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Formation of Simple Nitriles upon Glucosinolate Hydrolysis Affects Direct and Indirect Defense Against the Specialist Herbivore, *Pieris rapae*

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Abstract The glucosinolate–myrosinase system, found in plants of the order Brassicales, has long been considered an effective defense system against herbivores. The defensive potential of glucosinolates is mainly due to the products formed after myrosinase-catalyzed hydrolysis upon tissue damage. The most prominent hydrolysis products, the isothiocyanates, are toxic to a wide range of organisms, including herbivorous lepidopterans. In contrast, little is known about the biological activities of alternative hydrolysis products such as simple nitriles and epithionitriles that are formed at the expense of isothiocyanates in the presence of epithiospecifier proteins (ESPs). Here, we used transgenic *Arabidopsis thaliana* (Brassicaceae) plants overexpressing ESP (35S:*ESP* plants) to investigate the effects of simple nitriles on direct and indirect defense against the specialist cabbage white butterfly *Pieris rapae* L. (Lepidoptera, Pieridae). In the 35S:*ESP* plants, glucosinolates are hydrolyzed mainly to simple nitriles upon tissue disruption, while isothiocyanates are the predominant hydrolysis products in Columbia-0 (Col-0) wild-type plants. The parasitoid *Cotesia rubecula* (Hymenoptera, Braconidae), a specialist on *P. rapae* larvae, was significantly more attracted to *P. rapae*-infested 35S:*ESP* plants than to *P. rapae*-infested Col-0 wild-type plants in a wind tunnel setup. Furthermore, female *P. rapae* butterflies laid more eggs on Col-0 wild-type plants than on 35S:*ESP* plants when the plants had been damaged previously. However, when given a choice to feed on 35S:*ESP* or Col-0 plants, caterpillars did not discriminate between the two genotypes. Growth rate and developmental time were not significantly different between caterpillars that were reared on 35S:*ESP* or Col-0 plants. Thus, the production of simple nitriles instead of isothiocyanates, as catalyzed by ESP, can promote both direct and indirect defense against the specialist herbivore *P. rapae*.

Keywords Glucosinolate · Epithiospecifier protein · *Arabidopsis thaliana* · Nitrile · Isothiocyanate · Insect performance · *Pieris rapae* · Oviposition preference · *Cotesia rubecula* · Indirect defense

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Introduction

The glucosinolate–myrosinase system is one of the best-characterized antiherbivore defenses in the plant kingdom. Common to all families of the Brassicales order, it is found in agriculturally important *Brassica* crops and the model plant *Arabidopsis thaliana* (Fahey et al. 2001; Wittstock and Halkier 2002; Kliebenstein et al. 2005; Grubb and Abel 2006; Halkier and Gershenzon 2006). The system consists of glucosinolates, a group of amino acid-derived thioglucosides and myrosinases (thioglucoside glucohydrolases, EC 3.2.1.147) that hydrolyze the thioglucosidic bond of the glucosinolates yielding glucose and an unstable aglucone

