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Insect eggs suppress plant defence against chewing herbivores.

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SUMMARY

Plants activate direct and indirect defences in response to insect egg deposition. However, whether eggs can manipulate plant defence is unknown. In *Arabidopsis thaliana*, oviposition by the butterfly *Pieris brassicae* triggers cellular and molecular changes that are similar to the changes caused by biotrophic pathogens. In the present study, we found that the plant defence signal salicylic acid (SA) accumulates at the site of oviposition. This is unexpected since the SA pathway controls the defence against fungal and bacterial pathogens whereas it negatively interacts with the jasmonic acid (JA) pathway, which is crucial for the defence against herbivores. Application of *P. brassicae* or *Spodoptera littoralis* egg extract onto leaves reduced the induction of insect-responsive genes after challenge with caterpillars, suggesting that egg-derived elicitors suppress plant defence. Consequently, larval growth of the generalist herbivore *S. littoralis*, but not of the specialist *P. brassicae*, was significantly higher on plants treated with egg extract than on control plants. In contrast, suppression of gene induction and enhanced *S. littoralis* performance were not found in the SA-deficient mutant *sid2-1*, indicating that SA mediates this phenomenon. These data reveal an intriguing facet of the cross-talk between SA- and JA-signalling pathways and suggest that insects have evolved a way to suppress the induction of defence genes by laying eggs that release elicitors. We show here that egg-induced SA accumulation negatively interferes with the JA pathway and provides an advantage to generalist herbivores.

INTRODUCTION

Plants attacked by herbivorous insects develop various defence strategies (Howe and Jander 2008). In addition to the pre-existing physical and chemical barriers consisting of trichomes, thick secondary cell walls and toxic metabolites, plants deploy defences that are induced upon recognition of the enemy. As a sophisticated indirect mechanism, they emit volatiles to recruit predators of the attacker. This is superimposed on the production of antinutritive compounds, poisons, and defensive proteins that directly affect the performance of the herbivore (Howe and Jander 2008, Walling 2000). At the molecular level, herbivores trigger massive transcriptional changes that are mainly controlled by the JA pathway (De Vos *et al.* 2005, Kempema *et al.* 2007, Reymond *et al.* 2004). Accordingly, *Arabidopsis* mutants impaired in the JA response are more susceptible to insect feeding (Bodenhausen and Reymond 2007, McConn *et al.* 1997, Reymond *et al.* 2004, Dicke and Farmer 2004, Stotz *et al.* 2002).

Insect eggs are apparently a passive stage of herbivores but they nevertheless represent a real threat for the plant as they give rise to the feeding larvae. Direct and indirect responses to oviposition have been reported (Hilker and Meiners 2006). Direct defences include the growth of undifferentiated cells on pea pods, which elevate and thus increase the risk of desiccation and predation of weevil eggs (Doss *et al.* 2000), the production of the ovicidal substance benzyl benzoate in rice in response to eggs of white-backed plant hopper (Seino *et al.* 1996), or the development of a necrotic zone at the site of egg deposition in *Brassica nigra* and potato, which results in egg desiccation and mortality (Balbyshev and Lorenzen 1997, Shapiro and Devay 1987). Another direct defence is the production of oviposition deterrents that prevent female butterflies to lay more eggs on the

host (De Vos *et al.* 2008). Indirect defences comprise the emission of volatiles (mono- and sesquiterpenes) or the modification of the plant surface chemistry in response to oviposition, resulting in the attraction of egg parasitoids. These tri-trophic interactions have been observed in several species and with different predators (Fatouros *et al.* 2005, Hilker *et al.* 2002, Meiners and Hilker 2000).

There is thus evidence that plants can perceive egg deposition but the knowledge on the nature of the elicitors and on the cellular and molecular responses to oviposition is still limited. The only characterized elicitors of a direct defence are those responsible for the formation of tumour-like structures on pea pods. They are long-chain fatty-acid derived molecules called bruchins that are found in eggs and adults of bruchid beetles (Doss *et al.* 2000). Elicitors triggering the release of volatiles have not yet been identified but some are present in the oviduct secretions coating the egg (Hilker *et al.* 2005). For leaf surface changes that attract egg parasitoids, the elicitor is the anti-aphrodisiac male pheromone benzyl cyanide that is contained in the accessory gland secretion released by mated female butterflies (Fatouros *et al.* 2008). Some egg predators can even exploit this sexual signal to attach themselves to the mated female butterflies and locate the host plant (Huigens *et al.* 2009).

In response to oviposition, plants reduce their photosynthetic activity (Schroder *et al.* 2005) and the emission of ethylene (Schroder *et al.* 2007). Recently, we analysed the expression profile of *Arabidopsis* leaves after oviposition by two species of pierid butterflies. Eggs laid by the Large Cabbage White *Pieris brassicae* modified the expression of hundreds of genes, including defence and stress-related genes that are induced in plants undergoing programmed cell death. Furthermore, callose deposition, hydrogen peroxide

production and cell death occurred at the site of egg deposition and we showed that egg-derived elicitors triggered these events (Little *et al.* 2007). Moreover, necrotic zones were observed at the oviposition site in plants related to *Arabidopsis* (Bruessow and Reymond 2007).

The genes induced by oviposition include several known targets of SA. This signalling molecule is a potent inducer of pathogenesis-related genes and is involved in the resistance against biotrophic pathogens (Glazebrook 2005). Interestingly, it is known that SA- and JA-signalling pathways interact antagonistically (Beckers and Spoel 2006, Koornneef and Pieterse 2008). For example, there is evidence that SA can suppress JA-dependent defence responses (Cipollini *et al.* 2004, Doares *et al.* 1995, Gupta *et al.* 2000, Koornneef *et al.* 2008, Spoel *et al.* 2007, Spoel *et al.* 2003). The activation of the SA pathway weakens the plant response to attackers that are resisted through the JA-pathway, as nicely shown with a necrotrophic fungus (Spoel *et al.* 2007) or a chewing herbivore (Cipollini *et al.* 2004). This raised the question whether eggs trigger an accumulation of SA and whether this could in turn inhibit the defence responses against insect herbivores. We show here that SA levels increase at the site of oviposition and that the induction of herbivore-responsive genes is compromised after a further attack by chewing insects. Moreover, larvae of a generalist herbivore grow better on plants treated with egg extract, suggesting that eggs can manipulate plant defence signalling by interfering with the response to herbivores. These data unravel an intriguing phenomenon whereby insects may indirectly protect their offspring by releasing as yet unknown egg elicitors that target the defensive mechanism of the plant.

