Understanding the chemical ecology of Brassicaceae specialist herbivore *P. xylostella* and its predators

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by

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Certificate

This is to certify that this dissertation entitled ‘Understanding the chemical ecology of Brassicaceae specialist herbivore *P. xylostella* and its predators’ towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Mahendra Pawar at Indian Institute of Science Education and Research under the supervision of Dr. Sagar Pandit, Assistant Professor, Department of biology, IISER Pune during the academic year 2019-2020.

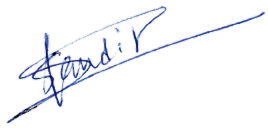
Mahendra Pawar

Dr. Sagar Pandit

Declaration

I hereby declare that the matter embodied in the report entitled 'Understanding the chemical ecology of Brassicaceae specialist herbivore *P. xylostella* and its predators' are the results of the work carried out by me at the Department of Biology Division, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Sagar Pandit and the same has not been submitted elsewhere for any other degree.

Mahendra Pawar



Dr. Sagar Pandit

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Abstract

Plant infochemistry plays crucial role in governing the tritrophic interactions. Biological pest control is an environmental friendly approach that uses the natural enemies to control the pest population. To establish a potential biocontrol agent it's crucial to understand the natural enemy's response to the plant-produced as well as herbivore-metabolized chemicals. Here we tried to investigate the effect of glucoraphanin and its metabolized forms produced by the diamondback moth larvae on plant-herbivore-natural enemy interactions to reveal the ecological significance of plant's defense and herbivore's phytotoxin detoxification mechanisms in the tritrophic interactions. Native Indian biological control agents for the diamondback moth were collected and identified based on the available keys. Plant-mediated RNA interference (PMRi) was used to silence the detoxification gene in *Plutella xylostella*. PMRi led to the accumulation of toxic isothiocyanates in gut, hemolymph and frass of *P. xylostella* larvae. These toxic isothiocyanates showed detrimental effects on growth of the herbivore. Three predatory spider species- *Hasarius adansoni, Plexippus paykulli* and *Wadicosa fidelis* were used to evaluate the effect of gene silencing on the behaviour and choice of the predators. Spiders showed uniform behavior to the changes in metabolite composition of their prey. Frass analysis of these spiders revealed that the spiders were able to detoxify the toxic isothiocyanates via mercapturic pathway. Thus, this case study demonstrates the ecological significance and metabolism of glucosinolates in plant-herbivore-natural enemy interaction. This study contributes valuable inputs towards devising integrated pest management strategies against the pest *P. xylostella*.

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1. Introduction

Plants and insects are considered to be co-evolved since they are living together for a long time. Plants being sessile in nature need robust defense strategies to defend themselves from insect herbivores. Plants demonstrate various defense strategies that include mechanical barriers and production of allelochemicals (War et al., 2012; Bennet and Wallsgrove, 1994). The mechanical barriers include structural defenses such as spinescence, trichomes, thick leaves, barks and waxy cuticles (Hanley et al., 2007). Allelochemicals are bioactive compounds that can interfere with physiology and affect the growth and survival of herbivore (Weir, Park, and Vivanco, 2004). Herbivores, in turn, have evolved many different methods such as detoxification, rapid excretion, and sequestration to counter the plant defenses (Heckel, 2018; Mello et al., 2002; Jhonson and Dowd, 1983). In addition to direct chemical defense, some of the host plant volatile chemicals can serve as a mode of indirect defense by attracting natural enemies of herbivores such as predators and parasitoids. Often, the plant's direct defense and indirect defense are examined individually. Many times the same chemical that is responsible for direct defense, also influences the behavior and development of the natural enemies (Gols et al., 2008; Thaler, 1999). Hence, it is important to study the tritrophic interactions involving plants, insect herbivores and their natural enemies together.

Brassicaceae family plants produce glucosinolates as major defense compounds. Brassicaceae family plants include agronomically important crop plants such as broccoli, cabbage, mustard and cauliflower. The general structure of glucosinolates contains a central carbon atom attached to a thio-glucose, sulfated aldoxime and a variable side chain. Glucosinolates are classified into three groups as aliphatic, indolic and aromatic glucosinolates based on the presence of characteristic side chain group (Agerbirk and Olsen, 2012). Intact glucosinolates themselves are not toxic to herbivores. Brassicaceae plants contain an enzyme called myrosinase (thioglucosidases). In plants, glucosinolates and enzyme myrosinase are stored in separate cellular compartments of the leaf to prevent self-intoxication (Ratzka et al., 2002). Glucosinolates are majorly stored in sulphur rich cells (S-cells), while

myrosinase is stored in myrosin cells present near stomata and in phloem idioblast (Koroleva et al., 2000; Andréasson et al., 2001). When an herbivore chews the leaves, the compartments rupture facilitating contact between enzyme myrosinase and intact glucosinolates. Deglycosylation of glucosinolates leads to the formation of aglucones which are unstable and they further rearrange to form toxic isothiocyanates (ITCs) (Haliker and Gershenzon, 2006). This is known as detonation of mustard oil bomb (Luthy and Matile, 1984). Isothiocyanates are lipophilic in nature and can enter the cells via passive diffusion. Due to the electrophilic nature of ITCs, they readily react with the nucleophiles in the cells and cause disruption in structure and function of the proteins (Brown and Hampton, 2011; Jeschke et al., 2016a).

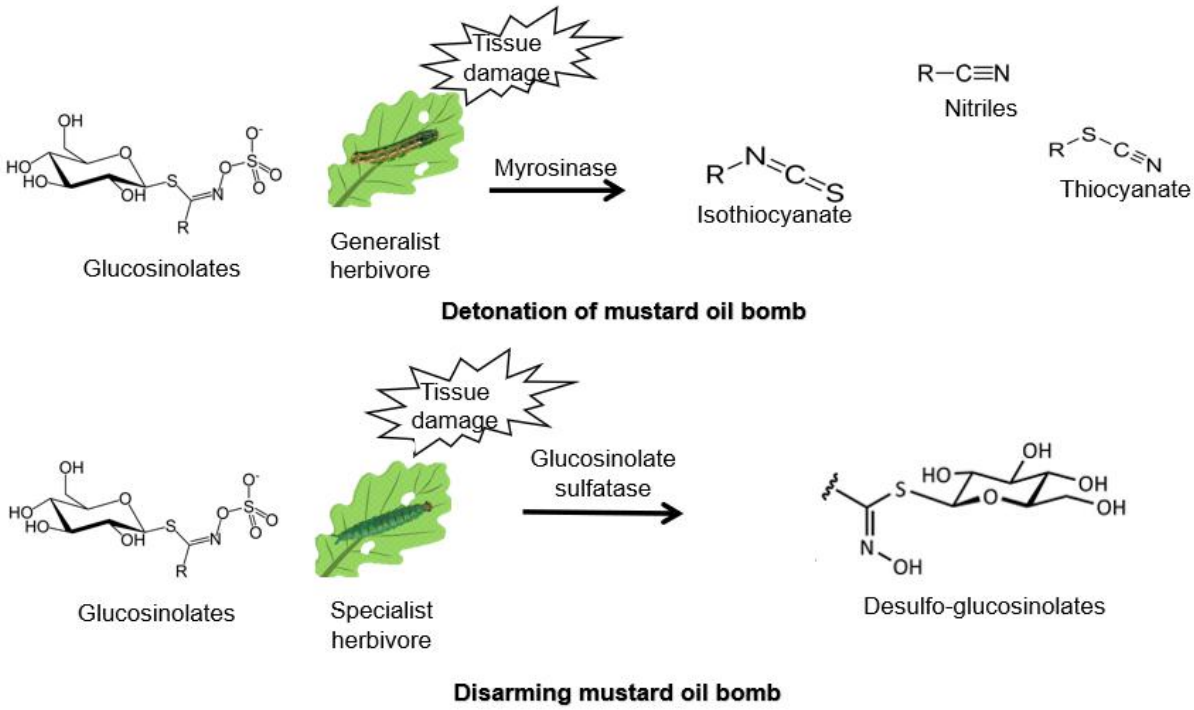


Fig 1: Schematic representation of glucosinolate-myrosinase system. When a generalist herbivore feeds on the leaf tissue, facilitating the formation of toxic isothiocyanates and other toxic products. But, in case of *P. xylostella* the glucosinolates are rapidly being desulfatized into non-toxic desulfoglucosinolates by the enzyme glucosinolate sulfatase.

P. xylostella is a globally found specialist insect pest of Brassicaceae crops and its larval stage is most devastating in nature (Macharia and Groote, 2005). Despite the toxic glucosinolates, these insects feed heavily on Brassicaceae

plants and cause severe damage to agriculturally important crops such as cabbage, cauliflower, broccoli and mustard. *P. xylostella* has evolved a counter-defense mechanism against its host's glucosinolate-myrosinase chemical defense system. It uses glucosinolate sulfatase (PxGSS) enzyme, which desulfatizes the glucosinolates and thus enzyme myrosinase cannot act on desulfoglucosinolates. This is known as disarming the mustard oil bomb (Ratzka et al., 2002). In spite of possessing the PxGSS, *P. xylostella*'s larval and pupal development times and moth fecundity were found to vary as a function of the glucosinolate composition of its hostplants (Sarfraz et al., 2009). Diamondback (DBM) female moths select the host plants after a careful assessment of their glucosinolate composition. Hence, the glucosinolates play crucial role in *P. xylostella*-host interaction.

The global estimated damage caused by DBM is around 4-5 billion US dollars (Zalucki et al., 2012). In Asia, this pest has known to damage the crops upto 90 % (Verkerk and Wright, 1996). *P. xylostella* is known to survive in varied temperatures ranging from 10°C to 35°C (Marchioro, 2011). *P. xylostella* completes its life-cycle from egg stage to adult moth in short span of 20-25 days (Golizadeh et al., 2009). Due to these factors, *P. xylostella* has become a destructive pest whose control remains a global problem. To control this pest population many synthetic pesticides were tried. But *P. xylostella* has acquired resistance to most of the known synthetic pesticides (Agboyi et al., 2016). Uses of chemical pesticides have many disadvantages in it. Spraying chemical pesticides contaminates the soil, water and nearby plants thus deteriorating the growth and quality of crops. Many chemical pesticides are harmful for the natural enemies of the herbivores (Aktar, 2009). As these pesticides are sprayed on the consumable crops, they adversely affect the human health (Hernández, 2013). Bt toxin has been used as substitute against chemical pesticides. *P. xylostella* has been reported to develop resistance against Bt toxins (Pardo et al., 2015). Hence, sustainable alternative strategies are required to control this pest population in the crop field. Biological pest control is one of the alternative approach which has been explored (Sarfraz, 2005).