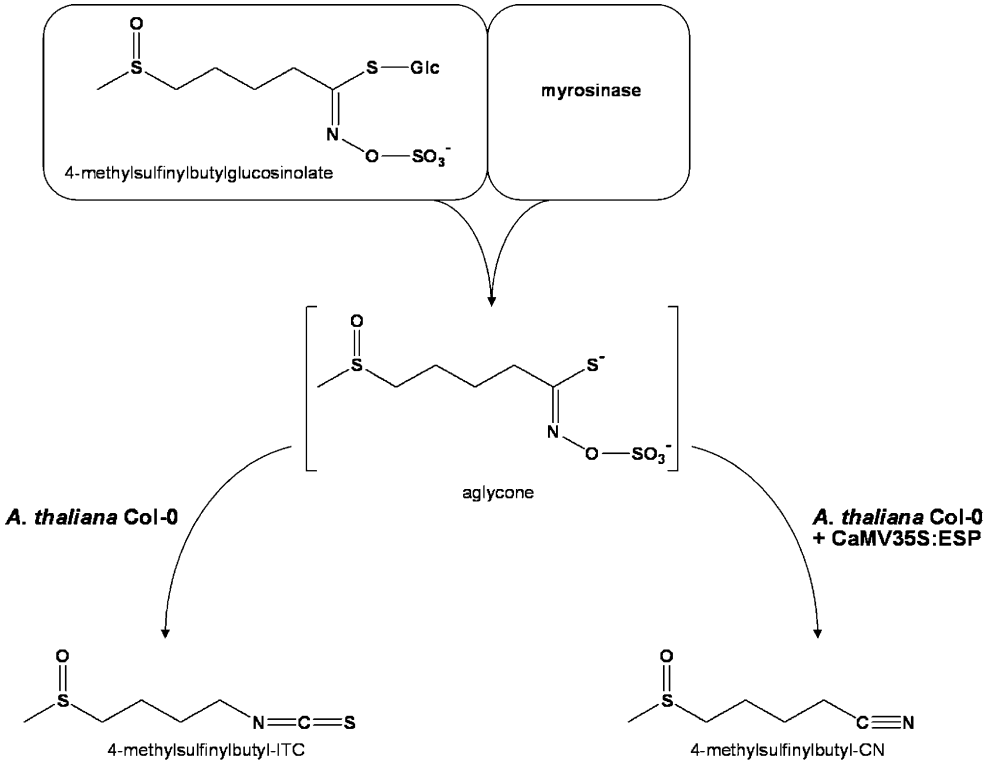

Fig. 1 Glucosinolate hydrolysis in *A. thaliana* Col-0 wild-type and 35S:*ESP* plants as shown for 4-methylsulfinylbutylglucosinolate, the main glucosinolate found in the Col-0 ecotype. Glucosinolates and their hydrolytic enzymes, the myrosinases, are stored in different cells in intact plant tissue. Damage to the tissue results in myrosinase-catalyzed hydrolysis of glucosinolates yielding glucose and unstable aglucones. In the ecotype Col-0, these aglucones predominantly rearrange into isothiocyanates due to the absence of a functional epithiospecifier protein. Expression of ESP from the *Arabidopsis* ecotype L*er* under the control of the constitutive CaMV35S promoter results in nitrile formation upon leaf damage

(Fig. 1) (Rask et al. 2000). Spontaneous rearrangement of the aglucone then leads to the formation of an isothiocyanate. Under the influence of specifier proteins that are present in many glucosinolate-containing plants including *Brassica napus* and some *Arabidopsis* accessions, alternative products such as simple nitriles and epithionitriles are formed at the expense of isothiocyanates (Bernardi et al. 2000; Foo et al. 2000; Lambrix et al. 2001; Wittstock and Burow 2007). Glucosinolate hydrolysis in intact plant tissue is prevented by spatial separation of glucosinolates and myrosinases through storage in different cells and thus occurs only upon tissue disruption, e.g., by a feeding herbivore (Andréasson and Jørgensen 2003).

The glucosinolate–myrosinase system is involved in defense responses against herbivorous insects of different feeding guilds (Kliebenstein et al. 2002; Agrawal and Kurashige 2003; Wittstock et al. 2003; Kliebenstein 2004; Mewis et al. 2005; Barth and Jander 2006; Kim and Jander 2007). Its role in direct defense has been attributed mainly to the isothiocyanates, which are the predominant hydrolysis products in many plant species. Isothiocyanates are toxic upon ingestion, contact, or when present in the gas phase (Agrawal and Kurashige 2003; Wittstock et al. 2003). However, the glucosinolate–myrosinase system appears to differentially affect specialist and generalist herbivores (Giamoustaris and Mithen 1995; Li et al. 2000). Several specialist insects have developed counteradaptations to circumvent the toxic effects of glucosinolates and their hydrolysis products (Müller et al. 2001; Alibadi et al. 2002; Ratzka et al. 2002; Wittstock et al. 2004; Agerbirk et al. 2006; Vergara et al. 2006; Wheat et al. 2007). Moreover, glucosinolates and isothiocyanates often serve as attractants or oviposition and feeding stimulants for these insects (Van Loon et al. 1992; Huang and Renwick 1994; Wittstock et al. 2003; Miles et al. 2005; Schoonhoven et al. 2005; Renwick et al. 2006; Barth and Jander 2006; Smallegange et al. 2007). Still, several studies have shown negative correlations between the glucosinolate content of the diet and the larval performance of herbivorous insects specialized on Brassicaceae (Mewis et al. 2005, 2006; Gols et al. 2007, 2008; Kim and Jander 2007).

Little is known about the biological activities of hydrolysis products other than isothiocyanates. In general, simple nitriles are considered less toxic than isothiocyanates (Lambrix et al. 2001; Wittstock et al. 2003; Burow et al. 2006b). For example, larvae of the generalist *Tricho-*

plusia ni (cabbage looper, Lepidoptera, Plusiinae) feed more, and larvae of the generalist *Spodoptera littoralis* (Egyptian cotton leafworm, Lepidoptera, Noctuidae) perform better on nitrile-producing *Arabidopsis* plants than on isothiocyanate-producing plants (Lambrix et al. 2001; Burow et al. 2006b; Zhang et al. 2006). Given that insect herbivores seem to suffer less from simple nitriles than from isothiocyanates, the ecological rationale for nitrile formation in the Brassicaceae is not easy to understand. Different scenarios can be drawn in which a plant may benefit from producing nitriles. For example, nitrile formation could be advantageous in direct and indirect defense against specialized herbivores. Direct plant defense negatively affects the feeding stages or the egg deposition of the herbivore, whereas indirect defense acts by recruiting natural enemies of the herbivore, such as predators and parasitoids (Hilker and Meiners 2002). Since isothiocyanates are often exploited as attractants, oviposition, or feeding stimulants by specialized herbivores (Rask et al. 2000; Renwick 2002; Wittstock et al. 2003; Renwick et al. 2006), plants that do not produce isothiocyanates upon damage may become less apparent to specialists. Alternatively, carnivorous insects such as predators and parasitoids may be differentially attracted by isothiocyanates and nitriles. A few studies have demonstrated that parasitoids are attracted to isothiocyanates (Titayavan and Altieri 1990; Pivnick 1993; Murchie et al. 1997; Bradburne and Mithen 2000; Reddy et al. 2002; Blande et al. 2007). However, parasitoid and predator performance is also known to correlate negatively with glucosinolate content (Harvey et al. 2003; Kazana et al. 2007; Gols et al. 2008). This negative relationship might favor a behavioral response of predators and parasitoids to plant volatiles that contain simple nitriles instead of isothiocyanates.