RESULTS

Elicitors are present in eggs of different insects

We recently reported that *P. brassicae* eggs activate the expression of hundreds of genes in *Arabidopsis* (Little *et al.* 2007). Using an *Arabidopsis* transgenic line containing the promoter of the defence gene *PR1* coupled to the β-glucuronidase (GUS) reporter gene, we showed that this activation was localized to the site of oviposition and could be mimicked by the application of soluble *P. brassicae* egg extracts (Little *et al.* 2007). To test whether this phenomenon also occurred with eggs of other insects we monitored the response of *Arabidopsis* GUS reporter lines to egg extract application. In addition to *PR1* we selected three other genes strongly induced by oviposition, a gene encoding a trypsin inhibitor (*TI*), a chitinase gene (*CHIT*), and a senescence-associated gene (*SAG13*). A strong and localized GUS staining was observed for all marker genes in response to egg extracts from the specialist *P. brassicae*, the generalist *Spodoptera littoralis*, and the non-herbivore *Drosophila melanogaster* (Figure 1a). In addition, we found that extracts of bacteria recovered from *P. brassicae* eggshells and extracts from the yeast *Saccharomyces cerevisiae* did not activate the marker genes (F. Bruessow, unpublished results). These results indicate that *Arabidopsis* recognizes one or several generic elicitors present in eggs of distantly related insects and that these molecules are probably insect-specific.

We then carried out an initial characterization of the egg-derived elicitor(s). Application of empty eggshells of freshly hatched *P. brassicae* larvae onto PR1::GUS plants for 72 hr did not cause a GUS staining, suggesting that the gene-induction activity resides within the embryo (Figure 1b). GUS staining was enhanced by a proteinase K

treatment of the egg extract and was conserved after filtration of the soluble fraction through a 3-kDa filter, indicating that the elicitor is a small molecule without enzymatic activity (Figure 1b). Finally, we found that the eliciting activity is enriched in the fraction containing total egg lipids (Figure 1b).

SA accumulates in the oviposited leaf

The SA pathway activates numerous genes that are induced in the oviposited leaf (Little *et al.* 2007). Furthermore *ICS1*, which encodes the key enzyme required for SA biosynthesis (Wildermuth *et al.* 2001), is strongly upregulated by oviposition (Little *et al.* 2007). We thus quantified SA in leaf discs underneath the eggs and in distal *Arabidopsis* leaves in response to oviposition by *P. brassicae* butterflies. The SA levels increased gradually in oviposited leaves during four days after oviposition, whereas these levels stayed almost constant in non-oviposited leaves. After four days, SA levels were more than 10-fold higher in oviposited leaves than in control leaves before oviposition, reaching ca. 21 μg/g FW (Figure 2a). Similarly, leaf discs from plants treated with *P. brassicae* egg extract accumulated high amounts of SA compared to untreated leaves, indicating that the treatment was mimicking the response to oviposition (Figure 2b). Given that SA-responsive genes are still induced in tissue adjacent to the oviposition sites (Little *et al.* 2007) and that considerable amounts of SA are found underneath the eggs, we can postulate that oviposited leaves contain significant levels of SA in the whole leaf.

SA has been detected in eggs of lepidopteran insects (Tooker and De Moraes 2007) and therefore this could be the source of the levels found in oviposited leaves. We

quantified SA in *P. brassicae* eggs laid on *Arabidopsis* leaves. SA levels did not change during 4 days after oviposition, averaging ca. 0.5 μg/g FW, which is about 25% of the basal levels of untreated *Arabidopsis* leaves (Figure S1). Since SA levels that accumulate in the leaves in response to oviposition are much higher than those founds in eggs, a simple transfer of SA from the egg to the plant cannot account for the observed accumulation. An alternative explanation is that a small amount of SA from the egg can induce the synthesis of SA in the plant. This is unlikely since SA does not regulate the expression of *ICS1* (Wildermuth *et al.* 2001). Thus, SA accumulation in response to oviposition likely results from *de novo* synthesis in the leaf.

Egg extract suppresses herbivore-induced gene expression

Since SA accumulated in the oviposited leaf and since the SA pathway is known to interfere with JA-dependent defence against insects, we tested the effect of a treatment with egg extract on the expression of insect-responsive genes. For that purpose, we selected 10 genes (Table S1) that are strongly induced in response to chewing herbivores and controlled by the JA pathway (Little *et al.* 2007, Reymond *et al.* 2004), and measured their expression by quantitative real-time PCR. Plants were treated for 5 days with *P. brassicae* egg extract and were then infested for 2 days with newly hatched larvae of the specialist *P. brassicae* or the generalist *S. littoralis*. As expected, all the selected genes were strongly induced after challenge with either *P. brassicae* or *S. littoralis* larvae in control plants that were not treated with egg extract. However, this induction was markedly reduced in leaves that had been pre-treated with egg-extract (Figure 3a,b). For example, the known anti-

herbivore gene *VSP2* showed a ca. 60-fold higher induction in untreated plants than in plants treated with egg extract. Overall, a similar suppression of gene expression was observed with either *P. brassicae* or *S. littoralis* larvae. This suppression was only observed in treated leaves. In distal leaves the induction of insect-responsive genes was similar, whether or not the plants were treated with egg extract (Figure S2). Furthermore, treatment with *S. littoralis* egg extract also suppressed defence gene expression in response to subsequent challenge with *S. littoralis* larvae, indicating that this phenomenon was conserved between two distantly related insects (Figure S3).

Interestingly, the suppression of insect-responsive genes was almost completely abolished in the SA-deficient mutant *sid2-1*. The large majority of the genes were equally induced by infestation with *S. littoralis* larvae in treated and untreated plants (Figure 3c). Thus, our data indicate that the suppression of defence gene expression in response to egg elicitors occurs locally and is largely controlled by SA.

To assess whether the SA-mediated suppression of insect-induced genes was only targeting the JA pathway, we monitored the expression of 5 JA-independent genes (Reymond *et al.* 2004) in plants challenged with *S. littoralis*. These genes were induced both by a treatment with egg extract or by *S. littoralis* feeding. However, a treatment with egg extract followed by a subsequent attack by insect larvae did not suppress their induction (Figure S4). These data suggest that egg-induced SA accumulation leads to a preferential inhibition of JA-dependent defence gene expression.