Different hymenopteran parasitoids were used as biocontrol agents to control the pest population. Host plant's glucosinolate composition was found to critically influence growth and development of parasitoids inside the *P. xylostella*'s larvae. Endoparasitoids *Diadegma fenestrale* and *Diadegma semiclausum* were found to be affected by the glucosinolate content of the diet of their host herbivores (Gols et al., 2009). Another parastoid, *D. insulare* showed differential growth rates in *P. xylostella* feeding on different *Brassica* species having varied glucosinolate contents (Sarfaraz, 2009). Parasitoids that were used for biocontrol agents were native to the temperate climates of Europe. When these same parasitoids were introduced in India, they were not successful due to difference in geoclimatic conditions (Talekar, 1997). Thus, all these reports suggest that plant chemistry and climatic conditions are crucial to the crop-DBM-natural enemy interaction. However, since most of the abovementioned findings have come from comparisons involving different *Brassica* species or different varieties of the same species and since the glucosinolate composition of *Brassica* plants is usually highly complex, these studies could not ascertain whether the effects could be attributed to the qualitative variations in the overall glucosinolate composition or quantitative variations in the major glucosinolates. For designing a robust pest control strategy, factors influencing the predator behavior and performance must be clearly understood, especially roles of at least the most common and abundant glucosinolates should be deciphered so that the *Brassica* breeding programs will obtain clear goals on whether to increase or decrease their concentrations in the upcoming varieties. Thus, understanding the tritrophic interaction will help us to establish the integrated pest management (IPM) approach to reduce the damage caused by *P. xylostella*.

In this project, we investigated the top-down (natural enemies) and bottom-up (plant chemical defense) forces acting on *P. xylostella* by using the combination of molecular techniques, performance assays and metabolomics. We performed LC-MS based glucosinolate profiling of nine native varieties of *Brassica* crops. Glucoraphanin, sinigrin and glucobrassicin were the major glucosinolates found in these *Brassica* crops. In order to study the effect of glucosinolates in tritrophic interaction, *Arabidopsis thaliana* was used as

model host-plant. Plant mediated RNAi was used to silence the *Pxgss1* (detoxification gene). Silencing the *Pxgss1* had a negative effect on the growth of *P. xylostella* larvae as seen by decreased weight of larvae fed on *Px*gss1 dsRNA infiltrated Col-0 plants (normal levels of glucosinolates). However, there was no negative effect seen on the growth of *P. xylostella* larvae fed on *Px*gss1 infiltrated *myb28myb29* (aliphatic glucosinolate deplete) plants. Instead, the *P. xylsotella* larval growth was higher when fed on *myb28myb2*9 (aliphatic glucosinolate deplete) plants as compared to larvae fed on Col-0 plants. *Pxgss1* silenced larvae had higher concentrations of isothiocyanates in the gut, hemolymph and frass. The accumulations of these toxic isothiocyanates were responsible for negative effect on growth of *P. xylostella* larvae. Spiders, earwigs and ladybird beetle were the most prevalent predators of *P. xylostella* larvae. No choice assays and behavioral assays were performed with three species of spiders to study the effect of altered glucosinolates on natural enemies of the *P. xylostella*. Predators showed uniform preference towards *P. xylostella* larvae fed on *Pxgss1* silenced and non-silenced Col-0 and *myb28myb29* (aliphatic glucosinolate deplete). The frass of the spiders contained conjugates of sulforaphane, suggesting that the spiders were able to detoxify the sulforaphane via general mercapturic acid pathway. Our work shows that, these predators are robust to any change in glucosinolate metabolism and composition unlike the previously reported parasitoids and natural enemies. These predators are well acclimatized to Indian tropical and sub-tropical climates. Hence, these predators can be employed as potential biocontrol agents.

2. Materials and methods

Plant material

Arabidopsis thaliana Columbia-0 (Col-0) accession (containing normal level of glucosinolates) and glucosinolate biosynthesis mutant *myb28myb29* (aliphatic glucosinolates deplete) were obtained from Arabidopsis Biological Research Centre (ABRC). They were germinated on Murashige and Skoog medium without sucrose (Hi-media) and later transferred to the soil mixture containing 1 % cocopeat, 1 % perlite, 1 % vermiculite and 1 % red soil. Plants were grown in a controlled short-day environment chamber at 22 °C, 70 % relative humidity, and a 10 h light: 14 h dark photoperiod with light intensity of 120-150 µmol m^{-2} s^{-1}. The VIGS plants were kept at long day conditions with 14 h light: 10 h dark photoperiod and provided with Hoagland solution (Hi-media, India) once a week. Commercially available *Brassica* crop seeds of native varieties were obtained and planted in a randomized manner in the IISER Pune field.

Insect culture

P. xylostella larvae were collected from *Brassica* crop fields in and around Pune and the culture was maintained on mustard seedlings *(Brassica napus)* which were grown in a short-day conditions at 26 °C, 65 % relative humidity and 16 h light and 8 h dark photoperiod. Adult diamondback moths were reared on 10 % sucrose solution. *P. xylostella* eggs required for the experiments were collected by prompting female moths to lay on a parafilm sheets pasted with *Brassica oleracea var. capitata* leaf extract placed in a cage. Parafilm sheets were transferred on the wild type and mutant *Arabidopsis* plants and the larvae were allowed to feed continuously on the plants.

Plant and insect tissue, RNA isolation and cDNA synthesis

Total RNA was isolated from homogenized Col-0 and *Px*gss1 infiltrated *A. thaliana* leaf tissues using manufacturer's protocol (RNA-Xpress reagent, HiMedia, India). cDNA was synthesized from this RNA by PrimeScript Reverse Transcriptase kits (Takara, Japan), using random hexamers.

Midgut of fourth instar *P. xylostella* larvae were dissected and pooled into TRIzol reagent (RNA XPress reagent, HiMedia), as *Pxgss1* is highly expressed in midgut (Heidel-Fischer, 2019). Midguts of larvae were crushed using glass beads and total RNA was extracted using manufacturer's protocol (RNA XPress reagent, HiMedia, India). cDNA was synthesized from this RNA by PrimeScript Reverse Transcriptase kits (Takara, Japan) using oligo dT primers.

Pxgss1 silencing construct for plant-mediated RNAi

A 526 bp fragment was used to silence the *Pxgss1* gene. *P. xylostella* contains three homologs of *Pxgss1* genes that show high sequence similarity with each other. The partial coding sequences of *P. xylostella Pxgss* genes were retrieved from NCBI (MG022096, MG022097 and MG022099). Another fragment of 127 base pair was designed that was unique to *Pxgss1* and would just silence the *Pxgss1.* 127 bp fragment of *Pxgss1* gene was amplified from total cDNA pool obtained from *Px*gss1 fragment specific primer pairs *Px*gss*1 F* (5'-CCTTGTGAGCTTCGTGACCTG-3') and *Px*gss1 *R* (5'-CAGACTTGGGGTCAGCGAC-3'). XbaI and XmaI restriction enzyme cutting sites were added to the ends of the amplified *Pxgss1* fragment using the primer pairs of *Px*gss1 *F-RE* (5'GCGGCGCCCGGGCCTTGTGAGCTTCGTGACCTG-3') and *Px*gss1 *R-RE* (5'-GCGGCGTCTAGACAGACTTGGGGTCAGCGAC-3'), and the fragment was further digested by XbaI and XmaI (Promega, USA). The pTRV1 (YL192) and pTRV2 (YL156) viral vectors were used to continuously produce dsRNA required for plant-mediated RNA interference. The restriction-digested 127 bp *Px*gss1 fragment was inserted in an antisense orientation into the multiple cloning site of XbaI-XmaI digested pTRV2 by T4 DNA ligase (Promega, USA). The cloned fragement was confirmed by sequencing. Phytoene desaturase (pds) fragment cloned in pTRV2 vector was used as a positive control to check the efficiency of virus induced gene silencing in plants.

