Supplementary Information

Identification and evolution of glucosinolate sulfatases in a specialist flea beetle

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Supplementary Figures and Tables



Supplementary Figure S1. Glucosinolate sulfatase activity in *Psylliodes chrysocephala* and *Phyllotreta armoraciae*. Crude protein extracts from the gut and the remaining body tissues were incubated with eight different glucosinolates. The extracted LC-MS/MS ion chromatogram of desulfo-sinalbin is shown. Remaining intact sinalbin in the assay is detected as desulfo-sinalbin due to in-source fragmentation in positive ionization mode.

	Signal peptide	
PcSulf1 PcSulf2 PcSulf3 PcSulf4 PcGSS1 PcGSS3 PcGSS3 PcGSS5 PxGSS1	MTWFRFVSLTLLNIYNIVENI - YFVINGISBE MPCTSAFTIFVTSALCSSMSFAEOPN MWESTSAFVTFSOISVAIGENTKSEKE MWESKVFTIFLGJGIMETOKPN MWESKVFTIFLGJGIMETOKPN MWESKVFTIFLGJV-GIMETOKPN MVUSKVFTIFLGJV-GIMETOKPN MADEWFMLHILGJV-GIMETOKPN MADEWFMLHILGJV-GIMETOKPN MADEWFMLHILGJV-GIMETOKPN MKPN	IS INADDIGNDUGFHGSNCIPTPNIDALAYNGIILNNYYVTAICTPSRSALMTGKYPIHGMOHAVUYGAEPEGLPLNE 114 IS UNADDIGNDUGFHGSNCIPTPNIDALAYNGIILNEYYHTSISTFBRAALUTGKYPHIGGOGSSSFBAARSLEPEGI IS BADDUGNDUGFHGSDEIPTNIDALSNGVILDESYTOJTCTPSRAALUTGKYPHIGGOGSSSFBAARSLEPEN II UNADDUGENDUGFHGSDEIPTNIDALAYNGVILDHYYTGSICTPSRAASLTGKYPHIGGOHAVUDESEPEGLELNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGSICTPSRAASLTGKYPHIGGOHAVUDESEPEGLELNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGSICTPSRAASLTGKYPHIGGOHAVUDESEPEGLELNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGSICTPSRAASLTGKYPHIGGOHAVUDESEPEGLELNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLELNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLELNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLELNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLELNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLENNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLENNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLENNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLENNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNHYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLENNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNEYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLENNE 104 IS MADDUGENDUGFHGSDEIPTNIDALAYNGVILNEYTGAICFPSRAASLTGKYPHIGGOHAVUDESEPEGLENNE 104 IS MADDUGENDUGFHGSDEIFTNIDALAYNGVILNENTGAUCTFPSRAASLTGKYPHIGGOHAVUDESEPEGLEDINE 104 IS MADDUGENDUGFHGSDEIFTNIDALAYNGVILNENTGAUCTFPSRAASLTGKYPHIGGOHAVUDESEPEGLEDINE 104 IS MADDUGENDUGFHGSDEIFTNIDALAYNGVILNENTGAUCTFPSRAASLTGKYPHIGGOHAVUDESEPEGEPINDELFNU
PcSulf1 PcSulf2 PcSulf3 PcSulf4 PcGSS2 PcGSS3 PcGSS5 PcGSS5 PxGSS1	KLIPOLICELCY ZNEUCKWHLGSYRKEYLDYFRGFKSE KLIPSYFKELCYSPULICKWHLGYSFKNFTFZERGFDTE FYMSEFLKKLCYSFNLCKKWHLGSEKKSYTFTRGFDTE TIMPOLIKONCYSFNLGSEKKSYTFTRGFDTE TIMPOLIKONCYSFNLGSEKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWHLGESKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWHLGESKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWHLGESKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWLGESKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWLGESKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWLGESKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWLGESKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWLGESKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWLGESKKSYTFTRGFDTE TIMPOLIKONCYTHSVCKWLGESKKSYTFTRGFDTE	GSMTGHDYNTHTAVESPGNGLDARRNMSVAYDLHGKYSTDUFT2GSVRITKNHNTTHEIFYLAHDANHSGN 227 GYNGGSASYDVIITTMRFNGRDYTGFLRNDGRALSESGKYRTDUFTDHTVKITMEDDSRSFTLMAAHANHSGN 227 GMTGYNGHSYEINVTSFIDAKKNFSGFDLHDGFOF0WOLGGYAFLBAKKSDTHERHDINGSFIFIAUGHEN GMTGYNGYTHMIKASFSDECUEFRRNI VWDAKGKYSTTLFTNEAVKLISENDINGWFLYAHAAHAGN 229 GWTLHDYTTHMIKASFSDECUEFRRNI VWDAKGKYSTTLFTNEAVKLISENDINGWFLYAHAAHAAHAGN 229 GYLLHDYTTHMIKADYANDTGYDFRRNI VWDAKGKYSTTLFTNEAVKLISENDINGWFLYAHAAAHAAHAA 219 GWTLGHDYTTHMIKADYANDTGYDFRRNI VWDAKGKYSTTLFTNEAVKLISENDINGWFLYAHAAAHAAHAA 219 GWTLGHDYTTHMIKASWSTEICUEFRRNI VWDAKGKYSTTLFTNEAVKLISENDINGWFLYAHAAAHAAHAA 219 GWTCGHDYYTHMI
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PcSulf1 PcSulf2 PcSulf3 PcSulf4 PcSS51 PcSS52 PcSS53 PcGS55 PxGS55 PxGS51	VWINFGEVILNFGTVIIA- VMINFGIECTKPDCSPLENN- VMIFMIECTKPDCSPLENN- VMINFGUICKKNN	Transmembrane domain 554 559 563 MISE IVVS IILFMIFGL-SKLADTEARKREKKRKSEALLSNNGA- 620 INIS IVIS IILFMVFGL-SKLADTEARREFENADALLRNNNS- 623 INII IILSS MILFMIFGL-SKLADTEARRERKEQKKKKALLSNNNYF 634 MIII IVVS IILFIVFRLSSKLADTKAKKREQKRKAETLLSNNSV- 614 MIII IVVS IILFIVFRLSSKLADTKAKKREQKRKAETLLSNNSV- 614 MIII IANSSTILFIVFRLSSKLADTKAKKREQKRKETLLSNNSV- 614 547

