Received: 14 July 2023 | Revised: 26 October 2023 | Accepted: 1 November 2023

DOI: 10.1111/pce.14765

ORIGINAL ARTICLE

Plant, Cell & Environment | WILEY

A butterfly egg-killing hypersensitive response in *Brassica nigra* is controlled by a single locus, PEK, containing a cluster of TIR-NBS-LRR receptor genes

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Funding information
Nederlandse Organisatie voor Wetenschappelijk Onderzoek

Abstract

Knowledge of plant recognition of insects is largely limited to a few resistance (*R*) genes against sap-sucking insects. Hypersensitive response (HR) characterizes monogenic plant traits relying on R genes in several pathosystems. HR-like cell death can be triggered by eggs of cabbage white butterflies (*Pieris* spp.), pests of cabbage crops (*Brassica* spp.), reducing egg survival and representing an effective plant resistance trait before feeding damage occurs. Here, we performed genetic mapping of HR-like cell death induced by *Pieris brassicae* eggs in the black mustard *Brassica nigra* (*B. nigra*). We show that HR-like cell death segregates as a Mendelian trait and identified a single dominant locus on chromosome B3, named *PEK* (*Pieris egg-killing*). Eleven genes are located in an approximately 50 kb region, including a cluster of genes encoding intracellular TIR-NBS-LRR (TNL) receptor proteins. The *PEK* locus is highly polymorphic between the parental accessions of our mapping populations and among *B. nigra* reference genomes. Our study is the first one to identify a single locus potentially involved in HR-like cell death induced by insect eggs in *B. nigra*. Further fine-mapping, comparative genomics and validation of the *PEK* locus will shed light on the role of these TNL receptors in egg-killing HR.

KEYWORDS
Brassica crops, bulk segregant analysis, cabbage white butterfly, egg deposition, *k*-mers, NLRs, plant–insect interaction, TNLs

1 | INTRODUCTION

Our mechanistic understanding of the initial recognition of herbivorous insects by plants is still limited. In contrast to recognition of plant pathogens, so far only a handful of resistance (*R*) genes have been identified against insect herbivores, encoding cell surface receptors (pattern recognition receptors, PRRs) and intracellular receptors (nucleotide-binding leucin rich-repeat, NLRs) (Kourelis & van der Hoorn, 2018; Snoeck et al., 2022). Known resistance traits against insects based on *R* genes are mainly limited to sap-sucking insects,

including gall midges (Bentur et al., 2016), aphids (Botha et al., 2005; Dogimont et al., 2014; Klingler et al., 2009; Nicolis & Venter, 2018; Rossi et al., 1998; Sun et al., 2020), whiteflies (Nombela, Williamson, & Muñiz, 2003) and planthoppers (Liu et al., 2015; Tamura et al., 2014; Zhao et al., 2016). Reports on *R* -genes mediating resistance to leaf-chewing insects are even more scarce, with few reports on PRRs associated with defence against caterpillars (Gilardoni et al., 2011; Hu et al., 2018; Steinbrenner et al., 2020). So far it has been shown that most insect–plant interactions are controlled by polygenic quantitative traits (Kliebenstein, 2017).

Given the lack of *R*-genes effective against leaf-chewing insects, resistance mechanisms targeting insect eggs have been proposed as a complementary defence strategy (Fatouros et al., 2016; Tamiru et al., 2015). Clearly, the recognition and killing of herbivorous 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), are pests of *Brassica* crops and a serious agricultural challenge (Kumar, 2017; Ryan et al., 2019). *Pieris* eggs induce a hypersensitive response (HR)-like cell death in various plants, mainly of the Brassicaceae family, resembling HR induced by pathogens (Caarls et al., 2023; Griese et al., 2021; Shapiro & De Vay, 1987). HR is a well-studied plant defence trait that is widespread in all higher plants, and activation occurs upon recognition of pathogen effectors by an *R* gene (Balint-Kurti, 2019; Dalio et al. 2020). However, the genes involved in detection and activation of insect egg-induced HR remain largely unknown. So far, two studies investigated the genetic basis of *P. brassicae* egg-induced HR-like cell death using the model species *A. thaliana* (Groux et al., 2021) and the crop *B. rapa* (Bassetti et al., 2022), both of which benefit from extensive resources for classical forward genetics. In *A. thaliana*, two genes associated with *P. brassicae* egg-induced HR-like cell death were identified, that is, *L-type Lectin Receptor Kinase-I.1* (*LecRK-I.1*) and *Glutamate receptor 2.7* (*GLR2.7*) (Groux, 2019; Groux et al., 2021). In *B. rapa*, three QTLs associated with cell death size induced by egg wash were identified (Bassetti et al., 2022). The QTLs found in *B. rapa* included many genes involved in plant immunity, but they underlined large genomic regions yet to be fine-mapped. A partial overlap among the loci identified in both plant species was suggested, given that *Bra*LecRK-I.1 is included within the *Pbc3* locus found in *B. rapa* (Bassetti et al., 2022). More research is clearly needed to understand the extent to which the genetic regulation of this plant-*Pieris* egg interaction is conserved between plant species.

Although these studies investigated the genetic basis of HR-like cell death, it is evident that the cell death observed in *A. thaliana* and *B. rapa* manifests as a light necrosis that has no or little effect on egg survival (Griese et al., 2021; Groux, 2019; Groux et al., 2021). In contrast, black mustard (*Brassica nigra* L.), a natural host of several *Pieris* spp., exhibits a severe HR-like cell death, spreading from the leaf abaxial side up to the adaxial side and correlating with a substantial mortality of different *Pieris* spp. eggs (Caarls et al., 2023; Griese et al., 2020, 2021). In natural populations of *B. nigra*, HR-like cell death reduces egg survival by more than 40% (Fatouros et al., 2014). Therefore, it represents a trait with the potential to enhance crop resistance against leaf-chewing insects for breeders, as it reduces the impact of subsequently feeding larvae hatching from eggs. Previously, we found that the severity and occurrence of egg-induced cell death in *B. nigra* differ between plants of the same accession and between accessions (Caarls et al., 2023; Griese et al., 2017; Pashalidou et al., 2015), suggesting natural variation therein. A solution of compounds derived from the eggs, an egg wash, was shown to mimic egg-induced responses in plants (Caarls et al., 2023) and it provides suitable treatment to screen large genetic populations.

