Indole-3-Acetonitrile Production from Indole Glucosinolates Deters Oviposition by Pieris rapae

Martin de Vos, Ksenia L. Kriksunov, and Georg Jander*

Boyce Thompson Institute for Plant Research, Ithaca, New York 14853

Like many crucifer-specialist herbivores, Pieris rapae uses the presence of glucosinolates as a signal for oviposition and larval feeding. Arabidopsis thaliana glucosinolate-related mutants provide a unique resource for studying the in vivo role of these compounds in affecting P. rapae oviposition. Low indole glucosinolate cyp79B2 cyp79B3 mutants received fewer eggs than wild type, confirming prior research showing that indole glucosinolates are an important oviposition cue. Transgenic plants overexpressing epithiospecifier protein, which shifts glucosinolate breakdown toward nitrile formation, are less attractive to ovipositing P. rapae females. Exogenous application of indol-3-ylmethylglucosinolate breakdown products to cyp79B2 cyp79B3 mutants showed that oviposition was increased by indole-3-carbinol and decreased by indole-3-acetonitrile (IAN). P. rapae larvae tolerate a cruciferous diet by using a gut enzyme to redirect glucosinolate breakdown toward less toxic nitriles, including IAN, rather than isothiocyanates. The presence of IAN in larval regurgitant contributes to reduced oviposition by adult females on larvae-infested plants. Therefore, production of nitriles via epithiospecifier protein in cruciferous plants, which makes the plants more sensitive to generalist herbivores, may be a counter-adaptive mechanism for reducing oviposition by P. rapae and perhaps other crucifer-specialist insects.

Crucifers and other plants in the order Caparales have an effective chemical defense that requires the hydrolysis of glucosinolates by myrosinase (β-thioglucoside glucohydrolase [TGG]; EC 3.2.1.147), leading to the formation of breakdown products that deter herbivory (for review, see Grubb and Abel, 2006; Halkier and Gershenzon, 2006). In contrast to generalist herbivores, which tend to avoid glucosinolates, Pieris rapae and other crucifer-feeding specialists recognize glucosinolates and their breakdown products as stimulants for feeding and oviposition. 2-Propenylglucosinolate, 2-phenylethylglucosinolate, and other glucosinolates applied to Vigna sinensis leaves stimulated feeding by P. rapae larvae (Renwick and Lopez, 1999; Miles et al., 2005). Host plant choice experiments with purified glucosinolates sprayed onto Phaseolus vulgaris showed that P. rapae oviposition is stimulated primarily by indole and aromatic glucosinolates (Huang and Renwick, 1993, 1994). Although intact indol-3-ylmethylglucosinolate (I3M) was the strongest P. rapae oviposition stimulant found in cabbage (Brassica oleracea) surface washes (Renwick et al., 1992; Van Loon et al., 1992), the chemical instability of indole glucosinolates suggests that the P. rapae were exposed to not only intact I3M but also breakdown products in these assays. Related experiments also showed that indole and aromatic glucosinolates elicit stronger responses than aliphatic glucosinolates from P. rapae tarsal chemoreceptor cells, which are used to test leaf surfaces prior to oviposition (Städlér et al., 1995).

Glucosinolates are produced constitutively in cruciferous plants, but their degradation is strictly regulated by the spatial separation of glucosinolates and myrosinase in the plant. Upon tissue rupture, myrosinase enzymes cleave glucosinolates, producing unstable thiohydroxamate-O-sulfonates, which can be broken down further to produce a wide variety of insect-deterrent compounds. The epithiospecifier protein (ESP) interacts with myrosinase and directs glucosinolate breakdown toward the formation of nitriles and epithionitriles at the expense of the generally more toxic isothiocyanates (Lambrix et al., 2001; Fig. 1). In the case of I3M, the predominant indole glucosinolate in Arabidopsis (Arabidopsis thaliana; Petersen et al., 2002; Brown et al., 2003), ESP promotes the formation of indole-3-acetonitrile (IAN) instead of indolymethylisothiocyanate as the primary breakdown product (Miao and Zentgraf, 2007; Burow et al., 2008). Indolymethylisothiocyanate, when it is produced in Arabidopsis and other crucifers, reacts rapidly to form indole-3-carbinol (I3C), a relatively stable I3M breakdown product (Agerbirk et al., 1998).

Natural variation in Arabidopsis sensitivity to feeding by the generalist Trichoplusia ni is strongly influenced by the presence or absence of ESP in the land races Columbia-0 (Col-0) and Landsberg erecta (Ler; Jander et al., 2001; Lambricht et al., 2001). Similarly,
transgenic overproduction of ESP in Arabidopsis increased growth of *Spodoptera littoralis*, another generalist lepidopteran herbivore (Burow et al., 2006b). As was demonstrated by addition of microencapsulated isothiocyanates to artificial diets, these compounds are also toxic to the crucifer-specialist *P. rapae* (Agrawal and Kurashige, 2003). However, when *P. rapae* larvae feed from intact plants, a nitrile specifier protein in the insect gut, which functions in an analogous manner to the plant-derived ESP, directs glucosinolate breakdown toward less toxic nitriles that are excreted in the frass (Wittstock et al., 2004).