Agrobacterium mediated infiltration of VIGS constructs

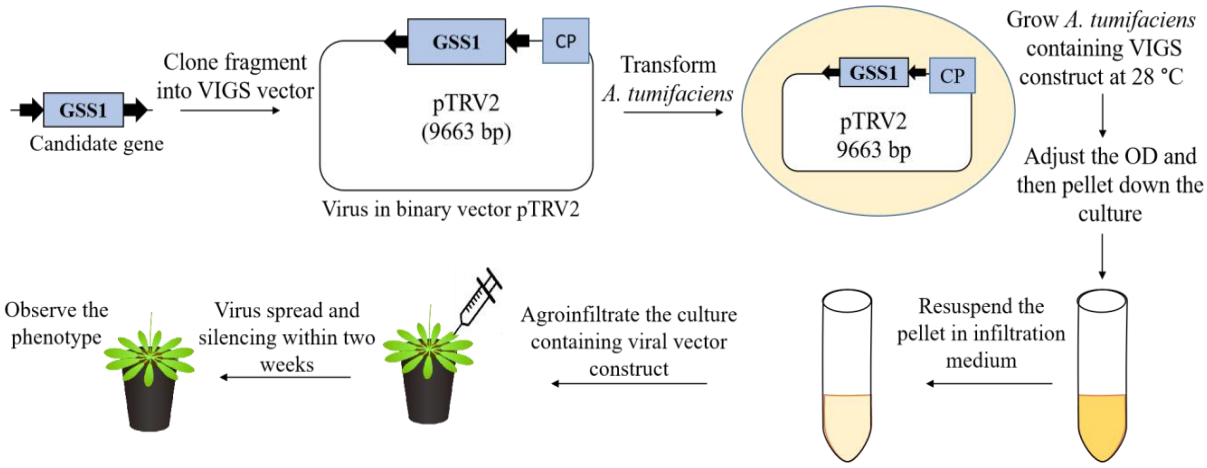


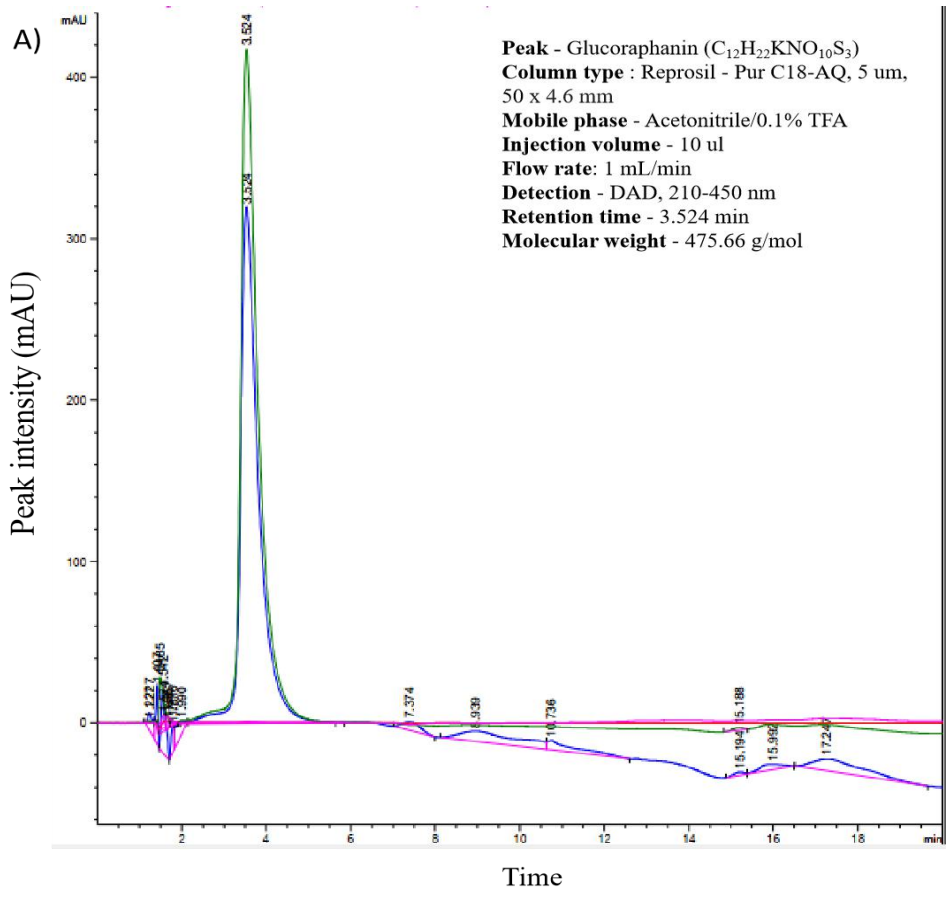
Fig 2: Schematic for VIGS work-flow

pTRV1 and pTRV2 containing *Px*gss1, pTRV2 empty vector, and pTRV2 containing *pds* constructs were transformed into GV3101 strain of *A. tumefaciens*. *A. tumefaciens* cultures were grown in Luria-Bertani (LB) medium containing 50 µg/ml kanamycin and 30 µg/ml rifampicin at 28 °C. After 48 h, *A. tumefaciens* cells were harvested by centrifugation (Eppendorf 5430 R, Germany) at 4,500 x g for 20 min. *A. tumefaciens* pellet was resuspended in infiltration medium containing 10 mM $MgCl_2$, 10 mM MES, and 200 uM acetosyringone in milli-Q water. OD_{600} adjusted to 1 with a UV/Vis spectrophotometer (Eppendorf, Germany), and incubated at room temperature for 4 h on test tube roller (205RM Tarsons, India). Equal volumes of *A. tumefaciens* containing pTRV1 and pTRV2 derivatives were mixed before infiltration in the plants. Infiltration was carried out on the abaxial surface of the leaves with 1 mL syringe (without needle) into four basal leaves of the five to six leaf stage of *A. thaliana* Col-0 and *myb28myb29* plants. Four weeks post infiltration, the total RNA from newly emerged leaves was isolated to check the viral spread.

HPLC-fractionation based purification of glucosinolates

10 g of broccoli seeds (Green magic) were homogenized in liquid nitrogen and extracted using 50 ml 80 % MS-grade methanol (JT Baker, USA). The extracts were loaded on DEAE Sephadex C25 (Sigma-Aldrich, USA) cation exchange column and washed with formic acid/iso-propanol/water (3:2:5) and

eluted with K_2SO_4 /3 % iso-propanol into methanol. The samples were concentrated using rotary evaporator and then analysed by high performance liquid chromatography, using C18 column (Gemini, 50 × 4.6 mm, 5 µm particle size). Glucoraphanin and glucobrassicin fractions were collected based on the retention time of the standards. The retention time for glucoraphanin standard was 3.54 minutes while the retention time for glucobrassicin standard was 14.05 minutes. The crude extract also has peaks around the same retention time as that of standards. The collected fractions were further verified and quantified using X500R UPLC/ESI/QTOF-MS.



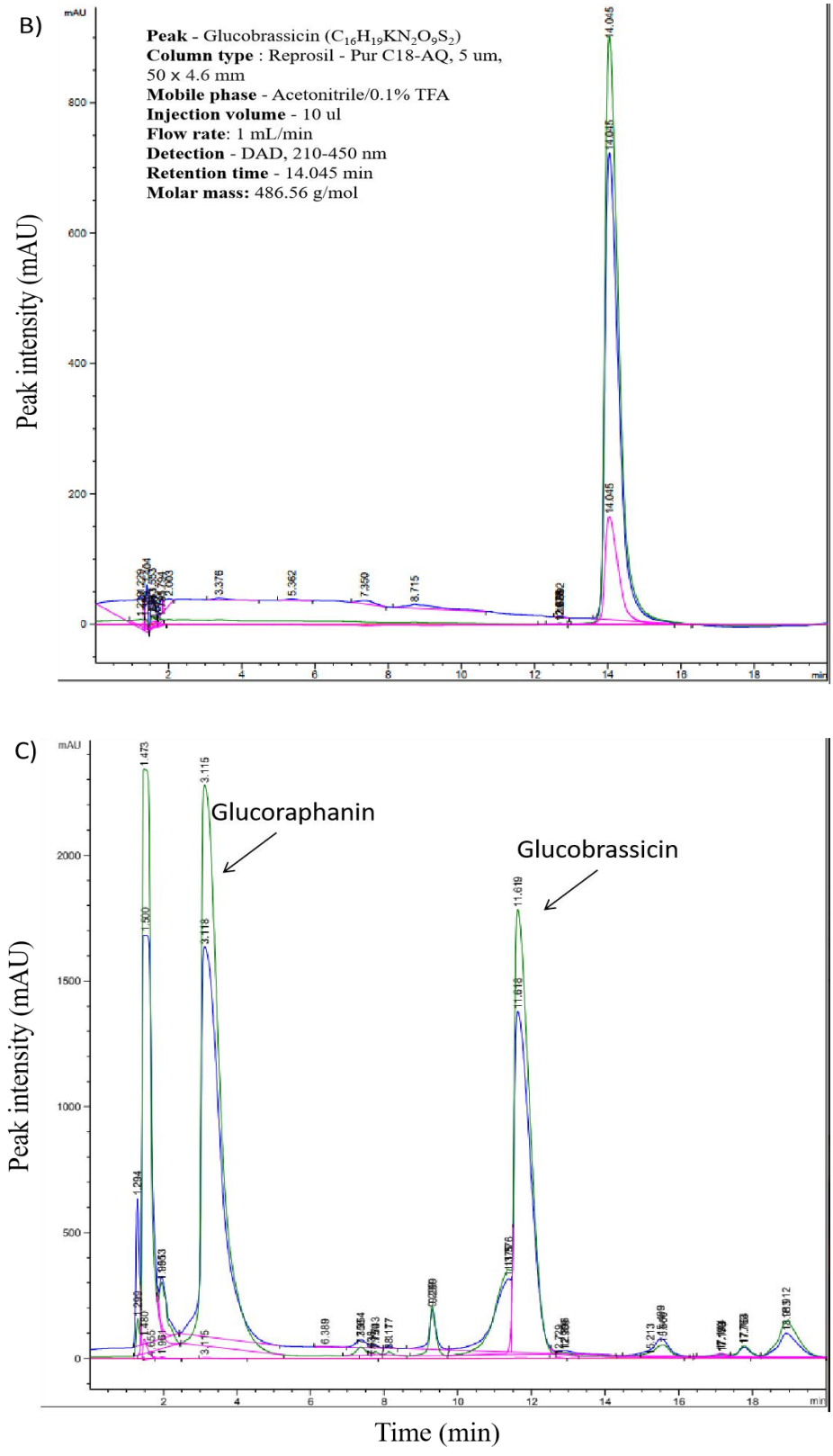


Fig 3: (A) and (B)Glucoraphanin and glucobrassicin standard chromatograms respectively, (C) Broccoli seed extract chromatogram. The glucosinolate fractions were collected based on the retention time of standards.

UPLC/ESI/QTOF-MS based glucosinolate profiling of plant tissue

Leaf tissues of *Brassica* crops and *Arabidopsis* were homogenized in liquid nitrogen. 100 mg of tissue was extracted in 1 ml of 80 % MS-grade methanol (JT Baker, USA) containing 0.2 µM sinigrin as an internal standard for *Arabidopsis* leaf tissue samples and 0.2 µM adonitol other *Brassica* leaf tissue samples. The samples were analysed by X500R UPLC/ESI/QTOF-MS (SCIEX) equipped with C18 column (Gemini, 50 × 4.6 mm, 5 µm particle size). Internal standard was used to monitor the response of the machine throughout the run. Glucoraphanin and glucobrassicin standards (Phytoplan, Germany) were used for relative quantification of intact glucosinolates. The standards were diluted from a range of 10 ug/ml to 7.8 ng/ml. Intact glucosinolates were analyzed in negative ion mode. Quantification of selected parent ions was achieved by multiple reaction monitoring (MRM). Parameters for LC-MS method were used from previous reports (Malka et al., 2016).

Time (min)	Mobile phase A MiliQ (0.2% FA)	Mobile phase B Acetonitrile
0	98.5	1.5
1	98.5	1.5
6	95	5
8	93	7
9	91.6	8.4
9.1	0	100
10	0	100
10.1	98.5	1.5
14	98.5	1.5

Table 1: Elution profile of intact and desulfoglucosinolates

Sr no	**Brassica crop**	**Variety**	**Abbrevation**
1	Radish 1.1	Ivory white	IWRDSH
2	Cauliflower	Kimaya	KMCBG
3	Naval kol	Neo F1	NVL KL
4	Cabbage 1.1	Syngenta	SYNCBG
5	Brocolli	Green magic	GMBRC
6	Radish 1.2	Red globe	RGRDSH
7	Cabbage 1.2	F1 dollar	F1CBG
8	Cabbage 1.3	Hybrid Saint	HSCBG
9	Mustard	Mahyco	MUS

Table 2 : *Brassica* crop variety and their abbreviation

Sr no.	Common Name	Chemical group	Chemical formula	ms ms fragments
1	Glucoraphanin	Aliphatic	$C_{12}H_{23}NO_{10}S_3$	436.046, 259.012, 195.033, 178.017,
2	Glucobrassicin	Indolic	$C_{16}H_{20}N_2O_9S_2$	447.054, 259.013, 205.044,
3	Progoitrin	Aliphatic	$C_{11}H_{19}NO_{10}S_2$	388.040, 353.071, 266.967, 160.841, 126.880
4	Giucoiberin	Aliphatic	$C_{11}H_{21}NO_{10}S_3$	423.039, 358.026, 259.012, 195.973,
5	Sinigrin	Aliphatic	$C_{10}H_{17}NO_9S_2$	358.027, 259.013, 161.986, 116.017, 74.099
6	Glucoraphenin	Aliphatic	$C_{12}H_{21}NO_{10}S_3$	434.022, 358.032, 274.99, 259.011, 172.811, 129.025
7	Gluconapin	Aliphatic	$C_{11}H_{19}NO_9S_2$	372.045, 292.365, 241.865, 145.037
8	Glucobrassicanapin	Aliphatic	$C_{12}H_{21}NO_9S_2$	386.058, 259.012, 195.032, 144.048
9	Glucoerucin	Aliphatic	$C_{12}H_{23}NO_9S_3$	339.035, 274.853, 258.810, 241.774, 194.063
10	Glucoibarin	Aliphatic	$C_{15}H_{29}NO_{10}S_3$	423.002, 374.038, 372.045, 191.0194, 180.051, 111.009
11	4 methoxyglucobrassicin	Indolic	$C_{17}H_{22}N_2O_{10}S_2$	447.062, 391.113, 259.011, 235.054, 145.0499
12	Gluconasturtiin	Aromatic	$C_{15}H_{21}NO_9S_2$	422.058, 274.990, 259.013, 180.049, 138.970
13	Glucobarbarin	Aromatic	$C_{15}H_{21}NO_{10}S_2$	439.233, 381.83, 293.68, 197.74

Table 3: List of glucosinolates analysed in *Brassica* crops and their ms-ms fragment patterns

For relative quantification of aliphatic and indolic glucosinolates in *Brassica* crops glucoraphanin and glucobrassicin standards were used. The ms-ms fragments of all the analyzed glucosinolates were confirmed with the previously reported fragments (Fabre et al, 2007; Sun et al, 2016).