Plants treated with egg extract are more susceptible to *S. littoralis* larvae

To test whether the suppression of insect-responsive genes had a consequence on insect performance, we measured the weight of larvae feeding on plants treated with *P. brassicae* egg extract. Plants were treated for 5 days with *P. brassicae* egg extract and were subsequently challenged for 8 days with newly hatched *P. brassicae* larvae. Surprisingly, the weight of larvae fed on treated or untreated plants did not differ significantly (Figure 4a). In contrast, larvae of the generalist herbivore *S. littoralis* gained significantly more weight on egg extract-treated plants than on untreated plants (Figure 4b). The enhanced susceptibility of egg extract-treated Col-0 plants was abolished in the *sid2-1* mutant. Indeed, *S. littoralis* larvae reached the same weight after 8 days of feeding on treated or untreated *sid2-1* plants (Figure 4c). We thus show here that treatment with egg extract triggers a potent, SA-mediated, suppression of defence against a generalist herbivore.

Suppression of plant defences by egg extract does not depend on NPR1

Studies with pathogens have shown that the regulatory protein NPR1 plays a crucial role in the inhibitory action of SA on the JA-dependent defences (Leon-Reyes *et al.* 2009, Spoel *et al.* 2007, Spoel *et al.* 2003). Therefore, we analysed whether NPR1 is also involved in the inhibition of insect-induced responses by egg-derived elicitors. When *npr1-1* mutants were challenged with *S. littoralis* larvae, insect-responsive genes were strongly upregulated. However, a pre-treatment with egg extract inhibited this induction significantly, similarly to Col-0 plants (Figure 5a). Accordingly, *npr1-1* plants pre-treated with egg extract were more susceptible to feeding by *S. littoralis* than untreated plants (Figure 5b). Collectively, our data thus indicate that the suppression of insect defences by egg extract requires the accumulation of SA but is independent of NPR1.

DISCUSSION

Our data provide strong evidence that egg-derived elicitors trigger the suppression of defences against chewing herbivores in *Arabidopsis*. This process is mediated by SA, as evidenced by the lack of gene suppression and the absence of enhanced insect susceptibility in *sid2-1* mutants. Several observations indicate that application of egg extract mimics the actual oviposition by *P. brassicae*. 1) PR1::GUS is activated similarly by oviposition and by application of egg extract (Little et al. 2007 and Figure 1, this work); 2) oviposition and egg extract treatment lead to a local accumulation of SA, with similar kinetics (Figure 2); and 3) whole-genome analysis of gene expression in response to oviposition and egg extract treatment yields overlapping transcript profiles (data not shown).

The observation that egg extracts from distantly related insect species activate the same marker genes, along with the finding that egg extracts from both a specialist and a generalist insect are effective in suppressing defence gene expression, is an indication that some generic molecules are recognized by the plant and induce a conserved response. This is analogous to the detection of microbe-associated molecular patterns (MAMPs) by the innate immune system of the plant (Boller and Felix 2009) and potentially broadens the list of exogenous cues that are recognized by this surveillance mechanism. Further purification and characterization of the *P. brassicae* elicitor(s) should shed light on the identity of these molecules.

It is remarkable that this egg elicitor-recognition system is used by the herbivore for its own benefit. Generally, plants have evolved mechanisms to detect MAMPs as a defense strategy and there is yet no example where the attacker obtains a benefit to be recognized. If egg detection is similar to MAMP-triggered immunity, we have to postulate that the egg

recognition by a MAMP/receptor pair evolved first as a defense mechanism for the plant and was subsequently hijacked by the herbivore for its own advantage.

We found that plants treated with egg extract were more susceptible to chewing larvae of the generalist herbivore *S. littoralis* but not to larvae of the specialist *P. brassicae*. The most straightforward explanation is that *P. brassicae,* a specialist of Brassicaceae species, is adapted to the defences of these plants. For example, larvae of several lepidopteran species including *P. rapae* and *P. brassicae* contain a nitrile-specifier gut protein that detoxifies the breakdown products of the glucosinolates, which are the major insect deterrents in Arabidopsis (Wittstock et al. 2004, Wheat et al. 2007). Moreover, we recently showed that larvae of the specialist *P. brassicae* performed equally well on wild-type *Arabidopsis* plants than on mutants that either lacked glucosinolates or that were impaired in the JA-pathway, whereas larvae of the generalist *S. littoralis* were much larger when feeding on the mutant plants (Schlaeppi *et al.* 2008). Alternatively, *P. brassicae* regurgitant might contain factors that suppress plant defenses. Hence, the oviposition-induced inhibition of JA-dependent defenses would be redundant with this activity and may explain why *P. brassicae* larvae perform equally well on treated and untreated plants.

It is intriguing that *P. brassicae* eggs have kept the ability to suppress herbivore defences without a significant increased performance of their own progeny. One reason could be that this trait preceded the acquisition of tolerance to induced defences, and therefore would have provided an advantage to the larvae early in evolution, before specialization and adaptation to its current host plants. Alternatively, the suppression of defences could still be beneficial for the larvae in less favourable conditions, for instance when the detoxification of defence compounds is less efficient due to high yields of toxins

produced in some Brassicaceae species. Finally, in addition to its role in the inhibition of the JA pathway, the egg-triggered accumulation of SA might have another, non-exclusive, role in facilitating the development of the larvae. Since the SA pathway is involved in the defence against microbial and fungal pathogens, it could be advantageous for the hatching larvae to stimulate SA production and therefore generate an uninfected leaf area that retains its full nutritive value. Interestingly, the SA levels measured in the oviposited leaves are in the same range as those that are found in leaves infected with bacterial, fungal, and oomycete biotrophs (Roetschi *et al.* 2001, Summermatter *et al.* 1995, Wildermuth *et al.* 2001). The combined accumulation of SA (this work) and the up-regulation of many pathogenesis-related genes at the site of oviposition (Little *et al.* 2007) support this hypothesis.

Our data show that eggs hijack the SA pathway for the benefit of the hatching larvae, but raise the question if SA accumulation is of direct benefit for the plant? Several SA-responsive genes might be involved in the control of the programmed cell death that is observed at the site of oviposition (Little *et al.* 2007). In some species, this response is exacerbated and results in death or falling of the eggs from the leaf surface (Balbyshev and Lorenzen 1997, Shapiro and Devay 1987). Other SA-induced genes might play a direct as yet unknown defensive role against the eggs. In addition, SA might be converted to the volatile methyl salicylate (MeSA), which has been shown to inhibit oviposition of the cabbage moth *Mamestra brassicae* (Ulland *et al.* 2008). It would be interesting to test whether oviposited *Arabidopsis* plants emit MeSA and whether this subsequently reduces further oviposition by *P. brassicae* butterflies.