Supplementary Figure S2. Amino acid sequence alignment of arylsulfatase-like enzymes (Sulfs) and glucosinolate sulfatases (GSSs) from *Psylliodes chrysocephala* and the *Plutella xylostella* GSS1 (*Px*GSS1). Two conserved signature sequences of arylsulfatases are marked with a bar above the alignment and catalytically active residues are marked with ▼. All enzymes possess an N-terminal signal peptide. The C-terminal transmembrane domain is marked with a black frame. The threshold for similarity (grey)/identity (black) shading was set to 70%.



Supplementary Figure S3. Detection of recombinant *Pc*Sulfs, *Pc*GSSs, and *Px*GSS1 by Western blotting. Proteins were heterologously expressed in *Sf*9 insect cells. Protein samples marked with an asterisk were concentrated by trichloroacetic acid (TCA) precipitation.



Supplementary Figure S4. Activity of recombinant sulfatases towards the general substrate 4-nitrocatechol sulfate. Proteins were heterologously expressed in *Sf*9 insect cells and non-transfected *Sf*9 cells served as a control. n.d., not detected. Means+SD of n=3 technical replicates.



Supplementary Figure S5. LC-MS/MS analyses of glucosinolate sulfatase activity assays using *Sf*9-expressed *Pc*GSS1 (a) and *Pc*GSS2 (b). Heterologously expressed enzymes were incubated with a mixture of eight glucosinolates. Extracted LC-MS/MS ion chromatograms of desulfo-sinalbin (a) and desulfo-indol-3-ylmethyl glucosinolate (desulfo-I3M glucosinolate) (b) are shown. Remaining intact glucosinolate in the assay was detected as desulfo-glucosinolate due to in-source fragmentation in positive ionization mode.



