR1* at 24 h (ANOVA followed by Tukey, $P < 0.001$), *PR1* induction by *M. brassicae* egg wash was comparable to the control treatment (Tukey, $P > 0.05$) (Fig. 4C, Supplementary Table S5). Incubation of leaf discs with *M. brassicae* egg wash induced a weak ethylene production compared to treatment with *P. brassicae* egg wash, with levels comparable to the ones previously observed in *B. nigra* plants lacking a visible cell death (Kruskal-Wallis: $H_2 = 14.87$, $P < 0.001$) (Fig. 4D). Overall, eggs of *M. brassicae* do not induce HR-like cell death and only a weak ROS response in *B. nigra*.

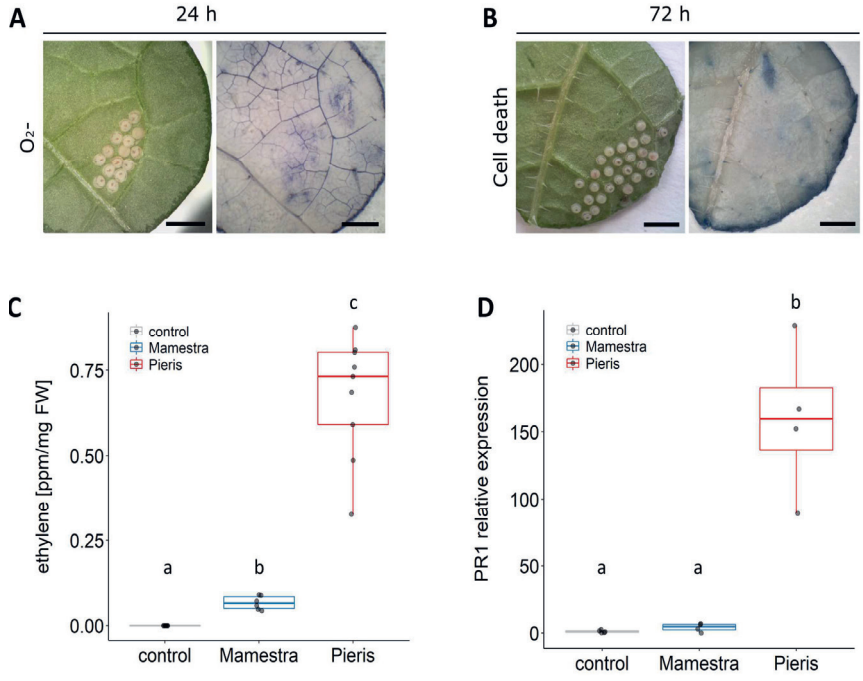


Figure 4. **Cellular and molecular responses induced by eggs and egg wash of *M. brassicae* in *B. nigra*.** A) Leaf of *B. nigra* oviposited on by *M. brassicae* eggs stained by NBT showing light O2•– accumulation underneath *M. brassicae* eggs. B) Trypan blue staining showed no cell death underneath eggs. C) PR1 expression in leaf tissue treated with control (MES buffer), *P. brassicae* wash or *M. brassicae* wash. Different letters indicate significant differences in mean PR1 expression (ANOVA followed by Tukey, $P < 0.001$). D) Ethylene production in *B. nigra* leaf in response to egg washes. Different letters indicate significant differences in mean production of ethylene (pairwise Wilcoxon test, $P < 0.01$). Magnification bar = 1 mm.

Comparison between HR-like cell death and cell death induced by other biotic stresses

Previously, we reported plant phenotypic variation for HR-like cell death in *B. nigra* and *B. rapa,* with accessions that either showed presence or absence of HR-like cell death (Griese *et al.* 2017, Bassetti *et al.* 2021). Here we investigated whether a *B. nigra* plant showing absence of egg-induced HR-like cell death was generally impaired in the ability to develop cell death against other biotic stresses. Thus, two *B. nigra* accessions that either consistently develop a strong HR-like response (SF48-O1) or no cell death at all (DG1-S1), were treated with egg wash and extracts of the Gram-negative hemibiotrophic bacterium *Xanthomonas campestris* pv. *campestris* (Xcc) and the necrotrophic fungi *Alternaria brassicicola* and *Rhizoctonia solani*. We found that DG1-S1, the accession unable to develop cell death upon egg wash, could still develop a cell death against pathogen extracts (Fig. 5). In other words,

egg wash induced different HR-like severity between the two accessions unlike the three pathogen extracts (Chi-square test, $P < 0.05$) (Fig. 5, Supplementary Fig. S2). Finally, we asked whether the difference responsiveness of *Brassica* spp. accessions correlated with variation in cell death at cellular level. Interestingly, TB staining revealed cell death on both *B. nigra* and *B. rapa* also in absence of a visible HR-like cell death (Supplementary Fig. S3).

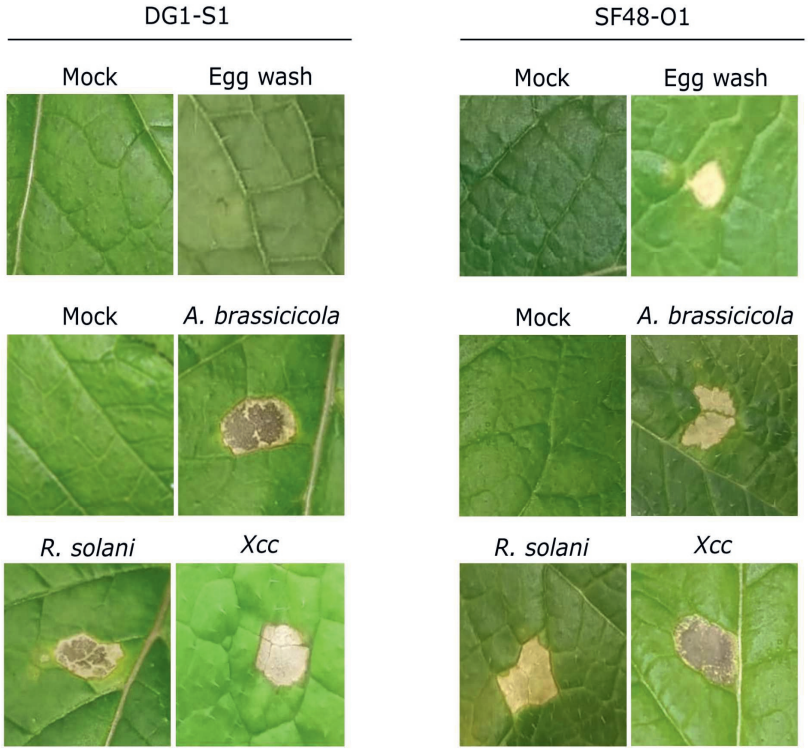


Figure 5. **Expression of HR-like cell death induced by *P. brassicae* egg wash and different pathogen extracts in two different *B. nigra* accessions.** Two *B. nigra* accessions showed difference in HR-like cell death phenotype. DG1-S1 (no HR-like) and SF48-O1 (HR-like). The two *B. nigra* accessions were also tested with pathogen extracts of *Alternaria brassicicola*, *Rhizoctonia solani* and *Xanthomonas campestris* pv. *campestris (Xcc)*. Egg wash was applied as droplets while pathogens extracts were infiltrated using a 1 ml syringe. Mock treatments were done with demineralized water. Lesions were scored and imaged 4 days after treatment.

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

We investigated whether the difference in HR responsiveness may be related to phytohormonal pathways. Thus, we quantified expression of marker genes induced by SA, such as *ICS1*, *PR1* and *PR2* (Little *et al.* 2007), and genes regulated by JA, such as *MYC2*, *VSP1* and *VSP2* (Reymond *et al.* 2004) in *B. nigra* accessions SF48-O1 and DG1-S1 across three different time points. Overall, all maker genes were upregulated upon egg wash treatment

compared to control. However, magnitude of expression of SA-related marker genes was significantly different between the two *B. nigra* accessions at different time points (Fig. 6). In contrast, 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 HR+ plants already at 6 h (ANOVA, $P < 0.01$). 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 HR+ plants (ANOVA, $P < 0.05$), and the difference with HR- plants 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 24 h in all treatments, but with no significant differences between the two accessions (ANOVA, $P > 0.05$).

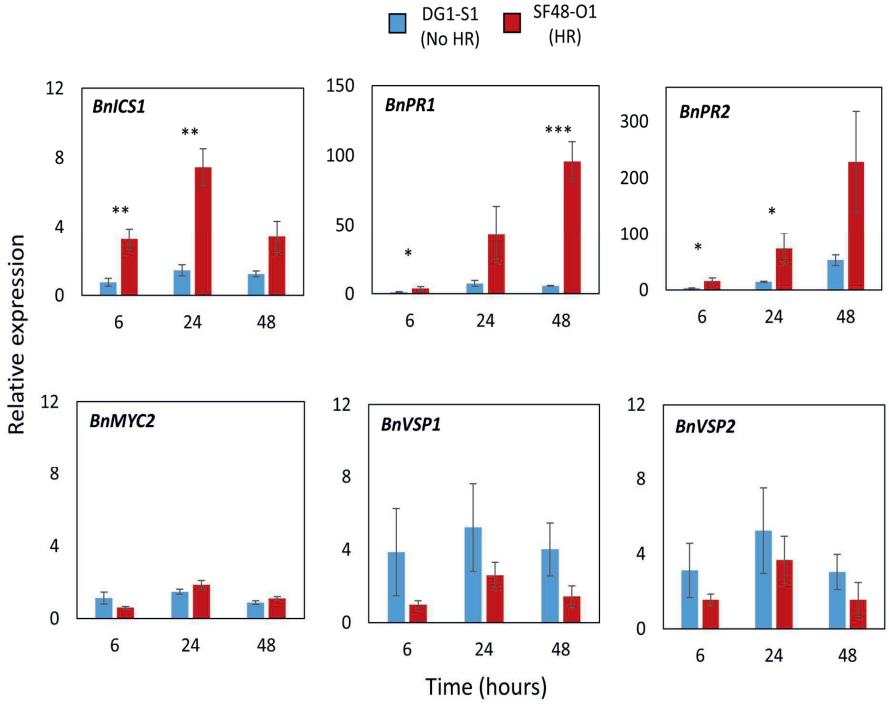


Figure 6. Expression of SA- and JA-related defense genes upon *P. brassicae* egg wash treatment in two different *B. nigra* accessions. Expression was measured with two treatments (egg wash, control), two accessions (DG1-S1, SF48-O1) and at three time points (6, 24 and 48 hours after treatment). Gene expression was measured by qPCR-RT and normalized to housekeeping gene *BnGADPH*. Expression levels are calculated relative to control treatment (water droplets). Means + SE of four biological replicates are shown. Asterisks indicate different P-values: * <0.05, ** <0.01, *** <0.001 (Dunn's multiple comparison test).

Discussion

In this study, we detected plant immune responses normally associated with pathogen-induced HR, such as production of ROS, callose deposition, cell death, and expression of SA-related defense genes, underneath eggs of *P. brassicae* in both its natural host plants, *B. nigra* and *B. rapa.* These immune responses are specifically induced by eggs and egg wash of the specialist *P. brassicae* and not by eggs of the generalist *M. brassicae*. Egg-induced HR-like cell death is specifically induced by egg-associated elicitors as *B. nigra* accession with no cell death can still develop a functional HR against pathogens. Moreover, *B. nigra* accessions developing a macroscopically visible cell death show an earlier and stronger upregulation of SA-related defense genes compared to plants lacking a visible cell death. Our results suggest that plants develop a general immune response in response to insect eggs while a visible HR-like cell death may be the result of genetic variation at plant immunity components that are specifically induced by *P. brassicae* eggs.

As a first objective, we assessed whether known early plant immunity responses are induced by *P. brassicae* eggs in both *B. nigra* and *B. rapa*. Histochemical stainings revealed that ROS accumulation, callose deposition and cell death anticipate the onset of the macroscopic HR-like cell death on leaves of both plant species. ROS and callose are present already 24 h after oviposition. Cell death is detectable 72 h after oviposition and occasionally already at 48 h. Previously, *P. brassicae* eggs laid on *A. thaliana* accession Col-0 were shown to induce ROS and cell death only at 72 h after oviposition, without developing a visible HR-like response (Little *et al.* 2007, Bruessow *et al.* 2010, Gouhier-Darimont *et al.* 2013). Further, we show that the upregulation of SA-related defense marker genes, such as *ICS1* which synthesizes the SA precursor isochorismate (Lefevere *et al.* 2020), and the pathogenesis-related genes *PR1* and *PR2* (Linthorst 1991), occurs already at 6 h after *P. brassicae* oviposition. So far, induction of *PR1* and *PR2* in *B. nigra* was reported only at 24 h and 72 h, respectively (Fatouros *et al.* 2014, 2015, Bonnet *et al.* 2017).

We then assessed the specificity of the response to *Pieris* eggs by comparing two *B. nigra* accessions which differ in their ability to develop HR-like cell death. We show that DG1-S1, a *B. nigra* accession lacking a visible HR-like cell death, is still able to induce a functional HR upon treatment with extracts of a pathogenic bacterium and two fungi. The phenotypic variation in egg-induced response between SF48 and DG1-S1 may then be associated with genetic variation at plant immunity genes that are specifically regulating the interaction between eggs and plants.

Currently, we do not know whether this variation regards genes involved in the perception of egg elicitors/EAMPs or rather genes involved in downstream signalling and/or defense genes. In this study, we only assess the differential expression of SA-related marker genes which are induced early and more strongly in *B. nigra* accession SF48. This may indicate that the development of a macroscopic cell death depends on a stronger induction of SA, a hypothesis that should be tested by measuring hormonal levels in both accessions. Nevertheless, it is likely that the ability to develop a macroscopic cell death in SF48 is also reliant on differential (quantitative) regulation of other cellular pathways, given the complex interplay of cellular processes that eventually lead to a HR (Mur *et al.* 2008). In the interaction between *S. dulcamara* and *S. exigua* eggs, plant variation in egg-induced chlorosis was associated with different levels of ROS but similar levels of SA hormone (Geuss *et al.* 2017). This corroborates the current knowledge that HR is not a monomorphic trait, but rather a general term to indicate a specific plant cell death with characteristic hallmarks that can vary across different plant-attacker interactions (Mur *et al.* 2008, Balint-Kurti 2019).

Future studies should also investigate the roles of the different components characterizing the plant immunity response against *Pieris* eggs. The production of ROS underneath eggs has been found so far in several plant-insect egg interactions (de Puysseleyr *et al.* 2011, Kim *et al.* 2012, Gouhier-Darimont *et al.* 2013, Bittner *et al.* 2017, Geuss *et al.* 2017, Das *et al.* 2021, Oates *et al.* 2021, Ojeda-Martinez *et al.* 2021) suggesting an integral role of ROS. Indeed, the transcription of ROS-related genes appears to be conserved across five phylogenetically distant plant-insect egg systems, also in absence of visible HR-like cell death (Lortzing *et al.* 2020). Nevertheless, mechanistic data are needed to elucidate the function of ROS in plant-egg interactions. In different pathosystems, ROS is thought to cause direct damage to the attackers` cells, as well to contribute to other regulatory functions related to gene transcription, redox, signalling, vesicle trafficking, lipid peroxidation and modulation of cell death (Torres 2010, Herrera-Vasquez *et al.* 2015, Waszczak *et al.* 2018). Furthermore, ROS appear to be also involved in signalling of plant defense responses against insect herbivores (Maffei *et al.* 2007, Erb & Reymond 2019). In some plant-egg interactions, it is proposed that ROS may contribute to a direct egg-killing effect (Bittner *et al.* 2017, Geuss *et al.* 2017). For example, leaves of a *Solanum dulcamara* accession developing a chlorosis under eggs of *S. exigua*, display also higher ROS concentration and lower egg hatching rate compared to accession lacking the chlorotic phenotype (Geuss *et al.* 2017). In the *P. brassicae-Brassica* spp. interaction, however, egg survival was shown to be affected by a reduced atmospheric

humidity at the oviposition site in presence of HR (Griese *et al.* 2017), while the role of ROS has not been investigated.

Our results expand the knowledge on plant defense mechanisms against eggs and support the hypothesis that *Brassica* plants can specifically recognize *Pieris* egg deposition. While plant responses to eggs have been described in different plant species (Reymond 2013, Hilker & Fatouros 2015), very few EAMPs have been identified so far (Hilker *et al.* 2005, Tamiru *et al.* 2011, Salerno *et al.* 2013; Stahl *et al.* 2020). We show that a water-soluble wash from *P. brassicae* eggs is sufficient to initiate a plant immune response as induced by eggs, which point to the presence of EAMPs in the butterfly secretions surrounding the eggs and underneath the eggs. Conversely, we found weak ROS accumulation and no cell death underneath *M. brassicae* eggs, and we observed neither *PR1* upregulation nor ethylene production in response to *M. brassicae* egg wash. The use of *P. brassicae* egg wash and the comparative study with a generalist moth allows us to hypothesize that *P. brassicae* eggs are specifically recognized by the plant. Recently, we reported that the induction of HR-like cell death and high *PR1* expression in *B. nigra* is specific to eggs of pierid butterflies of the Pierinae subfamily, such as *Pieris* spp. and *Anthocharis cardamines*, all butterflies that are specialists of brassicaceous species (Chatper 2, Griese *et al.* 2021). In this study, we added an extra layer by showing that lack of *PR1* expression under moth eggs is accompanied by a weak ROS and not cell death. Our results suggest that cell death, ethylene production and *PR1* gene expression, at least in *B. nigra*, are specific to *P. brassicae* eggs, and we hypothesize that these responses are activated after detection of one or more Pierinae-specific elicitor/EAMPs.

Currently, the chemical characterization of *Pieris* egg wash is undergoing and we do not have information on these putative Pierinae-specific EAMPs. Nevertheless, here we showed that application of phosphatidylcholines (PCs) does not induce a HR-like cell death in *B. nigra.* PCs were recently identified in the lipidic fraction of *P. brassicae* egg extract as EAMPs responsible for upregulation of *PR1* in *A. thaliana* (Stahl *et al.* 2020). Considering that PCs are essential components of cell membranes, they are possibly also responsible for the upregulation of *PR1* expression that was observed in response to egg extracts of different generalist insects (Bruessow *et al.* 2010). Thus, PCs may represent EAMPs that are conserved among insect eggs and that induce a sort of general plant immune response against insect egg deposition. Additional EAMPs that are specific to *Pieris* eggs may instead further induce the plant immune system leading to the development of a macroscopic HR-like cell death.

In conclusion, the HR-like cell death that is commonly observed in *Brassica* spp. under eggs of *Pieris* spp. appears to be the result of a regulated plant immune response. The response

is specifically induced by *Pieris* EAMPs that likely originate from the glue that surround the eggs. Genetic variation in *Brassica* spp. for HR-like phenotype will help in the future to identify novel molecular components that are still lacking in the description of plant-egg interactions.

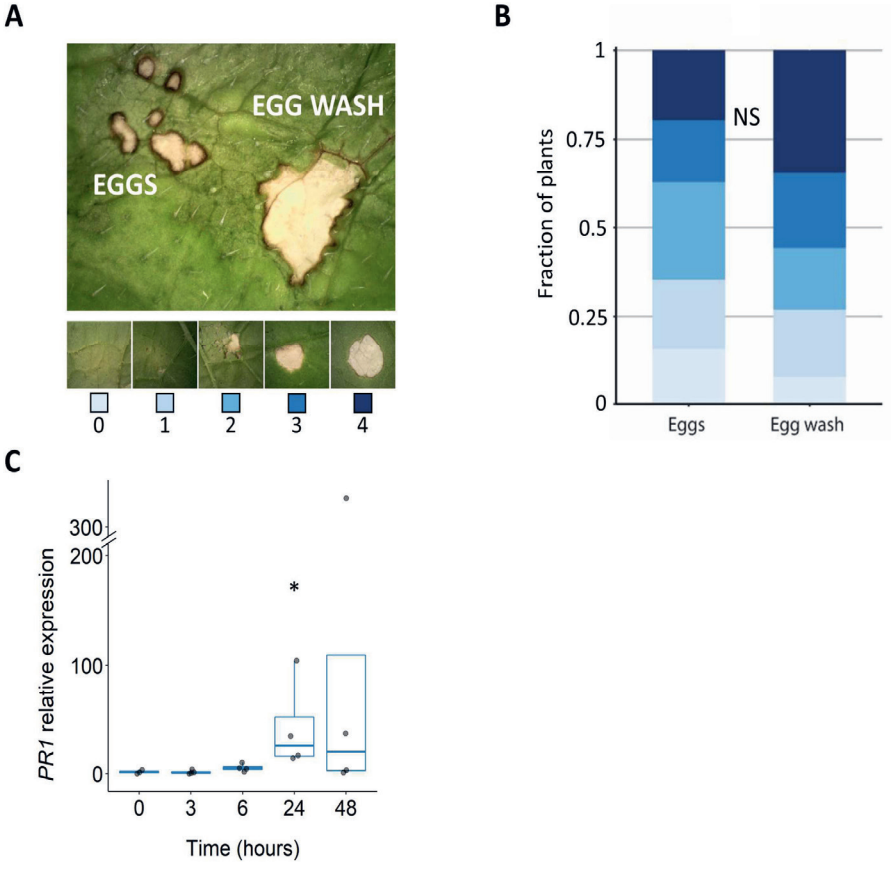
Acknowledgements

We are grateful to Sean Geurts and other employees of Unifarm (WUR) for rearing and caring of the plants used in the experiment. We thank Pieter Rouweler, André Gidding, and Frans van Aggelen for rearing of *P. brassicae* and *M. brassicae*. We thank Gabriella Bukovinszkine'Kiss for assistance with several experiments, and Arjen van der Peppel for help with ethylene measurements. Guusje Bonnema and Erik Poelman are thanked for seeds of *B. rapa* and *B. nigra*.

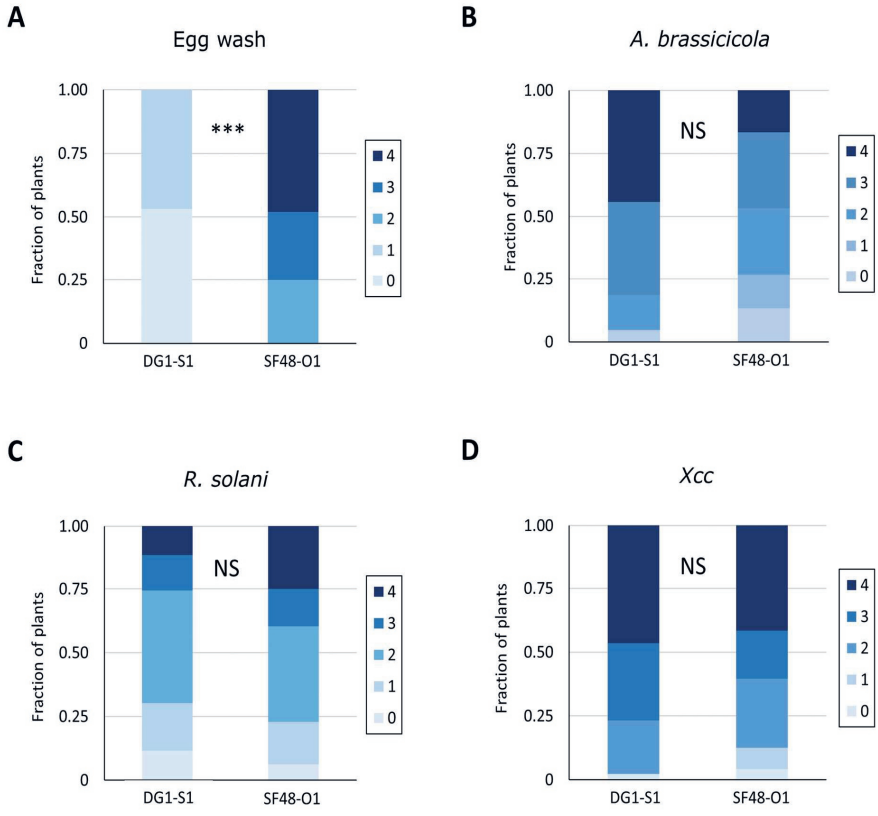
Availability of data and materials

The datasets supporting the conclusions of this article are temporary available at this link https://figshare.com/s/0dd88f0686ecb04d7657 (restricted access, to be released upon publication).

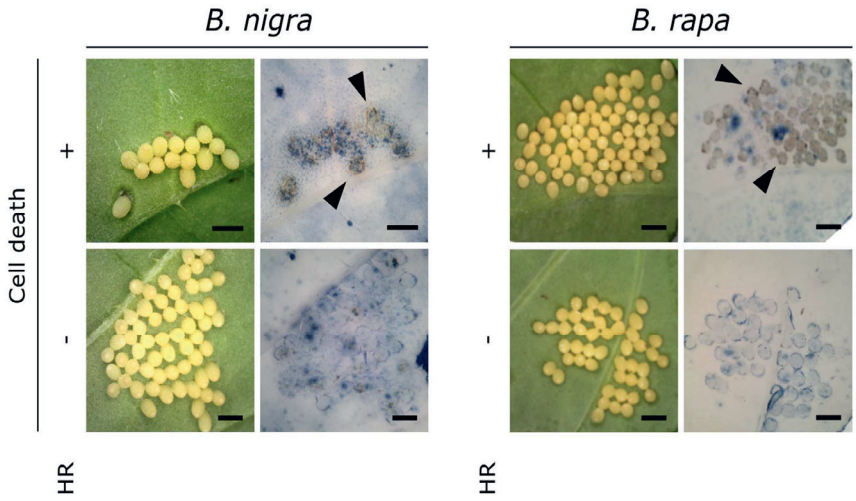
Supplementary figures



Supplementary Figure S1. *Pieris brassicae* eggs and egg wash induce comparable cell death in *B. nigra*. A) Both eggs and egg wash induce macroscopic HR-like cell death that are visually indistinguishable. Control treatments (MES buffer or demineralized water) did not induce cell death (not shown). B) Severity of HR-like cell death induced by egg wash and eggs are comparable when scored in categories. For classes score, see Figure 1A. C) *PR1* expression of leaves treated with egg wash. Each treatment consisted of four biological replicates, each including six leaf discs. Gene expression was measured by qRT-PCR and normalized to the housekeeping gene *BnGADPH*. Treatments were compared to untreated plants at 0 h time point (ANOVA followed by Dunnett's test, $P < 0.05$). Asterisks indicate different P-value ranges: * <0.05, ** <0.01, *** <0.001. NS = not significant.



Supplementary Figure S2. Severity of macroscopic cell death induced by egg wash and pathogen extracts. A) HR-like cell death induced by egg wash. B-D) HR lesions induced by extracts of *A. brassicicola, Rhizoctonia solani* and *Xanthomonas campestris* pv. c*ampestris* (*Xcc*). For each treatment/accession combination N=15. Treatments were applied on the abaxial side of the leaf, egg wash was applied as droplets, pathogens extracts were infiltrated with a syringe. Controls (water droplets or infiltrated) did not induce visible symptoms. Asterisks indicate different P-value ranges: * <0.05, ** <0.01, *** <0.001. NS = not significant.



Supplementary Figure S3. Eggs induce similar cell death in *B. nigra* and *B. rapa* irrespective of the visible HR-like cell death phenotype. *B. nigra* (lef) and *B. rapa* (right) leaves 72 h after oviposition show cell death (trypan blue staining). Fully developed HR-like cell death is visible underneath the trypan blue stains (top row). Black arrows indicate representative HR-like spots. Magnification bars = 1 mm.

Supplementary tables

Supplementary Table S1. Primer sequences used for real time qRT-PCR.

Gene	Forward (5' to 3')	Reverse (5' to 3')
*Bn*GADPH	GGAGCTGCCAAGGCTGTCGG	CCTTCAGATTCCTCCTTGATAGCC
*Bn*ICS1	CAGATTATTCTCCCGCAAGAAG	GGCGGAGGTTGAGATTTGAG
*Bn*MYC2	GTGGAATCGAGCAAGAGGAA	ATCGTTAACCACCGACATACT
*Bn*PR1	CGCCGACGGACTAAGAGGCG	ACACCTCGCTTTGCCACATCCA
*Bn*PR2	ATCGGACGTTGTGGCTCTTT	TTTGGGAACGTCGAGCATGA
*Bn*VSP1	CAGCTACCCCAACTGCAGAA	GCGGTACTGTTCGGAAGTGA
*Bn*VSP2	CATCAGCTACCCCAACTGCA	GCGGTACTGTTCGGAAGTGA

Supplementary Table S2. HR-like cell death (score ranging from 0 to 4) displayed by *B. nigra* plants elicited by different treatments. Plants in which the eggs or egg wash tested did induce a HR-like cell death("HR+") and plants in which they did not ("HR-") are counted. Data of four separate experiments is taken together of which the mean HR frequency and HR severity is shown. Different letters indicate there was no significant difference in HR severity (Kruskal-Wallis test).

Treatment	HR+	HR-	HR severity (mean + SE)	HR frequency (mean + SE)
Eggs	33	18	2.04 + 0.15 [a]	0.69 + 0.04
Egg wash	38	14	2.72 + 0.17 [a]	0.73 + 0.04

Supplementary Table S3. Relative expression of *PR1* gene in *B. nigra* after egg deposition or treatment with egg wash. N indicates biological replicates (separate plants). HR severity is mean of all tested plants scored at 72 hours after treatment or oviposition. PR1 relative expression is represented by mean + se. An asterisk indicates a significant difference in mean transcript levels compared to the 0 h timepoint for eggs (ANOVA: $F_{4,14} = 6.12$, $P = 0.005$) and after treatment with egg wash (ANOVA: $F_{4,14} = 4.03$, $P = 0.022$). There were no significant differences found in mean transcript levels between the treatments for each timepoint (Student's t-tests: $P > 0.05$).

Time point	Treatment	N	HR-like severity (mean)	*PR1* relative expression (mean + SE)
0 h	NA	4	NA	1.63 + 0.54
3 h	Eggs	4	2.50	0.67 + 0.15
	Egg wash	4	3.25	1.47 + 0.42
6 h	Eggs	4	3.00	55.62 + 20.99
	Egg wash	4	2.75	5.45 + 0.88
24 h	Eggs	4	3.25	140.34 + 54.57 *
	Egg wash	4	2.50	42.16 + 10.54 *
48 h	Eggs	4	2.75	131.93 + 32.98 *
	Egg wash	4	3.00	157.33 + 39.33

Supplementary Table S4. Ethylene production after egg wash treatment in no-HR and HR plants. *Brassica* nigra leaves responded with ethylene production after incubation with egg wash for 5 hours compared to incubation with control MES buffer (Wilcoxon rank sum test, $P < 0.001$). Control treatment was an incubation of leaves in MES buffer in which the egg wash was prepared. Plant HR was determined in plants after leaves were samples for the ethylene production assay by treating another leaf with egg wash and scoring spots 72 hours after treatment. N = number of plants used for assay.

HR-like phenotype	Treatment	N	HR-like severity (mean)	Ethylene production ($mean_{ppm}$ + SE)
No-HR	Control	10	0	0.00 + 0.00
	Egg wash	10	0.60	0.11 + 0.01
HR	Control	10	0	0.00 + 0.00
	Egg wash	10	2.30	0.42 + 0.01

Supplementary Table S5. Relative expression of *PR1* gene in *B. nigra* 24 hours after treatment with either *P. brassicae* or *M. brassicae* egg wash. Control is a treatment with MES buffer in which the egg wash was prepared. N indicates biological replicates (separate plants). *PR1* relative expression is represented by mean + se. Different letters indicate significant differences in mean *PR1* expression, ANOVA followed by Tukey, $P<0.001$.

Egg wash	N	*PR1* relative expression (mean + SE)
Control	5	1.24 + 0.19
Pieris brassicae	4	159.48 + 14.39
Mamestra brassicae	4	4.18 + 0.80

Chapter 4

Genetic analysis reveals three novel QTLs underpinning a butterfly egg-induced hypersensitive response-like cell death in *Brassica rapa*

Niccolò Bassetti[1], Lotte Caarls[1,3], Gabriella Bukovinszkine'Kiss[1,2], Mohamed El-Soda[5], Jeroen van Veen[1], Klaas Bouwmeester[1,4], Bas J. Zwaan[2], M. Eric Schranz[1], Guusje Bonnema[3], Nina E. Fatouros[1]

[1] Biosystematics Group, Wageningen University & Research, Wageningen, The Netherlands
[2] Laboratory of Genetics, Wageningen University & Research, Wageningen, The Netherlands
[3] Laboratory of Plant Breeding, Wageningen University & Research, Wageningen, The Netherlands
[4] Laboratory of Entomology, Wageningen University & Research, Wageningen, The Netherlands
[5] Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt

BMC Plant Biology (2022) 22:140 https://doi.org/10.1186/s12870-022-03522-y

Abstract

Cabbage white butterflies (*Pieris* spp.) can be severe pests of *Brassica* crops such as Chinese cabbage, Pak choi (*Brassica rapa*) or cabbages (*B. oleracea*). Eggs of *Pieris* spp. can induce a hypersensitive response-like (HR-like) cell death which reduces egg survival in the wild black mustard (*B. nigra*). Unravelling the genetic basis of this egg-killing trait in *Brassica* crops could improve crop resistance to herbivory, reducing major crop losses and pesticides use. Here we investigated the genetic architecture of a HR-like cell death induced by *P. brassicae* eggs in *B. rapa*. A germplasm screening of 56 *B. rapa* accessions, representing the genetic and geographical diversity of a *B. rapa* core collection, showed phenotypic variation for cell death. An image-based phenotyping protocol was developed to accurately measure size of HR-like cell death and was then used to identify two accessions that consistently showed weak (R-o-18) or strong cell death response (L58). Screening of 160 RILs derived from these two accessions resulted in three novel QTLs for *Pieris brassicae*-induced cell death on chromosomes A02 (Pbc1), A03 (Pbc2), and A06 (Pbc3). The three QTLs Pbc1–3 contain cell surface receptors, intracellular receptors and other genes involved in plant immunity processes, such as ROS accumulation and cell death formation. Synteny analysis with *A. thaliana* suggested that Pbc1 and Pbc2 are novel QTLs associated with this trait, while Pbc3 also contains an ortholog of LecRK-I.1, a gene of A. thaliana previously associated with cell death induced by a *P. brassicae* egg extract. This study provides the first genomic regions associated with the *Pieris* egg-induced HR-like cell death in a *Brassica* crop species. It is a step closer towards unravelling the genetic basis of an egg-killing crop resistance trait, paving the way for breeders to further fine-map and validate candidate genes.

Keywords
Plant immunity, Insect eggs, HR-like cell death, Germplasm screening, QTL mapping, Image-based phenotyping, Oviposition-induced defence, Pieridae, Brassicaceae

Introduction

Plant-insect interactions often start with herbivore egg deposition on plant tissues. Through millions of years of co-evolution with insects, plants have evolved mechanisms to recognize insect eggs as non-self to induce defence responses (Hilker & Fatouros 2015). Different egg-killing traits have been described, such as neoplasm formation (Doss *et al.* 2000, Petzold-Maxwell *et al.* 2011, Guess *et al.* 2017), secretion of toxic chemicals (Seino *et al.* 1996), tissue crushing (Desurmont *et al.* 2011), and hypersensitive response (HR)-like cell death (Shapiro & DeVay 1987, Balbyshev & Lorenzen 1997, Garza *et al.* 2001). Such defenses represent an additional component in the plant-insect arms race, but their potential for sustainable crop protection has so far largely been overlooked and underutilized (Fatouros *et al.* 2016).

Eggs of *Pieris* spp. butterflies (Lepidoptera: Pieridae) induce a HR-like cell death on their natural host plants belonging to the Brassicaceae family (Griese *et al.* 2021). The large cabbage white (*P. brassicae* L.) and the small cabbage white (*P. rapae* L.) represent major pests of *Brassica* crops worldwide (Kumar *et al.* 2017, Ryan *et al.* 2019). Despite available knowledge on plant defences against them, *Pieris* spp. are specialists well equipped for feeding on Brassicaceae (Nallu *et al.* 2018, Erb & Reymond 2019). Their caterpillars effectively detoxify the secondary metabolites produced by their host plants, so-called glucosinolates or "mustard oils" (De-la-Cruz *et al.* 2020, Wheat *et al.* 2007). Considering this, *Pieris* egg-induced HR-like cell death may represent a genuine and unexplored defense response of Brassicaceae against adapted specialist herbivores.

The HR-like cell death response was initially observed underneath eggs of *P. rapae* and *P. napi* deposited on leaves of wild populations of black mustard (*Brassica nigra* L.), on which it caused egg-killing by desiccating or dropping off (Shapiro & DeVay 1987). Later, this egg-killing trait was also observed underneath eggs of *P. brassicae* and, interestingly, it was found to work in concert with the attraction of egg parasitoid wasps through the release of oviposition-induced plant volatiles (Fatouros *et al.* 2012, 2014). Under field conditions, the synergistic effect of HR-like cell death and egg parasitism reduced up to 80% of *Pieris* egg survival on *B. nigra* (Fatouros *et al.* 2014). The direct egg-killing effect of the cell death seems to work mainly against singly laid eggs, irrespective of whether they are from solitary species such as *P. rapae* and *P. napi*, or from the gregarious species *P. brassicae* (Griese *et al.* 2017, Griese *et al.* 2021). These studies suggest that HR-like cell death may be an effective egg-killing trait, for which the plant molecular and genetic mechanisms are still poorly understood.

Most of the initial knowledge on the molecular aspects of the *Pieris* egg-plant interaction was obtained using the model plant *A. thaliana* (Reymond 2013). Upon *P. brassicae* oviposition, *A. thaliana* responds with reactive oxygen species (ROS) and cell death, further accompanied by the accumulation of phytohormone salicylic acid (SA) and the induction of the SA-responsive gene *PATHOGENESIS RELATED PROTEIN 1* (*PR1*) (Little *et al.* 2007, Bruessow *et al.* 2010). These responses were shown to be dependent on *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*), *ISOCHORISMATE SYNTHASE 1*/*SALICYLIC ACID INDUCTION DEFICIENT 2* (*ICS1/SID2*) and, partially, *NONEXPRESSER OF PR GENES 1* (*NPR1*), which are known signaling components of plant defense responses against biotrophic pathogens (Gouhier-Darimont *et al.* 2013). Transcriptomic studies in different plants species have confirmed that insect oviposition induces genes associated with SA- and ROS-mediated immune responses and *PR1* gene expression (Little *et al.* 2007, Fatouros *et al.* 2008, Firtzlaff *et al.* 2016, Baruah *et al.* 2017, Drok *et al.* 2018, Nallu *et al.* 2018, Lortzing *et al.* 2019, Valsamakis *et al.* 2020, Das *et al.* 2021), including in *B. nigra* and *B. rapa* (Fatouros *et al.* 2015, Caarls *et al.* 2021, Griese *et al.* 2021). Further, it has been suggested that there is a conserved transcriptional response amongst different plant-insect egg interactions (Lortzing *et al.* 2020).

The similarities between the plant defenses induced against insect eggs and biotrophic pathogens suggest that insect eggs are also recognized by the plant immune system (Erb & Meldau 2012, Reymond 2013), but it is yet not known how. The induction of plant defenses partly relies on the specific recognition of non-self-molecules released by biotic attackers that are detected by plasma membrane pattern recognition receptors (PRRs) (Couto & Zipfel 2016, Tang *et al.* 2017) or intracellular nucleotide binding leucin-rich repeat receptors (NLRs) (van den Burgh and Joosten 2019). Feeding of herbivorous insects induce plant immunity through the release of herbivore-associated molecular patterns (HAMPs) contained in oral secretions of insect larvae and/or damage-associated molecular patterns (DAMPs) resulting from damaged plant tissues (Acevedo *et al.* 2015). Both signals have been associated with the perception by different types of PRRs (Gust *et al.* 2017, Erb & Reymond 2019).

Contrary to cues of larval feeding, only a few insect egg-associated molecular patterns (EAMPs) have been identified (Doss *et al.* 2000, Fatouros *et al.* 2008, Hilker *et al.* 2005, Stahl *et al.* 2020). In *A. thaliana*, some candidate PRRs involved in perception of *P. brassicae* eggs were recently discovered. Several L-type lectin receptor-like kinases (LecRKs), a class of PRRs, were upregulated upon *P. brassicae* oviposition (Little *et al.* 2007). One of them, LecRK-I.8, was found to be required for the induction of downstream ROS production, cell

death and *PR1* expression (Gouhier-Darimont *et al.* 2013, 2019). More recently, a genome-wide association study (GWAS) in *A. thaliana* identified LecRK-I.1 as a candidate gene underlying one of two loci involved in the induction of cell death upon treatment with *P. brassicae* egg extract (Groux *et al.* 2021b).

To date, only a few studies have attempted to map genetic loci associated with insect oviposition-induced responses (Yang *et al.* 2014, Mariyammal *et al.* 2019, Tamiru *et al.* 2020, Groux *et al.* 2021b). A strong HR-like cell death that eventually leads to egg-killing has been mainly shown for plant species of the tribe Brassiceae (Lineage II), which includes wild species such as *Brassica nigra*, *Sinapis* spp., *Crambe* spp., as well as diverse *Brassica* crops such as *B. napus, B. oleracea* and *B. rapa* but not *A. thaliana* (Fatouros *et al.* 2014, Pashalidou *et al.* 2015a, Griese *et al.* 2021). Interestingly, species belonging to the tribe Brassiceae are known host plants for *Pieris* spp. while *A. thaliana* is not (Harvey *et al.* 2007).

Next to interspecific variation between Brassicaceae species we also identified intraspecific variation in HR-like cell death among accessions of several species (Fatouros *et al.* 2014, Griese *et al.* 2021), suggesting that genetic analysis to identify casual loci should be feasible. Up to now, classical forward genetics, such a linkage mapping and/or GWAS, helped to identify quantitative trait loci (QTLs) involved in both upstream (perception) and downstream mechanisms associated with plant resistance to insect feeding (Dogimont *et al.* 2014, Liu *et al.* 2015, Thoen *et al.* 2017, Gust *et al.* 2017, Nallu *et al.* 2018, Sun *et al.* 2020). Currently, genetic mapping efforts in *Brassica* crop species are increasingly made possible given a growing availability of high quality genomic and genetic resources (Belser *et al.* 2018, Zhang *et al.* 2018, Li *et al.* 2020, Lou *et al.* 2020). It is thus timely to use genetic approaches to unravel the genetics underlying plant-insect egg interactions in non-model species. Genetic mapping of insect egg-induced defenses in *Brassica* crops can help both the fundamental understanding of HR-like cell death and its applied use as novel defense trait in plant breeding.

Here we present the genetic analysis of *P. brassicae* butterfly egg-induced HR-like cell death in *Brassica rapa* by QTL mapping. First, we investigated the phenotypic variation for HR-like cell death within *B. rapa* germplasm using a core collection previously assembled and curated (Del Carpio *et al.* 2010, Zhao *et al.* 2010). Then, we assessed the robustness of the phenotype and we quantitatively measured cell death size with a novel image-based phenotyping protocol. We identified two accessions with a significant difference in size of HR-like cell death and we screened a recombinant inbred line (RIL) population resulting in the identification of three novel QTLs. This study provides the first QTLs and candidate genes

associated with butterfly egg-induced cell death in *B. rapa,* an important crop species and natural host plant of *Pieris* spp.

Material and methods

Plant material

For germplasm screening, 56 *Brassica rapa* L. (Brassicaceae) accessions were selected representing all major *B. rapa* crop types (e.g., Chinese cabbage, Pak choi, turnip, oil types), and to include different levels of genetic heterogeneity, such as feral populations, landraces, breeding material, and doubled haploid (DH) lines (Supplementary Fig. S1, Supplementary Table S1). Most *B. rapa* accessions and all DH lines were obtained from the core collection of Dr. Bonnema at Plant Breeding, Wageningen University and Research (Del Carpio *et al.* 2010, Zhao *et al.* 2010) with a few additional accessions obtained from the Centre for Genetic Resources (CGN, The Netherlands). A *B. nigra* accession previously reported to induce a strong HR-like cell death was used as positive control (Griese *et al.* 2017). After the germplasm screening, ten accessions, considered suitable as potential parents of biparental mapping population, were selected for a second HR-like cell death evaluation. Criteria for the selection were: the accession i) displays an HR-like cell death score at the extremes of the phenotypic distribution and is consistent across individual plants; ii) is fast flowering (< 1 year); iii) is self-compatible; iv) was multiplied by selfings or was used to generate a DH line (in order to have homozygous material to repeat experiments); and, v) it was preferably showing comparable leaf phenotypes to minimize the segregation of leaf morphological traits after crossing. Finally, a mapping population of 160 recombinant inbred lines (RILs), previously generated from a cross between the *B. rapa* DH lines L58 (caixin type, ssp. *parachinensis*) and R-o-18 (yellow sarson type, spp. *tricolaris*) was used for QTL mapping of HR-like cell death size (Bagheri *et al.* 2012).

Plant growing conditions

Plants were grown in a greenhouse compartment under standardized conditions (21/18 °C day/night minimum temperature, 16/8 h light/dark photoperiod; and 50–70% relative humidity). The daily maximum temperature was not controlled and subjected to some variation (max + 5 °C). Seeds were vernalized at 4 °C for 2 days and then sown in small trays with sowing soil (Lensli, Bleiswijk, The Netherlands). Seedlings were transplanted 1 week after

germination to 17 cm diameter pots with potting soil (Lensli, Bleiswijk, The Netherlands). Plants were grown for 5 weeks before being subjected to *P. brassicae* oviposition or treatment with egg wash.

Insect rearing

Pieris brassicae L. butterflies were obtained from a rearing facility of the Laboratory of Entomology, Wageningen University. Insects were kept in a greenhouse compartment (21 °C, 16/8 h dark photoperiod, 60-80% relative humidity). Larvae were reared on Brussel sprouts (*Brassica oleracea* var. *gemmifera* cv. Cyrus), while the adults were fed with a 10% honey solution and allowed to oviposit on the same plant.

Oviposition and egg wash treatment

Freshly eclosed *P. brassicae* female butterflies were allowed to mate, subsequently separated from the males, kept without plants for two days, and then used for no-choice oviposition experiments. Butterflies were allowed to freely oviposit on one *B. rapa* plant at the time in small cages each containing 2-4 female butterflies. A maximum of two egg clutches consisting of 10-20 eggs were laid on the two youngest fully developed leaves of each plant, while the other leaves were covered by a net. Then a new plant was placed into the cage. After every 4-5 egg-laden plants, mated female butterflies in each of the small cages were replaced to randomize the effect of insect genetic diversity.

For the egg wash treatment, *P. brassicae* egg clutches were collected from Brussel sprouts leaves within 24 h after oviposition. Eggs were carefully removed with a stainless-steel lab spatula and collected in an Eppendorf tube in a ratio of ~1000 eggs per 1 ml demineralized water. Eggs were incubated overnight at room temperature, after which the liquid phase was directly used or stored at -20 °C.