Artificial diet

A soy based diet prepared whose composition (for 500 ml) was as follows: Caesin 5 g, soy flour 15 g, Brewers' yeast 8.1 g, sucrose 17.5, vitamin premix 5 g, ascorbic acid 2 g, I-inositol 0.2 g, choline chloride 0.5 g, Wesson salt 5g, aureomycin 0.5 g, sorbic acid 1 g, methyl-P 1 g, cholesterol 1.25 g, oil 3.5 ml, KOH solution 2.5 ml, alphacel 2.5 g, agar 11.25 g, water 420 ml. The diet was stored at 4 °C until use.

Quantitative real-time PCR (qPCR)

To quantify *Pxgss1* silencing efficiency, *Pxgss1* RNA was isolated and cDNA was synthesized as mentioned above. qPCR was performed using SYBR Green kit (Takara, Japan). Ribosomal subunit protein S13 gene (*RSP13*) was used as internal control for normalization of *Pxgss1* transcripts. Relative quantification of transcripts was performed using standard curve method. The primer pairs used for qPCRs are mentioned in the table.

Sr. no	Name	Primer sequence (5'-3')
1	Pxgss1_QF	CATCAAGCTGTGGTGCTCCTC
2	Pxgss1_QR	CTGCCGTCAAGCTGCTCC
3	RPS13_QF	ATGTCGCTCGTAATACCGGAC
4	RPS13_QF	CAGTAGTGACGCATGCCTCG

Table 4: List of primer sequences used for qPCR

Larval weight measurements

To study the effects of *Pxgss1* silencing on the growth of *P. xylostella,* larval weights were measured throughout its developmental period. Eggs were collected and transferred on *Px*gss1 infiltrated and non-infiltrated Col-0 and *myb28myb29* plants. The larval weights were measured on 5th days post

hatching (dph), 7th dph and 9th dph. On the day of weighing, 30 larvae per each treatment were picked and weighed on fine weighing balance.

Complementation experiment

To determine whether sulforaphane was responsible for the decreased larval weights a complementation experiment was performed. Sulforaphane and glucoraphanin were added to the artificial diets at physiological concentrations of the leaf. Solvent control (60 % methanol) and water control were used as appropriate controls. Metabolites were added to the diet by 2 ul pipette and mixed homogeneously in the diet. *P. xylostella* larvae were fed on the diet containing different treatment and larval weights were measured. The diet was changed in alternate days.

***P. xylostella* tissue collection**

P. xylostella eggs were collected and transferred on wild-type control, empty vector, and *Px*gss1 infiltrated Col-0 and *myb28myb29* plants kept in a 30×15 cm plastic boxes in a controlled growth chamber. The plants in the boxes were changed as per the requirements. Tissues from 15 larvae were pooled to make one replicate for gut content and hemolymph. Early fourth instar larvae were dissected for hemolymph and gut content collection. Hemolymph was collected by 2 ul pipette by making a small scratch by needle on the lower posterior end of the larvae. Midguts were dissected under a dissecting microscope. Gut was washed and cleaned in TE buffer.

Metabolite extraction and UPLC/ESI/QTOF-MS analysis of *P. xylostella* larvae

Gut, hemolymph and frass samples collected for metabolomic analysis as mentioned in above section (‘*P.xylostella* tissue collection’) were homogenized in 100 ul extraction buffer (60 ml methanol, pH 3 adjusted by acetic acid) in bead beater for 3 minutes. Homogenized samples were centrifuged at 14000 RPM for 30 minutes at 4°C. The supernatant was transferred into insert vials and analysed by UPLC/ESI/QTOF-MS. Intact glucosinolates were analysed in negative ion mode while, desulfo-glucosinolates and isothiocyanates were analysed in positive ion

mode. Quantification of selected parent ions was achieved by multiple reaction monitoring method. Parameters for LC-MS method were used from previous reports (Sun et al., 2019). The elution profiles for intact and desulfo-glucosinolates were same as mentioned in the above section ('UPLC/ESI/QTOF-MS based glucosinolate profiling'). The elution profiles for sulforaphane and indole-3-carbinol are mentioned in the table 5 and 6 respectively.

Time (min)	Mobile phase A MiliQ (0.05 % FA)	Mobile phase B Acetonitrile
0	85	15
0.5	15	85
2.5	0	100
3.5	97	3
6	97	3

Table 5: Elution profile of sulforaphane and its conjugates

Time (min)	Mobile phase A MiliQ (0.05 % FA)	Mobile phase B Acetonitrile
0	90	10
0.5	10	90
6	10	90
6.1	0	100
7.5	0	100
7.6	90	10
10	90	10

Table 6: Elution profile of indole-3-carbinol and its conjugates

Predator collection and identification

Predators like spiders, bugs and earwigs were collected from the different crop fields and cultured in a controlled environment chamber at 22°C, 70% relative humidity, and a 16 h light: 8 h dark photoperiod. The predators were provided with different instars of *P. xylostella* larvae to check the predatory behavior. The most commonly found predators were short-listed and identified based on morphological keys. The morphological key used for identification of spiders is as follows-

A	***Hasarius adansoni***
Family	Anterior median eyes clearly lager than other eyes, like searchlights; field of eyes quadrangular in dorsal view; habitus as fig.
Genus	Cheliceral teeth as fig.; tibia of the male pedipalp as long as the bulbus or longer; epigyne with 2 small depressions, their margins half-moon-like sclerotised; in greenhouses.
Species	Male pedipalp as fig; tibia as long as cymbium, strikingly white haired; epigyne and cheliceral furrow margin as figs.

B	***Wadicosa fidelis***
Family	Connecting line of posterior lateral eyes - posterior median eyes intersects the middle line of the carapace in front of the anterior margin of the prosoma; position of the eyes as fig.; hunting spiders; females carry their egg sac attached to the spinnerets.
Genus	Tegulum with two prolateral processes (PTP); sternum in both sexes without spines; epigyne with median septum (MS) tapering backwards; epigyne with two separated fovea (F) in anterior position; body length 6-7 mm.
Species	Colouration variable, varying between dark brown, grey to almost black. Sternum dark. Opisthosoma with dark cardiac mark and light lateral markings; ventrally lighter. Body length male: 5.6-6.8 mm.

C	*Plexippus paykulli*
Family	Anterior median eyes clearly larger than other eyes, like search lights; field of eyes quadrangular in dorsal view (the posterior eyes of Salticidae may be difficult to recognize for beginners, due to their smallness, but they are always present.
Species	Prosoma dorsally brown with black hairs, sides sandy with black margins, in lower half with white hairs. Opisthosoma sandy, with dorsal white median band which is widened T-like in the posterior third. Palp: sandy, with white and yellow hairs, cymbium widened. Body length male: 6.85-9.1 mm.

Table 7: Taxonomy key used for identification of three spider species

No choice and behavioral assays

P. xylostella eggs were collected and transferred on wild-type control, empty vector, and *Px*gss1 infiltrated Col-0 and *myb28myb29* plants. Third instar larvae were used for the no choice and behavioral assays. Three predatory spiders; *Hasarius adansoni, Plexippus paykulli* and *Wadicosa fidelis* were used for all the assays. All the spiders used in the assays, were starved for 12 hours prior to the assays. Assays were performed in polypropylene containers. In no choice assay, a single larvae (fed on either wild-type control/ empty vector/ *Px*gss1 infiltrated Col-0 and *myb28myb29* plants) was provided to the predator. Number of *P. xylostella* larvae killed and consumed by the predators in 20 minute time span was calculated. In behavioral assay, each predator was presented four *P. xylostella* larvae (all four larvae raised on same treatment) and the number of *P. xylostella* larvae killed and consumed by the predators in 3 hour time span was noted.

Collection of predators' frass

To decipher predator's ability to metabolize the ingested glucosinolates, the frass of the predators was collected after the completion of abovementioned no choice and behavioral assays. The frass pellets were collected in methanol (60 %, pH 3) and was further analysed by UPLC/ESI/QTOF-MS. The same elution profiles mentioned in ('Metabolite extraction and UPLC/ESI/QTOF-MS analysis of *P. xylostella* larvae') section were used for analysis of metabolites in frass.

3. Results

3.1 Glucosinolate profiling of native varieties of *Brassica* crops

Glucosinolates are mainly classified as aliphatic, indolic and aromatic based on the presence of side chain groups (Redovnikovic, 2008; Halkier, 2006). Nearly, 140 different structures of glucosinolates are reported (Reichelt, 2002). The amount and profile of glucosinolates in *Brassica* crops differs based on various factors that include genotype of the cultivar, developmental stages of the plant, storage tissue, environmental factors such as soil nutrition, temperature, water, light, and agricultural methods (Park, 2013; Pek, 2012; Wang, 2012). Glucosinolates are well-known defense compounds against insect herbivores. In addition to that, few studies have suggested the role of glucosinolates as defense compounds in tritrophic interactions (Gols, 2008; D'Auria, 2005; Sarfraz, 2008; Hopkins, 2009). Though the reports suggest the role of glucosinolates in tritrophic interaction, it is not clear whether the effects shown by the glucosinolates are a result of qualitative variation in glucosinolate content or due to the difference in amount of major glucosinolates. At least studying the effect of most common and abundant glucosinolate will help in devising pest control strategies. The content of the candidate glucosinolate that has negative effect on the *P.xylsotella* larvae can be manipulated in the *Brassica* crops.

To study the effect of individual glucosinolate we first analyzed the major glucosinolates present in the native varieties of *Brassica* crops. *Brassica* crops were planted in the field in a randomized manner. Two months old leaf tissues were collected, snap frozen in liquid nitrogen and extracted in 80% methanol (JT Baker, USA) for metabolomic analysis. In total, 13 glucosinolates belonging to three different groups; aliphatic, indolic and aromatic were analysed using X500R UPLC/ESI/QTOF-MS. The intact glucosinolates were identified based on their unique ms-ms fragmentation patterns and retention time (Table 3). The ms-ms fragmentation patterns were compared and confirmed with the previously reported values in the literature (Fabre et al, 2007; Sun et al, 2016). Commercially available pure glucosinolate standards, glucoraphanin and glucobrassicin (Phytoplan, Germany) were used for the relative quantification of all the glucosinolates.

Glucoraphanin, glucobrassicin and sinigrin were the most commonly present glucosinolates in all the analysed *Brassica* crops (fig 4).

