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RESEARCH PAPER

Signalling of *Arabidopsis thaliana* response to *Pieris brassicae* eggs shares similarities with PAMP-triggered immunity

Caroline Gouhier-Darimont, André Schmiesing, Christelle Bonnet, Steve Lassueur and Philippe Reymond*

Department of Plant Molecular Biology, University of Lausanne, 1015 Lausanne, Switzerland

* To whom correspondence should be addressed. E-mail: Philippe.Reymond@unil.ch

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Abstract

Insect egg deposition activates plant defence, but very little is known about signalling events that control this response. In *Arabidopsis thaliana*, oviposition by *Pieris brassicae* triggers salicylic acid (SA) accumulation and induces the expression of defence genes. This is similar to the recognition of pathogen-associated molecular patterns (PAMPs), which are involved in PAMP-triggered immunity (PTI). Here, the involvement of known signalling components of PTI in response to oviposition was studied. Treatment with *P. brassicae* egg extract caused a rapid induction of early PAMP-responsive genes. In addition, expression of the defence gene *PR-1* required EDS1, SID2, and, partially, NPR1, thus implicating the SA pathway downstream of egg recognition. *PR-1* expression was triggered by a non-polar fraction of egg extract and by an oxidative burst modulated through the antagonistic action of EDS1 and NUDT7, but which did not depend on the NADPH oxidases RBOHD and RBOHF. Searching for receptors of egg-derived elicitors, a receptor-like kinase mutant, *lecRK-I.8*, was identified which shows a much reduced induction of *PR-1* in response to egg extract treatment. These results demonstrate the importance of the SA pathway in response to egg-derived elicitor(s) and unravel intriguing similarities between the detection of insect eggs and PTI in Arabidopsis.

Key words: *Arabidopsis thaliana*, oviposition, PAMP-triggered immunity, *Pieris brassicae*, *PR-1* expression, SA pathway.

Introduction

Eggs from herbivorous insects pose a serious threat for plants as they develop into feeding larvae. Consequently, plants have evolved exquisite strategies to respond to oviposition by producing direct and indirect defences (Hilker and Meiners, 2006). Several plant species develop a necrotic zone at the oviposition site, and this is often associated with increased egg mortality or a reduced larval survival rate (Shapiro and DeVay, 1987; Balbyshev and Lorenzen, 1997; Hilker and Meiners, 2006; Bruessow and Reymond, 2007). Indirect defences consist of the emission of volatiles in response to insect eggs, resulting in the attraction of egg parasitoids (Hilker *et al*., 2002). Modifications of plant surface chemistry by egg deposition can also be used by egg parasitoids as a cue to locate their host (Fatouros *et al*., 2008).

Plants are thus able to perceive egg deposition, but the chemical nature of egg-derived elicitors that trigger plant responses is still largely unknown (Hilker and Meiners, 2010). An elicitor responsible for the release of volatiles by the pine sawfly was isolated from oviduct secretions (Hilker *et al*., 2005). Formation of tumour-like structures on pea pods is caused by bruchins, long-chain fatty acid-derived molecules found in eggs of bruchid beetles (Doss *et al*., 2000). Anti-aphrodisiac male pheromones in the accessory gland secretions from mated female pierid butterflies cause leaf surface changes that attract egg parasitoids (Fatouros *et al*., 2008). In addition, very little is known about signalling pathways that control plant responses to oviposition. Eggs of the phytophagous mites *Tetranychus urticae* develop faster in the

tomato mutant *def-1*, which is deficient in jasmonic acid (JA) accumulation (Ament *et al*., 2004). JA treatment triggered the emission of volatiles that attract egg parasitoids, mimicking the response of plants to oviposition (Hilker *et al*., 2002). However, it is still unclear how other responses to oviposition, including necrosis and defence gene expression, are regulated.

Pieris brassicae, the Large White butterfly, is distributed worldwide, but is mainly found throughout Europe and Asia. It is a serious pest of cultivated *Brassica* vegetables and can cause substantial yield losses (Feltwell, 1978; Kular and Kumar, 2011). In recent years, *Arabidopsis thaliana* has become a useful model to gain molecular insights into the plant response to *Pieris* species (Reymond *et al*., 2004; de Vos *et al*., 2005). For the response to oviposition, it was shown that *P. brassicae* eggs trigger localized cell death, accumulation of callose, and production of reactive oxygen species (ROS) on leaves (Little *et al*., 2007). Importantly, the *Arabidopsis* transcriptome signature after oviposition was strikingly different from that observed after feeding by chewing larvae. *Pieris brassicae* eggs triggered expression changes similar to those observed during infection with biotroph pathogens, including the induction of defence genes (Little *et al*., 2007). Furthermore, it was found that eggs from distantly related insect species induce the expression of similar genes and that this activity is enriched in egg lipids (Little *et al*., 2007; Bruessow *et al*., 2010). This indicated that some generic egg-derived molecules are recognized by the plant and induce a conserved response, in analogy with the detection of pathogen-associated molecular patterns (PAMPs) by the plant innate immune system (Boller and Felix, 2009). The recognition of PAMPs by specific cell surface receptors initiates convergent signalling cascades that ultimately result in the expression of defence genes, a process called PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). Early PAMP-responsive genes are regulated by a combined activation of mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) (Boudsocq *et al*., 2010). In addition, PAMP recognition leads to a rapid oxidative burst and the production of ROS that are required for defence gene induction (Suzuki *et al*., 2011). In plants, ROS are often generated by the action of NADPH oxidases, also known as respiratory burst oxidase homologues (RBOHs), which produce O_2^- in the apoplast. Two *Arabidopsis* NADPH oxidases, RBOHD and RBOHF, play a key role in innate immunity and cell death regulation (Torres *et al*., 2002; Suzuki *et al*., 2011; Marino *et al*., 2012).

PAMP recognition is generally followed by the activation of the salicylic acid (SA) pathway (Vlot *et al*., 2009). Treatment with the bacterial PAMP flagellin induces SA accumulation, and the expression of a major proportion of PAMP-induced genes requires a functional SA pathway (Tsuda *et al*., 2008). One key upstream component of the SA pathway is Enhanced Disease Susceptibility1 (EDS1), which is an important regulator of PTI (Wiermer *et al*., 2005). EDS1 controls SA biosynthesis by isochorismate synthase SID2 (ICS1) (Wildermuth *et al*., 2001) and subsequent induction of defence genes against biotroph pathogens (Wiermer *et al*., 2005). In addition, EDS1 regulates the production of ROS that are often associated with biotic and abiotic stresses (Straus *et al*., 2010). Signalling downstream from SA is controlled by the key regulator Nonexpressor of PR genes1 (NPR1). Upon SA accumulation, a change in the cellular redox state triggers monomerization of NPR1 that is subsequently translocated to the nucleus where it activates transcription of defence genes (Vlot *et al*., 2009). NPR1 interacts with TGA bZIP transcription factors that bind to the *PR-1* promoter and have either positive or negative transcriptional activity (Kesarwani *et al*., 2007).

