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Glucosinolates and Their Hydrolysis Products in *Arabidopsis thaliana* Influence Performance and Feeding Choice of *Pieris rapae* and *Spodoptera exigua*

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Abstract

Glucosinolates and their hydrolysis products, found in plants of the order Brassicales, are well-known for their defensive properties against insect herbivores. *Arabidopsis thaliana* (Col-0) genetic lines with mutations that modify the type of glucosinolates (*i.e. myb28myb29* and *cyp79B2cyp79B3* are deficient in the production of aliphatic and indolyl glucosinolates, respectively) make it possible to test for the specific effects of these secondary chemicals on insect herbivores. The *Pad3* mutant (deficient in camalexin), which has a role in resistance to pathogens, was also tested. Likewise, the effects of different glucosinolate hydrolysis products can be evaluated using genetically modified (GM) lines of the wild type Col-0 ecotype, which naturally produces isothiocyanates. These GM lines include the nitrile-producing 35S:*ESP* and the double knockout *tgg1tgg2*, which virtually lacks hydrolysis products. In both no-choice and choice experiments, the crucifer specialist *Pieris rapae* was virtually unaffected by differences in the type of glucosinolates or hydrolysis products. In contrast, the generalist insect *Spodoptera exigua* had statistically significant increases in pupae/adult weight and faster developmental times when reared on mutants deficient in the production of aliphatic and indolyl glucosinolates and their hydrolysis products. There were no differences in the performance of either insect species when reared on wild type Col-0 or *Pad3*. Results from feeding choice trials showed that *Pieris rapae* had no statistically significant preference for any of the genetic lines. In contrast, *Spodoptera exigua* had a significant feeding preference for the double mutant *tgg1tgg2*. This study provides evidence that variation in the type of glucosinolates and their hydrolysis products can influence insect performance and feeding choices, and that responses are species-specific.

Keywords

Arabidopsis thaliana, Glucosinolates, Hydrolysis Products, Specialist and Generalist Insects

1. Introduction

Plants serve as a food source for a wide variety of insect herbivores across multiple families and feeding guilds. Leaf chewer insects, such as beetles (Coleoptera) and caterpillars (Lepidoptera), make up the vast majority of phytophagous insects [1]. Plants use an arsenal of defenses (mechanical and chemical) and life history strategies to avoid being damaged by herbivores, and phytophagous insects evolve adaptations to withstand plant defenses and obtain nutrition in an endless co-evolutionary arms race [2] [3]. Selective pressures inflicted by insect herbivores on plants have resulted in the evolution of a vast array of secondary chemicals with a crucial role in defense [4] [5]. While these chemicals can be effective in deterring or repelling generalist insect herbivores (*i.e.* those with a wider host range), specialist insect species (with a narrower host range) have been shown to be less affected by these chemicals [5] [6]; however, there are exceptions to these generalizations [7]. Overall, generalist insects are known to be more susceptible to the toxic effects of secondary chemicals, including decreased fitness and higher mortality [6] [8] [9] [10]. Specialist feeders have evolved multiple strategies to mitigate potential negative effects of the toxins, including detoxification mechanisms, sequestering of chemicals, and behavioral strategies [11] [12] [13] [14].

The ability of specialist insect herbivores to tolerate secondary chemical defenses within their host plants has been considered a key principle of the specialist-generalist paradigm [7]. According to this paradigm, specialist insects are relatively unaffected by secondary chemicals. However, there have been a number of recent studies suggesting contrary trends [13] [15] [16] [17] [18]. Differences in feeding guild [17] or synergistic effects of distinct types of secondary chemicals may be involved in differential responses of specialist insects [19]. In any case, there are pronounced differences in the effects of secondary chemicals on generalist and specialist insect species, primarily due to the capacities of specialists to circumvent or detoxify plant toxic compounds, which in turn, has led to the diversification of plant defenses against insect herbivores [8] [9] [20] [21].