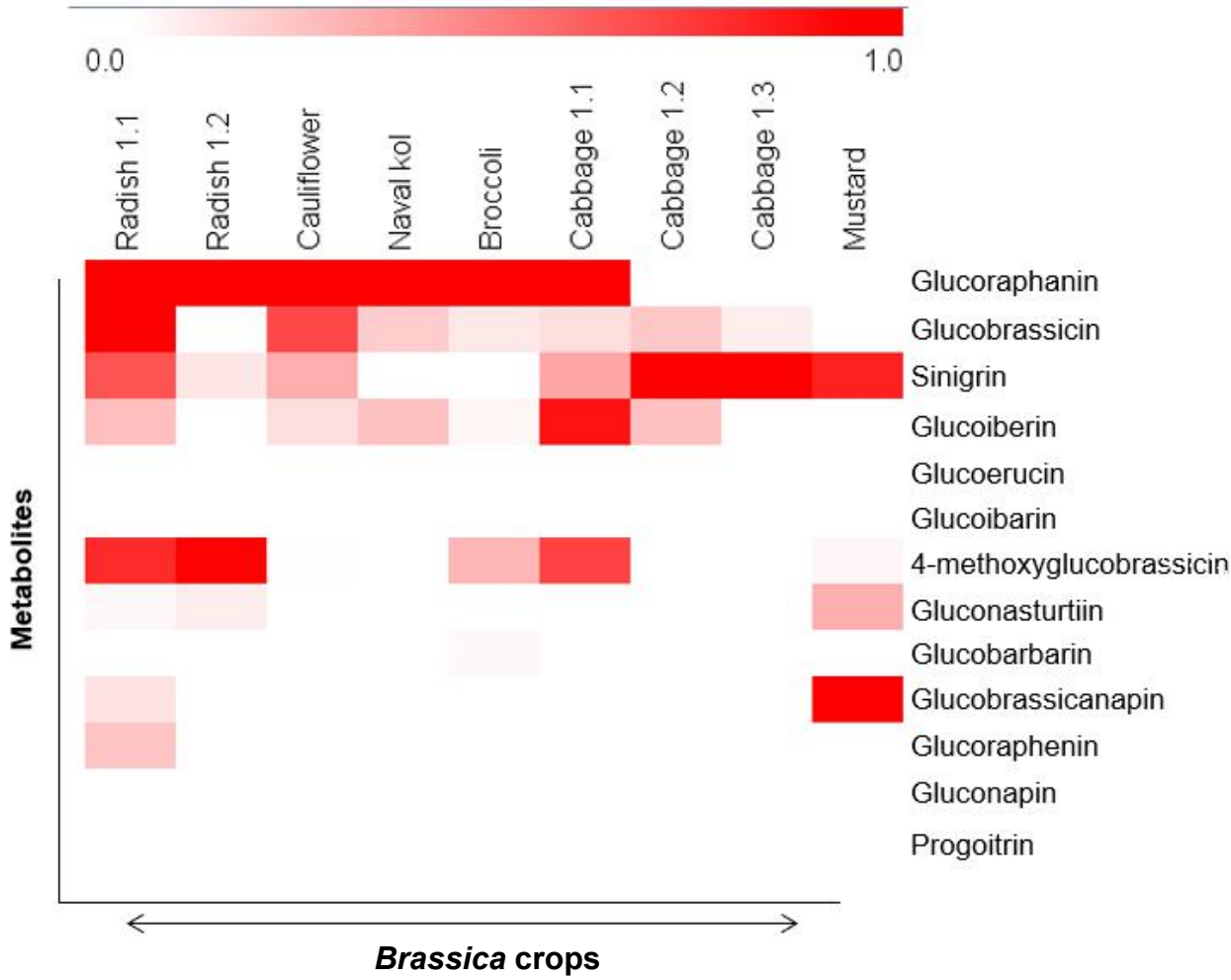


Fig 4: Glucoraphanin, glucobrassicin and sinigrin are the major glucosinolates found in the screened varieties. (A) Column represents different *Brassica* crops, row represents mean relative compound concentration in (nmol/g) of fresh weight. Values of each rows are normalized to the highest value of that row. The values range from 0 to 1 in a particular row, with highest value being assigned 1. The abbreviations used for different *Brassica* crops and their varieties are mentioned in table 2.

Navalkol (Neo F1), cauliflower (Kimaya), radish (ivory white and red globe) and broccoli (Green magic) contained glucoraphanin in highest quantity, while cabbage (F1 dollar and hybrid saint) and mustard (Mahyco) had sinigrin in higher amounts.

3.2 *A. thaliana* as a model host plant and metabolism of glucosinolates in *P. xlysotella*

Glucoraphanin, glucobrassicin and sinigrin were the most common glucosinolates found in the native varieties of *Brassica* crops. To begin with, we selected the most abundant and common glucosinolate-glucoraphanin as our candidate glucosinolate to study its role in tritrophic interaction. *A. thaliana* was selected as model host plant as it has several advantages such as; it belongs to Brassicaceae family, hence it contains glucosinolates as major defense compounds. Moreover, it contains only glucoraphanin (aliphatic glucosinolate) and glucobrassicin (indolic glucosinolate) as major glucosinolates in its shoot tissue (Brown, 2003; Reichelt, 2001). *A. thaliana* is amenable to genetic transformations and mutant lines with altered glucosinolate profiles are available (M Koornneef, 2010). *A. thaliana* has also been successfully used for plant mediated RNAi (Mao, 2007).

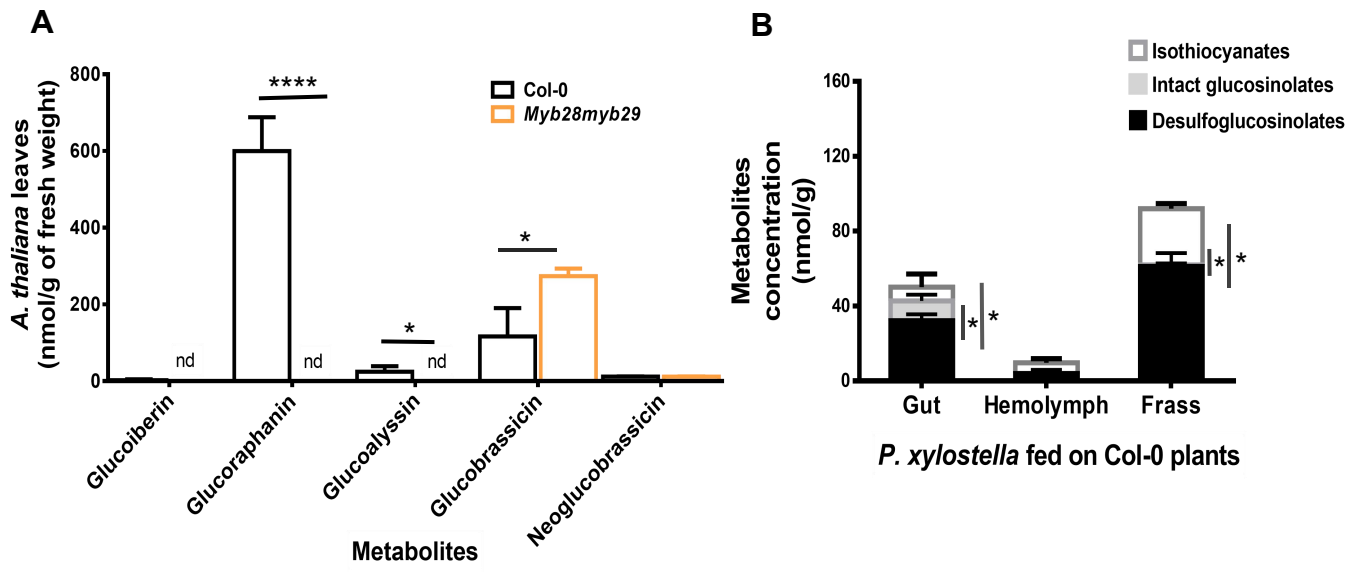


Fig 5: Major glucosinolates in *A. thaliana* and their metabolism in *P. xylostella* (A) Concentrations of four glucosinolates in (nmol/g) of fresh leaf tissues found in Col-0 and *myb28myb29* plants. (B) Concentrations of intact glucosinolates, desulfo-glucosinolates and isothiocyanates in gut, hemolymph and frass of *P. xylostella* larvae fed on wild type Col-0 plants. Significance was determined by Tukey HSD tests and one-way ANOVA.

To study the effect of glucoraphanin in tritrophic interactions we used Col-0 and *myb28myb29* (aliphatic glucosinolates deplete) mutant line. *Myb28myb29* (aliphatic glucosinolates deplete) mutant line was selected to study the effect of absence of glucoraphanin in the tritrophic interaction. To verify the major glucosinolates present in the Col-0 wild type *A. thaliana* plants we performed X500R UPLC/ESI/QTOF-MS based quantification of the leaf tissue. Analysis revealed the presence of glucoraphanin, glucobrassicin, glucoalyssin, neoglucobrassicin in the leaf tissue. Glucoraphanin had the highest concentration of 599 nmol/g of fresh weight followed by glucobrassicin with the concentration of 116 nmol/g of fresh weight. In Col-0, aliphatic glucosinolates glucoalyssin and glucoiberin were present in very trace amounts as compared to glucoraphanin. While indolic glucosinolate neoglucobrassicin was very low as compared to glucobrassicin. In case of *myb28myb29* mutant line, no aliphatic glucosinolates were detected. Indolic glucosinolates were present at normal concentrations.

To study the metabolism of glucoraphanin in the *P. xylostella, P. xylostella* eggs were transferred on the Col-0 wild type plants and larvae were allowed to continuously feed on the plants. Gut, hemolypmh and frass were collected and analysed using X500R UPLC/ESI/QTOF-MS. Intact glucoraphanin, desulfo-glucoraphanin and its's isothiocyanate sulforaphane were detected in the collected samples. Desulfoglucoraphanin was present in the highest quantity as compared to sulforaphane and intact glucoraphanin in gut, hemolymph and frass. Highest amounts of desulfoglucoraphanin were present in the frass, suggesting that most of the glucosinolates were desulfatized by PxGSS and excreted in the frass.

3.3 Plant mediated RNAi leads to decrease in transcript levels of *Pxgss1:*

P. xylostella larvae converted most of the ingested glucosinolates into desulfoglucosinolates. Desulfatization by *P. xylostella* is considered as a beneficial detoxification strategy (Ratzka et al., 2002; Matile, 1980). To understand the effect of desulfatization on performance and metabolism of *P. xylostella* we used reverse genetic approach. We employed plant mediated RNA interference (PMRi) to silence *Pxgss1* gene. Plant mediated RNA

interference (PMRi) is a powerful reverse genetic tool that is used for post transcriptional gene silencing. In PMRi, host plant is transformed with cDNA fragment of herbivore's target gene in an inverted way. The cDNA is constantly transcribed to produce dsRNA in the plant. The herbivore feeding on these plants will ingest the dsRNA. Once the dsRNA enters inside the insect's cells it will activate the RNAi pathway and cause silencing of the target gene. This method is very helpful in lepidopteran insects as they lack the RNA dependant RNA polymerase (RDRP) and SID protein (Gordon KHJ, 2007). In PMRi, the plants will continuously keep producing dsRNAs. As herbivores are feeding constantly on the plants, they will ingest these dsRNAs.

In PMRi, a 526 base pair fragment that would target the *Pxgss1* m-RNA was cloned into pTRV2 vector and transformed into *Agrobacterium.* The *Agrobacterium* containing the *Px*gss1 fragment was then infiltrated in 2-3 leaf stage Col-0 and *myb28myb29* (aliphatic deplete) *A. thaliana* plants. Some plants were also infiltrated with *A. tumifaciens* containing pTRV2 vector (without any fragment) as a negative control. To check the presence of *Px*gss1 fragment and virus spread in the plants, total RNA was isolated from the newly emerged non infiltrated *A. thaliana* leaves 30 days post infiltration. Presence of *Px*gss1 fragment post infiltration was confirmed by doing PCR using fragment specific primers (fig 6).