By avoiding oviposition on plants that are previously infested, adult female Lepidoptera can help to ensure that sufficient food will be available for their offspring. Both *P. rapae* and the closely related *Pieris brassicae* oviposit less readily on plants with feeding larvae (Rothschild and Schoonhoven, 1977), suggesting biochemical changes in the host plants or that the larvae, their frass, or regurgitant contain oviposition deterrents. Similarly, both species show reduced oviposition on plants that already carry conspecific eggs (Schoonhoven et al., 2005). Oviposition by *P. brassicae* and to a lesser extent *P. rapae* triggers defense-related gene expression changes in Arabidopsis (Little et al., 2007). Resulting metabolic changes in the plants or perhaps direct visual and chemical cues from the eggs themselves could provide signals that deter subsequent oviposition (Blakmeer et al., 1994).

*P. rapae* commonly infest weedy crucifers such as cabbage and *Arabis* (formerly *Arabis* *lyrata* (Mitchell-Olds, 2001; Agrawal and Kurashige, 2003) and have also been observed on both planted (Harvey et al., 2007) and natural (Geervliet, 1997) Arabidopsis populations. Although a winter annual growth habit may allow Arabidopsis to avoid herbivory by insects that require warmer temperatures, not all Arabidopsis exhibit this life cycle (Pigliucci, 2002). Particularly in more southern regions, there would be considerable overlap between Arabidopsis and Lepidoptera such as *P. rapae*, which overwinter as pupae and emerge as soon as it is warm enough for them to fly. For instance, a larva of an unknown lepidopteran species was observed feeding on a natural stand of Arabidopsis in North Carolina (Mauricio, 1998).

Although there is evidence that ovipositing *P. rapae* females are stimulated by indole glucosinolates (Huang and Renwick, 1993, 1994), this has not been proven through analysis of isogenic lines with and without indole glucosinolates. Furthermore, although the insects are perhaps more likely to be exposed to glucosinolate breakdown products rather than intact glucosinolates, evidence of an in vivo role for indole glucosinolate breakdown products in the oviposition response is still lacking. Therefore, we have made use of Arabidopsis mutants with blocked indole glucosinolate biosynthesis (*cyp79B2 cyp79B3*; Fig. 1), reduced myrosinase activity (*tgg1 tgg2*), and ESP overproduction (35S:ESP) to assess the role of indole glucosinolates and their breakdown products in *P. rapae* oviposition.

Surprisingly, the two primary indole-glucosinolate breakdown products showed contrasting effects on *P. rapae* oviposition, with I3C acting as a stimulant and IAN acting as a deterrent. Moreover, application of IAN-containing larval regurgitant reduced host plant attractiveness for ovipositing *P. rapae*. This suggests that ESP-expressing plants that produce nitriles may appear chemically similar to those infested by *P. rapae* and thereby deter oviposition by this specialist herbivore.

**RESULTS**

**Oviposition by *P. rapae* Is Dependent on Indole Glucosinolates**

Three-week-old wild-type and mutant Arabidopsis plants were used in a 24-h choice test to investigate the

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**Figure 1.** Biosynthesis and breakdown of I3M. Indole glucosinolates are derived from Trp, with CYP79B2 (At4g39950), CYP79B3 (At2g22330), CYP83B1 (At4g31500), C-S lyase (At2g20610), UGT74B1 (At1g24100), and AtST5a (At5g74100) being key enzymes in the biosynthetic pathway. Indole glucosinolate hydrolysis depends on myrosinases TGG1 (At5g26000) and TGG2 (At5g25980), which have redundant function. ESP (At1g54040) directs the breakdown of I3M toward IAN rather than 13C.
in vivo role of indole glucosinolates in the oviposition response of *P. rapae*. Female *P. rapae* deposit eggs individually on the leaf surface (Fig. 2A), allowing relative plant attractiveness to be assessed by counting eggs. Compared to wild-type plants, the low indole glucosinolate mutant (*cyp79B2 cyp79B3*) received significantly fewer eggs (Fig. 3A), indicating an in vivo role for indole glucosinolates in triggering oviposition by female *P. rapae*. Absence of camalexin, the major Arabidopsis phytoalexin, in *cyp79B2 cyp79B3* mutants (Glazebrook and Ausubel, 1994; Zhou et al., 1999; Schuhegger et al., 2006) could also influence *P. rapae* oviposition. However, no significant difference was observed when comparing the number of eggs deposited on wild-type Col-0 and the phytoalexin deficient3 (*pad3-1*) mutant plants over 24 h (Fig. 3B). Interestingly, oviposition on *atr1D atr2D* mutants, which have elevated indole glucosinolate content, was not significantly different from Col-0 plants (Fig. 3C), suggesting that wild-type levels of indole glucosinolates are sufficient as an oviposition cue. This is consistent with a prior report that, beyond an optimal concentration, addition of more glucosinolates either did not promote *P. rapae* oviposition or actually decreased it (Huang and Renwick, 1993).