Coevolutionary interactions between members of the Brassicaceae plant family and its specialist and generalist herbivores have been widely studied. Many of these interactions are mediated by glucosinolates, an important secondary chemical class found in this plant family [22] [23]. Upon insect herbivory, glucosinolates are hydrolyzed by enzymes called myrosinases to produce hydrolysis products, such as isothiocyanates and nitriles [6] [22] [23]. The glucosinolate/myrosinase system is also commonly known as the "mustard oil bomb" [6]. Glucosinolates and their hydrolysis products are generally considered to be toxic or feeding deterrents for generalist herbivorous insects, while simultaneously acting as oviposition and feeding stimulants for specialist insects [6] [9] [24] [25] [26]. This dichotomy has led to differential ecological effects on crucifer plants, because higher glucosinolate levels in plants can protect them against generalist insects but attract specialist insects [25]. While some studies have supported the specialist-generalist paradigm [9] [25] [27] [28], others have contested it [7] [13] [16] [17] [29]. Early studies on the effects of glucosinolates and their hydrolysis products were limited by the complexity of these chemicals and their interactions in experimental settings. For example, intact glucosinolates added to insect artificial diets cannot demonstrate the effects of the hydrolysis products on insects. Glucosinolate hydrolysis products are volatiles and may even react with components of the artificial diet [6]. However, the identification of specific genes with roles in plant defense (specifically related to the glucosinolate-myrosinase system) has led to the production of transgenic and mutant plants varying only in glucosinolate profiles or their hydrolysis products. These genetically modified lines are invaluable to assess the effects of these secondary chemicals on insect herbivores [6] [29].

The main goal of this study was to evaluate the role of glucosinolates and their hydrolysis products on a specialist and a generalist insect species. For this purpose, genetically modified (GM) lines of the model plant *Arabidopsis thaliana* differing only in a type of glucosinolate or hydrolysis product were used to assess the effects of these secondary compounds on the specialist *Pieris rapae* and the generalist *Spodoptera exigua*. It was hypothesized that the selected specialist and generalist species will likely differ in their performance and feeding preferences for the different *A. thaliana* GM lines as a result of their distinct physiological adaptations. The selected plant lines used in this study have the same genetic background as the wild type Col-0 plants from which they are derived. No-choice experiments, where larvae were placed on the different GM plants to complete their cycle, were used to assess the effects of different intact glucosinolates and hydrolysis products on fitness-related traits of the selected insect herbivores (traits related to growth and development). In addition, choice experiments were conducted to assess the feeding preferences of each lepidopteran species for different *A. thaliana* glucosinolate hydrolysis products. These experiments are important for addressing ecological questions related to insect preferences and how these chemicals may mediate plant-insect interactions and their coevolutionary adaptations.

2. Materials and Methods

2.1. Plant Genetic Lines and Growth Conditions

Arabidopsis thaliana is a self-fertilizing, annual plant of the Brassicaceae family, which includes broccoli, cabbage, cauliflower, and many other agriculturally important crucifer crops. Like other members of this plant family, *A. thaliana* produces glucosinolates, which are secondary chemicals that serve an important role in plant defense against insect herbivores. About 200 different glucosinolates have been identified within the Brassicaceae family [30]. Distinct glucosinolates (β-thioglucoside-N-hydroxysulfates) share the same basic chemical composition, a central carbon bound to a thioglucose group and a sulfate group, and a variable side chain derived from amino acid precursors [4]. When plant tissues are damaged, the enzyme myrosinase (β-thioglucoside glucohydrolase, TGG) comes into contact with the glucosinolates, and hydrolyzes them, producing isothiocyanates, nitriles, thiocyanates, and other glucosinolate hydrolysis products [4] [23]. The model plant *A. thaliana* is known to produce over 30 distinct glucosinolates, the composition of which varies depending on the ecotype [31] [32]. The wild type Col-0 and five genetically modified lines were used in this study to evaluate the effects of different glucosinolate profiles and hydrolysis products on the performance and feeding choice of two insect species. The ecotype Col-0 produces primarily isothiocyanates as a product of hydrolysis [6]. Each of the mutant lines used in this study (35S:*ESP*, *tgg1tgg2*, *cyp79B2cyp79B3*, *myb28myb29*, *and pad3*) contain the same genetic background as the wild type Col-0, differing only in their glucosinolate profile or hydrolysis products [24] [33] [34]. The transgenic line 35S:*ESP* overexpresses the epithiospecifier protein (ESP) found in another Arabidopsis ecotype, Landsberg erecta (Ler), which causes 4-methylsulfinylbutylglucosinolate (the most common glucosinolate found in Col-0 leaves) to be hydrolyzed into simple nitriles instead of the corresponding isothiocyanate [35]. The double mutant *tgg1tgg2* causes a deficiency in the two major myrosinases that are responsible for the hydrolysis of leaf glucosinolates in *A. thaliana*. Therefore, this double mutant produces almost no hydrolysis products [24].