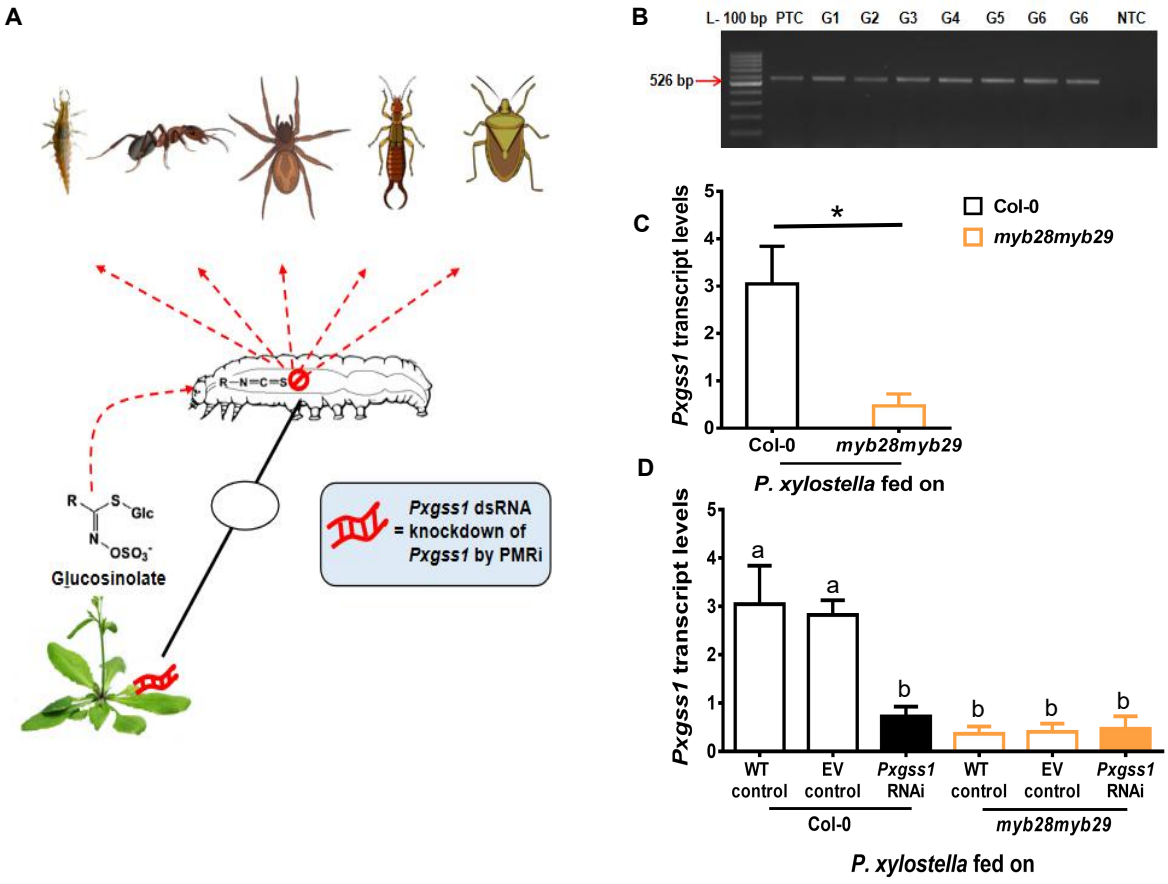


Fig 6: **PMRi reduces the *Pxgss1* transcript levels in the midgut of *P. xylostella***. (A) Schematic for plant mediated RNAi. A *Px*gss1 dsRNA fragment is introduced in plants by *Agrobacterium* infiltration. *P. xylostella* larvae continuously feeding on the plant containing dsRNA fragment will lead to silencing of *Pxgss1.* (B) Presence of *Px*gss1 dsRNA fragment in newly emerged leaves, 30 days post infiltration. G- *Px*gss1 infiltrated, PTC- positive template control, NTC- no template control. (C) Induction of *Pxgss1* transcript levels seen in *P. xylostella* larvae fed on Col-0 (plants containing aliphatic glucosinolates) as compared to *P. xylostella* larvae fed on *myb28myb29* (aliphatic glucosinolate deplete) plants ($F = 9.75$, p≤0.0259, n=6 for all bars). (D) *Pxgss1* transcipt (relative to ribosomal protein S13) levels in midgut of *P. xylostella* ($F = 11.01$, p≤0.0001, n=6 for all bars). Significance was determined by Tukey HSD tests and one-way ANOVA.

Pxgss1 is abundantly expressed in midgut tissue (Sun et al., 2019; Heidel-Fischer et al., 2019). To check the transcripts of *Pxgss1, P. xylostella* eggs were transferred on the plants and early fourth instar larvae were dissected to isolate total RNA from midgut tissue. The *Pxgss1* transcripts were

significantly lower (8 fold) in *P. xylostella* larvae fed on wild type *myb28myb29* (aliphatic deplete) plants as compared to Col-0 plants. This implies that *Pxgss1* is inducible and its induction depends on intake of aliphatic glucosinolates. Plant mediated RNAi led to significant down regulation of *Pxgss1* in *P. xylostella* larvae fed on Col-0 RNAi plants (fig 6). The *Pxgss1* transcripts were lowered by nearly 80 % in *P. xylostella* larvae fed on Col-0 RNAi plants as compared to those fed on control plants. As *Pxgss1* is inducible, the levels of *Pxgss1* transcripts were only 17 % in *P. xylostella* larvae fed on *myb28myb29* (aliphatic glucosinolates deplete) as compared to *P. xylostella* larvae fed on Col-0 (normal glucosinolate levels) (fig 6).

3.3 Silencing the *Pxgss1* has negative effects on the *P. xylostella's* larval growth

To evaluate the effects of *Pxgss1* silencing on the growth of *P. xylostella* larvae, the larval weights were measured at three time intervals 4 dph, 7 dph and 9 dph. *P. xylostella's* eggs were collected and transferred on the six different treatments of the plants. The larvae were continuously fed on the plants and their weights were measured at selected time intervals. Negative effects on the growth of larvae fed on Col-0 RNAi infiltrated plants were seen from the 7 dph (fig 7). This difference was further also seen on 9 dph. The larvae fed on *Px*gss1 infiltrated Col-0 plants gained only 62 % weight as compared to the larvae fed on all other treatments. The larval weight of *P. xylostella* larvae fed on aliphatic deplete *myb28myb29* plants was 25% greater than the average larval weight of *P. xylostella* larvae fed on Col-0 plants. This suggests that the aliphatic glucosinolates have some negative effect on the growth of *P. xylostella* larvae.

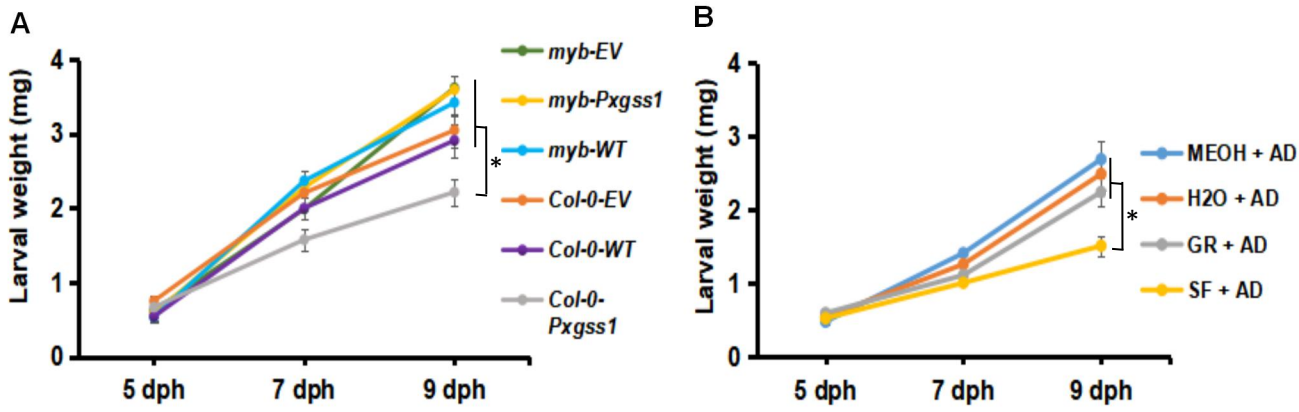


Fig 7: ***Pxgss1* silencing has negative effect on growth of *P. xylostella* larvae** (A) Larval weights were measured on 5 dph (days post hatching), 7 dph and 9 dph. There was reduction in larval weights of larvae fed on *Px*gss1 infiltrated Col-0 plants. 7 dph, $F\ (5, 174) = 4.007$, $p \leq 0.0018$; 9 dph, $F\ (5, 174) = 6.866$, $p \leq 0.0001$. (B) *P. xylostella* were fed on artifical diet spiked with sulforaphane, glucoraphanin, methanol and water. Larval weight measured on 5 dph, 7 dph and 9 dph. Significant reduction in weight of larvae fed on diet containing sulforaphane was seen on 9 dph. 9 dph, $F\ (3, 103) = 7.133$, $p \leq 0.0002$. Significance was determined by Tukey HSD tests and one-way ANOVA.

To check whether sulforaphane was responsible for negative effects on growth of *P. xylostella*, larvae were fed on artificial diet (AD) spiked with physiological concentrations of sulforaphane, AD with glucoraphanin, AD with methanol and AD with water were used as controls. The larvae feeding on different treatments were weighed on 4 dph, 7 dph and 9 dph. Significant reduction in weight of the larvae feeding on AD spiked with sulforaphane was seen as compared to other treatments. Thus, silencing the *Pxgss1* has negative effects on the *P. xylostella's* larval growth and sulforaphane is responsible for the reduction in the larval weight.

3.4 Silencing the *Pxgss1* changes the glucosinolate content in the *P. xylostella* larvae

To check the effect of *Pxgss1* silencing on glucosinolate metabolism, gut, hemolymph and frass of fourth instar larvae were collected and analysed. As

glucoraphanin is the most abundant glucosinolate in *A. thaliana,* its intact, desulfo- and isothiocyanate forms were analysed using X500R UPLC/ESI/QTOF-MS. Non-silenced *Pxgss1 P. xylostella* larvae with typical levels of glucosinolate sulfatase were able to desulfatize the glucosinolates into non toxic desulfo-glucosinolates.