Having observed that oviposition in *Arabidopsis* is associated with the release of egg-derived elicitors, a strong SA accumulation, and the up-regulation of similar sets of genes to those up-regulated during infection by biotroph pathogens (Little *et al*., 2007; Bruessow *et al*. 2010), it was decided to investigate whether perception of insect eggs shares common signalling components with PTI. Here, it is revealed that plants use a similar, but not identical, signalling machinery to respond to pathogens and insect eggs.

Materials and methods

Plant and insect growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col-0) plants were grown in a growth chamber as described previously (Reymond *et al*., 2004). The *sid2-1* mutant was obtained from Christiane Nawrath (University of Lausanne), *rbohD/F* from Miguel Angel Torres (Polytechnic University of Madrid), *eds1-2* and *nudt7-1* from Jane Parker (MPI for Plant Breeding Research, Koln), *tga2356* from Corné Pieterse (Utrecht University), and *npr1-1*, *rbohD* (SALK_109396), *rbohF* (SALK_034674), and *lecrk-I.8* (SALK_0066416) from the Nottingham *Arabidopsis* Stock Centre, All mutants are in the Col-0 background.

Pieris brassicae was reared on *Brassica oleracea* in a greenhouse (Reymond *et al*., 2000).

Treatments with egg extracts

Pieris brassicae eggs were collected and crushed with a pestle in Eppendorf tubes. After centrifugation (15 000 *g*, 3 min), the supernatant ('egg extract') was stored at –20 °C. Plants were 4 weeks old at the time of treatment. For each plant, two leaves were treated with 2 µl of egg extract. A total of four plants were used for each experiment. After the appropriate time, egg extract was gently removed with a paintbrush and treated leaves were stored in liquid nitrogen. Untreated plants were used as controls.

For extraction of total lipids, 1 ml of *P. brassicae* egg extract was transferred into a 50 ml Falcon tube and was mixed dropwise with 6.25 ml of $CHCl_3$/EtOH (1:1, v/v). The solution was placed on a shaker for 30 min and mixed with an additional 15 ml of $CHCl_3$/EtOH. The supernatant was evaporated in a speedvac and the dried material was dissolved in 25 ml of $CHCl_3$ and filtered through a funnel packed with cotton. After evaporation, the dried lipid extract (~18 mg) was then resuspended in 100 µl of dimethylsulphoxide (DMSO) and diluted with water to a final volume of 1 ml.

Solid-phase extraction (SPE) fractionation of egg lipids was done on a Sep-Pak C18-reverse phase cartridge (Waters AG, Baden, Switzerland). A 4 mg aliquot of lipids dissolved in 10% DMSO was loaded on the cartridge and eluted with 2 ml of 50% MeOH, followed by 2 ml of 80% MeOH, 2 ml of 100% MeOH, and 2 ml of 100% tetrahydrofuran. Fractions of 2 ml were collected, dried under a nitrogen flux, and resuspended with DMSO to a final concentration of 100 mg ml^{-1}. Each fraction was diluted 10× with water before treatment.

For treatment with pyrrolidine dithiocarbamate (PDTC), leaves were dipped for 10 s in a 100 µM solution 1 h before treatment with egg extract. PDTC treatment was then repeated every 24 h for 72 h. Untreated plants and plants only treated with PDTC were used as controls.

Insect bioassays

For oviposition tests, eight 5-week-old *Arabidopsis* Col-0 plants, eight *eds1-2* plants, and eight *nudt7-1* plants were placed in one cage containing *P. brassicae* butterflies for 4 h in the greenhouse. Plants were then transferred to a growth chamber for 5 d (20 °C, 65% relative humidity, 100 µmol m^{-2} s^{-1}, 10/14 h photoperiod) and the number of hatched eggs was measured. For each experiment, three cages were used and each experiment was replicated three times independently.

To test for the effect of the oviposition host genotype on subsequent larval performance, 30 freshly hatched larvae from eggs oviposited on Col-0, *eds1-2*, and *nudt7-1* were placed on 22 Col-0 plants in transparent plastic boxes and were allowed to feed for 7 d in a growth chamber. Larvae were then collected and weighed with a precision balance. This experiment was repeated three times independently.

Quantitative real-time PCR analysis

RNA extraction and the quantitative PCR procedure were published previously (Bruessow *et al.*, 2010). The list of gene-specific primers can be found in Supplementary Table S1 available at *JXB* online.

Histochemical stainings

Superoxide radical (O_2^-) was visualized with the sensitive dye nitroblue tetrazolium (NBT; Sigma, http://www.sigmaaldrich.com). After removal of egg extract, leaves were submerged in a solution containing 0.02% NBT and 10 mM NaN_3 for 4 h at room temperature in the dark. Hydrogen peroxide (H_2O_2) accumulation was measured with 3,3-diaminobenzidine (DAB; Sigma). Leaves were submerged in a 1.0 mg ml^{-1} DAB solution and incubated in the dark at room temperature for 6–8 h. For visualization of cell death, leaves were submerged in lactophenol trypan blue solution [5 ml of lactic acid, 10 ml of 50% glycerol, 1 mg of trypan blue (Sigma), and 5 ml of phenol] at 30 °C for 2–3 h. After each staining, leaves were destained for 10 min in boiling 95% ethanol.

Microscope images were saved as TIFF files and processed for densitometric quantification with ImageJ version 1.64 (NIH).

Results

Eggs up-regulate early PAMP-responsive genes

Since recognition of insect eggs by *Arabidopsis* displayed some elements of a PAMP response (Little *et al.*, 2007), the expression of early responsive genes specific for either the MAPK or the CDPK branch of the PTI response was measured. It was shown previously that treatment with egg extract closely mimics the effect of oviposition by *P. brassicae* (Bruessow *et al.*, 2010). *Arabidopsis* plants were thus treated with *P. brassicae* egg extract and RNA was extracted a few hours later. Up-regulation of *FRK1*, a MAPK-specific gene, of *CYP81F2*, a MAPK-dominant gene, of *NHL10*, a MAPK- and CDPK-regulated gene, and of *PHI1*, a CDPK-specific gene (Boudsocq *et al.*, 2010) was observed already 3 h after egg extract application and was stronger after 9 h of treatment (Fig. 1). Thus, these data indicate that *P. brassicae* egg-derived elicitors activate early genes that are common to the PTI response.

