Successful herbivore attack due to metabolic diversion of a plant chemical defense

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Plants protect themselves against herbivory with a diverse array of repellent or toxic secondary metabolites. However, many herbivorous insects have developed counteradaptations that enable them to feed on chemically defended plants without apparent negative effects. Here, we present evidence that larvae of the specialist insect, *Pieris rapae* (cabbage white butterfly, Lepidoptera: Pieridae), are biochemically adapted to the glucosinolate–myrosinase system, the major chemical defense of their host plants. The defensive function of the glucosinolate–myrosinase system results from the toxic isothiocyanates that are released when glucosinolates are hydrolyzed by myrosinases on tissue disruption. We show that the hydrolysis reaction is redirected toward the formation of nitriles instead of isothiocyanates if plant material is ingested by *P. rapae* larvae, and that the nitriles are excreted with the feces. The ability to form nitriles is due to a larval gut protein, designated nitrile-specifier protein, that by itself has no hydrolytic activity on glucosinolates and that is unrelated to any functionally characterized protein. Nitrile-specifier protein appears to be the key biochemical counteradaptation that allows *P. rapae* to feed with impunity on plants containing glucosinolates and myrosinases. This finding sheds light on the ecology and evolution of plant–insect interactions and suggests novel highly selective pest management strategies.

One of the best-studied groups of plant defense compounds are the glucosinolates (Fig. 1), amino acid-derived thioglycosides found in several plant families (1), including the agriculturally important crops of the Brassicaceae such as oilseed rape, cabbage, and broccoli and the model plant *Arabidopsis thaliana* (2). Glucosinolates cooccur with myrosinases (thioglucoside glucohydrolases, EC 3.2.3.1), and together these two components constitute an activated plant defense system known as the ''mustard oil bomb'' (3). On tissue damage, the nontoxic glucosinolates are hydrolyzed by myrosinases into biologically active derivatives (Fig. 1*A*). The outcome of the hydrolysis reaction depends on the structure of the glucosinolate side chain and the reaction conditions (4). The most common class of hydrolysis products, isothiocyanates (mustard oils), has frequently been shown to be highly toxic to both generalist and specialist insect herbivores (5, 6).

Despite the toxicity of isothiocyanates, several lepidopteran insect species use glucosinolate- and myrosinase-containing plants as hosts. Although the neurophysiological bases of host plant choice in these species have been studied extensively (7), relatively little is known about how they overcome the toxicity of their host plants. Among the well known specialist insect herbivores on glucosinolate-containing plants, *Pieris rapae* is one of the most abundant butterflies in Northern and Central Europe, and it has recently also become a pest in North America. *P. rapae* has been a model insect for studying herbivore host selection (7, 8), but the biochemical mechanism by which it copes with the glucosinolate–myrosinase system is still unknown. Here, we report on the identification of a larval gut protein from *P. rapae* that prevents formation of isothiocyanates by redirecting glucosinolate hydrolysis toward nitrile formation. Prevention of isothiocyanate formation by this protein appears to be the key biochemical mechanism by which *P. rapae* circumvents the toxicity of its host plants. Interestingly, this mechanism contrasts with that found in *Plutella xylostella* (9), another lepidopteran insect specialized on glucosinolate-containing plants.

Materials and Methods

Plants and Insects. *A. thaliana* [ecotype Columbia (Col-0)] was grown at 21°C and 55% humidity with a photoperiod of 10 h and *Sinapis alba* cv. SALVO at 25°C with a photoperiod of 18 h. *P. rapae* was reared on *Brassica oleracea gemmifera* cv. Rosella at 22°C and 70% humidity with a photoperiod of 16 h. The culture originated from individuals collected locally (Jena, 2001) and was supplemented with individuals provided by J. van Loon (Wageningen University, Wageningen, The Netherlands).

Intact Glucosinolates and Standards for Hydrolysis Product Analysis. Allylglucosinolate was from Sigma–Aldrich, and all other intact glucosinolates were isolated as described (10, 11). Benzyl isothiocyanate [1-(isothiocyanatomethyl)benzene], phenylacetonitrile, and 4-hydroxybenzyl alcohol standards were from Sigma–Aldrich; 4-hydroxyphenylacetonitrile and benzonitrile were from Merck; and allyl isothiocyanate (3-isothiocyanatoprop-1-ene) and but-3-enenitrile were from Fluka.

Isolation and Identification of 4-Hydroxyphenylacetonitrile Sulfate. 4-Hydroxyphenylacetonitrile sulfate, detected by sulfatase-catalyzed hydrolysis to 4-hydroxyphenylacetonitrile (12), was isolated from aqueous extracts of feces of *P. rapae* reared on *S. alba*. The extract was loaded onto a column of DEAE Sephadex A-25. Elution was carried out with 0.5 M K_2SO_4. After ethanol precipitation of K_2SO_4, the appropriate fractions were evaporated *in vacuo* at 40°C, and the residue was extracted with methanol at 60°C. The methanolic extract was evaporated to dryness, and the remaining material, dissolved in water, was subjected to preparative HPLC on a Shimadzu LC-10AT HPLC equipped with a Supelcosil LC ABZ column. A water (A)–methanol (B) gradient was used with 0% B for 10 min and 0–5% (vol/vol) B over 5 min. A colorless salt (7 mg), obtained after lyophilization of the appropriate fractions, was subjected to NMR and IR spectroscopy and electrospray ionization MS on a Bruker Avance (Billerica, MA) 400 spectrometer, a System 2000 Fourier transform–IR spectrophotometer (Perkin–Elmer), and a Bruker Esquire 3000+ ion trap mass spectrometer, respec-

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Abbreviations: NSP, nitrile-specifier protein; FID, flame ionization detection; LC-MS/MS, liquid chromatography–tandem MS.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY425622).