By using *sid2-1* plants we found that SA accumulation is required for the suppression of insect-induced defences but we have previously shown that the majority of egg-induced genes are still induced in *sid2-1* three days after oviposition (Little *et al.* 2007). This apparent discrepancy is nevertheless explained by the fact that the target genes are not the same. On one hand, oviposition directly controls the expression of hundreds of genes that include SA- and programmed cell death-related genes. On the other hand, oviposition is followed by an accumulation of SA that indirectly interferes with the induction of genes that are not induced by eggs but by chewing larvae.

We provide here strong evidence that egg-induced accumulation of endogenous SA suppresses JA-mediated defences against a generalist herbivore. Cross-talk between JA and SA signalling has been described in response to different attackers (Beckers and Spoel 2006, Koornneef and Pieterse 2008, Stout *et al.* 2006). Plants challenged with microbial pathogens, treated with SA, or treated with inducers of the SA pathway were more susceptible to herbivorous insects or to necrotrophic pathogens (Cipollini *et al.* 2004, Cui *et al.* 2005, Leon-Reyes *et al.* 2009, Spoel *et al.* 2007, Thaler *et al.* 1999). Wild tobacco and *Arabidopsis* mutants with elevated SA levels were more susceptible to feeding by *S. exigua* and *Trichoplusia ni* larvae, respectively (Cui *et al.* 2002, Rayapuram and Baldwin 2007). Infection with tobacco mosaic virus stimulated SA signalling in tobacco and consequently increased feeding by the hornworm *Manduca sexta* (Preston *et al.* 1999). At the molecular level, exogenous treatments with SA and JA have shown that SA inhibits the JA-induced expression of several defence genes (Doares *et al.* 1995, Niki *et al.* 1998) including the well-known JA markers *VSP2*, *PDF1.2*, and *LOX2* in *Arabidopsis* (Koornneef *et al.* 2008, Spoel *et al.* 2003).

The role of the antagonistic effect of SA on the JA-pathway is not fully understood but is thought to help the plant to optimize its response to aggressors of different lifestyles (Spoel and Dong 2008). Indeed, by inhibiting an inappropriate defence pathway, the plant could allocate more energy for an effective defence against a particular attacker. Our finding that egg-derived elicitors suppress plant defence against insects by activating the SA-JA cross-talk illustrates another modulation of the defence signalling network, but this time for the benefit of the attacker. Indeed, eggs are manipulating the plant to indirectly lower the defences against feeding larvae. A similar phenomenon has been reported for the phloem-feeding silverleaf whitefly (*Bemisia tabaci*). Nymph feeding on *Arabidopsis* induced SA-responsive genes and repressed JA-responsive genes. In addition, mutants that activated the SA pathway or were impaired in JA signalling allowed a faster nymph development (Zarate *et al.* 2007). It was proposed that nymphs suppressed JA defences via SA-JA cross-talk but endogenous SA levels were not measured. In another example, sorghum plants attacked by greenbug aphids activated SA-regulated genes although it was shown that JA-regulated defences were effective against aphids. Again, the SA-JA cross-talk was evoked to explain these intriguing results, albeit without experimental evidence (Zhu-Salzman *et al.* 2004). Other studies indicate that components in insect oral secretions (OS) can suppress plant defences (Kahl *et al.* 2000, Lawrence *et al.* 2007, Musser *et al.* 2002). *Arabidopsis* plants had higher levels of JA-dependent defences in response to *S. exigua* caterpillars with impaired salivary secretions compared to intact caterpillars (Weech *et al.* 2008). Interestingly, this suppression of defence responses was abolished in mutants unable to mount a systemic acquired resistance and might indicate that SA is involved in this phenomenon. In support of this hypothesis, treatment with *S. exigua* OS stimulated SA

accumulation in *N. attenuata* (Diezel *et al.* 2009). There is thus emerging evidence that insects or insect-derived cues (eggs or OS) are able to manipulate the SA-JA cross-talk to their advantage.

The molecular mechanisms of cross-talk between SA- and JA-pathways are not fully understood. The regulatory protein NPR1 was identified as a key component in the inhibitory action of SA on the JA-dependent defences (Spoel *et al.* 2003). NPR1 is active in the nucleus to regulate PR-gene expression (Spoel *et al.* 2009) but has an additional role in the cytosol where it is proposed to negatively interact with several components of the JA-pathway (Beckers and Spoel 2006, Spoel *et al.* 2003). Moreover, ethylene signalling was found to override the function of NPR1 in the SA-JA cross-talk (Leon-Reyes *et al.* 2009, Leon-Reyes *et al.* 2010). In addition, the transcriptional activator WRKY70 (Li *et al.* 2004), glutaredoxin (Ndamukong *et al.* 2007), and the fatty acid desaturase SSI2 (Kachroo *et al.* 2001) are important modulators of SA-JA cross-talk in *Arabidopsis*. In this study, we found that egg-induced defence suppression against a generalist herbivore is independent of NPR1. This finding confirms earlier studies with Arabidopsis that showed that the inhibition of JA-induced resistance to chewing insects still occurred in *npr1-1* plants treated with SA or with virulent bacterial pathogens (Cipollini *et al.* 2004, Cui *et al.* 2002). In addition, SA inhibited the induction of leaf trichomes, another component of resistance to herbivores, in an NPR1-independent way (Traw and Bergelson 2003). There is thus emerging evidence that the negative SA/JA cross-talk is controlled differently depending on the type of aggressor triggering the JA pathway. It will be interesting to elucidate the precise molecular mechanism of the suppression of insect-induced responses. We have found that the inhibition of JA-induced genes by egg extract was still observed after a

treatment with MeJA, suggesting that the negative SA/JA cross-talk operates downstream of JA biosynthesis (data not shown).

In summary, we present here an intriguing facet of the arms race between plants and insect herbivores in which an apparent inert stage of the insect, the egg, hijacks the SA-signalling pathway for the benefit of its progeny. It will be fascinating to see whether this phenomenon is widely distributed in nature and has evolved as a potent way of circumventing the defences of the plants.