The genetic lines *cyp79B2cyp79B3* and *myb28myb29* differ from Col-0 in their intact glucosinolate profiles. The double knockout *cyp79B2cyp79B3* has mutations in two genes that lead to the production of indole-3-acetaldoxime, and therefore, prevents the production of indolyl glucosinolates [36]. Likewise, the double mutant *myb28myb29* is deficient in the production of aliphatic glucosinolates [37]. Camalexin is an indole phytoalexin, which is also synthesized from indole-3-acetaldoxime, and thus, absent in *cyp79B2cyp79B3*. Finally, the *pad3* mutant is deficient only in the production of camalexin while leaving the pathway to indole production intact, thereby, serving as a control for the effects of indolyl glucosinolates [34] [36].

Seeds of the wild type Col-0 were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA) and the genetically modified lines were kindly supplied by Dr. Daniel Kliebenstein (University of California, Davis). Plants used for all experiments were stratified for five days at 5˚C in complete darkness before planting in 50-well flats using a commercial soil mix (ProMix, Premier Horticulture Inc., Quakertown, PA, USA). At two weeks old, plants were transferred to 10 cm pots and placed in growth chambers set at a 10/14 day/night photoperiod and a 23˚C/20˚C day/night temperature cycle. Experiments were conducted when plants were at the pre-bolting stage.

2.2. Insect Species

The generalist moth *Spodoptera exigua* (Lepidoptera: Noctuidae) and the specialist butterfly *Pieris rapae* (Lepidoptera: Pieridae) were used in both choice and no-choice experiments. Both *S. exigua* (commonly known as beet armyworm) and *P. rapae* (cabbage white butterfly) are common agricultural pests of cruciferous plants. The moth *S. exigua*, which originated in Southeast Asia, was first identified in Oregon (USA) in 1876, and by 1924, it had reached Florida [38]. Currently, *S. exigua* exists in 101 different countries [39]. The life cycle of this common insect pest can be completed in as few as 24 days, and during summer months this species can complete multiple life cycles [40]. The specialist butterfly *P. rapae*, a Palearctic native that is widespread across Europe and Asia, was first reported in North America in 1860 in Quebec City, Canada, and like *S. exigua*, quickly made its way south and was found on the gulf coast by 1886 [41]. The number of generations this species completes in a year depends on climatic conditions. Its life cycle can vary from as few as three weeks in warmer climates up to as many as six weeks [42]. Both *S. exigua* and *P. rapae* are resistant to numerous pesticides [43] [44] and cause substantial damage to agriculturally important crucifer crops, like cabbage and broccoli. However, while *P. rapae* feeds primarily on (and is adapted to) mustard oil containing crucifer plants, *S. exigua* larvae utilize a much broader food spectrum including vegetable, field and flower crops across 18 plant families [45].

Naïve *S. exigua* and *P. rapae* larvae used in the no-choice experiments were obtained from Benzon's Research Inc. (Carlisle, PA) and Carolina Biological Supply Co. (Burlington, NC), respectively, as 1st instars and were reared on artificial diet from the same supplier until they reached the 2nd instar stage, when they were placed on the experimental plants. In addition, naïve third-instar larvae of both insect species were used in the choice experiments (purchased from the same commercial suppliers). All insects were maintained on artificial diet in growth chambers set at a 23˚C/20˚C day/night temperature cycle and 10/14 day/night photoperiod.