**P. rapae** Oviposition Is Reduced by ESP Activity

Because it seemed likely that *P. rapae* would encounter glucosinolate breakdown products rather than intact glucosinolates on the leaf surface, where there is also expression of both myrosinase and ESP (Andreason et al., 2001; Husebye et al., 2002; Burow et al., 2007), we hypothesized that altered regulation of glucosinolate breakdown by ESP would influence *P. rapae* oviposition. Unlike in Col-0, the presence of ESP activity in Ler shifts the hydrolysis of glucosinolates toward production of nitriles (Lambrix et al., 2001). Direct comparison of oviposition on whole Col-0 and Ler plants was not feasible due to phenotypic differences in rosette size and flowering time that would strongly influence host plant choice by female *P. rapae*. However, in a detached-leaf assay (Fig. 2B), Col-0 leaves received significantly more eggs than Ler leaves (Fig. 3D), suggesting that a glucosinolate breakdown products produced via ESP could reduce *P. rapae* oviposition.

Analysis of methanol leaf surface washes was used to determine what indole glucosinolate breakdown products could be perceived by ovipositing *P. rapae*. IAN was 13-fold more abundant in Ler surface washes, whereas I3C was 5-fold more abundant in Col-0 surface washes (Fig. 4A). Control experiments showed increasing recovery of IAN from Col-0 leaves with wash times ranging from 5 to 20 s (Supplemental Fig. S1A). Recovery of IAN did not increase over time in 50% methanol surface washes. Spectrophotometric measurements showed very low absorption at 647 and 660 nm (chlorophyll A and B, respectively), which was not significantly different from blank controls (Supplemental Fig. S1B), indicating that there was no significant cell damage due to the methanol surface washes.

Because Ler and Col-0 plants differ in several aspects of glucosinolate biology (Kliebenstein et al., 2001; Lambrux et al., 2001), we used transgenic Col-0 overexpressing ESP from the cauliflower mosaic virus 35S promoter (35S:ESP; Burow et al., 2006b) in a more specific assay to study the effects of nitriles on *P. rapae* oviposition. Compared to empty-vector control plants, surface washes of 35S:ESP plants contained approximately 4-fold more IAN, whereas no significant difference was observed in the abundance of I3C (Fig. 4B). Although the ESP-overexpressing plants with high nitrile levels received significantly fewer *P. rapae* eggs (Fig. 3E), hatching success and larval weight gain were unaffected (Supplemental Fig. S2, A and B). Therefore, these data suggest that nitrile formation during glucosinolate breakdown can play a significant role in deterring oviposition.
ESP modulates glucosinolate breakdown through a direct interaction with myrosinase (Burrow et al., 2006a). Therefore, given the clear effects of ESP overproduction (Fig. 3E), it seemed likely that the absence of myrosinase in tgg1 tgg2 mutants (Barth and Jander, 2006) would also influence \textit{P. rapae} oviposition. Somewhat surprisingly, there was no significant difference in the number of eggs deposited on wild-type and tgg1 tgg2 mutant plants (Fig. 3F), even if the plants were mechanically damaged to promote glucosinolate breakdown prior to the oviposition assay (Fig. 3G).

Indole glucosinolates in damaged plant tissue undergo degradation that is independent of the TGG1 and TGG2 myrosinases (Barth and Jander, 2006). To investigate whether this myrosinase-independent breakdown influences \textit{P. rapae} oviposition, we performed pair-wise comparisons of oviposition on cyp79B2 cyp79B3 tgg1 tgg2 quadruple mutants with tgg1 tgg2 and cyp79B2 cyp79B3 double mutants. Figure 3H shows that tgg1 tgg2 plants are more attractive than the quadruple mutant, whereas cyp79B2 cyp79B3 were equally attractive (Fig. 3I). This indicates that TGG1 and TGG2-dependent breakdown of indole glucosinolates is not essential for the production of \textit{P. rapae} oviposition cues. Ovipositing \textit{P. rapae} females are deterred by ESP activity in the tgg1 tgg2 mutant background (Fig. 3J) but not by plants transformed with an empty vector (EV) control (Fig. 3K). Because the action of ESP depends on the physical interaction with myrosinase (Lambrix et al., 2001; Burrow et al., 2006a), this result indicates that some other myrosinase might break down indole glucosinolates in the tgg1 tgg2 mutant background. To test this hypothesis, indole glucosinolate breakdown products were measured in leaf surface washes of tgg1 tgg2 mutants, with and without ESP overexpression. Similar to results obtained for 35S:ESP and EV wild-type plants (Fig. 4B), there was a significantly elevated IAN in surface washes of 35S:ESP tgg1 tgg2 plants and no significant difference in I3C abundance (Fig. 4C).