In this study, we investigated the inheritance and genetic basis of *P. brassicae* egg-induced HR-like cell death in *B. nigra*. We found variation in the cell death between and within field-collected *B. nigra* accessions, and then used crosses between strong- and low-responding individual plants to study the inheritance of the trait. We found that *Pieris* butterfly egg-induced HR-like cell death in *B. nigra* segregates as a Mendelian trait, and then performed genetic mapping through bulk-segregant analysis paired with whole genome sequencing (BSA-seq). Recombinant analysis was used to further fine-map the genetic region and identify a single dominant locus of approximately 50 kb, which we named *Pieris* egg-killing (*PEK*). Within the *PEK* locus, a cluster of TIR-NBS-LRR (TNL) receptor genes showed ample polymorphisms between our parental lines and between different *B. nigra* genomes.

2 | MATERIALS AND METHODS

2.1 | Plant material

The inheritance of the HR-like cell death was studied using *Brassica nigra* L. accessions collected from a local population in the floodplain of the Rhine River near Wageningen, The Netherlands (N51.96, E05.68). The accessions SF3-O1, SF19-O1, SF25-O1, SF29-O1, SF47-O1 and SF48-O1 originated from one multiplication by open pollination ('O1') of accessions used in previous studies (Griese et al., 2017). The accessions DG1, DG12 and DG29 originated from open pollinated wild plants collected in 2018. A single DG1 plant showing no HR in response to eggs was selfed to obtain accession DG1-S1. A single DG1-S1 plant was then crossed with a single SF48-O1 plant to obtain an F1 population (Figure 2a). Single plants from the F_1 that showed HR-like cell death were backcrossed to other DG1-S1 plants to obtain segregating backcross families (BC_1). Selfing of individual plants was done to generate BC_1S_1 and BC_1S_2 populations.

Plants were grown in a greenhouse under standardized conditions (21°C day/18°C night, RH 50%–70%, LD 16:8 h). Seeds were vernalized at 4°C for 2 days to induce even germination and then

were sown in small trays with sowing soil (Lentse Potgrond). Seedlings were transplanted 1 week after germination into 17 cm diameter pots with potting soil (Lentse Potgrond). Plants were grown for 5 weeks before treatment with *P. brassicae* egg wash.

2.2 | Insect material

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%, LD 16:8 h). 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.

2.3 | Egg wash treatments

We used two different protocols for obtaining wash from *P. brassicae* eggs. For both types of egg washes, induction of HR-like cell death is comparable in severity as previously shown (Caarls et al., 2023). For the treatment of different *B. nigra* accessions (Figure 1), egg wash was prepared following a recently published protocol (Caarls et al., 2023). In short, *P. brassicae* eggs were collected on filter paper pinned underneath a *B. oleracea* leaf in a cage containing 20 mated females. Egg clutches laid on the paper were cut out and submersed in 1 mL 2-(N-morpholino) methanesulfonic acid (MES) buffer per 400 eggs, to be incubated overnight (16 h) without disturbance. The solution ('egg wash') was transferred into a new tube the next morning and stored at −20°C until use. For this experiment, two 10 µL drops of egg wash were applied to each of the two youngest fully developed leaves. Drops of an equivalent amount of MES buffer were applied as negative control.

For the treatment of genetic mapping populations and to compare egg-induced HR to pathogen-induced HR, a protocol resulting in a more concentrated egg wash was used (Bassetti et al., 2022). 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 demineralized water in a ratio of about 1000 eggs per 1 mL of water. After overnight incubation at room temperature, the liquid phase was transferred into a new tube and stored at −20°C until use. Then, two 5 µL drops of egg wash were applied to each of the two youngest fully developed leaves. Drops of an equivalent amount of demineralized water were applied as negative control.

2.4 | Pathogen extracts treatments

The fungal pathogens *Rhizoctonia solani* (Rs) and *Alternaria brassicicola* isolate MUCL2097 (Ab), were cultured on Potato Dextrose agar (PDA) plates at 25°C. To prepare fungal extracts, the mycelium was first grown until it covered a whole Petri dish (Ø 100 mm), then it was cut into mycelial plugs (1 cm^2), inoculated into 100 mL PDA liquid medium and incubated at 25°C, 200 rpm. Mycelium was harvested from PDA cultures after 1.5 weeks, dried from excess medium and placed into a 50 mL tube containing 5 mL demineralized water. All tubes were kept on ice and ultrasonicated six times for 1 min 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-calcium carbonate (YDC) agar plates at 28°C. To prepare bacterial extract, Xcc was grown overnight in 15 mL liquid LB medium at 28°C at 200 rpm. Cultures of Xcc with an OD600 of 0.6−0.7 were centrifuged for 10 min at 3000 rpm, resuspended in a minimal amount of demineralized 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. All pathogen extracts were infiltrated using a 1 mL syringe.

2.5 | Assessment of egg wash-induced cell death

Egg wash-induced HR-like cell death was scored for 'HR severity' on a scale from 0 to 4 as previously described (Caarls et al., 2023). The scale classes are: 0: no visible 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 beyond the treated spot.

2.6 | DNA extraction, pooling, sequencing

For each population of our crossing scheme, genomic DNA was extracted from previously sampled young leaves, which were snap frozen and stored at −80°C. DNA was extracted following a modified CTAB method from the Maloof lab (https://openwetware.org/wiki/Maloof_Lab:96well_CTAB). DNA concentration and purity were estimated with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). DNA integrity was assessed using a 1% agarose gel with ethidium bromide. Before sequencing, DNA concentration was measured with a Qubit Fluorometer (Invitrogen).

For the bulk segregant analysis paired with whole genome sequencing (BSA-seq), 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. Further, the three accessions that originated the initial crossing were also used for DNA isolation (the S parent DG1-S1, the R parent F1_#130 and the R 'donor' of HR SF48-O1) (Figure 2). For the whole genome sequencing experiment, 1 µg 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. Sequencing libraries were generated using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) following the manufacture's protocol. The genomic DNA was randomly fragmented to a size of 350 bp by Bioruptor, then DNA fragments were size-selected with sample purification beads. The selected fragments were then end-polished, A-tailed and ligated with the full-length adaptor. After these treatments, the fragments were filtered with beads. Finally, the library was analysed for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified using real-time PCR. Libraries were sequenced on an Illumina NovaSeq 6000 platform using 150 bp paired end reads.

