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The Effect of Glucosinolates on the Growth and Development of *Helicoverpa armigera* Larvae and the Expression of Midgut Sulfatase Genes

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Abstract: The plant–pest interaction and its mechanisms are a novel research direction for pest control. They provide molecular targets for developing new pesticides and targeted control measures to control insect herbivores. Glucosinolate is a large family of secondary substances found in cruciferous plants that are harmful to herbivorous insects. Specialist herbivores have developed specific anti-defense genes and detoxifying mechanisms against glucosinolate from the host plant, but how generalist herbivores respond to glucosinolate at the molecular level is unknown. In this study, we investigated the effects of different glucosinolate concentrations on the growth and development of *Helicoverpa armigera*. Moreover, the expression of sulfatase genes (*HaSulfs*) was also checked following exposure to glucosinolate concentrations. The developmental duration of larvae and prepupa of *H. armigera* was significantly increased by 14.79–25.03% after feeding glucosinolate compared to the control. Quantitative Real-Time PCR (RT-qPCR) was carried out to analyze the expression of *HaSulf* family genes in the midgut of fifth instar larvae of *H. armigera*. The results showed that the upregulated expression patterns of *HaSulf* family genes were diversified after feeding at different concentrations. The expression level of *HaSulf* was detected with the *HaSulf* antibody. Only the glucosinolate-fed larvae had a visible target band and were mainly distributed in the midgut wall. Taken together, glucosinolate can significantly affect the growth and development of *H. armigera* larvae. It can induce the expression of *HaSulf* in the midgut of *H. armigera* at gene and protein levels. This study could be useful to understand the development of plant-derived insecticides resistance in *H. armigera*.

Keywords: plant secondary metabolites; glucosinolate; cotton bollworm; sulfatase; growth and development

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1. Introduction

The cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), is a worldwide pest that causes severe damage to economic crops [1]. At present, chemical insecticides [2,3] and transgenic *Bacillus thuringiensis* (Bt) crops [4,5] are widely used to control them, but their long-term application has led to the gradual resistance of *H. armigera.* For many years, people have been guiding the rational use of pesticides to overcome or delay the development of resistance. However, ignoring the mutual restriction between chemical control and plants, plant secondary substances and pests may accelerate the development of pest resistance and reduction of crop resistance, leading to pest outbreaks [6–8].

The interaction of plant secondary metabolites and pests and their mechanisms is a major topic in scientific research. It is important to promote the selection of pest-targeting genes and the development of green pesticides [9–12].

Glucosinolate (GS) is a secondary metabolite of nitrogen and sulfur-containing anions widely present in cruciferous plants [13,14]. Glucosinolate can be catalyzed by myrosinase to produce a series of toxic hydrolysis products, including isothiocyanate (ITC), nitrile, thiocyanate, and other active substances. Some of these products poison the pests or inhibit them from laying eggs, thereby avoiding or reducing plant damage [15]. At present, there are more than 120 types of glucosinolate found in nature. The types and content of glucosinolate in cruciferous plants are the most abundant [16]. Studies have found that different varieties of cruciferous plants have different types of glucosinolate. The composition and content of glucosinolate vary greatly between parts of the same plant and at various stages of development [17]. This variation in glucosinolate quantity and composition also helps cruciferous vegetable crops to survive pests and other environmental stresses [18].

Herbivorous insects have been hampered to some extent by the diversity of the plant's complex and delicate glucosinolate-myrosinase defense mechanisms. However, cruciferous vegetable pests can feed host plants as part of a long-term co-evolutionary process. Several anti-defense mechanisms deal with glucosinolate-myrosinase defense mechanisms in plants [19–24]. The cabbage stem flea beetle (*Psylliodes chrysocephala*) and the specialist pest *Plutella xylostella* may desulfurize host plant glucosinolates via their glucosinolate sulfatase (GSS) [25,26]. As a result, the host plant myrosinase cannot recognize and hydrolyze the substrate glucosinolate and produce toxic secondary metabolic defense substances [27–29]. Sulfatase is also found in generalist herbivores. The expression of *Heliothis virescens* sulfatases was upregulated after feeding wild-type *Arabidopsis thaliana*, while the larvae weight increased significantly on the mutant *A. thaliana* without glucosinolate [30]. Sulfatase is an evolutionarily conservative enzyme found in both prokaryotic and eukaryotic organisms. The sulfatase activity is initiated by the sulfatase modification factor (SUMF1), which modifies a cysteine residue in the active center of sulfatase to formylglycine (FGLY) [31–33]. The functions of the SULF and SUMF genes in insects have not been thoroughly studied. The insect genome sequences provide an opportunity to examine the genes involved in the detoxification of plant glucosinolate [34]. In this study, eight *HaSulfs* and one *HaSumf1* were identified from the *H. armigera* genome. It was found that feeding *H. armigera* larvae on different concentrations of glucosinolate (above 4 ppm concentration range) can considerably restrict the growth and development of the larvae and can promote the expression of *HaSulfs* in the midgut tissue of the *H. armigera*. Our findings provide a theoretical foundation for developing new green pesticides and targeted control measures for *H. armigera*.