EXPERIMENTAL PROCEDURES

Plants and insects

Arabidopsis thaliana (Col-0), PR1::GUS (gift from Dr Allan Shapiro, Florida Gulf Coast University), the mutants *sid2-1* (gift from Dr Christiane Nawrath, University of Lausanne) and *npr1-1* (Nottingham Arabidopsis Stock Center, http://arabidopsis.info) in the Col-0 background were grown as described previously (Reymond *et al.* 2004). For GUS reporter lines, the promoter region of *TI* (At1g73260), *CHIT* (At2g43570), and *SAG13* (At2g29350) was amplified by PCR (for primer sequences, see Table S1), cloned into the GATEWAY donor vector pDONR/Zeo (Invitrogen, http://www.invitrogen.com), recombined into the destination vector pMDC162 cassette A (Curtis and Grossniklaus 2003), and the plasmids transferred to *Agrobacterium tumefasciens* strain pGV3101 (pMP90). Transgenic plants were obtained by floral dipping and selection of the seeds on 100 μg/ml hygromycin.

Rearing of *Pieris brassicae* (large white butterfly) was done on cabbage (*Brassica oleracea*) (Reymond *et al.* 2004). *Spodoptera littoralis* (Egyptian cotton worm) eggs were obtained from Syngenta (Stein, Switzerland). *Drosophila melanogaster* eggs were obtained from Dr Tadeusz Kawecki (University of Lausanne).

SA measurements

SA was quantified in excised leaf discs by HPLC as described previously (Garcion *et al.* 2008). Analyses were carried out on triplicate samples with good reproducibility and the results given are mean values. For each biological replicate, ca. 15 leaf discs of 10 mm diameter were collected on oviposited/treated or non-oviposited/untreated leaves from 10

plants. Fifteen egg batches (30-35 eggs/batch) from the same plants were analysed similarly.

Treatments with egg extracts

P. brassicae eggs laid on cabbage leaves were crushed with a pestle in Eppendorf tubes. After centrifugation (15'000 g, 3 min), the supernatant ("egg extract") was stored at -20°C. Egg extracts from *S. littoralis* and *D. melanogaster* were prepared similarly. For GUS activity, 2 µl of egg extract, corresponding to one egg batch of ca. 20-30 eggs, was spotted onto each leaf for 72 h. The extract was then gently removed with a paint brush and GUS staining was performed as described previously (Little *et al.* 2007). For protease treatment, 50 U of proteinase K immobilized on eupergit C beads (Fluka, http://www.sigmaaldrich.com) was added to 1 ml of egg extract. The sample was incubated for 20 min at 30°C with agitation at 600 rpm in a thermomixer (Eppendorf, http://www.eppendorf.com). The beads were then spun down and the promoter:GUS plants treated with 2 µl of the supernatant. For filtration, 100 µl of egg extract was placed in a 3 kDa-microcon filter (Millipore, http://www.millipore.com) and centrifuged for 60 minutes at 14’000 rpm at 20°C. Two µl of flow-through were applied onto the promoter:GUS plants. For total lipids extraction, 20 ml of chloroform/ethanol (1/1) was added drop wise to 1 ml of egg extract in an Erlenmeyer and incubated 1 hour at room temperature on a shaker. The clear supernatant was transferred to a beaker and the solvent evaporated to dryness on a steam bath. The extract was dissolved in pure chloroform and filtered through a funnel packed with cotton. Chloroform was evaporated under nitrogen and the lipid extract was

applied to promoter:GUS plants. Remaining eggshells of freshly hatched *P. brassicae* larvae were collected and mixed with distilled water before application onto promoter:GUS plants with a spatula.

For expression analyses and bioassays, two spots of 2 µl of egg extract were applied onto each leaf. In total, two leaves of 20 plants were treated. After 5 days, the egg extract was gently removed with a paint brush and one freshly hatched *P. brassicae* or two freshly hatched *S. littoralis* larvae were placed onto each plant. Plants were placed in Plexiglas boxes in a growth room as previously described (Bodenhausen and Reymond 2007). After two days of feeding, local and distal leaves from four plants were harvested for RNA analysis and the 16 remaining plants were left for another 6 days with the larvae. At the end of the experiment, larvae were collected and weighed. Controls consisted of untreated and/or uninfested plants.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen, http://www.qiagen.com). For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen, http://www.invitrogen.com) in a final volume of 25 µL. Each cDNA sample was generated in triplicate from each RNA and diluted 4-fold with water. Gene specific primers (Table S1) were designed to give amplicons between 70 and 200 bp in the 3' end of the cDNA strand. Primer efficiencies were assessed doing a 5 step dilution regression. qRT-CR analysis was performed in a final volume of 25 µl containing 2.5 µl of cDNA, 0.1 µM of each primer, 0.03 µM of reference

dye, and 2X Brilliant II Fast SYBR® Green QPCR Master Mix (Agilent, http://www.agilent.com). Reactions were generated in a real-time PCR machine (Mx3000P™, Agilent, http://www.agilent.com) with the following program: 95°C for 10 min; then 40 cycles comprising 10 s at 95°C, 20 s at 55°C and 17 s at 60°C. Relative mRNA abundance was normalized to the reference gene At2g28390 (*Arabidopsis* SAND family protein), which has been shown to be a superior reference gene for qRT-PCR (Czechowski *et al.* 2005), and expressed relative to the control sample. Values in Fig. 3 are the mean of three independent biological replicates.

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SUPPORTING INFORMATION

Figure S1. SA levels in *P. brassicae* eggs.

Figure S2. Expression of insect-induced genes in distal leaves.

Figure S3. Suppression of insect-induced genes in response to treatment with *S. littoralis* egg-extract.

Figure S4. Treatment with egg extract does not suppress the expression of JA-independent genes.

Table S1. List of primers used for real-time PCR and promoter:GUS constructs.

FIGURE LEGENDS

Figure 1. Egg-derived elicitors activate the expression of reporter genes.

(a) Application of 2 µl of egg extracts from different insect species onto leaves of *Arabidopsis* promoter:GUS lines. GUS expression was analysed by histochemical staining 72 h after treatment. *PR1, TI, CHIT*, and *SAG13* are egg-inducible genes. The top left panel is a photograph of an entire leaf, whereas the other panels are close-up views of the spotted area. *P. b.*, *Pieris brassicae*; *S. l.*, *Spodoptera littoralis*; *D. m.*, *Drosophila melanogaster*.

(b) Activation of the *PR1* promoter in response to application of eggshell preparation, egg extract treated with proteinase K, lipids extracted from the eggs, and egg extract filtered through a 3-kDa filter. GUS staining was performed 72 h after each treatment.

Figure 2. SA accumulates in response to oviposition by *P. brassicae*.