Experimental design

Germplasm screening was carried out in September 2017 by application of egg wash. Two 5 µl droplets of egg wash were applied on the two youngest fully developed leaves of each plant. Droplets of an equivalent amount of demineralised water were applied as negative control. Each genotype/accession was represented by 3–5 replicates (individual plants). Plants were arranged in a randomized complete block design with five blocks and one plant per accession within each block. Re-evaluation of ten homozygous lines, that were either inbred (CC-106, SO-040, BRO-127, IMB211, CC-168 BRO-030, CC-AO3, L58) or DH lines (R-o-

18, R500, BRO-030, CC-AO3, L58), was carried out in February 2018 using both no-choice oviposition and egg wash treatment. Plants were arranged in a randomized complete block design with two blocks and five plants per accession within each block. Three QTL mapping experiments were carried out in August/September 2018 using eggs deposited by *P. brassicae* females. The whole RIL population was grown three times over three consecutive weeks, each time with one replicate per RIL and three replicates for the two parents L58 and R-o-18. Validation of QTL effects and additive interactions was carried in September 2019 using twelve RILs (RIL_19, RIL_22, RIL_32, RIL_45, RIL_73, RIL_77, RIL_93, RIL_97, RIL_100, RIL_106, RIL_130, RIL_137) which were selected randomly for their contrasting genotypes at the peak markers of QTLs *Pbc1–3*. For all experiments with RILs, plants were subjected to no-choice oviposition as described above.

Assessment of HR-like cell death

Egg wash-induced HR-like cell death was scored in the germplasm screening on a scale from 0 to 3 with: 0, no visible cell death; 1, a grey/dark spot smaller than droplet size; 2, a black necrotic spot covering the whole treated area; 3, strong cell death visible also on the adaxial side (Supplementary Fig. S1a). For the re-evaluation of homozygous (inbred and DH) lines from ten selected accessions and for the QTL experiment, egg and egg wash-induced HR-like cell death size (area) was measured with an image-based phenotyping protocol (see next section). For all experiments, individual plants were assigned the highest HR-like cell death score or size out of all egg- and egg wash-treated spots. To account for variability in egg clutches size, cell death size measured underneath egg clutches was divided by the number of eggs in the clutch, and normalized to 10 eggs:

$$\text{cell death}_{clutch} = \frac{\text{cell death}}{n \text{ eggs}} * 10 \qquad (1)$$

Development of image-based phenotyping protocol

To obtain a reliable and reproducible quantification of egg- and egg wash-induced HR-like cell death size (area), we developed a custom image-based phenotyping protocol (Supplementary Fig. S2). Leaf discs containing egg clutches- or egg wash-treated spots were sampled with a cork borer of 6 mm diameter and placed in Petri dishes with 1% phytoagar (Duchefa Biochemie, Haarlem, The Netherlands) or wet filter paper (Supplementary Fig. S2a). Just prior to the sampling of the leaf disks with deposited egg clutches, eggs were counted and then gently removed with adhesive tape to prevent leaf damaging. Spots treated with egg wash

were sampled directly. Leaf discs were imaged with a Dino-Lite Edge Digital microscope (AnMo Electronics Corporation, Hsinchu, Taiwan) connected to a laptop (Supplementary Fig. S2b). Each leaf disc was imaged with the light polarizer filter "fully open" using the following settings: LED zone 2 and 4: ON; LED zone 1 and 3: OFF; autoexposure: ON; white balance: STANDARD; output file format: PNG; resolution: 2592 × 1944 pixel.

Image analysis was performed on Fiji with ImageJ v1.52 software (Schindelin *et al.* 2012) using the image segmentation plugin Trainable WEKA Segmentation v3.2.28 (Arganda-Carreras *et al.* 2017). Image analysis was executed through a custom Fiji macro script. In WEKA, the image segmentation was performed using the training features *Minimum*, *Maximum*, *Mean*, *Variance*, *Median* and the classifier algorithm *FastRandom- Forest*. For each leaf disc, first a classifier model was trained using a training set composed by representative image pixels that were labelled either as "healthy leaf tissue" or "HR-like cell death". The trained classifier was then applied to the whole image to generate an 8-bit seg- mentation of HR-like cell death spots (Additional file 1: Supplementary Fig. S2d). The 8-bit segmented HR-like area was finally measured in Fiji using the command Analyze particles with Area as measurement (Additional file 1: Supplementary Fig. S2e). The use of the WEKA automated segmentation resulted in more reproducible measurements of cell death size (Additional file 1: Supplementary Fig. S3).

Phenotypic data analysis

All data analyses were performed in R 3.5.3 (R Core Team 2021). Raw data were firstly checked for assumptions of normality (Shapiro-Wilk normality test) and homogeneity of variances (Fligner-Killeen test). Non-normal data were analyzed after data transformation (root square on cell death size) or with a non-parametric test (cell death score). Phenotypic data obtained from germplasm screening were not normally distributed and thus analyzed with the non-parametric Kruskall-Wallis test. *Post-hoc* analysis was conducted with the Dunn test using Benjamini-Hochberg correction as implemented by the *dunnTest* function from *FSA* package (Ogle *et al.* 2021). Phenotypic data from re-evaluation of ten *B. rapa* accessions were analyzed on square root-transformed data. HR-like cell death size was analyzed by using the following model:

$$y = \mu + Block + Block{:}Row + Block{:}Col + G + r_{error} \quad (2)$$

in which μ is the overall trait mean, *Block* is the blocking factor of the experimental design, *Row* and *Col* represents the spatial location of plant within a *Block*, *G* represents the *B. rapa*

accession/genotype. Parsimonious models were explored by stepwise removal of each factor and comparison of the full *versus* reduced model with a Likelihood Ratio Test. The most parsimonious model retained only *G* as factor. Finally, data were analyzed using one-way ANOVA, followed by Tukey`s HSD *post hoc* test with Benjamini–Hochberg correction with $\alpha < 0.01$ as implemented in *multcomp* package (Hothorn *et al.* 103). Phenotypic data from the QTL experiments and the re-evaluation of RILs were also analyzed on square root transformed data with the model in equation (2). Genotypic means of RILs across the three QTL experiments were calculated as the Best Linear Unbiased Estimator (BLUEs), using the mixed model:

$$BLUE = \mu + G + Exp + r_{error} \qquad (3)$$

in which μ is the overall trait mean, *G* represents the RIL genotype, *Exp* the QTL experiment (1-3). BLUEs were calculated by fitting *G* as fixed effect and *Exp* as random effect. The model was analyzed by REML procedure using the function *lmer* from *lme4* package (Bates *et al.* 2021).

Estimation of variance components

Variance components for genetic and experimental residual error were estimated with equation (2) fitted as mixed model with all factors included as random (Khan *et al.* 2019). The model was analyzed by REML procedure using the function *lmer* from *lme4* package (Bates *et al.* 2021). Classic broad-sense heritability (H^2) was calculated by using the estimated variance components with the formula $\sigma_G / (\sigma_G + \sigma_E)$ as previously described (Holland *et al.* 2003). Genetic and environmental coefficient of variation (CV) was calculated according to the equation:

$$CV = \sqrt{\sigma^2_X}/n * 100 \qquad (4)$$

in which *n* is the grand mean of the population, and σ^2_X is a variance component (σ^2_G or σ^2_E).

Linkage map construction and QTL analysis

A combined and denser genetic map for the RIL population L58 x R-o-18 was created using marker data previously generated in two separate studies, that is AFLP, SSR and SNP markers (Bagheri *et al.* 2012), and InDel PCR markers (Zhang *et al.* 2015) (Supplementary Tables S4-S5). We used Haldane's mapping function with default setting as implemented in JoinMap 4.0 (Kyazma), to convert recombination frequencies to centiMorgan (cM). The final linkage map constituted of 485 markers and 10 linkage groups corresponding to the 10

chromosomes of the *B. rapa* A genome for a total of 1154.44 cM (Supplementary Fig. S4, Supplementary Tables S6-S7). QTL analysis was performed using the R/qtl package in R 3.5.3 (Broman *et al.*2003). Genotype probabilities at positions not covered by the linkage map were estimated every 1 cM with the *calc.genoprob* function (step size = 1). First, single QTL models were searched with the *scanone* function using an interval mapping method (Haley-Knott regression). Subsequently, multiple-QTL model (MQM) interval mapping using Haley-Knott regression was performed to investigate multiple-QTL models which included the previously identified QTLs and additional (potential) QTLs with the *mqmscan* function. As separate analysis, MQM was also implemented with the *stepwiseqtl* function which gave similar results. Finally, epistatic additive and interactions were investigated with pairwise two-QTLs models as implemented in the *scantwo* command. For all analysis, LOD score significance threshold at 5% error rate was estimated with a 1000 permutations test.

Identification of candidate genes withing the QTLs regions

In order to investigate the gene content underlying the identified QTLs, the linkage map was anchored to the *B. rapa* reference genome Chiifu v3.0 (BRAD Brassica database, accessed on 30th August 2021). Sequences of the QTL-flanking markers were aligned to the reference genome using Geneious Prime v8 (Biomatters) to extract their physical location and all *B. rapa* genes contained within them. *B. rapa* gene functional annotation was assigned as the best match to *A. thaliana* protein database (genome TAIR 10) using BLAST+ v2.12.0 (E-value = 1e^{-5}). Candidate genes associated with plant defense, biotic stress and cell death, including cell surface and intracellular receptors, were manually searched within the description of the *A. thaliana* orthologs to each *B. rapa* gene.

Analysis of syntenic relationships between *B. rapa* and *Arabidopsis thaliana* genomes was performed using the comparative genomic tool SynMap on the CoGe web platform (Lyons *et al.* 2008, Haug-Baltzell *et al.* 2017). SynMap legacy version was used with the following settings: DAGChainer Options “Maximum distance between two matches (-D): 20 genes”; “Minimum number of aligned pairs (-A): 5 genes”; Merge Syntenic Blocks “Algorithm: Quota align merge”; Syntenic Depth “Algorithm: Quota Align”, “Ratio of coverage depth (*A. thaliana*) 1 -to- 3 (*B. rapa*)” , “Overlap distance 40”; Fractionation Bias “Run OFF”; CodeML “Calculate syntenic CDS pairs: Synonymous (Ks) substitution rate; “Color scheme: Rainbow 2”, “Max Value: 2”, “Log10 Transform: OFF”; Advance Options “Tandem duplication distance: 10”.

Results

Screening of a *B. rapa* core collection

As a first objective, we investigated whether there was intraspecific variation for HR-like cell death in our *B. rapa* core collection. Out of the whole collection, we screened a subset of 56 accessions representing geographical and morphological diversity within the collection (Supplementary Table S1). Plants were treated with *P. brassicae* egg wash, which was previously reported as a reliable egg-mimicking treatment in *B. nigra* (Caarls *et al.* 2021). The main phenotypic diversity in HR-like response among the *B. rapa* accessions was limited to variation in cell death size (Supplementary Fig. S1, Supplementary Table S2). Egg wash induced a cell death on most of the accessions which appeared as necrotic black/dark spots of varying size on the leaf abaxial side (score 1–2). However, the spots never developed into the fully expanded and brown necrotic tissue, also visible on the adaxial side (score 3) (Supplementary Fig. S1a). Such a strong cell death was only observed in the *B. nigra* accession included as positive control (Supplementary Fig. S1b). Nevertheless, we found differences in HR-like cell death between *B. rapa* accessions (Kruskal-Wallis: $\chi^2_{57} = 130.59$, $P < 0.001$).

Six accessions, i.e. CC-106, FT-086, MIZ-019, R500, R-o- 18 and VT-089, showed no cell death (score 0) in all biological replicates (Supplementary Fig. S1b). Most of the accessions developed only a weak response, with a within-accession variation between individual plants ranging from no cell death (score 0) to small dark necrotic spots (score 1). At the other end of the phenotypic distribution, eleven accessions developed an HR-like cell death of score 2 in most of the biological replicates (i.e. ZCT, PC-184, IMB211, L58, PC-078, CC-114, CC-048, CC-168, CC-050, CC-Z16, CC-058). A specific morphotype was not associated with HR-like cell death as most of the major crop types (Pak choi, turnip, oil types) were found at both extremes of the phenotypic distribution (Supplementary Fig. S1b). The only exception were the Chinese cabbage (CC) accessions, of which 8 out of 14 developed an HR-like cell death with large black/dark spots (score 2) on most of the biological replicates. Genetic heterogeneity of accessions appeared to be not associated with cell death variation as heterogeneous accessions and homogenous inbred lines and DH lines were found on both side of the phenotypic distribution.

Overall, we found statistical differences in HR-like cell death (Dunn's test, $P < 0.01$) between the accessions that showed no cell death (score 0) and the accessions that developed large dark necrotic spots (score 2) upon egg wash treatment (Supplementary Table S2). We then selected ten accessions either showing no response (CC-106, R-o-18, R500, SO-040), little

cell death (BRO-030) or a strong cell death (score 2) in at least few replicates (BRO-127, CC-AO3, IMB211, CC-168, L58) for a further evaluation of their cell death phenotype. These accessions were selected based on specific criteria (see Material and Methods), and also because they were available as homozygous lines; being either inbred due to repeated selfing (self-compatible accessions) or previously used to generate homozygous DH lines (self-incompatible accessions).

Image-based phenotyping of HR-like cell death size on selected *B. rapa* homozygous lines

The selected *B. rapa* homozygous lines (inbred and DH lines) were re-evaluated to assess the robustness of their HR-like cell death phenotype with the aim to identify ideal parental lines to generate biparental mapping populations. Plants were treated with both *P. brassicae* egg clutches (10-20 eggs) and egg wash droplets to compare to which extent the egg wash could mimic the HR-like cell death induced by eggs. Further, we measured HR-like cell death size as quantitative trait using an image-based phenotyping protocol (Supplementary Fig. S2-S3). Image analysis confirmed the differences in HR-like cell death between the selected accessions (Fig. 1, Supplementary Table S3). Overall, we found differences in mean cell death sizes in response to both eggs (ANOVA: $F_{74} = 8.55$, $P < 0.001$) and egg wash (ANOVA: $F_{74} = 15.88$, $P < 0.001$). The two accessions that developed the smallest HR-like response (CC-106, R-o-18) were statistically different in cell death size from the ones with the largest HR-like response (IMB211, L58) for both eggs and egg wash (Tukey`s HSD, $P < 0.01$).

Overall, accessions IMB211 and L58 showed the largest cell death size for both treatments (Fig. 1). In fact, mean cell death size induced by either eggs or egg wash were similar for IMB211 (1.20 and 1.24 mm^2, respectively) and L58 (1.23 and 1.33 mm^2, respectively). In contrast, accessions CC-AO3, CC-168 and SO-040 showed a cell death induced by eggs that was two to three times larger than the response induced by egg wash. To a lesser extent, R500, BRO-030 and BRO-127 also showed a higher cell death induced by eggs compared to egg wash. CC-106 and R-o-18 showed the smallest mean cell death underneath the eggs (0.07 and 0.24 mm^2, respectively), in contrast to the total absence of cell death upon egg wash treatment observed in the germplasm screening. Overall, the two treatments showed limited correlation across the ten accessions (Pearson's $r = 0.55$, $P < 0.001$), mainly because they resulted in comparable responses only for the accessions at the extremes of the distribution. The broad-sense heritability (H^2) was slightly lower for cell death size induced by eggs (0.47) than by egg wash (0.64) (Supplementary Table S3).

In summary, accessions with a small/intermediate HR-like response showed a larger cell death size under eggs compared to egg wash, while the overall ranking was similar. Thus, we concluded that using egg deposition worked better than egg wash to screen for HR-like cell death in order to not underestimate the cell death induced by low responsive *B. rapa* accessions. Overall, IMB211 and L58 were confirmed as lines with a strong HR-like cell death while CC-106, R-o-18, R500 confirmed to be lines with a weak cell death, validating the results of the germplasm screening. Out of these accessions, L58 and R-o-18 represented ideal candidates for crossings because of their self-compatibility, similar flowering time, and leaf size/shape. Thus, we used the L58 (♀) x R-o-18 (♂) RIL population that was previously generated by Bagheri *et al.* (2012) to pursue QTL linkage mapping.

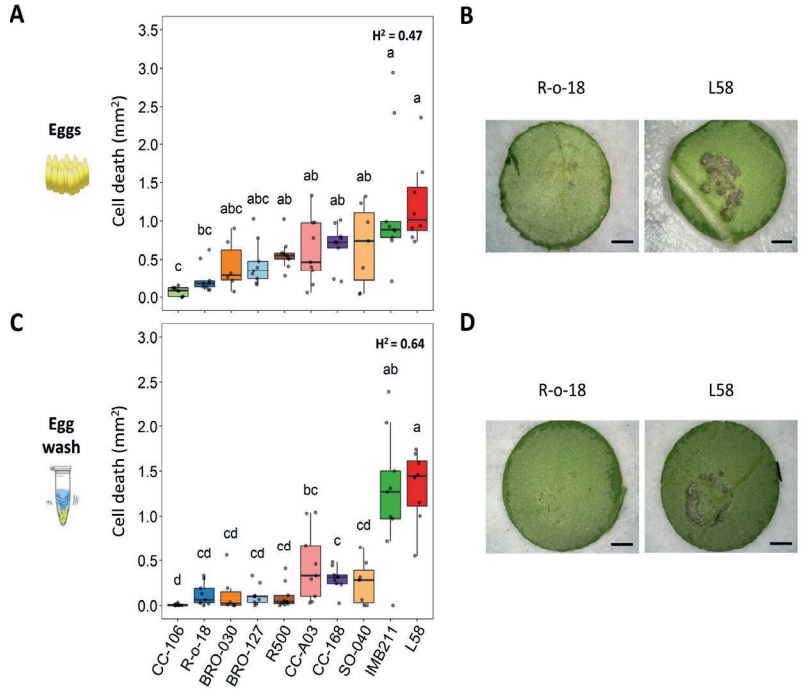


Figure 1. Phenotypic variation in hypersensitive response (HR)-like cell death size between ten *B. rapa* homozygous lines. A Cell death induced by 10–15 *P. brassicae* eggs. B Differential response on DH lines R-o-18 and L58 leaves underneath *P. brassicae* eggs. C Cell death upon spot-inoculation with 5 µl droplets of *P. brassicae* egg wash. D Differential response on DH lines R-o-18 and L58 leaves at egg washed-treated spots. For each accession, N = 6–10 plants were used for both experiments, each plant was treated with eggs or egg wash on two leaves. Cell death size was quantified using a custom image-based phenotyping protocol. Each plant was assigned the cell death of the most severe spot. Boxplots represents the interquartile range (1st and 3rd quantile) and the median, each dot represents a single plant. Letters report differences in mean size of HR-like cell death between accessions (Tukey's HSD test, $P < 0.01$). Broad-sense heritability (H^2) is indicated at top right corner of each graph. Magnification bars inside photos = 1 mm.

Phenotypic analysis and QTL mapping on a RIL population

The RIL population L58 x R-o-18 consisting of 160 lines (F10) was used to identify QTLs underlying *P. brassicae* egg-induced HR-like cell death. We generated a new linkage map combining markers from previous studies (Supplementary Tables S4-S5) (Bagheri *et al.* 2012, Zhang *et al.* 2015). The final genetic map consisted of 485 loci and covered a total of 1154.4 cM, with a mean density of 2.38 cM (Supplementary Fig. S4, Supplementary Tables S6-S7). Image-based phenotyping of egg-induced cell death from three experiments was used to estimate best linear unbiased estimators (BLUEs) of cell death size for each parental and RIL genotype. Overall, the parents R-o-18 and L58 showed BLUE values of 0.49 (SD = 0.4) and 1.53 (SD = 0.42) mm^2, respectively (Fig. 2, Table 1). Their within-accession variation in HR-like cell death size, i.e. their phenotypic range, was larger than what we observed in previous germplasm evaluations, thus resulting in a smaller difference in mean cell death size between the two parents. The RILs showed an approximate normal distribution of cell death size with a mean BLUE value of 0.77 (SD = 0.51) mm^2 (Fig. 2, Table 1). The RILs phenotypic distribution was skewed towards the R-o-18 phenotypic value and only seven RILs developed a cell death size larger than L58. The broad-sense heritability across the three experiments was similar to what was previously observed for egg-induced cell death size ($H^2 = 0.49$).

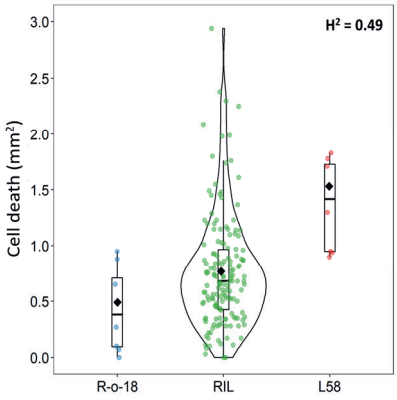


Figure 2. Phenotypic distribution of *P. brassicae* egg-induced cell death in the *B. rapa* RIL population L58 x R-o-18. Blue (R-o-18) and red (L58) dots indicate single plants used across three experiments ($N = 7$) and that were used to estimate single parental BLUE values. Green dots indicate a single BLUE value for each RIL ($N = 3$). Each plant was oviposited with two egg clutches and cell death size was quantified using a custom image-based phenotyping protocol. The largest cell death out of the two clutches was assigned to each plant. Boxplots represents the interquartile range (1st and 3rd quantile) and the median. Black diamonds represent mean BLUE value of the two parents and the whole RIL population. Broad-sense heritability (H^2) is indicated at top right corner of the graph.

Table 1. Summary statistics of cell death phenotypic data (BLUEs) of the L58 x R-o-18 RIL population.

Genotype	N	Range (mm^2)	Mean (mm^2)	SD [a] (mm^2)	H^2 [b]
L58	7	0.9 - 1.83	1.53	0.42	
R-o-18	7	0 - 0.95	0.49	0.40	0.49
RILs	160	0 - 2.94	0.77	0.51	

[a] SD standard deviation
[b] H^2 broad-sense heritability

A total of three QTLs associated with HR-like cell death size were identified on three *B. rapa* chromosomes using an interval mapping method (Haley-Knott regression). The QTLs were named *P. brassicae* egg-induced cell death (*Pbc*) (Fig. 3a, Table 2, Supplementary Fig. S5). First, phenotypic data (BLUEs) were analyzed using single-QTL models, resulting in the identification of two QTLs, i.e. *Pbc1* on chromosome A02 (LOD 5.63) and *Pbc3* on chromosome A06 (LOD 4.15). Additionally, multi-QTL model (MQM) detected another QTL, *Pbc2,* on chromosome A03 (LOD 3.33). Two-QTL models revealed absence of epistatic interactions from any pairwise comparison among *Pbc1-3*, and weak additive interactions between *Pbc1:Pbc2* and *Pbc1:Pbc3* (Supplementary Fig. S6). *Pbc1* explained 17.9% of the additive phenotypic variance, with BrID11121 as top marker (85.4 cM) and a 1.5-LOD confidence interval spanning about 27cM between markers 899118|9904922 and BrID11907 (Table 2). The minor QTLs *Pbc2* and *Pbc3* explained a smaller proportion of the additive phenotypic variance, 6.35% and 7.32% respectively, with BrID90099 (129.2 cM) and BrID90095 (63.9 cM) as respective top markers (Table 2). *Pbc1* was the only QTL with a 1.5-LOD confidence interval lying entirely above the LOD significance threshold (Fig 3a). As the RIL phenotypic distribution was skewed toward the R-o-18 values, we expected L58 alleles contributing to a larger cell death size for all QTLs. Interestingly, this was true only for *Pbc1* which showed opposite effect size compared to *Pbc2* and *Pbc3* (Fig. 3b). In fact, the allele of L58 contributed to an increase in HR-like size of 0.45 mm^2 for *Pbc1*, while the allele of R-o-18 determined an increase in HR-like of 0.27 mm^2 for *Pbc2* and 0.28 mm^2 *Pbc3* (Table 2).

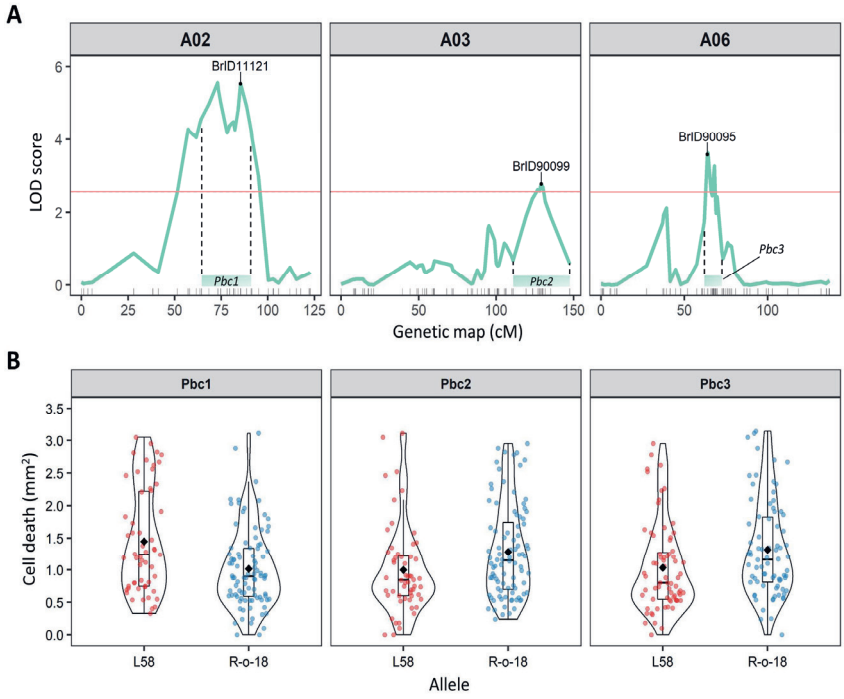


Figure 3. Chromosomal locations and allelic effects of three QTLs for *P. brassicae* egg-induced cell death (*Pbc*) in *B. rapa*. A LOD score of chromosomes A02, A03 and A06 from MQM mapping of HR-like cell death size induced by *Pieris* eggs on 160 RILs. Labels indicate the closest marker to the peak LOD score. LOD threshold is indicated with a dashed horizontal line (2.59 after 1000 permutations at 5% error rate). Marks on the x-axis indicate the position of makers on the genetic map. Colored boxes above markers indicate the 1.5-LOD confidence interval of each QTL. B Effect plots of each QTL. Cell death size across 160 RILs grouped by the parental allele (L58, red; R-o-18, blue). Black diamonds represent mean cell death size of all RILs within each allelic group.

Table 2. Quantitative trait loci associated with HR-like cell death size in the L58 x R-o-18 RIL population.

QTL	chr	LOD peak [a] (cM)	Peak marker (cM) (Mb)	1.5-LOD interval (cM) (Mb)	R^2 [b]	Effect [c] (mm^2)
Pbc1	A02	5.63 (86)	BrID11121 (85.4) (23.52)	899118|9904922 - BrID11907 (64.35 - 91.07) (8.65 – 25.29)	17.90	0.45
Pbc2	A03	3.33 (129)	BrID90099 (129.2) (31.58)	900988|9961556 - E3552M3 (110.88 – 147.46) (25.33 – 38.15) [d]	6.35	-0.27
Pbc3	A06	4.15 (64)	BrID90095 (63.9) (9.32)	BrID10649 - BrID90309 (61.94 - 72.63) (8.07 – 20.14)	7.32	-0.28

[a] LOD threshold of MQM estimated after 1000 permutations and 5% error rate was 2.59.
[b] R^2 indicates the percentage of additive phenotypic variance explained by each QTL.
[c] Effect size of each QTL, calculated as $\mu_A - \mu_B$, where μ_A is the mean of RILs with the L58 allele and μ_B is the mean of RILs with the R-o-18 allele.

Validation of QTL effects on few selected RIL lines

The validation of QTL effects was carried out on twelve selected RIL lines which showed contrasting genotypes at the peak markers of the three QTLs *Pbc1-3* (Supplementary Fig. S7). Overall, we observed differences in egg-induced cell death between RILs (ANOVA: $F_{11,24} = 5.06$, $P < 0.001$), mostly due to allelic differences at *Pbc1* (BrID11121), as the RILs with the L58 allele showed larger cell death size. Analysis of QTL effects with a three-way ANOVA showed a significant main effect for *Pbc1* ($F_{1, 32} = 84.02$, $P < 0.001$) and *Pbc3* ($F_{1, 32} = 10.91$, $P = 0.002$). The effect of *Pbc3* was only significant upon inclusion of *Pbc1* in the model (Fig. 4b), while no effect was detected for *Pbc2* ($F_{1, 32} = 3.05$, $P = 0.09$) (Fig. 4a). Analysis of the *Pbc1-Pbc3* haplotypes highlighted the large effect of the *Pbc1-L58* allele and a marginal effect of both *Pbc2-R-o-18* and *Pbc3-R-o-18* alleles.

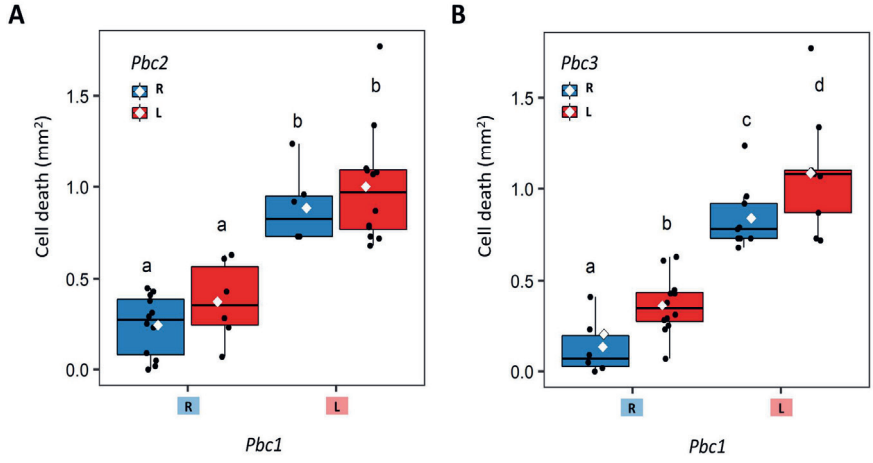


Figure 4. Validation of QTL effects and additive interactions for QTLs *Pbc1-3* on selected RILs. Twelve RILs ($N = 3$) with contrasting genotypes at the peak markers of QTLs *Pbc1–3* were selected randomly for a second phenotypic evaluation with *P. brassicae* egg clutches. RILs are grouped by genotype at the peak markers to show pairwise effects and additive interactions between QTLs. A) interaction between *Pbc1* (BrID11121) and *Pbc2* (BrID90099). B) interaction between *Pbc1* and *Pbc3* (BrID90095). Blue box with "L" = L58 allele, red box with "R" = R-o-18 allele. Boxplots represents the interquartile range (1st and 3rd quantile) and the median. White diamonds represent mean cell death of each QTL genotype. Letters report differences in mean size of HR-like cell death between haplotypes (Tukey's HSD test, $P < 0.01$).

Identification of candidate genes underlying the QTLs

We investigated the genomic locations of the three QTLs for potential candidate genes associated with HR-like cell death using the *B. rapa* reference genome cv. Chiifu (v3.0). We searched for annotated genes that encode for cell surface receptors (PRRs), intracellular receptors (NLRs), or that are involved in general plant defense mechanisms, such as ROS

production and cell death (Supplementary Tables S8-10). The QTL *Pbc1* showed the largest effect with the allele of the L58 parent contributing to a large cell death and it was located at the interval 8.65-25.29 Mb (± 1.5 LOD) on chromosome A02. This region contains 2012 annotated genes, of which 69 are related to the plant immunity and defense (Supplementary Table S8). Among them, we found 14 cell surface receptors (of both the RLK and RLP type) and 19 intracellular TIR-NBS-LRR (TNL) receptors. Sixteen of the TNLs are closely located in three clusters, at the intervals 12.47–12.55 Mb, 21.64–21.73 Mb and 22.68–22.99 Mb. Moreover, *Pbc1* also includes *B. rapa* homologs to three *RLKs* previously found to be upregulated upon oviposition in *A. thaliana* (Little *et al.* 2007): i.e., *BraA02g017190.3C* homolog of a LRKL domain-kinase protein (AT1G66880), *BraA02g022170.3C* and *BraA02g022180.3C* homologs of *NEMATODE-INDUCED LRR-RLK 1* (*NILR1*, AT1G74360) and *BraA02g033550.3C* and *BraA02g033570.3C*, homologs of *PBS1-LIKE 19* (AT5G47070). Further, *Pbc1* region includes genes involved in mediating cell death processes, such as *BraA02g032910.3C* and *BraA02g032940.3C* that are both homologs of *A. thaliana ACCELERATED CELL DEATH 11* (*ACD11*), and *BraA02g033670.3C*, a homolog of *BAX-INHIBITOR 1* (*BI-1*, AT5G47120).

The minor QTL *Pbc2* was located in the interval 25.34-38.15 Mb on A03 (± 1.5 LOD) and included 1594 genes in total, of which 49 being plant immunity-related genes (Supplementary Table S9). Within *Pbc2* we found different types of PRRs such as a cluster of 15 cysteine-rich RLKs (CRKs) at the interval 25.91-26.32 Mb and BraA03g059300.3C, homolog of *L-TYPE LECTIN RECEPTOR KINASE I.9* (*LecRK-I.9*). In this region we found also NLRs, specifically a cluster of four TIR-NBS-LRR at the interval 25.52-25.56 Mb. Further, this region included also BraA03g053480.3C and BraA03g057870.3C, homologs of two known regulators of plant immunity, i.e. *SUPPRESSOR-OF-NPR1 CONSTITUTIVE 4 (SNC4)* and *BRI1-ASSOCIATED RECEPTOR KINASE (BAK1)*, respectively, and two homologs of putative *RESPIRATORY BURST OXIDASE HOMOLOGUE G* (*RbohG*) genes. The third QTL, *Pbc3,* was located between 6.77 and 16.13 Mb on A06 (± 1.5 LOD). This region included a total of 2292 genes, of which 28 plant defense-related genes (Supplementary Table S10). Within *Pbc3* we found homologs of *RbohD* and *RbohJ*, different types of RLKs, i.e. homologs to two *WALL-ASSOCIATED RECEPTOR KINASES 1* (*WAK1*, AT1G21250) and *2* (*WAK2*, AT1G21270), and, interestingly, a cluster of four L-type LecRKs including LecRK-I.1, that was recently associated to *P. brassicae* egg extract-induced cell death in *A. thaliana* (Groux *et al.* 2021b).

Given that *Pbc3* appeared to overlap with one of the two loci identified in *A. thaliana* by Groux *et al.* (2021b), we investigated the syntenic relationship between *Pbc1-3* regions and *A. thaliana* genome. *Pbc1* was syntenic to regions on *A. thaliana* chromosomes 1, on the top of chromosome 4 and on middle of chromosome 5 (Supplementary Fig. S8). *Pbc2,* which was located to the bottom of chromosome A03, showed synteny to the bottom half of *A. thaliana* chromosome 4. *Pbc3*, which is located on the center of chromosome A06, was syntenic to regions on both *A. thaliana* chromosomes 3 and 5. Indeed, *Pbc3* was syntenic to the region of *A. thaliana* chromosome 3 that included LecRK-I.1, candidate gene associated with egg extract-induced cell death (Groux *et al.* 2021b). Overall, *Pbc1* and *Pbc2* represented novel loci mediating HR-like cell death as they did not show synteny to the two loci previously identified in *A. thaliana*.

Discussion

Here, we report the first genetic analysis of a butterfly egg-induced defence trait in an economically important crop, *B. rapa*. We showed intraspecific variation for *P. brassicae* egg-induced HR-like cell death within a *B. rapa* germplasm collection. By developing an automated image-based phenotyping protocol, we could accurately measure HR-like cell death size in a RIL population and identified three new QTLs associated with this trait. The three QTLs include many candidate genes that are involved in plant immunity processes such as extra- and intra-cellular receptors, ROS production, and cell death.

Genetic mapping of egg-induced cell death identified three QTLs, *Pbc1* (on chromosome A02), *Pbc2* (A03) and *Pbc3* (A06) that together explained about 31.5% of the phenotypic variance. Thus, in *B. rapa*, HR-like cell death size appears to be a polygenic trait similar to whitebacked planthopper egg-induced lesions in rice (Yang *et al.* 2014) or other pathogen-induced leaf necrotic symptoms (Corwin *et al.* 2016, Yates *et al.* 2019). None of the three QTLs reported here have been validated yet, for example by using alternative segregating populations. Nevertheless, *Pbc1* may represent a stable QTL as it explained the larger proportion of variance (17.9%), its confidence interval was entirely above the LOD threshold and it contributed to larger cell death with the allele of L58, the parent showing a stronger HR-like cell death. On the contrary, *Pbc2* and *Pbc3* represented minor QTLs, their LOD peaks were just above the LOD threshold, and their positive effect was due to alleles of R-o-18, the

parent showing a smaller HR-like cell death. The unexplained phenotypic variance may be due to other undetected minor QTLs for which we expect that L58 alleles contribute to a larger cell death. In fact, only few RILs showed transgressive segregation beyond the mean value of L58 while the phenotypic distribution of the whole population was skewed towards the value of R-o-18, the parent showing a small cell death. Future research should validate the stability of the QTLs identified in this study, their (epistatic) interactions, and the effect of the plant genetic background by using other genetic populations/association panels and/or testing different environments.

In this study, we implemented the first image-based phenotyping method to assess insect egg-induced cell death on plant tissues and to perform QTL mapping. So far, image-based methods were used for genetics studies of plant disease symptoms (Corwin *et al.* 2016, Stewart *et al.* 2017, Fordyce *et al.* 2018, Yates *et al.* 2019) or insect feeding damage (Kloth *et al.* 2017, Thoen *et al.* 2017, Visschers *et al.* 2019), but never for egg-induced responses. Nevertheless, our experiments showed an intermediate broad-sense heritability (H^2 = 0.47-0.49) which points at a considerable environmental effect on the trait that our current bioassays and/or phenotyping protocol could not disentangle. Insect genetic variation is known to contribute to low heritability of genetic studies of plant defense traits to insects (Kliebenstein 2017). Unfortunately, at the moment it is difficult to control for the genetic variation of the *P. brassicae* butterflies used in our bioassay, beyond using a large number of adults and refreshing them regularly during experiments. Our imaged-based phenotyping protocol allowed us to measure cell death size with increased precision (highly repeatable measurements) but it can possibly be improved in accuracy (measuring the true size of cell death). Alternatively, the measurement of cell death size could be combined with other cell death-related traits, e.g. severity (variation in lesion colour), to possibly determine traits with higher heritability and increase the power of QTL detection.

The three QTLs *Pbc1-3* provide a new source of candidate genes that will help to understand the molecular mechanisms underlying the interaction between *P. brassicae* eggs and *Brassica* host plants. Within these QTL regions, we identified different type of genes that are commonly involved in plant immunity processes, such as signalling/stress perception, ROS accumulation and cell death formation. Perception of pathogen-associated molecular patterns (PAMPs) by PRRs surface receptors triggers plant immunity (Couto & Zipfel 2016). Many PRRs belonging to different classes were found within the QTLs *Pbc1-3*, although involvement in plant immunity has been experimentally validated only for a few. For example, the *Pbc1* region includes LecRK-V.5 which was previously found to negatively modulate plant

immunity against biotrophic pathogens (Arnaud *et al.* 2012, Desclos-Theveniau *et al.* 2012, Wang *et al.* 2014). Further, *Pbc1* includes also orthologs of three (predicted) RLKs (AT1G66880, AT1G74360, AT5G47070), which were previously found to be upregulated upon *P. brassicae* oviposition in *A. thaliana* (Little *et al.* 2007). One of these three, AT1G74360, encodes the LRR-RLK *NILR1* that was found to be required for plant immunity to nematodes in a *BAK1*-dependent manner (Mendy *et al.* 2017). BAK1 is a known central regulator of pathogen-triggered immunity which works as a co-receptor of PRRs mediating the perception of many different biotic stresses (Roux *et al.* 2011), including feeding by insects, such as aphids and caterpillars (Yang *et al.* 2011, Vincent *et al.* 2017). Interestingly, one *B. rapa* ortholog of BAK1, BraA03g057870.3C, is present within our QTL *Pbc2*. Whether BAK1 is also involved in the regulation of defences against *Pieris* eggs is an intriguing question that awaits future experimental validation. *Pbc2* includes also many cysteine-rich RLKs (*CRK*s), of which *CRK5* was experimentally shown to mediate pathogen-induced cell death (Chen *et al.* 2004) and *CRK11* was upregulated upon *P. brassicae* egg deposition (Little 2007). Finally, QTL *Pbc3* includes a cluster of *B. rapa* orthologs of LecRK-I genes, among which LecRK-I.1, which was recently identified as one of the two *A. thaliana* loci associated with *Pieris* egg extract-induced cell death (Groux *et al.* 2021b). Considering the large confidence intervals of the QTL reported here, we cannot point yet at specific PRRs and/or RLKs as the casual genes responsible for the variation in HR-like cell death observed within our plant material.

Despite the many different RLKs present within the QTL *Pbc1-3*, it is still possible that variation in cell death size is dependent on other genetic mechanisms. Within our QTLs, we identified clusters of intracellular TIR-NBS-LRRs (TNLs) and a *B. rapa* homolog N REQUIREMENT GENE 1 (NRG1). NRG1 was shown to interact with ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and SENESCE-ASSOCIATED GENE 101 (SAG101) to form a protein complex that is required for TNL cell death signalling (Lapin *et al.* 2019, Sun *et al.* 2021). It is interesting to recall that EDS1 and PHYTOALEXIN DEFICIENT 4 (PAD4), which forms with EDS1 another complex that is also required for TNL-mediated cell death (Wagner *et al.* 2013, Sun *et al.* 2021), were upregulated in *A. thaliana* upon *P. brassicae* egg deposition (Little *et al.* 2007). Despite all this, it is still largely speculative to state that insect eggs may be perceived by intracellular TNLs in absence of evidences on whether and how egg-associated molecular patterns can get into contact with the inside of plant cells.

Some plant species have been shown to accumulate ROS underneath insect eggs, such as *A. thaliana, Pinus sylvestris* or *Brassica* spp. (Little *et al.* 2007, Bittner *et al.* 2017, Caarls

et al. 2021), while others use ROS accumulation to directly kill eggs such as *Solanum dulcamara* (Guess *et al.* 2017). Hence, it is suggested that ROS signalling in response to insect eggs is conserved in different plant species (Lortzing *et al.* 2020). We identified different NADPH oxidases (Rboh proteins) which are known to be involved in production of ROS (Torres 2010). Further, we found genes involved in cell death regulation such as the *B. rapa* orthologs to BI-1 and ACD11 within QTL *Pbc1*. While BI-1 is a known suppressor of H_2O_2-dependent cell death (Ishikawa *et al.* 2013) and has been associated with the cell death regulation in plant-powdery mildew interaction (Weis *et al.* 2013), ACD11 is involved in autoimmunity (Brodersen *et al.* 2002) and activation of cell death and defence responses (Li *et al.* 2019). Finally, we also found a homolog of ICS1/SID2 within *Pbc1*. This enzyme produces the precursor of SA (Lefevere *et al.* 2019), the main phytohormone so far associated with plant defences to insect eggs (Little *et al.* 2007, Bruessow *et al.* 2010, Gouhier-Darimont *et al.* 2013, Lortzing *et al.* 2019). Overall, the QTLs here presented include relatively large regions containing thousands of genes. Thus, future fine-mapping efforts are necessary to increase the resolution on the QTLs here reported and determine the exact genetic mechanisms of *P. brassicae* egg-induced cell death in *B. rapa*.

The identification of QTLs involved in HR-like cell death size in *B. rapa* also allows comparisons with the model plant *A. thaliana*. QTLs *Pbc1* and *Pbc2* showed no synteny to the two loci recently identified in *A. thaliana* (Groux *et al.* 2021b), thus representing novel genomic regions associated with *P. brassicae* egg-induced cell death. Interestingly, the QTL *Pbc3* is syntenic to the *A. thaliana* locus on chromosome 3 containing a cluster of clade I L-type LecRKs. The comparison in genetic architectures of HR-like cell death between the *B. rapa* and *A. thaliana* is intriguing but too premature to point at communalities and specificities. In fact, there are crucial differences in the plants' responses, experimental setup and plant genetic material employed by the different studies. HR-like cell death in *A. thaliana* was visually scored in discrete categories which accounted also for light/strong chlorosis and light/strong cell death (Groux *et al.* 2021b). On the contrary, in our study no chlorosis was observed and only cell death size was measured. Different phenotyping methods/parameters can affect genetic mapping results and indeed it was previously shown that shape and size of leaf disease symptoms can be genetically unliked in different pathosystems (Yates *et al.*2019, Méline *et al.* 2021). Moreover, bioassays on *A. thaliana* were conducted by treating plants with *P. brassicae* egg extract, which was shown to also induce cell death in *B. nigra* (Groux *et al.* 2021b). Nonetheless, egg extract likely contains lipidic compounds from the inner egg tissues and it is still unclear whether and how they are able to diffuse through the egg and reach the

leaf surface (Stahl *et al.* 2020). Clearly, more research is needed to further disentangle the genetic architectures of egg-induced cell death in *A. thaliana* and *B. rapa.*

It is remarkable that *B. rapa* showed a phenotypic variation in HR-like cell death that was limited to necrotic black spots varying in size. A similar mild cell death appearing as black spots was also observed underneath *P. brassicae* eggs on a limited number of *B. oleracea* accessions (Reymond 2013, Griese *et al.* 2021). This mild cell death contrasts sharply with the strong and severe cell death that we regularly observe on leaves of wild species of the tribe Brassiceae (Brassicaceae Lineage II), such as in *Brassica nigra*, *Sinapis* spp., and *Crambe* spp., which leads to reduced egg survival (Griese *et al.* 2017, 2021). The differences in HR-like cell death between wild Brassicaceae, e.g. *Brassica nigra*, and other *Brassica* crops raise questions on the role of crop domestication on this defence trait. *Brassica* crops are characterized by an extraordinary intraspecific diversity in morphotypes which differ significantly from the progenitor wild relatives as result of domestication (Cheng *et al.* 2016, Mabry *et al.* 2021, McAlvay *et al.* 2021). The selection for specific crop morphotypes, but also for quality traits, such as flavour, taste and storage, mostly targeted leaf morphological and/or biochemical traits, which often show trade-offs with overall plant defense traits (Turcotte *et al.* 2014, Whitehead *et al.* 2017). Whether similar trade-offs also impacted the HR-like cell death expressed by current *Brassica* crop types should be tested. Certainly, we cannot yet conclude to have captured the full extent of intraspecific phenotypic variation as we screened only 56 accessions from one representative *B. rapa* core collection (Del Capio *et al.* 2010, Zhao *et al.* 2010). Our choice aimed at encompassing the overall genetic diversity of the core collection while representing all species morphotypes. Nevertheless, we may have missed accessions with a stronger HR-like phenotype and/or alternative variants at the identified QTLs. In summary, future germplasm screenings should not only include more accessions, but also include *Brassica* wild material. What is the genetic basis for the differences in HR-like cell death severity between wild Brassicaceae, e.g. *Brassica nigra*, and other *Brassica* crops is a fascinating question that deserves to be addressed in future research.

The mild HR-like cell death observed in our *B. rapa* germplasm was shown to not affect egg survival (Griese *et al.* 2021). This is expected to have certain implications regarding its deployment as egg-killing defense trait in plant breeding. In future, screening of more germplasms within *Brassica* crops, including crop wild relatives, could still identify strong HR-like cell death. An alternative possibility for deploying it as crop defense trait could be via introgression from *B. nigra*. Interspecific introgression of other disease resistance traits is a viable option as it is already being pursued within the *Brassica* genus by using interspecific

crosses, embryo rescue, and marker-assisted selection (Diederichsen *et al.* 2009, Lv *et al.* 2020).