EDS1 is crucial for egg-induced defence gene expression

EDS1 activity is crucial for PTI-related ROS production and SA-dependent defence gene activation. This activity is antagonized by Nudix hydrolase7 (NUDT7), a pyrophosphohydrolase that is important for limiting oxidative stress (Straus *et al.*, 2010). The expression of *PR-1*, a well known marker of the SA pathway that is strongly up-regulated by oviposition (Little *et al.*, 2007), was thus followed in wild-type and *eds1-2* plants after treatment with *P. brassicae* egg extract. In Col-0, *PR-1* expression increased gradually from 24 h to 72 h after egg extract treatment, whereas this induction was almost completely abolished in *eds1-2* (Fig. 2A). In contrast, *nudt7-1* exhibited a much greater up-regulation of *PR-1* than Col-0, in line with the known antagonistic effect of NUDT7 on EDS1 activity. Similarly, the expression of several oviposition-induced genes was strongly reduced in *eds1-2*, including ICS1, a gene that is required for SA synthesis, thioredoxin-H5 (TRX5), a gene that regulates an important step of the SA-mediated defence response, a trypsin

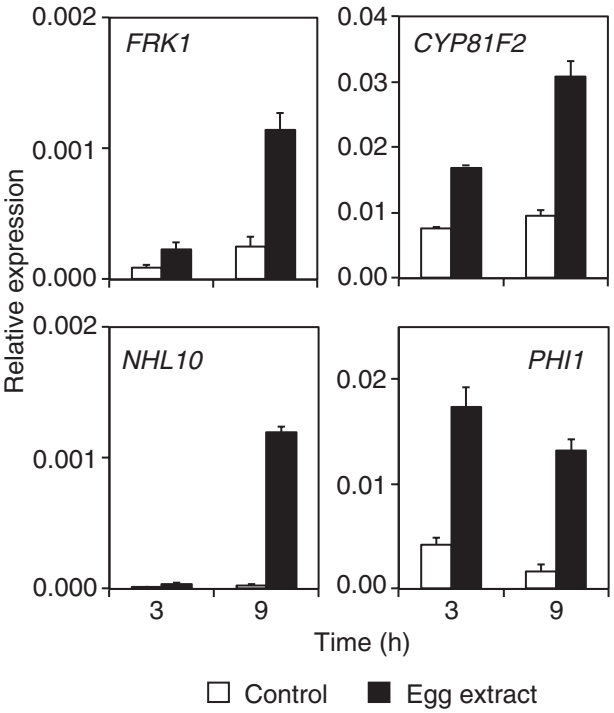

Fig. 1. Expression of early PAMP-responsive genes. Relative expression levels of *FRK1* (At2g19190), *CYP81F2* (At5g57220), *NHL10* (At2g35980), and *PHI1* (At1g35140) were analysed in *Arabidopsis* by quantitative PCR. Leaves were treated with 2 µl of *Pieris brassicae* egg extract for 3 h and 9 h before RNA extraction. Expression levels were normalized with respect to the housekeeping gene *EIF4A* (At3g13920). Data bars represent the mean (±SE) of three technical repeats. This experiment was repeated once with similar results.

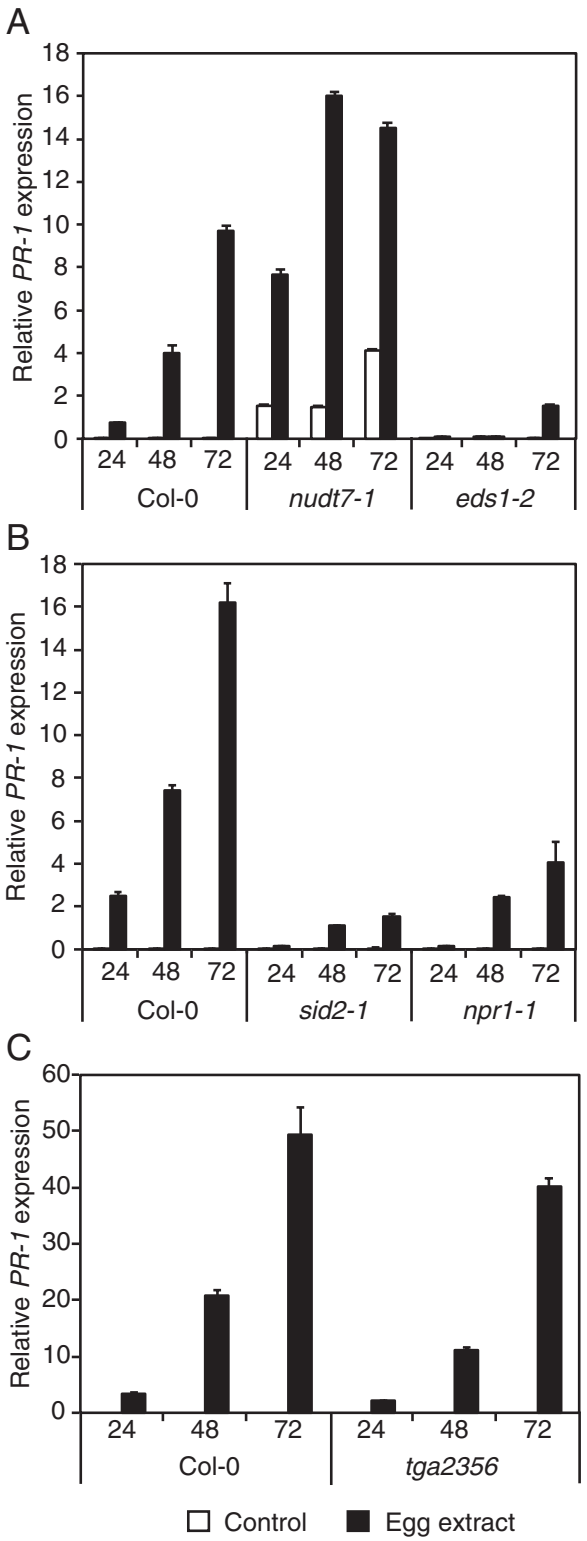

Fig. 2. *PR-1* expression in SA signalling mutants. The relative expression level of *PR-1* (At2g14610) was analysed by quantitative PCR. Leaves from Col-0, *nudt7-1*, and *eds1-2* (A), Col-0, *sid2-1*, and *npr1-1* (B), and Col-0 and the *tga2356* quadruple mutant (C) were treated with 2 µl of *P. brassicae* egg extract for 24, 48, and 72 h before RNA extraction. Expression levels were normalized with respect to the housekeeping gene *EIF4A*. Data bars represent the mean (±SE) of three technical repeats. Each experiment was repeated once with similar results.

inhibitor (TI), and a chitinase (CHIT) (Supplementary Fig. S1 at *JXB* online). These data indicate that EDS1 plays a key role in *Arabidopsis* response to oviposition and that EDS1 acts in concert with NUDT7 to control the expression of SA-dependent genes.

NPR1 controls egg-induced PR-1 expression independently of TGAs

NPR1 interacts with TGA transcription factors to control the expression of pathogenesis-related genes. Six members of this family have been shown to play a role in response to bacterial pathogens (Zhang *et al*., 2003; Kesarwani *et al*., 2007). Specifically, TGA2, TGA5, and TGA6 have a significant and redundant function in *PR-1* expression but require TGA3 for a more stringent activation (Zhang *et al*., 2003; Kesarwani *et al*., 2007; Blanco *et al*., 2009). *PR-1* expression was analysed in *npr1-1* and in the quadruple mutant *tga2356* in response to egg extract treatment. First, *npr1-1* showed a much reduced induction of *PR-1* compared with Col-0, although this reduction was not as pronounced as in *sid2-1* (Fig. 2B). Surprisingly, *PR-1* expression in *tga2356* followed wild-type accumulation from 1 d to 3 d after egg treatment (Fig. 2C). These results show that up-regulation of *PR-1* in response to egg treatment requires NPR1 but not TGA2, TGA3, TGA5, and TGA6.