2.3. No-Choice Experiments

Twenty *Arabidopsis thaliana* plants from each of the selected genetic lines (*i.e.* Col-0, 35S:*ESP*, *tgg1tgg2*, *myb28myb29*, *cyp79B2cyp79B3*, *and pad3*) were used in the no-choice experiments performed using both the generalist *S. exigua* and the specialist *P. rapae* species. One second instar-larva of each species was placed on each of the experimental plants. Each pot was then covered with a plastic cage with a mesh lid for aeration and to prevent insects from moving to a different plant. During the course of the experiment, plants were kept in growth chambers set at a 23˚C/20˚C day/night temperature cycle and 10/14 day/night photoperiod. Once daily, plants were inspected to assess larval status or the initiation of the pupal stage. Time to pupation was recorded. At pupation, insects were removed from the plant, weighed, placed in a petri dish, and returned to the growth chamber until adult eclosion. At eclosion, adults were flash-frozen, weighed and sexed.

2.4. Choice Experiments

Three *Arabidopsis thaliana* genetic lines were used in choice tests: Col-0, 35S:*ESP*, and *tgg1tgg2*. For each insect species, 60 choice trials were performed. All larvae used were in the late 3rd instar stage and had been reared only on artificial diet. For the experiment, one leaf from each genotype was placed equidistantly in a 14 cm petri-dish. Selected leaves for the experiment were matched for size within and among different trials. One larva was placed in the center of the petri-dish and left for 1 hour to make a choice, which was determined by observed feeding on the selected leaf. If a larva failed to choose a plant leaf within one hour, it was removed from the experiment and a new petri-dish was set up for a fresh larva.

2.5. Data Analyses

For the no-choice experiments, a one-way ANOVA was performed to test whether different glucosinolate profiles or hydrolysis products affected fitness-related traits of *S. exigua* and *P. rapae*. Selected variables included time to pupation, pupae and adult weight. A non-parametric test (Kruskal-Wallis) was used for the developmental variable because it was not normally distributed. Post-hoc multiple comparisons (protected Fisher's LSD test) were performed to evaluate significant differences in mean insect responses among genetic lines.

For the choice experiments, Chi-square tests were used to determine whether there was a significant difference between the frequencies of each genotype chosen by each insect species. All statistical analyses were performed using SAS (version 9.2, SAS Institute).

3. Results

3.1. No-Choice Experiments

For *P. rapae*, variation in glucosinolate profiles did not have a statistically significant effect on adult weight, but they significantly influenced pupal weight and days to pupation (**Figures 1(a)-(c)**). While there were no significant differences in pupal weight between wild type Col-0 and the other genetic lines, individuals reared on *myb28myb29* (~no aliphatic glucosinolates) had significantly lower weight compared to those reared on *cyp79B2cyp79B3* (~no indolyl glucosinolates and camalexin) or *pad3* (~no camalexin) (**Figure 1(a)**). Larvae of *P. rapae* reared on *cyp79B2cyp79B3* plants had a statistically significant delay to pupation compared to those reared on Col-0 or *pad3* (**Figure 1(c)**).

For *S. exigua*, variation in glucosinolate profiles had a statistically significant effect on all measured fitness-related traits (**Figures 1(d)-(f)**). Pupae weight of