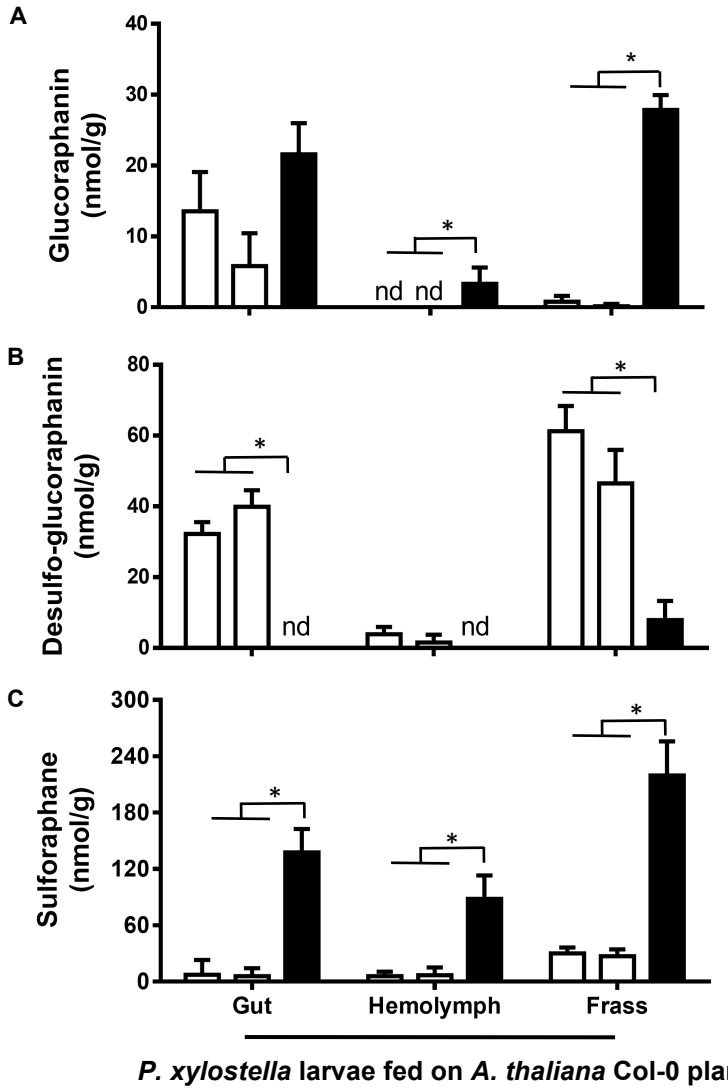


Fig 8: *Pxgss1* silencing leads to reduction in levels of desulfoglucoinolates and an increase in isothiocyanates concentrations in *P. xylostella*. (A) Intact glucoraphanin, gut *F(2,6)* = 9.155, p≤ 0.0150, hemolymph *F(2, 6)* = 22.00, p≤0.0017, frass *F(2, 6)* = 83.38, p ≤0.0001. (B) Desulfo-glucoraphanin, gut *F(2,6)* = 85.72, p≤0.0001, hemolymph, *F(2,6)* = 2.25, p≤0.1473, frass, *F(2,6)* = 30.37, p≤0.0001. (C) Sulforaphane gut *F(2, 6)* = 113.7, p≤0.0001, hemolymph *F(2, 6)* = 32.80, p≤0.0001, frass *F(2, 6) = 63.14,* p≤0.0001. Significance was determined by Tukey HSD tests and one-way ANOVA.

The *Pxgss1* silenced larvae were unable to desulfatize the ingested glucosinolates, which in turn led to an increase in formation of toxic isothiocyanates. Hence, *Pxgss1* silencing due to plant mediated RNAi, lead to significant decrease in desulfo-glucosinolates and also significant increase in isothiocyanates formation in gut, hemolymph and frass (fig 8). Frass of the larvae contained the highest amount of glucoraphanin, desulfo-glucoraphanin and sulforaphane. This indicates that the most of the glucoraphanin and its metabolites are excreted into the frass.

3.5 Identification and screening of native predators of *P. xylostella* larvae

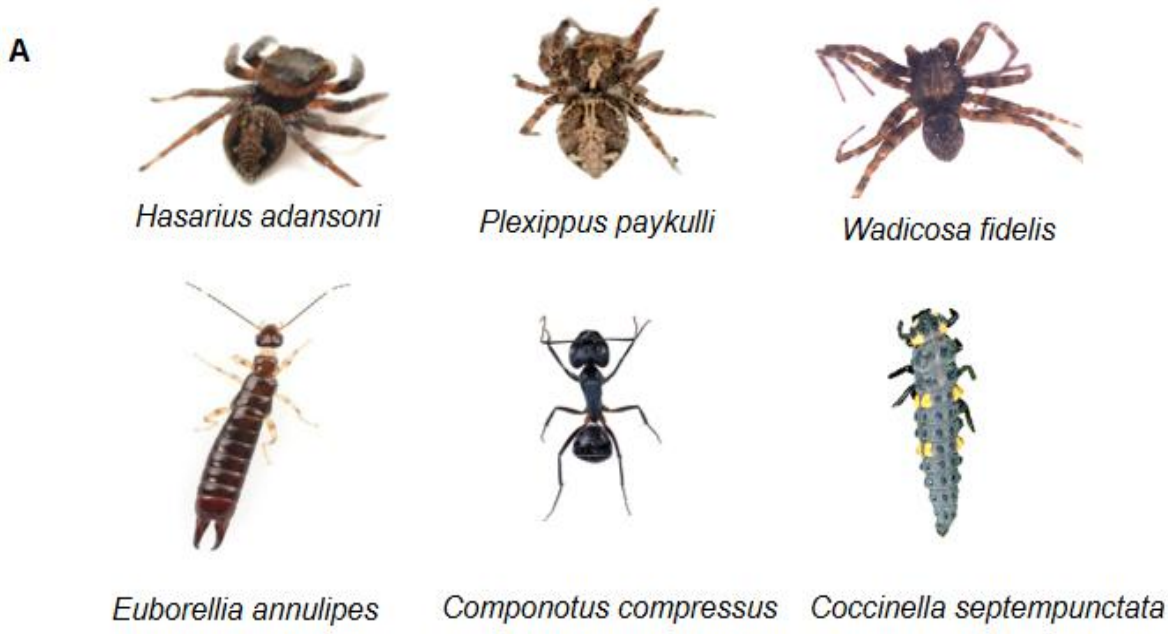


Fig 9: Spiders, earwigs, ants and the lady bug beetles were the most common predators of *P. xylostella* found in different *Brassica* crop fields

Possible predators of *P. xylostella* were collected from their natural habitats in different *Brassica* fields. In total, 18 predators were collected and checked if they preyed on *P. xylostella* larvae (table 8). Spiders, earwigs, beetles and ants were the major predators found in these fields (fig 9). The predators were identified using morphological keys. The key identified predators were spiders- *Wadicosa fidelis*, *Hasarius adansoni* and *Plexippus paykulli*, earwig-

Euborellia annulipes, Asian ladybug beetle larvae- *Coccinella septempunctata* and ants- *Componotus compressus* and *Myrmica spp*.

Predator	Preys on *P. xylostella*	Predator	Preys on *P. xylostella*
Spiders		**Beetle larvae**	
Plexippus paykulli	✓	*Coccinella septempunctata*	✓
Menemerus bivittatus	✗	*Cheilomenes sexmaculata*	✗
Telamonia dimidiata	✗	**Earwig**	
Hasarius adansoni	✓	*Euborellia annulipes*	✓
Wadicosa fidelis	✓	*Labidura riparia*	✗
Bugs		*Forcipula spp*	✓
Odontopus nigricornis	✗	**Ants**	
Antilochus conquebertii	✗	*Componotus compressus*	✓
Halys dentatus	✗	*Crematogaster spp*	✗
Chinavia hilaris	✗	*Myrmica spp*	✓
Lacewing			
Crysoperla carnea	✓		

Table 8: List of screened predators of *P. xylostella* larvae

3.6 Predators show uniform response to the altered glucosinolate metabolism:

It has been reported that the composition of glucosinolates in prey influences the development and choice of parasitoids and predators. To study the effect of the glucosinolates and its metabolized forms on third trophic level, no choice and behavioral assays were performed. In no choice assays, each predator was given a single *P. xylostella* larvae which was fed on *Px*gss1 infiltrated and non-infiltrated Col-0 and *myb28myb29* plants. Generally, when a prey is toxic to the predator it will consume it few times but later, the predator will be aversive towards that prey. So, in the behavioral assays each predator was provided with four *P. xylostella* larvae and the total number of larvae consumed in three hours was noted. The behavior of predators such as- prey assessment, prey capture and whether it partially or completely ingests the prey was observed. Two jumping spiders- *Hasarius adansoni* and *Plexippus paykulli* and a wolf spider- *Wadicosa fidelis* were used for no choice and behavior assays. Percentage of *P. xylostella* larvae captured and killed by the predators was calculated in no choice assays. In case of behavior assays, average number of *P. xylostella* larvae consumed by the spiders in three hours were noted. All the tested predators showed invariable preference towards *P. xylostella* larvae containing different metabolomic content (fig 10). Hence, the spiders were insensitive to the glucoraphanin and its metabolized forms.

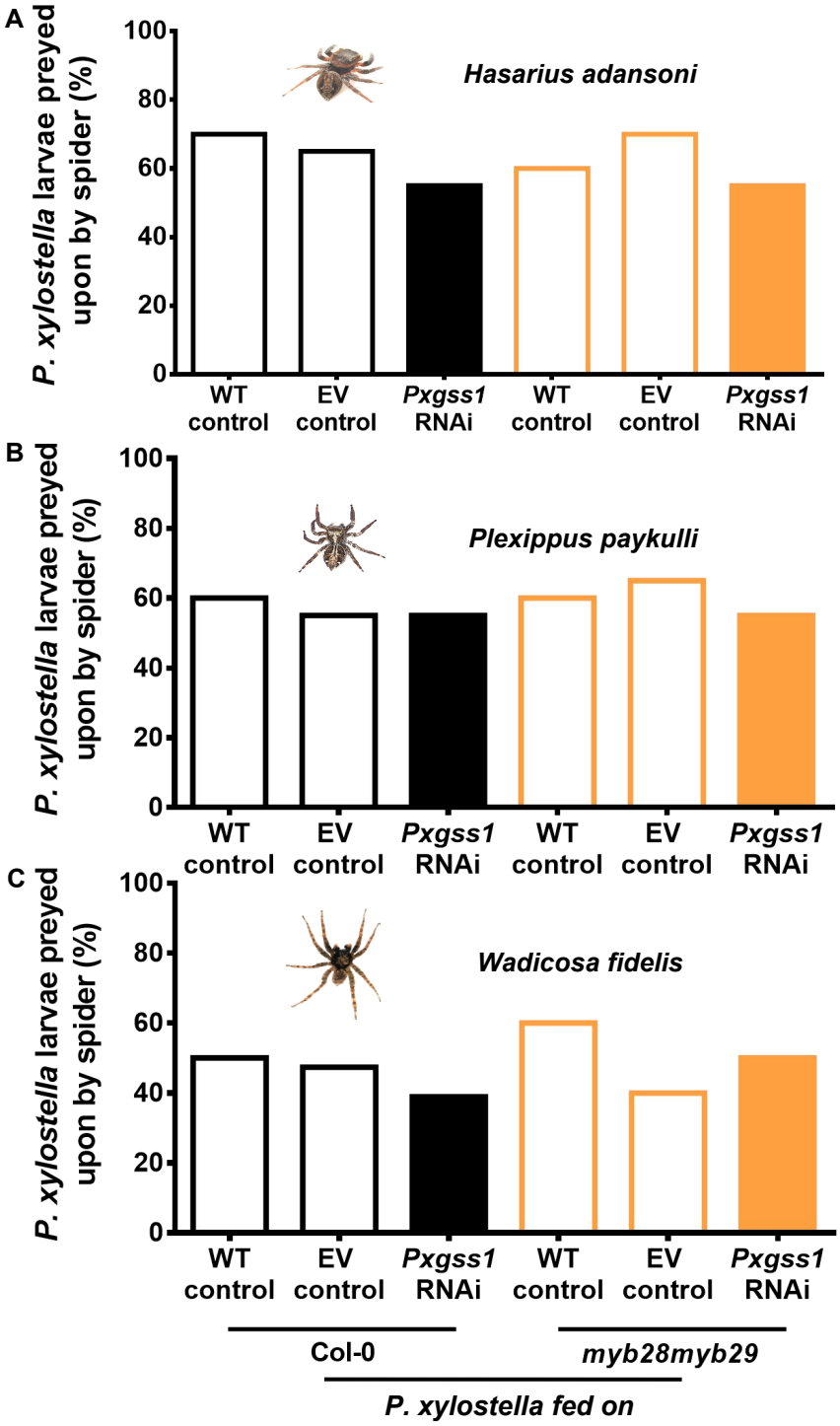


Fig 10: No choice assay: Predators showed uniform preference towards *P. xylostella* larvae fed on *Pxgss1* silenced and non-silenced Col-0 and *myb28myb29* (aliphatic glucosinolate deplete). (A) *Hasarius adansoni*, (B) *Plexippus paykulli*, (C) *Wadicosa fidelis.* Significance was determined by two-sided proportions test.