EDS1 and SA modulate ROS accumulation

The production of two major ROS, O_2^- and H_2O_2, as well as cell death, in response to egg extract treatment was further explored. Leaves were stained with NBT, which preferentially detects O_2^-, with DAB, which reveals the presence of H_2O_2, or with trypan blue, which accumulates in dead cells. Treatment with egg extract triggered a strong accumulation of O_2^-, H_2O_2, and cell death in Col-0 (Fig. 3). Whereas O_2^- accumulation was similar to that in the wild type in *eds1-2*, *nudt7-1*, and in the SA-deficient mutant *sid2-1*, H_2O_2 and cell death were significantly diminished in *eds1-2* and *sid2-1* and, in contrast, significantly increased in *nudt7-1* (Fig. 3; Supplementary Fig. S3 at *JXB* online). These results indicate that both EDS1 and SA are required to generate H_2O_2 downstream of O_2^-, and that this process is under the negative regulation of NUDT7.

To investigate the relationship between ROS production and *PR-1* expression after egg treatment, the ROS scavenger PDTC was used. PDTC strongly diminished egg-induced up-regulation of *PR-1*, suggesting that ROS production is required for defence gene expression in response to oviposition (Fig. 4A). To confirm that *PR-1* induction was dependent on SA-derived ROS accumulation, *sid2-1* was treated with egg extract in the presence or absence of PDTC. There was only a weak induction of *PR-1* by egg extract treatment in *sid2-1* compared with Col-0, and this weak expression was further attenuated by PDTC treatment, indicating that this residual induction was also dependent on ROS accumulation (Fig. 4A). The implication of RBOHD and RBOHF was then tested. Single mutants *rbohD* and *rbohF*

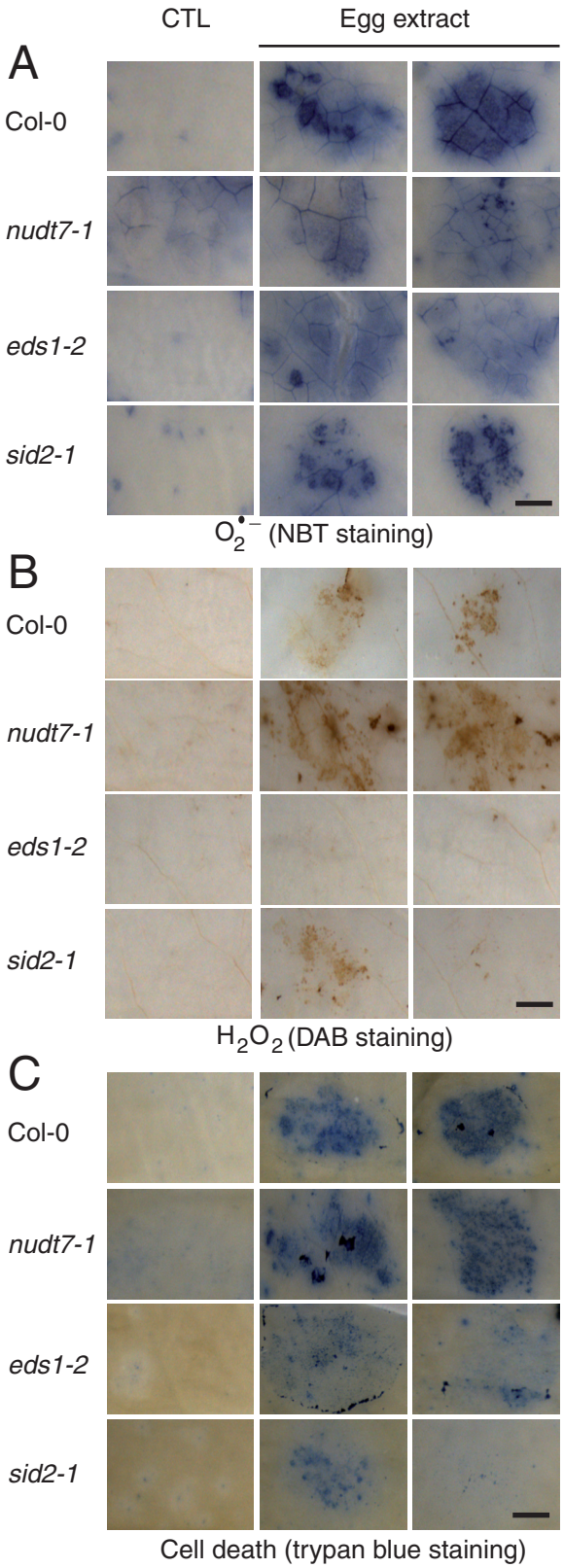

Fig. 3. Accumulation of reactive oxygen species (ROS) and cell death in SA signalling mutants. Leaves from Col-0, *nudt7-1*, *eds1-2*, and *sid2-1* plants were treated with 2 µl of *P. brassicae* egg extract for 72 h. Histochemical staining of leaves with nitroblue tetrazolium (NBT) to detect O_2^- (A), 3,3-diaminobenzidine (DAB) to detect H_2O_2 (B), and trypan blue to detect cell death (D) was performed. Control plants (CTL) were not treated and stained after 72 h. For all stainings, leaves from 3–5 different plants per genotype were used. Panels are close-up photographs of the spotted area. This experiment was repeated twice with similar results. All images were taken with the same magnification. Bar=1 mm.