Supplementary Figure S6. Mutation of the asparagine residue at position 125 does not affect glucosinolate sulfatase (GSS) activity of *Pc*GSS3 and *Pc*GSS4 towards the substrate 3-butenyl glucosinolate. *Pc*GSS3, *Pc*GSS3 N125H, *Pc*GSS4, and *Pc*GSS4 N125H were heterologously expressed in High FiveTM insect cells and incubated with eight different glucosinolate substrates for 2 h at 35°C. Means+SD of n=3 technical replicates.



Supplementary Figure S7. Analysis of selection pressures acting on *PcGSS* and *PcSulf4* genes and other members of the coleopteran Sulf4 clade. The Maximum-Likelihood tree of coleopteran Sulf4 and GSS enzymes was constructed using MEGA 7.0.14, rooted with *Tc*Sulf4. The two major active *GSS* are shown in bold. Panels (**a**) to (**f**) show the series of models that were used to determine whether different selection pressures act on the two *PcGSS* subclades (**a** and **b**), on the entire *PcGSS* clade and the ancestral *PcSulf4* gene (**c** and **d**), and on the *PcGSS/PcSulf4* clade, the *PaSulf4/PsSulf4* clade, and other coleopteran *Sulf4* genes (**e** and **f**). The ω ratios calculated with the final three-ratio model are shown on the same tree in (**g**). All clades are under purifying selection. *Pc, Psylliodes chrysocephala; Pa, Phyllotreta armoraciae; Ps, Phyllotreta striolata; Dp, Dendroctonus ponderosae; Ld, Leptinotarsa decemlineata; Tc, Tribolium castaneum.*



Supplementary Figure S8. Alignment of signature sequences I and II from coleopteran and lepidopteran aryIsulfatase-like enzymes (Sulfs) and glucosinolate sulfatases (GSS). Catalytically active residues are marked with ▼. The threshold for similarity (grey)/identity (black) shading was set to 80%. Coleoptera: *Pc, Psylliodes chrysocephala*; *Pa, Phyllotreta armoraciae*; *Ps, Phyllotreta striolata*; *Dp, Dendroctonus ponderosae*; *Ld, Leptinotarsa decemlineata*; *Tc, Tribolium castaneum*; Lepidoptera: *Bm, Bombyx mori*; *Ms, Manduca sexta*; *Px, Plutella xylostella.*

Gene	Newly emerged adults		7 day-old adults fed on Sinapis alba (n=5)		7 day-old adults fed on Brassica rapa (n=4)	
PcSulf1	t=1.346	<i>P</i> =0.271	t=-0.227	<i>P</i> =0.832	t=-6.824	<i>P</i> =0.006
PcSulf2	<i>t</i> =-5.098	<i>P</i> =0.015	<i>t</i> =-34.550	<i>P</i> <0.001	<i>t</i> =-19.129	<i>P</i> <0.001
PcSulf3	<i>t</i> =-8.736	<i>P</i> =0.003	<i>t</i> =-54.822	<i>P</i> <0.001	<i>t</i> =-11.728	<i>P</i> =0.001
PcSulf4	<i>t</i> =-10.722	<i>P</i> =0.002	<i>t</i> =-9.245	<i>P</i> <0.001	<i>t</i> =-57.978	<i>P</i> <0.001
PcGSS1	<i>t</i> =18.188	<i>P</i> <0.001	<i>t</i> =6.643	<i>P</i> =0.003	<i>t</i> =2.666	<i>P</i> =0.076
PcGSS2	<i>t</i> =2.444	<i>P</i> =0.092	<i>t</i> =2.569	<i>P</i> =0.062	<i>t</i> =3.554	<i>P</i> =0.038
PcGSS4	<i>t</i> =11.017	<i>P</i> =0.002	<i>t</i> =14.360	<i>P</i> <0.001	<i>t</i> =-4.991	<i>P</i> =0.015
PcGSS5	<i>t</i> =12.013	<i>P</i> =0.001	<i>t</i> =11.033	<i>P</i> <0.001	<i>t</i> =5.446	<i>P</i> =0.012

Supplementary Table S1. Comparison of *PcSulf* and *PcGSS* gene expression in the gut and the rest body of *Psylliodes chrysocephala* by paired *t*-test.