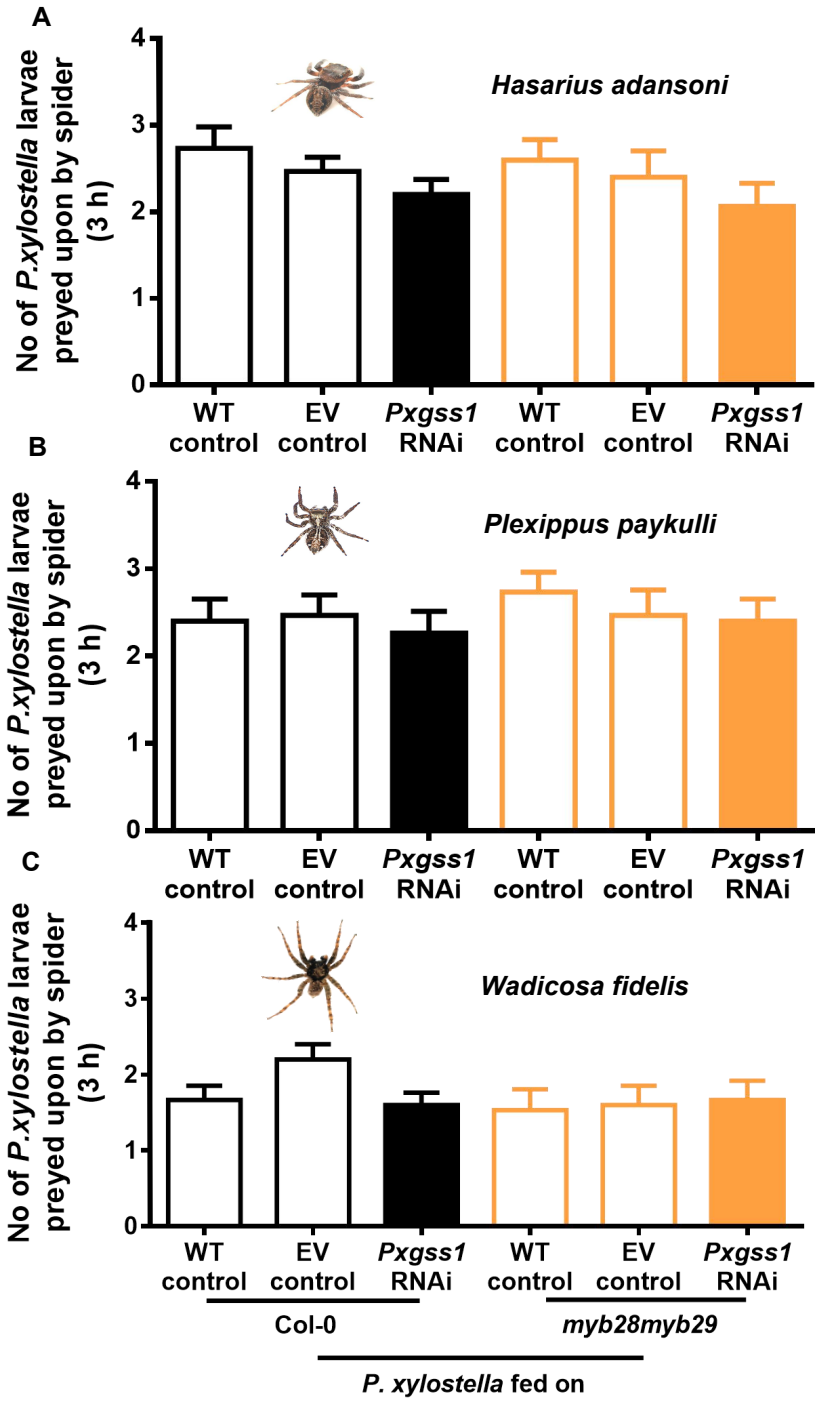


Fig 11: Behaviour assay: Predators showed similar behavior towards *P. xylostella* larvae fed on *Pxgss1* silenced and non-silenced Col-0 and *myb28myb29* plants. In each assay four larvae fed on each plant line were provided to the spider. The number of larvae killed by the spider in a period of three hours was noted. (A) *Hasarius adansoni*, (B) *Plexippus paykulli*, (C) *Wadicosa fidelis.* Significance was determined by two-sided proportions test.

3.7 Metabolomics of predators frass

As there were no observable negative effect of isothiocyanates on the spiders, we hypothesized that the spiders might be able to detoxify or metabolize the toxic isothiocyanates. To test this hypothesis, we collected and analysed the frass of spiders that preyed on *Pxgss1* silenced and non silenced *P. xylostella* larvae.

Sr no.	Compound name	Abbrevation	$[M+H]^+$ m/z	Relative amount
1	Sulforaphane	SF	179.4	0.47
2	Sulforaphane-cysteine-glycine	SF-Cys-Gly	355.8	0.46
3	Sulforaphane-cysteine	SF-Cys	298.4	0.05
4	Sulforaphane-glutathione	SF-GSH	484.8	0.02

Table 9: Sulforaphane and its conjugates found in the frass of the predatory spiders.

Sulforaphane, sulforaphane-acetyl-cysteine, sulforaphane-cysteine and sulforaphane-glutathione were detected in the frass of the spiders that preyed on *Pxgss1* silenced *P. xylostella* larvae (fig 12). These isothiocyanate conjugates are a part of mercapturic acid pathway. Mercapturic acid pathway is an isothiocyanate detoxification pathway found in some organisms that feed on isothiocyanate containing diet (Schramm, 2012; Sun et al., 2019). Hence, this suggests that the spiders were able to detoxify the toxic sulforaphane via conjugation with different amino acids. Only desulfoglucoraphanin and no other conjugates were detected in frass of the spiders fed on non silenced *Pxgss1 P. xylostella* larvae.

4. Discussion and conclusion

As plants produce allelochemicals to deter the herbivore, the herbivores have come up with certain counter-adaptations to overcome the defense mounted by the plants. Hence, there is an evolutionary arms race between the two. Insect herbivores have several counter-adaptations that include detoxification, rapid excretion and sequestration. Detoxification is the process that primarily involves enzymes that neutralize the toxic effects of the allelochemicals (Schoonhoven et al., 2005). But production of these enzymes entails energetic costs. The herbivore has to distribute some of its energy from other essential biological functions to the detoxification process. Hence, there is a always a detoxification and growth trade-off (Cresswell et al., 1992; Karban and Agrawal, 2002). To minimize the costs of detoxification, often the genes associated with the detoxification are inducible (X Li, 2002, YE Zhang 2012). Initially, *Pxgss* was assumed to be constitutively expressed (Ratzka et al., 2002, Winde and Wittstock, 2011). Here, we and others (Heidel-Fischer et al., 2019, Sun et al., 2019) have observed that glucosinolate sulfatase gene is inducible and its induction is dependent upon dietary intake of glucosinolates.

PMRi is a powerful technique which is gaining popularity in crop protection field (Mao, 2007). Here, we used PMRi to silence the *P. xylostella's* detoxification gene glucosinolate sulfatase. Silencing the *Pxgss1* gene had negative effects on the growth of *P. xylostella's* larvae as seen by decreased larval weight of *P. xylostella* larvae fed on *Pxgss1* silenced Col-0 plants. Hence, this suggests that desulfation is certainly a detoxification process. *Pxgss1* silencing caused changes in the overall metabolic content of the larvae. The larvae fed on *Pxgss1* silenced plants had higher concentrations of isothiocyanates in their gut, hemolymph and frass. Artificial diet spiked with physiological concentrations of sulforaphane showed a reduction in *P. xylostella's* larval weights. Thus, sulforaphane was responsible for the negative effects on the growth of *P. xylostella's* larvae. Our achievement of efficiently targeting the *Pxgss1* gene using PMRi can be further expanded to other *Brassica* crops inorder to control the population of *P. xylostella.*

Several herbivores are known to metabolize the allelochemicals for their protection against the natural enemies (Kazana et al., 2007, Müller et al., 2001, Kos et al., 2011). *D. insulare* larvae showed differential growth rate when fed on *P. xylostella* feeding on different *Brassica* species having varied glucosinolate contents (Sarfraz et al., 2008). Endoparasitoids, *D. fenestrale* and *D. semiclausum* had differential performance based on the *P. xylostella's* diet (Gols, 2008). Apart from parasitoids, various predators like European fire ant and birds also select their prey *P. xylostella* larvae based on their diet's glucosinolate composition (Hopkins et al., 2009). Hence, it's possible that the presence of glucosinolates and its different forms in the herbivore might have an effect on the natural enemies. Parasitoids used for biocontrol agents were well suited to the temperate climates of Europe. The same parasitoids when introduced were not successful due to difference in geoclimatic conditions (Talekar, 1997). Understanding of tritrophic interactions has the capability to help the agricultural system directly. Significant biocontrol of plant pests can be applied by the third trophic level, given an ample population of natural enemies.

Hence, we screened and identified the native abundant predators of *P. xylostella* that are well acclimatized to the tropical climates of India. We observed and recorded the behaviour of three species of predatory spiders which were fed with *P. xylostella* larvae that in turn were fed on different *A. thaliana* lines differing in glucosinolate content. Spiders showed invariable preference towards *P. xylostella* larvae containing different glucosinolate content. This might be due to the fact that the predators were able to detoxify the ingested isothiocyanates via mercapturic acid pathway. These spiders are robust to any change in glucosinolate composition unlike the previously reported natural enemies (Hopkins et al., 2009). Hence, these spiders being native and well adapted to the environment can be potentially used for controlling the population of the *P. xylostella.* In future, sulforaphane can be sprayed on the plant tissue and these spiders can be employed as biocontrol agents to curb the damaged caused by the pest. Interestingly, the isothiocyanate sulforaphane is known for its anticancer and antioxidant properties in humans (Su et al., 2018).

5. References

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