Conclusion

We report the identification of the first QTLs associated with a HR-like cell death induced by *Pieris* butterfly eggs in the economically important crop *B. rapa*. Our study confirms that plant genetic factors are involved in the elicitation of a HR-like cell death, a plant defense response against insect eggs. This work provides the basis for further identification of genes mediating the interaction between butterfly eggs and plants. Future studies should validate the QTLs by screening other genetic populations and/or association panels. Fine-mapping of the identified QTLs would then help to increase the resolution of the loci and further elucidate the genetic regulation of the egg-induced HR-like cell death.

Acknowledgements

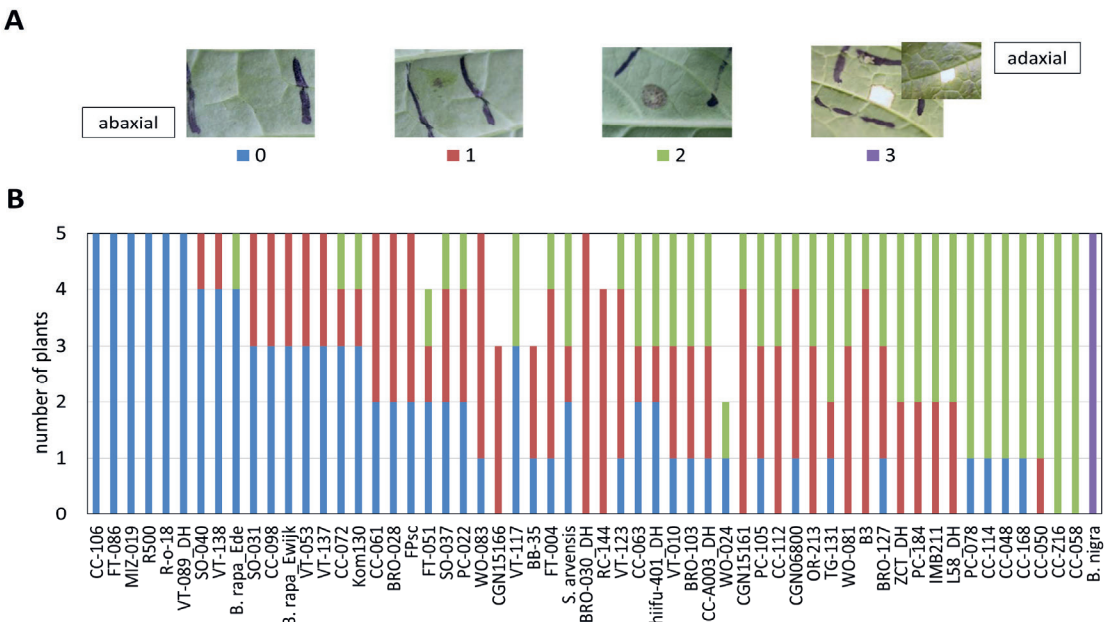
We are thankful to: Johan Bucher (WUR Laboratory of Plant Breeding) for providing us seeds of the *B. rapa* core collection and the Centre for Genetic Resources the Netherlands (CGN) for providing additional accessions; the employees of Unifarm (WUR) for taking care of the plants used in our experiments; Pieter Rouweler and Andre Gidding for rearing of *Pieris brassicae*; Eline Ampt for the introduction to ImageJ. We are grateful to Dr. Ben Vosman for his input on the experimental designs of both germplasm and RILs screening. Oviposition on the RIL population was possible thanks the help of Dr. Thijmen Breeschoten, Frank Becker, Marco Busscher, Nynke Groendijk-Wilders, Dr. Deedi Sogbohossou and Xiuji Wang. We finally thank Dr. Marcos Malosetti and Roel van Bezouw for help with statistical analysis and QTL mapping.

Availability of data and materials

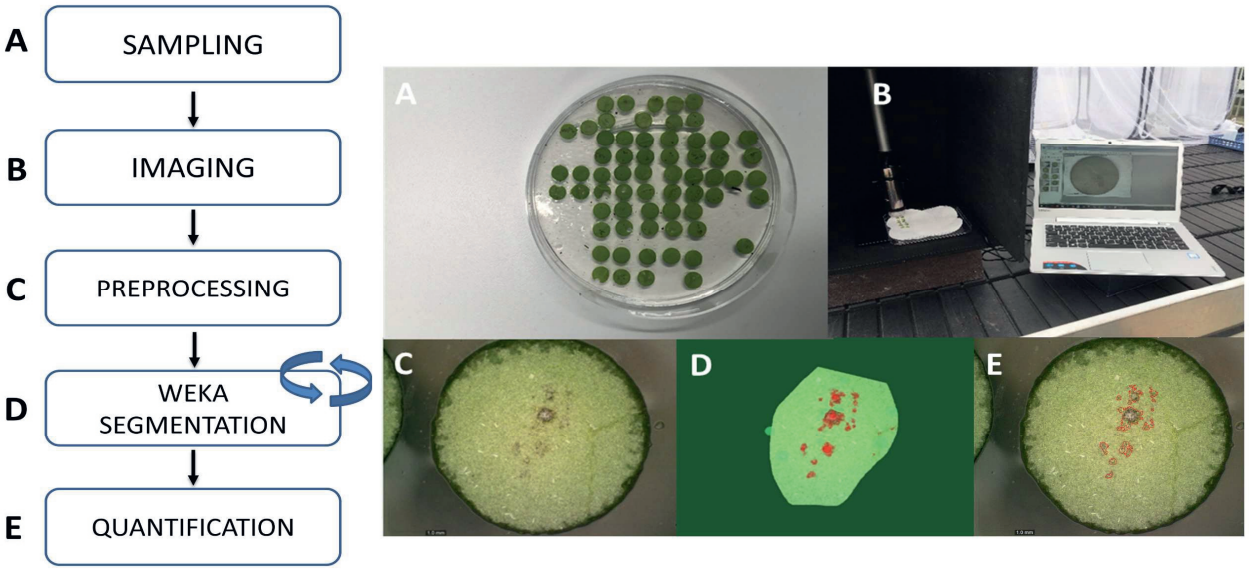
All data generated or analyzed during this study are included in this published article (Additional file 3). Datasets and scripts used for data analysis are also available in a Zenodo repository (https://doi.org/10.5281/zenodo.6014948).

Supplementary figures

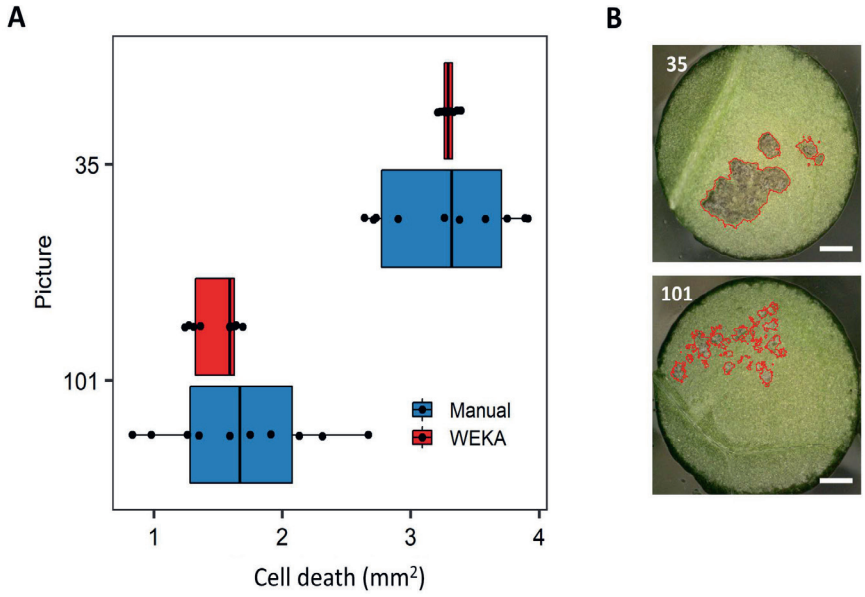
Supplementary tables are available online as Additional file 1 at https://doi.org/10.1186/s12870-022-03522-y.



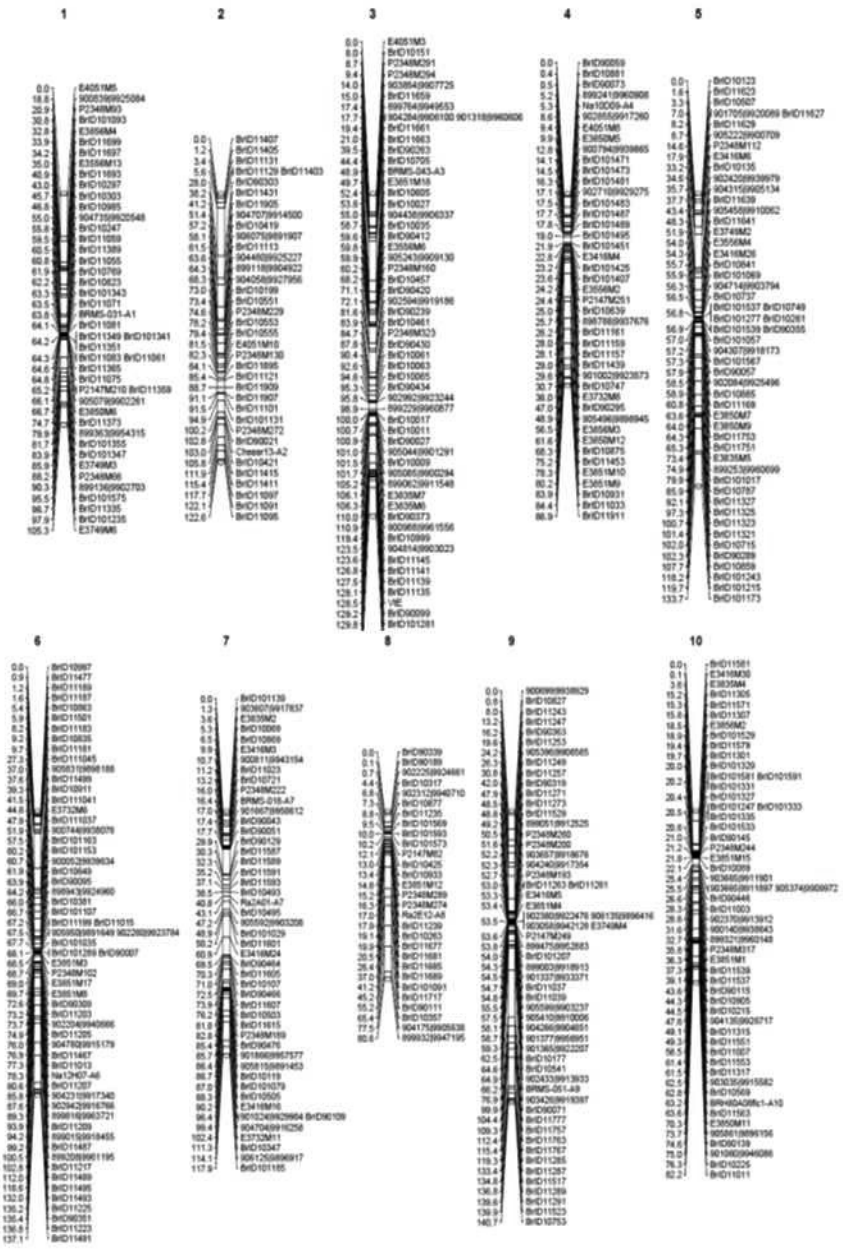
Supplementary Figure S1. Germplasm screening of 56 B. rapa accessions reveals variation in HR-like cell death. (A) Cell death induced by egg wash treatment was scored with a scale from 0 to 3 where: 0, no visible cell death (blue); 1, small grey/dark spot smaller than the treatment spot (red); 2, black necrotic spot covering the whole treated area (green); 3, strong cell death visible also on the adaxial side (purple). (B) Distribution of HR-like cell death scores within each B. rapa accession (N = 3-5). Plants were treated with two 5 µl P. brassicae egg wash droplets on the abaxial side of two fully developed leaves. Each plant was assigned the score of the spot with the most severe cell death.



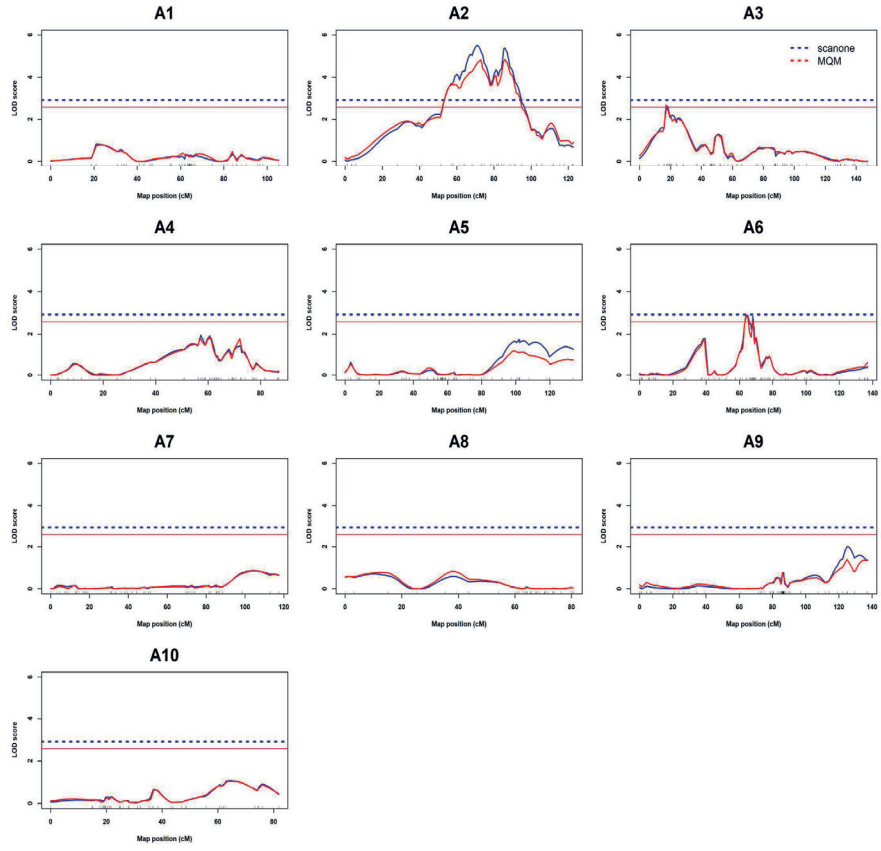
Supplementary Figure S2. Image-based phenotyping protocol developed to quantify HR-like cell death size on leaves. (A) Leaf discs are sampled from a plant on the spots with either eggs or egg wash and stored on Petri dishes with 1% phytoagar or wet filter paper. The choice of Petri dishes substrate should aim to keep the sample moist, to reduce background light reflections and to achieve high quality imaging. **(B)** Leaf discs are imaged with a DinoLite electronic microscope connected to a laptop. This light-weight setup is suitable to perform imaged-based phenotyping directly at the experimental site (greenhouse or field). Further, DinoLite microscope includes a polarizer light that helps to reduce light reflections on the samples. **(C)** Images are imported in Fiji and different pre-processing steps can be performed to maximize the contrast between the HR-like cell death, or other phenotypes of interest, and the background healthy leaf tissue. **(D)** The Fiji plugin Trainable WEKA Segmentation is used to obtain a machine learning-based image segmentation. This step can be repeated to increase the segmentation accuracy. **(E)** The segmented HR-like cell death is finally quantified. Highlighting the measured area with a red border helps to visually assess the segmentation accuracy.



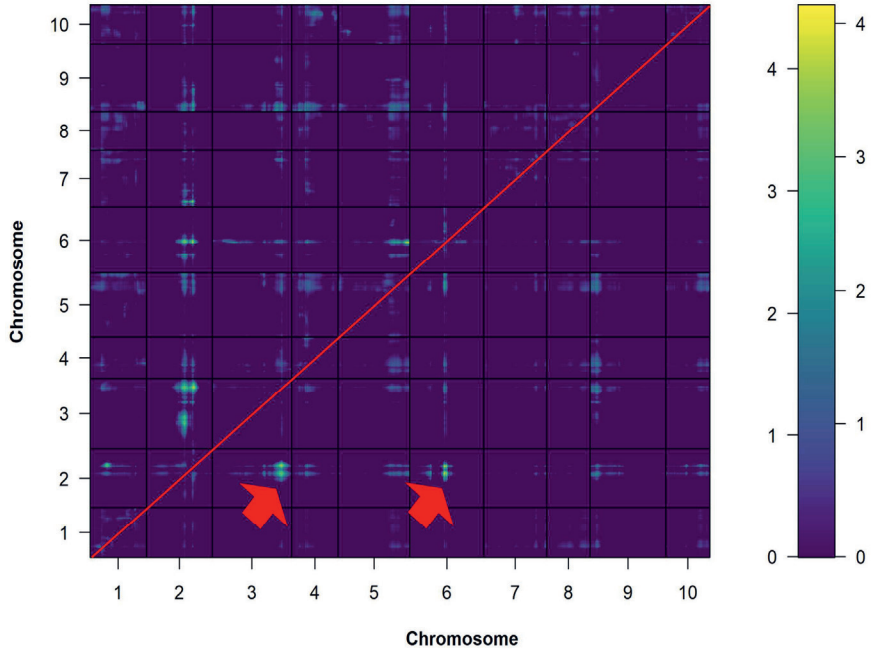
Supplementary Figure S3. Replicability of two image segmentation methods to quantify HR-like cell death size. (A) Leaves with either large cell death (sample "35") or weak cell death (sample "101") were used to compare the two methods. "Manual" indicates an image manual thresholding based on the pixels color distribution. "WEKA" indicates an automated segmentation performed with the Trainable WEKA Segmentation plugin in Fiji. Each image was re-analysed ten times with each method (N=10). (B) One representative result of a WEKA segmentation of cell death for both picture 35 and 101. Red margins indicate the area that was quantified. Magnification bars inside photos = 1 mm.



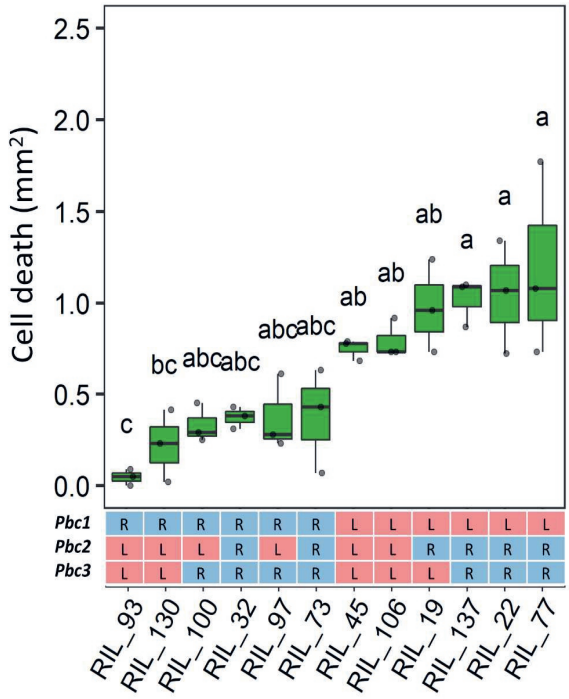
Supplementary Figure S4. Genetic linkage map for the L58 x R-o-18 RIL population. 485 markers are grouped in 10 linkage groups which represents the 10 B. rapa chromosomes (*Brassica* A genome).



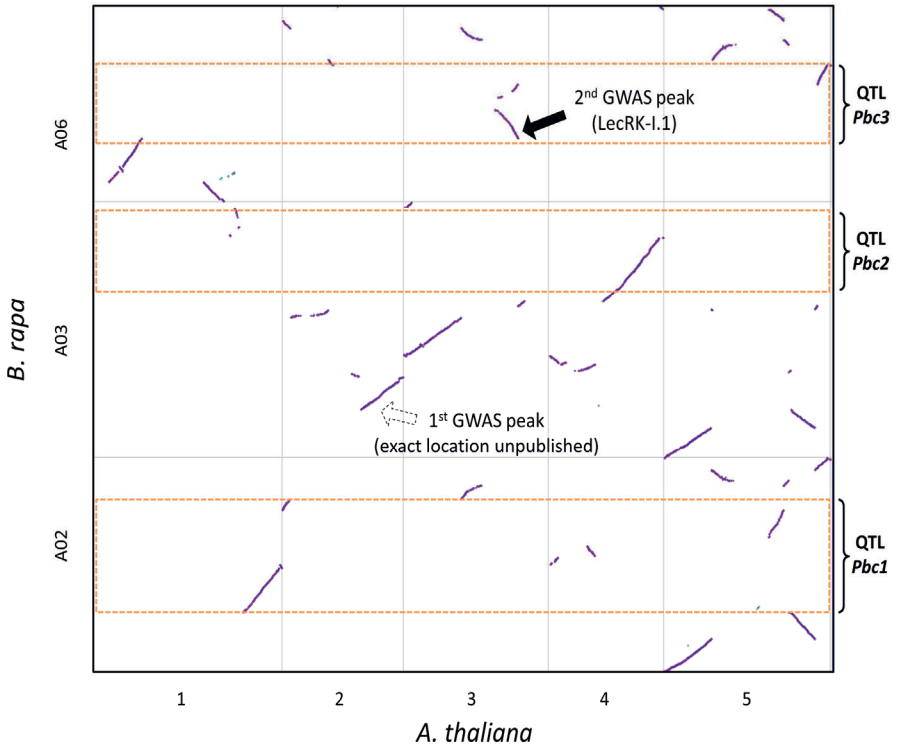
Supplementary Figure S5. Quantitative trait loci for HR-like cell death size in the L58 x R-o-18 RIL population. LOD scores profiles of single-QTL models (blue) and MQM models (red) are represented. LOD significance thresholds after 1000 permutation at 5% error rate were, respectively, 2.90 and 2.59 and they are indicated with dashed lines.



Supplementary Figure S6. Heatmap of genome-wide LOD scores of two-QTL models to investigate epistatic interaction and additive effects. Upper triangle shows LOD scores of models testing epistatic interaction effects, lower triangle shows LOD scores of models testing only additive effects (interaction excluded from the model). Only significant additive effects were detected between *Pbc1*:*Pbc3* and *Pbc1:Pbc6* (red arrows). LOD score thresholds were 6 for interaction models and 3.92 for additive models. All LOD thresholds were calculated after 1000 permutations at 5% error rate. Axes represent the 10 *B. rapa* chromosomes. LOD scores intensity are colored according to the colour bar (right side of the panel). Left side of the bar indicates LOD scores intensity for the upper triangle, right side indicates LOD scores intensity for the lower triangle.



Supplementary Figure S7. Phenotypic distribution of HR-like cell death in twelve selected RILs to validate QTL effects. Twelve RILs with contrasting genotypes at the peak markers of QTLs *Pbc1-3* were selected randomly for a second phenotypic evaluation. Colored boxes below each boxplot represent the RIL genotype at the peak markers for *Pbc1* (BrID11121), *Pbc2* (BrID90099) and *Pbc3* (BrID90095). Blue box with "L" = L58 allele, red box with "R" = R-o-18 allele. Each RIL (N = 3) was oviposited with two egg clutches and cell death size was quantified using a custom image-based phenotyping protocol. The largest cell death out of the two clutches was assigned to each plant. Boxplots represents the interquartile range (1st and 3rd quantile) and the median. Letters report differences in mean size of HR-like cell death between RILs (Tukey's HSD test, $P < 0.01$).



Supplementary Figure S8. Synteny analysis between *B. rapa* quantitative trait loci for cell death size and *A. thaliana*. Dotplot showing the syntenic relationship between *B. rapa* chromosomes A02, A03, A06 (y-axis) and *A. thaliana* (At) chromosomes 1-5 (x-axis). Regions highlighted are the QTLs *Pbc*1-3 (±1.5 LOD interval) identified in this study (rectangles with orange dashed lines). The two arrows represent the locations of two loci identified in a GWAS study in A. thaliana (Groux *et al.* 2021b). The dashed white arrow indicates an approximate location for the locus on chromosome At 2 as the exact location has not been released by the authors. The black arrow indicates the exact location of a LecRK-I cluster, including LecRK-I.1, on chromosome At 3. Axes represent increasing nucleotides positions starting at 0 bp on the bottom right corner for each chromosome.

Supplementary tables

Supplementary tables are available online as Additional file 2 at https://doi.org/10.1186/s12870-022-03522-y.

Supplementary Table S1. List of *B. rapa* accessions used for the germplasm screening.

Supplementary Table S2. Evaluation of 56 *B. rapa* accessions for *P. brassicae* egg wash-induced necrosis.

Accession	*B. rapa* subtaxa	Morphotype group	N	Cell death score (Mean)	Dunn`s test [a]
CC-106	subsp. pekinensis	Chinese Cabbage	5	0	a
FT-086	subsp. rapa	Fodder Turnip	5	0	a
MIZ-019	subsp. nipposinica	Mizuna	5	0	ab
R500	subsp. trilocularis	Yellow Sarson	5	0	abc
R-o-18_DH	subsp. trilocularis	Yellow Sarson	5	0	abc
VT-089_DH	subsp. rapa	Vegetable Turnip	5	0	abc
SO-040	subsp. oleifera	Spring Oil	5	0.2	abcd
VT-138	subsp. rapa	Vegetable Turnip	5	0.2	abcd
*B. rapa*_Ede	-	-	5	0.4	abcde
*B. rapa*_Ewijk	-	-	5	0.4	abcde
cCC-098	subsp. pekinensis	Chinese Cabbage	5	0.4	abcde
SO-031	subsp. oleifera	Spring Oil	5	0.4	abcde
VT-053	subsp. rapa	Vegetable Turnip	5	0.4	abcde
VT-137	subsp. rapa	Vegetable Turnip	5	0.4	abcde
BRO-028	var. ruvo	Broccoletto	5	0.6	abcde
CC-061	subsp. pekinensis	Chinese Cabbage	5	0.6	abcde
CC-072	subsp. pekinensis	Chinese Cabbage	5	0.6	abcde
FPsc	subsp. oleifera	Rapid cycling	5	0.6	abcde
KOM-130	var. perviridis	Komatsuna	5	0.6	abcde
BB-35	-	-	3	0.6	abcdef
FT-051	subsp. rapa	Fodder Turnip	4	0.75	abcde
PC-022	subsp. chinensis	Pak Choi	5	0.8	abcde
SO-037	subsp. oleifera	Spring Oil	5	0.8	abcde
VT-117	subsp. rapa	Vegetable Turnip	5	0.8	abcdef
WO-083	subsp. oleifera	Winter Oil	5	0.8	abcde
BRO-030_DH	var. ruvo	Broccoletto	5	1	abcdef

Supplementary Table S2. Continued.

CC-063	subsp. pekinensis	Chinese Cabbage	5	1	abcdef
CGN06800	subsp. rapa	Fodder turnip	5	1	abcdef
Chiifu-401_DH	subsp. pekinensis	Chinese Cabbage	5	1	abcdef
FT-004	subsp. rapa	Fodder Turnip	5	1	abcdef
CGN15166	var. ruvo	Broccoletto	3	1	abcde
RC-144	subsp. oleifera	Rapid cycling	4	1	abcdef
S. arvensis	-	-	5	1	abcdef
VT-123	subsp. rapa	Vegetable Turnip	5	1	abcdef
WO-024	subsp. oleifera	Winter Oil	2	1	abcdef
B3	subsp. oleifera	Rapid cycling	5	1.2	abcdef
BRO-103	var. ruvo	Broccoletto(Caixin)	5	1.2	abcdef
BRO-127	var. ruvo	Broccoletto(Turnip Geen)	5	1.2	abcdef
CC-A003_DH	subsp. pekinensis	Chinese Cabbage	5	1.2	abcdef
CGN15161	var. ruvo	Broccoletto	5	1.2	abcdef
PC-105	var. narinosa	Wutacai	5	1.2	abcdef
VT-010	subsp. rapa	Vegetable Turnip	5	1.2	abcdef
CC-112	subsp. pekinensis	Chinese Cabbage	5	1.4	abcdef
OR-213	subsp. oleifera	Chinese Oil Rape	5	1.4	abcdef
TG-131	var. perviridis	Turnip Green	5	1.4	abcdef
WO-081	subsp. oleifera	Winter Oil	5	1.4	abcdef
PC-184	subsp. chinensis	Pak Choi	5	1.6	abcdef
ZCT_DH	var. purpuraria	Zi Tai Cai	5	1.6	abcdef
IMB211	subsp. oleifera	Rapid cycling	5	1.6	bcdef
L58_DH	var. parachinensis	Caixin	5	1.6	bcdef
PC-078	var. parachinensis	Caixin	5	1.6	bcdef
CC-048	subsp. pekinensis	Chinese Cabbage	5	1.6	cdef
CC-114	subsp. pekinensis	Chinese Cabbage	5	1.6	cdef
CC-168	subsp. pekinensis	Chinese Cabbage	5	1.6	def
CC-050	subsp. pekinensis	Chinese Cabbage	5	1.8	def
CC-058	subsp. pekinensis	Chinese Cabbage	5	2	ef
CC-Z16	subsp. pekinensis	Chinese Cabbage	5	2	ef
B. nigra	-	-	5	3.0	-

[a] Dunn`s post hoc test with Benjamini–Hochberg correction, $\alpha < 0.05$

Supplementary Table S3. Summary statistics of *B. rapa* homozygous lines re-evaluated for egg- and egg wash-induced cell death.

Genotype	genetic background	N	Eggs-induced cell death (mm^2)					Egg wash-induced cell death (mm^2)				
			Range	Mean	SD [a]	se [b]	Tukey`s HSD [c]	Range	Mean	SD [a]	se [b]	Tukey`s HSD [c]
CC-106	inbred	9	0 - 0.15	0.07	0.06	0.02	a	0 - 0.03	0.01	0.01	0.00	a
R-o-18	inbred	9	0.09 - 0.63	0.24	0.19	0.06	ab	0 - 0.33	0.12	0.13	0.04	ab
BRO-030	DH	6	0.07 - 0.9	0.41	0.33	0.13	abc	0 - 0.56	0.13	0.22	0.09	ab
BRO-127	inbred	9	0.16 - 1.03	0.43	0.29	0.10	abc	0 - 0.33	0.11	0.11	0.04	ab
R500	DH	9	0.28 - 1.03	0.57	0.20	0.07	bc	0 - 0.41	0.10	0.14	0.05	ab
CC-A03	DH	9	0.06 - 1.33	0.61	0.43	0.14	bc	0.03 - 1.04	0.44	0.39	0.13	bc
CC-168	inbred	9	0.2 - 1.01	0.68	0.28	0.09	bc	0.02 - 0.48	0.30	0.13	0.04	b
SO-040	inbred	7	0.04 - 1.32	0.68	0.53	0.20	bc	0 - 0.65	0.25	0.25	0.09	ab
IMB211	inbred	9	0.2 - 2.94	1.20	0.88	0.29	c	0 - 2.39	1.24	0.70	0.23	cd
L58	DH	8	0.73 - 2.36	1.23	0.55	0.19	c	0.55 - 1.74	1.33	0.40	0.14	d
B. nigra	heterogeneous wild material	6	0.92 - 4.22	2.76	1.11	0.45	-	2.77 - 8.77	6.00	2.40	0.98	-
genetic CV				33.6					70.19			
environmental CV				35.92					52.94			
broad-sense heritability (H^2)				0.47					0.64			

[a] standard deviation
[b] standard error
[c] Tukey`s HSD post hoc test with Benjamini–Hochberg correction, $\alpha < 0.01$

Supplementary Table S4. List of SNPs that were used to design PCR markers and construct a genetic map. (available online)

Supplementary Table S5. List of InDels that were used to design PCR markers and construct a genetic map. (available online)

Supplementary Table S6. Genetic linkage map constructed with the L58 x R-o-18 RIL population. (available online)

Supplementary Table S7. Summary of the L58 x R-o-18 RIL population genetic map.

Chr.	Loci	Length (cM)	Ave. spacing (cM)	Max spacing (cM)
A01	45	105.3	2.3	18.8
A02	37	122.6	3.3	22.4
A03	60	147.5	2.5	18.5
A04	43	86.9	2.0	11.0
A05	53	133.7	2.5	15.3
A06	60	137.1	2.3	17.7
A07	48	117.9	2.5	12.2
A08	29	80.6	2.8	12.0
A09	56	140.7	2.5	23.0
A10	54	82.2	1.5	11.6
whole genome	485	1154.4	2.4	-

Supplementary Table S8. Candidate genes related to plant immunity found within the regions of QTLs *Pbc1* (A02).

*B. rapa*_gene_ID	CDS_start	CDS_end	strand	Ath gene_ID	Ath gene symbol	Ath gene_name	Ath_gene_description_Araport11	Differentially expressed in *A. thaliana* [a]
BraA02g017190.3C	9,310,042	9,315,781	+	AT1G66880			Protein kinase superfamily protein;(source:Araport11)	+
BraA02g017210.3C	9,384,825	9,390,857	-	AT1G66930	LRK10L2	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR- LIKE PROTEIN KINASE-LIKE2 (LRK10L2)	Protein kinase superfamily protein;(source:Araport11)	
BraA02g017220.3C	9,391,818	9,395,793	-	AT1G66910			Protein kinase superfamily protein;(source:Araport11)	
BraA02g017240.3C	9,414,259	9,414,805	+	AT1G66980	SNC4	SUPPRESSOR OF NPR1-1 CONSTITUTIVE 4 (SNC4)	Encodes SNC4 (suppressor of npr1-1, constitutive 4), an atypical receptor-like kinase with two predicted extracellular glycerophosphoryl diester phosphodiesterase domains.	
BraA02g017800.3C	9,871,029	9,873,008	-	AT3G59700	LECRK-V.5	L-TYPE LECTIN RECEPTOR KINASE V.5 (LECRK-V.5)	Member of Receptor kinase-like protein family. Represses stomatal immunity induced by Pseudomonas syringae pv. tomato DC3000.	
BraA02g017820.3C	9,883,372	9,883,974	-	AT1G67740	PSBY	PHOTOSYSTEM II BY (PSBY)	PsbY precursor (psbY) mRNA. This single nuclear gene is imported into the chloroplasts where it is processed into two integral membrane proteins with identical topology (PsbY-1 and PsbY-2). The protein appears to bind manganese. Important for the redox control of cytochrome b559.	-
BraA02g018300.3C	10,204,414	10,207,710	-	AT1G68400			leucine-rich repeat transmembrane protein kinase family protein;(source:Araport11)	
BraA02g018310.3C	10,209,611	10,210,977	+	AT5G22690			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)	
BraA02g018320.3C	10,210,980	10,212,950	+	AT5G46450			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)	
BraA02g019010.3C	10,770,440	10,771,566	+	AT1G69170	SBP	SQUAMOSA PROMOTER BINDING PROTEIN (SBP)-DOMAIN TRANSCRIPTION FACTOR 6 (SPL6)	Encodes SPL6. Required for the resistance mediated by the TIR-NB-LRR RPS4 against Pseudomonas syringae carrying the avrRps4 effector. Transcriptome analysis indicates that SPL6 positively regulates a subset of defense genes.	
BraA02g019610.3C	11,253,607	11,255,013	+	AT1G70110	LECRK-V.1	L-TYPE LECTIN RECEPTOR KINASE V.1 (LECRK-V.1)	Concanavalin A-like lectin protein kinase family protein;(source:Araport11)	
BraA02g021090.3C	12,340,910	12,344,545	+	AT1G72520	LOX4	LIPOXYGENASE 4 (LOX4)	PLAT/LH2 domain-containing lipoxygenase family protein;(source:Araport11)	
BraA02g021280.3C	12,469,541	12,470,035	-	AT1G72890			Disease resistance protein (TIR-NBS class);(source:Araport11)	
BraA02g021310.3C	12,511,331	12,515,265	+	AT4G09420			Disease resistance protein (TIR-NBS class);(source:Araport11)	
BraA02g021320.3C	12,522,687	12,524,510	+	AT1G72870			Disease resistance protein (TIR-NBS class);(source:Araport11)	

BraA02g021370.3C	12,551,634	12,553,319	+	AT1G72890			Disease resistance protein (TIR-NBS class);(source:Araport11)	
BraA02g021620.3C	12,644,425	12,645,072	+	AT1G73260	KTI1	KUNITZ TRYPSIN INHIBITOR 1 (KTI1)	Encodes a trypsin inhibitor involved in modulating programmed cell death in plant-pathogen interactions.	+
BraA02g021630.3C	12,646,584	12,647,207	+	AT1G73260	KTI1	KUNITZ TRYPSIN INHIBITOR 1 (KTI1)	Encodes a trypsin inhibitor involved in modulating programmed cell death in plant-pathogen interactions.	+
BraA02g021640.3C	12,647,654	12,648,247	+	AT1G73260	KTI1	KUNITZ TRYPSIN INHIBITOR 1 (KTI1)	Encodes a trypsin inhibitor involved in modulating programmed cell death in plant-pathogen interactions.	+
BraA02g021680.3C	12,660,670	12,661,311	-	AT1G73260	KTI1	KUNITZ TRYPSIN INHIBITOR 1 (KTI1)	Encodes a trypsin inhibitor involved in modulating programmed cell death in plant-pathogen interactions.	+
BraA02g021690.3C	12,665,224	12,665,838	-	AT1G73260	KTI1	KUNITZ TRYPSIN INHIBITOR 1 (KTI1)	Encodes a trypsin inhibitor involved in modulating programmed cell death in plant-pathogen interactions.	+
BraA02g021700.3C	12,667,738	12,668,064	-	AT1G73260	KTI1	KUNITZ TRYPSIN INHIBITOR 1 (KTI1)	Encodes a trypsin inhibitor involved in modulating programmed cell death in plant-pathogen interactions.	+
BraA02g021710.3C	12,671,508	12,671,822	-	AT1G73260	KTI1	KUNITZ TRYPSIN INHIBITOR 1 (KTI1)	Encodes a trypsin inhibitor involved in modulating programmed cell death in plant-pathogen interactions.	+
BraA02g021750.3C	12,694,402	12,695,040	-	AT1G73260	KTI1	KUNITZ TRYPSIN INHIBITOR 1 (KTI1)	Encodes a trypsin inhibitor involved in modulating programmed cell death in plant-pathogen interactions.	+
BraA02g021800.3C	12,722,062	12,722,982	-	AT1G73500	MKK9	MAP KINASE KINASE 9 (MKK9)	member of MAP Kinase Kinase family. Autophosphorylates and also phosphorylates MPK3 and MPK6. Independently involved in ethylene and calmalexin biosynthesis. Induces transcription of ACS2, ACS6, ERF1, ERF2, ERF5, ERF6, CYP79B2, CYP79B3, CYP71A13 and PAD3.	
BraA02g021870.3C	12,765,148	12,768,137	-	AT1G73680	ALPHA DOX2	ALPHA DIOXYGENASE (ALPHA DOX2)	Encodes an alpha dioxygenase. Recombinant protein catalyzes the conversion of a wide range of fatty acids into 2(R)-hydroperoxy derivatives.	+
BraA02g022150.3C	12,960,935	12,964,417	-	AT1G74190	RLP15	RECEPTOR LIKE PROTEIN 15 (RLP15)	receptor like protein 15;(source:Araport11)	
BraA02g022160.3C	12,968,040	12,977,187	-	AT1G74190	RLP15	RECEPTOR LIKE PROTEIN 15 (RLP15)	receptor like protein 15;(source:Araport11)	
BraA02g022170.3C	12,978,848	12,981,109	-	AT1G74360	NILR1	NEMATODE-INDUCED LRR-RLK 1 (NILR1)	NILR1 encodes a serine/threonine kinase involved in defense response to nematodes.	+
BraA02g022180.3C	12,983,483	12,986,073	-	AT1G74360	NILR1	NEMATODE-INDUCED LRR-RLK 1 (NILR1)	NILR1 encodes a serine/threonine kinase involved in defense response to nematodes.	+
BraA02g022190.3C	12,987,394	12,988,985	-	AT1G74190	RLP15	RECEPTOR LIKE PROTEIN 15 (RLP15)	receptor like protein 15;(source:Araport11)	
BraA02g022480.3C	13,140,025	13,144,356	+	AT1G74710	ICS1 / SID2 / EDS16	ISOCHORISMATE SYNTHASE 1 (ICS1), SALICYLIC ACID INDUCTION DEFICIENT 2 (SID2), ENHANCED DISEASE SUSCEPTIBILITY TO ERYSIPHE ORONTII 16 (EDS16)	Encodes a protein with isochorismate synthase activity. Mutants fail to accumulate salicylic acid. Its function may be redundant with that of ICS2 (AT1G18870).	+
BraA02g023090.3C	13,525,814	13,526,148	+	AT5G44420	PDF1.2	PLANT DEFENSIN 1.2 (PDF1.2)	Encodes an ethylene- and jasmonate-responsive plant defensin. mRNA levels are not responsive to salicylic acid treatment; although jasmonate and salicylic acid can act synergistically to enhance the expression of this gene.	

BraA02g023100.3C	13,544,412	13,544,763	+	AT2G26020	PDF1.2b	PLANT DEFENSIN 1.2B (PDF1.2b)	Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant defensin (PDF) family	
BraA02g024100.3C	14,280,073	14,282,028	-	AT1G77470	RFC3	REPLICATION FACTOR C SUBUNIT 3 (RFC3)	Encodes a protein with high homology to the Replication Factor C, Subunit 3 (RFC3) of yeast and other eukaryotes. rfc3 mutants are hypersensitive to salicylic acid and exhibit enhanced induction of PR genes and resistance against virulent oomycete Hyaloperonospora arabidopsidis Noco2. The enhanced pathogen resistance in the mutant is NPR1-independent.	
BraA02g025510.3C	15,325,371	15,327,458	-	AT1G80840	WRKY40	WRKY DNA-BINDING PROTEIN 40 (WRKY40)	Pathogen-induced transcription factor. Binds W-box sequences in vitro. Forms protein complexes with itself and with WRKY40 and WRKY60. Coexpression with WRKY18 or WRKY60 made plants more susceptible to both P. syringae and B. cinerea. WRKY18, WRKY40, and WRKY60 have partially redundant roles in response to the hemibiotrophic bacterial pathogen Pseudomonas syringae and the necrotrophic fungal pathogen Botrytis cinerea, with WRKY18 playing a more important role than the other two. The mRNA is cell-to-cell mobile.	
BraA02g025720.3C	15,434,172	15,436,304	+	AT1G80600	WIN1	HOPW1-1-INTERACTING 1 (WIN1)	Encodes HopW1-1-Interacting protein 1 (WIN1). Interacts with the P. syringae effector HopW1-1. WIN1 is a putative acetylornithine transaminase. Modulates plant defenses against bacteria. Three WIN proteins are identified so far (WIN1: AT1G80600; WIN2: AT4G31750; WIN3: AT5G13320). Mediates red-light inhibition of seed germination.	
BraA02g025760.3C	15,460,827	15,464,259	+	AT1G80600	WIN1	HOPW1-1-INTERACTING 1 (WIN1)	Encodes HopW1-1-Interacting protein 1 (WIN1). Interacts with the P. syringae effector HopW1-1. WIN1 is a putative acetylornithine transaminase. Modulates plant defenses against bacteria. Three WIN proteins are identified so far (WIN1: AT1G80600; WIN2: AT4G31750; WIN3: AT5G13320). Mediates red-light inhibition of seed germination.	
BraA02g027760.3C	17,114,539	17,115,774	-	AT4G12560	CPR1	CONSTITUTIVE EXPRESSER OF PR GENES 1 (CPR1)	Encodes CPR1 (Constitutive Expresser of PR Genes 1, also known as CPR30), a F-Box protein that functions as a negative regulator of defense response and targets resistance proteins.	
BraA02g028160.3C	17,509,574	17,510,393	-	AT3G61190	BAP1	BON ASSOCIATION PROTEIN 1 (BAP1)	Encodes a protein with a C2 domain that binds to BON1 in yeast two hybrid analyses. Its ability to bind to phospholipids is enhanced by calcium ions. Involved in maintaining cell homeostasis.	+
BraA02g028480.3C	17,773,276	17,778,494	+	AT4G11230	RBOHI	(RBOHI)	NADPH-oxidase RbohI is expressed highly in seeds and roots. Mutants have inreased sensitivity to osmotic stress suggesting a role in mediating cellular response to stress in roots.	
BraA02g030140.3C	20,211,140	20,213,155	-	AT2G37710	LECRK-IV.1	L-TYPE LECTIN RECEPTOR KINASE IV.1 (LECRK-IV.1)	Induced in response to Salicylic acid. The mRNA is cell-to-cell mobile.	+
BraA02g031160.3C	21,266,944	21,272,875	+	AT5G40910			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)	

BraA02g031530.3C	21,553,166	21,556,336	-	AT5G20480	EFR	EF-TU RECEPTOR (EFR)	Encodes a predicted leucine-rich repeat receptor kinase (LRR-RLK). Functions as the receptor for bacterial PAMP (pathogen associated molecular patterns) EF-Tu.
BraA02g031540.3C	21,560,904	21,563,918	-	AT5G20480	EFR	EF-TU RECEPTOR (EFR)	Encodes a predicted leucine-rich repeat receptor kinase (LRR-RLK). Functions as the receptor for bacterial PAMP (pathogen associated molecular patterns) EF-Tu.
BraA02g031580.3C	21,627,986	21,629,903	+	AT5G45110	NPR3	NPR1-LIKE PROTEIN 3 (NPR3)	Encodes NPR3, a paralog of NPR1. Involved in negative regulation of defense responses against bacterial and oomycete pathogens. npr3 mutants has elevated level of PR1 expression. Interacts with TGA2, TGA3, TGA5 and TGA6 in yeast two hybrid assays. NPR3 and NPR4 are receptors for the immune signal salicylic acid. The mRNA is cell-to-cell mobile.
BraA02g031600.3C	21,639,494	21,640,470	+	AT5G45220			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)
BraA02g031610.3C	21,640,978	21,643,148	-	AT4G36140			disease resistance protein (TIR-NBS-LRR class);(source:Araport11)
BraA02g031620.3C	21,645,948	21,646,487	-	AT5G45210			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)
BraA02g031630.3C	21,668,513	21,669,121	+	AT5G45250	RPS4	RESISTANT TO P. SYRINGAE 4 (RPS4)	RPS4 belongs to the Toll/interleukin-1 receptor (TIR)-nucleotide binding site (NBS)-Leu-rich repeat (LRR) class of disease resistance (R) genes. Confers specific resistance to Pseudomonas syringae pv. tomato carrying the avirulence gene AvrRPS4. Produces alternative transcripts with truncated open reading frames.
BraA02g031640.3C	21,671,827	21,677,399	+	AT4G36150			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)
BraA02g031660.3C	21,690,398	21,694,675	-	AT5G45230			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)
BraA02g031710.3C	21,721,969	21,730,200	+	AT5G45240			Disease resistance protein (TIR-NBS-LRR class);(source:Araport11)
BraA02g032320.3C	22,258,772	22,260,232	+	AT5G45930	CHLI2	MAGNESIUM CHELATASE I2 (CHLI2)	encodes a second Chl I gene (CHLI2), a subunit of magnesium chelatase which is required for chlorophyll biosynthesis. Has ATPase activity, regulated by TRX-f. Involved in the assembly of the Mg chelatase complex.
BraA02g032770.3C	22,615,923	22,619,579	+	AT5G46330	FLS2	FLAGELLIN-SENSITIVE 2 (FLS2)	Encodes a leucine-rich repeat serine/threonine protein kinase that is expressed ubiquitously. FLS2 is involved in MAP kinase signalling relay involved in innate immunity. Essential in the perception of flagellin, a potent elicitor of the defense response. FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. The mRNA is cell-to-cell mobile.
BraA02g032840.3C	22,687,229	22,684,805	+	AT5G46270			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)
BraA02g032850.3C	22,689,939	22,690,406	+	AT5G46450			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)
BraA02g032880.3C	22,697,547	22,702,197	+	AT5G46450			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)

BraA02g032890.3C	22,708,626	22,712,851	+	AT4G08450			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)	
BraA02g032910.3C	22,719,912	22,724,151	+	AT2G34690	ACD11	ACCELERATED CELL DEATH 11 (ACD11)	Gene product transports the glycolipid precursor sphingosine between membranes in vitro. Mutant constitutively expresses defense-related genes that accompany the hypersensitive response normally triggered by avirulent pathogens. The mRNA is cell-to-cell mobile.	
BraA02g032920.3C	22,727,424	22,731,700	+	AT4G08450			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)	
BraA02g032940.3C	22,741,872	22,744,650	+	AT2G34690	ACD11	ACCELERATED CELL DEATH 11 (ACD11)	Gene product transports the glycolipid precursor sphingosine between membranes in vitro. Mutant constitutively expresses defense-related genes that accompany the hypersensitive response normally triggered by avirulent pathogens. The mRNA is cell-to-cell mobile.	
BraA02g033300.3C	22,988,009	22,991,378	-	AT5G46270			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)	
BraA02g033550.3C	23,278,997	23,283,157	-	AT5G47070	PBL19	PBS1-LIKE 19 (PBL19)	Encodes a member of the RLCK VII-4 subfamily of receptor-like cytoplasmic kinases that has been shown to phosphorylate MAPKKK5 Ser-599 and MEKK1 Ser-603, both players in PRR-mediated resistance to bacterial and fungal pathogens.	+
BraA02g033570.3C	23,283,854	23,284,859	-	AT5G47070	PBL19	PBS1-LIKE 19 (PBL19)	Encodes a member of the RLCK VII-4 subfamily of receptor-like cytoplasmic kinases that has been shown to phosphorylate MAPKKK5 Ser-599 and MEKK1 Ser-603, both players in PRR-mediated resistance to bacterial and fungal pathogens.	+
BraA02g033670.3C	23,401,799	23,403,162	+	AT5G47120	BI1	BAX INHIBITOR 1 (BI1)	Encodes BI-1, a homolog of mammalian Bax inhibitor 1. Functions as an attenuator of biotic and abiotic types of cell death. Bax-induced cell death can be downregulated by ectopically expressing AtBI in planta. The mRNA is cell-to-cell mobile.	
BraA02g036020.3C	25,028,210	25,028,770	-	AT2G03300	ATTX12	(ATTX12)	TX12 is a Toll/Interleukin-1 receptor domain containing protein. Misexpression results in ectopic activation of defense response genes.	
BraA02g036040.3C	25,058,968	25,059,593	-	AT2G03300	ATTX12	(ATTX12)	TX12 is a Toll/Interleukin-1 receptor domain containing protein. Misexpression results in ectopic activation of defense response genes.	
BraA02g036050.3C	25,072,116	25,072,696	-	AT2G03300	ATTX12	(ATTX12)	TX12 is a Toll/Interleukin-1 receptor domain containing protein. Misexpression results in ectopic activation of defense response genes.	