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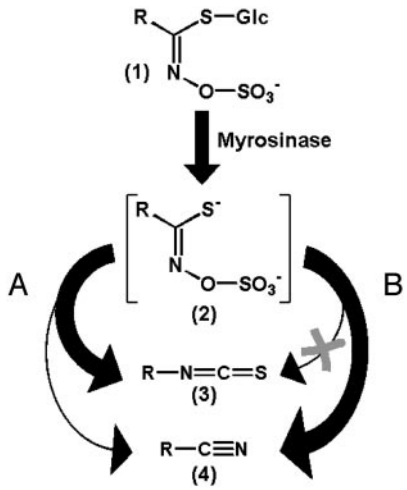

Fig. 1. The glucosinolate–myrosinase system in plant defense and as manipulated by the *P. rapae* NSP. In damaged plant tissue, glucosinolates (1) are hydrolyzed by myrosinases yielding glucose and unstable aglucones (2). (*A*) In mechanically damaged plant tissue, the aglucones rearrange spontaneously into isothiocyanates [mustard oils (3)], which have frequently been shown to act as insecticides. Depending on the structure of the glucosinolate side chain R and the reaction conditions, nitriles (4) and other hydrolysis products can also be formed. (*B*) In plant material ingested by *P. rapae* larvae, the aglucones (2) do not undergo the rearrangement to the toxic isothiocyanates (3) but instead form nitriles (4) caused by the presence of NSP.

tively. UV spectra were recorded with a Shimadzu SPD-M10AVP diode array UV detector. The content of 4-hydroxyphenylacetonitrile sulfate in feces was determined as described (12) and compared to the 4-hydroxybenzylglucosinolate content of *S. alba* leaves. Leaves from designated positions on *S. alba* plants were divided in halves along their midveins. One-half was fed to *P. rapae* larvae conditioned on *S. alba*, the other half was lyophilized and analyzed for its glucosinolate content as described (12). Allylglucosinolate was used as an internal standard.

Feeding Experiments with [^{14}C]4-Hydroxybenzylglucosinolate and Identification of [^{14}C]4-Hydroxyphenylacetonitrile Sulfate. [^{14}C]4-Hydroxybenzylglucosinolate (103 Bq/nmol, 80 Bq/μl) U-^{14}C-labeled in the aglucone moiety was prepared as described (13) by using transgenic *A. thaliana* CaMV35S::CYP79A1 (14). Detached rosette leaves of *A. thaliana* Col-0 that had absorbed 20–50 μl of a 1:1 mixture of [^{14}C]4-hydroxybenzylglucosinolate and Murashige–Skoog medium were fed to late instar *P. rapae* larvae. Control experiments showed that intact [^{14}C]4-hydroxybenzylglucosinolate accounted for at least 50% of the radioactivity in the treated leaves. After complete consumption of the leaves, feces, larvae, and plant residues were extracted in 50% (vol/vol) methanol. All extracts were analyzed by liquid scintillation counting and by TLC.

The major radioactive metabolite was analyzed by applying the feces extract, either alone or spiked with a nonradioactive extract of feces from *P. rapae* fed *S. alba*, to DEAE Sephadex A-25. Elution with water after sulfatase treatment (12) recovered 86–89% of the loaded radioactivity in the eluate. The eluate was analyzed by HPLC on an Agilent HP1100 (Agilent Technologies, Palo Alto, CA) series instrument with a UV detector (229 nm) and a flow-through radioactivity monitor (Radiomatic 500 TR, Packard) using a Supelcosil LC-18-DB column and a water (A)–methanol (B) gradient with 0% B for 10 min, 0–40% (vol/vol) B over 35 min, 40– (vol/vol) 100% B over 0.5 min at a flow rate of 0.25 ml/min at 25°C. Ultima-Flo AP scintillation fluid (Packard) was used in a ratio of 4:1 (vol/vol) to column eluent. The counting efficiency for ^{14}C was 75%.

In parallel, the composition of extracts of feces of *P. rapae* fed either [^{14}C]4-hydroxybenzylglucosinolate-treated *A. thaliana* or untreated *S. alba* leaves was analyzed with or without prior sulfatase treatment. Aliquots of the extracts and of a 1:1 mixture of the extracts were allowed to evaporate overnight at room temperature. Then, one set of replicates was redissolved in 20 mM Mes, pH 6.0, and the other in 20 mM Mes, pH 6.0, containing sulfatase solution. After incubation for at least 2 h, the samples were analyzed by HPLC with UV and radioactivity detection as described above.

Analysis of 4-Hydroxybenzylglucosinolate Hydrolysis Products in *S. alba*. Leaves of 3- to 5-week-old *S. alba* plants were divided in halves along their midvein. One-half of each leaf was analyzed for glucosinolates as described (12), and the other half was macerated in water. After 10 min at room temperature, the samples were boiled for 1 min and, after addition of the internal standard allylglucosinolate, for another 2 min followed by homogenization. Aliquots of these extracts were analyzed for 4-hydroxyphenylacetonitrile essentially as described (12). Quantification of remaining glucosinolate and proof of the absence of 4-hydroxyphenylacetonitrile sulfate were carried out on an aliquot of the extract treated as for glucosinolate analysis (12).