2.7 | *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*-mers 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 such as unions, intersections or differences between *k*-mer tables of different samples. An additional filtering step was carried out to retain *R* haplotype-specific *k*-mers. The sequencing yielded 14.4 Gb raw data for the *R* bulk and an approximate 24x depth considering a haploid *B. nigra* genome of about 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 about 12x. Thus, we decided to retain *k*-mers with a depth between 10x and 20x which represented *k*-mers derived from R 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 three mismatches (Li & Durbin, 2009). The number of mapped *k*-mers per 1 Mb bins was counted using bedtools v2.25 (Quinlan & Hall, 2010).

2.8 | Kompetitive allele specific PCR (KASP) markers genotyping

KASP 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 single nucleotide polymorphisms (SNPs) identified by *k*-mers of the R haplotype. KASP genotyping assays were performed according to the manufacturer's instructions (LGC Genomics). 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 10 mM 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).

2.9 | Variant calling within *PEK* locus

SNPs and Insertion/Deletion (InDels) variants were called using a workflow based on GATK Best Practices (DePristo et al., 2011). Reads were aligned to the *B. nigra* reference genome C2 v1.0, to which the mitochondrial sequence (Genbank accession no. NC_029182) and chloroplast sequence (Genbank accession no. NC_030450) were added, using BWA mem v0.7.17 with default parameters. The resulting alignment files were sorted and indexed using SAMtools v.1.11 (Li & Durbin, 2009). Alignment files were filtered to restrict variant calling to the *PEK* locus. Duplicate read pairs were marked using the MarkDuplicates tool of the GATK suite v.4.1.9.0. Variants (SNPs and InDels) were detected using GATK HaplotypeCaller on each sample on a window of 1.5 Mb around the *PEK* locus (position 6–7.5 Mb). 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 in agreement with our genetic model were retained: (i) heterozygous in resistant (R) material; (ii) homozygous DG1-S1 allele in susceptible (S) material. The functional effect of the retained variants was predicted using SnpEff with default parameters (Cingolani et al., 2012).

Comparison of copy number variations (CNVs) at the *PEK* locus between three *B. nigra* reference genomes was performed with the tool GEvo on the CoGe web platform (Lyons et al., 2008). All genomes were accessed in September 2021 from the following databases: *B. nigra* genomes NI100 v2.0 and C2 v1.0 were downloaded from http://cruciferseq.ca/ (Perumal et al., 2020), genome Sangam v1.0 from http://brassicadb.cn/ (Paritosh et al., 2020). Pairwise alignment of genomes was performed with default settings: Alignment algorithm '(B)LastZ: Large regions'; Word size: 8; Gap start penalty: 400; Gap extend penalty: 30; Chaining: NO; Score threshold: 3000; Max threshold: 0; Minimum high-scoring segment pairs (HSP) length: 50. CNVs were identified by visualizing the region between markers flanking *PEK* locus (M27 and M28) and identifying conserved regions/genes that were highlighted as HSP.

2.10 | Comparative genomics of *PEK* locus between *B. nigra*, *A. thaliana* and *B. rapa*

Synteny analysis between the *B. nigra PEK* locus and *A. thaliana* and *B. rapa* genomes was performed using the tool SynMap on the CoGe web platform (Haug-Baltzell et al., 2017; Lyons et al., 2008). SynMap legacy version was used with similar settings as previously described (Bassetti, 2022). All genomes were accessed in September 2021 from the following databases: *B. nigra* genomes NI100 v2.0 and C2 v1.0 were downloaded from http://cruciferseq.ca/, genome Sangam v1.0 from http://brassicadb.cn/, *B. rapa* genome Chiifu v3 from http://brassicadb.cn/, *A. thaliana* Col-0 vTAIR10 from https://www.arabidopsis.org/.

2.11 | Statistical analysis of phenotypic data and marker-trait association

All statistical analyses were conducted on R v4.0.1 (R Core Team, 2021). Scoring of HR-like cell death in severity categories were analysed with a non-parametric method (Kruskal–Wallis test) on HR scores (0, 1, 2, 3 and 4) and different accessions were included as categorical fixed factors. Differences in mean HR severity were tested using Kruskal–Wallis test, followed by Wilcoxon rank sum test with Benjamini–Hochberg correction. χ^2 tests were used to test the goodness of fit of the segregation of phenotypic data and KASP markers data. Marker-trait association was analysed with the R/qtl package (Broman et al., 2003) using the *scanone* function for binary data (presence/absence of HR).

3 | RESULTS

3.1 | Natural variation in egg-induced HR between *B. nigra* accessions

To study natural variation in egg-induced HR-like cell death under controlled conditions, we collected seeds from a local *B. nigra* population that previously showed variation in HR-like cell death in field conditions (Griese et al., 2017). Induction of HR-like cell death was tested using treatment with an egg wash, which was previously shown to mimic *Pieris* eggs (Caarls et al., 2023). Overall, we observed variation between plants of each accession in the occurrence and severity of the HR-like cell death (Figure 1). In addition, we found significant differences between accessions in the HR-like cell death severity response (Kruskall–Wallis test, $p < 0.05$). In some accessions, for example SF48-O1, almost all plants showed an HR (score 2–4) and for some, for example, DG1, only one out of seven plants responded with an HR to the wash. The variation in egg-induced HR within and between accessions suggested the existence of genetic variation within the population that could be used for genetic mapping.

3.2 | Variation in egg-induced HR is independent of cell death induced by other biotic stresses

We hypothesized that a *B. nigra* plant not showing HR-like cell death in response to *Pieris* egg wash would still be able to develop cell death against other biotic stresses. In other words, the variation between *B. nigra* accessions in their response to egg wash treatment would be consequence of differential recognition of eggs compounds. We tested this hypothesis using two *B. nigra* accessions that either consistently develop a strong HR-like response (SF48-O1) or no cell death at all (DG1-S1). We treated them with egg wash and extracts of the Gram-negative hemibiotrophic bacterium *X. campestris* pv. *campestris* (Xcc) and the necrotrophic fungi *A. brassicicola* and *R. solani*. DG1-S1, the accession unable to develop cell death upon egg wash treatment, could still develop a cell death against pathogen extracts (Supporting Information S1: Figure 1). Thus, *Pieris* egg wash induced different HR-like severity between the two accessions unlike the three pathogen extracts (Kruskal–Wallis test, $p < 0.01$) (Figure 1 and Supporting Information S1: Figure 2).