**Oviposition Is Differentially Affected by Indole Glucosinolate Breakdown Products**

Whereas wild-type Col-0 produces I3C as the primary I3M breakdown product, plants expressing ESP produce primarily IAN (Miao and Zentgraf, 2007; Burrow et al., 2008). To determine whether these two metabolites differentially affect \textit{P. rapae} oviposition, cyp79B2 cyp79B3 plants, which contain low indole glucosinolate levels, were sprayed with IAN and I3C for oviposition experiments. Compared to mock-treated control plants, cyp79B2 cyp79B3 plants treated with 1 mM IAN received fewer eggs (Fig. 5A), indicating that IAN deters oviposition. Because IAN is volatile, the presence of this compound on the leaves during the entire experiment was confirmed with a surface wash of the plants 24 h after treatment (Fig. 5B). IAN deters \textit{P. rapae} oviposition over a wide range of concentrations, with a 0.01 mM IAN application still having a significant deterrent effect (Fig. 5C). IAN concentration in leaf surface washes of ESP-expressing plants (i.e. \textit{Ler} and 35S:ESP; Fig. 4, A and B) is within the range of exogenous IAN applications that were tested.

In contrast to IAN addition, I3C-treated cyp79B2 cyp79B3 plants received significantly more eggs than wild-type controls, indicating that this indole glucosinolate breakdown product is attractive to female \textit{P. rapae} (Fig. 5D). I3C was abundant in surface washes 24 h after spraying Arabidopsis plants with 1 mM I3C (Fig. 5E). Because this I3C concentration greatly exceeded that found in surface washes of untreated Col-0 leaves (Fig. 4A), we also tested the oviposition response of \textit{P. rapae} on leaves that were treated with 100-fold less I3C. Compared to mock-treated leaves, oviposition was higher on cyp79B2 cyp79B3 leaves treated with 0.01 mM I3C (Fig. 4F), showing that, like IAN, I3C functions as an oviposition cue over a wide range of concentrations.

**IAN in \textit{P. rapae} Regurgitant Deters Oviposition**

It was demonstrated previously that female \textit{P. rapae} avoid ovipositing on plants infested with conspecific larvae (Rothschild and Schoonhoven, 1977). We observed this phenomenon with wild-type, but not cyp79B2 cyp79B3 mutant, plants (Fig. 6). The deterrent effects of larvae-infested plants (Fig. 6) and exogenous IAN (Fig. 5) suggested that IAN in larval regurgitant or frass could contribute to the avoidance of infested plants by female \textit{P. rapae}.