[a] Genes differentially expressed in *A.thaliana* upon *P. brassicae* egg deposition (Little et al. 2007). + induced genes, - repressed genes

Supplementary Table S9. Candidate genes related to plant immunity found within the regions of QTLs *Pbc2* (A03).

B. rapa gene_ID	CDS_start	CDS_end	strand	Ath gene_ID	Ath gene symbol	Ath gene_name	Ath_gene description_Araport11	Differentially expressed in *A. thaliana* [a]
BraA03g049830.3C	25,524,902	25,527,938	-	AT5G11250	BNT1	BNT1 (BURNOUT1)	Encodes an atypical TIR-NBS-LRR protein that is involved in stress responses. Loss of function alleles overproduce stress hormones JA,SA, ABA, and ET.	
BraA03g049840.3C	25,544,676	25,545,344	-	AT5G11250	BNT1	BNT1 (BURNOUT1)	Encodes an atypical TIR-NBS-LRR protein that is involved in stress responses. Loss of function alleles overproduce stress hormones JA,SA, ABA, and ET.	
BraA03g049860.3C	25,547,960	25,559,336	-	AT5G11250	BNT1	BNT1 (BURNOUT1)	Encodes an atypical TIR-NBS-LRR protein that is involved in stress responses. Loss of function alleles overproduce stress hormones JA,SA, ABA, and ET.	
BraA03g049870.3C	25,559,821	25,560,459	-	AT5G11250	BNT1	BNT1 (BURNOUT1)	Encodes an atypical TIR-NBS-LRR protein that is involved in stress responses. Loss of function alleles overproduce stress hormones JA,SA, ABA, and ET.	
BraA03g050150.3C	25,735,726	25,737,094	-	AT4G22305	SOBER1	SUPPRESSOR OF AVRBST-ELICITED RESISTANCE 1 (SOBER1)	Encodes SOBER1, a carboxylesterase that inhibits AvrBsT-triggered phenotypes in Arabidopsis. SOBER1 was formerly linked to AT4G22300 but this locus was split in the TAIR10 annotation into AT4G22300 and AT4G22305. AT4G22300 is now known as TIPSY1 and AT4G22305 corresponds to SOBER1.	
BraA03g050440.3C	25,910,041	25,916,082	-	AT4G23190	CRK11	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 11 (CRK11)	Encodes putative receptor-like protein kinase that is induced by the soil-borne vascular bacteria, Ralstonia solanacearum. Naming convention from Chen et al 2003 (PMID 14756307)	+
BraA03g050780.3C	26,139,993	26,142,632	-	AT4G23130	CRK5 / RLK6	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 5 (CRK5)	Encodes a receptor-like protein kinase. Naming convention from Chen et al 2003 (PMID 14756307)	
BraA03g050810.3C	26,154,222	26,154,389	-	AT4G23130	CRK5 / RLK6	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 5 (CRK5)	Encodes a receptor-like protein kinase. Naming convention from Chen et al 2003 (PMID 14756307)	

BraA03g050820.3C	26,159,479	26,159,889	-	-	-	-	-	
BraA03g050830.3C	26,160,202	26,160,483	-	AT4G23130	CRK5 / RLK6	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 5 (CRK5)	Encodes a receptor-like protein kinase. Naming convention from Chen et al 2003 (PMID 14756307)	
BraA03g050840.3C	26,161,559	26,164,031	-	AT4G23130	CRK5 / RLK6	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 5 (CRK5)	Encodes a receptor-like protein kinase. Naming convention from Chen et al 2003 (PMID 14756307)	
BraA03g050870.3C	26,178,672	26,181,777	+	AT4G23230	CRK15	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 15 (CRK15)	Encodes a cysteine-rich receptor-like protein kinase.	
BraA03g050880.3C	26,184,573	26,187,049	+	AT4G23180	CRK10 / RLK4	(RLK4);CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 10 (CRK10)	Encodes a receptor-like protein kinase. Naming convention from Chen et al 2003 (PMID 14756307) The mRNA is cell-to-cell mobile.	
BraA03g050890.3C	26,187,836	26,190,371	-	AT4G23230	CRK15	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 15 (CRK15)	Encodes a cysteine-rich receptor-like protein kinase.	
BraA03g050900.3C	26,191,764	26,194,343	-	AT4G23230	CRK15	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 15 (CRK15)	Encodes a cysteine-rich receptor-like protein kinase.	
BraA03g050910.3C	26,195,108	26,199,173	-	AT4G23240	CRK16	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 16 (CRK16)	Encodes a cysteine-rich receptor-like protein kinase.	
BraA03g050930.3C	26,206,749	26,215,889	-	AT4G23190	CRK11	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 11 (CRK11)	Encodes putative receptor-like protein kinase that is induced by the soil-borne vascular bacteria, Ralstonia solanacearum. Naming convention from Chen et al 2003 (PMID 14756307)	+
BraA03g050970.3C	26,236,189	26,239,279	-	AT4G23290	CRK21	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 21 (CRK21)	Encodes a cysteine-rich receptor-like protein kinase.	
BraA03g050980.3C	26,240,029	26,243,721	+	AT4G23190	CRK11	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 11 (CRK11)	Encodes putative receptor-like protein kinase that is induced by the soil-borne vascular bacteria, Ralstonia solanacearum. Naming convention from Chen et al 2003 (PMID 14756307)	+

BraA03g051160.3C	26,343,578	26,345,704	+	AT4G23570	SGT1A	(SGT1A)	Closely related to SGT1B, may function in SCF(TIR1) mediated protein degradation. AtSGT1a and AtSGT1b are functionally redundant in the resistance to pathogenes.
BraA03g051210.3C	26,382,174	26,383,787	-	AT4G23160	CRK8	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 8 (CRK8)	Encodes a cysteine-rich receptor-like protein kinase.
BraA03g051420.3C	26,517,381	26,519,377	+	AT4G23740			Leucine-rich repeat protein kinase family protein;(source:Araport11)
BraA03g051750.3C	26,735,503	26,738,128	-	AT4G24250	MLO13	MILDEW RESISTANCE LOCUS O 13 (MLO13)	A member of a large family of seven-transmembrane domain proteins specific to plants, homologs of the barley mildew resistance locus o (MLO) protein.
BraA03g052240.3C	26,990,097	26,995,181	-	AT4G25090	ATRBOHG	(ATRBOHG)	Riboflavin synthase-like superfamily protein;(source:Araport11)
BraA03g052260.3C	27,001,094	27,005,285	-	AT4G25090	ATRBOHG	(ATRBOHG)	Riboflavin synthase-like superfamily protein;(source:Araport11)
BraA03g053480.3C	27,684,636	27,692,192	-	AT1G66980	SNC4	SUPPRESSOR OF NPR1-1 CONSTITUTIVE 4 (SNC4)	Encodes SNC4 (suppressor of npr1-1, constitutive 4), an atypical receptor-like kinase with two predicted extracellular glycerophosphoryl diester phosphodiesterase domains.
BraA03g053580.3C	27,720,009	27,723,038	+	AT4G27190			NB-ARC domain-containing disease resistance protein;(source:Araport11)
BraA03g054050.3C	28,020,791	28,021,855	-	AT4G23160	CRK8	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 8 (CRK8)	Encodes a cysteine-rich receptor-like protein kinase.
BraA03g054480.3C	28,274,125	28,274,346	+	AT4G28460	PREPIP1	PRECURSOR OF PAMP-INDUCED PEPTIDE 1 (PREPIP1)	Activates immune responses through RECEPTOR-LIKE KINASE7 (RLK7). Induces stomatal closure is dependent on RLK7 and the transcription of genes involved in SA production and SA-dependent stomatal closure. SA promotes the flg22-induced expression of PIP1 preligand, prePIP1.
BraA03g054950.3C	28,603,617	28,607,604	+	AT4G29180	RHS16	ROOT HAIR SPECIFIC 16 (RHS16)	root hair specific 16;(source:Araport11)
BraA03g055010.3C	28,670,837	28,672,054	+	AT4G29240			Leucine-rich repeat (LRR) family protein;(source:Araport11)
BraA03g056620.3C	29,742,036	29,742,581	-	AT4G31470			CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein;(source:Araport11)

BraA03g056960.3C	29,894,685	29,895,836	+	AT4G31800	WRKY18	WRKY DNA-BINDING PROTEIN 18 (WRKY18)	-	
BraA03g057870.3C	30,464,435	30,466,983	-	AT4G33430	BAK1 / SERK3	BRI1-ASSOCIATED RECEPTOR KINASE (BAK1), SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3	Leu-rich receptor Serine/threonine protein kinase. Component of BR signaling that interacts with BRI1 in vitro and in vivo to form a heterodimer.	
BraA03g058050.3C	30,531,482	30,531,973	+	AT4G33720	ATCAPE3	(ATCAPE3)	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein;(source:Araport11)	
BraA03g058060.3C	30,558,067	30,558,579	+	AT5G26130	ATCAPE8	(ATCAPE8)	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein;(source:Araport11)	
BraA03g058070.3C	30,558,997	30,559,488	-	AT4G33720	ATCAPE3	(ATCAPE3)	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein;(source:Araport11)	
BraA03g058080.3C	30,560,624	30,561,727	-	AT3G24610			Galactose oxidase/kelch repeat superfamily protein;(source:Araport11)	
BraA03g058090.3C	30,574,098	30,574,586	+	AT5G26130	ATCAPE8	(ATCAPE8)	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein;(source:Araport11)	
BraA03g058100.3C	30,587,983	30,588,474	-	AT4G33720	ATCAPE3	(ATCAPE3)	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein;(source:Araport11)	
BraA03g058110.3C	30,590,069	30,590,560	+	AT4G33720	ATCAPE3	(ATCAPE3)	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein;(source:Araport11)	
BraA03g058660.3C	31,025,619	31,026,925	-	AT4G34180	CYCLASE1	CYCLASE1 (CYCLASE1)	Encodes a cyclase-family protein that is a negative regulator of cell death that regulates pathogen-induced symptom development.	
BraA03g059300.3C	31,462,328	31,462,654	+	AT5G60300	LecRK-I.9	L-TYPE LECTIN RECEPTOR KINASE I.9	Encodes a legume-type lectin receptor kinase that is structurally distinct from the mammalian extracellular ATP receptors and acts as an extracellular ATP receptor in Arabidopsis.	
BraA03g059560.3C	31,615,391	31,617,657	+	AT4G35600	CST	CAST AWAY (CST)	Encodes a receptor-like cytoplasmic kinase that acts as a spatial inhibitor of cell separation.	+

BraA03g059570.3C	31,618,407	31,620,594	+	AT4G35600	CST	CAST AWAY (CST)	Encodes a receptor-like cytoplasmic kinase that acts as a spatial inhibitor of cell separation.	+
BraA03g059700.3C	31,716,103	31,719,675	-	AT4G35790	PLDDELTA	PHOSPHOLIPASE D DELTA (PLDDELTA)	Encodes a protein with phospholipase D activity. Involved in phospolipase metabolism. Mutants are affected in hydrogen peroxide mediated cell death.	
BraA03g060530.3C	32,233,023	32,236,390	+	AT2G14080			Disease resistance protein (TIR-NBS-LRR class) family;(source:Araport11)	
BraA03g060710.3C	32,351,392	32,353,567	-	AT4G37460	SRFR1	SUPPRESSOR OF RPS4-RLD 1 (SRFR1)	Encodes a tetratricopeptide repeat domain containing protein that shows sequence similarity to those of transcriptional repressors in other organisms. Involved in mediating effector-triggered immunity.	
BraA03g065090.3C	37,519,162	37,520,535	+	AT2G01820	TMK3	TRANSMEMBRANE KINASE 3 (TMK3)	Transmembrane kinase (TMK), member of the plant receptor-like kinase (RLK) family.	

[a] Genes differentially expressed in *A.thaliana* upon *P. brassicae* egg deposition (Little et al. 2007). + induced genes, - repressed genes

Supplementary Table S10. Candidate genes related to plant immunity found within the regions of QTLs *Pbc3* (A06).

B. rapa gene_ID	CDS_start	CDS_end	strand	Ath gene_ID	Ath gene symbol	Ath gene_name	Ath_gene Description Araport11	Differentially expressed in *A. thaliana* [a]
BraA06g016660.3C	9,140,552	9,143,308	+	AT1G21270	WAK2	WALL-ASSOCIATED KINASE 2 (WAK2)	cytoplasmic serine/threonine protein kinase induced by salicylic acid. mutant plants exhibit a loss of cell expansion and dependence on sugars and salts for seedling growth, affecting the expression and activity of vacuolar invertase.	
BraA06g016670.3C	9,144,788	9,147,046	+	AT1G21270	WAK2	WALL-ASSOCIATED KINASE 2 (WAK2)	cytoplasmic serine/threonine protein kinase induced by salicylic acid. mutant plants exhibit a loss of cell expansion and dependence on sugars and salts for seedling growth, affecting the expression and activity of vacuolar invertase.	
BraA06g016680.3C	9,147,767	9,150,489	+	AT1G21250	WAK1	CELL WALL-ASSOCIATED KINASE 1 (WAK1)	Encodes a cell wall-associated kinase that interacts with AtGRP3 and may function as a signaling receptor of extracellular matrix component such as oligogalacturonides. The mRNA is cell-to-cell mobile.	
BraA06g016710.3C	9,154,069	9,156,430	+	AT1G21270	WAK2	WALL-ASSOCIATED KINASE 2 (WAK2)	cytoplasmic serine/threonine protein kinase induced by salicylic acid. mutant plants exhibit a loss of cell expansion and dependence on sugars and salts for seedling growth, affecting the expression and activity of vacuolar invertase.	
BraA06g017730.3C	9,837,092	9,838,847	+	AT3G49340			Cysteine proteinases superfamily protein;(source:Araport11)	+
BraA06g018260.3C	10,219,644	10,223,450	-	AT5G17680			disease resistance protein (TIR-NBS-LRR class);(source:Araport11)	
BraA06g019390.3C	11,073,295	11,076,417	-	AT3G47090			Leucine-rich repeat protein kinase family protein;(source:Araport11)	+
BraA06g019740.3C	11,356,257	11,359,436	+	AT3G47110			Leucine-rich repeat protein kinase family protein;(source:Araport11)	
BraA06g020160.3C	11,591,291	11,593,369	+	AT3G46710			NB-ARC domain-containing disease resistance protein;(source:Araport11)	
BraA06g020760.3C	12,041,763	12,047,543	+	AT3G45810	RBOHJ	RESPIRATORY BURST OXIDASE HOMOLOG J (RBOHJ)	ferric reductase-like transmembrane component family protein;(source:Araport11)	

BraA06g020930.3C	12,168,134	12,170,134	+	AT3G45440	LECRK-I.6	L-TYPE LECTIN RECEPTOR KINASE I.6 (LECRK-I.6)	Concanavalin A-like lectin protein kinase family protein;(source:Araport11)	
BraA06g020940.3C	12,172,059	12,174,152	+	AT3G45430	LECRK-I.5	L-TYPE LECTIN RECEPTOR KINASE I.5 (LECRK-I.5)	Extracellular ATP transmembrane receptor involved in innate immunity.	
BraA06g020950.3C	12,174,942	12,177,005	+	AT3G45410	LECRK-I.3	L-TYPE LECTIN RECEPTOR KINASE I.3 (LECRK-I.3)	encodes a receptor-like kinase that has serine/threonine kinase activity whose expression is induced by high salt stress. This induction is inhibited by tobacco ethylene receptor.	
BraA06g020970.3C	12,181,177	12,183,225	+	AT3G45330	LECRK-I.1	L-TYPE LECTIN RECEPTOR KINASE I.1 (LECRK-I.1)	Concanavalin A-like lectin protein kinase family protein;(source:Araport11)	
BraA06g025040.3C	17,457,543	17,458,970	-	AT3G49340			Cysteine proteinases superfamily protein;(source:Araport11)	+
BraA06g027150.3C	19,029,527	19,032,113	+	AT5G63710			Leucine-rich repeat protein kinase family protein;(source:Araport11)	
BraA06g029150.3C	20,207,869	20,208,355	+	AT1G12660			Predicted to encode a PR (pathogenesis-related) protein. Belongs to the plant thionin (PR-13) family with the following members: At1g66100, At5g36910, At1g72260, At2g15010, At1g12663, At1g12660.	
BraA06g033020.3C	22,603,492	22,606,584	+	AT5G25930			kinase family with leucine-rich repeat domain-containing protein;(source:Araport11)	+
BraA06g033030.3C	22,615,386	22,618,176	+	AT5G25930			kinase family with leucine-rich repeat domain-containing protein;(source:Araport11)	+
BraA06g033040.3C	22,621,485	22,622,531	+	AT5G25930			kinase family with leucine-rich repeat domain-containing protein;(source:Araport11)	+
BraA06g033140.3C	22,658,303	22,661,082	+	AT5G66900	NRG1.1	N REQUIREMENT GENE 1.1 (NRG1.1)	RPW8 -CNL gene is required for signal transduction of TNLs; functionally redundant to NRG1.2. Exhibits autoimmunity.	
BraA06g035180.3C	23,826,323	23,828,325	-	AT1G53350			Disease resistance protein (CC-NBS-LRR class) family;(source:Araport11)	
BraA06g035420.3C	23,931,074	23,932,901	+	AT5G48657			defense protein-like protein;(source:Araport11)	
BraA06g035630.3C	24,016,862	24,019,138	+	AT5G48380	BIR1	BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (BIR1)	Encodes a BAK1-interacting receptor-like kinase named BIR1. Negatively regulates multiple plant resistance signaling pathways, one of which is the SOBIR1(AT2G31880)-dependent pathway.	+

BraA06g035910.3C	24,157,803	24,162,074	-	AT5G47910	RBOHD	RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD)	NADPH/respiratory burst oxidase protein D (RbohD).Interacts with AtrbohF gene to fine tune the spatial control of ROI production and hypersensitive response to cell in and around infection site. The mRNA is cell-to-cell mobile.	
BraA06g036610.3C	24,549,355	24,551,449	+	AT3G29030	EXPA5	EXPANSIN A5 (EXPA5)	Encodes an expansin. Naming convention from the Expansin Working Group (Kende et al, 2004. Plant Mol Bio)	-
BraA06g036660.3C	24,571,138	24,573,589	+	AT3G28930	AIG2	AVRRPT2-INDUCED GENE 2 (AIG2)	avrRpt2-induced gene that exhibits RPS2- and avrRpt2-dependent induction early after infection with Pseudomonas syringae pv maculicola strain ES4326 carrying avrRpt2	+
BraA06g036670.3C	24,574,970	24,575,871	+	AT3G28930	AIG2	AVRRPT2-INDUCED GENE 2 (AIG2)	avrRpt2-induced gene that exhibits RPS2- and avrRpt2-dependent induction early after infection with Pseudomonas syringae pv maculicola strain ES4326 carrying avrRpt2	+

[a] Genes differentially expressed in *A.thaliana at three time points (24 h, 48 h, 72 h) after P. brassicae* egg deposition (Little et al. 2007). + induced genes, - repressed genes

Chapter 5

Pieris brassicae egg-induced cell death in *Brassica nigra* is mediated by a single locus containing a cluster of TIR-NBS-LRR receptors

Niccolò Bassetti[1], Lotte Caarls[1,2], Patrick Verbaarschot[1], Tim Bongers[1], Thijmen van der Loop[1], Nam Hoang[1], Klaas Bouwmeester[1,3], Guusje Bonnema[2], Bas J. Zwaan[4], M. Eric Schranz[1], Nina E. Fatouros[1]

[1] Biosystematics Group, Wageningen University & Research, Wageningen, The Netherlands
[2] Laboratory of Plant Breeding, Wageningen University & Research, Wageningen, The Netherlands
[3] Laboratory of Entomology, Wageningen University & Research, Wageningen, The Netherlands
[4] Laboratory of Genetics, Wageningen University & Research, Wageningen, The Netherlands

Abstract

A hypersensitive response (HR) characterizes monogenic qualitative resistance traits in several pathosystems. Its role in resistance to insects is relatively understudied and limited to a few resistance (*R*) gene-based defense responses against piercing-sucking insects. The hypersensitive response (HR)-like cell death induced by egg deposition of cabbage white butterflies (*Pieris* spp.) in *Brassica* spp. reduces egg survival and represents an effective resistance trait before that feeding larvae emerges. However, its implementation as defence trait is conditional on the understanding of its genetic basis. In this study, we found that *P. brassicae* egg-induced HR segregates as a Mendelian trait in wild accessions of black mustard *B. nigra* L. Through bulk-segregant analysis coupled with whole-genome sequencing (BSA-seq), we identified a single dominant locus on chromosome B3 which we named *PEK (Pieris* egg-killing). Fine-mapping through recombinant analysis restricted the *PEK* locus to a ~50 kb region that contains several tandemly duplicated genes, including a cluster of potential candidate resistance TIR-NBS-LRR (TNL) receptor proteins. We found that *PEK* is polymorphic between the parental accessions of our crossing scheme and shows copy number variants (CNVs) of TNL genes among *B. nigra* reference genomes. These results highlight the need for a complete *de novo* assembly of the *PEK* locus from our parental accessions to precisely fine map the causal locus and/or polymorphism. Further fine-mapping of the *PEK* locus will reveal whether the TNLs are responsible for the HR phenotype, while studying the diversity of the locus among Brassicaceae will shed light on the evolutionary basis of HR.

Keywords
Crop wild relatives (CWR), *Brassica* crops, cabbage white butterfly, plant-insect interaction, Bulk Segregant Analysis, *k*-mers, nucleotide-binding leucin rich-repeat (NLR)

Introduction

Insect pests represent a major threat for global food security. Each year up to ~40% of crop yields are lost due to biotic stresses and a considerable portion of this loss is caused by herbivorous insects (IPCC Secretariat 2021). One reason for pest success is that crop plants, especially modern improved varieties, possess reduced physical and chemical defenses compared to their crop-wild relatives (Olsen & Wendel 2013, Chen *et al.* 2015). Improvement of pest resistance traits can greatly benefit from tapping into the genetic variation of crop wild relatives (CWR) to restore traits lost through domestication and/or breeding (Palmgreen *et al.* 2015). To this purpose, introgression and deployment of crop resistance traits from CWR requires a thorough understanding of the genetic and molecular basis of plant-herbivore interactions (Schuman & Baldwin 2016, Erb & Reymond 2019).

Most of plant defenses against feeding insect herbivores appear to be controlled by polygenic quantitative traits (Kliebenstein 2017). Conversely, monogenic qualitative resistance, often based on different types of hypersensitive response (HR), is more common against phytopathogens and it is controlled by resistance (*R*) genes, which are mostly (semi)dominant loci (Kourelis & Van Der Hoorn 2018). More than 300 *R* genes have been cloned, the majority being cell surface (pattern recognition receptors, PRRs) or intracellular receptors (nucleotide-binding leucin rich-repeat, NLRs) (Kourelis & Van Der Hoorn 2018). However, only a handful of *R* genes, of both PRRs and NLRs type, are effective against insect herbivores, mainly limited to piercing-sucking insects such as gall midges (Harris *et al.*2012; Bentur *et al.*2016), aphids (Rossi *et al.*1998; Botha *et al.*2005; Klingler *et al.*2009; Dogimont *et al.*2014; Nicolis and Venter, 2018; Sun *et al.*2020), whiteflies (Nombela *et al.*2003), and planthoppers (Tamura *et al.*2014; Liu *et al.*2015; Zhao *et al.*2016). While defense to chewing caterpillars is polygenic, a few reports suggest that PRR surface receptors also mediate these defenses (Gilardoni *et al.* 2011, Hu *et al.* 2018, Steinbrenner *et al.* 2021). Nonetheless, quantitative resistance involving multiple quantitative trait loci (QTLs) seem to be more common against chewing herbivores (Thoen *et al.* 2017, Nallu *et al.* 2018). Arguably, the characterization of the molecular mechanisms underlying these small effect QTLs is not feasible (Broekgaarden *et al.* 2011). Given the lack of effective *R* genes against chewing insects, resistance mechanisms targeting the insect eggs have been proposed as complementary defence strategy (Tamiru *et al.* 2015, Fatouros, *et al.* 2016). Clearly, the recognition and killing of insect eggs is advantageous to plants as it prevents the destructive feeding by the hatching

larvae (Hilker & Fatouros 2015, 2016). The investigation of egg-killing traits thus represents an alternative and unexplored source of novel *R* genes to increase crop resistance to pests (Fatouros *et al.* 2016).

Cabbage white butterflies, such as the gregarious *Pieris brassicae* and the solitary *P. rapae* (Lepidoptera: Pieridae), can be pests of crucifer crops (*Brassica* spp.) and a serious agricultural challenge (Kumar *et al.* 2017, Ryan *et al.* 2019). *Pieris* eggs induce a HR-like cell death in host plants of the Brassicaceae family resembling a HR induced by pathogens (Shapiro 1987, Caarls *et al.* 2021, Griese 2021, Chapter 3). Under field conditions, a severe HR-like cell death (hereafter simply "HR") reduce egg survival up to >40% on wild black mustard *B. nigra* (Griese *et al.* 2021, Chapter 2). Furthermore, egg deposition induces the emission of oviposition-induced plant volatiles (OIPVs) which promotes egg parasitism by *Trichogramma* spp. wasps (Fatouros *et al.* 2014). The synergistic effect of the two traits contributed to killing of up to ~80% of *Pieris* eggs on wild *B. nigra.* (Fatouros *et al.* 2014). The egg-killing effect of the HR was also observed in greenhouse experiments against singly laid *P. brassicae* eggs (Griese *et al.* 2017, Griese *et al.* 2021, Chapter 2). Thus, egg-induced cell death represents a relatively easy-to-score trait with a high potential as a novel plant defense against eggs, hence reducing the impact of later potential larval herbivory. While it is known that plants respond to *Pieris* eggs with a salicylic acid (SA)-dependent immune response (Little *et al.* 2007, Bruessow *et al.* 2010, Bonnet *et al.* 2017, Caarls *et al.* 2021, Chapter 3), the genetic and molecular mechanisms of egg-induced HR remain understudied.

Brassica nigra represents an ideal plant species to study egg-induced HR because the phenotype is strong, stable, easy to score, and with a proven egg-killing effect (Chapter 3, Caarls *et al.* 2021). Previous investigations into the genetic basis of HR-like cell death were conducted using the model species *A. thaliana* (Groux *et al.* 2021b) and on the crop *B. rapa* (Bassetti 2022, Chapter 4) which both benefit from extensive resources for classical forward genetics. A genome-wide association study (GWAS) in *A thaliana* identified two loci, a L-type *lectin receptor-like kinase-I.1* (*LecRK-I.1*) and a putative Ca^{2+} channel *glutamate receptor 2.7* (*GLR2.7*) (Groux *et al.* 2019, 2021b). The second study was a QTL mapping in *B. rapa* which identified three QTLs *Pbc1-3* associated with cell death size (Bassetti *et al.* 2022). The QTLs included many genes involved in plant immunity, but they underlined large genomic regions which await to be fine-mapped. A partial overlap among the loci identified in both studies was also suggested, given that *Bra*LecRK-I.1 is included within *Pbc3* (Bassetti *et al.* 2022). Still, it is also evident that egg-induced HR is a polygenic trait involving different components of plant immune signalling, many of which may be unique to certain plant species. The HR-like cell

death so far observed in *A. thaliana* and *B. rapa* appeared weaker and it had no or little effect on *P. brassicae* egg survival (Groux *et al.*2019, Griese *et al.* 2021). In contrast, *B. nigra* shows a severe HR, spreading from the leaf abaxial up to the adaxial side, which is required for a substantial egg-killing (Griese *et al.*2021, Caarls *et al.* 2021, Chapters 2 and 4). This difference in phenotypes suggests that *B. nigra* may represent a more suitable model to study egg-induced HR as it may reveal a unique genetic regulation of the trait. Further, it allows comparative studies with other *Brassica* crops which may have lost the trait during domestication and/or selection (Griese *et al.* 2021, Chapter 2). Indeed, crossings between *Brassica* cultivars and/or species are commonly done for introgression of resistance traits (Katche *et al.* 2019, Lv *et al.* 2020), including from crop wild relatives (CWR) into crops.

In this study, we investigated the inheritance and genetic basis of *P. brassicae* egg-induced HR cell death that appears to be specific to *B. nigra*. We hypothesized that the trait may have a different genetic basis than previously shown in *A. thaliana* and *B. rapa*. Given the lack of advanced genetic populations, we used plant material collected from wild populations to study the inheritance of the trait. We performed genetic mapping through bulk-segregant analysis paired with whole genome sequencing (BSA-seq). We found that the HR induced by *Pieris* butterfly eggs in *B. nigra* segregates as a Mendelian trait and we identified a single ~50 kb locus which we named *Pieris* egg-killing cell death (*PEK*). Within *PEK*, a gene cluster of TIR-NBS-LRRs, a type of NLR receptors, showed copy number variants (CNVs) between different *B. nigra* genomes. We propose that that variation at the cluster of TNLs is responsible for the egg-induced HR phenotype considering that in other pathosystems TNLs often underline *R*-gene resistance based on HR.

Material and methods

Plant and insect materials

The inheritance of the HR-like cell death was studied using *B. nigra* accessions collected from a local population in the floodplain of the Rhine River near to Wageningen, The Netherlands (51°57'38.6"N 5°40'45.3"E). The accession SF48-O1 shows a severe HR-like cell death and it is originated from multiplication by open pollination of accession SF48 (Griese 2017). Accession DG1 showed instead weak or no HR-like cell death. A single DG1 plant was crossed with a single SF48-O1 plant to obtain a F_1 population (Fig. 1a). Single plants from the F_1 that showed HR-like cell death were backcrossed to other DG1 plants to obtain segregating

backcross families (BC_1). Selfing of individual plants generation of BC_1-S_1 and BC_1-S_2 populations.

Plants were grown in a greenhouse under standardized conditions (21° day / 18° night, RH 50-70%, LD 16:8 h). Seeds were vernalized at 4° C for two days to induce even germination and then were sown in small trays with sowing soil (Lentse potgrond, Lent, The Netherlands). Seedlings were transplanted one week after germination into 17 cm diameter pots with potting soil (Lentse potgrond, Lent, The Netherlands). Plants were grown for five weeks before treatment with *P. brassicae* egg wash.

Pieris brassicae L. (Lepidoptera: Pieridae) butterflies were obtained from a rearing of the Laboratory of Entomology, Wageningen University. Insects were kept in a greenhouse under standardized conditions (21 ± 4° C, RH: 60 – 80 %, L16: D8). Larvae were reared on Brussel sprout (*Brassica oleracea* var. *gemmifera*) cv. Cyrus, while the adults were fed with a 10 % honey solution and allowed to oviposit on clean plants of the same genotype.

Plant treatments

Egg wash was prepared following a recently published protocol (Bassetti *et al.* 2021, Chapter 4). In brief, *P. brassicae* egg clutches were collected from Brussel sprout leaves within 24 h after oviposition. Eggs were carefully removed with a stainless-steel lab spatula without breaking them and placed in an Eppendorf tube together with demineralised water in a ratio of ~1000 eggs per 1 ml of water. After an overnight incubation at room temperature, the liquid phase was retained and stored at -20 °C. For all experiments, two 5 µl drops of egg wash were applied to each of the youngest two fully developed leaves. Drops of an equivalent amount of demineralised water were applied as positive control.

Assessment of egg wash-induced cell death

Egg wash-induced HR-like cell death was scored on a scale from 0 to 4 as previously described (Caarls 2021): 0, no visual response; 1, brown spots underneath egg wash-treated spot, only visible at abaxial side leaf; 2, cell death also visible at adaxial side of leaf, spot smaller than 2 mm diameter; 3; cell death covering the size of the egg wash-treated spot; 4, cell death spreading lesion beyond the treated spot.

DNA extraction, pooling, sequencing

For all the population of our crossing scheme, Genomic DNA was extracted from young leaves previously sampled, snap frozen and stored at -80C. DNA was extracted following a

modified CTAB method from Maloof lab (https://openwetware.org/wiki/Maloof_Lab:96well_CTAB). DNA concentration and purity was estimated with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, MA, USA). The DNA integrity was confirmed was assessed using a 1% agarose gel with ethidium bromide. Prior to sequencing, DNA concentration was measured with a Qubit Fluorometer (Invitrogen, MA, USA).

For the bulk segregant analysis (BSA) resistant (R, plants showing HR) and susceptible (S, plants without HR) bulks (n = 10) were prepared by pooling equal amounts of DNA from each individual plant. For the WGS experiment, 1 ug of genomic DNA of each sample (three accessions and two bulks) was used for library preparation. Library preparation and whole genome sequencing were carried out by Novogene (Cambridge, UK). Sequencing libraries were generated using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, UK) following the manufactures' protocol. The genomic DNA was randomly fragmented to a size of 350bp by Bioruptor, then DNA fragments were narrowly size selected with sample purification beads. The selected fragments were then end-polished, A-tailed, and ligated with the full-length adapter. After these treatments, these fragments were filtered with beads. At last, the library was analysed for size distribution by Agilent 2100 Bioanalyzer (Agilent technologies, CA, USA) and quantified using real-time PCR. Libraries were sequenced on an Illumina NovaSeq 6000 platform using 150 bp paired end (PE) reads.

_K_-mer based genetic mapping

K-mer based genetic mapping was performed following the recommendations of Comparative subsequence sets analysis (CoSSA) workflows (Prodhomme *et al.* 2019). First, sequencing read quality was assessed with FastQC (Andrews 2010). Then, *k*-mer tables were built with a *k*-mer size of 31 nucleotides using GlistMaker of the GenomeTester4 v4.0 suite (Kaplinski *et al.* 2015). *k*-mer that occurred only once were removed as likely resulting from sequencing errors. To identify resistant (*R*) haplotype-specific *k*-mers, GlistCompare of GenomeTester4 was used to perform basic set operations as unions, intersections of differences between *k*-mer tables of different samples (Supplementary Fig. S1). An additional filtering step was carried out to retain *R* haplotype-specific *k*-mers. The sequencing yielded 14.4 Gb for the *R* bulk and an approximate 24x depth considering a haploid *B. nigra* genome of ~550 Mb. Given that *B. nigra* genome is diploid (2n = 2x = 16) and assuming uniform sequencing coverage, *k*-mers originated from the *R* haplotype should have sequencing depth of ~12x. Thus, we decided to retain *k*-mers with a depth between 10x and 20x which represented *k*-mers

derived from R-specific haplotype. Retained *k*-mers were aligned to *B. nigra* reference genomes NI100 v2.0, C2 v1.0 and Sangam v1.0 using BWA *aln* (v0.7.17) allowing 3 mismatches (Li & Durbin 2009). The number of mapped *k*-mers mapped per 1 Mb bins were counted using bedtools v2.25 (Quinlan & Hall 2010).

KASP markers genotyping

Kompetitive Allele Specific Polymorphisms (KASP) PCR markers were used to validate the results of *k*-mer based genetic mapping. For each sample, DNA concentration was adjusted to 5-50 ng/µl. Primers were designed on the sequences flanking the SNPs identified by *k*-mers of the R-specific haplotype. KASP genotyping assays were performed according to the manufacturer's instructions (LGC Genomics, UK). In brief, 2 µL DNA at a concentration of 5–50 ng/µL was added to 96-well plate with KASP PCR mix (5 µL 2× KASP Master mix, 0.6 µL 10mM primer mix, 2.4 Milli-Q water). The PCR was performed in a CFX96 Touch Real-Time PCR Detection System combined with CFX Maestro Software for data reading (Bio-Rad, Hercules, CA, USA).

Variant calling within *PEK* locus

Reads were aligned to a modified *B. nigra* reference genome C2 (v1.0), which also included the mitochondrial sequence (Genbank accession no. NC_029182) and chloroplast sequence (Genbank accession no. NC_030450) using BWA mem (v0.7.17) with default parameters. The resulting alignment files were sorted and indexed using samtools (v.1.11). Alignment files were filtered to restric variant calling to the *PEK* locus. Variants were called using a workflow based on GATK Best Practices (De Pristo et al. 2011). Duplicate read pairs were marked using the MarkDuplicates tool of the GATK suite (v.4.1.9.0). Variants (SNPs and InDels) were called in each sample on a window of x Mb around the *PEK* locus using GATK HaplotypeCaller. Samples were then jointly genotyped using GATK GenomicsDBImport and GATK GenotypeGVCFs, with default parameters. SNPs filtration was performed with the following parameters: QD < 2, QUAL < 30, SOR > 3, FS > 60, MQ < 40, MQRankSum < -12.5, ReadPosRankSum < -8. InDels filtration was performed with the following parameters: QD < 2, QUAL < 30, SOR > 3, FS > 200, ReadPosRankSum < -20. Finally, only variants that were in agreement with our genetic model were retained, that is heterozygous in resistant material and homozygous DG1-S1 allele in susceptible (HR-) material. The functionals effects of the retained variants was predicted using SnpEff with default parameters (Cingolani *et al.* 2012).

RNAseq of parental accessions SF48-O1 and DG1-S1

The gene expression experiment for RNA-seq was the same as described in Chapter 3. Five weeks old *B. nigra* DG1-S1 or *B. nigra* SF48-O1 plants were treated with egg wash and demineralized water (negative control) and sampled at 6 h, 24 h and 48 h after treatment. For each accession 15 plants were treated on last two fully developed leaves with two 5 µl spots for each treatment. Treated spots were sampled as leaf disks (6 mm Ø) and leaf discs were stored at -80C until RNA extraction. For each treatment combination (two accessions, two treatments, three time points), three samples were pooled to finally obtain five biological replicates. In order to confirm the HR phenotype of each plant, each plant was treated with two extra egg wash spots and scored after five days.

RNA extraction was carried out using Direct-zol RNA Miniprep Kit (Zymo Research, CA, USA) following manufacturer`s protocol. For each sample, genomic DNA was removed during RNA extraction protocol. RNA purity was checked with NanoDrop ND-1000 spectrophotometer (Thermo Scientific, MA, USA), RNA integrity was checked on by a 2100 Agilent Bioanalyser with a plant RNA NanoChip assay (Agilent Technologies, CA, USA) and RNA concentration was assessed with a Qubit Fluorometer (Invitrogen, MA, USA).

RNA library preparation and sequencing were carried out by Novogene (Cambridge, UK). A strand-specific PCR free library was prepared following the TrueSeq dUTP method. Libraries were sequenced on an Illumina NovaSeq 6000 platform using 150 bp paired end (PE) reads. Sequencing yielded an average of 13 Gb (~43.3 million PE reads) per sample.

RNAseq data analysis

Read quality before and after trimming was assessed with FastQC (Andrews 2010) and multiQC (Ewels *et al.* 2016). Read were processed with Trimmomatic v0.39 (Bolger *et al.* 2014), with the following parameters: ILLUMINACLIP:2:30:10; SLIDINGWINDOW:4:20; MINLEN:75. Trimmed reads were aligned to *B. nigra* genome C2 v1.0 using HISAT2 v2.2.1 (Kim *et al.* 2019) with default settings and transcript abundance was quantified with StringTie v2.2.0 (Pertea *et al.* 2015). Fragments per kilobase of transcript per million fragments mapped (FPKM) were calculated using the Ballgown package v2.22.0 (Fu *et al.* 2020) in RStudio v4.0.1 (R Core Team 2021). The resulting FPKM values were then filtered on a sum(row) > 0 to remove non-expressed genes, and the remaining transcripts were considered as “expressed” and retained for downstream analysis. Differential gene expression analysis was performed in

DESeq2 v1.30.1 (Love *et al.* 2014). Log_2(fold change) were determined using DESeq2 with default settings. This was calculated by comparing pairs of egg wash treated samples and water droplet control samples within an accession (Table 1). The resulting Log_2(fold change) per gene of a pair was then filtered with a FDR-adjusted p value < 0.05 and an absolute Log_2(fold change) ≥ 1. *A. thaliana* orthologs were assigned to each *B. nigra* gene using BLASTp v2.12.0 (Camacho *et al.* 2009) with settings *-eval* $1e^{-10}$ and *-max_target_seqs* 1. *Brassica. nigra* genes for which no confident blastp hit was found were subjected to an additional BLASTn search with similar settings.

Statistical analysis of phenotypic data

All statistical analyses were conducted on R v4.0.1 (R Core Team 2021). Chi-square tests were used to test the goodness of fit of the segregation of phenotypic data and KASP markers data. Kruskal–Wallis tests were performed to validate the association of the KASP markers with HR-like cell death phenotype.

Comparative genomics of *PEK* locus

Syntenic relationships of the *PEK* locus region between the three complete *B. nigra* genomes (NI100 v2.0, C2 v1.0 and Sangam v1.0) were performed on the CoGe web platform (https://genomevolution.org/coge/) using the SynMap tool (Lyons *et al.* 2008, Haug-Baltzell *et al.* 2017). The legacy version of SynMap was used with the following settings: DAGChainer Options "Maximum distance between two matches (-D): 20 genes; Minimum number of aligned pairs (-A): 5 genes"; Merge Syntenic Blocks "Algorithm: Quota align merge"; Fractionation Bias "Windows size: 100 genes, Fractionation bias calculation: Use all genes in target genome"; CodeML "Calculate syntenic CDS pairs: Synonymous (Ks) substitution rate; Color scheme: Max value 2, Log10 OFF"; Advance Options "Tandem duplication distance: 10".

Results

HR is inherited as single dominant Mendelian trait

Previously, we reported phenotypic variation for *P. brassicae* egg-induced HR in a local population of *B. nigra* (Griese *et al.* 2017, Caarls *et al.* 2021). We repeatedly observed that no visible HR (score 0) or a weak cell death (score 0-1) did not affect egg survival, hence these plants were considered susceptible (S). Conversely, a strong cell death (score 2-4) was considered resistant (R) because it reduces egg survival (Chapter 2, Griese *et al.* 2017, 2021, Fatouros *et al.* 2014). We studied the inheritance of *P. brassicae* egg-induced HR in a crossing scheme derived from a cross between accessions DG1-S1 and SF48-O1 (Fig. 1a). The parental accessions consistently showed contrasting phenotypes (Fig. 1a), with DG1-S1 showing no HR (score 0-1) and SF48 showing a strong HR (score 2-4) upon treatment with egg wash, which we previously showed to mimic *Pieris* eggs (Chapter 3. Caarls 2021). When HR was scored as presence/absence in the F_1 population (F1-1, n = 150), it segregated with a clear bimodal distribution with a 1:1 ratio between plants without and with HR ($\chi 2$ test, $P > 0.05$) (Fig. 1b, Table 1). Segregation in the F_1 was not surprising considering that wild *B. nigra* plants are self-incompatible and thus highly heterozygous. A backcross population (BC1-3, n = 66) between a resistant F_1 plant and the susceptible parent (DG1-S1) showed again a 1:1 segregation (Fig. 1b, Table 1). Recurring of a 1:1 segregation pattern resembled the outcome of a test-cross for a single heterozygous locus. This was confirmed after selfing a resistant BC_1 plant with HR which resulted in a BC_1S_1 population (BC1S1-1, n = 695) showing a 3:1 ratio between resistant and susceptible plants (Fig. 1b, Table 1). Overall, the segregation of *P. brassicae* egg-induced HR in our crossing scheme was consistent with a Mendelian trait controlled by a single dominant locus, which should be heterozygous in the HR donor *B. nigra* accession SF48-O1.

Table 1. Segregation ratios of egg-induced phenotypes in *B. nigra* populations used for genetic mapping. Plants without HR are considered susceptible (S), with HR are instead resistant (R).