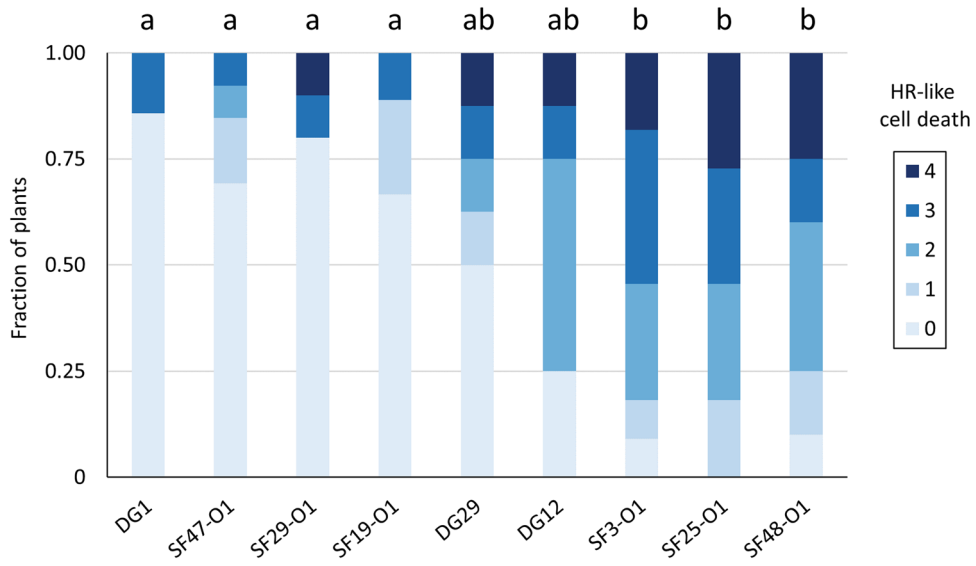

FIGURE 1 Intraspecific variation of HR-like cell death in *Brassica nigra* accessions. HR-like cell death induced by egg wash in nine accessions. Upon drop inoculation with egg wash on the abaxial side, symptoms were scored in classes: 0, no visible response; 1, brown spots underneath egg wash-treated 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. Controls with MES buffer did not induce visible symptoms as previously reported (Caarls et al., 2023). For each treatment/accession combination $n = 7–20$. Different letters indicate significant difference between accessions (Kruskall–Wallis test, p-value < 0.05). HR, hypersensitive response. [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Egg-killing HR is inherited as a single dominant Mendelian locus

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 that were selected from our germplasm screening (Figure 2a). The parental accessions consistently showed contrasting phenotypes, particularly with DG1-S1 showing no/weak HR (score 0–1) and SF48-O1 showing a strong HR (score 2–4) upon treatment with egg wash. A single plant with the HR phenotype of interest was chosen randomly from each accession before crossing. When egg-killing 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 (χ^2 test, $p > 0.05$) (Figure 2b and Table 1). Observing segregation in the F_1 was not surprising given that wild *B. nigra* are self-incompatible and thus highly heterozygous. Further, it suggested that HR was controlled by heterozygous loci at least in the R donor accession. 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 (χ^2 test, $p > 0.05$) (Figure 2b and Table 1). The reoccurrence of a 1:1 segregation pattern recalled the outcome of a test-cross for a single heterozygous locus. Indeed, selfing a resistant BC_1 plant with HR resulted in a BC_1S_1 population (BC1S1-1, $n = 695$) with a 3:1 ratio between R and S plants (χ^2 test, $p > 0.05$) (Figure 2b and Table 1). Overall, the segregation of *P. brassicae* egg-killing HR in our crossing scheme was consistent with a trait controlled by a single dominant Mendelian locus, which was expected to be heterozygous in the R donor SF48-O1 plant expressing HR.