\textit{P. rapae} larvae make use of gut-specific nitrile specifier protein to direct breakdown of glucosinolates to nitriles rather than the more toxic isothiocyanates, an adaptation that results in the presence of glucosinolate-derived nitriles in larval frass (Wittstock et al., 2004). Compared to larvae feeding on cyp79B2 cyp79B3 plants, both regurgitant (Fig. 7A) and frass (Fig. 7B) from larvae feeding on Col-0 plants contain significantly higher amounts of IAN. During feeding bouts, lepidopteran larvae typically apply small amounts of regurgitant onto the feeding site (Truitt and Paré, 2004). Application of 2 \mu L fresh regurgitant from Col-0-fed larvae to cyp79B2 cyp79B3 leaves in a detached-leaf assay (Fig. 2B) showed that this regurgitant acts as a significant deterrent for \textit{P. rapae} oviposition (Fig. 7C). The regurgitant applied to a single leaf (approximately 25 \mu g cm\textsuperscript{-2} IAN) was comparable to IAN levels detected in surface washes of \textit{Ler} (10.6 \mu g cm\textsuperscript{-2} IAN) and 35S:ESP (6.4 \mu g cm\textsuperscript{-2} IAN), or cyp79B2 cyp79B3 plants sprayed with 1 mM IAN (44.2 \mu g cm\textsuperscript{-2} IAN). A direct comparison of regurgitant from cyp79B2 cyp79B3-fed and Col-0-fed larvae showed that the deterrent effect requires the presence of an indole-derived compound in the host plant tissue (Fig. 7D). Addition of approximately 5 mg of larval frass, which has lower IAN levels than regurgitant (Fig. 7, A and B), to a detached-leaf assay (Fig. 2B) did not have a significant effect on \textit{P. rapae} oviposition (cyp79B2 cyp79B3 leaves...
Figure 3. *P. rapae* oviposition on wild-type Arabidopsis and mutants with altered glucosinolate profiles. A, Oviposition by a single adult *P. rapae* female over 24 h in choice tests comparing Col-0 and mutant plants with low indole glucosinolate levels (*cyp79B2* *cyp79B3*; *n* = 14). B, Oviposition choice test comparing Col-0 and camalexin-deficient mutant *pad3-1* plants (*n* = 15). C, Oviposition choice test comparing Col-0 and mutant plants with high indole glucosinolate levels (*atr1D* *atr2D*; *n* = 11). D, Oviposition choice test (3 h) comparing Col-0 and Ler (*n* = 30). E, Oviposition choice test comparing ESP-overexpressing and Col-0 control plants (*n* = 22). F and G, Oviposition choice test comparing Col-0 and mutant plants lacking myrosinase activity (*tgg1* *tgg2*; *n* = 13) and mechanically damaged Col-0 and *tgg1* *tgg2* mutant plants (*n* = 19). H and I, Oviposition choice tests comparing the quadruple mutant *cyp79B2* *cyp79B3* *tgg1* *tgg2* with *tgg1* *tgg2* (*n* = 20) or *cyp79B2* *cyp79B3* (*n* = 15). J and K, Oviposition choice test comparing 35S:ESP *tgg1* *tgg2* plants (*n* = 25) and EV control *tgg1* *tgg2* plants (*n* = 7) with *tgg1* *tgg2* plants. Mean ± s, all comparisons for significance are paired *t* tests, except D, which shows an unpaired *t* test.
without frass, 6.6 ± 2.0 eggs; cyp79B2 cyp79B3 leaves with frass from Col-0-fed larvae, 5.2 ± 1.4 eggs; n = 20; P = 0.36; unpaired t test).

**DISCUSSION**

Taken together, our results lead us to propose an ecological role for nitrile formation in Arabidopsis, whereby some accessions can reduce *P. rapae* oviposition by modulating indole glucosinolate breakdown with ESP. Oviposition experiments with cyp79B2 cyp79B3 double mutants (Fig. 3A), which are almost completely devoid of indole glucosinolates (Zhao...
et al., 2002), suggest that these glucosinolates or their breakdown products serve as positive signals for *P. rapae* oviposition. However, we cannot completely rule out the possibility that altered production of auxin or other, as yet unknown indole metabolites that are metabolically downstream of CYP79B2 and CYP79B3 also contribute to the reduction in *P. rapae* oviposition on *cyp79B2 cyp79B3* mutants.

Both a transgenic line overexpressing ESP and the ESP-producing *Ler* land race release more IAN in surface washes (Fig. 4, A and B) and are less attractive oviposition sites than wild-type Col-0 (Fig. 3, D and E). For as yet unknown reasons, ESP overexpression causes changes in the Arabidopsis glucosinolate profile (Burow et al., 2006b). In particular, the abundance of intact I3M is decreased (Supplemental Fig. S3), suggesting that ESP overproduction increases I3M turnover in transgenic plants. Concomitant with the I3M decrease, IAN abundance is significantly increased in surface washes of 35S:ESP plants, but I3C remains unchanged (Fig. 4, B and C). Therefore, consistent with the known function of ESP, a greater percentage of the I3M is being converted to IAN in the 35S:ESP transgenic plants.

Together, decreased oviposition on both *cyp79B2 cyp79B3* and 35S:ESP plants (Fig. 3, A and E) showed that, although some indole compounds are oviposition stimulants, the nitrile breakdown products of indole glucosinolates are deterrent. This hypothesis was verified by showing that, when added to *cyp79B2 cyp79B3* leaves, I3C stimulates oviposition, but IAN represses it (Fig. 5). However, neither I3C nor IAN influenced *P. rapae* oviposition when applied to green paper (Traynier and Truscott, 1991), suggesting that these chemical signals must be associated with other oviposition cues in plant leaves or that reaction with other leaf constituents produces the actual attractive and deterrent compounds. Leaf surface washes show that IAN and I3C could be encountered by ovipositing *P. rapae* (Fig. 4). Although it is possible that leakage of cell contents is induced by surface washes (Reifenrath et al., 2005), the lack of chlorophyll release under our experimental conditions (Supplemental Fig. S1B) shows that there is no significant damage of epidermal cells.