Because the glucosinolate–myrosinase system is an activated plant defense, the role of specific glucosinolate hydrolysis products is difficult to study in experimental settings. Plants that differ in the type of hydrolysis products they form often also differ in other properties such as glucosinolate content or myrosinase activity. The use of synthetic compounds alone or in mixtures to compare the defensive role of certain hydrolysis products also can be misleading due to differences in volatility. Moreover, many of them cannot be obtained as pure compounds. However, the availability of molecular tools to genetically manipulate model plants such as *Arabidopsis* provide an opportunity to overcome these difficulties (e.g., Degenhardt et al. 2003; Snoeren et al. 2007). The use of transgenic *Arabidopsis* lines that have been modified in glucosinolate hydrolysis provide a more natural system to compare the effects of naturally occurring relative and absolute amounts of members of different types of glucosinolate hydrolysis products.

Arabidopsis accessions differ in the type of glucosinolate hydrolysis products they produce upon tissue damage. This is due to allelic variation at the locus that encodes the epithiospecifier protein (ESP, Lambrix et al. 2001). For example, plants of the Landsberg *erecta* (L*er*) accession possess a functional *ESP* gene and produce predominantly simple nitriles upon tissue disruption, while isothiocyanates are the major glucosinolate hydrolysis products of the Columbia-0 (Col-0) accession that lacks functional ESP (Lambrix et al. 2001). We used a transgenic line of *Arabidopsis* Col-0, which expresses the *ESP* cDNA from the L*er* accession under the control of the CaMV35S promoter (35S:*ESP* plants; Burow et al. 2006b). In the 35S:*ESP* plants, glucosinolates are predominantly hydrolyzed to simple nitriles, whereas Col-0 wild-type plants mainly produce isothiocyanates upon damage (Burow et al. 2006b). For example, in Col-0 wild-type plants, the hydrolysis of 4-methylsulfinylbutylglucosinolate, the most abundant glucosinolate in the rosette leaves of this ecotype, leads to the formation of 4-methylsulfinylbutyl isothiocyanate. If ESP is overexpressed, however, the corresponding nitrile is produced (Fig. 1). However, neither the glucosinolate profile, the myrosinase activity levels, nor the morphology is altered in 35S:*ESP* plants compared with Col-0 wild type (Burow et al. 2006b).

In the present study, we used the 35S:*ESP* plants to investigate the role of simple nitriles in the interaction between *Arabidopsis*, the specialist cabbage white butterfly *Pieris rapae* L. (Lepidoptera, Pieridae), and its specialist larval endoparasitoid *Cotesia rubecula* (Marshall) (Hymenoptera, Braconidae). *P. rapae* is one of the most abundant butterflies in Northern and Central Europe and is neurophysiologically and biochemically adapted to using glucosinolate-containing plants as its sole hosts (Renwick 2002; Wittstock et al. 2004; Schoonhoven et al. 2005; Braby and Trueman 2006, and references therein). Most interestingly, caterpillars of *P. rapae* excrete simple nitriles in their feces (Agelopoulos et al. 1995) due to the action of a midgut nitrile-specifier protein that directs the hydrolysis of ingested glucosinolates from isothiocyanate towards simple nitrile formation (Wittstock et al. 2004; Burow et al. 2006a). *P. rapae* is able to complete its development on *Arabidopsis* (Van Loon et al. 2000; Harvey et al. 2007).

Volatiles of different plant species, including *Arabidopsis*, that are infested with *P. rapae* caterpillars are known to attract *C. rubecula* females (Agelopoulos and Keller 1994a; Geervliet et al. 1994, 1996; Van Poecke et al. 2001). The headspace of *P. rapae*-infested *Arabidopsis* plants contains nitriles that may originate from the frass of the caterpillars or the wounded plant tissue (Van Poecke et al. 2001; Van Poecke 2007; Wittstock et al. 2004). The feces of *P. rapae* caterpillars are attractive to *C. rubecula* from a distance, and their headspace contains predominantly nitriles (Agelopoulos

et al. 1995; Wittstock et al. 2004). Electrophysiological studies have demonstrated that antennae of *C. rubecula* respond to certain isothiocyanates and nitriles (J.J.A. van Loon, unpublished results; Smid et al. 2002). Therefore, we used wind tunnel experiments to test whether *Arabidopsis* plants that overexpress ESP are more attractive to *C. rubecula* than the isothiocyanate-producing Col-0 wild-type plants.

Isothiocyanates are involved in long-distance host recognition by a number of specialized insect herbivores (reviewed in Wittstock et al. 2003). Therefore, we investigated whether the higher proportion of nitriles formed in 35S:*ESP* plants as compared to wild-type plants influences the oviposition behavior of *P. rapae* butterflies. Glucosinolates stimulate egg deposition by *P. rapae* upon contact, but in nature butterflies might also be exposed to glucosinolate hydrolysis products, e.g., when host plants are already infested with feeding caterpillars (e.g., Huang et al. 1993, 1994). Additionally, we tested whether the feeding preference and performance of *P. rapae* differs between 35S:*ESP* plants and Col-0 wild-type plants. Despite the known glucosinolate detoxification mechanism in the midgut of *P. rapae* caterpillars, high concentrations of isolated isothiocyanates reduce *P. rapae* caterpillar growth and survival (Agrawal and Kurashige 2003). Combining these diverse bioassays, we aimed at obtaining a better insight into the contribution of nitriles to the relationships of *P. rapae* with its host plant and its enemies.