(a) SA was quantified in *Arabidopsis* leaves during four days after oviposition. Black bars indicate oviposited leaves and white bars indicate non-oviposited leaves. For each biological replicate, ca. 15 leaf discs of 10 mm diameter were collected on oviposited or non-oviposited leaves from 10 plants. Each oviposited leaf contained a batch of 30-35 eggs. Values (±SE) are the mean of three biological replicates.

(b) SA was quantified in Arabidopsis leaves during four days after application of 2 µl of *P. brassicae* egg extract. For each biological replicate, ca. 15 leaf discs of 10 mm diameter were collected. Black bars indicate treated leaves and white bars indicate non-treated leaves. Values (±SE) are the mean of three biological replicates.

Figure 3. Treatment with egg extract suppresses insect-induced genes.

(a, b) Col-0 *Arabidopsis* plants were treated for 5 days with *P. brassicae* egg extract and subsequently challenged for 48 h with *P. brassicae* larvae (a) or with *S. littoralis* larvae (b). (c) *Arabidopsis sid2-1* plants treated with *P. brassicae* egg extract and challenged with *S. littoralis* larvae.

Expression of insect-responsive genes was measured by real-time PCR. Values are normalized to the reference gene and expressed relative to unchallenged control samples. White bars indicate untreated plants. Black bars indicate plants treated with egg extract. Values (±SE) are the mean of three biological replicates.

Figure 4. Treatment with egg extract increases plant susceptibility to a generalist herbivore. Freshly hatched larvae of the specialist *P. brassicae* (a) and of the generalist *S. littoralis* (b, c) were placed on *Arabidopsis* plants and larval weight was measured after eight days of feeding. (c) *sid2-1* plants were challenged with *S. littoralis* larvae.

White bars indicate untreated control plants challenged with insects. Black bars indicate plants treated with *P. brassicae* egg extract 5 days before insect challenge. The number of larvae used in each experiment is shown in the bars. Error bars indicate SE. Asterisks indicate statistically significant differences compared to the control (Student's *t* test, α = 0.001). n. s., not significant. Similar results were observed in several independent replicate experiments.

Figure 5. Suppression of defences by egg extract is NPR1-independent.

(a) *npr1-1* plants were treated for 5 days with *P. brassicae* egg extract and subsequently challenged for 48 h with *S. littoralis* larvae. Expression of insect-responsive genes was measured by real-time PCR. Values are normalized to the reference gene and expressed relative to unchallenged control samples. White bars indicate untreated plants. Black bars indicate plants treated with egg extract. Values (±SE) are the mean of three biological replicates. Control experiments with Col-0 are in Figure 3b.

(b) Freshly hatched *S. littoralis* larvae were placed on *npr1-1* plants and larval weight was measured after eight days of feeding. White bars indicate untreated control plants challenged with insects. Black bars indicate plants treated with *P. brassicae* egg extract 5 days before insect challenge. The number of larvae used in each experiment is shown in the

bars. Error bars indicate SE. Asterisks indicate statistically significant differences compared to the control (Student's *t* test, $\alpha = 0.001$). Similar results were observed in several independent replicate experiments. Control experiment with Col-0 is in Figure 4b.