I3C is less volatile than IAN, but reactions with other plant metabolites (Agerbirk et al., 1998; Staub et al., 2002) could reduce the effective concentration of I3C that remains after application onto Arabidopsis leaves. Assays of macerated Arabidopsis tissue showed very little free I3C but abundant I3C adducts that were formed with ascorbate, glutathione, and amino acids (J.H. Kim and G. Jander, unpublished data). Therefore, even though a significant amount of I3C persists after being sprayed on the leaf surface (Fig. 5E), at this point we cannot rule out the possibility that *P. rapae* oviposition is attracted by other metabolites that are formed by a reaction with I3C.
Assessment of leaf surface chemistry most likely occurs when *Pieris* butterflies drum leaves with their front tarsi prior to oviposition (Terofal, 1965; Schoonhoven et al., 2005). However, microscopic examination of the leaf surface did not reveal any physical damage (A. Renwick, personal communication). Given that *P. rapae* tarsi contain chemoreceptors that are particularly sensitive to aromatic and indole glucosinolates (Städler et al., 1995), it will be interesting to determine whether these same chemoreceptors are also sensitive to I3C and/or IAN. In contrast to *P. rapae*, which responds most strongly to aromatic and indole glucosinolates, the related species *Pieris napi oleracea* shows a preference for aliphatic glucosinolates during oviposition (Huang and Renwick, 1994). *P. napi oleracea* also uses glucosinolate detoxification enzymes to produce nitriles in the gut (Agerbirk et al., 2006); one might predict that the respective isothiocyanate and nitrile breakdown products of aliphatic glucosinolates would differentially affect oviposition by this species.

For ovipositing female *Pieris*, there is likely a significant selective advantage to using IAN or other nitriles as signals for avoiding plants with conspecific larvae. Prior larval feeding would have a deleterious effect on food availability, nutritional quality, and host defense responses. Compared to uninfested plants, Arabidopsis plants that were previously infested by *P. rapae* larvae showed increased resistance to subsequent attack by larvae of the same species (De Vos et al., 2006). This can be partly explained by the fact that *P. rapae* feeding induces a jasmonate burst (Reymond et al., 2000; De Vos et al., 2005), which would up-regulate plant defense pathways. Exogenous addition of jasmonate also reduces *P. rapae* oviposition on cabbage (Bruinsma et al., 2007).

Given the effects of ESP overproduction on IAN formation (Fig. 4B), the observation that *P. rapae* oviposition was unaffected by *tgg1 tgg2* myrosinase knockout mutations (Fig. 3, F and G) was somewhat surprising. ESP activity leads to increased IAN production (Fig. 4, B and C) and reduced oviposition by *P. rapae* even in a *tgg1 tgg2* background (Fig. 3I). Comparison of *P. rapae* oviposition on the *cyp79B2 cyp79B3 tgg1 tgg2* and *tgg1 tgg2* plants shows that the latter are significantly more attractive to adult female *P. rapae* (Fig. 3H). One possible explanation is that *tgg1 tgg2* mutants may lack both positive and negative oviposition stimuli and that integration of these signals results in equal attractiveness for *tgg1 tgg2* and Col-0 plants. Otherwise, ESP might interact with an as yet unknown thioglucosidase to activate the breakdown of indole glucosinolates. Consistent with this hypothesis, indole glucosinolates, unlike aliphatic glucosinolates, suffer significant breakdown in macerated tissue of *tgg1 tgg2* mutants (Barth and Jander, 2006), the relative amounts of I3C and IAN that are produced in macerated tissue are similar in *tgg1 tgg2* and wild-type Col-0 (J.H. Kim and G. Jander, unpublished data), and 35S:ESP increases IAN abundance in surface washes of *tgg1 tgg2* mutant plants (Fig. 4C). At least 40 Arabidopsis genes are predicted to encode functional myrosinases and other β-glucosidases (Xu et al., 2004), and it is quite possible that one or more of these enzymes interacts with ESP and contributes to indole glucosinolate breakdown in the foliage of *tgg1 tgg2* mutant plants.

The apparent paradox of Arabidopsis producing I3C, a compound that promotes *P. rapae* herbivory, can be explained if ovipositing *P. rapae* are taking advantage of a defense system to which their own larvae are resistant but which deters other potential herbivores and pathogens. I3C is derived directly from indolylmethylisothiocyanate (Fig. 1), and isothiocyanate production generally contributes to herbivore and pathogen resistance in crucifers (Donkin et al., 1995; Lambrix et al., 2001; Agrawal and Kurashige, 2003). Further natural selection for I3C production could be provided by I3C adducts, some of which are deterrent to herbivorous insects (J.H. Kim and G. Jander, unpublished data).