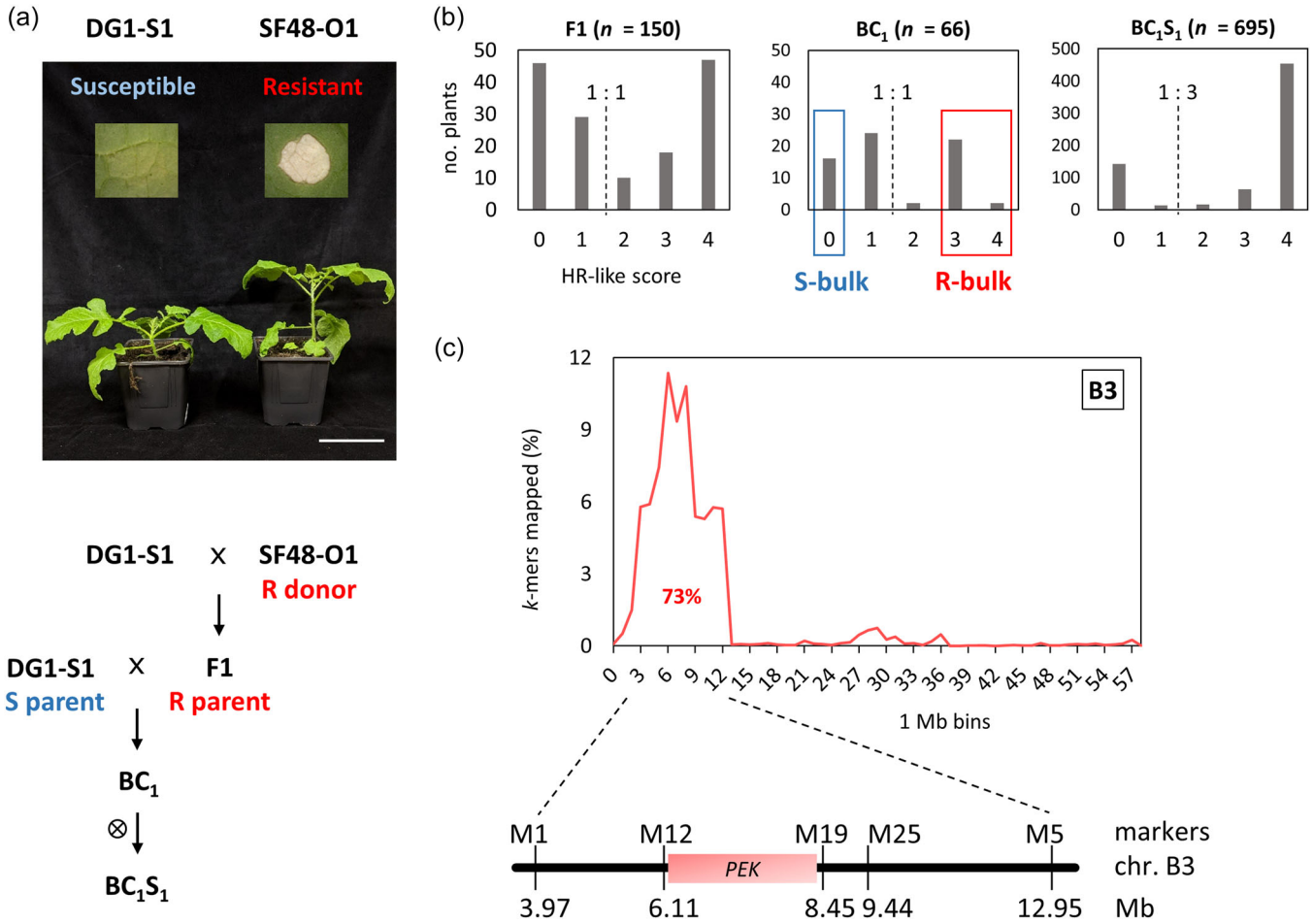

FIGURE 2 Butterfly egg-killing HR segregates as a Mendelian trait and it is mapped to a single locus on chromosome B3. (a) *Brassica nigra* parental accessions, 5-week old plants and relative HR phenotype (above). Crossing scheme used to study inheritance of HR-like induced by *Pieris brassicae* egg wash (below). A single plant with the HR phenotype of interest was chosen randomly from each accession before crossing. Scale bar = 10 cm. (b) HR phenotypic distribution within each population. (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 mapped R haplotype *k*-mer set. A single peak consisting of approximately 73% of the mapped *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). HR, hypersensitive response; KASP, kompetitive allele specific polymorphisms. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Segregation ratios of phenotypes in the populations used for genetic mapping of *PEK* locus.

		Observed		Expected			
Population	Generation	R	S	R	S	Ratio (R:S)	χ^2 test (*p*-value)
F1-1	F1	75	75	75	75	1: 1	1
BC1-3	BC_1	26	40	33	33	1: 1	0.085
BC1S1-1	BC_1S_1	533	155	516	172	3: 1	0.134

Note: For each population, plants were treated with egg wash and scored as resistant (R) with a HR scores 2–4 and susceptible (S) with a HR score 0–1. Phenotypic distribution was analysed with a χ^2 test.

Abbreviation: HR, hypersensitive response.

A single locus genetic model was further supported by the phenotypic segregation in other crosses involving selfings of parental plants, F_2 and BC_1 populations (Supporting Information S1: Table 1). Selfing of the susceptible parent DG1-S1 resulted in progeny with no HR (DG1-S2, $n = 15$). Similarly, a backcross between an individual F_1 plant with no HR and DG1-S1 (BC1−5, $n = 70$) resulted also in progenies with no HR. These results 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 in population BC1-3. Finally, F_2 populations derived from resistant F_1 plants showed a 3:1 segregation as expected (F2−2, $n = 8$; F2−3, $n = 12$).

3.4 | Genetic mapping of egg-killing HR to a single *PEK* locus through BSA-seq

We performed bulk segregant analysis coupled with whole genome sequencing (BSA-seq) on the backcross population BC1-3 (Figure 2b). We generated two bulks ($n = 10$) with either susceptible plants (S-Bulk) or 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') were sequenced using Illumina 150 bp paired-end reads yielding between 14 and 22 Gb data for each sample (Supporting Information S1: Table 2). As we estimated a haploid genome size of our *B. nigra* material of approximately 550 Mb, our sequencing resulted in a read depth between 26x and 35x for each diploid genome, thus a coverage ranging between 13x and 17.5x for 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 R parent F1_#130, homozygous in the S parent DG1-S1 and segregating 1:1 in the backcross population BC1-3. Thus, we expected a single resistant (R) allele in the R parent 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 (Supporting Information S1: Figure 3). The resulting R haplotype-specific *k*-mer set was filtered to retain unique *k*-mers with a frequency similar to half of the sequencing depth for a haploid genome (15x ± 5) to discard *k*-mers derived from repeated regions (Supporting Information S1: Table 3).

The unique R haplotype-specific *k*-mers were aligned to the *B. nigra* reference genome C2 (Supporting Information S1: Figure 4). Approximately 85% of the R-specific *k*-mers were successfully aligned, while the rest were likely sequences from our plant material that were too divergent or absent from the *B. nigra* reference genome. A unique single peak consisting of approximately 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 to 13 Mb (Figure 2c and Supporting Information S1: Figure 4). We named this locus *Pieris*-egg killing (*PEK*). All other *k*-mers (approximately 27%) mapped at a very low frequency, below 0.5% for each 1 Mb bin, throughout the rest of the genome (Supporting Information S1: Figure 4). Similarly, alignment of R haplotype-specific *k*-mers to other *B. nigra* genomes also resulted in a single peak of aligned R haplotype-specific *k*-mers, namely on chromosome B3 of accession NI100 (Supporting Information S1: Figure 5) and chromosome B7 of accession Sangam (Supporting Information S1: Figure 6). The location of *PEK* on a different chromosome in accession Sangam was likely due to a different chromosome naming by the authors of the genome, as we found that chromosome B7 of accession Sangam is perfectly syntenic to chromosome B3 of C2 and NI100 accessions (Supporting Information S1: Figure S7). In conclusion, a BSA-seq approach allowed us to map the HR cell death trait on a BC_1 population and identified a single genetic locus, *PEK*, on chromosome B3. This confirms the genetic model that we hypothesized based on the phenotypic segregation.

3.5 | *PEK* locus is associated with egg-killing HR

To validate the *PEK* locus identified by BSA-seq on chromosome B3, we designed KASP markers that targeted the whole 10 Mb region (Supporting Information S1: Table 4). We genotyped the entire BC_1 population (BC1−3, $n = 66$) with five KASP markers. Each KASP marker cosegregated with the HR cell death phenotype, without showing segregation distortion (χ^2 test, $p > 0.05$) (Supporting Information S1: Table 5). 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 (Figure 2c). Thus, validation with KASP markers confirmed that HR was associated with heterozygosity at the *PEK* locus on chromosome B3.