Analysis of Glucosinolate Hydrolysis Products by GC. Dichloromethane extracts of aqueous solutions were dried over Na_2SO_4 and concentrated to $\approx$150 μl in a nitrogen stream. Samples were analyzed by GC-MS and GC with flame ionization detection (FID) using an Agilent 6890 series gas chromatograph with an HP5MS column, splitless injection at 200°C, and temperature programs I (45°C for 3 min, 12°C per min to 230°C, 60°C per min to 280°C, 3 min final hold), II (45°C for 3 min, 12°C per min to 96°C, 18°C per min to 150°C, 60°C per min to 270°C, 3-min final hold), or III (as I, but with 35°C starting temperature). MS and FID were carried out as described (15). Product identification was done by comparison of MS spectra and retention times with those of authentic standards or hydrolysis products of authentic glucosinolate standards or with published MS spectra (16). Quantification of benzyl isothiocyanate and phenylacetonitrile was done by GC-MS with selected ion monitoring based on peak area relative to the internal standard benzonitrile using experimentally determined response factors. The recoveries of benzyl isothiocyanate, phenylacetonitrile, and benzonitrile on extraction from the water phase were 57 $\pm$ 6%, 53 $\pm$ 12%, and 58 $\pm$ 6%, respectively (means $\pm$ SD, n = 9). For quantification, equal recovery was assumed. Other compounds were quantified by GC-FID as described (15).

Analysis of Glucosinolate Hydrolysis Products in Extracts of *A. thaliana* Leaves and *P. rapae* Feces. Two *A. thaliana* rosette leaves from 6-week-old plants (100–160 mg) were macerated in 500 μl of water. After 5 min at room temperature (allowing complete glucosinolate hydrolysis), 25 μl of benzonitrile (100 ng/μl in methanol) was added, and the samples were extracted with a total of 4 ml of dichloromethane in three extraction steps. Larvae conditioned on *A. thaliana* (Col-0) for 3 days were starved for 3 h and then fed rosette leaves of *A. thaliana* (Col-0). Feces were collected continuously into sealed glass vials. Aliquots of 110–160 mg of feces were ground in 500 μl of dichloromethane. After addition of 500 μl of water and 25 μl of benzonitrile (100 ng/μl in methanol), the samples were shaken vigorously. Phases were separated, the water phase was extracted twice with an equal volume of dichloromethane, and the organic phases were pooled. Dichloromethane extracts were analyzed by GC-MS and GC-FID (program I).

Preparation of Protein Extracts. Larvae of *P. rapae* (fourth or fifth instar) were starved for 16 h. The larvae were dissected into heads, midguts, hindguts, and remaining bodies in 50 mM Tris·HCl, pH

7.5. Green material in midgut and hindgut was removed. The tissues were washed with 50 mM Tris·HCl, pH 7.5, and homogenized in the same buffer. The 20,000 × *g* supernatants were used as crude extracts. Protein content was determined with Bio-Rad Protein Reagent by using BSA as a standard.

Nitrile-Specifier Protein (NSP) Assay. Assays were done in 50 mM Tris·HCl, pH 7.5, containing 1 mM benzylglucosinolate and an appropriate amount of protein extract or fraction thereof in a total volume of 500 μl at room temperature. The reaction was started by addition of myrosinase (thioglucosidase, 278 units/g, Sigma–Aldrich) to a final concentration of 50 μg/ml. At the end of the incubation time (typically 40 min), 50 μl of benzonitrile (100 ng/μl in methanol) were added, the assay mixtures were extracted with 2 × 750 μl of dichloromethane and analyzed by GC-MS and GC-FID (program II). NSP activity was expressed as nanomols phenylacetonitrile formed per minute. Under these conditions, myrosinase-catalyzed hydrolysis in the absence of NSP was linear over 3 h. For quantitative analyses, the amount of NSP added to the assay mixture was adjusted so the reaction yielded benzyl isothiocyanate and phenylacetonitrile. To test substrate specificity, benzylglucosinolate in the assay mixture was replaced by other intact glucosinolates (1 mM final concentration), and GC analysis was done with program III. To test whether benzyl isothiocyanate was a substrate for NSP, benzyl isothiocyanate (0.075, 0.75, and 1.5 mM) was incubated with midgut extract in the presence or absence of benzylglucosinolate and myrosinase. Methanol used as a solvent for benzyl isothiocyanate did not influence the hydrolysis of benzylglucosinolate by myrosinase and NSP at the concentrations used (<2%).