Supplementary Table S2. Comparison of *PcGSS* gene expression in newly emerged adults and after feeding for seven days on *Sinapis alba* and *Brassica rapa*, respectively.

Gene	Method	Statistics	P value
PcGSS1	Kruskal-Wallis test	<i>H</i> =8.523	<i>P</i> =0.005
PcGSS2	Kruskal-Wallis test	<i>H</i> =2.443	<i>P</i> =0.319
PcGSS4	ANOVA	<i>F</i> =4.145	<i>P</i> =0.046
PcGSS5	ANOVA	<i>F</i> =1.143	<i>P</i> =0.354

Supplementary Table S3. Comparison of *PcGSS* gene expression and GSS

activity in gut tissue of adult Psylliodes chrysocephala injected with dsRNA

targeting *PcGSS1*, *PcGSS2*, and *IMPI*, respectively.

Gene	Transformation	Method	Statistics	P value
PcGSS1	Log ₁₀	ANOVA	F=75.988	<i>P</i> <0.001
PcGSS2	-	Kruskal-Wallis test	<i>H</i> =9.231	<i>P</i> =0.01
PcGSS4	-	ANOVA	<i>F</i> =12.662	<i>P</i> <0.001
PcGSS5	-	ANOVA <i>F</i> =58.117		<i>P</i> <0.001
Desulfo-glucosinolate				
desulfo-3But	-	Kruskal-Wallis test	<i>H</i> =1.801	<i>P</i> =0.406
desulfo-4MSOB	Log ₁₀ (x+0.01)	ANOVA	F=5.065	<i>P</i> =0.014
desulfo-4MTB	Log ₁₀ (x+0.01)	ANOVA	F=2.486	<i>P</i> =0.102
desulfo-I3M	Log ₁₀	ANOVA	<i>F</i> =10.552	<i>P</i> <0.001
desulfo-2PE	-	Kruskal-Wallis test	<i>H</i> =0.113	<i>P</i> =0.954
desulfo-Benzyl	Log ₁₀	ANOVA	<i>F</i> =12.514	<i>P</i> <0.001
desulfo-Sinalbin	Log ₁₀	ANOVA	<i>F</i> =21.001	<i>P</i> <0.001

3But, 3-butenyl; 4MSOB, 4-methylsulfinylbutyl; 4MTB, 4-methylthiobutyl; I3M, indol-3-ylmethyl; 2PE, 2-phenylethyl

Supplementary Table S4. LC-MS/MS parameters for multiple reaction monitoring

Compound	Q1 [<i>m/z</i>]	Q3 [<i>m/z</i>]	DP (V)	EP (V)	CE (V)	CXP (V)
desulfo-2OH3But	310.0	148.0	30	5	15	5
desulfo-3But	294.0	132.0	30	5	15	5
desulfo-4MSOB	358.0	196.0	30	5	15	5
desulfo-4MTB	342.0	180.0	30	5	15	5
desulfo-I3M	369.0	207.0	30	5	15	5
desulfo-2PE	344.1	182.1	30	5	15	5
desulfo-Benzyl	330.0	168.0	30	5	15	5
desulfo-Sinalbin	346.0	184.0	30	5	15	5
Sinalbin	424.0	95.9	-65	-5	-60	0

(MRM) of desulfo-glucosinolates and sinalbin.

2OH3But, 2-hydroxy-3-butenyl; 3But, 3-butenyl; 4MSOB, 4-methylsulfinylbutyl; 4MTB, 4-methylthiobutyl; I3M, indol-3-ylmethyl; 2PE, 2-phenylethyl