Methods and Materials

Plants *A. thaliana* Col-0 and the transgenic line 35S:*ESP* (2.6, T4; in the Col-0 background; Burow et al. 2006b) used for wind tunnel and oviposition experiments were grown from seed in soil (Lentse Potgrond, Cuijk, the Netherlands) in a controlled-climate room at 21 ± 1°C, a L8:D16-h photoperiod, 80–110μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and 55 ± 5% relative humidity (RH). Two-week-old seedlings were transferred to pots (Teku, Pöppelmann, Lohne, Germany) containing the same soil. Plants used for the experiments were 4–6-week-old and in the vegetative stage. Plants used to test the feeding preference and larval performance of *P. rapae* were grown under similar conditions for 4–5weeks except that the photoperiod was a L10:D14-h photoperiod and the relative humidity was 60 ± 5%.

Insects A continuous rearing of *P. rapae* for oviposition preference tests was maintained on Brussels sprouts plants in a climatized room at 21 ± 1°C, 60 ± 10% RH and an L16:D8-h photoperiod. *P. rapae* larvae used for feeding preference tests and larval performance tests were raised under similar conditions except that relative humidity was 75 ± 5%. The parasitoid *C. rubecula* was reared on *P. rapae* caterpillars feeding on Brussels sprouts plants in a greenhouse at 24 ± 4°C, 60 ± 20% RH, and a L16:D8-h photoperiod. For experiments, *C. rubecula* pupae were collected and kept in a cage in a climate cabinet (23 ± 1°C, 60 ± 10% RH, and L16:D8-h photoperiod). Emerging wasps were provided with water and honey. Male and female wasps were kept together until the experiment.

Plant Treatments for Parasitoid and Oviposition Experiments Plants were either artificially damaged or infested with ten first-instar *P. rapae* caterpillars. Plants were infested for 24 h and were kept in a climate chamber at 21 ± 1°C, a L8:D16-h photoperiod, 80–110μmol m^{-2} s^{-1} photosynthetically active radiation, and 55 ± 5% RH until the experiments. Artificially damaged plants were obtained by punching a ~7-mm^2 hole into each of six leaves of a plant right before the experiment. Intact *Arabidopsis* plants served as controls.

Wind Tunnel Experiments Behavioral choice experiments with the parasitoid *C. rubecula* were done with a wind tunnel setup (25 ± 1°C, 60 ± 10% RH, 35μmol m^{-2} s^{-1} PAR) as described by (Geervliet et al. 1994). The wind speed was adjusted to 0.2m s^{-1}. Two-choice experiments were conducted by placing two odor sources each consisting of six *Arabidopsis* plants at the upwind end of the tunnel as described by Van Poecke et al. (2001). Naïve *C. rubecula* females (without oviposition experience) were separated from males 3h prior to the experiment and transferred to another cage that was placed in the experimental room to acclimatize the parasitoids to the new environment. The wasps were individually introduced into the wind tunnel on *Arabidopsis* leaves of the treatments that they were also exposed to in the wind tunnel. These leaves were used in an alternating way, i.e., in the case that *P. rapae*-infested Col-0 wild-type was tested against caterpillar-infested 35S:*ESP* plants, the first, third, fifth, etc. wasp was introduced on an Col-0 wild-type leaf from which the caterpillars and their products had been carefully removed. Consequently, the second, fourth, sixth, etc. tested parasitoid was introduced on an 35S:*ESP* leaf from which the caterpillars and their products had been carefully removed as well. This increases the general behavioral response of the parasitoids to plant cues but likely does not induce a shift of preference (Kaiser and Cardé 1992; Bleeker et al. 2006; Smid 2006). Wasps were allowed to walk onto the leaves themselves. The leaf with the wasp was placed at the middle of the release cylinder, which was 60cm downwind from the two odor sources. As soon as the parasitoid had left the leaf for a few seconds, the leaf was carefully removed with tweezers without

disturbing the parasitoid. The flight behavior of the wasps was observed. Flights that resulted in a landing on one of the two odor sources were recorded as a "choice." Parasitoids that did not leave the release cylinder or landed on other parts of the wind tunnel within 10min were recorded as "no choice." Every parasitoid was used once. In order to correct for unforeseen asymmetry in the setup, the position of the odor sources was swapped after five tested parasitoids. After testing ten parasitoids, the odor sources were replaced with new ones. Every experiment was repeated at least five times over the course of several days. In order to check whether parasitoid discrimination was influenced by the amount of the caterpillar feeding, the leaf area removed by caterpillars from three infested Col-0 wild-type plants and three 35S:*ESP* plants from three experimental days was analyzed using the program ImageJ 1.37v (http://rsb.info.nih.gov/ij/).