We then proceeded with fine-mapping *PEK* locus through recombinant analysis using a BC_1S_1 population (BC1S1-1, $n = 695$) that was generated by selfing a resistant BC1−3 plant with heterozygous genotype (*PEK*-SF48/*pek*-DG1), thus carrying both SF48-O1 and DG1-S1 alleles. The whole BC_1S_1 population was genotyped with five KASP markers (M1, M13, M19, M25, M5) spanning the 10 Mb 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 (Figure 3a). 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 (Figure 3b). 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 were homozygous for *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, on a about 50 kb interval on chromosome B3 (Figure 3b).

We performed an additional recombinant screening on four BC_1S_2 populations that were generated from selfing four R plants of BC_1S_1 population with heterozygous genotype (*PEK*-SF48/*pek*-DG1) at the locus (Supporting Information S1: Figure 8). All four BC_1S_2 populations showed the expected 3:1 phenotypic segregation ratio between R and S plants (Supporting Information S1: Table 1). Informative recombinants between M12 and M15 were identified in all populations (7 in BC1S2-2, 12 in BC1S2-3, 2 in BC1S2-4, 8 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 3' border of the locus.

3.6 | Main candidate genes within *PEK* are TNL receptors arranged in a cluster

To identify candidate genes for the HR phenotype, we checked the annotations within the *PEK* locus in the *B. nigra* reference genome C2. The region contains 11 genes (Figure 3c) 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-LRRs (TNLs) receptor proteins. A fourth 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 (Cui et al., 2015). Thus, they represent main candidate genes for the HR phenotype. Additionally, 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) across the coding regions of the eleven genes, but we could not pinpoint putative casual variants associated to the trait (Supporting Information S1: Table 6).

TNLs are often also characterized by CNVs and genomic rearrangements as the result of tandem duplications, unequal crossing over and gene conversion (Kuang et al., 2004). We therefore scanned the locus diversity between the three available *B. nigra* reference genomes. Indeed, we found extensive CNVs for some of the genes in the *PEK* locus when comparing the available *B. nigra* genomes NI100, C2 and Sangam (Supporting Information S1: Figure 9). Specifically, TNLs within the *PEK* locus were present in two copies in NI100, four copies in C2 and seven copies in Sangam. Similarly, we found CNVs also for *Bn* MAP1D which is present in two copies in NI100, three copies in C2 and four copies in Sangam. Collectively, our data showed that the *PEK* locus is highly polymorphic among available *B. nigra* genomes, suggesting that similar genetic variation may be present also in our *B. nigra* material.

Finally, we investigated whether the *PEK* locus overlaps with loci that were previously associated with *P. brassicae* egg-induced cell death in *A. thaliana* (Groux et al., 2021) and *B. rapa* (Bassetti et al., 2022). Overall, *PEK* appeared syntenic to the distal 5' end of *A.*

TABLE 2 Segregation ratios and association with HR phenotype of five markers used to genotype the whole BC_1S_1 population ($n = 695$).

KASP marker	Position on B3 (Mb)	Observed			Expected (1:2:1)			χ^2 test (p-value)	Association with HR (LOD score)[a]
		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	72.8
M13	6.53	173	341	159	168.25	336.5	168.25	0.702	105.3
M19	8.45	166	354	158	169.50	339	169.5	0.469	84
M25	9.43	161	362	156	169.75	339.5	169.75	0.217	66.1
M5	12.95	156	363	151	167.50	335	167.5	0.093	50.5

Note: Alleles at each KASP marker are given a symbol based on the phenotype of the accession from which they derived: 'R' is for the SF48-O1 allele, the resistant parent. 'S' is for the DG1-S1 allele, the susceptible parent.

Abbreviations: HR, hypersensitive response; KASP, kompetitive allele specific polymorphisms.

[a]LOD threshold of MQM for a significant marker-trait association was estimated 1.86 after 1000 permutations and 5% error rate.

TABLE 3 List of genes included within the *PEK* locus in the ***B**rassica nigra* genome C2.

				A. thaliana homolog		
***B. nigra* gene ID**	**Start CDS (bp)**	**End CDS (bp)**	**Strand**	**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[a]	-	Membrane protein
				AT4G37040	MAP1D	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[b]	-	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
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[b]	-	Multiple TIR-NBS-LRR
BniB03g015520.1C2	6 456 006	6 469 883	+	TIR-NBS-LRR[b]	-	Multiple TIR-NBS-LRR

Note: *B. nigra* gene IDs within PEK locus as annotated in genome C2. Start and end location of coding sequence (CDS) is given. *A. thaliana* homologs were assigned with BLASTp (*E*-value = 1e−5).

[a]This *B. nigra* gene appears to be a fusion between two *A. thaliana* genes.

[b]These *B. nigra* genes are canonical TIR-NBS-LRRs, hence it is difficult to assign an *A. thaliana* ortholog without in-depth phylogenetic analysis.

thaliana chromosome 4 (Supporting Information S1: Figure 10a) and to the centre of *B. rapa* chromosome A08 (Supporting Information S1: Figure 10b). None of these regions were previously associated with butterfly egg-induced responses, as genes *AtLecRK-I.1* (AT3G45330) and *AtGLR2.7* (AT2G29120) located on *A. thaliana* chromosomes 2 and 3 (Groux, 2019; Groux et al., 2021), while *B. rapa* three QTLs *Pbc1-3* were located on chromosomes A02, A03 and A06 (Bassetti et al., 2022). In conclusion, the *PEK* locus here identified represents a novel genomic region in *B. nigra* associated with a butterfly egg-induced HR which results in egg-killing.

4 | DISCUSSION

HR induced by *Pieris* spp. egg deposition is an appealing model system to study the interaction between plants and insect eggs, as well as an effective plant defence trait against chewing herbivores. Here, we report for the first time that *P. brassicae* egg-killing HR-like cell death in *B. nigra* segregates as a Mendelian dominant trait underlined by a single locus, which we named *PEK*. Through BSA-seq and fine-mapping, we located the *PEK* locus within an interval of approximately 50 kb on the proximal arm of *B. nigra* chromosome B3. This locus contains many duplicated genes, including a gene cluster encoding several intracellular TIR-NBS-LRR (TNL) receptors. These are the only genes in this locus associated with plant immunity according to the available literature. So far, no TNL receptors have been shown to be involved in immunity responses against insects. It is thus intriguing to further understand whether they are involved in direct recognition of an egg-derived component and/or in the signalling downstream of egg recognition.