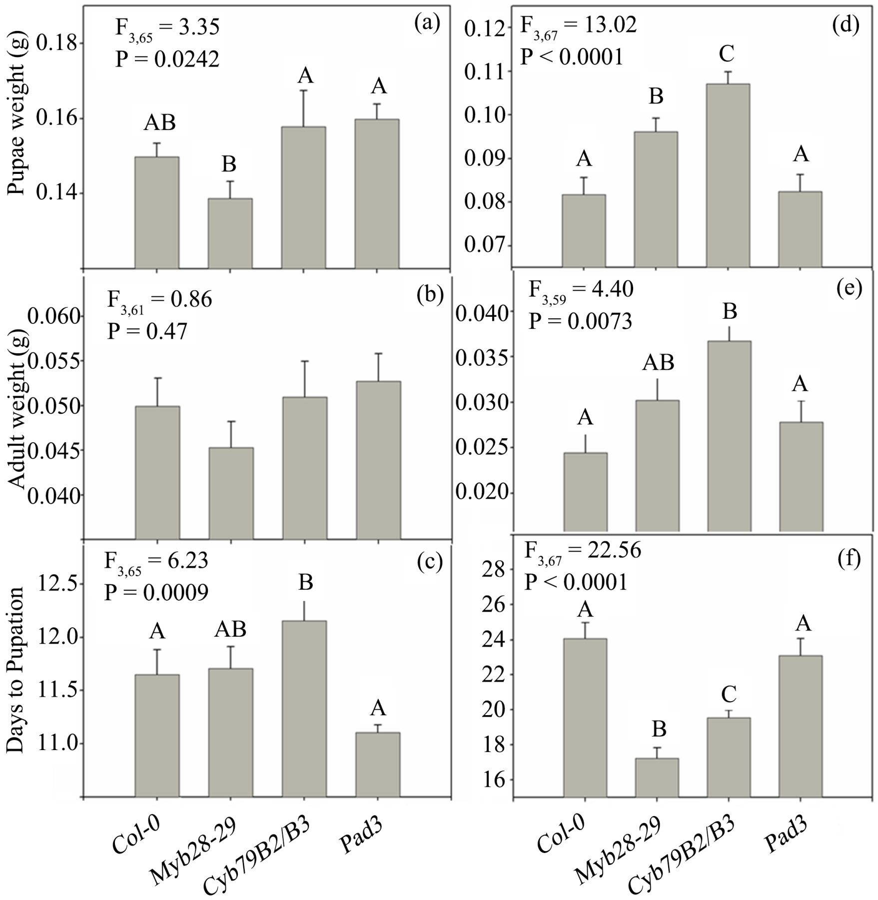

Figure 1. Effects of different *Arabidopsis thaliana* genetic lines differing in their intact glucosinolate profiles on fitness-related traits of *Pieris rapae* ((a)-(c)) and *Spodoptera exigua* ((d)-(f)). Different letters on bars denote statistically significant differences among means (P < 0.05).

individuals reared on *myb28myb29* and *cyp79B2cyp79B3* were significantly higher than those reared on Col-0 and *pad3* (**Figure 1(d)**). These trends were maintained for adult weights, but significant only for individuals reared on *cyp79B2cyp79B3* (**Figure 1(e)**). Time to pupation was significantly shorter for *S. exigua* raised on *myb28myb29* and *cyp79B2cyp79B3* than individuals reared on Col-0 or *pad3* (**Figure 1(f)**).

There was a statistically significant effect of variation in glucosinolate hydrolysis products on fitness-related traits of *S. exigua*, but not on *P. rapae* (**Figure 2**). Pupal and adult weight of *S. exigua* were significantly higher for individuals reared on 35S:*ESP* and *tgg1tgg2* plants (which produce nitriles or ~no hydrolysis products, respectively) than those raised on wild type Col-0 (**Figure 2(d)** and **Figure 2(e)**). Time to pupation was significantly shorter for larvae reared on the 35S:*ESP* or *tgg1tgg2* mutants than on wild type Col-0 plants (**Figure 2(f)**). It is worth noting that performance of *S. exigua* was maximized (highest pupal weight and shortest developmental time) when raised on *tgg1tgg2* plants, which virtually lack hydrolysis products (**Figure 2(d)** and **Figure 2(f)**).