Purification of NSP. Extracts of up to 550 midguts of *P. rapae* were ultracentrifuged at 120,000 × *g* for 40 min. The precipitate obtained on addition of $(NH_4)_2SO_4$ to the 120,000 × *g* supernatant [30–70% $(NH_4)_2SO_4$ saturation] was dissolved in 50 mM Tris·HCl, pH 7.5, dialyzed against the same buffer, and applied to a column of Fast Flow DEAE-Sepharose (Sigma–Aldrich) equilibrated with 50 mM Tris·HCl, pH 7.5, containing 50 mM NaCl. After elution with a gradient of NaCl in 50 mM Tris·HCl, pH 7.5, fractions with NSP activity were brought to a concentration of 1 M $(NH_4)_2SO_4$ and applied to a column of HP phenylsepharose (Amersham Pharmacia Biosciences) equilibrated in 50 mM Tris·HCl, pH 7.5, containing 1 M $(NH_4)_2SO_4$. Elution was accomplished with a gradient of $(NH_4)_2SO_4$ in 50 mM Tris·HCl, pH 7.5. Fractions with NSP activity were chromatographed on a MonoQ column HR 5/5 (Amersham Pharmacia Biosciences) equilibrated with 50 mM Tris·HCl, pH 7.5, containing 50 mM NaCl. Active fractions eluted with a gradient of NaCl in 50 mM Tris·HCl, pH 7.5, were further purified on a Superdex 200 Highload Prep Grade 16/60 column (Amersham Pharmacia Biosciences) equilibrated in 50 mM Tris·HCl, pH 7.5, containing 100 mM NaCl. Fractions were analyzed by SDS/PAGE on Tris-SDS/PAGE gels (12%) after boiling for 5 min with standard loading buffer. Tris-PAGE gels (8.25%) prepared without addition of SDS were used for protein analysis under native conditions. For isolation of functional NSP from native PAGE gels, one lane of the gel was stained with Coomassie blue. The remaining gel was cut into 12 horizontal strips, which were extracted with water. The samples were concentrated and desalted by using Microsep 30-K ultrafiltration devices (Pall Life Sciences, Ann Arbor, MI), analyzed for NSP activity, and reanalyzed on SDS/PAGE and native PAGE gels.

Preparation of *P. rapae* cDNA Libraries. For isolation of RNA from larval tissue, five male and five female fourth instar larvae were dissected in 100 mM Tris·HCl, pH 7.5. RNA and poly(A)+ mRNA from Malpighian tubules, fat body, midgut, head, and remaining body was isolated with standard methods. Double-stranded full-length-enriched cDNA was generated by primer extension with the SMART cDNA library construction kit (Clontech) according to the manufacturer's protocol. The cDNA was digested with *Sfi*I and ligated to the *Sfi*I-digested pDNR-Lib plasmid vector (Clontech). One cDNA library was generated for each of the five tissue types.

Generation of a *P. rapae* EST Database. Single-pass sequencing of the 5′ termini of cDNA clones from each of the cDNA libraries was carried out on an ABI 3700 automatic DNA sequencer (PE Applied Biosystems). Vector clipping and quality trimming were done as described (17). BLAST searches were conducted on a local server by using the National Center for Biotechnology Information BLASTALL program.

Amino Acid Sequence Analysis of Proteins. Protein bands were excised from Coomassie-blue-stained SDS/PAGE and native PAGE gels. In-gel digestion, analysis by liquid chromatography–tandem MS (LC-MS/MS), and peptide identifications were done as described (18) by using a database of translated ESTs of the *P. rapae* cDNA libraries. N-terminal amino acid sequencing by Edman degradation was performed at TopLab (Martinsried, Germany) on protein bands blotted onto poly(vinylidene difluoride) membranes. The PrNSP sequence was analyzed by the programs TMPREDICT (19), PSORT II (20), and DNASTAR (DNASTAR, Madison, WI).

Heterologous Expression in *Escherichia coli*. The cDNA encoding the extracellular d03c03 protein predicted by PSORT II (20) was amplified from the EST clone d03c03 by PCR by using the primers ExNSPF (5′-ATGAAGCCTCGTCTTTTCGAGACCT-3′) and ExNSPR (5′-CTGGCCGTAAAGGGCAG-3′), and cloned into the pCR-T7/CT-TOPO expression vector (Invitrogen) in frame with the C-terminal His-tag. The correct insert sequence was verified by sequencing. Cultures of *E. coli* BL21(DE3) pLysS (Invitrogen) harboring the d03c03 expression construct or empty pCR-T7/CT-TOPO vector were diluted to an OD_{600} of 0.1 with terrific broth supplemented with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol and grown to an OD_{600} of 0.4–0.6 at 18°C and 220 rpm before isopropyl-1-thio-β-D-galactopyranoside was added (1 mM). After 16 h, bacterial cells were extracted in 50 mM Tris·HCl, pH 7.5, by sonication. The 20,000 × *g* supernatants were used as crude *E. coli* extracts.

Expression Analysis by RT-PCR. Ten *P. rapae* fourth instar larvae were dissected in 100 mM Tris·HCl, pH 8, into heads, midguts, fat bodies and hindguts with Malpighian tubules, and remaining bodies. Total RNA was isolated from these tissues and from adults. First-strand cDNA was synthesized from 2 μg of total RNA by using the Omniscript RT-Kit (Qiagen, Chatsworth, CA) and oligo d(T) primers according to the manufacturer's instructions. *PrNSP*-specific primer pairs (NSP03-IF, 5′-TCATGAAAAGCTTAGACGCCTTG-3′; NSP03-IR, 5′-ACTCATCGAACTGGTTTTGGAA-3′) were multiplexed with primers for elongation factor 1-α (EF-1α) used as a control (PEF1α-F, 5′-ACATTGTCGTCATTGGACAC-3′; PEF1α-R, 5′-AGTGTGAAAGCGAGAAGAGC-3′). PCRs were set up in 1× reaction buffer supplemented with 0.2 μl of first-strand cDNA/0.2 mM of each dNTP/1 unit of *Taq* polymerase (Qiagen)/20 pmol of each *PrNSP*-specific primer/10 pmol of each EF-1α primer in a total volume of 25 μl. Cycling conditions were 94°C for 2 min followed by 29 cycles of 94°C (15 s), 56°C (15 s), and 72°C (40 s), followed by 72°C for 7 min on a GeneAmp PCR System 9700 thermal cycler (PE Applied Biosystems).