Oviposition Preference Test Freshly emerged *P. rapae* adults were transferred to a large cage (67 × 100 × 75cm) in a greenhouse compartment at 24 ± 4°C, 60 ± 20% RH, and an L16:D8-h photoperiod. Butterflies were provided with 10% sucrose solution. Three to 5 days after emergence, one male and one female butterfly were transferred to each of the oviposition cages (67 × 50 × 75cm) in the same greenhouse compartment. Each butterfly couple was also provided with a 10% sucrose solution. In addition to natural daylight, the cages were illuminated by sodium vapor lamps (SON-T, 500W, Philips, the Netherlands) from 10:00a.m. to 4:00p.m. At 48h prior to the experiment, a single untreated Brussels sprouts leaf was placed in each cage as an oviposition substrate. After 6h, the leaf was removed. On the experimental day between 10:00a.m. and 11:00a.m., a transgenic 35S:*ESP* plant and a Col-0 wild-type plant were placed approximately 40cm apart from each other in each cage. In one experiment, both plants were intact, and in a second experiment both plants were artificially damaged. *P. rapae* was allowed to lay eggs on the two plants for 5 h. The plants were then removed, and the eggs laid were counted. The experiments were conducted in several cages at the same time and on several days per treatment, each replicate with new plants and butterflies.

P. rapae *Feeding Preference Test* Feeding choice tests were carried out in a climate chamber with an L16:D8-h photoperiod, 75 ± 5% RH, and a temperature of 21 ± 1°C. For each replicate, three Col-0 plants and three 35S:*ESP* plants were alternately arranged in a circle. A single *P. rapae* third instar was released in the center of each arena at the level of the leaf rosettes, and the larvae were allowed to feed for 24 h. A total of 18 replicates was carried out on three experimental days with independently grown sets of plants. Leaf areas removed from the three wild-type plants and the three 35S:*ESP* plants in each arena were measured. Leaf rosettes were digitally photographed with a reference mark before and after the experiments to calculate the removed leaf area using the program ImageJ 1.37v (http://rsb.info.nih.gov/ij/).

P. rapae *Performance and Developmental Studies* To compare the performance of *P. rapae* on the 35S:*ESP* plants and Col-0 wild-type plants, larvae were reared from emergence to pupation on 5-week-old 35S:*ESP* plants in a climate chamber (21 ± 1°C, L16:D8-h photoperiod, 75 ± 5% relative humidity). Single newly emerged larvae (<24 h old) were placed on individual plants that were then covered with perforated plastic bread bags to prevent insects from escaping. The weight of each larva was recorded on day 7 after hatching, when the larvae were transferred to fresh plants for the first time, and again on day 10. The larvae were transferred to new plants three to four times depending on their consumption rate. One to 2 days after pupation, the pupae were removed from the plants and placed individually in small ventilated plastic vessels. The emergence of adults was monitored every day for a period of 30 days.

Statistical Analysis In the wind tunnel experiments, a *binomial test* was used to analyze whether the behavioral choices of the parasitoids differed from a 50:50 distribution between the two odor sources. Parasitoids that did not make a choice were excluded from the statistical analysis. To check whether introducing the parasitoids on different leaves influenced the subsequent choice of the parasitoids, a *McNemar test* for marginal homogeneity was applied. In the oviposition choice tests, most individuals of *P. rapae* laid eggs on both the Col-0 wild-type and the 35S:*ESP* plant. The number of eggs on each treatment per individual were considered as a paired sample and were analyzed with the nonparametric *Wilcoxon signed ranks test*. In addition, we also analyzed the egg incidence by using a *sign test*. For the feeding choice experiments with *P. rapae*, the removed leaf area from each of the three Col-0 wild-type and the three 35S:*ESP* plants of each replicate were pooled, and a mean was calculated. A *Wilcoxon signed ranks test* was applied to test whether means were statistically different. *Mann–Whitney U tests* were applied to test for differences in performance-related parameters between Col-0 wild-type and 35S:*ESP* plants. All tests are described by Glantz (2005), and for some tests the statistical software package SPSS 12.0.1 (SPSS Inc., Chicago, IL, USA) was used.

Results

Behavioral Response of C. rubecula *to Volatiles of ESP Overexpressing and Wild-Type Arabidopsis Plants. P.*

rapae-caterpillar-infested 35S:*ESP* plants attracted more *C. rubecula* parasitoids than caterpillar-infested Col-0 plants in a two-choice wind tunnel setup (*binomial test*, $p < 0.05$; Fig. 2). However, when artificially damaged before the experiment, 35S:*ESP* plants attracted significantly fewer parasitoids than caterpillar-infested Col-0 wild-type plants (*binomial test*, $p < 0.05$; Fig. 2). When caterpillar-infested 35S:*ESP* plants were compared with artificially damaged ones, more parasitoids landed on the caterpillar-infested plants (*binomial test*, $p < 0.005$; Fig. 2). However, artificially damaged 35S:*ESP* plants were more attractive to *C. rubecula* than intact plants of the same line (*binomial test*, $p < 0.001$; Fig. 2). The introduction of the parasitoids to the wind tunnel on leaves of either Col-0 or 35S:*ESP* plants did not bias their subsequent choice but stimulated the general response of the wasps to plant volatiles (data not shown, *McNemar test*, $p > 0.05$). The leaf area removed by the caterpillars did not differ between 35S:*ESP* and Col-0 wild-type plants (35S:*ESP*: $1.62 \pm 0.11 cm^2$, Col-0: $1.61 \pm 0.22 cm^2$; mean ± SE, $N = 18$; *Mann–Whitney U test*, $p > 0.4$).