Genetic variation in the foliar glucosinolate content of naturalized populations of Arabidopsis (Mauricio, 1998) suggests that this trait is under evolutionary selection. Similarly, natural variation in the expression of ESP and EPITHIOSPECIFIER MODIFIER PROTEIN1 (Zhang et al., 2006) indicates that there is selective pressure for maintaining both nitrile and isothiocyanate production in Arabidopsis populations. Sequencing of the ESP locus from several Arabidopsis accessions showed that ESP activity has been lost at least twice, once through absence of gene expression and once through a deletion in the coding region (Lambrix et al., 2001). Nevertheless, even though isothiocyanates are toxic to larvae of *P. rapae* and other Lepidoptera (Wadleigh and Yu, 1987; Li et al., 2000; Lambrix et al., 2001; Agrawal and Kurashige, 2003), many crucifers express nitrile-forming ESP.

Given that isothiocyanates are generally more toxic to insects than nitriles, it is perhaps surprising that ESP production is maintained in many isolates of Arabidopsis and other crucifers. Although the avoidance of IAN by ovipositing *P. rapae* can select for continued nitrile production, this is unlikely the only environmental cue that favors ESP expression. Additional natural selection that could account for the continued nitrile production by cruciferous plants includes: (1) IAN inhibits the growth of some phytopathogenic fungi (Pedras et al., 2002); (2) other, as yet unknown pathogens may be more sensitive to nitriles rather than isothiocyanates; (3) nitriles have toxic effects on some insects (Peterson et al., 2000; Galletti et al., 2001); (4) ESP-catalyzed nitrile production may allow plants to avoid specialist herbivores that use isothiocyanates as host recognition cues (Hovanitz et al., 1963; Bartlet et al., 1993; Ekborn, 1998; Rojas, 1999; Renwick et al., 2006); (5) volatile nitriles could provide an indirect defensive benefit, perhaps by alerting parasitoids and predators to the presence of insect herbivores; and (6) release of isothiocyanates rather than nitriles in response to damage might be more toxic to the plants themselves. Nevertheless, by demonstrating that nitrile
production by ESP-producing Arabidopsis deters *P. rapae* oviposition, we have proved strong evidence for selective pressure that can favor the production of nitriles in cruciferous plants, even though these are otherwise less deterrent than isothiocyanates for many insect herbivores.

**MATERIALS AND METHODS**

**Plant Growth and Insect Rearing**

Seeds of wild-type Arabidopsis (*Arabidopsis thaliana*) land races Col-0 and Ler were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). Col-0 transgenic lines overexpressing ESP (line 37.3; Burow et al., 2006b) and EV (line 7.1) control transformants were kindly supplied by U. Wittstock and M. Burow (University of Braunschweig, Braunschweig, Germany). The Col-0 *tgg1* *tgg2* mutant was described previously (Barth and Jander, 2006). The Col-0 *atr1D* *atr2D* and Col-0 *cyp79B2* *cyp79B3* mutant lines were kindly supplied by J. Bender (Johns Hopkins University, Baltimore, MD) and C. Celenza (Boston University, Boston).

Seeds were kept in 0.1% Phytagar (Invitrogen) for 24 h at 4°C prior to planting on Cornell mix (Landry et al., 1995) with Osmocote fertilizer (Scotts). Plants were grown in Conviron growth chambers in 20-× 40-cm nursery flats at a photosynthetic photon flux density of 200 μmol m⁻² s⁻¹ and a 16-h photoperiod. The temperature in the chambers was 23°C and the relative humidity was 50%. Plants were grown for an additional 3 weeks and used in experiments before flowering.

A colony of *Pieris rapae* (kind gift of M. del Campo, Cornell University, Ithaca, NY) was maintained on cabbage (*Brassica oleracea*) plants var. Wisconsin Golden Acre (Seedway) under the same growth chamber conditions as those that were homozygous for the absence of glucosinolate breakdown following damage to identify those that were homozygous *tgg1* *tgg2* and control plants. Subsequently, leaves of these plants were sprayed with 1 mM I3C, 0.01 mM I3C, 1 mM IAN, 0.01 mM IAN, or 80% methanol solvent control, and the number of eggs after 24 h was used as a measure of the attractiveness to adult female *P. rapae*.

**Genetic Crosses**

Arabidopsis crosses were performed as described by Weigel and Glazebrook (2002). A quadruple mutant (*cyp79B2* *cyp79B3* *tgg1* *tgg2*) was made by crossing *cyp79B2* *cyp79B3* and *lgg1* *lgg2* plants. Individual F₁ seedlings were germinated on Murashige and Skoog medium supplemented with kanamycin (25 μg mL⁻¹) to select for *tgg1* *tgg2* T-DNA insertions, transferred to soil, and screened by HPLC (Kim and Jander, 2007) for the low indole glucosinolate profile associated with *cyp79B2* *cyp79B3*. Subsequently, leaves of these plants were screened by HPLC for lack of breakdown of aliphatic glucosinolates upon tissue rupture. Similarly, crosses were made between *tgg1* *tgg2* mutant plants and plants overexpressing 35S:ESP or transformed with an EV. Plants were first selected on Murashige and Skoog agar supplemented with gentamycin (100 μg mL⁻¹) to select for the 35S:ESP construct and subsequently screened for the absence of glucosinolate breakdown following damage to identify those that were homozygous *tgg1* *tgg2* (see Supplemental Fig. S3, A–C, for glucosinolate profiles).