Population	Generation	Observed		Expected			χ^2 test (P-value)
		R	S	R	S	Ratio	
F1-1	F_1	75	75	75	75	1 : 1	1.000
BC1-3	BC_1	26	40	33	33	1 : 1	0.085
BCS1-1	BC_1S_1	533	155	516	172	3 : 1	0.134

The single locus model was further supported by the phenotypic segregation in other crosses involving selfings of parental plants and in F_2 and BC_1 populations (Supplementary Table S1). Selfing of the susceptible parent DG1-S1 resulted in progeny with no HR (DG1-S2, n = 15). Similarly, a backcross between F_1 plants with no HR and DG1-S1 (BC1-5, n = 70) resulted also in progenies with no HR. These results further suggested that absence of HR resulted from fixing a homozygous recessive allele at a single locus. Conversely, backcrosses between F_1 with HR-like and DG1-S1 showed again a 1:1 segregation (BC1-4, n = 69; BC1-6, n = 70), as previously observed. Finally, F_2s derived from resistant F_1s showed a 3:1 segregation as expected (F2-2, n = 8; F2-3, n = 12).

BSA-seq on BC_1 confirmed HR as single Mendelian locus

To identify genomic regions associated with *Pieris* egg-induced HR, we performed a Bulk segregant analysis coupled with whole genome sequencing (BSA-seq) on the backcross population BC1-3 (Fig. 1B). We generated two bulks (n = 10) with either susceptible plants (S-Bulk) or with resistant plants (R-Bulk). Genomic DNA of S-bulk, R-bulk, the S parent (DG1-S1), the R parent (F1_#130) and the donor of HR (SF48-O1, "R donor") was sequenced using Illumina 150 bp paired-end reads yielding between 14 and 22 Gb data for each sample (Supplementary Table S2). As we estimated a haploid genome size of our *B. nigra* material of ~550 Mb, our sequencing resulted in a read depth between 26x and 35x for each diploid genome of parents, thus a coverage ranging between 13x and 17.5x of a haploid genome. Given the heterozygosity in our plant material, we performed a *k*-mer based BSA-seq approach (CoSSA) which was recently developed for a highly heterozygous tetraploid potato (Prodhomme *et al.* 2019). Our genetic model pointed at a monogenic dominant locus which was heterozygous in the backcross population BC1-3, thus carrying a single resistant (R) allele conferring HR. Accordingly, we first generated *k*-mer tables (k = 31) for each sample independently. Then we selected *k*-mers from the R allele ("R haplotype") by using basic set algebra to retain *k*-mers that were unique to the R-bulk and originated from R parent and R donor (Supplementary Fig. S1). The resulting R haplotype-specific *k*-mer set was filtered to retain unique *k*-mers with a frequency similar to half of sequencing depth for a haploid genome (15x + 5) to discard *k*-mers derived from repeated regions (Supplementary Table S3).

The unique R haplotype-specific *k*-mers were aligned to 1 Mb bins of the *B. nigra* reference genome C2 (Supplementary Fig. S2). Approximately ~85% of the R-specific *k*-mers were successfully aligned, while the rest likely represented sequences from our plant material that were too divergent or absent from the *B. nigra* reference genome. A unique single peak

consisting of ~73% of the R haplotype-specific *k*-mers was found in a 10 Mb interval on the proximal end of chromosome B3, spanning from 3 Mb to 13 Mb (Fig. 1c). All other *k*-mers (~27%) mapped at a very low frequency (below 0.4% for each 1 Mb bin) throughout the rest of the genome (Supplementary Fig. S2). Similarly, alignment of R haplotype-specific *k*-mers to other *B. nigra* genomes resulted also on a single peak, namely on chromosome B3 of accession NI100 and chromosome B7 of accession Sangam (not shown). We found that chromosome B7 of Sangam is perfectly syntenic to chromosome B3 of C2 and NI100 (Supplementary Fig. S3). In conclusion, a BSA-seq approach allowed us to map the HR cell death trait in a BC_1 population and identified a single genetic locus on chromosome B3, which confirms the genetic model that we hypothesized based on the phenotypic segregation. We named the newly identified locus *Pieris*-egg killing cell death (*PEK*) locus.

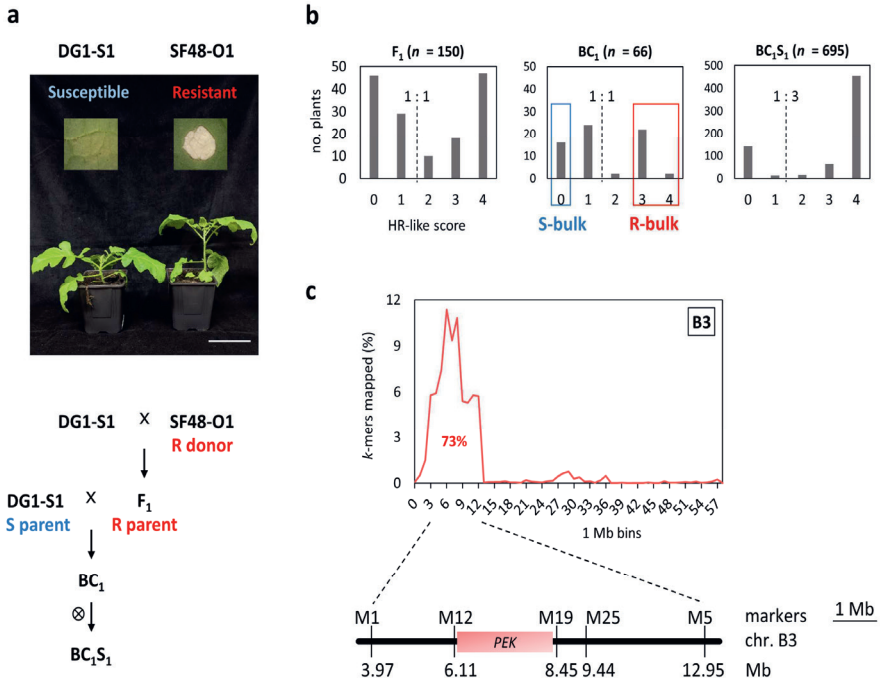


Figure 1. *P. brassicae* eggs-induced HR segregates as a Mendelian trait and it is mapped to a single locus on chromosome B3. a) B. nigra parental accessions as five-weeks old plants and relative HR phenotype (above). Crossing scheme used to study inheritance of HR (below). Magnification bar = 10 cm. b) Phenotypic distribution of populations obtained from the crossing scheme. c) Distribution of *k*-mers unique to resistant (R) samples mapped on chromosome B3 (genome C2). *k*-mers are plotted on each 1 Mb bin as percentage of the total *k*-mer set. A single peak consisting of ~73% *k*-mers located the *PEK* locus at interval 3-13 Mb (top panel). Validation of the locus was carried out with KASP markers on the BC_1 population (n = 66) and four informative recombinants restricted the *PEK* locus between 6.11 and 8.45 Mb (bottom panel).

Validation of *PEK* locus on chromosome B3 by marker analysis

To validate the *PEK* locus identified by BSA-seq on the top of chromosome B3, we designed KASP markers that targeted the whole 10 Mb region (Supplementary Table S4). We genotyped the entire BC1 population (BC1-3, $n = 66$) with five KASP markers. Each KASP marker co-segregated with the HR cell death phenotype (Kruskall-Wallis test, $P < 0.05$), without showing segregation distortion (χ^2 test, $P > 0.05$) (Supplementary Table S5). The S parent DG1-S1 and susceptible BC_1 plants were homozygous (*pek*-DG1/*pek*-DG1) while the R donor SF48-O1, the R parent F1_#130 and resistant BC_1 plants were heterozygous (*PEK*-SF48/*pek*-DG1). Four informative recombinants between M1 and M25 were then genotyped with additional KASP markers, which restricted the locus to the interval 6.11-8.45 Mb between M12 and M19 (Fig. 1c). Validation with KASP markers confirmed that HR was associated with heterozygosity at the *PEK* locus on chromosome B3.

Table 2. Segregation ratios and association with HR phenotype of five markers used to genotype the whole BC_1S_1 population (n = 695). Alleles at each marker are given a symbol based on the phenotype of the accession from which they derive: "R" is for the SF48-O1 allele (*PEK*-SF48), from the resistant parent. "S" is for the DG1-S1 allele (*pek*-DG1) from the susceptible parent.

Marker	position on chr. B3 (Mb)	Observed			Expected (1:2:1)			χ^2 test (P-value)	Association with HR (LOD score)
		R/R	*R/S*	*S/S*	*R/R*	*R/S*	*S/S*		
M1	3.97	170	346	159	168.75	337.5	168.75	0.675	73.4
M13	6.53	173	341	159	168.25	336.5	168.25	0.704	106.5
M19	8.45	166	354	158	169.50	339	169.5	0.469	81.5
M25	9.44	161	362	156	169.75	339.5	169.75	0.217	65.8
M5	12.95	156	363	151	167.50	335	167.5	0.093	53.6

Recombinant analysis on BC_1S_1 fine mapped the locus on ~50 kb interval

We then proceeded with fine-mapping the *PEK* locus using a BC_1S_1 population (BC1S1-1, $n = 695$) that was generated by selfing a resistant BC1-3 plant with the heterozygous genotype at the *PEK* locus carrying both SF48-O1 allele and DG1-S1 allele (*PEK*-SF48/*pek*-DG1). The whole BC_1S_1 population was genotyped with five KASP markers (M1, M13, M19, M25, M5) spanning the interval. Each marker showed a 1:2:1 allelic segregation ratio (χ^2 test, $P > 0.05$) which was expected given the 3:1 phenotypic segregation ratio between R and S plants (Table 2). The markers were placed on a genetic map of 20.6 cM with a total recombination rate of 2.54 cM/Mb (Fig. 2a). As previously observed in BC1-3, all markers covering the region were associated with HR and marker M13 (6.06 Mb) showed the highest

LOD score (Table 2). In total, we could identify 114 recombinants between markers M1 and M5, of which 64 informative recombinants between markers M1 and M19 (Fig. 2b). These 64 plants showed recombination between heterozygous (*PEK-SF48/pek*-DG1) and homozygous (*pek*-DG1/*pek*-DG1) genotypes. Interestingly, all the susceptible BC_1S_1 plants had homozygous *pek*-DG1 allele at marker M13. Additional KASP markers were designed between M1 and M19 and four key recombinants (Haplotypes 6 and 7) restricted the *PEK* locus between M27 and M28. Although we did not find a maker co-segregating with the HR phenotype, fine-mapping restricted the *PEK* locus to a ~50 kb interval on chromosome B3.

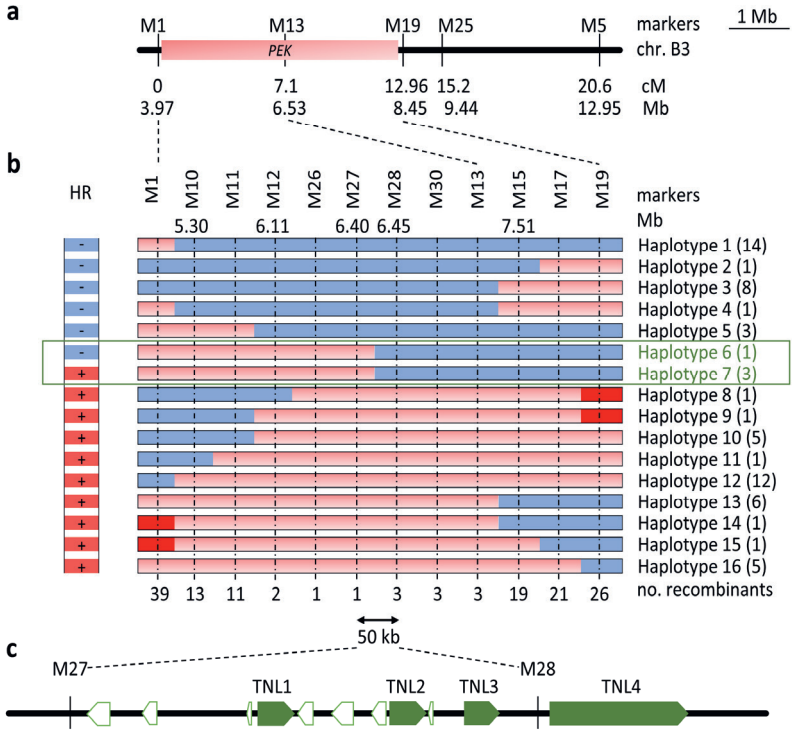


Figure 2. Fine-mapping restricted the *PEK* locus to a ~50 kb interval. a) Fine-mapping with five makers on a BC_1S_1 population (n = 695) located the *PEK* locus between 3.97 and 8.45 Mb. Marker names, genetic distance and physical distance are given. b) Genotype and phenotype of 64 recombinants used for recombinant analysis. Recombinants with same genotype are represented as a single haplotype. Blue bars represent homozygous DG1-S1 allele (S/S), light red heterozygous (R/S), dark red homozygous SF48-O1 genotype (R/R). Phenotype of each haplotype (HR presence/absence) is shown on the left side, "-" means susceptible (no HR), "+" means resistant (HR). Numbers under each dashed line indicate the recombinants between each marker and the phenotype. Informative recombinants for fine-mapping are indicated in green (Haplotype 6 & 7). c) *PEK* locus is fine-mapped to a ~50 kb interval (genome C2 v1.0) containing eleven genes and a cluster of four TIR-NBS-LRR (green). *B. nigra* gene names are reported only for the two genes at the border of the locus, full list of all genes is reported in Table 3.

We performed an additional recombinant screening on four BC_1S_2 populations that were generated from BC_1S_1 plants with heterozygous genotype (*PEK-SF48/pek-DG1*) at the locus (Supplementary Fig. S4). All four BC_1S_2s showed the expected 3:1 phenotypic segregation ratio between R and S plants (Supplementary Table S1). Informative recombinants between M12 and M15 were identified in all populations (7 in BC1S2-2, 12 in BC1S2-3, 2 in BC1S2-4, 9 in BC1S2-5) and genotyped with additional markers (M26, M27, M28, M30). In all populations, S plants had homozygous *pek*-DG1 allele at markers M26, M27 and M28. One R recombinant in population BC1S2-3 was homozygous *pek*-DG1/*pek*-DG1 at M28, thus confirming the right border of the locus.

Candidate genes within the *PEK* locus

To identify candidate genes for the HR phenotype, we checked the annotations within the *PEK* locus in the reference genome C2. The region contains 11 genes with many duplicated loci such as three *B. nigra* homologs of a methionine aminopeptidase 1D (MAP1D, AT4G3700), three homologs of an unknown membrane protein (AT4G37030) and three TIR-NBS-LRR. A fourth TIR-NBS-LRR (TNL) is present just outside marker M28. Based on gene annotations, the four TNLs are the only genes that we could associate to known plant immunity functions. Thus, they represent good candidate genes for the HR phenotype. Further, we used the sequencing data from the BSA-seq to study the variation within the *PEK* locus. In total we identified 785 variants (SNPs and InDels) within each of the eleven genes, but we could not pinpoint putative variants associated to the trait (Supplementary Table S6).

Table 3. List of genes included within the *Pek* locus in the *B. nigra* genome C2.

***B. nigra* gene ID (genome C2)**	**start CDS (bp)**	**end_CDS (bp)**	**std[a]**	***A. thaliana* homolog**		
				gene ID	**gene symbol**	**gene description**
BniB03g015420.1C2	6,411,323	6,409,090	-	AT4G37080	-	Protein of unknown function
BniB03g015430.1C2	6,414,903	6,416,090	-	AT4G37030 [b] AT4G37040	- MAP1D	Membrane protein Methionine (Met) aminopeptidase
BniB03g015440.1C2	6,425,471	6,425,693	-	AT4G37030	-	Membrane protein
BniB03g015450.1C2	6,426,485	6,430,131	+	TIR-NBS-LRR [c]	-	multiple TIR-NBS-LRR
BniB03g015460.1C2	6,430,513	6,432,096	-	AT4G37040	MAP1D	Met aminopeptidase
BniB03g015470.1C2	6,436,216	6,433,822	-	AT4G37030	-	Membrane protein

Table 3. Continued.

BniB03g015480.1C2	6,439,477	6,437,932	-	AT4G37020	-	eukaryotic initiation factor 4A-III
BniB03g015490.1C2	6,439,814	6,443,405	+	TIR-NBS-LRR [b]	-	multiple TIR-NBS-LRR
BniB03g015500.1C2	6,443,871	6,444,160	-	AT4G37040	MAP1D	Met aminopeptidase
BniB03g015510.1C2	6,447,293	6,450,764	+	TIR-NBS-LRR [c]	-	multiple TIR-NBS-LRR
BniB03g015520.1C2	6,456,006	6,469,883	+	TIR-NBS-LRR [c]	-	multiple TIR-NBS-LRR

[a] DNA strand.
[b] This B. nigra gene appears to be a fusion between two A. thaliana genes.
[c] These B. nigra genes are canonical TIR-NBS-LRRs, hence it`s difficult to assign an Ath ortholog without in-depth phylogenetic analysis.

To further restrict the candidate genes within the *PEK* locus, we investigated gene expression levels through RNA-seq on the two parental accessions, SF48-O1 and DG1-S1, upon challenge with *P. brassicae* egg wash. Nine out of eleven genes within the PEK locus were expressed in both parents except BniB03g015440.1C2 (annotated as membrane protein), which was not expressed at all, and BniB03g015500.1C2 (*Bn*MAP1D), which was expressed solely in SF48-O1. Further, we compared differential gene expression between mock and egg wash treatment for the two accessions. Three genes were downregulated upon treatment with egg wash, albeit with a greater magnitude in SF48-O1 than in DG1-S1: BniB03g015420.1C2 (protein unknown function), BniB03g015430.1C2 (membrane protein), BniB03g015460.1C2 (*Bn*MAP1D) (Fig. 3a). Conversely, each of the TNL showed a distinct expression profile (Fig. 3b). BniB03g015450.1C2 (TNL1) showed higher expression levels in SF48-O1 than in DG1-S1, although it was not differentially expressed compared to mock treatment. BniB03g015490.1C2 (TNL2) was not differentially expressed in both accessions (not shown). BniB03g015510.1C2 (TNL3) was transiently downregulated in SF48-O1 at 6 h and 24 h after treatment, while expression was stable in DG1-S1. Finally, BniB03g015520.1C2 (TNL4) was upregulated in SF48-O1 at each time point, albeit at low expression levels. In summary, we could identify six genes within the *PEK* locus that showed differential expression in response to egg wash and between the two parents.

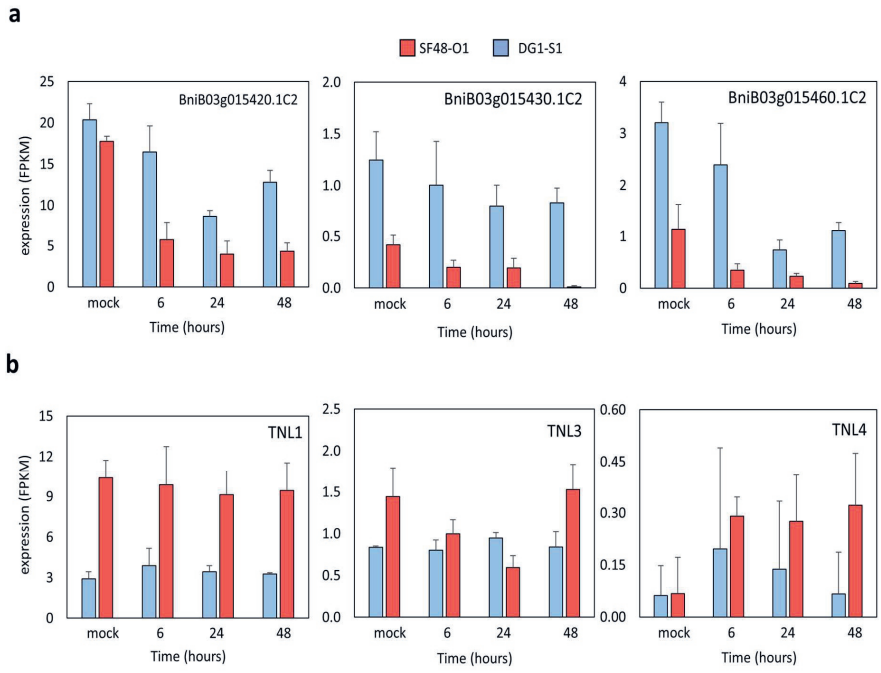


Figure 3. **Differential expression of genes within *PEK* locus from RNA-seq.** Six out eleven genes from the locus were differentially expressed between the parents SF48-O1 and DG1-S1. a) Genes downregulated in SF48-O1: protein of unknown function (BniB03g015420), a membrane protein (BniB03g015430) and *Bni*MAP1D (BniB03g015460). b) Genes upregulated in SF48-O1 are TIR-NBS-LRR receptors: TNL1 (BniB03g015450), TNL3 (BniB03g015510) and TNL4 (BniB03g015520). Bar plots represent mean + standard error of three samples.

PEK locus shows copy number variation (CNVs) among *B. nigra* genomes

The *PEK* locus contained multiple duplicated genes, including a cluster of TIR-NBS-LRR, a class of NLR intracellular receptors. NLRs are often organized in genomic clusters as the result of tandem duplications, unequal crossing over and gene conversion (Kuang *et al.* 2004). Thus, we suspected that the locus may be highly dynamic and polymorphic among *B. nigra* genomes. Indeed, we found extensive copy number variations (CNVs) for some of the genes when comparing the available *B. nigra* genomes NI100, C2, and Sangam (Fig. 4). Specifically, the TNL was present in two copies in NI100, four copies in C2, and seven copies in Sangam. Similarly, we found CNV also for *Bn*MAP1D which is present in two copies in NI100, three copies in C2, and four copies in Sangam (Fig. 4). Collectively, our data showed that the *PEK* locus is highly polymorphic among *B. nigra* genomes.

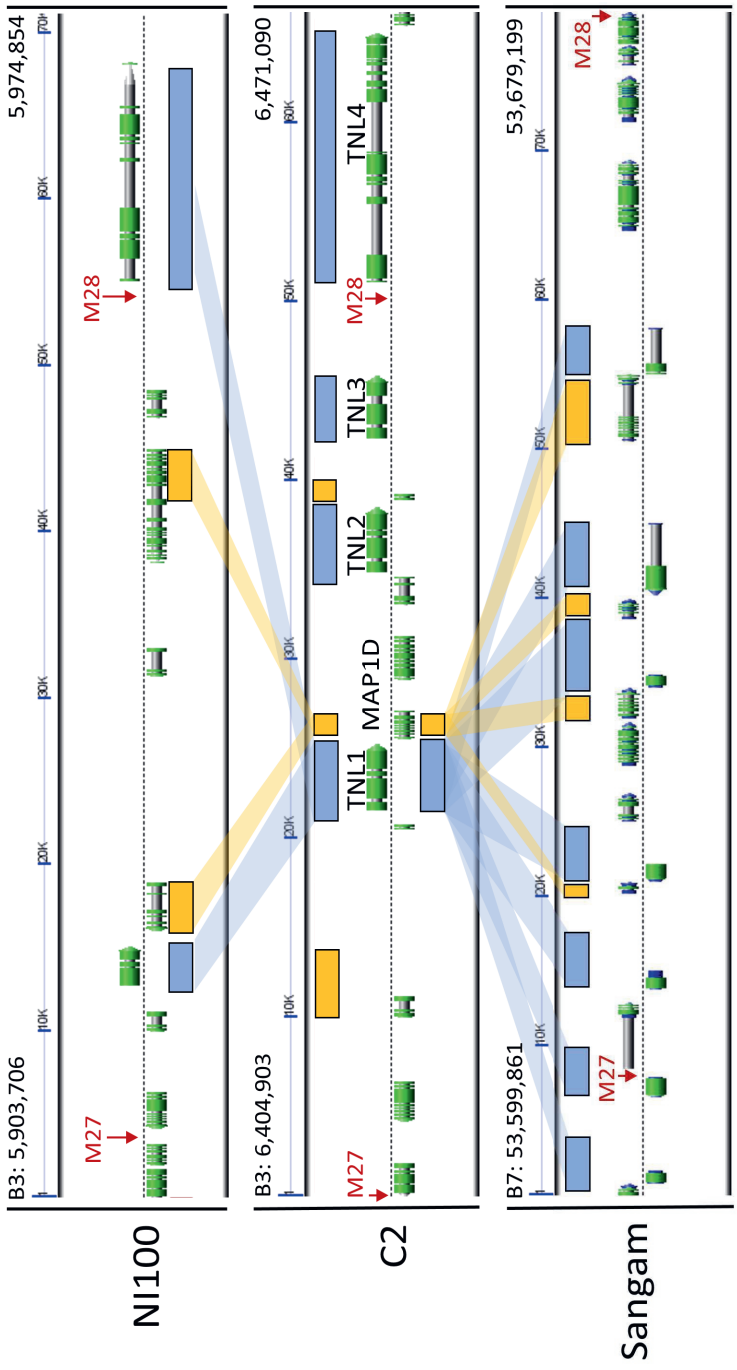


Figure 4. Comparative genomics of the *PEK* locus among three *B. nigra* genomes. Variation in gene content within the locus is evident for the TNLs (shaded blue) and *Bni*MAP1Ds (shaded in orange). Microsynteny was inferred in CoGe using (B)LastZ algorithm with default settings. Only High-scoring Segment Pair (HSP) for genes of interest are reported (blue and yellow). Locations of markers M27 and M28 that flank the locus are indicated in red.

Discussion

The HR induced by *Pieris* spp. egg deposition is both an appealing model system to study the interaction between plants and insect eggs and an effective plant defence trait to herbivores. Here, we report for the first time that *P. brassicae* egg-killing HR-like cell death segregates in *B. nigra* as a Mendelian dominant trait underlined by a single locus. Through BSA-seq and fine-mapping, we identified the *PEK* locus, a ~50kb interval on the proximal arm of chromosome B3. Six out of eleven genes within the *PEK* locus were differentially expressed in the two parental accessions of our crossing scheme. Among these genes, a cluster of TIR-NBS-LRR (TNLs) intracellular receptors are the only genes associated with plant immunity and may be considered likely candidate genes. Finally, we found that the *PEK* locus is dynamic and highly polymorphic as it presents copy number variation (CNVs) among three *B. nigra* genomes.

Segregation of the HR phenotype throughout our crossing scheme supported the evidence for a Mendelian trait underlined by a single dominant locus. As we crossed two highly heterozygous wild *B. nigra* accessions, we observed in the F_1 phenotypic segregation of different morphological traits. Nevertheless, segregation of HR was consistent with a single dominant locus originating from a heterozygous donor plant: we showed a 1:1 segregation ratio of F_1 and BC_1 populations, followed by a 3:1 segregation ratio of F_2, BC_1S_1 and BC_1S_2 derived from selfings of heterozygous resistant plants. Accordingly, selfing of plants without HR resulted in progenies that were also unable to develop HR. We successfully identified the *PEK* locus using a BSA approach which it already proven to be advantageous for quickly identifying single Mendelian loci in genetic populations with little recombination (Liu *et al.* 2012, Chang *et al.* 2018), even when using heterozygous species (Dakour *et al.* 2018, Prodhomme *et al.* 2019, Clot *et al.* 2020). So far, HR has been frequently associated with monogenic qualitative resistance to bacteria, fungi, nematodes and viruses (Kourelis & Van Der Hoorn 2018). In plant-insect interactions, however, HR seemed less prominent as defense response and mostly occurring against cell content feeders such as aphids, gall midges or planthoppers (Botha *et al.* 2006, Klinger *et al.* 2009, Himabindu *et al.* 2010, Stuart *et al.* 2012). It is thus remarkable that an HR cell death evolved to target eggs and that it is also underlined by a single major effect locus as previously shown mainly for HR-based resistance traits against pathogens.

Through recombinant analysis, we fine mapped the *PEK* locus to a ~50 kb region. Further fine-mapping in BC_1S_2 populations did not increase the resolution into the locus, likely

due to the small size of the populations. Yet it confirmed the borders flanking the region. *PEK* contains eleven genes, among which a cluster of intracellular receptors of the TNL type. TNLs are often associated with perception and signalling of plant immunity against pathogens (Cui *et al.* 2015), and that many cloned *R* genes providing resistance based on HR are actually TNLS or other NLRs (Kourelis & Van Der Hoorn 2018), The other genes within the *PEK* locus were annotated either as "unknown function", as an unspecified "membrane proteins" (BniB03g15430.C2) or were orthologs of a methionine aminopeptidase 1D (MAP1D, AT4G37030). MAP1D is an enzyme responsible for the cleavage of the initiator Methionine residue at the N-terminal of proteins (Ross *et al.* 2005). MAPs have been indicated as first step required for the stabilization and/or degradation of chloroplasts proteins (Apel *et al.* 2010), but a putative involvement in plant defense is yet to be proven. Given the involvement of TNLs in plant immunity, we consider the TNLs within the *PEK* locus as the main candidate genes for butterfly egg-induced HR.

Out of the eleven genes within the *PEK* locus, six genes, including three TNLs, were differentially expressed upon treatment with egg wash between the two parental accessions. Interestingly, one TNL (TNL1, BniB03g014450.C2) was consistently highly expressed in SF48-O1, the parent expressing HR, and not in the other parent DG1-S1. A second TNL (TNL4, BniB03g015520.C2) was upregulated in SF48-O1 and downregulated in DG1-S1. A few of the other genes within the locus were also differentially expressed, namely downregulated in SF48-O1 compared to DG1-S1. While the involvement of MAP proteins in plant immunity has not been proven, TNLs are fundamental regulators of the early signalling of plant immunity (Monteiro & Nishimura 2018) and can be regarded as main candidate genes for the *PEK* locus. We used differential expression to pinpoint functional TNLs that were potentially involved in eliciting HR. Yet, it is worth to recall that NLR activity may not necessarily be dependent on transcript abundance, but also on other mechanisms such as alternative splicing, post-transcriptional and post-translational modifications and protein levels (Lai & Eulgem 2017).

Identification of the casual gene through further fine-mapping and investigation of the variants casual of the HR phenotype will require to resolve the genomic structure of the *PEK* locus in our plant material. In fact, although we used our short-read sequencing data to characterize sequence variants (SNPs and InDels) between the two parental lines, interpretation of the data may be misleading for different reasons. First, our variant calling may be inaccurate as the gene content within the three available *B. nigra* genomes may not be accurately representative of our plant material. Second, variation in disease resistance traits underlined by

NLR loci may also be explained by presence/absence variations (PAVs) or copy number variations (CNVs) (Barragan & Weigel 2021). Considering that we found CNVs among three *B. nigra* reference genomes, we suspect that this type of variation also occurs within our plant material. Finally, clusters of NLR loci are often subjected to increased/suppressed recombination (Chin *et al.* 2001, Choi *et al.* 2013) or unequal crossing overs (Kuang *et al.* 2004). This can complicate fine-mapping in absence of the exact genome assembly of the plant material being studied. Complex NLR loci formed by different tandem repeated genes are increasingly being resolved using long-read sequencing technologies (Read *et al.* 2020, Chovelon *et al.* 2021, Wang *et al.* 2021a). Similar approaches will be certainly crucial to disentangle the genomic structure of the *PEK* locus.

Previously, we showed that *B. nigra* expresses a strong HR in response to *Pieris* spp. eggs resulting in egg-killing and that this response is consistently stronger compared to the responses observed in other Brassicaceae species (Griese *et al.* 2021, Chapter 2). Given that genomic data are available for most of the species used in that study, an improved assembly of the *PEK* locus will enable us to study whether the locus genetic diversity across the plant family correlates with interspecific diversity in HR phenotype. For example, *A. thaliana* shows natural variation for *Pieris* egg-induced HR that appears weaker than what we observed in *B. nigra* and that does not affect egg survival (Groux *et al.* 2021b). This weak type of HR could be the result of lower selective pressure by the herbivore since *A. thaliana* is not a host of *Pieris* spp. (Harvey *et al.* 2007) and only occasionally a host of *Anthocaris cardamines* (Wiklund 1984). Similarly, *B. rapa* crop morphotypes show a comparable weak HR (Bassetti *et al.* 2022). In this case, it could be the result of a different mechanism, for example negative selection during domestication as often occurs to many inducible defence traits (Turcotte *et al.* 2014, Whitehead *et al.* 2017). Given the high variability and diversification of loci containing cell-surface or intracellular receptors, the characterization of genetic diversity across a broad phylogenetic context can help to resolve the macroevolutionary history of these loci and generate hypothesis on putative functional domains (Wang *et al.* 2021a, Snoeck *et al.* 2022).

In conclusion, here we report that intraspecific variation for HR induced by *P. brassicae* eggs is associated with a single locus in *B. nigra*. Through classical forward genetics we identified the *PEK* locus, which contains a cluster of TNL receptors. The locus is highly polymorphic between our accessions and between available genomes, implying the need for improved genome assembly before future fine-mapping. This future work will enable cloning and functional testing of the first plant gene involved in defense against insect eggs.

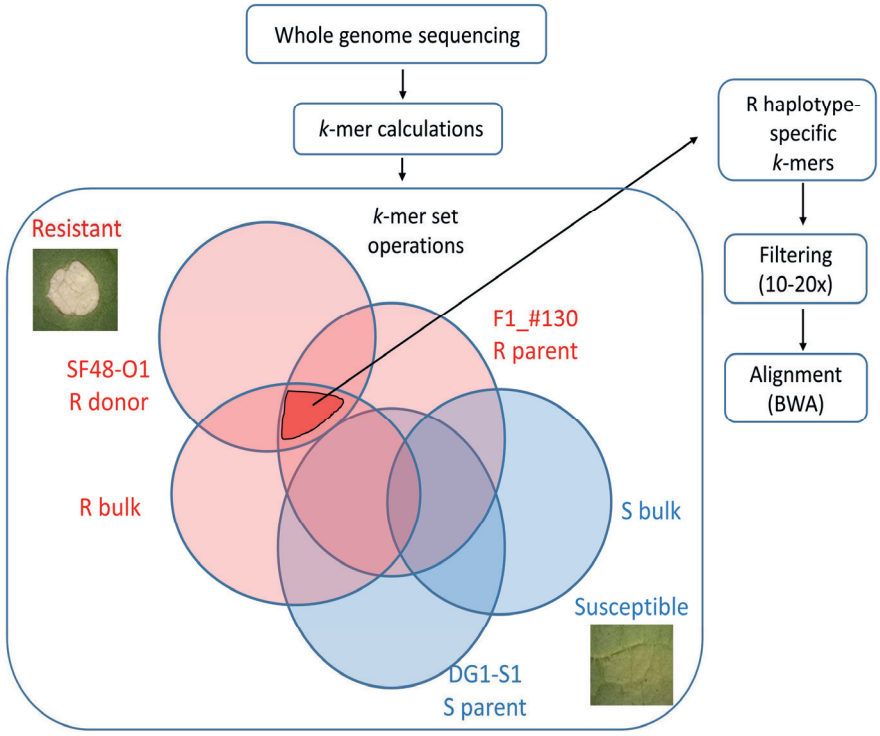
Acknowledgements

We are grateful to Charlotte Prodhomme and Corentin Clot for the introduction to CoSSA pipeline for *k*-mer based genetic mapping. Thanks to Chengcheng Cai and Robin van Velzen for support with whole genome sequencing data. Many thanks to Unifarm for caring wonderfully of experimental plants and butterfly rearing.

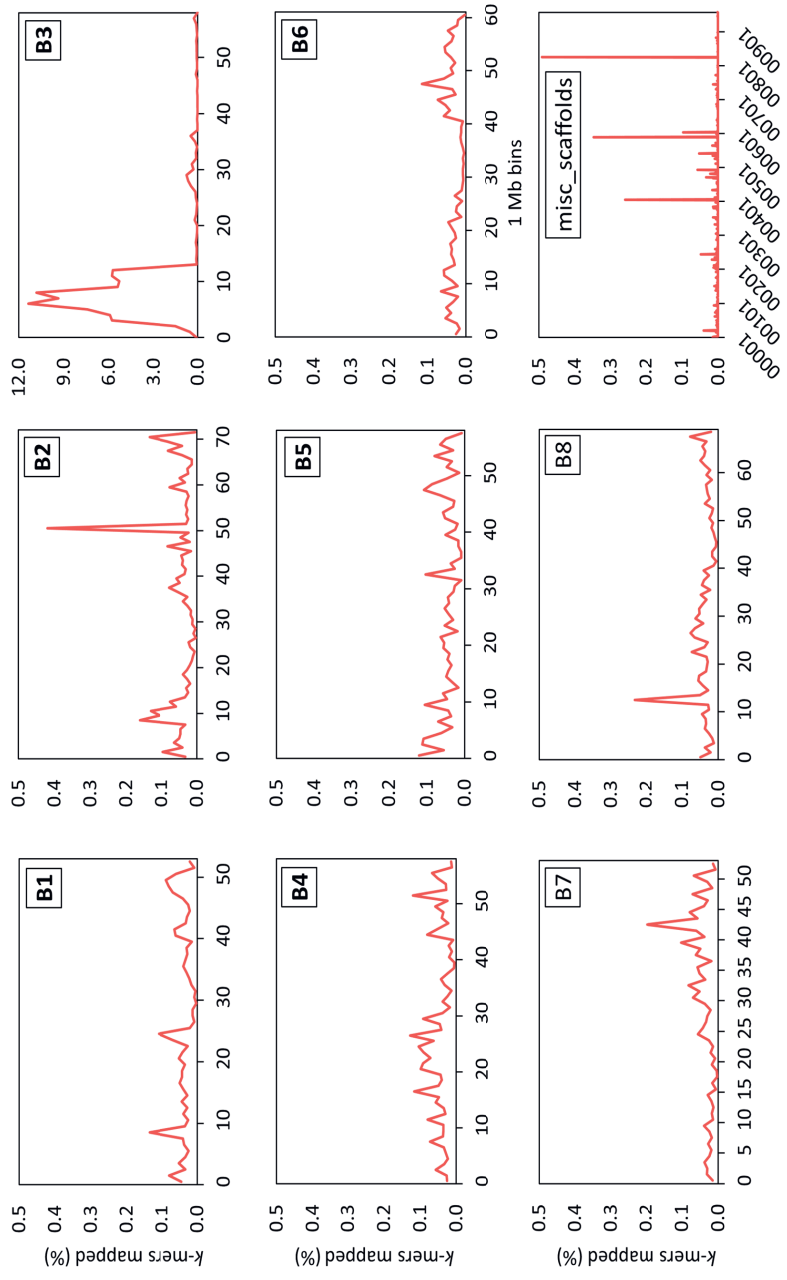
Availability of data and materials

The datasets supporting the conclusions of this article are temporary available at this link https://figshare.com/s/71f9776de96bae6d26df (restricted access, to be released upon publication).

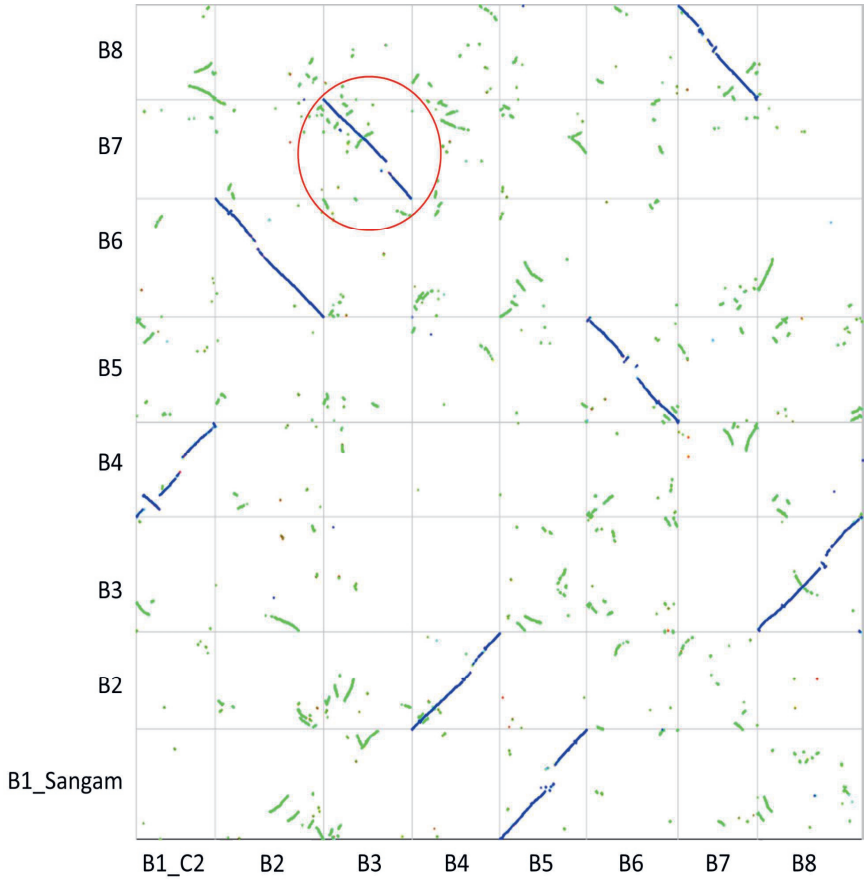
Supplementary figures



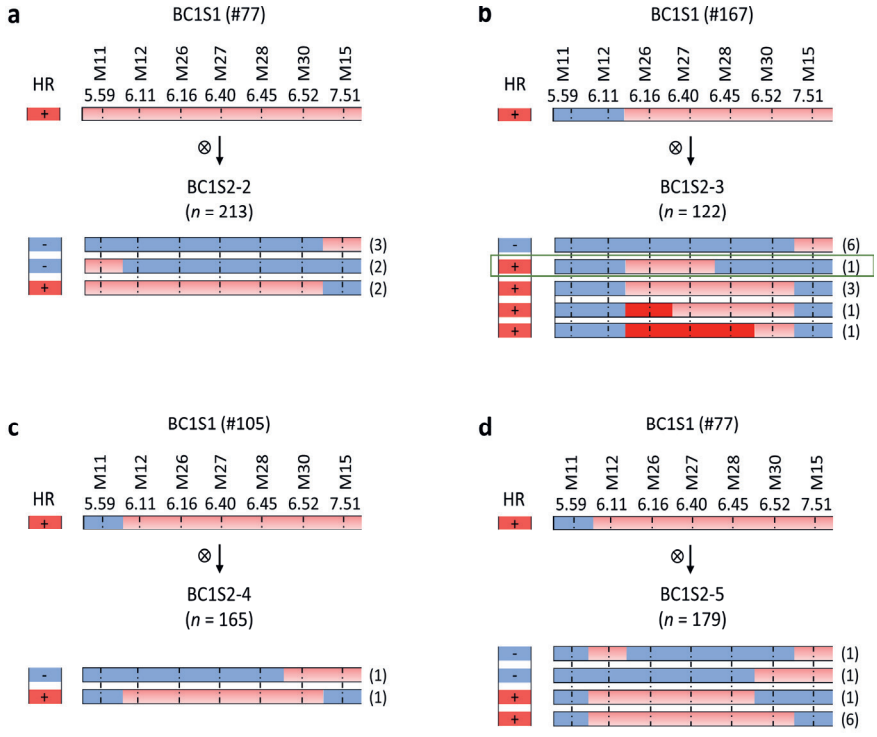
Supplementary Figure S1. Workflow of the BSA-seq strategy. *k*-mer set operations (subtraction, intersections) between the bulks and the parents to obtain the final *k*-mers set unique to resistant (R) haplotype. This set was then used for alignment to the *B. nigra* reference genomes. Sample names are color coded based on the phenotype with resistance (R) in red and susceptible (S) in light blue.



Supplementary Figure S2. Mapping of k-mers across whole genome C2. Distribution of *k*-mers unique to resistant (R) samples mapped on *B. nigra* chromosomes. *k*-mers are plotted on each 1 Mb bin as percentage of the total *k*-mer set.



Supplementary Figure S3. Syntenic relationship between genomes C2 (x-axis) and Sangam (y-axis). Dotplot showing the syntenic relationship between *B. nigra* genomes Sangam v1.0 (y-axis) and C2 v1.0 (x-axis). Blue dots represent main orthology. The analysis revealed different naming of the chromosomes between the two assemblies. *B. nigra* chromosome B3 of genome C2 corresponds to the inverted chromosome B7 of genome Sangam (red circle). Similar results were obtained were comparing NI100 v2.0 and Sangam v1.0.



Supplementary Figure S4 Recombinant analysis in populations BC1S2. Four BC_1S_2 populations were generated by selfing BC_1S_1 plants with resistant phenotype and heterozygous genotype (*PEK*-SF48/*pek*-DG1). a) BC1S1-2 (*n* = 213). b) BC1S1-2 (*n* = 213). c) BC1S1-2 (*n* = 213). b) BC1S1-2 (*n* = 213). In each panel, phenotype and genotype are given for parental plant and informative recombinants. Recombinants with same genotype are represented a single haplotype. Blue bars represent homozygous DG1-S1 allele (S/S), light red heterozygous (R/S), dark red homozygous SF48-O1 genotype (R/R). Phenotype of each haplotype (HR presence/absence) is shown on the left side, "-" means susceptible (no HR), "+" means resistant (HR).

Supplementary tables

Supplementary Table S1. Segregation ratios of phenotypes in all populations used in this study.

Populations	Parent phenotype	Observed		Expected		Ratio	χ^2 test (P-value)
		R	S	R	S		
Selfing of parents							
DG1-S1	S	0	15	0	15	0 : 1	1
SF48-S1	R	7	0	5	2	3 : 1	0.127
F_1							
F1-1	S x R	75	75	75	75	1 : 1	1
F_2							
F2-2	R	7	1	5	2	3 : 1	0.67
F2-3	R	8	4	9	3	3 : 1	0.44
F2-4	S	0	20	0	20	0 : 1	1
BC_1							
BC1-3	S x R	26	40	33	33		0.085
BC1-4	R x S	30	39	35	35	1 : 1	0.279
BC1-5	S x S	0	70	0	70	0 : 1	1
BC1-6	S x R	43	27	35	35	1 : 1	0.056
BC_1S_1							
BC1S1-1	R	533	155	516	172	3 : 1	0.134
BC_2							
BC1S2-2	R	161	52	160	53	3 : 1	0.843
BC1S2-3	R	99	24	92	31	3 : 1	0.159
BC1S2-4	R	129	42	128	43	3 : 1	0.895
BC1S2-5	R	130	51	136	45	3 : 1	0.324

Supplementary Table S2. Summary statistics of DNA sequencing output.

Sample	Sample phenotype	Raw reads (#)	Raw data (Gb)	Effective (%)	Error (%)	Q20 (%)	Q30 (%)	GC (%)	Seq depth[a]
SF48-O1	R donor	60,445,870	18.1	99.72	0.03	97.67	93.13	38.56	33.0
DG1-S1	S parent	48,138,460	14.4	99.64	0.03	97.7	93.23	38.3	26.3
F1_#130	R parent	63,861,175	19.2	99.82	0.03	97.66	93.16	38.44	34.8
S bulk	S bulk	72,563,113	21.8	98.91	0.03	97.5	92.75	37.95	39.6
R bulk	R bulk	47,950,987	14.4	99.35	0.03	97.81	93.43	37.85	26.2

[a] sequencing depth calculated for a diploid genome

Supplementary Table S3. Summary statistics of *k*-mer sets generated during CoSSA analysis.

k-mer set	Sample	Raw reads (#)	without depth cut-off		depth cut-off [2]		depth cut-off [10-22]	
			Nt	Nu	Nt	Nu	Nt	Nu
R donor	SF48-O1	60,445,870	14,502,074,260	938,264,871	14,049,317,059	485,507,670	93	39
S parent	DG1-S1	48,138,460	11,549,256,165	843,222,658	11,186,200,217	480,166,710	93	38
R parent	F1_#130	63,861,175	15,321,446,171	1,276,045,455	14,612,159,950	566,759,234	93	38
S bulk	S_bulk_0	72,563,113	17,409,135,143	1,158,873,187	16,824,880,962	574,619,006	93	38
R bulk	R_bulk_4	47,950,987	10,499,947,214	867,436,937	10,168,163,382	535,653,105	93	38

Nu = number of unique *k*-mers
Nt = number of total *k*-mers

Supplementary Table S4. Sequences flanking the SNPs that were used to design KASP markers. Order, position and chromosome where the SNPs are located are given for three B. nigra genomes.