Oviposition Preference of P. rapae *P. rapae* lays individual eggs that are usually distributed over the plants. In general, *P. rapae* females began to lay eggs soon after being exposed to the experimental plants, and they deposited eggs on both the transgenic and the wild-type plant in more than 99.5% of all replicates. When plants were undamaged, ovipositing butterflies did not discriminate between 35S:*ESP* and Col-0 wild-type plants (Fig. 3): the average number of eggs laid per female did not differ between plant genotypes (*Wilcoxon signed ranks test*, $p > 0.05$, $N = 18$, Fig. 3). In 56% of the cases, the butterflies laid more eggs on the wild-type plant, while in 44% they deposited more eggs on the 35S:*ESP* plant (*sign test*, $p > 0.05$, $N = 18$). However, the situation changed when plants were artificially damaged before being exposed to the butterflies. In this case, the butterflies significantly preferred to lay eggs on wild-type plants. In 81% of the cases, *P. rapae* laid more eggs on Col-0 plants than on the 35S:*ESP* plants (*sign test*, $p < 0.01$, $N = 22$, Fig. 3). This trend is also reflected in the significantly larger number of eggs laid per female on wild-type plants (Fig. 3). Overall, when plants were damaged, the nitrile-producing 35S:*ESP* plants were significantly less attractive for egg deposition by *P. rapae* than the isothiocyanate-producing wild-type Col-0.

Feeding Choice Experiments and Performance of P. rapae The next step was to test whether caterpillars show the same preference as adult female butterflies. In a multiple-choice experiment, young *P. rapae* caterpillars were allowed to choose between three 35S:*ESP* plants and three Col-0 wild-type plants for 24h. All tested caterpillars fed on both the transgenic and the wild-type plants. There was no significant difference in the mean leaf area removed between 35S:*ESP* and wild-type plants (35S:*ESP*: 7.0 ± 4.7 cm^2, Col-0: 6.1 ± 3.6 cm^2; $N = 18$; *Mann–Whitney U test*, $p > 0.05$). Furthermore, we analyzed the performance of *p. rapae* caterpillars on the 35S:*ESP* line and on wild-type plants. We recorded the larval weight after only 7 and 10

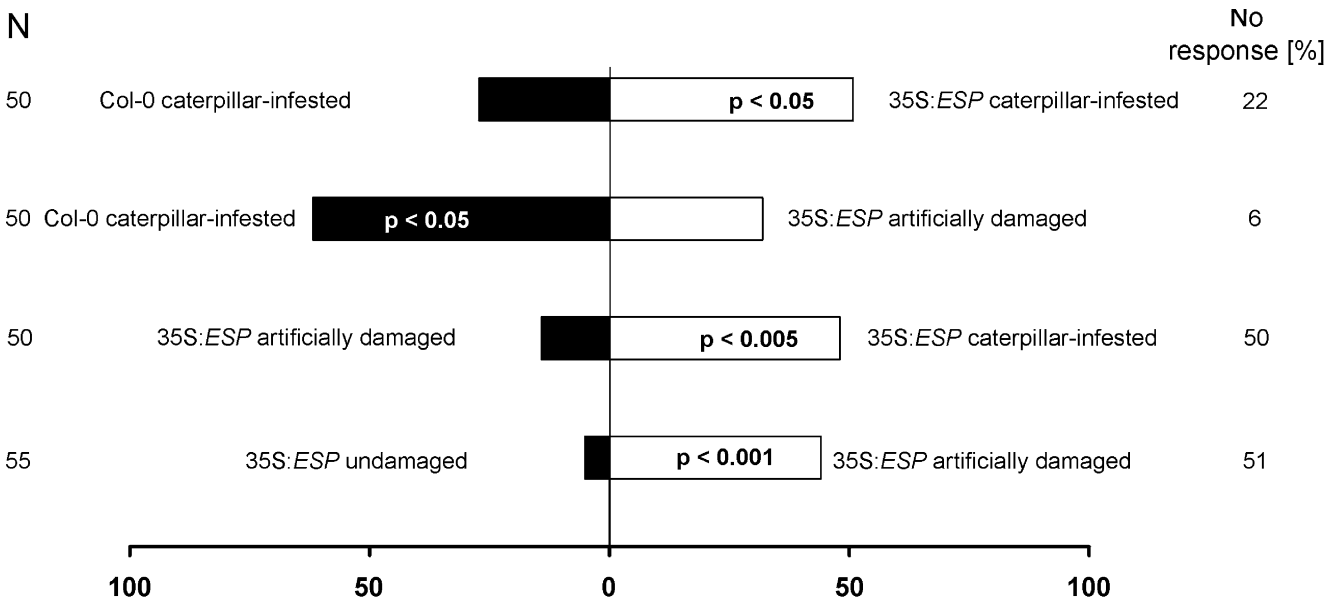

Fig. 2 Response of *C. rubecula* to odors of wild-type *Arabidopsis* Col-0 and transgenic 35S:*ESP* plants in a two-choice wind tunnel setup. Plants were either intact, artificially damaged right before the experiment, or infested with *P. rapae* caterpillars for 24 h. Parasitoids that did not make a choice were not included in the analysis. Choices between odor sources were analyzed using a *binomial test*