**Leaf Surface Washes and Identification of Intact Glucosinolates**

Fresh leaves (approximately 0.5 g) were harvested from Col-0, Ler, *cyp79B2* *cyp79B3*, 35S:ESP, and EV plants and immediately dipped into 2 mL of 100% methanol for 20 s, while keeping the cut petiole out of the solution. IAN was identified by HPLC with a UV (280 nm) and a fluorescence detector (excitation 275 nm, emission 350 nm). To test whether the leaves were damaged during these surface washes, IAN levels were observed after leaf dips of 5, 10, 15, and 20 s in either 50% or 100% methanol. In addition, chlorophyll A (647 nm) and B (660 nm) were detected photospectrophotometrically in the leaf surface washes. Intact glucosinolates were extracted as described by Kim and Jander (2007). Briefly, the leaf surface washes were bound to a Sephadex anion-exchange column, treated with 25 mM sulfamate, and eluted with 80% methanol. Desulfoglucosinolates were detected with a Waters 2695 HPLC and a Waters 2996 photodiode array detector at 229 nm, using sinigrin as an internal standard to compensate for losses during extraction.

**Extraction and Identification of IAN in Larval Regurgitant and Frass**

Third- and fourth-instar *P. rapae* larvae were starved for 10 h and subsequently allowed to feed from Arabidopsis Col-0 and *cyp79B2* *cyp79B3* plants. Fresh frass and regurgitant, collected into 100% methanol by applying moderate pressure onto larvae using flexible forceps, were processed immediately. IAN in frass and regurgitant was identified by comparing the HPLC retention time and absorption using a fluorescence detector (excitation 275 nm, emission 350 nm) with that of commercially available IAN.

**Oviposition Choice Experiments with *P. rapae***

Oviposition response experiments with *P. rapae* were always performed in a paired setup, where the Arabidopsis lines being compared were grown together at the same time in the same pot. One fertilized female *P. rapae* was provided four plants (two of each genotype) for 24 h in a 45- × 45- × 45-cm cage. Host preference was assessed by counting the number of eggs laid in 24 h. For Ler plants, which have morphological differences relative to Col-0, attractiveness was assessed using a detached-leaf assay. An equal leaf area (0.75 cm²) of each genotype being compared was mounted on the top a 200-mL Erlenmeyer flask with Parafilm (Alcan Packaging; Fig. 2B). Subsequently, these flasks were transferred to a cage with approximately 25 female *P. rapae* for 3 h. Oviposition preference was determined by counting the number of eggs deposited on the Erlenmeyer flask around leaf material. Both experimental setups give similar results for a comparison between Col-0 and *cyp79B2* *cyp79B3*. The same experimental setup was used to determine the effect of larval regurgitant on oviposition. Leaves of the low indole glucosinolate mutant *cyp79B2* *cyp79B3* were treated with 2 μL of freshly collected regurgitate. Oviposition preference was determined by counting eggs after 3 h.

To assess the attractiveness of DIC and IAN, *cyp79B2* *cyp79B3* plants were sprayed with 1 mM DIC, 0.01 mM DIC, 1 mM IAN, 0.01 mM IAN, or 80% methanol solvent control, and the number of eggs after 24 h was used as a measure of the attractiveness to adult female *P. rapae*.

**Statistical Analysis**

All results of choice experiments were tested for statistical significance in a paired t test (*α = 0.05*) for whole plants or with an unpaired t test (*α = 0.05*) for the detached-leaf assays, using SPSS10 for Windows (SPSS, 2005).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Effect of solvent concentration and duration of leaf surface dip on the detection of IAN and release of chlorophyll.

**Supplemental Figure S2.** Hatching success and larval growth on 35S:ESP and control plants.

**Supplemental Figure S3.** Glucosinolate content in progeny from crosses between 35S:ESP and *tgg1* *tgg2* plants.

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**LITERATURE CITED**


Bartlet E, Blight MM, Williams IH (1993) The responses of the cabbage seed weevil (Ceutorhynchus assimilis) to the odor of oisleed rape (Brassica napus) and to some volatile isothiocyanates. Entomol Exp Appl 68: 295–302


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Terofal F (1965) Zum Problem der Wirtsspezifität bei Pieriden (Lep.). Mitt Münch Ent Ges 55: 1–76