Marker	Sequences_flanking_SNP (genome NI100)	NI100			C2			Sangam		
		order	position (bp)	chr.	order	position (bp)	chr.	order	position (bp)	chr.
M1	GATTTCGCGACGAATCTCCGAGAAGTCATA[C/G]ATACGGCGGTTGTTAGGGAGATGAAGGAAC	1	3,725,356	B3	1	3,975,746	B3	31	55,732,150	B7
M10	CAAGGGGTACGGGTTTGTTAGATTTGGGGA[C/T]GAGAGTGAGCAGATAAGGGCTATGA	2	5,082,479	B3	2	5,308,705	B3	30	54,474,552	B7
M11	GCATCCGAAGAATAATAGAAGAAGAAGATC[A/T]GAGTCATGGATACAATGCCATCAAGTATCT	3	5,310,203	B3	3	5,597,765	B3	-	-	-
M12	TTTTGTGGTCAGTGTCCAAGAGAAACATTT[C/T]CTTAGGCCTAATCTCTCATTATTAAGCCAA	4	5,787,425	B3	4	6,113,005	B3	29	53,797,852	B7
M26	AGCCAAGAACTGCGGCTCGTTATTCTTAGC[A/C]GAAGTAGTAAACCCAACGAACACCAACGAG	5	5,833,709	B3	5	6,167,571	B3	28	53,742,858	B7
M27	TACCAAGCTAACCAAAAAACATGGTATGAG[G/A]CAAGAATGCCGACAACAAAAAACAAATGAG	6	5,907,557	B3	6	6,408,748	B3	27	53,675,083	B7
M28	TTCAATGCCTTCTCATTGTCAGGCAGCTTT[A/G]TTGCTTGCGTCCAAAGTAACAAGAACACAT	7	5,957,223	B3	7	6,451,769	B3	26	53,612,687	B7
M29	GCAACTGATCAGATTGAAGGGAAATCCCAA[T/C]AAAATCCTTCTCCCTCGGCTAATAGCCACA	8	5,997,900	B3	-	-	-	-	-	-
M30	GATCTACTTACGGACATTCTTGTGGTAAGA[T/C]ATTTTAGTCTGTGTTATTCTTAATTCATAC	9	6,062,627	B3	8	6,528,001	B3	25	53,506,770	B7
M13	AAAAGTTGAAACTAGAAATTGACGAGAAAA[C/T]CGGACAAGACCGTTTGATCGAGGAAACAGA	10	6,064,706	B3	9	6,530,000	B3	24	53,504,710	B7
M31	CTTTTCATACTTCATAGTATACGTATATTT[C/A]TTATACACGTATATTCATTTTGTTAACTAA	11	6,172,817	B3	-	-	-	23	53,388,460	B7
M32	AAGCTCCATGAACCTGTGTCACAGAGAGTG[C/T]GATTTTACATCAAATAATTGTTGAAGCAGC	12	6,305,405	B3	10	6,747,538	B3	22	53,266,741	B7

Supplementary Table S4. Continued.

Marker	Sequences_flanking_SNP (genome NI100)	NI100			C2			Sangam		
		order	position (bp)	chr.	order	position (bp)	chr.	order	position (bp)	chr.
M14	TAGTCTTCATGAAAACAAGAAGCATATATT[C/T]GTTCTTTGAAACATATATATAATTTGGAAT	13	6,331,605	B3	11	6,776,411	B3	21	53,241,877	B7
M33	CCTCTCGAACTCCTTGACATGATACCTGAG[A/G]TACCGATCGATGGTCGTGACCTGCATCAGC	14	6,589,682	B3	12	6,991,439	B3	20	52,996,523	B7
M2	GTAATGTAATTCAAACATTCATAATTTTAA[T/A]TTAGGATTTCAATAATATAGAAATCTTCTT	15	6,716,683	B3	13	7,109,896	B3	19	52,848,999	B7
M34	CTTGGGTTGTTTCCTCGGCCCTGGCCCTCA[A/T]CAGCCGACGTATCTGAAGGTAGTCGGTTTC	16	6,739,374	B3	14	7,129,268	B3	18	52,829,587	B7
M35	CTAACAAAGATTGAGAATGCAACATATACA[C/T]CCTCTGAACTCCATGCAAGAGATTAAAATA	17	6,902,975	B3	-	-	B3	-	52,673,178	B7
M36	TATAAATATTTAATTTGTGTATGTTTTTAA[C/T]TTTATTATATAAATTCATCTTGCGCACAC	18	6,980,214	B3	15	7,349,858	B3	17	52,617,320	B7
M37	TTTGAGCAATCACAGATATCTCAAGTCCAC[T/A]AGTCTCCCAGGGAAACTTCCGCCTGAGTTG	19	7,154,738	B3	16	7,517,094	B3	16	52,456,166	B7
M15	TGAGGATATCAAACTAAAAAATCCGTCCCT[T/G]TTTCAGGATTTTGAGGAATGTGAGTATGTC	20	7,156,703	B3	17	7,519,512	B3	15	52,454,193	B7
M17	CACGCGTTCAACGGTTACATTGGTGGAACC[G/A]CGACCGACAGAGATACAATCGTCTCCTGTT	21	7,407,280	B3	18	7,776,813	B3	14	52,196,999	B7
M18	GGACGCCGATCATGCGTTTGACGCAACGAT[A/T]GAGGAGCTATGCAAGAATCTCTACGAGTTG	22	7,775,715	B3	19	8,159,458	B3	13	51,874,869	B7
M16	TTTCTTTCCTGGTTCCAACTTTATTATATT[C/T]TTTTAACGGATTCCAACTTTTTTTCTTATT	23	7,782,491	B3	20	8,170,230	B3	12	51,863,256	B7
M19	GTCGCCGACGGTGGAAGAACCGTCGCCGTA[T/C]GAGACCTCGTAGAGACACGTGGCGTTTCTG	24	8,054,816	B3	21	8,453,666	B3	11	51,574,511	B7
M20	GGCAATGGCCTCGTCAAAGGCTTGCTTGGC[C/T]AAATGGCAAGCTCTGCAATAGTTTTCC	25	8,165,744	B3	22	8,602,439	B3	10	51,442,655	B7

Supplementary Table S4. Continued.

Marker	Sequences_flanking_SNP (genome NI100)	NI100			C2			Sangam		
		order	position (bp)	chr.	order	position (bp)	chr.	order	position (bp)	chr.
M21	ATCTCCCACGCTAAAGGCCTTTACGTTTGA[T/C]GATCTAAAGACAGCCACTAGAAACTTCAGA	26	8,335,955	B3	23	8,755,517	B3	9	51,300,931	B7
M22	AGCAACATTCACCGTCGCTCCTGGGGACGA[T/C]ATGACAGCTAGCTGCCTGGCGTACACCTTT	27	8,437,420	B3	24	8,861,977	B3	8	51,134,074	B7
M23	GAGAACCCCCAACGAAGAGCACGAGCCGGA[A/G]CACTATGATGATCTCAGCTTCACTTTAACC	28	8,617,101	B3	25	9,092,037	B3	7	50,940,153	B7
M24	TCCACATCAATGAACAAGTAGAAAAAATAT[C/T]GAGAAAGGGAGAGTGTATGGCTGACGGTAT	29	8,825,386	B3	26	9,291,785	B3	6	50,741,742	B7
M3	GAGAAGTCCCTTCTCCAAATAAACCTTTTG[C/T]GAGACGCTATCAAATGATTGATTCCTCCCT	30	8,921,765	B3	27	9,379,180	B3	5	50,633,779	B7
M25	TGTGGTGTTAAACGGGTCCGAATATATTGC[C/T]TTTTTCCAAGCCTGGAGAGCAATGTAAGCT	31	8,985,102	B3	28	9,439,399	B3	4	50,573,260	B7
M4	ATCTTTTATAACATTCTTCTACGTAACAAG[C/G]TGACACGGACCAAGGTCTATGCGTTAGTAT	32	10,828,336	B3	29	11,816,023	B3	-	-	-
M5	CAAACATTCAAGATCATCATCTTCATCCTG[G/A]AAAACCCAACTAAGTCAAACCGACTAAGAA	33	11,848,590	B3	30	12,955,154	B3	3	47,628,938	B7
M6	AGCTTCACCATTGGTCCAATCTCTAACCGT[T/A]GCCTTAACCAAGGTCGGGTTTTGACTAATC	34	12,664,392	B3	31	13,955,455	B3	2	46,826,962	B7
M7	GCTCCACCGCTCGCCGCCGCCGCCGCATTA[T/G]TTGCCGCCTTTGCCATCAGTGTCCTGCATC	35	16,987,677	B3	32	19,116,130	B3	1	42,354,871	B7

Supplementary Table S5. Segregation ratios and association with HR phenotype of five markers used to genotype the whole BC_1 population (n = 66). Alleles at each marker are given a symbol based on the phenotype of the accession from which they derive: "R" is for the SF48-O1 allele, the resistant parent. "S" is for the DG1-S1 allele, the susceptible parent.

Marker	position on B3 (Mb)	Observed		Expected (1:1) [a]		χ^2 test (P-value)	Kruskall-Walllis (P-value)
		R/S	*S/S*	*R/S*	*S/S*		
M1	3.97	22	43	26	40	0.359	
M12	6.11	23	42			0.504	
M19	8.45	22	43			0.359	
M25	9.44	20	42			0.223	
M5	12.95	21	44			0.243	

[a] Expected genotype ratios deviates from exact 1:1 ratio because to reflect the ratio of R:S plants in the BC_1 population.

Supplementary Table S6. Sequence variants (SNPs and InDels) identified in the *PEK* locus. Variants were detected using DNA short-read sequencing data. Effects of variants on the protein were predicted using SnpEff: "high" indicates severe effect on the protein (e.g. frameshift, splice acceptor/donor variant, start lost, stop gained/lost etc.), "moderate" indicates moderate effect (e.g. inframe insertion/deletion, missense variant), "low" indicates low effect (e.g. synonymous variant), "modifier" indicates variants outside coding regions (e.g. upstream/downstream CDS, intron/intergenic variant).

B. nigra gene ID	*Ath* ortholog	HIGH	MODERATE	LOW	MODIFIER	Total
BniB03g015420.1C2	AT4G37080	0	3	4	3	10
BniB03g015430.1C2	AT4G37030 + AT4G37040	2	5	1	0	8
BniB03g015440.1C2	AT4G37030	1	1	1	1	4
BniB03g015450.1C2	TIR-NBS-LRR	5	64	21	13	103
BniB03g015460.1C2	AT4G37040	6	11	10	17	44
BniB03g015470.1C2	AT4G37030	0	4	7	3	14
BniB03g015480.1C2	AT4G37020	2	4	12	22	40
BniB03g015490.1C2	TIR-NBS-LRR	8	27	27	0	62
BniB03g015500.1C2	AT4G37040	0	0	0	0	0
BniB03g015510.1C2	TIR-NBS-LRR	5	51	44	32	132
BniB03g015520.1C2	TIR-NBS-LRR	5	56	47	260	368

Chapter 6

General discussion

The aim of my thesis was to investigate the mechanisms and genetic basis of the hypersensitive response-like cell death (hereafter abbreviated as "HR-like" for simplicity) induced by eggs of *Pieris* spp. butterflies on Brassicaceae plants. Different approaches were used in order to: (1) elucidate the occurrence of HR-like in a macroevolutionary context, (2) characterize physiological and molecular plant immunity responses associated with the HR-like and, finally, (3) disentangle its genetic basis, both in a crop species (*Brassica rapa*) and in one of its wild relatives (*B. nigra)*. Specific aspects related to the main findings of my thesis were discussed in the individual research Chapters. In this final Chapter, I will first synthesize the main findings of my thesis. Then, I will discuss additional implications in a broader scientific context. I will, in fact, consider the knowledge generated by this research on mechanisms and genetic basis of egg-induced HR-like within the framework of the plant immunity system. Further, I will discuss the implications of genetic variation at resistance loci in the context of complex plant genomes. Finally, I will discuss the opportunity to study variation at the *PEK* locus in the context of linking molecular genetics with ecology and evolution.

Summary of main findings

The research presented in my thesis started by investigating the occurrence of HR-like within a macroevolutionary context. The well-established co-evolutionary dynamics between Pieridae butterflies and Brassicales plants is driven by the evolution of plant chemical defenses (i.e. glucosinolates) and the reciprocal counteradaptations of insect detoxification mechanisms (Wheat *et al.* 2007, Edger *et al.* 2015). In **Chapter 2**, I aimed to explore the overlooked egg-induced HR-like cell death trait as a potential adaptation in the butterfly-plant arms race. I found that HR-like occurs throughout the Brassicaceae family but not within Cleomaceae. A single gain of the trait followed by multiple losses throughout the family seemed the most parsimonious explanation, although I could not yet exclude multiple independent origins of egg-induced cell death. Further, it is evident that a strong egg-killing HR-like which reduces egg fitness is restricted to the Brassiceae tribe, which includes *P. brassicae* natural hosts from the *Brassica* genus and close relatives. On the insect side, a strong egg-killing HR-like is exclusively induced by eggs of butterflies of subfamily Pierinae that are specialists of Brassicaceae. In conclusion, I proposed that HR may have arisen as novel plant adaptation to defend plants against specialist butterflies that evolved effective glucosinolate detoxification mechanisms.

In **Chapter 3**, plant immunity responses associated with HR-like cell death were characterized more in depth at physiological and molecular levels. I used a comparative framework by testing eggs of the specialist *P. brassicae* and the generalist cabbage moth *Mamestra brassicae* on a crop, *B. rapa*, and its wild relative, *B. nigra*. Overall, the plant response to *P. brassicae* eggs is preceded by accumulation of ROS, callose deposition, cell death and *PR1* expression in both plant species, including accessions unable to express a visible macroscopic HR-like. These plant immunity responses are, however, weak and/or absent under eggs of *M. brassicae*. I showed that an egg wash made of egg-associated secretions is sufficient to induce a cell death comparable to eggs, unlike previously described egg elicitors phosphatidylcholines (PCs). Finally, I showed that plant accessions unable to express a macroscopic HR-like against *Pieris* eggs are nevertheless still able to induce a functional HR against pathogenic bacteria and fungi. I concluded that plant phenotypic variation in HR-like results from genetic variation at dedicated plant immune pathways that are specifically induced by components from eggs of adapted specialist butterflies.

Given the existence of plant genetic variation in HR-like**,** I attempted to disentangle the trait genetic architecture in two plant species. In **Chapter 4**, I carried out a germplasm screening of a *B. rapa* core collection followed by genetic mapping in a RIL population. HR-like in *B. rapa* is a polygenic trait as I identified three QTLs *Pieris brassicae*-induced cell death (*Pbc*). The QTLs *Pbc1-3* explained about a third of the genetic variation and included different types of genes related to plant immunity. Finally, I discussed the implications for fine-mapping of the loci and exploitation of HR-like as a defense trait in crop breeding.

As it was evident that HR-like in *B. rapa* is weaker compared to *B. nigra,* I decided to map HR-like by using crosses of *B. nigra* accessions from a local population (**Chapter 5**). HR-like segregated as a Mendelian trait throughout our crossing scheme in *B. nigra*. Through bulk-segregant analysis coupled with whole-genome sequencing (BSA-seq) and subsequent fine-mapping I identified a single *Pieris brassicae* egg-killing (*PEK)* locus. The locus interestingly includes a cluster of TIR-NBS-LRR receptors (TNLs), a type of NLR genes that are associated with HR-based plant defences. I showed that *PEK* locus has sequences polymorphisms (SNPs and InDels) between our parental accessions but also copy number variants (CNVs) between currently sequenced *B. nigra* genomes. This extensive variation at the locus implies the need to develop an improved genome assembly of our *B. nigra* material in order to perform further fine-mapping and identify casual genes and/or variants. Future research is needed to reveal whether the TNLs within the *PEK* locus are actually involved in detection and/or signalling of the HR-like induced by butterfly eggs.

Plant immune response against *Pieris* eggs

Threat by herbivorous insect eggs is an exciting novel aspect to study plant immune responses, which already accounts for an ever-increasing list of biotic stresses such as bacteria, fungi, oomycetes, viruses, phytoplasma, parasitic plants, nematodes, and insect herbivory (Kourelis & Van Der Hoorn 2018). Plant defenses against insect eggs are ubiquitous across taxonomic clades but the molecular understanding is still lacking for most of them (Hilker & Fatouros 2015, Fatouros *et al.* 2016). Currently, the interaction between *Pieris* eggs and *A. thaliana* is the most investigated system at the molecular level (Reymond 2013). Most of the research presented in this thesis aimed to complement this system by investigating *Brassica* spp. which are natural *Pieris* hosts and express a HR-like cell death against eggs.

Prior to my thesis, research in *A. thaliana* showed that the plant response to *Pieris* eggs differs considerably at the transcriptional level from the response to larval feeding (Little *et al.* 2013, Nallu *et al.* 2018). The uniqueness of the plant response to *P. brassicae* eggs involves physiological (ROS, cell death, callose) and SA-related signalling components which are typically observed in the basal immune response against biotrophic pathogens, known as pathogen-triggered immunity (PTI) (Little *et al.* 2007, Bruessow *et al.* 2010, Gouhier-Darimont *et al.* 2013). Furthermore, early PTI signalling mechanisms such as Ca^{2+} fluxes and MPK3/6 activation were also recently reported in *A. thaliana* (Groux 2019). A PTI response must involve some sort of perception by plant cells and, indeed, *P. brassicae* eggs induce upregulation of many PRRs, specifically of LecRKs type (Little *et al.* 2007), while the expression of the PTI-marker *PR1* is partly dependent on the surface receptor *LecRK-I.8* (Gouhier-Darimont *et al.* 2019). Given the occurrence of these plant immune responses, it was hypothesized that plants respond to *P. brassicae* eggs upon the perception of egg-derived elicitors, possibly conserved among different insect species. These elicitors were termed alternatively egg-associated molecular patterns (EAMPs) to mirror the nomenclature already in used with pathogens (PAMPs), herbivores (HAMPs) or damage-associated elicitors (DAMPs) (Erb & Reymond 2019, Reymond 2021). Recently, different types of phosphatidylcholines (PCs), a common component of cellular membranes, were identified as EAMPs inducing plant immune responses (Stahl *et al.* 2020).

While these studies were conducted on the *A. thaliana* accession Col-0 which lacks a macroscopic HR-like, my research aimed to establish a link between the plant immune responses against *Pieris* oviposition and a macroscopically visible HR phenotype as developed by *Brassica* spp. My results indicated that the HR eliciting activity is restricted to the egg glue secretions surrounding the eggs (Caarls *et al.* 2021, Griese *et al.* 2021, Chapter 2), while PCs

do not induce HR (Chapter 3). Similar to what is observed in *A. thaliana,* PTI responses are also induced by *Pieris* eggs in both *B. nigra* and *B. rapa* (Chapter 3). These responses precede the development of a visible HR-like and the difference between accessions showing or lacking HR-like seems to be rather quantitative as HR-displaying accessions show stronger and longer-lasting responses based on the expression of *PR1* and other SA-related marker genes. The difference between accessions with or without HR is suggested to depend also on a quantitative regulation of sphingolipids (Groux *et al.* 2021a), a known regulator of cell death (Townley *et al.* 2005, Huby *et al.* 2020). Despite these similarities with known pathogen-induced PTI and HR, egg-induced HR-like is putatively resulting from a specific induction of distinct pathways as our *B. nigra* accessions lacking egg-induced HR can still express a functional HR against bacteria or fungi (Chapter 3). This is not completely surprising given that HR is a not a monomorphic trait but rather a series of similar-looking cell deaths with own distinct physiological, molecular and metabolic markers (Mur *et al.* 2008, Künstler *et al.* 2016).

Finally, it is worth mentioning that HR can represent a dispensable trait in the overall basal plant immune response, as it is unnecessary to achieve plant resistance against certain pathogens (Yu *et al.* 1998, Bendahmane *et al.* 1999, Gassmann 2005). In the *Pieris* egg-plant-interaction, however, a severe HR-like cell death is required to kill eggs and, thus, to act as resistance to herbivorous insects (Griese *et al.* 2017, Chapter 2).

Genetic architecture of egg-induced HR

The hypersensitive response (HR) has been traditionally linked to the gene-for-gene concept, in which a dominant resistant gene (*R* gene) of plants detects a cognate dominant avirulence gene (*Avr* gene) of an attacker (Flor 1971). In most cases, HR are monogenic traits underlined by single R genes and classical forward genetics often resulted in cloning of membrane-associated pattern recognition receptors (PRRs) or cytosolic nucleotide-binding leucin-rich repeat receptor (NLRs) (Kourelis & Van Der Hoorn 2018, Schultink & Steinbrenner 2021). From a genetic perspective, unless a gene-for-gene interaction is proven, necrotic symptoms arising from biotic interactions should rather be defined as "HR-like" cell death. Nevertheless, the outcome of forward genetics on phenotypes consisting in necrotic lesions can be hard to disentangle given that HR, and more in general programmed cell death (PCD), are regulated by many different pathways (Mur *et al.* 2008). Indeed, signalling components of PTI and ETI responses, which often precede the onset of HR, are proven to be polymorphic in natural populations (Vetter, Karasov, and Bergelson 2016). It is thus unsurprising that necrotic lesions as result of a biotic stress are frequently quantitative and multigenic, a complex

interaction of small-effect loci dependent on host and attacker genotypes (Corwin *et al.* 2016, Soltis *et al.* 2019, Yates *et al.* 2019).

In view of these considerations, what did I learn so far on the genetic architecture of *Pieris* egg-induced HR-like cell death? Natural variation in HR-like within *A. thaliana* discovered two main loci, the putative Ca^{2+} channel *glutamate receptor 2.7* (*GLR2.7*) and the surface receptor *LecRK-I.1* (Groux 2019, Groux *et al.* 2021b). These loci were identified after egg-induced cell death was scored as a discrete trait using a few symptom classes. Interestingly, scoring the phenotype as 'presence/absence of cell death' gave instead slightly different results with the identification of *GLR2.7* and *LOH2*, an enzyme involved in synthesis of the sphingolipid ceramide (Groux 2019). These genes are plausible candidates as they can be easily associated with the immune response accompanying egg-induced HR (see previous section). Nevertheless, these loci explain only part of the total phenotypic variance, hinting at more undetected loci which may be located on other pathways.

In my study on *B. rapa*, HR-like was measured as 'cell death size' which clearly displayed as a quantitative trait with a continuous distribution among the germplasm and within a biparental segregating population (Chapter 4). The small-to-medium effect QTLs *Pcb1-3* that I identified also explained only part of the phenotypic variance. Genes within the three QTL intervals are involved in different plant immunity pathways, including clusters of PRRs and NLRs. However, the underlying genomic regions are still too large to point at reliable candidate genes. Overall, variation in egg-induced cell death in both *A. thaliana* and *B. rapa* seems to be regulated as a quantitative polygenic trait. In contrast, HR-like segregates in our *B. nigra* crossing scheme as a single dominant trait which I fine-mapped to the *PEK* locus (Chapter 5). A possible explanation can be that a single locus with a major effect on the phenotype masks subtle differences of multiple small effect QTLs. Additionally, I may have isolated a single major effect locus because of the use of bulk-segregant analysis, which does not always identify all loci underlying a trait compared to canonical QTL mapping (Haase *et al.* 2015). A more biological sound explanation, yet to be tested, is that the Toll-interleukin 1 receptor domain NLRs (TNLs) that are located within the *PEK* locus are receptors involved in either direct or indirect perception of elicitors/EAMPs originating from the eggs. In summary, while the results of these three genetic studies provide interesting candidate genes, it is still premature to draw conclusions on the genetic architecture of egg-induced HR-like cell death. In fact it is worth to recall that these studies differed in the use of host plants, egg treatments for the bioassays, scoring methods of HR and genetic populations.

A model for plant perception of *Pieris* eggs

The identification of a cluster of TNLs within the *PEK* locus (Chapter 5) allows me to hypothesize on how *Pieris* eggs may be perceived by plants. So far, TNLs and other NLRs have been associated with qualitative disease resistance traits, often based on HR, and indeed most of the cloned resistance (R) genes are NLRs (Kourelis & Van Der Hoorn 2018). Their main function is to activate defense upon perception of "effector proteins", which broadly indicates a diversified array of proteins generally injected inside plant cells by attackers to modulate and/or suppress the initial plant PTI response (Toruño *et al.* 2016, Basu *et al.* 2017). NLRs either directly or indirectly detect these effectors at different cellular localizations to trigger so-called effector-triggered immunity (ETI) which often leads to HR (Monteiro & Nishimura 2018). Assuming that the TNLs within the *PEK* locus are responsible for egg-induced HR, how can I explain a role for an intracellular receptor within the plant-egg interaction? This scenario implies that such "egg effectors" should be able to diffuse from the egg glue through the cell wall. Interestingly, ongoing efforts suggest that the egg-associated elicitor of HR may not be a protein but rather a terpene-derived metabolite (Caarls *et al.* in prep). Alternatively, the TNLs within *PEK* may not be involved in egg recognition but rather in sensing perturbations of cellular homeostasis (Cui *et al.* 2015). For example, the TNL *SNC1* of *A. thaliana* activates upon misregulation of MPK3/6 signalling and unregulated SA accumulation (Wang *et al.* 2013). Furthermore, certain autoimmune phenotypes in which cell death is regulated by sphingolipids appears to be monitored also by a TNL (Palma *et al.* 2010, Berkey *et al.* 2012).

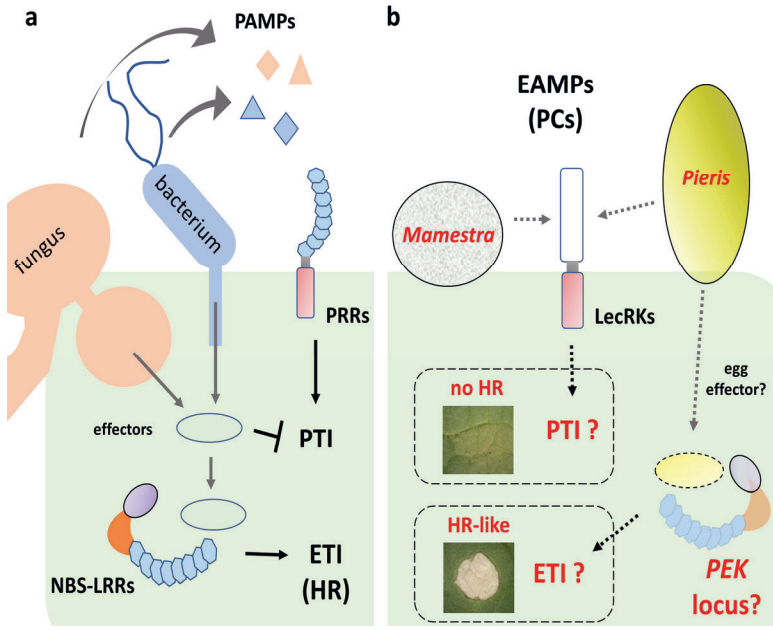


Figure 1. Model of plant immune system (a) and putative perception of Lepidoptera eggs (b). a) Plant immunity is triggered by the recognition of biotic attackers via multiple classes of protein receptors (Dangl & Jones 2006). For a detailed description of the immunity see General Introduction (Chapter 1). Briefly, membrane-associated receptors (PRRs) recognize PAMPs from microbial cells, which are conserved molecules that are required for microbial cell integrity. Recognition leads to a first layer of immunity, known as PTI. Host-adapted attackers can modulate and/or suppress PTI through the secretion of effector proteins. Plants evolved, nonetheless, a second layer of immunity (ETI) which is triggered through cytosolic nucleotide-binding leucin-reach repeat containing receptors (NBS-LRRs or NLRs). ETI is often associated with HR. b) General elicitors or egg-associated molecular pattern (EAMPs), that are conserved among Lepidoptera eggs, e.g. PCs, induce a weak plant immune repose resembling PTI. The recognition is mediated by different LecRKs in *A. thaliana* (Gouhier-Darimont *et al.* 2019, Groux *et al.* 2021). *Pieris* eggs instead induce a HR-like cell death in *Brassica* spp, putatively mediated by TNLs within the *PEK* locus. Dashed lines indicate putative signalling connections. Red text indicates elements of the model studied in this thesis.

Considering my results and the most recent literature, I can propose the following working model to explain the interaction between plants and *Pieris* eggs (Fig. 1). Lepidoptera eggs are perceived by the plant immune system similarly to the recognition of bacteria, fungi, and other biotic stresses. Egg elicitors that are conserved across Lepidoptera species, also known as egg-associated molecular patterns (EAMPs), are detected by plant cell surface receptors (PRRs). For example, *LecRK-I.1* and *LecRK-I.8* were identified in *A. thaliana*. This first general immune response encompasses the PTI phenotypes that I observe in plants lacking HR and under eggs of generalists, e.g. *M. brassicae* or non-adapted Pierid butterflies (Chapter 2 & 3). An example of such conserved elicitors/EAMPs are the phosphatidylcholines (PCs),

phospholipids which are present in all insect eggs (Stahl *et al.* 2020). PCs are, however, not sufficient to elicit HR in *B. nigra* and, further, the elicitor(s) of HR is present in the glue-like secretion surrounding the eggs (Chapter 3). One or more of these compounds, perhaps egg effector proteins, are detected by unknown plant receptors that trigger a second immune response leading to a macroscopic HR-like cell death which acts as a defence trait. This second immune response may be mediated in *B. nigra* by the TNLs located within the *PEK* locus (Chapter 5).

The proposed model is inspired by the gene-for-gene concept which implies a tight co-evolutionary trajectory between plant receptor *R* genes and attacker`s effector *Avr* genes. In simplified terms, as a new *R* gene allelic variant (or a new *R* gene) arises to detect an existing attacker`s *Avr* gene, a selective pressure is applied so that new *Avr* allelic variants (or a new *Avr* gene) can arise and spread through the population (Jones & Dangl 2006). The continuous birth-death of R and Avr genes, together with the fixation of alleles which increase fitness, determines a structuring of wild populations in resistant/susceptible plants and virulent/avirulent attackers, also known as "races" (pathogens) or "biotypes" (insects). The fixation of R genes is one of the foundations of plant breeding to achieve superior resistant cultivars in agroecosystems. However, R genes in wild populations are usually polymorphic and under balancing selection (Karasov *et al.* 2014, Stam *et al.* 2016). In our system, I show that the *PEK* locus is heterozygous in the wild *B. nigra* accession I used in our crossing scheme. Considering that *B. nigra* is an outcrosser, heterozygosity at the locus is likely maintained in natural populations, an hypothesis that should be tested in the future. On the insect side, however, I did not find yet *Pieris* spp. natural variation in the induction of HR, questioning whether "biotypes" may exist at all. Clearly, the studies presented here, are limited to one population of both plant and butterfly and more sampling of different natural populations is needed in the future. Overall, whether the principles of the gene-for-gene framework apply to the plant-*Pieris* egg system requires a deeper understanding of the molecular mechanisms behind this interaction.

NLR loci in plant genomes: need for long-read sequencing

The association of *Pieris* egg-induced HR-like cell death to the *PEK* locus, which is highly polymorphic between the current available *B. nigra* genomes (Chapter 5), has relevant implications for understanding the genetic basis of the trait and its (macro)evolutionary history. From a plant breeding perspective, the molecular markers flanking the *PEK* locus may be sufficient to attempt the introgression of HR-like trait into elite *Brassica* crops lines, as

interspecific crosses between *Brassica* crops and secondary/tertiary gene pools are sometimes done for other resistance traits (Katche *et al.* 2019, Hu *et al.* 2021, Singh *et al.* 2021). However, copy number variations and, even possible structural variations, usually complicates fine mapping of NLR loci beyond the flanking markers. From a scientific perspective, moreover, it is important to known whether the variation at this locus could also explain interspecific phenotypic variation in HR, for example within the *Brassica* clade or even within the whole Brassicaceae family. In this thesis I report evidences that HR-like cell death is stronger and determines egg-killing mostly in species of the *Brassica* clade (Chapter 2). Further, I observed variation within the *Brassica* clade, as the wild *B. nigra* and other wild species of genera *Crambe* and *Sinapis,* clearly express a stronger HR resulting in egg-killing compared to that of *Brassica* crops (Chapter 2), including the *B. rapa* germplasm that was screened (Chapter 4). It is intriguing to correlate this pattern to variation at the *PEK* locus. A similar comparative genomics approach has recently been carried out to understand the evolution and specificity of the receptor *INR*, a Fabaceae-specific PRR that recognizes the peptide inceptin, a HAMP found in the oral secretion of Lepidoptera larvae (Steinbrenner *et al.* 2020, Snoeck *et al.* 2022).

In order to enable similar comparative genomics studies, some characteristics intrinsic to the biology of both surface receptors (PRRs) and intracellular receptors (NLRs) call for more reliable assembly of the underlying loci. NLR proteins are ubiquitous in plants and experienced a massive expansion and lineage diversification during the evolution from green algae to land plants (Shao *et al.* 2019). This tremendous diversification is likely the result of adaptation to a multitude of biotic stresses plants are facing in the environment. The genomic arrangement in clusters of tandem duplicates is considered another main feature of NLRs (van Wersch and Li 2019). The evolutionary consequences are that many linked, highly similar genes can generate an incredible functional diversity through unequal crossovers and/or gene conversions with non-orthologs genes (Kuang *et al.* 2004, Wicker *et al.* 2007). As a result, casual polymorphisms at NLR loci can range from allelic series due to SNPs and/or InDels, to presence/absence variants (PAV), copy number variants (CNVs) including complex genome rearrangements, to protein modularity and even acquisition of non-canonical integrated domains (ID) (Dolatabadian and Fernando 2022). NLR loci are hardly ever well represented by a single plant genome and, further, are likely to be misassembled (Barragan & Weigel 2021). Indeed, the current era of pangenomes is revealing how NLRs are overrepresented in the “accessory” fraction of the genome, the one that is shared by only some accessions (Golicz *et al.* 2016, Montenegro *et al.* 2017, Hurgobin *et al.* 2018). The current solution is the use of third-generation long read sequencing, e.g. Oxford Nanopore or PacBio, to achieve improved

assemblies and annotations of whole genome NLR repertoire ("NLRome") (Van de Weyer *et al.* 2019, Seong *et al.*2020) or pangenomes (Song *et al.* 2020, Liu *et al.* 2020, Athiyannan *et al.* 2022).

Resolving the genomic structure of the *PEK* locus will be a fundamental step to enable further genetic and evolutionary studies. First, it will facilitate to pinpoint the causal variation through fine-mapping similarly to what it has been done on other NLR-containing loci (Read *et al.* 2020, Chovelon *et al.* 2021, Wang *et al.* 2021a, Athiyannan *et al.* 2022). A well annotated locus will then provide the background to study the causal mutations/variants responsible for the HR phenotype through silencing, knock-outs and study of protein domains (Monino-Lopez *et al.* 2021). Functional validation of genes in *Brassica* spp., is still quite cumbersome, with the exception of *B. oleracea* and *B. napus*. Still, candidate gene validation could be approached via improving virus-induced gene silencing protocols (Zheng *et al.* 2010, J. Yu *et al.* 2018, K. Bouwmeester, personal communication) or heterologous expression in *A. thaliana* or *N. benthamiana* which are used as model to study the ability of PRRs and NLRs to induce HR (Wu *et al.* 2017, Shangguan *et al.* 2018). Finally, a correct assembly will make possible accurate genotyping of the locus that is required for population genetics studies aimed at characterizing the locus diversity in an ecological context (Stam *et al.* 2016, Kourelis *et al.* 2020).

Future perspectives

My thesis addressed research questions about the genetic basis of egg-induced HR-like cell death and its putative evolutionary trajectory. More work is clearly needed to pursue both endeavours but the results here presented already outline a few directions for further investigation. Recent reports sketch an alarming decline in insect populations (Forister, Pelton, and Black 2019, Wagner *et al.* 2021), partly due to novel pesticides not being as selective as once assumed (Stokstad 2018). Therefore, research on plant-insect interaction should not only respond to curiosity-driven questions but also to a societal mandate to explore neglected insect resistance traits. From an applied perspective, it is thus worth to investigate the potential of egg-induced HR as a novel defense trait targeting herbivore insects. The cell death induced by *Pieris* eggs has a direct egg-killing effect which affects only a fraction of the oviposited eggs (Griese *et al.* 2021) Thus, future studies should also consider how HR correlates with indirect defenses, i.e. priming against incoming herbivory (Pashalidou *et al.* 2015b), attraction of egg/larval parasitoids (Fatouros *et al.* 2014) and even systemic acquire resistance (SAR) against biotrophic pathogens (Hilfiker *et al.* 2014, Orlovskis and Reymond 2020). This

comprehensive assessment could perhaps elaborate a "resistance score to pests" which considers both egg and larval stages. On the other side, markers associated with the *PEK* locus can already be used to introgress the HR trait from *B. nigra* into *Brassica* crops and assess its reliability and effectiveness in field conditions.

The identification of the *PEK* locus presented here should obviously be complemented in the future by an improved genomic assembly with long read sequencing. A correct assembly of the locus will in fact enable fine mapping and isolation of the casual gene, a fundamental step to understand the mechanism underlying variation in HR in our *B. nigra* material. Moreover, this effort will benefit other studies on genetic diversity and population genetics within and between wild *B. nigra* populations. If the TNLs within the *PEK* locus will be validated, the investigation of diversity at the locus could be further facilitated by means of resistance gene enrichment sequencing (RenSeq) technology, which relies on conserved sequences within NLR to perform a targeted R gene-enriched sequencing approach (Jupe *et al.* 2013).

From an evolutionary perspective, it is extremely intriguing to further study the role of egg-induced HR it the plant-butterfly co-evolutionary context. First, the results presented in Chapter 2 could be complemented by investigating variation at the *PEK* locus in other Brassicaceae genomes. In other words, syntenic loci in other Brassicaceae genomes should be identified to assess whether the existence and type of genomic variations which could potentially represent the genetic basis underlying the clade-specific occurrence of HR that I observed across the plant family (Chapter 2).

In conclusion, my thesis reports and discusses evolutionary, physiological, and genetic aspects concerning a HR-like cell death response induced in plants by insect eggs. My work shows how the interaction between *Brassica* plants and *Pieris* eggs is a suitable model to study plant-insect egg interaction in the future.

References

A

Acevedo FE, Peiffer M, Tan CW, Stanley BA, Stanley A, Wang J *et al.* (2017) Fall armyworm-associated gut bacteria modulate plant defense responses. *Mol Plant-Microbe Interact* 30: 127–137

Acevedo FE, Rivera-Vega LJ, Chung SH, Ray S, Felton GW (2015) Cues from chewing insects - the intersection of DAMPs, HAMPs, MAMPs and effectors. *Curr Opin Plant Biol* 26: 80–86

Adler FR, Karban R. (1994) Defended fortresses or moving targets? Another model of inducible defenses inspired by military metaphors. *Am Nat* 144: 813–832

Ali JG, Agrawal AA (2012) Specialist versus generalist insect herbivores and plant defense. *Trends Plant Sci* 17: 293–302

Al-Shehbaz IA (2012) A generic and tribal synopsis of the Brassicaceae (Cruciferae). *Taxon* 61: 931–954

Albani MC, Castaings L, Wötzel S, Mateos JL, Wunder J, Wang R, Reymond M, Coupland G (2012) PEP1 of *Arabis alpina* is encoded by two overlapping genes that contribute to natural genetic variation in perennial flowering. *PLOS Genet* 8 (12): e1003130.

Altmann S, Muino JM, Lortzing V, Brandt R, Himmelbach A, Altschmied L, Hilker M (2018) Transcriptomic basis for reinforcement of elm antiherbivore defence mediated by insect egg deposition. *Mol Ecol* 27: 4901–4915

Andrews S (2010) FastQC: a quality control tool for high throughput sequence data

Apel W, Schulze WX, Bock R (2010) Identification of protein stability determinants in chloroplasts. *Plant J* 63: 636–650

Arganda-Carreras I, Kaynig V, Rueden C, Eliceiri KW, Schindelin J, Cardona A *et al.* (2017) Trainable Weka Segmentation: A machine learning tool for microscopy pixel classification. *Bioinformatics* 33: 2424–2426

Arnaud D, Desclos-Theveniau M, Zimmerli L (2012) Disease resistance to *Pectobacterium carotovorum* is negatively modulated by the *Arabidopsis* lectin receptor kinase LecRK-V.5. *Plant Signal Behav* 7: 1070–1072

Athiyannan N, Abrouk M, Boshoff WHP, Cauet S, Rodde N, Kudrna D, Mohammed N, *et al.* (2022) Long-Read Genome Sequencing of Bread Wheat Facilitates Disease Resistance Gene Cloning *Nat Genet* 54 (3): 1–5

Austel N, Eilers EJ, Meiners T, Hilker M (2016) Elm leaves “warned” by insect egg deposition reduce survival of hatching larvae by a shift in their quantitative leaf metabolite pattern. *Plant Cell Environ* 39: 366–376

B

Bagheri H, El-Soda M, van Oorschot I, Hanhart C, Bonnema G, van den Bosch TJ *et al.* (2012) Genetic analysis of morphological traits in a new, versatile, rapid-cycling *Brassica rap*a recombinant inbred line population. *Front Plant Sci* 3: 183

Balbyshev NF, Lorenzen JH (1997) Hypersensitivity and egg drop: a novel mechanism of host plant resistance to colorado potato beetle (Coleoptera: Chrysomelidae). *J Econ Entomol* 90: 652–657

Balint-Kurti P (2019) The plant hypersensitive response: concepts, control and consequences. *Mol Plant Pathol* 20: 1163–1178

Bandoly M, Grichnik R, Hilker M, Steppuhn A (2016) Priming of anti-herbivore defence in Nicotiana attenuata by insect oviposition: herbivore-specific effects. *Plant Cell Environ* 39 (4), 848-859

Bar-On YM, Phillips R, Milo R (2018) The biomass distribution on Earth. *Proc Natl Acad Sci U S A* 115: 6506–6511

Barragan AC, Weigel D (2021) Plant NLR diversity: the known unknowns of pan-NLRomes. *Plant Cell* 33: 814–831

Baruah IK, Panda D, Jagadale MV, Das DJ, Acharjee S, Sen P, Sarmah BK (2017) Bruchid egg induced transcript dynamics in developing seeds of black gram (*Vigna mungo*). PLoS One 12: e0176337

Bassetti N, Caarls L, Bukovinszkine'Kiss G, El-Soda M, van Veen J, Bouwmeester K, Zwaan BJ, Schranz ME, Bonnema G, Fatouros NE (2022) Genetic analysis reveals three novel QTLs underpinning a butterfly egg-induced hypersensitive response-like cell death in *Brassica rapa*. *BMC Plant Biol* 22: 1–17

Basu S, Varsani S, and Louis J. (2018) Altering Plant Defenses: HAMPs and Effector Arsenal of Chewing Herbivores. *Mol Plant Microbe Interact* (31) 1: 13-21

Bates D, Maechler M, Bolker B, Walker S. (2021) lme4: linear mixed-effects models using eigen and S4. https://github.com/lme4/lme4/

Becerra JX. (2015) Macroevolutionary and geographical intensification of chemical defense in plants driven by insect herbivore selection pressure. *CurrOpin Insect Sci* 8: 15–21

Beilstein MA, Al-Shehbaz IA, Kellogg EA. (2006) Brassicaceae phylogeny andtrichome evolution. *Am J Bot* 93: 607–619

Beilstein MA, Al-Shehbaz IA, Mathews S, Kellogg EA. (2008) Brassicaceae phylogeny inferred from phytochrome A and *ndhF* sequence data: tribes andtrichomes revisited. *Am J Bot* 95: 1307–1327

Belser C, Istace B, Denis E, Dubarry M, Baurens FC, Falentin C *et al.* (2018) Chromosome-scale assemblies of plant genomes using nanopore long reads and optical maps. *Nat Plants* 4: 879–887

Bendahmane, Abdelhafid, Konstantin Kanyuka, and David C. Baulcombe. (1999) The Rx gene from potato controls separate virus resistance and cell death responses. *Plant Cell* 11 (5): 781–91

Bentur JS, Rawat N, Divya D, Sinha DK, Agarrwal R, Atray I, Nair S (2016) Rice–gall midge interactions: Battle for survival. *J Insect Physiol* 84: 40–49

Berenbaum M. (1983) Coumarins and caterpillars: a case for coevolution. *Evolution* 37: 163–179

Berkey, Robert, Dipti Bendigeri, and Shunyuan Xiao (2012) Sphingolipids and plant defense/disease: the 'death' connection and beyond. *Front Plant Sci* 3: 68

Beyaert I, Köpke D, Stiller J, Hammerbacher A, Yoneya K, Schmidt A, Gershenzon J, Hilker M (2011) Can insect egg deposition "warn" a plant of future feeding damage by herbivorous larvae? *Proc R Soc B Biol Sci* 279: 101–108

Biomatters, Geneious Prime. https://www.geneious.com/

Bittner N, Trauer-Kizilelma U, Hilker M (2017) Early plant defence against insect attack: involvement of reactive oxygen species in plant responses to insect egg deposition. *Planta* 245: 993–1007

Blenn B, Bandoly M, Küffner A, Otte T, Geiselhardt S, Fatouros NE, Hilker M (2012) Insect Egg Deposition Induces Indirect Defense and Epicuticular Wax Changes in *Arabidopsis thaliana*. *J Chem Ecol* 38: 882–892

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120

Boller T, Felix G (2009) A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors. *Annu Rev Plant Biol* 60: 379–406

Bonnet C, Lassueur S, Ponzio C, Gols R, Dicke M, Reymond P (2017) Combined biotic stresses trigger similar transcriptomic responses but contrasting resistance against a chewing herbivore in *Brassica nigra*. *BMC Plant Biol* 17: 1–14

Botha AM, Li Y, Lapitan NLV (2005) Cereal host interactions with Russian wheat aphid: A review. *J Plant Interact* 1: 211–222

Braby MF, Trueman JW. 2006. Evolution of larval host plant associations and adaptive radiation in pierid butterflies. *J Evol Biol* 19: 1677–1690

BRAD Brassica Database. http://brassicadb.cn/#/Download/. Accessed on August 2021.