Results

Nitriles in *P. rapae* Feces Are the Major Glucosinolate Metabolites. Feces of *P. rapae* larvae fed leaves of *S. alba*, a host plant of *P. rapae* that accumulates 4-hydroxybenzylglucosinolate, contain an appar-

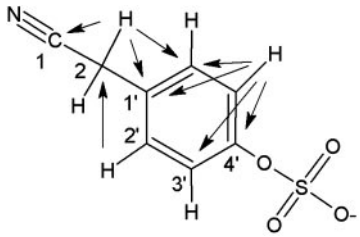

Fig. 2. Spectroscopic identification of 4-hydroxyphenylacetonitrile sulfate isolated from *P. rapae* feces. Structure and numbering system of 4-hydroxyphenylacetonitrile sulfate are depicted. The signals in NMR, MS, and IR spectra were assigned as follows: ^{1}H NMR (D_2O, 400 MHz): δ 3.94 (*s*, H2); 7.32 (''*d*'', J = 9 Hz, H3′ + H5′); 7.43 (''*d*'', J = 9 Hz, H2′ + H6′). ^{13}C NMR (D_2O, 100 MHz): δ 23.1 (C2); 120.8 (C1); 122.9 (C3′ + C5′); 129.3 (C1′); 130.3 (C2′ + C6′); 151.5 (C4′). Selected heteronuclear multiple bond correlation interactions are indicated by arrows. MS, *m/z*: 463 $[M^{2+}K]^-$; 447 $[M^{2+}Na]^-$; 212 $[M]^-$; 132 $[M\text{-}SO_3]^-$. MS/MS confirmed *m/z* 132 as a fragmentation product of *m/z* 212. IR, ν_{max}: 2,246 cm^{-1} (w, CN). UV, λ_{max}: 208 nm (100%), 260 nm (4%).

ent glucosinolate derivative that is converted to 4-hydroxyphenylacetonitrile on incubation with sulfatase (12). We isolated this compound from feces extracts and identified it as the sulfate ester of 4-hydroxyphenylacetonitrile by NMR and IR spectroscopy and MS (Fig. 2). The content of this compound in feces was comparable to the levels of 4-hydroxybenzylglucosinolate in *S. alba* leaves (40–200 μmol·g^{-1} dry weight) and correlated with the 4-hydroxybenzylglucosinolate content in the leaves ($R^2 = 0.63$, $n = 13$). In contrast, extracts of mechanically disrupted *S. alba* leaves did not contain 4-hydroxyphenylacetonitrile sulfate, and 4-hydroxyphenylacetonitrile accounted for only 1 ± 0.5% (mean ± SD, $n = 6$) of the hydrolyzed 4-hydroxybenzylglucosinolate.

The metabolic origin of 4-hydroxyphenylacetonitrile sulfate in the feces was demonstrated in *P. rapae* larvae fed leaves of *A. thaliana* (Col-0) that had taken up 4-hydroxybenzylglucosinolate U-^{14}C-labeled in the aglucone moiety. Of the total radioactivity recovered from larvae and feces, 95% was found in the feces. The feces extract contained one major radioactive metabolite that accounted for 87% of the radioactivity in the extract. Coelution of this metabolite with 4-hydroxyphenylacetonitrile sulfate and its quantitative conversion to a compound coeluting with 4-hydroxyphenylacetonitrile by sulfatase treatment confirmed its identity as [U-^{14}C]4-hydroxyphenylacetonitrile sulfate. This proved that the sulfate ester of 4-hydroxyphenylacetonitrile was derived from ingested 4-hydroxybenzylglucosinolate. In contrast, incubation of 4-hydroxybenzylglucosinolate with mechanically disrupted leaves of *A. thaliana* (Col-0) results in the formation of the corresponding isothiocyanate, 4-hydroxybenzyl isothiocyanate [4-(isothiocyanatomethyl)phenol], and derived compounds as the major hydrolysis products (14).

To assess the metabolism of other types of glucosinolates, feces of *P. rapae* larvae were analyzed that had fed on leaves of untreated *A. thaliana* (Col-0), which naturally contain glucosinolates with aliphatic side chains (21, 22) Again, the nitrile derivatives of the major leaf glucosinolates, but not the isothiocyanates, were detected in the feces (Fig. 3*A*), even though isothiocyanates were the major hydrolysis products of aliphatic glucosinolates in mechanically disrupted *A. thaliana* leaves (Fig. 3*B*). Taken together, the results of the feeding experiments suggested a quantitative conversion of ingested glucosinolates into nitriles by *P. rapae*. Because nitriles were only minor glucosinolate hydrolysis products in mechanically disrupted plant tissues, it was concluded that the formation of nitriles is due to processing by the larvae.