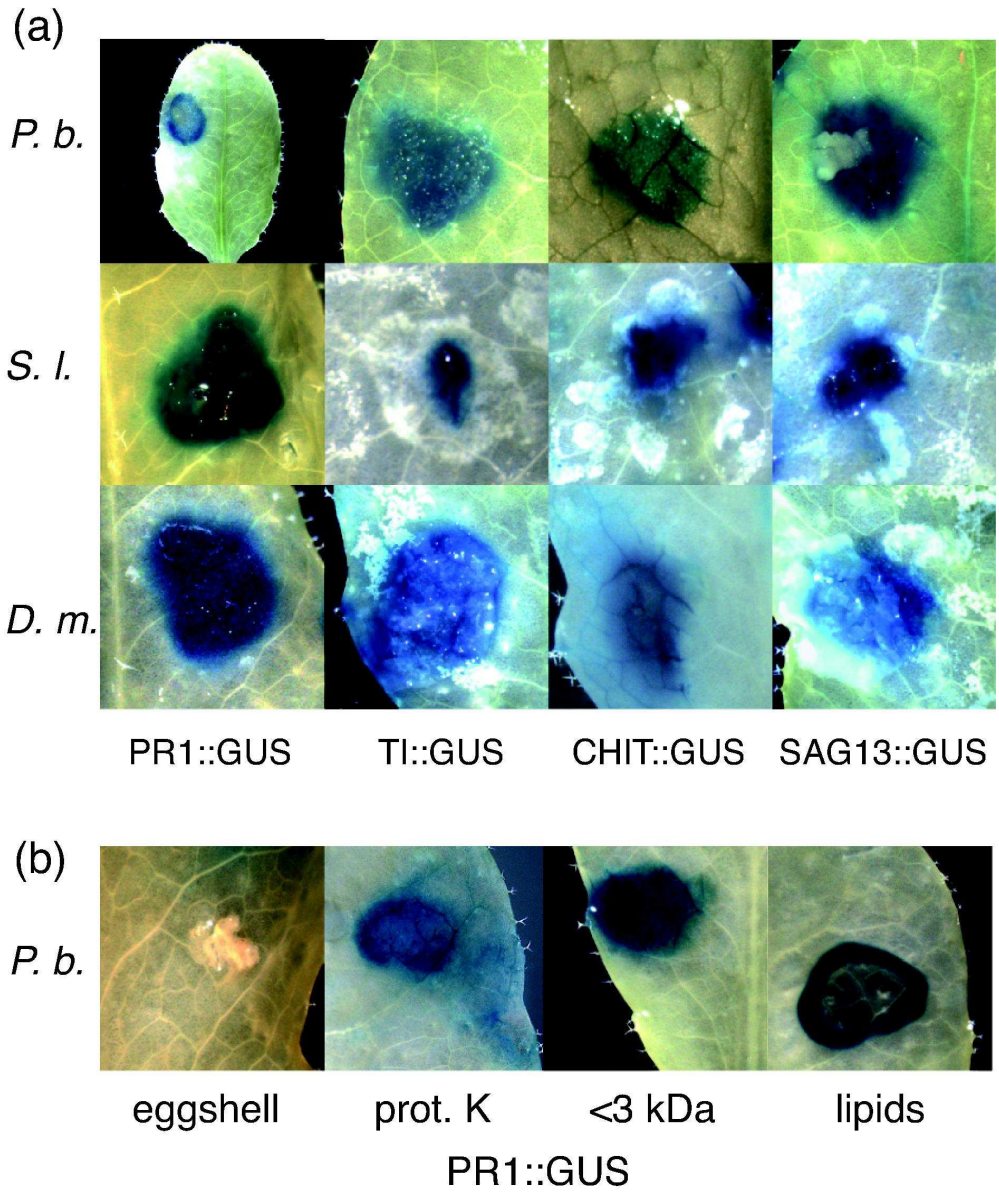

Figure 1
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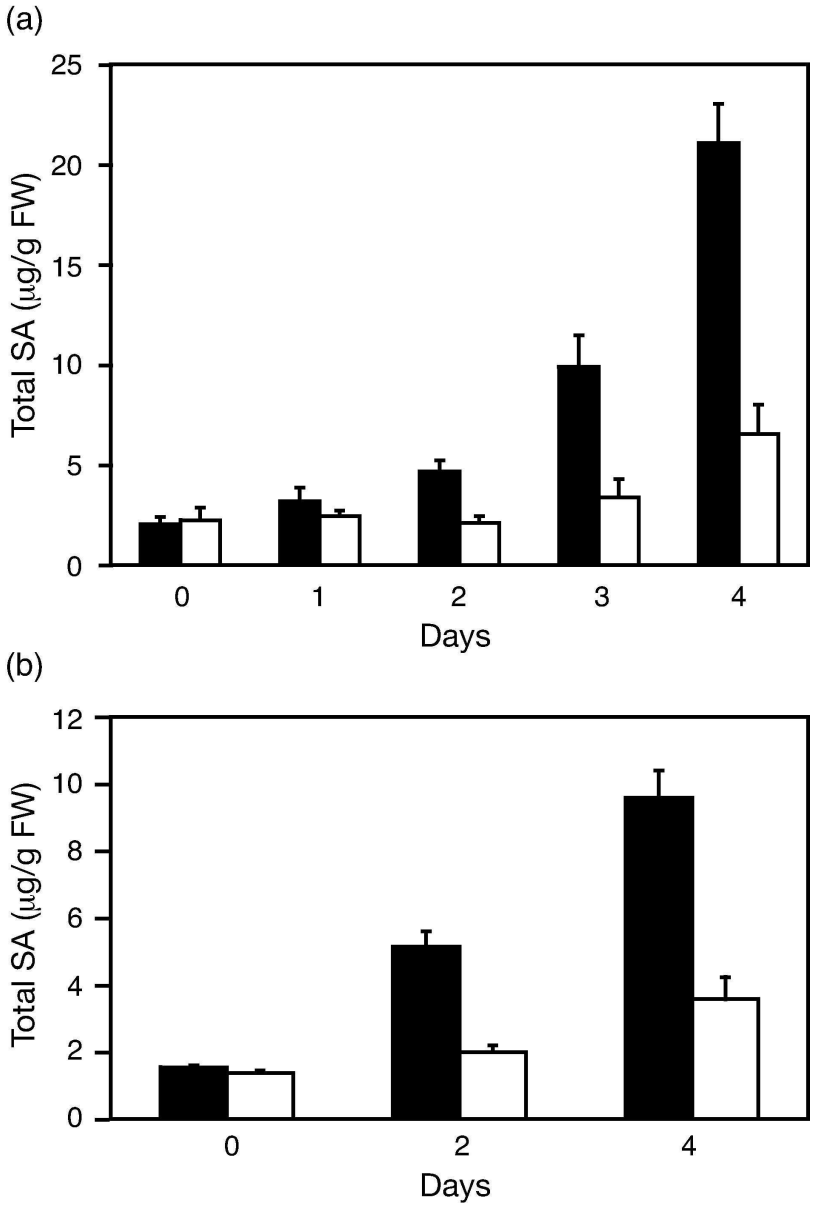

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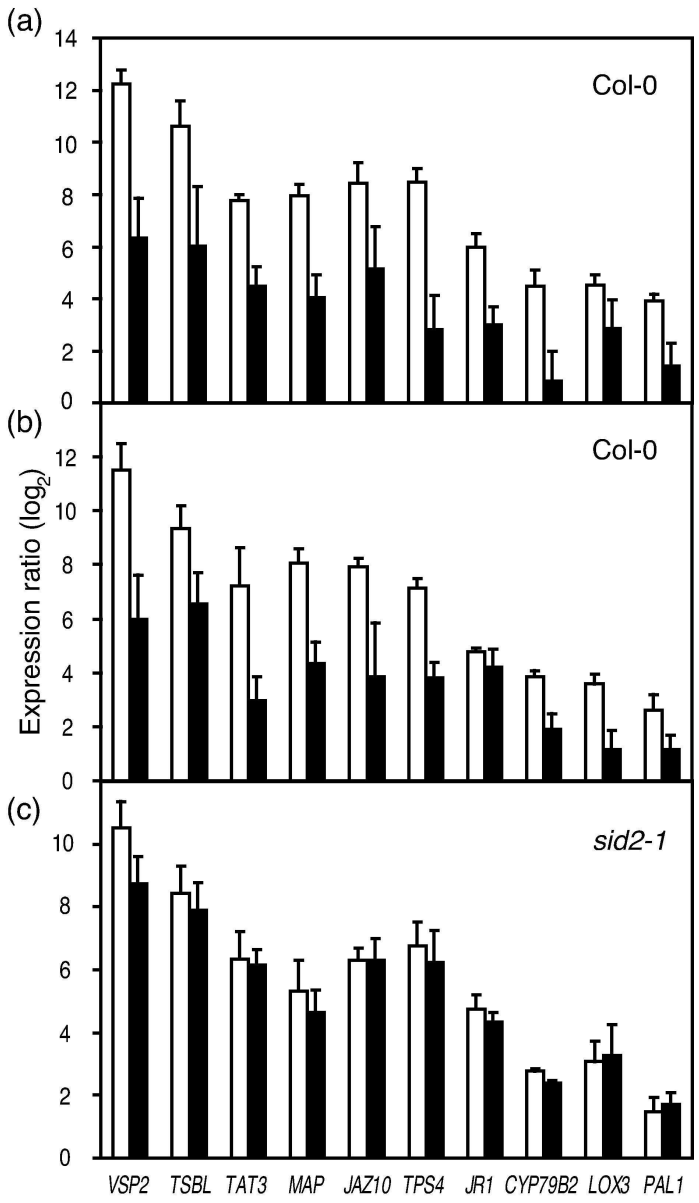