Segregation of the HR phenotype throughout our crossing scheme supported the evidence for a Mendelian trait controlled by a single dominant locus. As we crossed two plants from heterogenous wild *B. nigra* accessions, we observed phenotypic segregation of different morphological traits in the F_1 population. Segregation of HR was consistent with a single dominant locus originating from a heterozygous donor resistant (R) plant. In fact, we found 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 (S plants) resulted in progenies that were also unable to develop HR. We successfully identified the *PEK* locus using a BSA-seq approach which was already proven to be advantageous for quickly identifying Mendelian loci in genetic populations with little recombination (Liu et al., 2012), as well as in highly heterozygous species (Dakouri et al., 2018; Prodhomme et al., 2019). 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 and mostly occurring against cell content feeders such as aphids, gall midges or planthoppers (Botha et al., 2005; Himabindu et al., 2010; Klingler et al., 2009; Stuart et al., 2012). It is thus remarkable that an HR cell death evolved to target butterfly eggs and, also, that it is underlined by a single major effect locus as previously shown for HR-based resistance traits against pathogens and sap-sucking insects.

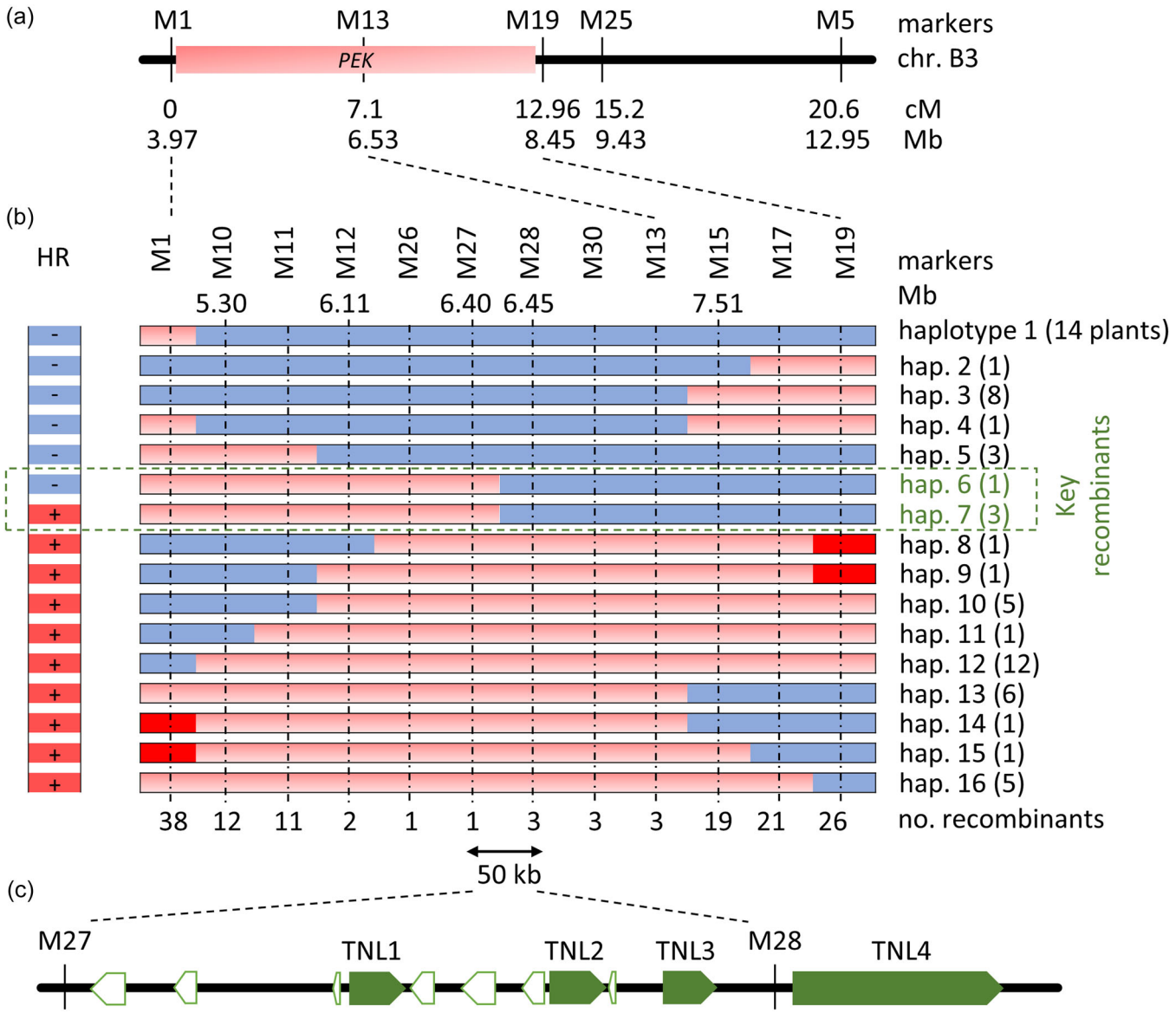

FIGURE 3 *PEK* locus is fine-mapped to a about 50 kb interval including a cluster of TIR-NBS-LRR. (a) Fine mapping with five makers on a BC_1S_1 population ($n = 695$) located the *PEK* locus between 3.97 and 8.45 Mb. Marker names, genetic distance (cM) and physical distance are given. (b) Genotype and phenotype of 64 recombinants. Recombinants with the same genotype are represented by a single haplotype ('hap.'). Key recombinants that located the *PEK* locus are indicated within a dashed green line. Blue bars represent homozygous DG-S1 allele (S/S), and light red bars represent heterozygous (R/S), dark red bars represent homozygous SF48-O1 genotype (R/R). The phenotype of each haplotype is shown on the left side: '-' means susceptible (S, no HR), '+' means resistant (R, strong HR). Numbers under each dashed line indicate the number of recombinants between each marker and the phenotype. (c) *PEK* locus is fine-mapped to a about 50 kb interval (genome C2 v1.0) containing 11 genes, among which four *TIR-NBS-LRRs* that are arranged in a cluster (green). A full list of genes within the *PEK* locus is reported in Table 3. HR, hypersensitive response. [Color figure can be viewed at wileyonlinelibrary.com]

Through recombinant analysis, we fine mapped the *PEK* locus to an about 50 kb region. Further fine-mapping in BC_1S_2 populations did not increase the resolution into the locus, likely because of small populations size. *PEK* contains 11 genes, among which a cluster of intracellular receptors of the TNL type. 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 defence is yet to be proven. On the other hand, given the involvement of TNLs in perception and signalling of plant immunity against pathogens (Cui et al., 2015), and that cloned *R* genes providing resistance based on HR are often NLRs (Kourelis & van der Hoorn, 2018), these TNLs could be considered as the main candidate genes for *Pieris* egg-induced HR-like cell death.