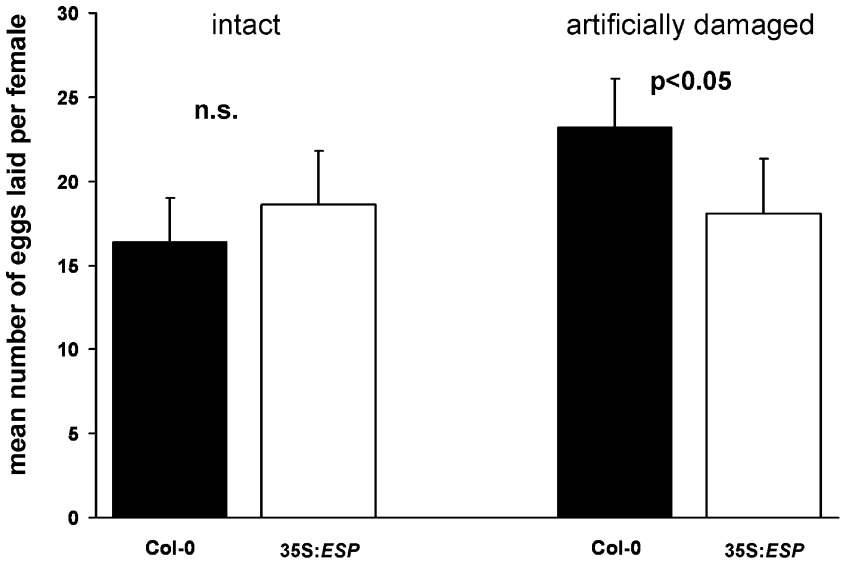

Fig. 3 Oviposition by *P. rapae* on wild-type *Arabidopsis* Col-0 (*black bars*) and transgenic 35S:*ESP* plants (*white bars*). Plants were either intact (*left*) or artificially damaged (*right*) before the experiment. Depicted is the mean number of eggs laid per female+standard error (analyzed using the *Wilcoxon signed ranks test* (N=18 with intact plants, N=22 with damaged plants)

days as differences in food quality at this early stage have a particularly strong effect on caterpillar growth and development (J.J.A. van Loon, personal communication). However, larval weight of caterpillars did not significantly differ between the plant genotypes at day 7 or 10 (Table 1). Likewise, pupal weight, developmental time until pupation, and the total developmental time were not significantly different between *P. rapae* caterpillars that fed on either 35S:*ESP* or Col-0 wild-type plants (Table 1).

Discussion

Our study demonstrates that glucosinolate-containing plants can benefit from producing predominantly simple nitriles instead of isothiocyanates upon herbivore damage despite the fact that simple nitriles are less toxic than isothiocyanates to insect herbivores. The experiments described employed a transgenic 35S:*ESP* line that upon tissue maceration hydrolyzes glucosinolates to simple nitriles rather than to isothiocyanates as in Col-0 wild-type plants (Burow et al. 2006b). Despite the differences in glucosinolate hydrolysis, there were no significant alterations between 35S:*ESP* and wild-type plants in glucosinolate content and composition and myrosinase activity (Burow et al. 2006b). The nitrile-producing 35S:*ESP* plants attracted significantly more *C. rubecula* parasitoids than Col-0 wild-type plants when both were infested by *P. rapae* (Fig. 2).

Simple nitriles are not only released by plants but are also the predominant volatiles emitted by the frass of *P. rapae* caterpillars, which is known to attract *C. rubecula* from a distance (Agelopoulos and Keller 1994b; Geervliet et al. 1994; Agelopoulos et al. 1995; Wittstock et al. 2004). Hence, nitrile-producing plants such as 35S:*ESP* plants may indicate the presence of feeding and defecating host larvae to reinforce the response of the parasitoids to *P. rapae*-induced volatiles (Fig. 2). This explanation is supported by a previous study showing that a combination of volatiles from *P. rapae*-infested cabbage and *P. rapae* feces was more attractive to *C. rubecula* than those of caterpillar-infested plants alone depending on the infestation level (Agelopoulos et al. 1995). In the present study, significantly more parasitoids were attracted to infested 35S:*ESP* and infested Col-0 plants compared to artificially damaged 35S:*ESP* plants. This demonstrates that the volatile blend emitted upon herbivory, including plant volatiles and volatiles derived from the herbivore, is more attractive to *C. rubecula* than volatiles emitted from the plant after mechanical damage (Fig. 2). Volatile blends of mechanically damaged 35S:*ESP* plants are most likely dominated by green leaf volatiles and simple nitriles, whereas the blend of caterpillar-infested plants is more complex, including also terpenoids and methyl salicylate (Van Poecke et al. 2001; Burow et al. 2006b). Indeed, *C. rubecula* parasitoids show electroantennogram responses to terpenoids and methyl salicylate in addition to responses to green leaf volatiles (Smid et al. 2002), indicating that these compounds have an important role in attracting the parasitoids as well. That the attraction of parasitoids is beneficial to the reproductive fitness of *A. thaliana* was demonstrated previously (Van Loon et al. 2000).