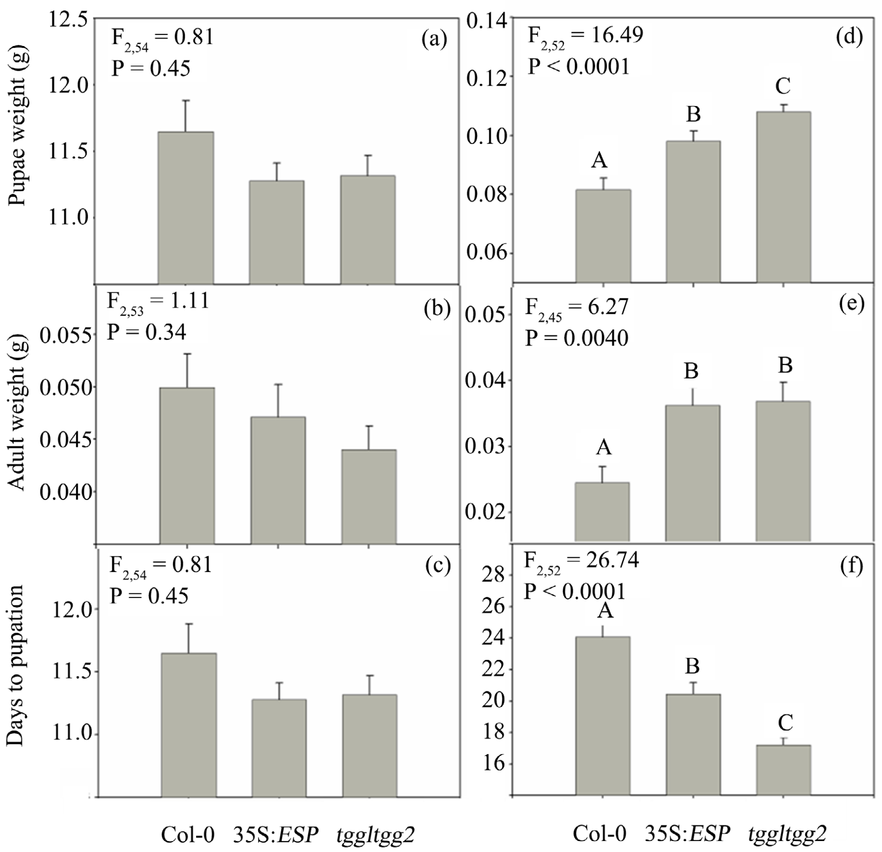

Figure 2. Effects of different *Arabidopsis thaliana* genetic lines differing in their glucosinolate hydrolysis products on fitness-related traits of *Pieris rapae* ((a)-(c)) and *Spodoptera exigua* ((d)-(f)). Different letters on bars denote statistically significant differences among means ($P < 0.05$).

3.2. Choice Experiments

Variation in glucosinolate hydrolysis products significantly affected the feeding choices of *S. exigua*, but not those of *P. rapae* larvae. There was a statistically significant preference of *S. exigua* larvae to feed on the double mutant *tgg1tgg2* over the nitrile producing 35S:*ESP* or the wild type Col-0 (**Figure 3**).

4. Discussion

This study provides experimental evidence that variation in intact glucosinolate profiles and their hydrolysis products can differentially affect fitness-related traits and feeding choices of common insect pests, such as *P. rapae* and *S. exigua*. In accordance with the specialist/generalist paradigm, the specialist *P. rapae* was less affected by the type of glucosinolate or product of hydrolysis than the generalist *S. exigua.* Performance of *S. exigua* was negatively impacted when individuals were reared on genetic lines that produce normal levels of both aliphatic and indolyl glucosinolates (wild type Col-0 and *pad3*). Likewise, *S. exigua* larvae preferred to feed on leaves of the double mutant *tgg1tgg2*, which virtually lacks hydrolysis products. In contrast, the specialist *P. rapae* was mostly unaffected by differences in intact glucosinolates and their hydrolysis products. However, a longer developmental time was found for larvae reared on *cyp79B2cyp79B3* (~no indolyl glucosinolates) compared with those raised on wild-type Col-0 and *pad3.* This result suggests that the presence of indolyl glucosinolates may be involved in *P. rapae* larval development. Longer developmental times are considered to be detrimental because it increases larval exposure to predators and reduces the number of generations that can be completed per year. It is known that glucosinolates can act as feeding and oviposition stimulants for specialist insect herbivores [46]; therefore, variation in the type of glucosinolate can potentially affect insect-fitness related traits. Other reasons for variation in insect performance may be related to lag responses, costs associated with detoxification and/or behavioral strategies leading to decreased glucosinolate induction [7] [14] [28] [29] [47]. Results of this study indicate that while the specialist/generalist paradigm may generally apply, specialist insects can also be susceptible to plant intraspecific variation in secondary chemistry.