The Larval Midgut Possesses NSP Activity. The metabolism of benzylglucosinolate was studied *in vitro* with *P. rapae* extracts and a commercial myrosinase preparation (Fig. 4 *A–F*). On incubation of

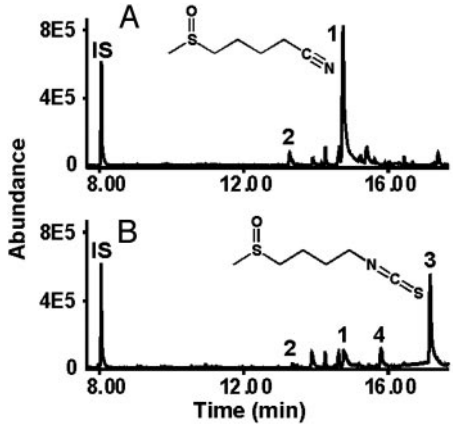

Fig. 3. Metabolism of glucosinolates in larvae of *P. rapae*. Analyses of glucosinolate hydrolysis products in feces of larvae fed *A. thaliana* (Col-0) rosette leaves (*A*) and in a comparable batch of leaves after mechanical disruption (*B*). Feces were extracted in a mixture of dichloromethane and water, whereas an equal amount of rosette leaves was macerated in water, and the aqueous phase was extracted with dichloromethane after 5 min. The organic phases were subjected to GC-MS. **1**, 5-(methylsulfinyl)pentanenitrile; **2**, 4-(methylsulfinyl)butanenitrile; **3**, 4-(methylsulfinyl)butyl isothiocyanate (1-isothiocyanato-4-(methylsulfinyl)butane); **4**, 3-(methylsulfinyl)propyl isothiocyanate (1-isothiocyanato-3-(methylsulfinyl)propane; IS, internal standard. Sections of total ion chromatograms are depicted.

benzylglucosinolate with myrosinase at pH 7.5, the pH measured in the midgut lumen of *P. rapae* larvae (data not shown and ref. 23), benzyl isothiocyanate was the only detected hydrolysis product. However, if larval midgut extract was also added, the corresponding nitrile, phenylacetonitrile, was formed instead of the isothiocya-

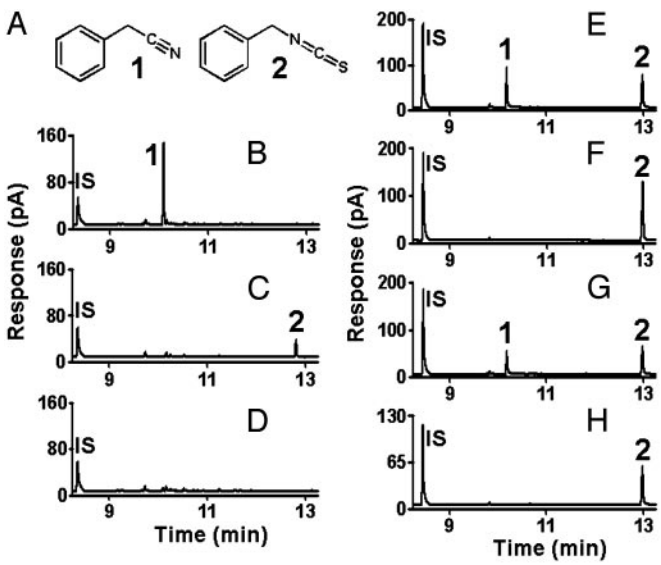

Fig. 4. Metabolism of benzylglucosinolate *in vitro* by myrosinase, *P. rapae* extracts, and heterologously expressed PrNSP. (*A*) Chemical structures of the hydrolysis products of benzylglucosinolate, phenylacetonitrile 1 and benzyl isothiocyanate 2. (*B–F*) Benzylglucosinolate was incubated with concentrated (*B–D*) or diluted (*E*) midgut extract (*C*, boiled midgut extract) in the presence (*B*, *C*, and *E*) or absence (*D*) of a commercial myrosinase preparation, or with myrosinase only (*F*). (*G* and *H*) Benzylglucosinolate was incubated with an extract of *E. coli* harboring a *PrNSP* expression construct (*G*) or the empty expression vector (*H*) in the presence of myrosinase. Assays were extracted with dichloromethane after 1-h (*B–F*) or 2-h (*G* and *H*) incubation at room temperature, and the organic phases were analyzed by GC-MS and GC-FID. Sections of GC-FID chromatograms are depicted. IS, internal standard.

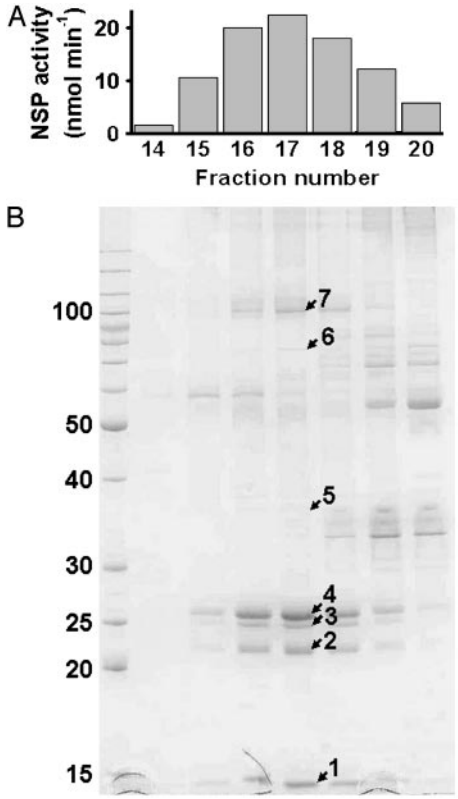

Fig. 5. Correlation of NSP activity with protein bands on SDS/PAGE after size exclusion chromatography of a purified NSP preparation. A plot showing total NSP activity per fraction (*A*) is superimposed with an image of a Coomassie-blue-stained SDS/PAGE gel showing the protein composition of each fraction (*B*). The eluate was collected in 2-ml fractions that were analyzed for NSP activity and by SDS/PAGE (an equivalent of 500 μl of fraction was loaded per lane). Arrows indicate bands analyzed by LC-MS/MS. The numbers on the left indicate the sizes of the molecular mass marker bands in kilodaltons.