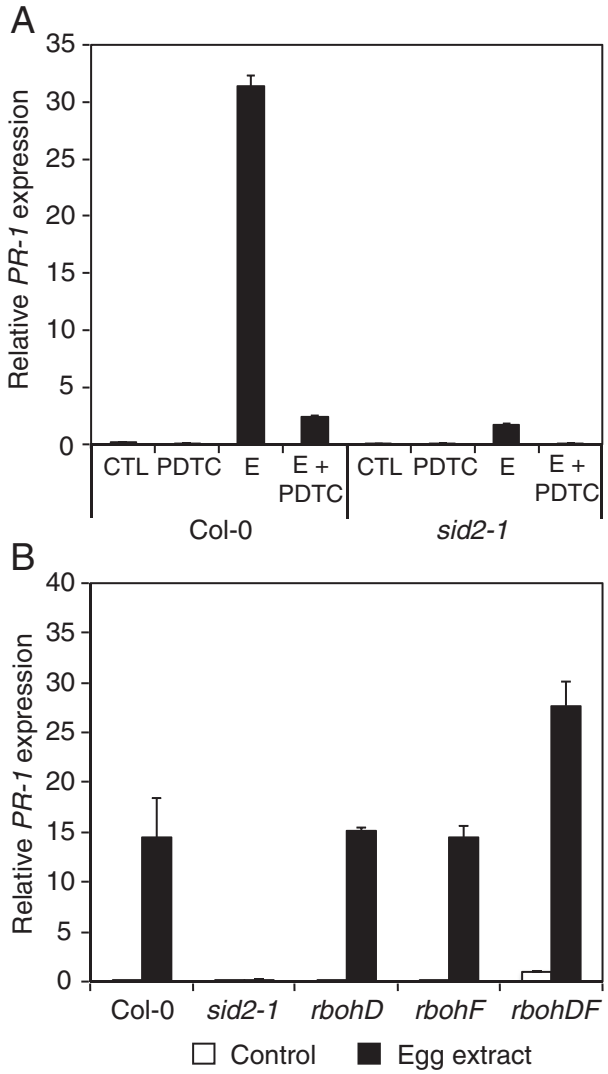

Fig. 4. Role of ROS in egg-induced *PR-1* expression. The relative expression level of *PR-1* was analysed by quantitative PCR. Expression levels were normalized with respect to the housekeeping gene *EIF4A*. Data bars represent the mean (±SE) of three technical repeats. Each experiment was repeated once with similar results. (A) Leaves from Col-0 and *sid2-1* plants were treated with 2 µl of *P. brassicae* egg extract for 72 h in the presence or absence of the ROS scavenger pyrrolidine dithiocarbamate (PDTC). CTL, untreated plants; PDTC, plants treated with PDTC; E, plants treated with egg extract; E + PDTC, plants treated with both egg extract and PDTC. (B) Leaves from Col-0, *rbohD*, *rbohF*, double mutant *rbohD/F*, and *sid2-1* plants were treated with 2 µl of *P. brassicae* egg extract for 72 h.

as well as the double mutant *rbohD/F* exhibited wild-type production of ROS and dead cells in response to egg extract (Supplementary Figs S2, S3 at *JXB* online). In addition, up-regulation of *PR-1* was not affected in these mutants, suggesting that RBOHD and RBOHF do not play a role in signalling events triggered by oviposition (Fig. 4B). Thus, the data show that defence gene expression in response to *P. brassicae* eggs requires an oxidative burst that depends on EDS1 and SA.

Partial purification of P. brassicae *egg extract*

In a preliminary attempt to characterize the chemical nature of egg-derived elicitors, lipids were extracted from *P. brassicae* eggs and fractionated by SPE. Similarly to crude egg extract, total lipids and a fraction eluted with 100% MeOH strongly activated *Arabidopsis* β-glucuronidase (GUS) reporter lines containing the promoter of *PR-1*, *TI*, and *SAG13*, which are genes induced by egg extract treatment (Bruessow *et al*., 2010). GUS staining was precisely confined to the site of application, suggesting that egg-derived elicitors are recognized at the site of oviposition (Supplementary Fig. S4 at *JXB* online). In addition, *PR-1* expression was quantitated and it was shown that *P. brassicae* egg extract, total egg lipids, and the active SPE fraction induced the expression of *PR-1* after 24 h, whereas a treatment with either water or 10% DMSO was inactive (Fig. 5). The active SPE fraction was also shown to induce early PAMP-responsive genes (Supplementary Fig. S5).

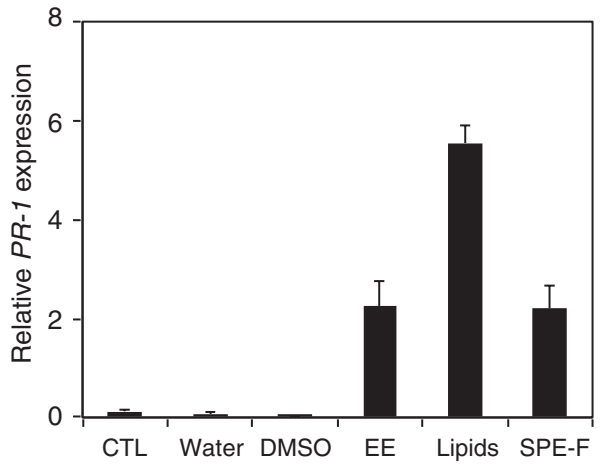

Fig. 5. Purified egg lipids activate *PR-1* gene expression. Plants were treated with 2 µl of *P. brassicae* egg extract, total egg lipids, and a fraction eluting at 100% MeOH from a solid-phase extraction column (SPE-F) for 24 h. Untreated plants (CTL) and plants treated with 2 µl of water or 10% DMSO were used as controls. The relative expression level of *PR-1* was analysed by quantitative PCR. Expression levels were normalized with respect to the housekeeping gene *EIF4A*. Data bars represent the mean (±SE) of three technical repeats. The experiment was repeated once with similar results.

Involvement of a L-type lectin receptor kinase in egg perception

PAMPs are generally perceived by receptor-like kinases (RLKs) at the plasma membrane. FLS2, the receptor for flagellin, was identified through a genetic screen (Gomez-Gomez and Boller, 2000), whereas CERK1, the receptor for chitin, was identified through a homology search with a rice oligosaccharide-binding protein (Miya *et al*., 2007). For the bacterial PAMP EF-Tu, its receptor EFR was discovered based on the observation that the *EFR* gene was induced after treatment with EF-Tu (Zipfel *et al*., 2006). Given that >600 RLKs are present in *Arabidopsis* (Shiu *et al*., 2004) and without having a chemically characterized egg elicitor, it was reasoned that one strategy to search for a potential receptor was to concentrate on RLKs that are induced by egg extract. It was previously reported that *P. brassicae* egg extract triggers a significant induction of 41 RLK genes (Little *et al*., 2007). T-DNA insertion lines were thus obtained from all candidate genes and *PR-1* expression was monitored in response to egg extract treatment (not shown). From all tested mutant lines, only one showed a clear and consistent reduced response to egg extract. The mutated gene encodes *LecRK-I.8*, which is an L-type lectin receptor kinase. The *lecrk-I.8* mutant has a T-DNA inserted in the middle of the *LecRK-I.8* coding sequence and has no detectable transcript (Fig. 6A, B). When treated with egg extract for 24, 48, and 72 h, lecrk-I.8 plants showed a strong, although not complete, reduction of *PR-1* expression compared with Col-0 (Fig. 6C), suggesting that this RLK plays a role in the perception of egg-derived elicitors.

Discussion

Pieris brassicae butterflies deposit eggs underneath *Arabidopsis* leaves by gently gluing them to the surface without apparent damage. Here it was shown that treatment with egg extract induces the expression of early PAMP-responsive genes within 3 h, indicating that there is a fast recognition of egg-derived elicitors by the plant. In addition, it was found that a fraction from purified egg lipids is able to induce *PR-1* and early PAMP-responsive genes, strongly suggesting that the activation of the SA pathway is triggered by egg-derived elicitor(s) of a non-polar nature. Since *P. brassicae* egg deposition does not cause wounding, these egg elicitors must freely cross the hydrophobic layer of plant cuticle, move through the cell wall, and reach the plasma membrane where potential receptors are located. Although previous observations that *P. brassicae* eggshells do not activate PR-1::GUS and that egg extracts from widely divergent insect species are similarly active (Bruessow *et al*., 2010) strongly suggest that egg-derived elicitors must be contained in the inner part of the egg and do not come from surface contaminants, it cannot be formally excluded that conserved endosymbionts or endogenous viruses are the source of elicitors. Further purification and identification of a pure elicitor will answer this question.