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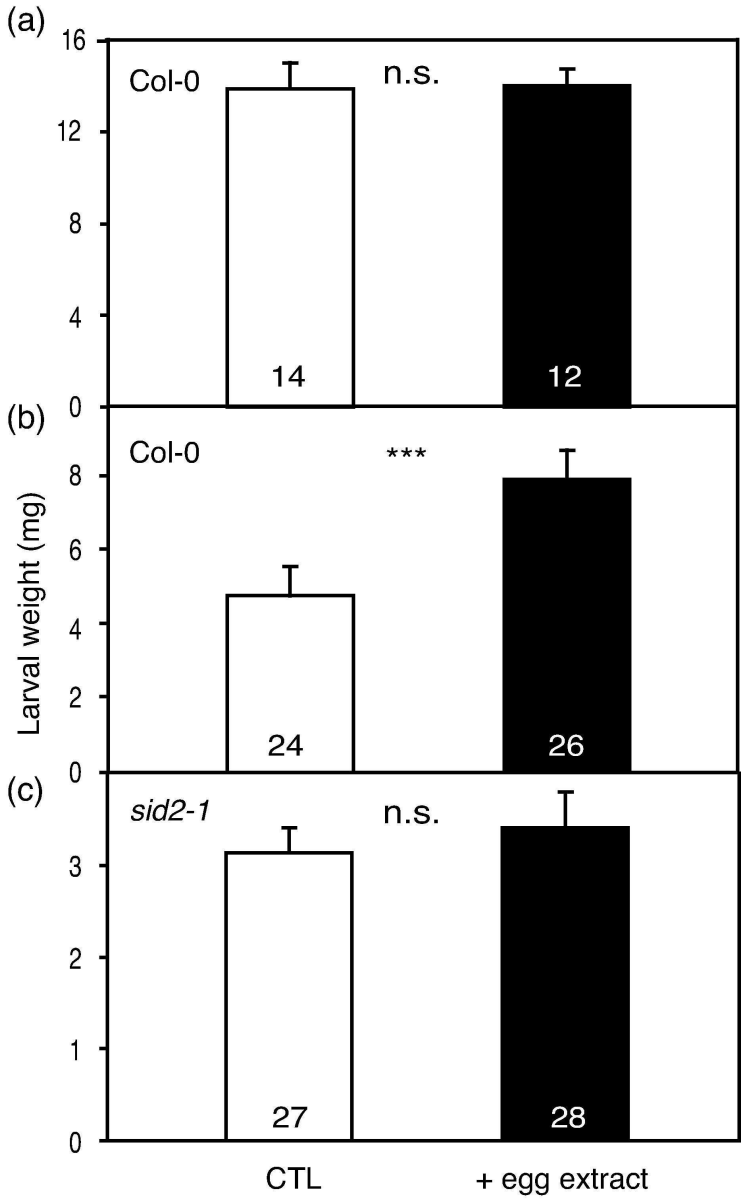

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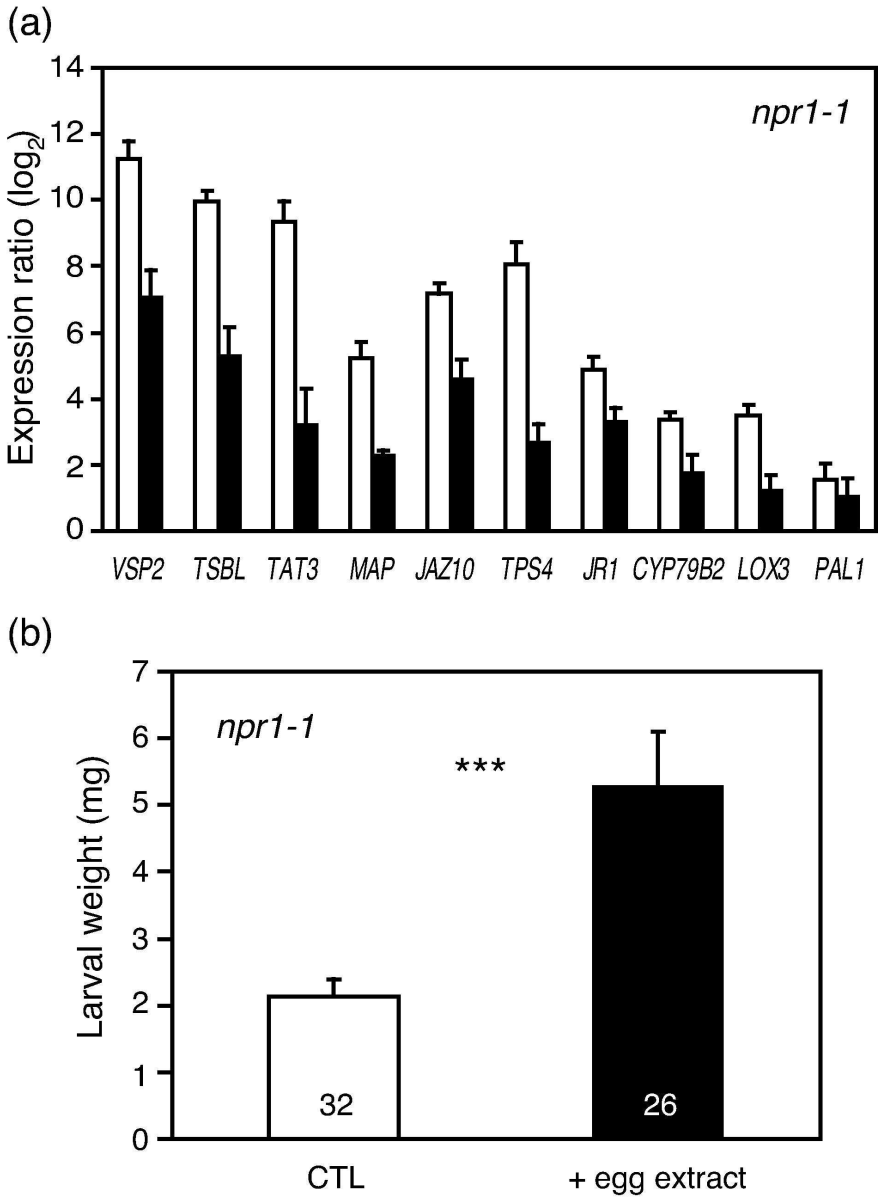

Figure 5
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