Brodersen P, Petersen M, Pike HM, Olszak B, Skov S, Ødum N *et al.* (2002) Knockout of *Arabidopsis ACCELERATED-CELL-DEATH11* encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes Dev*; 16: 490–502

Broekgaarden C, Snoeren TAL, Dicke M, Vosman B (2011) Exploiting natural variation to identify insect-resistance genes. *Plant Biotechnol* J 9: 819–825

Broman KW, Wu H, Sen S, Churchill G. (2003) R/qtl: QTL mapping in experimental crosses. *Bioinformatics*, 19: 889-890

Bruessow F, Gouhier-Darimont C, Buchala A, Metraux JP, Reymond P (2010) Insect eggs suppress plant defence against chewing herbivores. *Plant J* 62: 876–885

Bürger M, Chory J (2019) Stressed Out About Hormones: How Plants Orchestrate Immunity. *Cell Host Microbe* 26: 163–172

Bustos-Segura C, Poelman EH, Reichelt M, Gershenzon J, Gols R (2017) Intraspecific chemical diversity among neighbouring plants correlates positively with plant size and herbivore load but negatively with herbivore damage. *Ecol Lett* 20: 87-97

C

Caarls L, Bassetti N, van Doesburg F, Verbaarschot P, van Loon JJA, Schranz ME, Fatouros NE (2021) Deciphering *Brassica* plant defence responses to cabbage white butterfly egg-associated molecular patterns. *bioRxiv* Preprint at https://www.biorxiv.org/content/10.1101/2021.03.29.437462v1

Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009) BLAST+: Architecture and applications. *BMC Bioinformatics* 10: 1–9

Campos ML, De Souza CM, De Oliveira KBS, Dias SC, Franco OL (2018) The role of antimicrobial peptides in plant immunity *J Exp Bot* 69: 4997–5011

Chang A, Lamara M, Wei Y, Hu H, Parkin IAP, Gossen BD, Peng G, Yu F (2019) Clubroot resistance gene Rcr6 in *Brassica nigra* resides in a genomic region homologous to chromosome A08 in *B. rapa*. *BMC Plant Biol* 19: 224

Chen K, Fan B, Du L, Chen Z (2004) Activation of hypersensitive cell death by pathogen-induced receptor-like protein kinases from *Arabidopsis*. *Plant Mol Biol* 56: 271–283.

Chen YH, Gols R, Benrey B (2015) Crop domestication and its impact on naturally selected trophic interactions. *Annu Rev Entomol* 60: 35–58

Cheng F, Sun R, Hou X, Zheng H, Zhang F, Zhang Y *et al.* (2016) Subgenome parallel selection is associated with morphotype diversification and convergent crop domestication in *Brassica rapa* and *Brassica oleracea*. *Nat Genet* 48: 1218–1224

Chin DB, Arroyo-Garcia R, Ochoa OE, Kesseli RV, Lavelle DO, Michelmore RW (2001) Recombination and spontaneous mutation at the major cluster of resistance genes in lettuce (*Lactuca sativa*). *Genetics* 157: 831–849

Choi K, Zhao X, Kelly KA, Venn O, Higgins JD, Yelina NE, Hardcastle TJ, Ziolkowski PA, Copenhaver GP, Franklin FCH, *et al. (*2013) *Arabidopsis* meiotic crossover hot spots overlap with H2A.Z nucleosomes at gene promoters. *Nat Genet* 45: 1327–1338

Chovelon V, Feriche-Linares R, Barreau G, Chadoeuf J, Callot C, Gautier V, Le Paslier MC, Berad A, Faivre-Rampant P, Lagnel J, *et al. (*2021) Building a cluster of NLR genes conferring resistance to pests and pathogens: the story of the *Vat* gene cluster in cucurbits. *Hortic Res* 8: 1–12

Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden DM (2012) A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w^{1118}; *iso-2*; *iso-3*. Fly 6: 80–92

Clark BR, Faeth SH. (1998) The evolution of egg clustering in butterflies: a test of the egg desiccation hypothesis. *Evol Ecol* 12: 543–552

Clot CR, Polzer C, Prodhomme C, Schuit C, Engelen CJM, Hutten RCB, van Eck HJ (2020). The origin and widespread occurrence of Sli-based self-compatibility in potato. *Theor Appl Genet* 133: 2713–2728

CoGe: Comparative Genomics. https://genomevolution.org/coge/. Accessed on August 2021

Cohen JA (1985) Differences and similarities in cardenolide contents of queen and monarch butterflies in Florida and their ecological and evolutionary implications. *J Chem Ecol* 11: 85–103

Coll NS, Vercammen D, Smidler A, Clover C, van Breusegem F, Dangl JL, Epple P (2010) *Arabidopsis* type I metacaspases control cell death. *Science* 330 (6009): 1393–1397

Cook DE, Mesarich CH, Thomma BPHJ (2015) Understanding Plant Immunity as a Surveillance System to Detect Invasion. *Annu Rev Phytopathol* 53: 541-563

Corwin JA, Copeland D, Feusier J, Subedy A, Eshbaugh R, Palmer C *et al.* (2016) The quantitative basis of the *Arabidopsis* innate immune system to endemic pathogens depends on pathogen genetics. *PLoS Genet* 12: e1005789

Corwin JA, Subedy A, Eshbaugh R, Kliebenstein DJ (2016) Expansive Phenotypic Landscape of *Botrytis Cinerea* Shows Differential Contribution of Genetic Diversity and Plasticity. *Mol Plant Microbe Interact* 29 (4): 287–98

Couto D, Zipfel C (2016) Regulation of pattern recognition receptor signalling in plants *Nat Rev Immunol* 16: 537–552

Cui H, Tsuda K, Parker JE (2015) Effector-triggered immunity: From pathogen perception to robust defense *Annu Rev Plant Biol* 66: 487–511

D

Dakouri A, Zhang X, Peng G, Falk KC, Gossen BD, Strelkov SE, Yu F (2018) Analysis of genome-wide variants through bulked segregant RNA sequencing reveals a major gene for resistance to *Plasmodiophora brassicae* in *Brassica oleracea*. *Sci Rep* 8: 1–10

Dalio RJD, Paschoal D, Arena GD, Magalhães DM, Oliveira TS, Merfa MV, Maximo HJ, Machado MA (2021) Hypersensitive response: From NLR pathogen recognition to cell death response. *Ann Appl Biol* 178: 268–280

Das D, Baruah IK, Panda D, Paswan RR, Acharjee S, Sarmah BK (2021) Bruchid beetle ovipositioning mediated defense responses in black gram pods. *BMC Plant Biol* 21: 1–22

Daudi A, O'Brien JA (2012) Detection of hydrogen peroxide by DAB staining in *Arabidopsis* leaves. *Bio-protocol* 2 (18): e263

de Puysseleyr V, Höfte M, de Clercq P (2011) Ovipositing Orius laevigatus increase tomato resistance against *Frankliniella occidentalis* feeding by inducing the wound response. *Arthropod Plant Interact* 5: 71–80

Del Carpio DP, Basnet RK, de Vos RCH, Maliepaard C, Visser R, Bonnema G. (2011) The patterns of population differentiation in a *Brassica rapa* core collection. *Theor Appl Genet* 122: 1105–1118

De-la-Cruz IM, Velázquez-Márquez S, Núñez-Farfán J (2020) What do we know about the genetic basis of plant defensive responses to herbivores? A Minireview. In: *Evol Ecol Plant-Herbivore Inter*. Springer, Cham (CH), 295–314

Delaux PM, Schornack S (2021) Plant evolution driven by interactions with symbiotic and pathogenic microbes. *Science* 371 (6531)

Depristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, Philippakis AA, Del Angel G, Rivas MA, Hanna M, *et al.* (2011) A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet* 435 (43): 491–498

Desclos-Theveniau M, Arnaud D, Huang TY, Lin GJC, Chen WY, Lin YC *et al.* (2012) The *Arabidopsis* lectin receptor kinase LecRK-V.5 represses stomatal immunity induced by *Pseudomonas syringae* pv. *tomato* DC3000. *PLoS Pathog* 8: e1002513

Despres L, David JP, Gallet C (2007) The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol Evol* 22: 298– 307

Desurmont GA, Weston PA (2011) Aggregative oviposition of a phytophagous beetle overcomes egg-crushing plant defences. *Ecol Entomol* 36: 335–343

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the "cry for help." *Trends Plant Sci* 15: 167–175

Dickman MB, Fluhr R (2013) Centrality of host cell death in plant-microbe interactions. *Annu Rev Phytopathol* 51: 543–570

Diederichsen E, Frauen M, Linders EGA, Hatakeyama K, Hirai M (2009) Status and perspectives of clubroot resistance breeding in crucifer crops. *J Plant Growth Regul* 28: 265–281

Dodds P, Thrall P (2009) Recognition events and host-pathogen co-evolution in gene-for-gene resistance to flax rust. *Funct Plant Biol* 36: 395–408

Dogimont C, Chovelon V, Pauquet J, Boualem A, Bendahmane A (2014) The *Vat* locus encodes for a CC-NBS-LRR protein that confers resistance to *Aphis gossypii* infestation and *A. gossypii*-mediated virus resistance. *Plant J* 80: 993–1004

Dolatabadian A, Fernando WGD (2022) Genomic Variations and Mutational Events Associated with Plant–Pathogen Interactions *Biology* 11 (3): 421

Doss RP, Oliver JE, Proebsting WM, Potter SW, Kuy S, Clement SL *et al.* (2000) Bruchins: Insect-derived plant regulators that stimulate neoplasm formation. *Proc Natl Acad Sci U S A* 97: 6218–6223

Douglas AE (2018) Strategies for Enhanced Crop Resistance to Insect Pests. *Annu Rev Plant Biol* 69: 637–660

Drok S, Bandoly M, Stelzer S, Lortzing T, Steppuhn A (2018) Moth oviposition shapes the species-specific transcriptional and phytohormonal response of *Nicotiana attenuata* to larval feeding. *Sci Rep* 8: 1–14

E

Edger PP, Heidel-Fischer HM, Bekaert M, Rota J, Glöckner G, Platts AE, Heckel DG, Der JP, Wafula EK, Tang M *et al.* (2015). The butterfly plant arms-race escalated by gene and genome duplications. *Proc Natl Acad Sci U S A* 112: 8362–8366.

Ehrlich PR, Raven PH. (1964) Butterflies and plants: a study in coevolution. *Evolution* 18: 586–608

Erb M, Meldau S, Howe GA (2012) Role of phytohormones in insect-specific plant reactions. *Trends Plant Sci* 17: 250–259

Erb M, Robert CAM (2016) Sequestration of plant secondary metabolites by insect herbivores: molecular mechanisms and ecological consequences. *Curr Opin Insect Sci* 14: 8–11

Erb M, Reymond P (2019) Molecular interactions between plants and insect herbivores. *Annu Rev Plant Biol* 70: 527–557

Ewels P, Magnusson M, Lundin S, Käller M (2016) MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* 32: 3047–3048

F

Fan L, Chae E, Gust AA, Nürnberger T (2017) Isolation of novel MAMP-like activities and identification of cognate pattern recognition receptors in *Arabidopsis thaliana* using next-generation sequencing (NGS)–based mapping. *Curr Protoc Plant Biol* 2: 173–189

Fatouros NE, Broekgaarden C, Bukovinszkine'Kiss G, van Loon JJA, Mumm R, Huigens ME, Dicke M, Hilker M (2008) Male-derived butterfly anti-aphrodisiac mediates induced indirect plant defense. *Proc Natl Acad Sci U S A* 105: 10033–10038

Fatouros NE, Pashalidou FG, Cordero WVA, van Loon JJA, Mumm R, Dicke M, Hilker M, Huigens ME (2009) Anti-aphrodisiac compounds of male butterflies increase the risk of egg parasitoid attack by inducing plant synomone production. *J Chem Ecol* 35: 1373–1381

Fatouros NE, Lucas-Barbosa D, Weldegergis BT, Pashalidou FG, van Loon JJA, Dicke M, Harvey JA, Gols R, Huigens ME (2012) Plant volatiles induced by herbivore egg deposition affect insects of different trophic levels. *PLoS One* 7: e43607

Fatouros NE, Pineda A, Huigens ME, Broekgaarden C, Shimwela MM, Figueroa Candia IA, Verbaarschot P, Bukovinszky T (2014) Synergistic effects of direct and indirect defences on herbivore egg survival in a wild crucifer. *Proc R Soc B Biol Sci* 281: 20141254

Fatouros NE, Paniagua Voirol LR, Drizou F, Doan QT, Pineda A, Frago E, van Loon JJ (2015) Role of Large Cabbage White butterfly male-derived compounds in elicitation of direct and indirect egg-killing defenses in the black mustard. *Front Plant Sci* 6: 794

Fatouros NE, Cusumano A, Danchin EGJ, Colazza S (2016) Prospects of herbivore egg-killing plant defenses for sustainable crop protection. *Ecol Evol* 6: 6906–6918

Fernandes GW (1990) Hypersensitivity: A Neglected Plant Resistance Mechanism Against Insect Herbivores. *Environ Entomol* 19: 1173–1182

Fernández-Bautista N, Domínguez-Núñez JA, Moreno MMC, Berrocal-Lobo M (2016) Plant Tissue Trypan Blue Staining During Phytopathogen Infection. *Bio-protocol* 6 (24): e2078

Firtzlaff V, Oberländer J, Geiselhardt S, Hilker M, Kunze R (2016) Pre-exposure of *Arabidopsis* to the abiotic or biotic environmental stimuli "chilling" or "insect eggs" exhibits different transcriptomic responses to herbivory. *Sci Rep* 6: 1–13

Flor HH (1971) Current status of the gene-fob-gene concept *Annu Rev Phytopathol* 9: 275–296

Fordyce RF, Soltis NE, Caseys C, Gwinner R, Corwin JA, Atwell S *et al.* (2018) Digital imaging combined with genome-wide association mapping links loci to plant-pathogen interaction traits. *Plant Physiol* 178: 1406–1422

Forister ML, Pelton EM, Black SH (2019) Declines in Insect Abundance and Diversity: We Know Enough to Act Now. *Conserv Sci Pract* 1 (8): 1–8

Futuyma DJ, Agrawal AA (2009) Macroevolution and the biological diversity of plants and herbivores. *Proc Natl Acad Sci U S A* 106: 18054–18061

G

Garza R, Vera J, Cardona C, Barcenas N, Singh SP (2001) Hypersensitive response of beans to *Apion godmani* (Coleoptera: Curculionidae). *J Econ Entomol* 94: 958–962

Gassmann W (2005) Natural variation in the *Arabidopsis* response to the avirulence gene hopPsyA uncouples the hypersensitive response from disease resistance *Mol Plant Microbe Interact* 18 (10): 1054–60

Gazzani S, Li M, Maistri S, Scarponi E, Graziola M, Barbaro E, Wunder J, Furini A, Saedler H, Varotto C (2009) Evolution of MIR168 paralogs in Brassicaceae. *BMC Evol Biol* 9 (1): 62

Geuss D, Stelzer S, Lortzing T, Steppuhn A (2017) *Solanum dulcamara*'s response to eggs of an insect herbivore comprises ovicidal hydrogen peroxide production. *Plant Cell Environ* 40: 2663–2677

Geuss D, Lortzing T, Schwachtje J, Kopka J, Steppuhn A (2018) Oviposition by *Spodoptera exigua* on solanum dulcamara alters the plant's response to herbivory and impairs larval performance. *Int J Mol Sci* 19: 4008

Gilardoni PA, Hettenhausen C, Baldwin IT, Bonaventure G (2011) *Nicotiana attenuata LECTIN RECEPTOR KINASE1* suppresses the insect-mediated inhibition of induced defense responses during *Manduca sexta* herbivory. *Plant Cell* 23: 3512–3532

Golicz AA, Bayer PE, Barker GC, Edger PP, Kim HR, Martinez PA, Chan CKK *et al.* (2016) The pangenome of an agronomically important crop plant *Brassica oleracea*. *Nat Comm* 7 (1): 1–8

Gouhier-Darimont C, Schmiesing A, Bonnet C, Lassueur S, Reymond P (2013) Signalling of *Arabidopsis thaliana* response to *Pieris brassicae* eggs shares similarities with PAMP-triggered immunity. *J Exp Bot* 64: 665–674

Gouhier-Darimont C, Stahl E, Glauser G, Reymond P (2019) The *Arabidopsis* Lectin Receptor Kinase Lecrk-I.8 is involved in insect egg perception. *Front Plant Sci* 10: 623

Gould F, Brown ZS, Kuzma J (2018) Wicked evolution: Can we address the sociobiological dilemma of pesticide resistance? Science 360: (6390) 728–732

Graser G, Schneider B, Oldham NJ, Gershenzon J (2000) The methionine chain elongation pathway in the biosynthesis of glucosinolates in *Eruca sativa* (Brassicaceae). *Arch Bioch Biophys* 378: 411–419

Griese E, Dicke M, Hilker M, Fatouros NE (2017) Plant response to butterfly eggs: inducibility, severity and success of egg-killing leaf necrosis depends on plant genotype and egg clustering. *Sci Rep* 7: 7316

Griese E, Caarls L, Bassetti N, Mohammadin S, Verbaarschot P, Bukovinszkine'Kiss G, Poelman EH, Gols R, Schranz ME, Fatouros NE (2021) Insect egg-killing: a new front on the evolutionary arms-race between brassicaceous plants and pierid butterflies. *New Phytol* 230: 341–353

Griese E, Pineda A, Pashalidou FG, Iradi EP, Hilker M, Dicke M, Fatouros NE (2020) Plant responses to butterfly oviposition partly explain preference–performance relationships on different brassicaceous species. *Oecologia* 192: 463–475

Grimaldi D (1999) The co-radiations of pollinating insects and Angiosperms in the Cretaceous. *Ann Missouri Bot Gard* 86: 373–406

Grimaldi D, Engel MS (2005) Evolution of the Insects, 1st ed., Cambridge University Press, Cambridge

Groux R (2019) Molecular mechanisms of insect egg-triggered cell death Thèse de doctorat ès sciences de la vie (PhD), Université de Lausanne

Groux R, Fouillen L, Mongrand S, Reymond P (2021a) Sphingolipids are involved in *Pieris brassicae* egg-induced cell death in *Arabidopsis thaliana*. *bioRxiv* Preprint at https://www.biorxiv.org/content/10.1101/2021.07.09.451813v1

Groux R, Stahl E, Gouhier-Darimont C, Kerdaffrec E, Jimenez-Sandoval P, Santiago J, Reymond P (2021b) *Arabidopsis* natural variation in insect egg-induced cell death reveals a role for *LECTIN RECEPTOR KINASE-I.1 Plant Physiol* 185: 240–255

Guo X, Liu J, Hao G, Zhang L, Mao K, Wang X, Zhang D, Ma T, Hu Q, Al-Shehbaz IA *et al.* (2017) Plastome phylogeny and early diversification of Brassicaceae. *BMC Genomics* 18: 176

Gust AA, Nürnberger T (2020) A plant surface receptor for sensing insect herbivory. *Proc Natl Acad Sci U S A* 117: 32839–32841

Gust AA, Pruitt R, Nürnberger T (2017) Sensing Danger: Key to Activating Plant Immunity. *Trends Plant Sci* 22: 779–791

H

Haase NJ, Beissinger T, Hirsch CN, Vaillancourt B, Deshpande S, Barry K, Buell RC, Kaeppler SM, de Leon N. (2015) Shared Genomic Regions between Derivatives of a Large Segregating Population of Maize Identified Using Bulked Segregant Analysis Sequencing and Traditional Linkage Analysis *G3: Genes, Genomes, Genetics* 5 (8): 1593–1602

Hall JC, Sytsma KJ, Iltis HH (2002) Phylogeny of Capparaceae and Brassicaceae based on chloroplast sequence data *Am J Bot* 89: 1826–1842

Harris MO, Freeman TP, Anderson KM, Harmon JP, Moore JA, Payne SA, Rohfritsch O, Stuart JJ (2012) Hessian fly avirulence gene loss-of-function defeats plant resistance without compromising the larva's ability to induce a gall tissue. *Entomol Exp Appl* 145: 238–249

Harvey JA, Witjes LMA, Benkirane M, Duyts H, Wagenaar R (2007) Nutritional suitability and ecological relevance of *Arabidopsis thaliana* and *Brassica oleracea* as foodplants for the cabbage butterfly, *Pieris rapae*. *Plant Ecol* 189: 117–126

Haug-Baltzell A, Stephens SA, Davey S, Scheidegger CE, Lyons E (2017) SynMap2 and SynMap3D: Web-based whole-genome synteny browsers. *Bioinformatics* 33: 2197–2198

Heidel-Fischer HM, Kirsch R, Reichelt M, Ahn S-J, Wielsch N, Baxter SW, Heckel DG, Vogel H, Kroymann J (2019) An insect counteradaptation against host plant defenses evolved through concerted neofunctionalization. *MolBiol Evol* 36: 930–941

Heidel-Fischer HM, Vogel H (2015) Molecular mechanisms of insect adaptationto plant secondary compounds. *Curr Opin Insect Sci* 8: 8–14

Herrera-Vásquez A, Salinas P, Holuigue L (2015) Salicylic acid and reactive oxygen species interplay in the transcriptional control of defense genes expression. *Front Plant Sci* 6: 171

Hilfiker O, Groux, R, Bruessow, F, Kiefer, K, Zeier, J, & Reymond, P. (2014). Insect eggs induce a systemic acquired resistance in Arabidopsis. *Plant J* 80 (6), 1085-1094

Hilker M, Kobs C, Varama M, Schrank K (2002) Insect egg deposition induces *Pinus sylvestris* to attract egg parasitoids. *J Exp Biol* 205: 455–461

Hilker M, Stein C, Schröder R, Varama M, Mumm R (2005) Insect egg deposition induces defence responses in *Pinus sylvestris*: characterisation of the elicitor. *J Exp Biol* 208: 1849–1854

Hilker M, Fatouros NE (2015) Plant responses to insect egg deposition. *Annu Rev Entomol* 60: 493–515

Hilker M, Fatouros NE (2016) Resisting the onset of herbivore attack: plants perceive and respond to insect eggs. *Curr Opin Plant Biol* 32: 9–16

Himabindu K, Suneetha K, Sama VSAK, Bentur JS (2010) A new rice gall midge resistance gene in the breeding line CR57-MR1523, mapping with flanking markers and development of NILs. *Euphytica* 174: 179–187

Holland JB, Nyquist WE, Cervantes-Martínez CT , Janick J (2003) Estimating and interpreting heritability for plant breeding: an update. *Plant Breed Rev*, 22

Hothorn T, Bretz F, Westfall P (2021) multcomp: simultaneous inference in general parametric models. https://cran.r-project.org/package=multcomp

Hu L, Ye M, Kuai P, Ye M, Erb M, Lou Y (2018) OsLRR-RLK1, an early responsive leucine-rich repeat receptor-like kinase, initiates rice defense responses against a chewing herbivore. *New Phytol* 219: 1097–1111

Hu, Dandan, Jinjie Jing, Rod J. Snowdon, Annaliese S. Mason, Jinxiong Shen, Jinling Meng, and Jun Zou (2021) Exploring the gene pool of *Brassica napus* by genomics-based approaches. *Plant Biotechnol J* 19 (9): 1693–1712

Huang CH, Sun R, Hu Y, Zeng L, Zhang N, Cai L, Zhang Q, Koch MA, Al-Shehbaz IA, Edger PP *et al.* (2015) Resolution of Brassicaceae phylogeny using nuclear genes uncovers nested radiations and supports convergent morphological evolution. *Mol Biol Evol* 33: 394–412

Huby, Eloïse, Johnathan A. Napier, Fabienne Baillieul, Louise V. Michaelson, and Sandrine Dhondt-Cordelier (2020) Sphingolipids: towards an integrated view of metabolism during the plant stress response. *New Phytol* 225 (2): 659–70

Hundacker J, Bittner N, Weise C, Bröhan G, Varama M, Hilker M (2022) Pine defense against eggs of an herbivorous sawfly is elicited by an annexin-like protein present in egg-associated secretion. *Plant Cell Environ* 45: 1033–1048

Hurgobin B, Agnieszka A. Golicz, Philipp E. Bayer, Chon Kit Kenneth Chan, Soodeh Tirnaz, Aria Dolatabadian, Sarah V. Schiessl, *et al.* (2018) Homoeologous exchange is a major cause of gene presence/absence variation in the amphidiploid *Brassica napus*. *Plant Biotechnol J* 16 (7): 1265–74

I

IPCC Secretariat (2021) Scientific review of the impact of climate change on plant pests. A global challenge to prevent and mitigate plant-pest risks in agriculture, forestry and ecosystems. *FAO on behalf of the IPPC Secretariat*

Ishikawa T, Uchimiya H, Kawai-Yamada M (2003) The role of plant Bax inhibitor-1 in suppressing H_2O_2-induced cell death. In: *Methods in Enzymology*. Academic Press, Cambridge (UK), 239–256

J

Jones JDG, Dangl JL (2006) The plant immune system. *Nature* 444: 323–329

Jones JDG, Vance RE, Dangl JL (2016) Intracellular innate immune surveillance devices in plants and animals. *Science* 354 (6316): aaf6395

Jupe F, Witek K, Verweij W, Śliwka J, Pritchard L, Etherington GJ, Maclean D *et al.* (2013) Resistance gene enrichment sequencing (RenSeq) enables reannotation of the NB-LRR gene family from sequenced plant genomes and rapid mapping of resistance loci in segregating populations. *Plant J* 76 (3): 530–44

K

Kaplinski L, Lepamets M, Remm M (2015) Genome Tester4: A toolkit for performing basic set operations - union, intersection and complement on k-mer lists. *Gigascience* 4 (1): s13742-015

Karasov TL, Kniskern JM, Gao L, DeYoung BJ, Ding J, Dubiella U, Lastra RO, *et al.* (2014) The long-term maintenance of a resistance polymorphism through diffuse interactions. *Nature* 512 (7515): 436–40

Katche E, Quezada-Martinez D, Katche EI, Vasquez-Teuber P, Mason AS (2019) Interspecific hybridization for *Brassica* crop improvement. *Crop Breeding, Genet Genomics* 1 (1)

Kawahara AY, Plotkin D, Espeland M, Meusemann K, Toussaint EFA, Donath A, Gimnich F, Frandsen PB, Zwick A, Dos Reis M *et al.* (2019) Phylogenomics reveals the evolutionary timing and pattern of butterflies and moths. *Proc Natl Acad Sci, U S A* 116: 22657– 22663

Kenrick P, Crane PR (1997) The origin and early evolution of plants on land. *Nature* 389: 33–39

Kessler A (2015) The information landscape of plant constitutive and induced secondary metabolite production. *Curr Opin Insect Sci* 8: 47–53

Kew Royal Botanic Gardens (2020) State of the World's Plants and Fungi

Khan MS, Yin X, van der Putten PEL, Jansen HJ, van Eck HJ, van Eeuwijk FA *et al.* (2019) A model-based approach to analyse genetic variation in potato using standard cultivars and a segregating population. II. Tuber bulking and resource use efficiency. *F Crop Res* 242: 107582

Kim D, Paggi JM, Park C, Bennett C, Salzberg SL (2019) Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat Biotechnol* 2019 378 37: 907–915

Kim J, Tooker JF, Luthe DS, De Moraes CM, Felton GW (2012) Insect eggs can enhance wound response in plants: a study system of tomato *Solanum lycopersicum* L. and *Helicoverpa zea* Boddie. *PLoS One* 7: e37420

Kliebenstein DJ, Kroymann J, Mitchell-Olds T (2005) The glucosinolate-myrosinase system in an ecological and evolutionary context. *Curr Opin Plant Biol* 8: 264–271

Kliebenstein DJ (2017) Quantitative genetics and genomics of plant resistance to insects. *Annu Plant Rev* 47: 235–262

Klingler JP, Nair RM, Edwards OR, Singh KB (2009) A single gene, *AIN*, in *Medicago truncatula* mediates a hypersensitive response to both bluegreen aphid and pea aphid, but confers resistance only to bluegreen aphid. *J Exp Bot* 60: 4115–4127

Kloth KJ, Busscher-Lange J, Wiegers GL, Kruijer W, Buijs G, Meyer RC *et al.* (2017) *SIEVE ELEMENT-LINING CHAPERONE1* restricts aphid feeding on *Arabidopsis* during heat stress. *Plant Cell* 29: 2450–2464

Kourelis J, van der Hoorn RAL (2018) Defended to the nines: 25 years of resistance gene cloning identifies nine mechanisms for R protein function. *Plant Cell* 30: 285–299

Kourelis J, Malik S, Mattinson O, Krauter S, Kahlon PS, Paulus JK, and van der Hoorn RAL (2020) Evolution of a guarded decoy protease and its receptor in solanaceous plants. *Nat Comm* 11 (1): 1–15

Kuang H, Woo SS, Meyers BC, Nevo E, Michelmore RW (2004) Multiple genetic processes result in heterogeneous rates of evolution within the major cluster disease resistance genes in lettuce. *Plant Cell* 16: 2870–2894

Kulik A, Noirot E, Grandperret V, Bourque S, Fromentin J, Salloignon P, Truntzer C, Dobrowolska G, Simon-Plas F, Wendehenne D (2015) Interplays between nitric oxide and reactive oxygen species in cryptogein signalling. *Plant Cell Environ* 38: 331–348

Kumar S, Sarwan Kumar C (2017) Assessment of avoidable yield losses in crop brassicas by insect-pests. *J Entomol Zool Stud* 5: 1814–1818

Künstler A, Bacsó R, Gullner G, Hafez YM, Király L (2016) Staying alive - is cell death dispensable for plant disease resistance during the hypersensitive response? *Physiol Mol Plant Pathol* 93: 75–84

Kyazma. JoinMap. https://www.kyazma.nl/index.php/JoinMap/

L

Lai Y, Eulgem T (2018) Transcript-level expression control of plant NLR genes. *Mol Plant Pathol* 19: 1267–1281

Lapin D, Kovacova V, Sun X, Dongus JA, Bhandari D, Von Born P *et al.* (2019) A coevolved EDS1-SAG101-NRG1 module mediates cell death signaling by TIR-domain immune receptors. *Plant Cell* 31: 2430–2455

Lefevere H, Bauters L, Gheysen G (2020) Salicylic acid biosynthesis in plants. *Front Plant Sci* 11: 338

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25: 1754–1760

Li Q, Ai G, Shen D, Zou F, Wang J, Bai T *et al.* (2019) A *Phytophthora capsici* effector targets ACD11 binding partners that regulate ROS-mediated defense response in *Arabidopsis*. *Mol Plant* 12: 565–581.

Li Y, Liu GF, Ma LM, Liu TK, Zhang CW, Xiao D *et al.* (2020) A chromosome-level reference genome of non-heading Chinese cabbage [*Brassica campestris* (syn. *Brassica rapa*) ssp. *chinensis*]. *Hortic Res* 7: 1–13

Linthorst HJM (1991) Pathogenesis-related proteins of plants. *CRC Crit Rev Plant Sci* 10: 123–150

Little D, Gouhier-Darimont C, Bruessow F, Reymond P (2007) Oviposition by pierid butterflies triggers defense responses in Arabidopsis. *Plant Physiol* 143: 784–800

Liu S, Yeh CT, Tang HM, Nettleton D, Schnable PS (2012) Gene mapping via bulked segregant RNA-Seq (BSR-Seq). *PLoS One* 7: e36406

Li Y, Huang Y, Bergelson J, Nordborg M, Borevitz JO (2010) Association mapping of local climate-sensitive quantitative trait loci in Arabidopsis thaliana. *Proc Natl Acad Sci* 107 (49): 21199-21204

Liu Y, Wu H, Chen H, Liu Y, He J, Kang H *et al.* (2015) A gene cluster encoding lectin receptor kinases confers broad-spectrum and durable insect resistance in rice. *Nat Biotechnol* 33: 301–307

Liu, Yucheng, Huilong Du, Pengcheng Li, Yanting Shen, Hua Peng, Shulin Liu, Guo An Zhou, *et al.* (2020) Pan-genome of wild and cultivated soybeans. *Cell* 182 (1): 162-176.e13

Lortzing T, Kunze R, Steppuhn A, Hilker M, Lortzing V (2020) *Arabidopsis*, tobacco, nightshade and elm take insect eggs as herbivore alarm and show similar transcriptomic alarm responses. *Sci Rep* 10: 1–16

Lortzing V, Oberländer J, Lortzing T, Tohge T, Steppuhn A, Kunze R, Hilker M (2019) Insect egg deposition renders plant defence against hatching larvae more effective in a salicylic acid-dependent manner. *Plant Cell Environ* 42: 1019–1032

Lou P, Woody S, Greenham K, VanBuren R, Colle M, Edger PP *et al.* (2020) Genetic and genomic resources to study natural variation in *Brassica rapa*. *Plant Direct* 4 (12): e00285

Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15: 1–21

Lv H, Fang Z, Yang L, Zhang Y, Wang Y (2020) An update on the arsenal: mining resistance genes for disease management of *Brassica* crops in the genomic era. *Hortic Res* 7: 34

Lyons E, Pedersen B, Kane J, Alam M, Ming R, Tang H, Wang X, Bowers J, Paterson A, Lisch D, *et al.* (2008) Finding and comparing syntenic regions among Arabidopsis and the outgroups papaya, poplar, and grape: CoGe with rosids. *Plant Physiol* 148: 1772–1781

M

Mabry ME, Turner-Hissong SD, Gallagher EY, McAlvay AC, An H, Edger PP *et al.* (2021) The evolutionary history of wild, domesticated, and feral *Brassica oleracea* (Brassicaceae). *Mol Biol Evol* 38: 4419–4434

Maffei ME, Mithöfer A, Boland W (2007) Before gene expression: early events in plant–insect interaction. *Trends Plant Sci* 12: 310–316

Malcolm SB, Brower LP (1989) Evolutionary and ecological implications of cardenolide sequestration in the monarch butterfly. *Experientia* 45 (3): 284–295

Mariyammal I, Seram D, Samyuktha SM, Karthikeyan A, Dhasarathan M, Murukarthick J *et al.* (2019) QTL mapping in *Vigna radiata* × *Vigna umbellata* population uncovers major genomic regions associated with bruchid resistance. *Mol Breed* 39: 1–13

McAlvay AC, Ragsdale AP, Mabry ME, Qi X, Bird KA, Velasco P *et al.* (2021) *Brassica rapa* domestication: untangling wild and feral forms and convergence of crop morphotypes. *Mol Biol Evol* 38: 3358–3372

Méline V, Caldwell DL, Kim B-S, Baireddy S, Yang C, Sparks EE *et al.* (2021) Image-based assessment of plant disease progression identifies new genetic loci for resistance. *bioRxiv* Preprint at https://www.biorxiv.org/content/10.1101/2021.07.13.452064v2

Mendy B, Wang'ombe MW, Radakovic ZS, Holbein J, Ilyas M, Chopra D *et al.* (2017) *Arabidopsis* leucine-rich repeat receptor–like kinase NILR1 is required for induction of innate immunity to parasitic nematodes. *PLoS Pathog* 13: e1006284

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. *Gateway Computing Environments Workshop (GCE)*. 1-8

Mitter C, Farrell B, Wiegmann B (1988) The phylogenetic study of adaptive zones: has phytophagy promoted insect diversification? *Am Nat* 132: 107–128

Mohammadin S, Edger PP, Pires JC, Schranz ME (2015) Positionally-conserved but sequence-diverged: identification of long non-coding RNAs in the Brassicaceae and Cleomaceae. *BMC Plant Biol* 15: 217

Mohammadin S, Wang W, Liu T, Moazzeni H, Ertugrul K, Uysal T, Christodoulou CS, Edger PP, Pires JC, Wright SI, *et al.* (2018) Genome-wide nucleotide diversity and associations with geography, ploidy level and glucosinolate profiles in *Aethionema arabicum* (Brassicaceae). *Plant Syst Evol* 304(5): 619-630

Monino-Lopez, Daniel, Maarten Nijenhuis, Linda Kodde, Sophien Kamoun, Hamed Salehian, Kyrylo Schentsnyi, Remco Stam, *et al.* (2021) Allelic variants of the NLR protein Rpi-chc1 differentially recognize members of the *Phytophthora infestans* PexRD12/31 effector superfamily through the leucine-rich repeat domain. *Plant J* 107 (1): 182–97

Monteiro F, Nishimura MT (2018) Structural, functional, and genomic diversity of plant NLR proteins: An evolved resource for rational engineering of plant immunity. *Annu Rev Phytopathol* 56: 243–267

Montenegro, JD, Golicz AA, Bayer PE, Hurgobin B, Lee HT, Chan CKK, Visendi P, *et al.* (2017) The pangenome of hexaploid bread wheat. *Plant J* 90 (5): 1007–13

Mueller ND, Gerber JS, Johnston M, Ray DK, Ramankutty N, Foley JA (2012) Closing yield gaps through nutrient and water management. *Nature* 490: 254–257

Mukhtar MS, McCormack ME, Argueso CT, Pajerowska-Mukhtar KM (2016) Pathogen tactics to manipulate plant cell death. *Curr Biol* 26: R608–R619

Mur LAJ, Kenton P, Lloyd AJ, Ougham H, Prats E (2008) The hypersensitive response; the centenary is upon us but how much do we know? *J Exp Bot* 59 (3): 501–520

N

Nallu S, Hill JA, Don K, Sahagun C, Zhang W, Meslin C *et al.* (2018) The molecular genetic basis of herbivory between butterflies and their host plants. *Nat Ecol Evol* 2: 1418–1427

Ngou BPM, Ahn H-K, Ding P, Jones JDG (2021) Mutual potentiation of plant immunity by cell-surface and intracellular receptors. *Nature* 592: 110–115

Ngou BPM, Ding P, Jones JDG (2022) Thirty years of resistance: Zig-zag through the plant immune system. *Plant Cell* 34: 1447–1478

Nicolis V, Venter E (2018) Silencing of a unique integrated domain nucleotide-binding leucine-rich repeat gene in wheat abolishes *Diuraphis noxia* resistance. *Mol Plant-Microbe Interact* 31: 940–950

Nicolopoulou-Stamati P, Maipas S, Kotampasi C, Stamatis P, Hens L (2016) Chemical pesticides and human health: the urgent need for a new concept in agriculture. *Front Public Heal* 4: 148

Nombela G, Williamson VM, Muñiz M (2003) The root-knot nematode resistance gene Mi-1.2 of tomato is responsible for resistance against the whitefly *Bemisia tabaci*. *Mol Plant-Microbe Interact* 16: 645–649

O

O'Brien JA, Daudi A, Butt VS, Bolwell GP (2012) Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta* 236: 765–779

Oates CN, Denby KJ, Myburg AA, Slippers B, Naidoo S (2021) Insect egg-induced physiological changes and transcriptional reprogramming leading to gall formation. *Plant Cell Environ* 44: 535–547

Ogle D, Doll J, Wheeler P, Dinno A (2021) FSA: simple fisheries stock assessment methods. https://github.com/droglenc/FSA

Ojeda-Martinez D, Martinez M, Diaz I, Estrella Santamaria M (2021) Spider mite egg extract modifies *Arabidopsis* response to future infestations. *Sci Rep* 11: 1–17

Olsen KM, Wendel JF (2013) A bountiful harvest: Genomic insights into crop domestication phenotypes. *Annu Rev Plant Biol* 64: 47–70

Oñate-Sánchez L, Vicente-Carbajosa J (2008) DNA-free RNA isolation protocols for *Arabidopsis* thaliana, including seeds and siliques. *BMC Res Notes* 1 (1): 1-7

Orlovskis Z, Reymond P (2020). *Pieris brassicae* eggs trigger interplant systemic acquired resistance against a foliar pathogen in Arabidopsis. *New Phytol* 228 (5): 1652-1661

P

Palma K, Thorgrimsen S, Malinovsky FG, Fiil BK, Nielsen HB, Brodersen P, Hofius D, Petersen M, Mundy J. (2010) Autoimmunity in *Arabidopsis acd11* is mediated by epigenetic regulation of an immune receptor. *PLOS Pathogens* 6 (10): e1001137

Palmgren MG, Edenbrandt AK, Vedel SE, Andersen MM, Landes X, Østerberg JT, Falhof J, Olsen LI, Christensen SB, Sandøe P, *et al. (*2015) Are we ready for back-to-nature crop breeding? *Trends Plant Sci* 20: 155–164

Paniagua Voirol LR, Valsamakis G, Lortzing V, Weinhold A, Johnston PR, Fatouros NE, Kunze R, Hilker M (2020) Plant responses to insect eggs are notinduced by egg-associated microbes, but by a secretion attached to the eggs. *Plant, Cell & Environment* 43: 1815–1826

Pashalidou FG, Lucas-Barbosa D, van Loon JJA, Dicke M, Fatouros NE (2013) Phenotypic plasticity of plant response to herbivore eggs: effects on resistance to caterpillars and plant development. *Ecology* 94: 702–713

Pashalidou FG, Fatouros NE, van Loon JJA, Dicke M, Gols R (2015a) Plant-mediated effects of butterfly egg deposition on subsequent caterpillar and pupal development, across different species of wild Brassicaceae. *Ecol Entomol* 40: 444–450

Pashalidou FG, Frago E, Griese E, Poelman EH, van Loon JJA, Dicke M, Fatouros NE (2015b) Early herbivore alert matters: plant-mediated effects of egg deposition on higher trophic levels benefit plant fitness. *Ecol Lett* 18:927–936

Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL (2015) StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat Biotechnol* 33: 290–295

Petzold-Maxwell J, Wong S, Arellano C, Gould F (2011) Host plant direct defence against eggs of its specialist herbivore, *Heliothis subflexa*. *Ecol Entomol* 36: 700–708

Pieterse CMJ, van der Does D, Zamioudis C, Leon-Reyes A, van Wees SCM (2012) Hormonal modulation of plant immunity. *Annu Rev Cell Dev Biol* 28: 489–521

Prodhomme C, Esselink D, Borm T, Visser RGF, van Eck HJ, Vossen JH (2019) Comparative Subsequence Sets Analysis (CoSSA) is a robust approach to identify haplotype specific SNPs; Mapping and pedigree analysis of a potato wart disease resistance gene *Sen3*. *Plant Methods* 15: 1–20

Q

Quinlan AR, Hall IM (2010) BEDTools: A flexible suite of utilities for comparing genomic features. Bioinformatics 26: 841–842

R

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Rask L, Andrėasson E, Ekbom B, Eriksson S, Pontoppidan B, Meijer J (2000) Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *PlantMol Biol* 42: 93–114

Ratnasingham S, Hebert PDN (2007) BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). *Mol Ecol Notes* 7(3): 355-364

Read AC, Moscou MJ, Zimin AV, Pertea G, Meyer RS, Purugganan MD, Leach JE, Triplett LR, Salzberg SL, Bogdanove AJ (2020) Genome assembly and characterization of a complex zfBED-NLR gene-containing disease resistance locus in Carolina Gold Select rice with Nanopore sequencing. *PLOS Genet* 16: e1008571

Reymond P, Bodenhausen N, van Poecke RMP, Krishnamurthy V, Dicke M, Farmer EE (2004) A conserved transcript pattern in response to a specialist and a generalist herbivore. *Plant Cell* 16: 3132–47

Reymond P (2013) Perception, signaling and molecular basis of oviposition-mediated plant responses. *Planta* 238: 247–258

Reymond P (2021) Receptor kinases in plant responses to herbivory. *Curr Opin Biotechnol* 70: 143–150

Ross S, Giglione C, Pierre M, Espagne C, Meinnel T (2005) Functional and developmental impact of cytosolic protein N-terminal Methionine excision in *Arabidopsis. Plant Physiol* 137: 623–637

Rossi M, Goggin FL, Milligan SB, Kaloshian I, Ullman DE, Williamson VM (1998) The nematode resistance gene Mi of tomato confers resistance against the potato aphid. *Proc Natl Acad Sci U S A* 95: 9750–9754

Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, Holton N *et al.* (2011) The *Arabidopsis* leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell* 23: 2440–2455

Ryan SF, Lombaert E, Espeset A, Vila R, Talavera G, Dinca V *et al.* (2019) Global invasion history of the agricultural pest butterfly *Pieris rapae* revealed with genomics and citizen science. *Proc Natl Acad Sci* 116: 20015–20024

S

Salerno G, De Santis F, Iacovone A, Bin F, Conti E (2013) Short-range cues mediate parasitoid searching behavior on maize: The role of oviposition-induced plant synomones. *Biol Control* 64: 247–254

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T *et al.* (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9: 676–682.