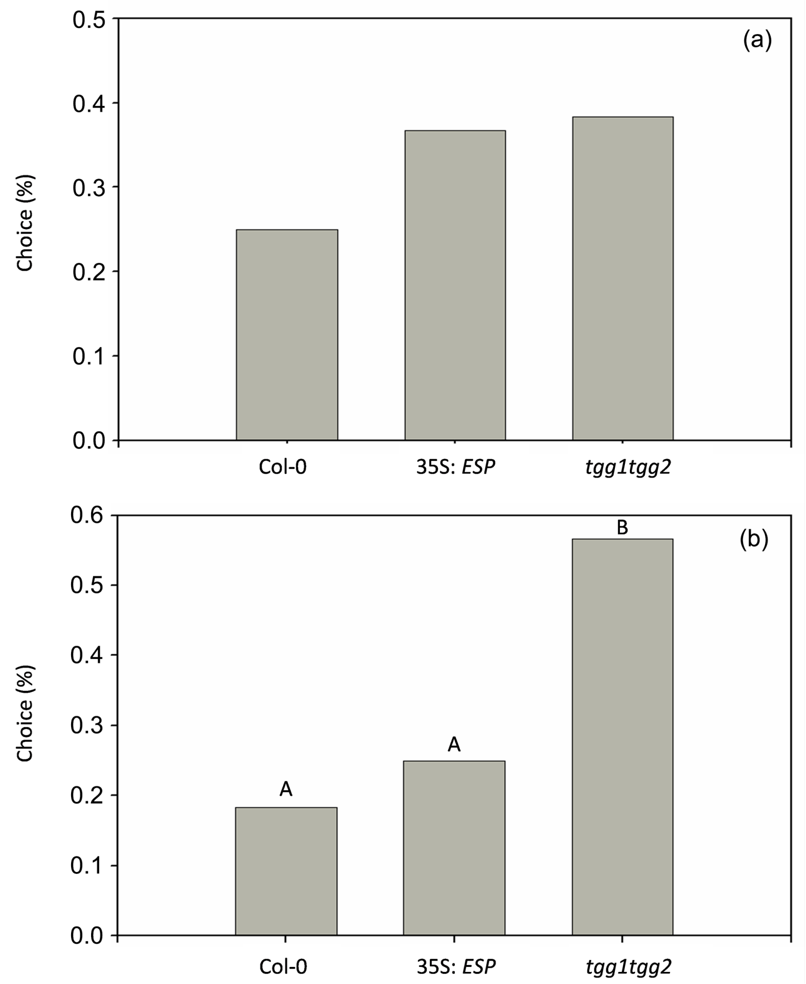

Figure 3. Feeding choice of *Pieris rapae* (a) and *Spodoptera exigua* (b) larvae exposed to *Arabidopsis thaliana* leaves differing in glucosinolate hydrolysis products. Different letters on bars denote statistically significant differences among means (P < 0.05).