nate. With excess amounts of midgut extract added, only the nitrile and no isothiocyanate was detected. No nitrile formation was observed in assays containing extracts of the rest of the body (not shown) or boiled midgut extract (Fig. 4*C*), indicating the involvement of a gut protein. Without addition of myrosinase, no hydrolysis products were formed in the presence of midgut extract (Fig. 4*D*) or extracts of the remaining body parts (not shown), consistent with the absence of a larval myrosinase activity. Nitrile formation

MKGVVVFLALAALGSAKPRLFETFQDHFKHFVDISNALEGAHWRRQQGQ
GYTPNPEYIAMLNKLGKLDLEQMFDDLKKEPEVQDIIEYLDTLTIDADY
YVENLQWIIQKLYVNDTNGIPGVEFHHRTRRHPMTGTTMTTALADTVSM
LPIRQLRATFNEKMAKNALFRNAIEGLKSERFLTLYKALWKNEAFLKVS
NILADCDFDLKYVFEELAVSLLGQNHPIQVTFQIQFDEFLDIIVNMESP
HWLRQLGGYKSFPEFMKSLDALSKTKLIDHYPRMNSASPAFKKVDAFLK
RHGIFVAYWIDRFDFLTEYFQKNDQAIGCNEIVPTEESHRSRRHPMTGR
TMHTFLADTVSLLPIRQLQSLFNEKMETNAIFKKAIEGVRNDEFKELYN
DLWTDDAWLDLVDELQTKYDLDLKYAVETLAPALYGQNVPLYISFQTQF
DEFVEIILAKAGDSIKSLIKAYKQNDAFRATLDTLDNTVFIQLYQDLRK
LNVFQTLDAYWKKHDVYVPYYIDRFEYLTYRLNTNVSEVGELEIKQSAG
QDITPSGTTMADFFADVVKILPKTELAALYEKKMSDNTVFSTAVNSLKS
EEGKKLYNDLWENRTFQAVANAYANNDFNFRYIFETFVPALYGQ

Fig. 6. Amino acid sequence of *P. rapae* NSP. A predicted extracellular targeting signal is shown in green. The protein contains two complete amino acid repeats (shown in red and blue) covering a stretch of ≈200 aa each and one incomplete repeat (shown in orange). Peptide sequences identified by LC-MS/MS analyses are underlined.

Table 1. N-terminal amino acid sequences of proteins obtained from purified NSP by Edman degradation

Band no.	Size, kDa	N-terminal sequence	Protein identification/position
1	14	KPRLF	d03c03 protein/17–21
2	22	HPMTGTTMT	d03c03 protein/130–138
4	26	HPMTGTTMTTALAD	d03c03 protein/130–143
		IVGGSPSQHGFFP	Similar to protease from *Drosophila melanogaster*

The position of the identified peptides within the amino acid sequence deduced from the cDNA sequence of the *P. rapae* EST d03c03 is given with the starting Met counted as position 1. Band numbers refer to Fig. 5. Note the predicted signal peptide cleavage site of the d03c03 protein (PrNSP) between amino acids 16 and 17 (Fig. 6).

was not detected on incubation of benzyl isothiocyanate with midgut extract (not shown). These results suggested the presence of a proteinaceous factor in the larval midgut that redirects myrosinase-catalyzed glucosinolate hydrolysis favoring nitrile instead of isothiocyanate formation. We designated this factor as an NSP with reference to the epithiospecifier protein that facilitates epithionitrile and nitrile formation in certain plants (15, 24, 25). Besides benzylglucosinolate, other glucosinolate substrates [4-hydroxybenzylglucosinolate, 4-(methylthio)butylglucosinolate, allylglucosinolate, and 3-butenylglucosinolate] were also converted into nitriles in the presence of midgut extracts and myrosinase (not shown). An initial survey of other lepidopteran insects showed the presence of NSP activity in larvae of the closely related *P. brassicae*, which also feed on plants containing glucosinolates and myrosinases, but its absence in larvae of the generalist lepidopteran herbivore *Spodoptera littoralis* reared on *A. thaliana*.

NSP Is Unrelated to Any Functionally Characterized Protein. To investigate NSP in more detail, the activity was purified from larval midgut extracts by four chromatographic steps. Purified NSP migrated as one sharp band on native PAGE and gave a consistent pattern of seven protein bands between 14 and 100 kDa on SDS/PAGE (Fig. 5). Tryptic peptides obtained from the native and SDS/PAGE bands were subjected to LC-MS/MS analyses. Searches within a database of 8,000 *P. rapae* ESTs showed that the MS data obtained from peptides of all analyzed bands matched a single *P. rapae* EST designated d03c03 with a sequence coverage of 43% (Fig. 6). This suggested that the protein corresponding to the EST d03c03 fragments on SDS/PAGE. No other sequences matching the mass spectral data were found in either the *P. rapae* EST or the National Center for Biotechnology Information database. Amino acid sequencing of selected protein bands by Edman degradation confirmed the