In an attempt to identify a receptor for egg-derived elicitors, it was found that the RLK LecRK-I.8 is required for

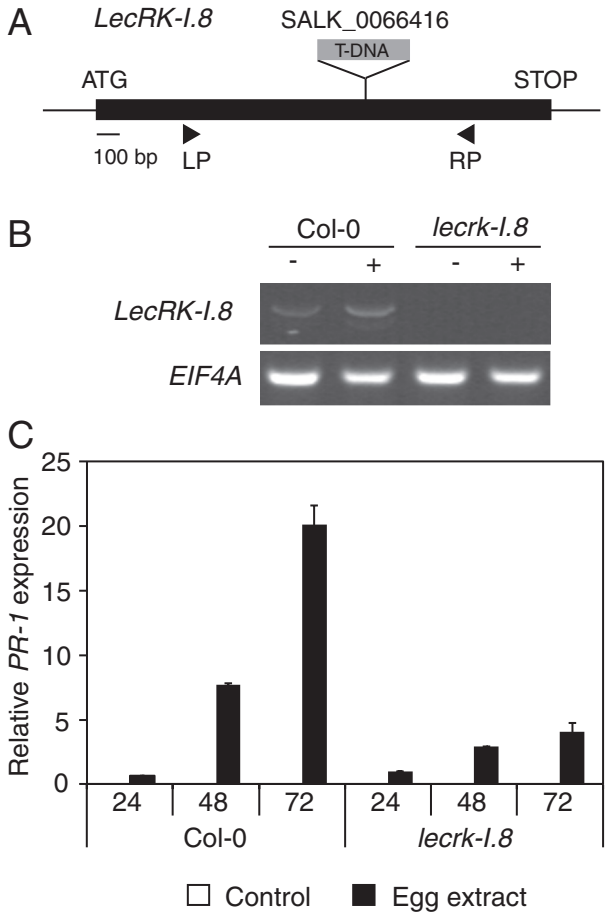

Fig. 6. Involvement of an L-type lectin receptor kinase in egg perception. (A) Gene structure of *LecRK-I.8* (At5g60280) showing the T-DNA insertion site of the mutant studied here. The positions of primers used for RT-PCR are shown. (B) Analysis of *LecRK-I.8* expression in Col-0 and in the T-DNA insertion line *lecrk-I.8* by RT-PCR. EIF4A was used as a control. Plants were treated with 2 µl of *P. brassicae* egg extract for 72 h (+). Untreated plants were used as controls (–). (C) The relative expression level of *PR-1* was analysed by quantitative PCR. Leaves from Col-0 and *lecrk-I.8* were treated with 2 µl of *P. brassicae* egg extract for 24, 48, and 72 h before RNA extraction. Expression levels were normalized with respect to the housekeeping gene *EIF4A*. Data bars represent the mean (±SE) of three technical repeats. This experiment was repeated once with similar results.

egg-induced *PR-1* induction. This receptor belongs to a class of L-type lectin receptor kinases consisting of 45 members. Several LecRKs are differentially expressed during growth and development and are induced upon treatment with elicitors and pathogens (Bouwmeester and Govers, 2009). Considering the lectin nature of the extracellular domain, LecRKs are postulated to interact with carbohydrate-containing ligands, but the presence of a conserved hydrophobic pocket does not exclude other ligands (Barre *et al.*, 2002). The observation that *lecrk-I.8* does not display a complete lack of *PR-1* induction suggests that other RLKs are required for egg perception. Genetic redundancy might explain this finding since *LecRK-I.8* is part of a cluster of five closely related LecRLK genes on chromosome 5. Alternatively, heterodimers between LecRK-I.8 and other co-receptors might form a ligand–receptor complex, and the disruption of one component might not totally abolish the response. Further characterization of egg-derived elicitors and the demonstration of their interaction with LecRK-I.8 or other RLKs will be crucial to understand early phases of egg detection by the plant.

Although the JA pathway is central for response to chewing larvae in *Arabidopsis* (Reymond *et al.*, 2004; Howe and Jander, 2008), SA accumulation under *P. brassicae* eggs (Bruessow *et al.*, 2010) suggested, however, that this pathway was involved in response to oviposition. Indeed, it is shown here that EDS1, SID2, and NPR1, essential components of the SA pathway, are necessary for the induction of defence genes in response to egg treatment. This is remarkable in the sense that two developmental stages of the same species, *P. brassicae* eggs and larvae, trigger two antagonistic signal transduction pathways. There are, however, examples where egg deposition is accompanied by wounding of the leaf. In pine trees, oviposition by the pine sawfly induces the release of plant volatiles that are attractive to egg parasitoids (Hilker *et al.*, 2002). In this case, needle slitting by gravid females occurs prior to egg deposition. The observation that JA treatment mimics the release of volatiles suggested that this hormone may be involved in indirect defence against eggs (Hilker *et al.*, 2002). However, SA accumulation was not measured in these plants and, since wounding activates the JA pathway, the specific contribution of JA to the signalling of oviposition remains to be determined.

In contrast to the well established involvement of ROS in plant–pathogen interactions, evidence for a role for ROS in plant–insect interactions is still preliminary (Giovanini *et al.*, 2006; Maffei *et al.*, 2006; Kerchev *et al.*, 2012). Although RBOHD and RBOHF have been implicated in PTI (Torres *et al.*, 2002; Marino *et al.*, 2012), it was found that treatment with egg extract induced wild-type ROS production and *PR-1* gene expression in a *rbohD*/*F* double mutant. There are 10 RBOHs in *Arabidopsis* but, unlike RBOHD and RBOHF, other RBOH homologues are mainly expressed in roots and rarely respond to stress in aerial parts (Suzuki *et al.*, 2011). Reduction of molecular oxygen to O_2^- can, however, occur through other mechanisms in plant cells. Photosystem I in the chloroplasts and Complex I and III of the electron transport chain in mitochondria generate O_2^- in response to excessive electron flow (Mittler *et al.*, 2004), whereas cell wall class III peroxidases can catalyse O_2^- production in the apoplast (Liszkay *et al.*, 2003). It was found that egg-induced accumulation of H_2O_2 and cell death, but not O_2^-, requires EDS1 and is under the negative control of NUDT7. This result is strikingly similar to the role of EDS1 and NUDT7 in photo-oxidative stress responses where they modulate the balance between chloroplast-derived O_2^- and H_2O_2 through SA (Straus *et al.*, 2010). Interestingly, insect egg deposition was shown to reduce photosynthetic activity (Schröder *et al.*, 2005) and the expression of photosynthesis-related genes (Little *et al.*, 2007). A decreased photosynthetic efficiency at the site of oviposition might lead to incomplete conversion of absorbed light energy by the photosystems, with the

consequence that excess excitation energy would be dissipated in the form of ROS, including O_2^-. Clearly, the data link ROS accumulation to egg-induced gene expression, but more work will be needed to identify the source and mechanisms of ROS production in response to oviposition.