Previous studies testing the specialist/generalist paradigm have yielded some mixed results. While some studies have demonstrated significant differences in how plant secondary chemistry affect generalist and specialist insect herbivores [8] [9] [33] [48], others suggest that specialists can also be affected, albeit to a lesser extent [13] [17] [24] [39]. A previous study by Bidart-Bouzat and Kliebenstein [17] found that plant responses to herbivory by six insect species (*i.e.* genome-wide transcriptional responses including major defense pathways and glucosinolate levels) were influenced more by the insect taxa/feeding guild (lepidopteran leaf chewers versus aphid phloem feeders) rather than by their ecological specialization (specialists vs. generalists). Within taxon, plant responses to lepidopteran herbivores were also not shaped by "herbivore specialization or phylogenetic history." These were important findings that challenge the classical predictions related to the specialist/generalist paradigm and suggest that plant-insect interactions may be shaped by different ecological and evolutionary adaptations in distinct environments. This topic has been further reviewed by Ali and Agrawal [7], highlighting the importance of insect feeding guilds influencing different aspects of plant responses and suggestions for further research related to specialist vs. generalist insects.

Variation in glucosinolate profiles in *A. thaliana* ecotypes has been attributed to the selection pressures exerted on individual plants and populations by distinct insect herbivores [31]. For example, in a study on the impact of generalist and specialist insects on the selection of *A. thaliana* populations for distinct glucosinolate profiles, Arany *et al.* [9] hypothesized that if generalist and specialist herbivores exert a contrasting selection pressure on *A. thaliana*, they should perform differently on those plants according to their chemical composition. Indeed, they found that insect responses were dependent on secondary chemistry and that the insects' feeding guilds and ecological specializations may also impose selection on plant secondary chemistry. In this study, the distinct glucosinolate profiles differentially affected the specialist *P. rapae* and the generalist *S. exigua*. While *P. rapae* was minimally affected, *S. exigua* performed better on mutants with defective production of aliphatic or indolyl glucosinolates than on Col-0 or *pad3* plants. This result is in accordance with a previous study by Müller *et al.* [33], where *S. exigua* experienced an increase in fitness on both the *myb28myb29* and the *cyp79B2cyp79B3* mutants, and even higher on the double mutant *cyp79B2cyp79B3 myb28myb29*, which does not produce either indolyl or aliphatic glucosinolates. This demonstrates that not only indolyl and aliphatic glucosinolates significantly influence insect responses, but also that there is an additive effect of these glucosinolates on this generalist herbivore.

As observed with intact glucosinolates, the specialist *P. rapae* was not significantly affected by variation in glucosinolate hydrolysis products either. Conversely, the generalist *S. exigua* was significantly influenced by plants producing different hydrolysis products in both no-choice and choice experiments, as predicted by specialist/generalist theory. Performance of *S. exigua* was enhanced when reared on the nitrile producing 35S:*ESP* and maximized on the double mutant *tgg1tgg2* (which lacks indolyl and aliphatic glucosinolates) compared to that on Col-0 plants. The wild type Col-0 produces isothiocyanates, which are known to be toxic to some generalist insects [24] [28] [49] [50]. In a previous study, increased performance of a generalist lepidopteran (*Trichoplusia ni*) was also detected when reared on the double mutant *tgg1tgg2* compared to on 35S:*ESP* or wild type Col-0 plants, but growth and survival of the specialist *Plutella xylostella* was not affected by the different genetic lines [51]. Furthermore, in choice experiments, the generalist *S. exigua* larvae preferred to feed on *tgg1tgg2* plants devoid of hydrolysis products than on 35S:*ESP* and wild type Col-0 plants. While nitriles produced by 35S:*ESP* plants may be less toxic to *S. exigua* than isothiocyanates in Col-0, this generalist insect still preferred to avoid glucosinolate hydrolysis products in a choice situation. Findings from this study were in agreement with previous research supporting the argument that variation in plant glucosinolate profiles and their hydrolysis products can have differential effects on generalist and specialist insect species. More research is needed on the potential synergistic effects of different glucosinolates on insects. While isothiocyanates are probably one of the most frequently studied hydrolysis products of glucosinolates, feeding choice trials in this study provide evidence that nitriles could play a larger role than expected in repelling generalist insects.

5. Conclusion

Results from this study provide further evidence that variation in glucosinolate hydrolysis profiles can impact the performance of insect herbivores and their feeding preferences in a species-specific way. This outcome likely results from distinct selective pressures and coevolutionary trajectories between different insect species and their host plants.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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