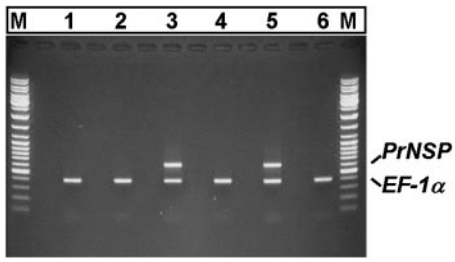

Fig. 7. Expression analysis of *PrNSP* by RT-PCR. Agarose gel shows RT-PCR products obtained from different tissues of fourth instar *P. rapae* larvae. Lane 1, head; lane 2, body (without head, gut, fat body, and Malpighian tubules); lane 3, midgut; lane 4, fat body; lane 5, hindgut with Malpighian tubules; lane 6, adults. The *PrNSP* transcript is represented by a PCR band of 650 bp, the *EF-1α* transcript (control) by a band of 400 bp. M, DNA ladder.

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results of the LC-MS/MS analyses and revealed that one of the bands contained an additional protein with an N-terminal sequence similar to a serine protease (Table 1) that was not represented in our *P. rapae* EST database and that might be responsible for the observed fragmentation.

The full-length cDNA sequence of EST d03c03 revealed an ORF of 1,896 bp, corresponding to a polypeptide of 632 aa (Fig. 6) and a molecular mass of 73 kDa. When the d03c03 cDNA was expressed in *E. coli*, extracts of bacteria harboring the d03c03 expression construct contained a protein of ≈70 kDa on SDS/PAGE with NSP activity that was absent from controls (Fig. 4 *G* and *H*). Thus, the EST d03c03 encodes an NSP that we refer to as PrNSP.

PrNSP does not possess any significant amino acid sequence similarity to the plant epithiospecifier protein (15) but has similarity to a group of insect gut proteins with unknown function that have been characterized as major allergens (26). It shows highest sequence similarity to the CrPII allergen (27) from the cockroach *Periplaneta americana* (24% identity, 45% similarity), to the amino acid sequence derived from an uncharacterized EST from a midgut cDNA library of the cotton bollworm, *Helicoverpa armigera* (GenBank accession no. BU038722; 26% identity, 51% similarity), and to the ANG12 protein from the mosquito *Anopheles gambiae* (GenBank accession no. CAA80505; 21% identity, 42% similarity). The cockroach allergens are found in the digestive tract, are excreted into the feces, and act as allergens if inhaled (26). The *ANG12* gene encodes a midgut protein of female mosquitoes and is induced after a blood meal [H. M. Mueller and A. Crisanti, (GenBank accession no. CAA80505)].

PrNSP Expression Is Tissue-Specifically and Developmentally Controlled. Transcript encoding PrNSP was detected in larval midgut tissue as well as in hindgut tissue with attached Malpighian tubules but not in the remaining body parts (Fig. 7). Hence, transcript accumulation coincided with the localization of NSP activity in *P. rapae* larvae. Transcripts were detected in all larval instars but not in *P. rapae* eggs or adults. *PrNSP* transcription is thus confined to digestive tissues and to developmental stages in which *P. rapae* feeds on tissue containing glucosinolates and myrosinases.

Discussion

The identification of PrNSP and the observed excretion of nitriles demonstrate that *P. rapae* larvae are able to biochemically counter the glucosinolate–myrosinase defense system of their host plants. The NSP activity in their guts redirects glucosinolate hydrolysis toward nitrile formation circumventing the generation of isothiocyanates (Fig. 1), which are highly reactive compounds toxic to a broad range of organisms (5) including *P. rapae* (6). Nitriles are generally less toxic than isothiocyanates (5), and larvae of *P. rapae* excrete nitriles in the feces unmodified [e.g., 5-(methylsulfinyl)pentanenitrile)] or after further metabolism (e.g., 4-hydroxyphenylacetonitrile).

The mechanism of PrNSP activity is still unclear. Because this protein does not have hydrolytic activity on glucosinolates in the absence of myrosinase (Fig. 4), it may serve as a cofactor that binds to the plant myrosinase and alters the direction of glucosinolate hydrolysis. Alternatively, it may use the glucosinolate–aglucone as a direct substrate for nitrile formation, a mechanism that would also require tight coupling with the myrosinase-catalyzed reaction. In either case, the function of this insect protein is realized only in conjunction with the activity of myrosinase, a plant enzyme.

Most known biochemical mechanisms used by insects to overcome plant chemical defenses involve the detoxification of secondary plant products (28). However, a two-component defense such as the glucosinolate–myrosinase system appears to offer additional targets for herbivore interference. Rather than metabolizing the toxic hydrolysis products of glucosinolates, *P. rapae* instead redirects the hydrolysis process to form nitriles. This mechanism is fundamentally different from that reported for another lepidopteran herbivore specialized on glucosinolate and myrosinase-containing plants, *Plutella xylostella*, which desulfates glucosinolates to metabolites that can no longer act as substrates for myrosinases (9). Thus, the complexity of the glucosinolate–myrosinase system is reflected in the diversity of mechanisms for insect herbivore counteradaptation.

PrNSP appears to be the key biochemical counteradaptation that allows *P. rapae* to feed on plants containing glucosinolates and myrosinases with impunity. Future research may shed light on its evolutionary origin and mechanism as well as suggest possibilities to block the action of NSP as a basis for the development of novel highly selective strategies to control *P. rapae* and *P. brassicae*, serious pests on crops of the Brassicaceae family.

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