Schoonhoven LM, van Loon JJA van, Dicke M (2005) Insect-plant biology, 2nd ed. NV., Oxford University Press, Oxford (UK)

Schultink, Alex, and Adam D. Steinbrenner. (2021) A playbook for developing disease-resistant crops through immune receptor identification and transfer. *Curr Opin Plant Biol* 62: 102089

Schuman MC, Baldwin IT (2016) The layers of plant responses to insect herbivores. *Annu Rev Entomol* 61: 373–394

Seino Y, Suzuki Y, Sogawa K. (1996) An ovicidal substance produced by rice plants in response to oviposition by the whitebacked planthopper, *Sogatella furcifera* (Horváth) (Homoptera: Delphacidae). *Appl Entomol Zool* 31: 467–473

Seong K, Seo E, Witek K, Li M, Staskawicz B (2020) Evolution of NLR resistance genes with non canonical N-terminal domains in wild tomato species. *New Phytol* 227 (5): 1530–43

Shangguan X, Zhang J, Liu B, Zhao Y, Wang H, Wang Z, Guo J, *et al.* 2018. A mucin-like protein of planthopper is required for feeding and induces immunity response in plants. *Plant Physiol* 176 (1): 552–65

Shao ZQ, Xue JY, Wang Q, Wang B, Chen JQ (2019) Revisiting the origin of plant NBS-LRR genes. *Trends Plant Sci* 24 (1): 9–12

Shapiro AM, DeVay JE (1987) Hypersensitivity reaction of *Brassica nigra* L. (Cruciferae) kills eggs of *Pieris* butterflies (Lepidoptera, Pieridae). *Oecologia* 71:631–632

Saharan GS, Naresh KM, Meena PD (2021) Molecular Mechanism of Crucifer's Host-Resistance. Springer, Singapore

Snoeck S, Abramson BW, Garcia AGK, Egan AN, Michael TP, Steinbrenner AD (2022) Evolutionary gain and loss of a plant pattern-recognition receptor for HAMP recognition. *bioRxiv* Preprint at https://www.biorxiv.org/content/10.1101/2022.03.30.484633v1

Snoeck S, Guayazán-Palacios N, Steinbrenner AD (2022) Molecular tug-of-war: Plant immune recognition of herbivory. *Plant Cell* 34 (5): 1497-1513

Soltis NE, Atwell S, Shi G, Fordyce R, Gwinner R, Gao D, Shafi A, Kliebenstein DJ (2019) Interactions of tomato and *Botrytis cinerea* genetic diversity: parsing the contributions of host differentiation, domestication, and pathogen variation. *Plant Cell* 31 (2): 502–19

Song JM, Guan Z, Hu J, Guo C, Yang Z, Wang S, Liu D, *et al.* (2020) Eight high-quality genomes reveal pan-genome architecture and ecotype differentiation of *Brassica napus*. *Nature Plants* 6 (1): 34–45

St.Clair DA (2010) Quantitative Disease Resistance and Quantitative Resistance Loci in Breeding. *Annu Rev Phytopathol* 48: 247–268

Stahl E, Hilfiker O, Reymond P (2018) Plant–arthropod interactions: who is the winner? *Plant J* 93: 703–728

Stahl E, Brillatz T, Ferreira Queiroz E, Marcourt L, Schmiesing A, Hilfiker O, Riezman I, Riezman H, Wolfender J-L, Reymond P (2020) Phosphatidylcholines from *Pieris brassicae* eggs activate an immune response in *Arabidopsis*. *eLife* 9: e60293

Stam R, Scheikl D, Tellier A (2016) Pooled enrichment sequencing identifies diversity and evolutionary pressures at NLR resistance genes within a wild tomato population. *Genome Biol Evol* 8 (5): 1501–15

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30(9): 1312-1313

Stamp NE (1980) Egg deposition patterns in butterflies: why do some species cluster their eggs rather than deposit them singly? *Am Nat* 115:367–380

Steinbrenner AD, Munoz-Amatriain M, Chaparro AF, Jessica Montserrat Aguilar-Venegas, Lo S, Okuda S, Glauser G, Dongiovanni J, Shi D, Hall M, *et al.* (2020) A receptor-like protein mediates plant immune responses to herbivore-associated molecular patterns. *Proc Natl Acad Sci U S A* 117: 31510–31518

Stewart E, Croll D, Lendenmann MH, Sanchez-Vallet A, Hartmann FE, Palma-Guerrero J *et al.* (2017) Quantitative trait locus mapping reveals complex genetic architecture of quantitative virulence in the wheat pathogen *Zymoseptoria tritici*. *Mol Plant Pathol* 19: 201–216

Stokstad E (2018) European Union Expands Ban of Three Neonicotinoid Pesticides. *Science* (10)

Stork NE (2018) How many species of insects and ther terrestrial arthropods are there on Earth? *Annu Rev Entomol* 63: 31–45

Strong DR, Lawton JH, Southwood RS (1984) Insects on plants : community patterns and mechanisms. Blackwell Scientific, Oxford (UK)

Stuart JJ, Chen M-S, Shukle R, Harris MO (2012) Gall midges (Hessian flies) as plant pathogens. *Annu Rev Phytopathol* 50: 339–357

Sun M, Voorrips RE, van't Westende W, van Kaauwen M, Visser RGF, Vosman B. Aphid resistance in *Capsicum* maps to a locus containing LRR-RLK gene analogues. (2020) *Theor Appl Genet* 133: 227–237

Sun X, Lapin D, Feehan JM, Stolze SC, Kramer K, Dongus JA *et al.* (2021) Pathogen effector recognition-dependent association of NRG1 with EDS1 and SAG101 in TNL receptor immunity. *Nat Commun* 12: 1–15

Swain T (1977) Secondary compounds as protective agents. *Ann Rev Plant Physiol* 28: 479–501

T

Tamiru A, Bruce TJA, Woodcock CM, Caulfield JC, Midega CAO, Ogol CKPO, Mayon P, Birkett MA, Pickett JA, Khan ZR (2011) Maize landraces recruit egg and larval parasitoids in response to egg deposition by a herbivore. *Ecol Lett* 14: 1075–1083

Tamiru A, Khan ZR, Bruce TJ (2015) New directions for improving crop resistance to insects by breeding for egg induced defence. Curr Opin Insect Sci 9: 51–55

Tamiru A, Paliwal R, Manthi SJ, Odeny DA, Midega CAO, Khan ZR *et al.* (2020) Genome wide association analysis of a stemborer egg induced "call-for-help" defence trait in maize. *Sci Rep* 10: 1–12

Tamura Y, Hattori M, Yoshioka H, Yoshioka M, Takahashi A, Wu J, Sentoku N, Yasui H (2014) Map-based cloning and characterization of a brown planthopper resistance gene BPH26 from *Oryza sativa* L. ssp. *indica* cultivar ADR52. *Sci Rep* 4: 1–8

Tang D, Wang G, Zhou JM (2017) Receptor kinases in plant-pathogen interactions: More than pattern recognition. *Plant Cell* 29: 618–637

Thoen MPM, Davila Olivas NH, Kloth KJ, Coolen S, Huang PP, Aarts MGM *et al.* (2017) Genetic architecture of plant stress resistance: multi-trait genome-wide association mapping. *New Phytol* 213: 1346–1362

Thompson JN (1989) Concepts of coevolution. *Trends Ecol Evol* 4: 179–183

Thor K (2019) Calcium-nutrient and messenger. Front Plant Sci 10: 440

Tilman D, Cassman KG, Matson PA, Naylor R, Polasky S (2002) Agricultural sustainability and intensive production practices. *Nature* 418: 671–677

Tolman T, Lewington R. (2009) Collins butterfly guide: The most complete field guide to the butterflies of Britain and Europe. Harper Collins, London (UK)

Tolman T. (2001) Photographic Guide to the Butterflies of Britain & Europe. Oxford University Press, Oxford (UK)

Torres MA (2010) ROS in biotic interactions. *Physiol Plant* 138: 414–429

Toruño TY, Stergiopoulos I, Coaker G (2016) Plant-Pathogen Effectors: Cellular Probes Interfering with Plant Defenses in Spatial and Temporal Manners. *Annu Rev Phytopathol* 54: 419–441

Townley HE, McDonald K, Jenkins GI, Knight MR, Leaver CJ (2005) Ceramides induce programmed cell death in *Arabidopsis* cells in a calcium-dependent manner. *Biol Chem* 386 (2): 161–66

Turcotte MM, Turley NE, Johnson MTJ (2014) The impact of domestication on resistance to two generalist herbivores across 29 independent domestication events. New Phytol 204: 671–681

Turcotte MM, Turley NE, Johnson MTJ (2014) The impact of domestication on resistance to two generalist herbivores across 29 independent domestication events. *New Phytol* 204: 671–681

V

Valsamakis G, Bittner N, Fatouros NE, Kunze R, Hilker M, Lortzing V (2020) Priming by timing: Arabidopsis thaliana adjusts its priming response to Lepidoptera eggs to the time of larval hatching. *Front Plant Sci* 11: 1969

van der Burgh AM, Joosten MHAJ (2019) Plant immunity: thinking outside and inside the box. *Trends Plant Sci* 24: 587–601

Vetter M, Karasov TL, Bergelson J (2016) Differentiation between MAMP triggered defenses in *Arabidopsis thaliana*. *PLoS Genet* 12 (6): e1006068

Vincent TR, Avramova M, Canham J, Higgins P, Bilkey N, Mugford ST *et al.* (2017) Interplay of plasma membrane and vacuolar ion channels, together with BAK1, elicits rapid cytosolic calcium elevations in *Arabidopsis* during aphid feeding. *Plant Cell* 29: 1460–1479

Visschers IGS, Peters JL, Timmermans LLH, Edwards E, Ferrater JB, Balatero CH *et al.* (2019) Resistance to three thrips species in *Capsicum* spp. depends on site conditions and geographic regions. *J Appl Entomol* 143: 929–941

W

Wagner D, Doak P (2017) Oviposition, larval survival and leaf damage by the willow leaf blotch miner, *Micrurapteryx salicifoliella*, in relation to leaf trichomes across 10 *Salix* species. *Ecol Entomol* 42: 629–635

Wagner S, Stuttmann J, Rietz S, Guerois R, Brunstein E, Bautor J *et al.* (2013) Structural basis for signaling by exclusive EDS1 heteromeric complexes with SAG101 or PAD4 in plant innate immunity. *Cell Host Microbe* 14: 619–630

Wagner DL, Grames EM, Forister ML, Berenbaum MR, Stopak S. 2021. Insect decline in the Anthropocene: death by a thousand cuts. *Proc Natl Acad Sci U S A* 118 (2): 1–10

Wahlberg N, Rota J, Braby MF, Pierce NE, Wheat CW (2014) Revised systematics and higher classification of pierid butterflies (Lepidoptera: Pieridae) based on molecular data. *Zool Scr* 43: 641–650

Walden N, German DA, Wolf EM, Kiefer M, Rigault P, Huang XC, Kiefer C, Schmickl R, Franzke A, Neuffer B, *et al. (*2020) Nested whole-genome duplications coincide with diversification and high morphological disparity in Brassicaceae. *Nat Commun* 11: 1–12

Wang W, Chen L, Fengler K, Bolar J, Llaca V, Wang X, Clark CB, Fleury TJ, Myrvold J, Oneal D, *et al. (*2021a) A giant NLR gene confers broad-spectrum resistance to *Phytophthora sojae* in soybean. *Nat Commun* 12: 6–13

Wang Y, Bouwmeester K, Beseh P, Shan W, Govers F. (2014) Phenotypic analyses of *Arabidopsis* T-DNA insertion lines and expression profiling reveal that multiple L-type lectin receptor kinases are involved in plant immunity. *Mol Plant-Microbe Interact* 27: 1390–1402.

Wang Z, Huang J, Nie L, Hu Y, Zhang N, Guo Q, Guo J, Du B, Zhu L, He G, *et al. (*2021b) Molecular and functional analysis of a brown planthopper resistance protein with two nucleotide-binding site domains. *J Exp Bot* 72: 2657–2671

Wang H, Lu Y, Liu P, Wen W, Zhang J, Ge X, Xia Y (2013) The ammonium/nitrate ratio is an input signal in the temperature-modulated, *SNC1*-Mediated and *EDS1*-dependent autoimmunity of *nudt6-2 nudt7*. *Plant J* 73 (2): 262–75

Waszczak C, Carmody M, Kangasjärvi J (2018) Reactive oxygen species in plant signaling. *Ann Rev Plant Biol* 69: 209-236

Wegener R, Schulz S, Meiners T, Hadwich K, Hilker M (2001) Analysis of volatiles induced by oviposition of elm leaf beetle *Xanthogaleruca luteola* on *Ulmus minor*. *J Chem Ecol* 27: 499–515

Weis C, Pfeilmeier S, Glawischnig E, Isono E, Pachl F, Hahne H *et al.* (2013) Co-immunoprecipitation-based identification of putative BAX INHIBITOR-1-interacting proteins involved in cell death regulation and plant–powdery mildew interactions. *Mol Plant Pathol* 14: 791–802

van Wersch S, Li X (2019) Stronger when together: clustering of plant NLR disease resistance genes. *Trends in Plant Science* 24 (8): 688–99

van de Weyer AL, Monteiro F, Furzer OJ, Nishimura MT, Cevik V, Witek K, Jones JDG, Dangl JL, Weigel D, Bemm F (2019) A species-wide inventory of NLR genes and alleles in *Arabidopsis* thaliana. *Cell* 178 (5): 1260-1272

Wheat CW, Vogel H, Wittstock U, Braby MF, Underwood D, Mitchell-Olds T (2007) The genetic basis of a plant-insect coevolutionary key innovation. *Proc Natl Acad Sci U S A* 104: 20427–20431

Whitehead SR, Turcotte MM, Poveda K (2017) Domestication impacts on plant-herbivore interactions: a meta-analysis. *Philos Trans R Soc B Biol Sci* 372: 20160034

Wicker T, Yahiaoui N, Keller B (2007) Illegitimate recombination is a major evolutionary mechanism for initiating size variation in plant resistance genes. *Plant J* 51 (4): 631–41

Wiklund, C. (1984). Egg-laying patterns in butterflies in relation to their phenology and the visual apparency and abundance of their host plants. *Oecologia* 23-29

Wiemers M, Chazot N, Wheat CW, Schweiger O, Wahlberg N (2020) A complete time-calibrated multi-gene phylogeny of the European butterflies. *ZooKeys* 938: 97–124

Wiklund C, Friberg M (2009) The evolutionary ecology of generalization: among-year variation in host plant use and offspring survival in a butterfly. *Ecology* 90: 3406–3417

Wilkinson SW, Mageroslashy MH, Lopez Sanchez A, Smith LM, Furci L, Cotton TEA, Krokene P, Ton J (2019) Surviving in a hostile world: plant strategies to resist pests and diseases. *Annu Rev Phytopathol* 57: 505–529

Winde I, Wittstock U (2011) Insect herbivore counteradaptations to the plant glucosinolate–myrosinase system. *Phytochemistry* 72: 1566–1575

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

Winkler IS, Mitter C, Scheffer SJ (2009) Repeated climate-linked host shifts have promoted diversification in a temperate clade of leaf-mining flies. *Proc Natl Acad Sci U S A* 106: 18103–18108

Wittstock U, Agerbirk N, Stauber EJ, Olsen CE, Hippler M, Mitchell-Olds T,Gershenzon J, Vogel H (2004) Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proceedings of the National Academy of Sciences, USA* 101: 4859–4864

Wu CH, Abd-El-Haliem A, Bozkurt TO, Belhaj K, Terauchi R, Vossen JH, Kamoun S (2017) NLR network mediates immunity to diverse plant pathogens. *Proc Natl Acad Sci U S A* 114 (30): 8113–18

X

Xue J, Lenman M, Falk A, Rask L (1992) The glucosinolate-degrading enzyme myrosinase in Brassicaceae is encoded by a gene family *Plant Mol Biol* 18: 387–398

Y

Yamasaki M, Yoshimura A, Yasui H (2003) Genetic basis of ovicidal response to whitebacked planthopper (*Sogatella furcifera* Horváth) in rice (*Oryza sativa* L.). *Mol Breed* 12: 133–143

Yang D-H, Hettenhausen C, Baldwin IT, Wu J. (2011) BAK1 regulates the accumulation of jasmonic acid and the levels of trypsin proteinase inhibitors in *Nicotiana attenuata*'s responses to herbivory. *J Exp Bot* 2011; 62: 641–652.

Yang JO, Nakayama N, Toda K, Tebayashi S, Kim CS (2014) Structural determination of elicitors in *Sogatella furcifera* (Horváth) that induce Japonica rice plant varieties (*Oryza sativa* L.) to produce an ovicidal substance against *S. furcifera* eggs. *Biosci Biotechnol Biochem* 78: 937–942

Yang Y, Xu J, Leng Y, Xiong G, Hu J, Zhang G *et al.* (2014) Quantitative trait loci identification, fine mapping and gene expression profiling for ovicidal response to whitebacked planthopper (*Sogatella furcifera* Horváth) in rice (*Oryza sativa* L.). *BMC Plant Biol* 14: 145

Yates S, Mikaberidze A, Krattinger SG, Abrouk M, Hund A, Yu K *et al.* (2019) Precision phenotyping reveals novel loci for quantitative resistance to septoria tritici blotch. *Plant Phenomics* 2019 2019.

Yu IC, Parker j, Bent AF (1998) Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis dnd1* mutant. *Proc Natl Acad Sci U S A* 95 (13): 7819–24

Yu J, Yang XD, Wang Q, Gao LW, Yang Y, Xiao D, Liu TK, Li Y, Hou XL, Zhang CW (2018) Efficient virus-induced gene silencing in *Brassica rapa* using a Turnip Yellow Mosaic Virus vector. *Biol Plant* 62 (4): 826–34

Yuan M, Jiang Z, Bi G, Nomura K, Liu M, Wang Y, Cai B, Zhou J-M, He SY, Xin X-F (2021) Pattern-recognition receptors are required for NLR-mediated plant immunity. *Nature* 592: 105–109

Z

Zhang L, Cai X, Wu J, Liu M, Grob S, Cheng F *et al.* (2018) Improved *Brassica rapa* reference genome by single-molecule sequencing and chromosome conformation capture technologies *Hortic Res* 5: 1–11

Zhang X, Meng L, Liu B, Hu Y, Cheng F, Liang J *et al.* (2015) A transposon insertion in FLOWERING LOCUS T is associated with delayed flowering in *Brassica rapa*. *Plant Sci* 241: 211–220

Zhao J, Artemyeva A, Del Carpio DP, Basnet RK, Zhang N, Gao J *et al.* (2010) Design of a *Brassica rapa* core collection for association mapping studies. *Genome* 53: 884–898

Zhao Y, Huang J, Wang Z, Jing S, Wang Y, Ouyang Y, Cai B, Xin X-F, Liu X, Zhang C, *et al.* (2016) Allelic diversity in an NLR gene BPH9 enables rice to combat planthopper variation. *Proc Natl Acad Sci U S A* 113(45): 12850-12855

Zheng SJ, Snoeren TAL, Hogewoning, van Loon JJA, Dicke M (2010) Disruption of plant carotenoid biosynthesis through virus-induced gene silencing affects viposition behaviour of the butterfly *Pieris rapae*. *New Phytol* 186 (3): 733–45

List of abbreviations

QTL: quantitative trait locus

HR: hypersensitive response

ROS: reactive oxygen species

SA: salicylic acid

JA: jasmonic acid

PRR: pattern recognition receptor

RLP: receptor-like protein

RLK: receptor-like kinase

LecRK: L-type lectin receptor kinase

CRK: cysteine-rich receptor kinase

WAK: wall-associated receptor kinase

NLR: nucleotide-binding leucin-rich repeat receptor

TIR-NBS-LRR (or TNL): Toll-interleukin 1 receptor domain NLRs

EAMP: egg-associated molecular pattern

PAMP: pathogen-associated molecular pattern

DAMP: damage-associated molecular pattern

HAMP: herbivore-associated molecular pattern

PTI: pathogen-triggered immunity

ETI: effector-triggered immunity

Summary

Summary

Over the course of millions of years of coevolution with their attackers, plants evolved an innate immune system that is finely tuned to recognize specific signals and induce defences. Plant defences to pathogens (bacteria, fungi, oomycetes, viruses) and herbivores (insects, nematodes) have been extensively investigated at both the phenotypic and molecular level. Other threats, for example insect eggs, have been largely overlooked despite representing the first contact between plants and insects. Eggs of *Pieris* spp. butterflies (Lepidoptera: Pieridae) induce a hypersensitive response (HR)-like cell death on their natural host plants belonging to the Brassicaceae family. As the cell death reduces egg survival, it may represent a potential adaptive trait in the ongoing arms race between glucosinolate-containing plants and their specialist butterflies. Moreover, as *Pieris* spp. represent a pest of *Brassica* crops in agricultural settings, a cell death targeting eggs may also act as an additional defense trait for crop breeding. To address both aspects, it is first needed to further understand the genetic basis of this egg-induced cell death. The aim of my dissertation is to explore the (macro)evolutionary and genetic basis of a HR-like cell death induced by *Pieris* spp. eggs.

The well-established co-evolutionary dynamics between Pieridae butterflies and Brassicales plants is driven by the plant chemical defenses (i.e. glucosinolates) and the reciprocal insect detoxification mechanisms. Thus, in **Chapter 2** I aimed to explore the overlooked egg-induced HR-like cell death as a potential adaptation in the context of this butterfly-plant arms race. I found that *P. brassicae* eggs induce a HR-like cell death in a few species of Brassicaceae family but not in the Cleomaceae. Further, egg-induced cell death was mostly present in the genus *Aethionema,* which is the sister lineage to the core Brassicaceae, and in species of the Brassicae tribe, which includes *P. brassicae* natural hosts from the *Brassica* genus and close relatives. As HR-like cell death appeared restricted to host species of *Pieris* spp., I tested whether the trait was specifically induced by eggs of butterflies that are adapted to glucosinolates. Indeed, butterflies of the Pierinae subfamily that feed on Brassicaceae induced cell death unlike other butterflies and moths. In conclusion, I proposed that HR may have arisen as novel plant adaptation to defend plants against butterflies that evolved effective glucosinolate detoxification mechanisms. In addition, as cell death is mostly effective against single eggs, I discussed possible butterflies counteradaptations such as egg clustering, oviposition on inflorescence and host-shift.

In **Chapter 3**, plant immune responses associated with HR-like cell death were studied more in depth at the physiological and molecular level. I aimed to investigate the specificity of egg-induced cell death by comparing eggs of a specialist butterfly (*P. brassicae)* and the generalist cabbage moth (*Mamestra brassicae)* on a crop (*B. rapa)* and its wild relative (*B. nigra)*. Eggs of *P. brassicae* induced a more severe macroscopic HR-like cell death in *B. nigra* than in *B. rapa.* Nevertheless, both plant species developed similar immune responses such as accumulation of ROS, callose deposition, cell death and *PR1* expression, which were also present in plant accessions unable to express a macroscopic HR-like. On the contrary, eggs of *M. brassicae* did not induce any visible cell death and plant immune responses were weak and/or absent. Further, I showed that an egg wash made with secretions surrounding *P. brassicae* eggs was sufficient to induce cell death. The HR-like cell death induced by *P. brassicae* eggs was specific to eggs as plant accessions that did not develop cell death under eggs were still able to induce a functional HR against pathogenic bacteria and fungi. These findings showed that *Brassica* spp. plant immune system induces a HR-like cell death upon specific recognition of elicitors originated from eggs of an adapted specialist butterfly.

In **Chapter 4**, I aimed to assess the potential of HR-like cell death as a defence trait in a crop (*B. rapa*) and to unravel its genetic architecture. First, I carried out a germplasm screening of 56 *B. rapa* accessions and I found phenotypic variation for cell death size. Further, I developed an imaged-based phenotyping protocol to accurately measure cell death size and I used it to re-assess a few accessions to identify potential parents suitable for crosses. Two accessions consistently showed contrasting cell death phenotype and a RIL population was used for genetic mapping. HR-like in *B. rapa* is a polygenic trait as I identified three QTLs *Pieris brassicae*-induced cell death (*Pbc*) which explained about a third of the genetic variation and included different types of genes related to plant immunity. Nevertheless, the HR-like cell death observed in *B. rapa* appeared weaker compared to the one developed by *B. nigra*. Finally, I discussed implications of these results for fine mapping of the *Pbc* loci and implications for exploitation of HR-like cell death as defense trait in crop breeding.

In **Chapter 5**, I investigated the genetic basis of *P. brassicae* egg-induced HR-like cell death using *B. nigra* accessions collected from a local wild population. The cell death segregated as a Mendelian trait throughout our crossing scheme. Through bulk segregant analysis followed by fine mapping I identified a single *Pieris brassicae* egg-killing (*PEK)* locus. Interestingly, the locus is underlined by a region including a cluster of TIR-NBS-LRR receptors (TNLs), a type of NLR genes that are associated with HR-based plant defences

against pathogens. I showed that *PEK* locus has sequences polymorphisms (SNPs and InDels) between our parental accessions but also copy number variants (CNVs) between currently sequenced *B. nigra* genomes. Given the extensive variation at the *PEK* locus, I discussed the need to develop an improved genome assembly of our *B. nigra* material in order to perform further fine mapping and identify casual genes and/or variants. Resolving of the structure of the *PEK* locus will allow to study whether the TNLs within the PEK locus are actually involved in detection and/or signalling of the HR-like induced by butterfly eggs.

In conclusion, this thesis investigated the evolutionary and genetic basis of a cell death induced by butterfly eggs in its host plants. The findings presented here suggest that plants induce a cell death-based defense response upon specific recognition of eggs from adapted butterflies. The identification of genetic loci associated with egg-induced cell death helps in further understanding the molecular basis of plant-egg interaction. Future validation of these loci may provide testable hypotheses in order to link genetic mechanisms to the evolution of HR-like across plant phylogeny.

Acknowledgments

Acknowledgments

Here we are, the most read section of each PhD thesis regardless of the research presented. Nonetheless, the toughest section to write as words fall short while trying to express my gratitude for the fantastic souls who I had the pleasure to share this journey with.

This thesis would not have been possible without the guidance and the unconditional confidence of the "Fab Four": **Nina, Eric, Bas** and **Guusje**. I wholeheartedly thank you all for your support and teachings which certainly shaped my personal and intellectual growth. **Nina,** your perseverance and motivation in moving your lifelong research topic into unknown scientific domains is truly inspiring. Your contagious energy helped me to move forward through the many lows I encountered, but it also urged me to immediately celebrate the occasional highs. **Eric**, you are a true multidisciplinary scientist. Your lateral thinking and relaxed attitude are the qualities that everyone should desire from a promoter. The way you connect the most diverse research topics and mold them into a successful research career will never stop amazing me. **Bas,** I admire your clarity of thinking. Sometimes, joining a meeting for less than half hour was enough for you to ask a formidable question. I will bring with me your usual comment "when you are stuck, you just need to lay back and wonder". **Guusje**, you have been my first scientist mentor during my MSc, and I was pleased to have you also on board for my PhD. I enjoy your Dutch frankness which helped me multiple times to compromise between what was possible and what was feasible. Thank you all, I will miss our biweekly meeting that often evolved into a wonderful brainstorming (jam) session. I certainly have left enough notes to work out a second PhD.

Lotte, your knowledge and skills perfectly complemented my background throughout our project. I learnt the most from you on how to conduct experimental work, from setup to execution. But also how not to get lost in conferences. I`ll be forever grateful for this. Your attention for details, knowledge of literature, assertiveness (and humor?) are quintessential features of the successful scientist you are. Hope you will forgive me for the countless hours you helped me screening plants in the greenhouse. **Klaas**, you were gradually integrated in our project and at time I felt you were a fifth supervisor. I could not ask for anyone better! Thanks for all the knowledge on molecular biology, plant immunity, Dutch nature that you shared with me over the years. You were the most critical commenter on the manuscripts, and it certainly helped me to become a better writer.

Thanks to the many external members of our project User Committee: **Bart, Martin**, **Colette**, **Roland**, **Joke**, **Joris**, **Marcos**, **Nelson**, **Alejandro, Jan**, **Tjeerd**. Your curiosity and critical questions during our progress meeting often helped us to clarify our research process. Further, thanks to Jan and Tjeerd for supporting the project with in-kind and/or monetary contributions.

To the external members of the Thesis Committee: **Joop van Loon**, **Anke Steppuhn**, **Like Fokkens** and **Merijn Kant,** thanks for taking the time to critically read my thesis. Hopefully we will have a pleasant and insightful discussion during the defense.

I am also grateful to the **Graduate School Experimental Plant Sciences** for its continuous effort to support PhD candidates and contribute to their personal and professional formation financing state-of-the-art courses.

The amazing research assistance at Biosystematics and Genetics labs is what made most of the research here presented actually feasible. **Gabriella**, **Corrie, José, Patrick, Frank**, **Francisca** thanks for the many times you helped me with: doing anything in the greenhouse (**Gabriella**); learning something on seeds (**Corrie**) or microscopes (**José**); setting up gene expression experiments or running molecular markers (**Patrick**); discussing any molecular biology topic often diverging into cooking & gastronomy (**Frank,** after all still a matter of protocols, right?). **Francisca**, thanks for your help with cloning for our VIGS project, too bad it did not make it into this thesis. Bonus point for Patrick, Frank and **Diego** (former postdoc at Genetics), as a music lover, it was great to listen to your very diverse playlists during endless sessions in the greenhouse or at the lab bench. Patrick, sorry for all that "experimental" jazz! Finally, many thanks to **Johan** (Lab of Plant Breeding) for his tips on growing and selfing Brassicas, **Pieter** (Lab of Entomology) for the countless eggs and butterflies he provided, and Unifarm - particularly **Sean** and **Jorik -** for taking care of our plants and for the many laughs in the greenhouse despite their terror for our butterflies regularly escaping into the corridors**.**

Over the last years I had the opportunity to collaborate with excellent scientists with whom I share an interest for most of the disciplines touched upon by this thesis. Thanks **Eddie** for laying the foundation for the study of genetic variation for egg-induced HR and, later, for involving me in setting up the screening of Brassicaceae spp. Thanks **Roel** for the countless discussions on statistical genetics and QTL mapping. **Mohamed**, I am happy that you collaborated on the QTL mapping in *B. rapa* although we never met in person - hopefully we will in the future! **Miguel**, thanks for your lessons on histochemical stainings and fine mapping. Thanks to **Clorentin** and **Chengcheng** for starting me out with Linux, genomics, *k*-mers. And thanks **Robin** for the discussions on variant calling and comparative genomics. **Nam**, I enjoyed

your help with the transcriptomics data shortly after you just moved to The Netherlands, in the middle of a pandemic and with your own project to start. Your dedication to science is inspirational.

As I often heard at Biosystematics: "research is only half of the goal of University, the other half being teaching". This statement became clearer to me only after I became involved in assisting the glorious "Pyrenees course". **Lars**, **Roel¸ Casper** I`ll be forever grateful to you for showing me the beauty of being a teacher, while engaging students on field excursions over botany and ecology. **Nina**, **Sabrina**, **Eva**, **Corné,** thanks for showing me wonderful insects and attempting to teach me their taxonomy too.

Academic teaching does not manifest itself only with courses, but also with mentoring students, which I discovered being an art on its own. **Jeroen**, **Salomè**, **Martijn**, **Leonne**, **Aoxiang**, **Ewan**, **Tim**, **Rick**, **Thijmen van der Loop** thanks for your patience, dedication and curiosity. Certainly, I learned from you as much I taught you.

The last six years at the Biosystematics Group were a blast. I will always cherish the many memories of our lunch breaks, lab outings and discussions ranging from science to the next character to be draw on the chalk board of our "Starbucks". To the core Group members during my PhD, **Wilma**, **Freek**, **Lars**, **Nynke**, **Roel**, **Casper, Sabrina**, **Kitty, Carla, Marco, Patrick, Klaas, Robin, Tinde, Setareh**, **Sara**, **Dêêdi**, **Tao Zhao**, **Floris**, **Thijmen Breeschoten**, **Wei**, **Eddie**, **Liana**, **Wouter Makkinje**, **Eva,** thanks a lot for this. The last two years brought also new lymph to the ever growing tree of Biosystematics. **Jordy**, **Tao Feng**, **Marieke**, **Cloe**, **Nam**, **Anastasia**, **Li**, **Siva**, **Qiong**, **Lei**, **Alejandra**, **Nicholaas**, **Pamela**, we haven`t spent much time together because of Covid and my (never ending) writing retreat, but I really enjoyed the few sporadic chats we had!

Life is Radix was also made more pleasant by the frequent exchanges with our neighbors of Laboratory of Genetics and CGN. Thank you all for all the chats, breaks, laughs and the various shared dinners/BBQs we had. Worth to mention the four "musketeers" **Renè Tom**, **Roel** and **Jitpanu**. It was great to talk about plant & genetics with you. If I could predict the future of plant science based on your profiles, it will be bright, full of innovation and "loud". Thanks to **Diego** and **Francesco** for spreading their sparkling Italian temperament from the lab to the coffee corner, you made me feel at home every day.

Finally, I`d like to express my gratitude to my friends, family, and partner for the good times together, their support and continuous interest ("are you still working with cabbages and butterflies?"). Life in Wageningen was sweet and pleasantly unpredictable thanks to the many "Droefies" I met along the way: **Maria**, **Mara**, **Georgios**, **Berta** & **Giulio**, **Jordi**, **Ramon**,

Jacopo, **Silvia**, **Adrià** & **Angel**, **Santos** & **Lucia** & **Angela**, **Johanna**, **Dina**, **Tossa**, **Cristina** & **Juan**, **Ko**, **Enrico Orsi** & **Patricia**, **Carlo**, **Philipp**, **Tommi** & **Titta**, **Sam** & **Elise**, **Lukas** & **Ina**, **Guidoriccio** & **Janne** & **Amelie**, **Alessio** & **Alina**. Later in Utrecht I had the pleasure to share meals, laughs (and multiple lockdowns) with **Emma**, **Wouter Bursma**, **Sophie** and **Fleur**. Thanks for teaching me Dutch and contributing to our "Blijelaan". Eternal gratitude also to the "Amsterdammers" who dare every now and then to go "outside the Ring" to bring some novelty into our lives: **Pia**, **Jesse**, **Kayleigh** & **Brandon, Maryse** and **Gloria**.

Most of my time outside of my PhD was undeniably devoted to making music. **Santos**, **Tim**, **Lana, Julian**, I had the most exiting leisure moments of last years with our Flotsam Metric. Hopefully we will get back on stage one day.

Grazie ai cari amici rimasti a casa, che non mi fanno mai mancare la leggerezza di essere Toscano. **Franci, Raffa** & **Elise**, **Clodi & Anita**, **Nando**, **Marco**, **Nicco & Lucia**, **Lorenzo Orsi**, **Guido & Michela**, **Claudia,** ogni volta che torno a trovarvi è come se non me ne fossi mai andato. A voi dico: "Hasta siempre vitellones!". Ai Valdorciani **Ame**, **Cecco**, **Menca**, **Gloria**, **Disio**, **Giovi**, **Bucci** & **Eva**, grazie per farmi sentire sempre a casa, in quella nostra terra fatta di bellezza ed umanità.

Caro **babbo Paolo** e **mamma Paola**, questo traguardo è anche il frutto dei sacrifici che avete fatto per supportare le mie scelte. Grazie per avermi insegnato a portare le cose a termine stringendo i denti ma con il sorriso. Ai **nonni Gino** & **Luigina**, **Giotto** & **Anna** e agli zii **Massimo** & **Paola**, **Gianni** & **Michela**, grazie per il vostro costante amore ed affetto così forte da superare la distanza che ci separa. Alle giovani di casa **Viola**, **Aurora**, **Bianca**, auguro il meglio per un futuro luminoso. Infine **Dande**, mia amata sorella, grazie per il tuo infinito amore e per la tua impredicibilità che ci tiene vivi. E per mantenere quell`angolo di agricoltura felice che è la Biofattoria l`Upupa.

Dank aan mijn Nederlandse familie **Toos & Koos, Hans & Sonja**, **Jeroen & Patricia**, als ik me hier thuis voel, is dat ook dankzij jullie vriendelijkheid en liefde.

Uiteindelijk **Donna**, mijn liefde. Alles is nu klaar en ik zal je niet meer dwingen tot te korte nachten. Je bent bij mij bij elke ademhaling. En ik zou willen dat het nog een tijd duurt.

About the author
Publications
Education statement

About the author

Niccolò Bassetti was born in Siena, Italy, on the 7th May 1989, and grew up in Val d`Orcia between dusty old villages, rolling hills and his grandparents` farm. Early memories of his whereabouts recount of "expeditions" to collect plants, fossils and minerals; impressing family guests with names of dinosaurs and astronomical objects; singing unknown Italian pop songs. Looking back, those were just the early signs of his ongoing passion for science and music. He learnt the importance of asking critical questions the hard way, when he discovered that the eggs of *Pterodactylus* that were raised by a family`s friend did not actually exist.

During the scientific lyceum in Montepulciano he matured an interest for social issues which kept him awake at night thinking how to combine natural sciences and societal impact. In 2008, he then enrolled in a BSc in Agricultural Sciences at the University of Florence which he completed with a BSc research on the water requirements for Tuscan agriculture in relation of climate change under supervision of Prof. Dr. Simone Orlandini. After a short experience as an agronomist, he started an MSc in Plant Sciences at Wageningen University in 2013, to specialize in plant genetics and pathology. A growing interest in data science made him complete a first MSc research on the transcriptional regulation of *Brassica rapa* turnip formation with dr. Guusje Bonnema (Lab. of Plant Breeding, Wageningen University), which resulted in a publication (Liu, Bassetti *et al.*, 2019). Eager to learn more on molecular biology, he then conducted a MSc internship at Keygene BV with Dr. Martin de Vos, working on gene validation for an important *Brassica* pathogen.

In 2016, Niccolò started his PhD project at Biosystematics Group (Wageningen University) supervised by Dr. Nina Fatouros, Dr. Guusje Bonnema, Prof. Dr. Bas Zwaan and Prof. Dr. Eric Schranz. For this project he worked on the evolution and genetic basis of a butterfly-egg induced cell death in *Brassica* species. Working at Biosystematics Group brought him in contact with the world of evolution and ecology, in a way closing the circle with his child interests for the natural world. The main finding of his PhD research are presented in this thesis.

Publications

Bassetti N, Caarls L, Verbaarschot P, Bongers T, van der Loop T, Hoang N, Zwaan BJ, Schranz ME, Bonnema G, Fatouros NE. Pieris brassicae egg-induced cell death in Brassica nigra is mediated by a single locus containing a cluster of TIR-NBS-LRR receptors. *In prep.*

Bassetti N, Caarls L, Bukovinszkine'Kiss G, El-Soda M, van Veen J, Bouwmeester K, Zwaan BJ, Schranz ME, Bonnema G, Fatouros NE (2022) Genetic analysis reveals three novel QTLs underpinning a butterfly egg-induced hypersensitive response-like cell death in Brassica rapa. *BMC Plant Biology*, 22 (1): 140. doi:10.1186/s12870-022-03522-y

Griese E*, Caarls L*, **Bassetti N**, Mohammadin S, Verbaarschot P, Bukovinszkine'Kiss G, Poelman EH, Gols R, Schranz ME, Fatouros NE (2021) Insect egg-killing: a new front on the evolutionary arms-race between brassicaceous plants and pierid butterflies. *New Phytologist*, 230: 341-353. doi:10.1111/nph.17145

Caarls L, **Bassetti N**, van Doesburg F, Verbaarschot P, van Loon JJA, Schranz, ME, Fatouros, NE (2021) Deciphering Brassica plant defence responses to cabbage white butterfly egg-associated molecular patterns. Preprint at *bioRxiv*, doi: https://doi.org/10.1101/2021.03.29.437462

Liu M, **Bassetti N**, Petrasch S, Zhang N, Bucher J, Shen S, Zhao J, Bonnema G (2019) What makes turnips: anatomy, physiology and transcriptome during early stages of its hypocotyl-tuber development. *Horticulture Research*, 6 (38), https://doi.org/10.1038/s41438-019-0119-5

Thorin T*, **Bassetti N*** (2016) The entrepreneurial way to integrate social responsibility and economic profitability into agri-business in East Africa. *Gewasbescherming: mededelingenblad van de Nederlandse Planteziektenkundige Vereniging in samenwerking met de Coordinatiecommissie Onkruidonderzoek NRLO 47*, 47 (1): 5-7

*Contributed equally to this study and shared first authorship

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Niccolò Bassetti
Date: 03 October 2022
Group: Biosystematics
University: Wageningen University & Research

1) Start-Up Phase	*date*	*cp*
► **First presentation of your project** "Genetic basis of the hypersensitive response-like necrosis induced by Pierid butterfly eggs in *Brassica rapa*", Biosystematics group	15 Mar 2017	1.5
► **Writing or rewriting a project proposal**		
► **MSc courses**		
Subtotal Start-Up Phase		1.5

2) Scientific Exposure	*date*	*cp*
► **EPS PhD student days**		
EPS PhD student days "Get2Gether", Soest, NL	9-10 Feb 2017	0.6
EPS PhD student days "Get2Gether", Soest, NL	15-16 Feb 2018	0.6
► **EPS theme symposia**		
EPS Theme 2 'Interactions between Plants and Biotic Agents' together with the Willie Commelin Scholten Day, Wageningen, NL	23 Jan 2017	0.3
EPS Theme 2 'Interactions between Plants and Biotic Agents' together with the Willie Commelin Scholten Day, Amsterdam, NL	24 Jan 2018	0.3
EPS Theme 2 'Interactions between Plants and Biotic Agents' together with the Willie Commelin Scholten Day, Wageningen, NL	1 Feb 2019	0.3
EPS Theme 2 'Interactions between Plants and Biotic Agents' together with the Willie Commelin Scholten Day, online	9 Feb 2021	0.2
EPS Theme 4 'Genome Biology', Wageningen, NL	13 Dec 2019	0.3
EPS Theme 4 'Genome Biology', online	11 Dec 2020	0.2
EPS Theme 4 'Genome Biology', online	17 Jan 2022	0.3
► **Lunteren Days and other national platforms**		
Annual meeting of the Netherlands Entomological Society, Ede, NL	16 Dec 2016	0.3
Annual meeting of the Netherlands Entomological Society, Ede, NL	15 Dec 2017	0.3
Annual meeting of the Netherlands Entomological Society, Ede, NL	17 Dec 2018	0.3
Annual meeting of the Netherlands Entomological Society, online	18 Dec 2020	0.2
Annual Meeting "Experimental Plant Sciences", Lunteren, NL	10-11 Apr 2017	0.6
Annual Meeting "Experimental Plant Sciences", Lunteren, NL	8-9 Apr 2019	0.6
Annual Meeting "Experimental Plant Sciences", online	12-13 Apr 2021	0.5
Annual Meeting "Experimental Plant Sciences", Lunteren, NL	11-12 Apr 2022	0.6
2nd Conference of the Netherlands Society for Evolutionary Biology (NLSEB), Ede, NL	16 Apr 2019	0.3
► **Seminars (series), workshops and symposia**		
Seminars:		
Martin Kaltenpoth, "Outsourcing immunity: Microbial symbionts for pathogen defense in insects"	7 Apr 2017	0.1
Martin Cann, "The immune receptor Rx1 remodels chromatin and chromatin interactors in immunity"	11 Jul 2017	0.1
Urs Wyss, "Highlights of hidden insect-worlds"	2 Oct 2017	0.1

Timothy Bilton, "Statistical methods for analysing low-coverage genotyping-by-sequencing data"	13 Jul 2018	0.1
Yan Wang, "A leucine-rich repeat receptor-like protein as PAMP receptor recognising XEG1, a Phytophthora glycoside hydrolase 12"	9 Sep 2018	0.1
Ronnie de Jonge, "Microbial small molecules - weapons of plant subversion"	20 Feb 2019	0.1
Hiroshi Maeda, "Exploiting Evolutionary Diversification of Primary Metabolic Enzymes"	4 Mar 2019	0.1
Ivan Baxter, "Mind the GxE=P: elemental content in plants"	9 Dec 2019	0.1
Bregje Wertheim, "Evolving immunity: genomic basis of evolution and variation in parasitoid resistance"	19 Jan 2017	0.1
Mike Singer, "One butterfly species tumbles off an adaptive peak and enters a lethal trap in the course of six host shifts observed across half a century"	12 Apr 2017	0.1
Richard Lenski, "Dynamics of Adaptation and Genome Evolution in a Long-Term Experiment"	31 Aug 2017	0.1
Christa Testerink, "Being flexible in time of stress"	15 Sep 2017	0.1
Workshops:		
EPS 12th Plant-Insect Interactions Workshop, Wageningen,NL	7 Nov 2017	0.3
EPS 13th Plant-Insect Interactions Workshop, Leiden, NL	9 Oct 2018	0.3
EPS 14th Plant-Insect Interactions Workshop, Amsterdam, NL	14 Nov 2019	0.3
GeneSprout Initiative Workshop Plants & Patents, Wageningen, NL	21 Oct 2019	0.2
Workshop Breeding for diversity, Wageningen, NL	30 Oct 2019	0.2
Symposia:		
1st WURomics symposium: Technology-Driven Innovation for Plant Breeding, Wageningen, NL	15 Dec 2016	0.3
Farewell Symposium for prof. Ton Bisseling, "The Undergrond Labyrinth: Roots, Friends and Foes", Wageningen, NL	8 Feb 2017	0.2
Mini symposium on insect-microbe-plant interactions, Wageningen, NL	7 Apr 2017	0.2
Genotype to Phenotype Modelling of Plant Adaptation, Wageningen, NL	16 Nov 2017	0.3
Breeding data: statistical advances in modern plant breeding, Wageningen, NL	16 Oct 2018	0.3
► **Seminar plus**		
► **International symposia and congresses**		
16th Symposium on Insect-Plant interactions (SIP16), Tours, FR	2-6 Jul 2017	1.3
26th Biannual International Plant Resistance to Insects Symposium (IPRI 18), Rothamsted Institute, Harpenden, UK	7-9 Mar 2018	0.9
CRC 973 Symposium "Bridging Ecology and Molecular Biology: Organismic Responses to Recurring Stress", Berlin, DE	9-11 Apr 2018	0.9
18th Congress of International Society for Molecular Plant-Microbe Interactions (MPMI 2019), Glasgow, Scotland, UK	14-18 Jul 2019	1.4
► **Presentations**		
"Towards the genetic basis of the hypersensitive response induced by *Pieris* spp. eggs in *Brassica rapa*" (Poster), at SIP16, Tours, FR	2-6 Jul 2017	1.0
"Hypersensitive response-like necrosis induced by *Pieris* eggs in *Brassica* spp." (Oral), at Annual meeting of the Netherlands Entomological Society, Ede, NL	15 Dec 2017	1.0
"The hypersensitive response-like necrosis induced by *Pieris* spp. eggs in *Brassica rapa*: road towards the mechanisms" (Poster), at IPRI 18, Rothamsted Institute, Harpenden, UK	7-9 Mar 2018	1.0
"The hypersensitive response-like necrosis induced by *Pieris* spp. eggs in *Brassica rapa*: road towards the mechanisms" (Oral), at CRC 973 Symposium "Bridging Ecology and Molecular Biology: Organismic Responses to Recurring Stress", Berlin, DE	9-11 Apr 2018	1.0

"Plants killing insect eggs: unravelling the genetic mechanism of *Pieris* egg-induced necrosis in *Brassica* crops" (Oral), at Annual Meeting "Experimental Plant Sciences", Lunteren, NL	8-9 Apr 2019	1.0
"Egg-killing plants: unravelling the genetic mechanisms of a butterfly egg-induced necrosis in *Brassica* crops" (Poster), at MPMI 2019, Glasgow, Scotland, UK	14-18 Jul 2019	1.0
► **3rd year interview**		
► **Excursions**		
EPS PhD online company visit to Genetwister Technologies BV	22 Mar 2022	0.2
Subtotal Scientific Exposure		21.3

3) In-Depth Studies	*date*	*cp*
► **Advanced scientific courses & workshops**		
EPS course 'Data Analyses and Visualizations in R', Wageningen, NL	8-9 May 2018	0.6
EPS course 'The power of RNA-seq', Wageningen, NL	11-13 Jun 2018	0.9
EPS course 'Transcription factors and transcriptional regulation', Wageningen, NL	13-15 Dec 2021	1.0
EPS workshop & 3rd WURomics symposium 'Advances in Food & Plant Metabolomics', Wageningen, NL	10-12 Dec 2019	0.9
► **Journal club**		
Biosystematics Group literature discussion	2017-2018	1.5
► **Individual research training**		
Subtotal In-Depth Studies		4.9

4) Personal Development	*date*	*cp*
► **General skill training courses**		
WGS PhD Competence Assessment, Wageningen, NL	19 Apr 2017	0.3
EPS Introduction Course, Wageningen, NL	26 Sep 2017	0.3
WGS course 'Project and Time Management', Wageningen, NL	10 Oct - 21 Nov 2017	1.5
WGS course 'Supervising BSc & MSc thesis students, Wageningen, NL	19-20 Sep 2019	0.6
WGS course 'Scientific Writing', Wageningen, NL	Jan - Feb 2020	1.8
WGS course 'Adobe InDesign Essential Training, online	9-10 Nov 2020	0.6
WGS course 'Scientific Artwork, Data visualisation and Infographics with Adobe Illustrator, online	1-9 Feb 2021	0.6
WGS course 'Career Orientation', Wageningen, NL	5 Oct - 2 Nov 2021	1.5
WGS PhD Workshop Carousel 2019, Wageningen, NL	24 May 2019	0.3
► **Organisation of meetings, PhD courses or outreach activities**		
► **Membership of EPS PhD Council**		
Subtotal Personal Development		7.5

5) Teaching & Supervision Duties	*date*	*cp*
► **Courses**		
Webs of Terrestrial Diversity	2017, 2018, 2019	3.0
► **Supervision of BSc/MSc students**		
MSc thesis Jeroen van Veen	2018	
MSc thesis Thijmen van der Loop	2022	
BSc thesis Salomè Rinzema	2018	

BSc thesis Aoxiang Xin	2020	
HBO Internship Martijn Flipsen	2019	
HBO internship Leonne van den Enden	2020	
HBO internship Ewan van Eijden	2020	
HBO internship Tim Bongers	2021	
HBO internship Rick Faesen	2021	3.0
Subtotal Teaching & Supervision Duties		6.0

TOTAL NUMBER OF CREDIT POINTS*	**41.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

The research described in this thesis was financially supported by the Toegepaste en Technische Wetenschappen (TTW), which is part of the Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), through the NWO/TTW VIDI grant no. 14854 awarded to dr. Nina E. Fatouros, and partly co-founded (<10%) by seed companies Syngenta and Hazera (Vilmorin & Cie group).

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design: Niccolò Bassetti

Layout design: Niccolò Bassetti

Printing: ProefschriftMaken (NL)