Many parasitoids change their behavioral preference to certain infochemicals by associative learning of these odors after a rewarding experience, e.g., an oviposition (Turlings et al. 1993; Vet et al. 1995). Also, *C. rubecula* can learn associatively to respond to certain plant odors either after an oviposition experience or by simply having contact with

Table 1 Performance of *P. rapae* on *Arabidopsis* Col-0 and 35S:*ESP*

	Col-0 wt (*N*=39)	35S:*ESP* (*N*=37)	Mann–Whitney *U* test (*p* value)
Larval weight on day 7 [mg]	24±2.1[a]	27±1.8	>0.05
Larval weight on day 10 [mg]	150±7.7	157±5.8	>0.05
Pupal weight [mg]	146±2.1	141±3.0	>0.05
Time until pupation [d]	12.3±0.2	12.2±0.2	>0.05
Total developmental time [d]	19.4±0.2	19.2±0.2	>0.05

[a] Data are presented as means±standard error

host products. However, unlike the situation in the closely related parasitoid species *Cotesia glomerata*, learning in *C. rubecula* results in an increased behavioral response towards the learned odor but does not shift an innate preference towards another odor (Bleeker et al. 2006; Smid 2006). This is confirmed by results of our study because introducing the parasitoids into the wind tunnel on a host-damaged leaf, alternating between a wild-type and a transgenic leaf, did not bias the subsequent choice to the leaf type they previously experienced. Moreover, giving *C. rubecula* females an oviposition experience in the presence of the odor of one of the two plant types would likely not result in a preference for that learned odor (Bleeker et al. 2006; Smid 2006).

P. rapae females laid significantly fewer eggs on mechanically damaged 35S:*ESP* plants than on mechanically damaged Col-0 wild-type plants. However, there was no preference when plants were undamaged suggesting that glucosinolate hydrolysis products rather than the glucosinolates themselves influenced plant acceptance by *P. rapae* (Fig. 3). De Vos et al. (2008) showed that ovipositing *P. rapae* preferred isothiocyanate-producing *Arabidopsis* Col-0 plants over 35S:*ESP* plants, even when the plants were not damaged. Numerous studies have shown that the nonvolatile glucosinolates serve as oviposition stimulants for *P. rapae* and closely related species (overview given by Chew and Renwick 1995; Hern et al. 1996; Schoonhoven et al. 2005). However, given that most glucosinolate hydrolysis products are volatile, while the parent glucosinolates are not, the hydrolysis products might be important cues for *P. rapae* at an earlier stage of the host location process. In fact, isothiocyanates in particular are known to attract many specialist herbivorous species (reviewed by Wittstock et al. 2003) and even to stimulate oviposition (Renwick et al. 2006). On the other hand, only a few studies have demonstrated that nitriles attract herbivores, and in general these were less attractive than isothiocyanates (Pivnick et al. 1992; Bartlet et al. 1997; Smart and Blight 2000; De Vos et al. 2008). Whether the preference of *P. rapae* to oviposit on isothiocyanate-emitting wild-type plants is due to the repellent effect of nitriles or to the stimulating effect of isothiocyanates or both remains to be investigated. However, oviposition preference of *P. rapae* for isothiocyanate-emitting wild-type plants is likely to benefit the butterflies either because isothiocyanates would indicate the presence of a (glucosinolate-containing) host plant or because nitrile emission could indicate a host plant that is already infested with conspecific larvae. Butterflies that avoid laying eggs on already infested host plants should reduce intraspecific competition and parasitism (Thompson and Pellmyr 1991). In fact, *P. rapae* is known to avoid laying eggs on plants that are infested with conspecific larvae or that carry conspecific eggs (Rothschild and Schoonhoven 1977; Schoonhoven et al. 1990; Sato et al. 1999). Nitriles, acting as indicators of feeding and defecating larvae, may not only signify the presence of competitors but may also hint at increased levels of glucosinolates and other defense compounds in the host plant as a result of induction that have a negative influence on the performance of the offspring (e.g., Agrawal and Kurashige 2003; Mewis et al. 2005, 2006; Gols et al. 2008). Accordingly, cabbage plants that were treated with jasmonic acid, a key hormone involved in induced plant defense, received significantly fewer eggs from *P. rapae* than respective control plants (Bruinsma et al. 2007).

The preference of *P. rapae* to oviposit on plants emitting isothiocyanates is likely due to the fact that isothiocyanate release indicates a functioning glucosinolate–myrosinase system. As many herbivores are not as well adapted to the glucosinolate–myrosinase system, the preference for a glucosinolate-containing plant by *P. rapae* butterflies is one key to avoid interspecific competition. Our present results show that *P. rapae* performed just as well on the isothiocyanate-producing Col-0 wild-type as on the nitrile-producing 35S:*ESP* line. In contrast, generalist herbivores are known to perform significantly better on nitrile-producing plants vs. isothiocyanate-producing plants (Lambrix et al. 2001; Burow et al. 2006b). *P. rapae* butterflies thus may be able to use isothiocyanates to select competitor-sparse food plants for their offspring (De Vos et al. 2008). To recap, gravid *P. rapae* females foraging for suitable host plants may have a number of reasons to prefer isothiocyanate-producing vs. nitrile-producing plants as an oviposition site. The release of nitriles upon damage may signal a higher risk of interspecific and intraspecific competition, an increased chance of parasitism, and a poorer substrate for larval

development. Alternatively, nitrile-producing plants might receive fewer eggs due to the lack of isothiocyanates as oviposition stimulus.

In summary, this paper has demonstrated that *A. thaliana* plants producing simple nitriles rather than isothiocyanates are better defended against the specialist herbivore, *P. rapae*, because of reduced oviposition and increased attraction of a specialist larval parasitoid. In contrast, isothiocyanate-emitting plants appear to be better defended against generalist herbivores. These findings provide a rationale for the existence of a polymorphism among *A. thaliana* ecotypes (Lambrix et al. 2001). The selective balance between these glucosinolate hydrolysis phenotypes may shift depending on the proportion of specialist to generalist herbivores present.

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