It was found that NPR1 is a major regulator of egg-induced gene expression. Induction of *PR-1* by egg extract was severely compromised in *npr1-1* but, however, not to the extent observed in *sid2-1*. A residual *PR-1* expression in *npr1-1* could be due to partial genetic redundancy. There are five NPR1 paralogues in *Arabidopsis* and they were shown to contribute quantitatively to SA responses (Canet *et al*., 2010). For instance, NPR3 and NPR4 negatively regulate *PR* gene expression (Zhang *et al*., 2006), illustrating a complex interplay of positive and negative activities of NPR factors. Indeed, NPR3 and NPR4 were recently found to bind SA directly with different affinities, and NPR3 and NPR4 are postulated to mediate NPR1 stability differently depending on SA levels (Fu *et al*., 2012).

Although NPR1 was reported to interact with TGA factors to activate SA-dependent gene expression, egg extract treatment induced *PR-1* to wild-type levels in the *tga2356* quadruple mutant, suggesting that factors other than TGAs are required for this induction. WRKY proteins represent another class of transcription factors that are involved in defence responses, and some WRKYs are direct targets of NPR1 (Wang *et al*., 2006). It was previously found that several WRKYs, including some NPR1 targets, are induced by *P. brassicae* oviposition (Little *et al*., 2007). These factors might thus play a specific role in egg-induced defence gene expression.

It was recently reported that egg-induced SA accumulation leads to a suppression of defence against chewing larvae by negatively interfering with the JA pathway (Bruessow *et al*., 2010). This finding raised the intriguing hypothesis that during evolution eggs have hijacked a pre-existing SA pathway for the benefit of their progeny. However, this left open the question on the initial role of SA in response to oviposition. Egg viability was tested in SA signalling mutants but, surprisingly, it was observed that the egg hatching rate was not significantly different between Col-0, *eds1-2*, and *nudt7-1* plants (Supplementary Fig. S6A at *JXB* online). In addition, *P. brassicae* larvae that emerged from eggs oviposited on these plants did not show any significant difference in weight gain when transferred to Col-0 plants for 7 d (Supplementary Fig. S6B). Thus, it looks like the activation of SA-dependent defences in Col-0 is not crucial for embryo development and further performance of hatching larvae. Although *Arabidopsis* does not display a strong necrotic zone at the oviposition site, other plant species develop a more intense response that can lead to egg mortality, egg dropping from the leaf, or a reduced larval survival rate (Shapiro and DeVay, 1987; Balbyshev and Lorenzen, 1997; Hilker and Meiners, 2006). One important role for the SA pathway might be to control the development of a hypersensitive-like response under the eggs. Activation of the SA pathway might be attenuated in *Arabidopsis* and not efficient enough to have measurable effects on egg viability. Whether the egg-induced SA pathway is only beneficial for the attacker or makes a significant contribution to defence against eggs will need to be further addressed.

In conclusion, a model depicting the current understanding of oviposition signalling in *Arabidopsis* is proposed that is based on existing knowledge of PTI and the SA pathway (Fig. 7). Eggs deposited on leaves release as yet unknown egg-associated molecular patterns (EAMPs) that are recognized by cell surface receptor(s), including potentially LecRK-I.8. This triggers a PTI-like response that involves early MAPK- and CPK-dependent signalling and a burst of O_2^-. EDS1 stimulates the accumulation of SA, thus favouring the conversion of O_2^- to H_2O_2. This leads to a change in the cellular redox state that alters NPR1 conformation, which is then translocated to the nucleus where its association with transcription factors controls the expression of defence genes. Although this pathway shares several known components of PTI, there are some differences that distinguish egg perception from bacterial infection. Indeed, it is shown here that RBOHD and RBOHF are not responsible for O_2^- accumulation and that TGA factors are dispensable for *PR-1* induction. Future work will be necessary to identify the chemical nature of EAMPs and their plant receptors, how and where ROS are generated, and which downstream transcription factors regulate the expression of SA-dependent genes. It is however remarkable

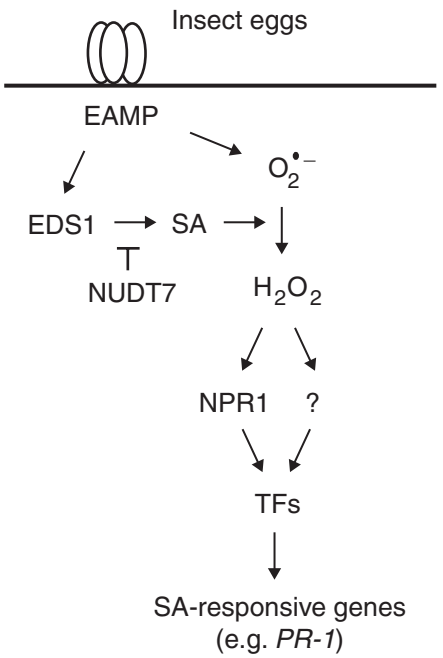

Fig. 7. Model for signalling of egg-induced gene expression in *Arabidopsis*. Upon egg oviposition, as yet unknown egg-associated molecular patterns (EAMP) are recognized by plant surface receptors and activate EDS1-dependent SA accumulation. SA promotes the conversion of EAMP-induced superoxide (O_2^-) to hydrogen peroxide (H_2O_2), which in turn leads to the translocation of NPR1 to the nucleus. Association of NPR1 with as yet unknown transcription factors (TFs) controls the up-regulation of *PR-1* and other SA-dependent genes. Eggs also activate an NPR1-independent induction of SA-responsive genes. In the model, NUDT7 acts as a negative regulator of EDS1 activity.

that plants have evolved a similar perception machinery to detect insect eggs, fungi, and bacteria.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. EDS1 is required for egg-induced expression of SA signalling genes.

Figure S2. Egg-induced ROS and cell death accumulation in *rbohD* and *rbohF* mutants.

Figure S3. Quantification of ROS and cell death accumulation.

Figure S4. Purified egg extracts activate the expression of reporter genes.

Figure S5. Purified egg extracts activate the expression of early PAMP-responsive genes.

Figure S6. Egg viability in SA signalling mutants.

Table S1. List of primers used in this study.

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References

Ament K, Kant MR, Sabelis MW, Haring MA, Schuurink RC. 2004. Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. *Plant Physiology* **135,** 2025–2037.

Balbyshev N, Lorenzen J. 1997. Hypersensitivity and egg drop: a novel mechanism of host plant resistance to Colorado potato beetle (Coleoptera: Chrysomelidae). *Journal of Economic Entomology* **90,** 652–657.