Nonetheless, it is still too premature to speculate on a potential perception of egg elicitors by a TNL, which is a type of receptor are so far only known to bind proteins (Monteiro & Nishimura, 2018), while ongoing efforts suggest that the egg-associated elicitor of HR is not a protein (Caarls et al., 2023). Alternatively, the TNLs within *PEK* may not be involved in direct egg recognition but rather in sensing perturbations of cellular homoeostasis (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 by a TNL (Berkey et al., 2012; Palma et al., 2010). Further fine-mapping and functional studies are necessary to elucidate whether the TNLs or other genes within *PEK* locus are responsible for the egg-induced HR phenotype.

PEK is a novel locus associated with *Pieris* spp. egg-induced HR-like cell death and it shows no synteny with any of the loci previously identified in *A. thaliana* (Groux et al., 2021) and *B. rapa* (Bassetti et al., 2022). This could be explained by the stronger HR cell death phenotype in *B. nigra* compared to other plant species. In fact, the severe egg-killing HR that we regularly observe in *B. nigra* so far was never foundneither in *B. rapa* nor in *A. thaliana* (Bassetti et al., 2022; Fatouros et al., 2014; Griese et al., 2021; Groux et al., 2021). Our results point to a different type of regulation of egg-induced HR in *B. nigra*. Nevertheless, a slight difference in the phenotyping method between the studies should also be noted. In this study, we mapped presence/absence of HR-like cell death, while previous studies focused on variation in lesion size treated as continuous or discrete trait. Additionally, the study in *A. thaliana* made use of an egg extract treatment containing compounds from the lipidic egg phase (Stahl et al., 2020), which we showed to not induce HR-like cell death in *B. nigra*, unlike the water-soluble phase contained in egg wash used in our studies (Caarls et al., 2023).

The strong HR developed by *B. nigra* in response to *Pieris* spp. eggs, which results in egg-killing, offers promising application in *Brassica* crops. From a plant breeding perspective, the molecular markers flanking the *PEK* locus may be sufficient to introgress egg-killing HR-like cell death into elite *Brassica* crops lines, as interspecific crosses between *Brassica* crop cultivars and crop wild relatives from secondary/tertiary gene pools have been successful for other resistance traits (Hu et al., 2021; Katche et al., 2019; Lv et al., 2020). From a scientific perspective, the identification of a locus associated with a plant trait with direct effect on herbivore survival will certainly help to explain its evolutionary basis and genetic architecture. Recent studies, in fact, showed that the characterization of genetic diversity of loci containing PRR or NLR receptors, such as *PEK*, across a broad phylogenetic context helped to resolve the macroevolutionary history of pathogen and insect resistance traits and to generate hypotheses on putative functional domains of candidate genes (Snoeck et al., 2022; Wang et al., 2021). Such studies may potentially reveal different types of polymorphisms at the *PEK* locus, as the interspecific variation in HR may have arisen from differences in life-history traits of plant species and/or exposure to selective pressure by insect eggs. For example, the weak type of HR developed by *A. thaliana* could be the result of lower selective pressure by the herbivore since as a short-day flowering species it is not a typical host of *Pieris* spp. (Harvey et al., 2007) and only occasionally of *Anthocharis cardamines* (Wiklund, 1984). Conversely, *B. rapa* crop morphotypes may show a weak HR as the result of a different mechanism (Bassetti, 2022). For example, crop domestication has often led to reduction in inducible defences (Chen et al., 2015; Turcotte et al., 2014; Whitehead et al., 2017).

In this study, many SNPs and InDels were found between our parental accessions. Additionally, we found CNVs at the locus between *B. nigra* reference genomes. The identification of abundant polymorphisms within *PEK* will likely require de novo assembly of the locus within our plant material. The high diversification in causal polymorphisms at NLR loci is well described (Dolatabadian & Fernando, 2022) and has evolved through a massive expansion and lineage diversification that allows adaptation to a multitude of biotic stresses (Shao et al., 2019). Consequently, NLR loci within a species are rarely well represented by a single plant genome and, further, are likely to be misassembled (Barragan & Weigel, 2021). It is thus fundamental to characterize the genomic context of the *PEK* locus in our plant material, and across the Brassicaceae, to study whether the locus' genetic diversity across the plant family correlates with interspecific variation in HR phenotype.

In conclusion, we report here that intraspecific variation for HR in *B. nigra* induced by *P. brassicae* eggs is associated with a single locus. Through classical forward genetics we identified the *PEK* locus, which contains a cluster of *TNL* receptor genes. The locus appears highly polymorphic between the known *B. nigra* genomes and we expect this to be the case also for our accessions. An improved genome assembly of the locus is thus a prerequisite to future fine-mapping endeavours. This future work will likely enable cloning and functional testing of the first *B. nigra* gene involved in defence against insect eggs.

ACKNOWLEDGEMENTS

We are grateful to Dr. Charlotte Prodhomme and Corentin Clot for insightful discussion on the CoSSA pipeline settings for *k*-mer based genetic mapping. Thanks to Dr. Chengcheng Cai and Dr. Robin van Velzen for discussing whole genome sequencing data analysis. Further, we are grateful to Dr. Isobel Parkin and Dr. Kumar Paritosh for access to early versions of *B. nigra* genomes. We are grateful to the employees of Unifarm (WUR) for rearing and caring of the plants used in the experiment. We thank Pieter Rouweler, André Gidding and the late Frans van Aggelen for rearing *Pieris brassicae*. This research was made possible by support of the Dutch Technology Foundation TTW, which is part of the Dutch Research Council (NWO) (NWO/TTW VIDI grant 14854 to N. E. F.).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sets supporting the conclusions of this article are available in a Zenodo repository (https://doi.org/10.5281/zenodo.8131352). All

raw sequencing data have been deposited in the European Nucleotide Achieve (ENA) under accession number PRJEB64240.

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How to cite this article: Bassetti, N., Caarls, L., Bouwmeester, K., Verbaarschot, P., Eijden, E., Zwaan, B. J. et al. (2023) A butterfly egg-killing hypersensitive response in *Brassica nigra* is controlled by a single locus, PEK, containing a cluster of TIR-NBS-LRR receptor genes. *Plant, Cell & Environment*, 1–14. https://doi.org/10.1111/pce.14765