Barre A, Hervé C, Lescure B, Rougé P. 2002. Lectin receptor kinases in plants. *Critical Reviews in Plant Sciences* **21,** 379–399.

Blanco F, Salinas P, Cecchini NM, Jordana X, van Hummelen P, Alvarez ME, Holuigue L. 2009. Early genomic responses to salicylic acid in *Arabidopsis*. *Plant Molecular Biology* **70,** 79–102.

Boller T, Felix G. 2009. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology* **60,** 379–406.

Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, Bush J, Cheng S-H, Sheen J. 2010. Differential innate immune signalling via Ca(2$^+$) sensor protein kinases. *Nature* **464,** 418–422.

Bouwmeester K, Govers F. 2009. *Arabidopsis* L-type lectin receptor kinases: phylogeny, classification, and expression profiles. *Journal of Experimental Botany* **60,** 4383–4396.

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

Bruessow F, Reymond P. 2007. Oviposition-induced changes in *Arabidopsis* genome expression: anticipating your enemy? *Plant Signaling and Behavior* **2,** 165–167.

Canet JV, Dobón A, Roig A, Tornero P. 2010. Structure–function analysis of *npr1* alleles in *Arabidopsis* reveals a role for its paralogs in the perception of salicylic acid. *Plant, Cell and Environment* **33,** 1911–1922.

De Vos M, Van Oosten VR, Van Poecke RMP, *et al.* 2005. Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions* **18,** 923–937

Doss RP, Oliver JE, Proebsting WM, Potter SW, Kuy S, Clement SL, Williamson RT, Carney JR, DeVilbiss ED. 2000. Bruchins: insect-derived plant regulators that stimulate neoplasm formation. *Proceedings of the National Academy of Sciences, USA* **97,** 6218–6223.

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. *Proceedings of the National Academy of Sciences, USA* **105,** 10033–10038.

Feltwell J. 1978. The depredations of the Large White Butterfly (*Pieris brassicae*) (Pieridae). *Journal of Research on the Lepidoptera* **17,** 218–225.

Fu ZQ, Yan S, Saleh A, *et al.* 2012. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. *Nature* **486,** 228–232.

Giovanini MP, Puthoff DP, Nemacheck JA, Mittapalli O, Saltzmann KD, Ohm HW, Shukle RH, Williams CE. 2006. Gene-for-gene defense of wheat against the Hessian fly lacks a classical oxidative burst. *Molecular Plant-Microbe Interactions* **19,** 1023–1033.

Gómez-Gómez L, Boller T. 2000. FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Molecular Cell* **5,** 1003–1011.

Hilker M, Kobs C, Varama M, Schrank K. 2002. Insect egg deposition induces *Pinus sylvestris* to attract egg parasitoids. *Journal of Experimental Biology* **205,** 455–461.

Hilker M, Meiners T. 2006. Early herbivore alert: insect eggs induce plant defense. *Journal of Chemical Ecology* **32,** 1379–1397.

Hilker M, Meiners T. 2010. How do plants 'notice' attack by herbivorous arthropods? *Biological Reviews* **85,** 267–280.

Hilker M, Stein C, Schröder R, Varama M, Mumm R. 2005. Insect egg deposition induces defence responses in *Pinus sylvestris*: characterisation of the elicitor. *Journal of Experimental Biology* **208,** 1849–1854.

Howe GA, Jander G. 2008. Plant immunity to insect herbivores. *Annual Review of Plant Biology* **59,** 41–66.

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

Kerchev PI, Fenton B, Foyer CH, Hancock RD. 2012. Plant responses to insect herbivory: interactions between photosynthesis, reactive oxygen species and hormonal signalling pathways. *Plant, Cell and Environment* **35,** 441–453.

Kesarwani M, Yoo J, Dong X. 2007. Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in *Arabidopsis*. *Plant Physiology* **144,** 336–346.

Kular JS, Kumar S. 2011. Quantification of avoidable yield losses in oilseed *Brassica* caused by insect pests. *Journal of Plant Protection Research* **51,** 38–43.

Liszkay A, Kenk B, Schopfer P. 2003. Evidence for the involvement of cell wall peroxidase in the generation of hydroxyl radicals mediating extension growth. *Planta* **217,** 658–667.

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

Maffei ME, Mithoefer A, Arimura G-I, *et al.* 2006. Effects of feeding *Spodoptera littoralis* on lima bean leaves. III. Membrane depolarization and involvement of hydrogen peroxide. *Plant Physiology* **140,** 1022–1035.

Marino D, Dunand C, Puppo A, Pauly N. 2012. A burst of plant NADPH oxidases. *Trends in Plant Sciences* **17,** 9–15.

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. 2004. Reactive oxygen gene network of plants. *Trends in Plant Sciences* **9,** 490–498.

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **104,** 19613–19618.

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. *The Plant Cell* **16,** 3132–3147.

Reymond P, Weber H, Damond M, Farmer EE. 2000. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *The Plant Cell* **12,** 707–720.

Schröder R, Forstreuter M, Hilker M. 2005. A plant notices insect egg deposition and changes its rate of photosynthesis. *Plant Physiology* **138,** 470–477.

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

Shiu S-H, Karlowski WM, Pan R, Tzeng Y-H, Mayer KFX, Li W-H. 2004. Comparative analysis of the receptor-like kinase family in *Arabidopsis* and rice. *The Plant Cell* **16,** 1220–1234.

Straus MR, Rietz S, Ver Loren van Themaat E, Bartsch M, Parker JE. 2010. Salicylic acid antagonism of EDS1-driven cell death is important for immune and oxidative stress responses in *Arabidopsis*. *The Plant Journal* **62,** 628–640.

Suzuki N, Miller G, Morales J, Shulaev V, Torres MA, Mittler R. 2011. Respiratory burst oxidases: the engines of ROS signaling. *Current Opinion in Plant Biology* **14,** 691–699.

Torres MA, Dangl JL, Jones JDG. 2002. *Arabidopsis* gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. *Proceedings of the National Academy of Sciences, USA* **99,** 517–522.

Tsuda K, Sato M, Glazebrook J, Cohen JD, Katagiri F. 2008. Interplay between MAMP-triggered and SA-mediated defense responses. *The Plant Journal* **53,** 763–775.

Vlot AC, Dempsey DA, Klessig DF. 2009. Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology* **47,** 177–206.

Wang D, Amornsiripanitch N, Dong X. 2006. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathogens* **2,** e123.

Wiermer M, Feys BJ, Parker JE. 2005. Plant immunity: the EDS1 regulatory node. *Current Opinion in Plant Biology* **8,** 383–389.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414,** 562–565.

Zhang Y, Cheng YT, Qu N, Zhao Q, Bi D, Li X. 2006. Negative regulation of defense responses in *Arabidopsis* by two NPR1 paralogs. *The Plant Journal* **48,** 647–656.

Zhang Y, Tessaro MJ, Lassner M, Li X. 2003. Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *The Plant Cell* **15,** 2647–2653.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JDG, Boller T, Felix G. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* **125,** 749–760.