2. Materials and Methods

2.1. Materials

The sinigrin hydrate (>99.0%, TLC Grade), Extraction kit (TransZol Up Plus RNA Kit), Reverse Transcription Kit (TransScript One-Step gDNA Removal and cDNA Synthesis Super Mix), and Fluorescence Quantitative kit TransStart® Green qPCR SuperMix were purchased from Beijing TransGen Biotech. The ethanol (100%), trichloromethane, and RNase-free water were all domestic chemical reagents. The primer synthesis and sequencing were performed by Sangon Biotech (Shanghai) Co., Ltd.

The *H. armigera* strain used in this study was obtained from the Key Laboratory of Bioresource Genetic Engineering of Xinjiang University, China. *Helicoverpa armigera* larvae were reared on artificial breeding. The adults were fed with a honey solution at 27 ± 2 °C, 60% ± 10% relative humidity, and L:D = 14: 10 h in a greenhouse without exposure to insecticides.

2.2. Bioassay

In bioassays experiments, seven glucosinolate concentrations (0, 2, 4, 20, 40, 80, 160 ppm) were used. Hundred-second instar larvae of same age were used for each

concentration. The developmental duration from larva to pupae and survival rate under different glucosinolate concentrations were observed. The weight of the fifth instar larvae and pupa were checked regularly.

2.3. Identification and Phylogenetic Tree Construction of HaSulf

The sulftases sequence was downloaded by searching the conserved protein domain database at NCBI (http://www.ncbi.nlm.nih.gov/ (accessed on 1 June 2017)). MEGA7 software was used to align all sequences and build a phylogenetic tree using the maximum likelihood technique. Bootstrap values for 1000 replicates were calculated with MEGA7. The BLAST approach was used to identify *SULFs* and *SUMFs* genes from the genomes of *H. armigera*, *P. xylostella*, and *Bombyx mori*. Through https://jaspar.genereg.net/analysis, the transcription binding site of the promoter region was predicted [35].

2.4. Expression of HaSulfs and HaSumf1

The RNA-seq data of *HaSulfs* and *HaSumf1* were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/ (accessed on 1 June 2017)). The RT-qPCR was performed using SYBR-green fluorescence and gene-specific primers to determine the expression levels of HaSulfs and HaSumf1 fed with different glucosinolate concentrations. Five biological and two technical replicates were used in RT-qPCR.

The midgut of fifth instar *H. armigera* larvae fed with different concentrations of glucosinolates was dissected and collected (24–48 h after molting). The TransZol Up Plus RNA Kit was used to extract the total RNA from the midgut following the recommended protocol. cDNAs were synthesized using 1.0 µg total RNA by PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology, Dalian, China). RT-qPCR reactions were performed using TransStart®Green qPCR SuperMix reagent in a Real-Time PCR System (Applied Biosystems, US Applied Biosystems, Waltham, MA, USA) following the manufacturer's instructions. PCR conditions were 94 °C for 30 s, 40 cycles of 94 °C for 5 s, 60 °C for 15 s, and 72 °C for 10 s, then 95 °C for 15 s and 60 °C for 1 min.

The relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ method. The ribosomal protein L15 (HaRPL15) gene was used as an internal reference. The primers sequences for RT-qPCR are listed in Table 1.

Table 1. The primers used for RT-qPCR.

Gene Name	Primer (5′-3′)
HaRPS15 qPCR F	CTGAGGTCGATGAAACTCTC
HaRPS15 qPCR R	CTCCATGAGTTGCTCATTG
HaSumf1 qPCR F	GCCAAAGATGGTTATGAAGG
HaSumf1 qPCR R	ATTCCCACACATTGCCAG
HaSulf1 qPCR F	GTTCGCTTCCGACAATGGAG
HaSulf1 qPCR R	GCATCAACCCTTGCCAAACT
HaSulf2 qPCR F	CGGTGATATAGTTGCAGCTTT
HaSulf2 qPCR R	CTCCACAATAACCCGACAACC
HaSulf5a qPCR F	ATGGAGTCCGCTACTGAAG
HaSulf5a qPCR R	ACACTAACATCGCCACCAG
HaSulf5b qPCR F	GTTGCTTCCACAGTTCCTG
HaSulf5b qPCR R	CGAATCCTCGGTTCATAGG

2.5. Determination of Protein Production in the Midgut

The proteins were extracted from the midgut wall and midgut contents of the fifth instar larvae of *H. armigera* when treated with 4 ppm glucosinolates (24–48 h after molting). In addition, 4 ppm of gossypol and methyl jasmonate were fed to *H. armigera* as controls for xenobiotics, and the production of *HaSulf* protein was measured. The Western blot step was performed as follows: (1) SDS-PAGE electrophoresis was used to separate the different protein samples, which was then transferred to a PVDF membrane; (2) samples were blocked with 5% skimmed milk powder overnight at 4 °C, then washed 3 times with

PBST for 5 min each time; (3) the primary antibody was incubated as follows: The primary antibody was diluted at a ratio of 1:10 and blocked for 2 h at 37 °C. The primary antibody removed and washed three times with PBST for five minutes each time; (4) the secondary antibody was incubated as follows: 2% skimmed milk powder was used, diluted (1:100) with goat anti-mouse secondary antibody (HRP-labeled), incubated at 37 °C for 2 h, and washed 3 times with PBS for 5 min each time; (5) DAB color development: 50 µL of freshly prepared color development solution was added dropwise to the membrane, darkened the color development for 10 min, and then immediately washed with distilled water to stop the reaction.

2.6. Statistical Analysis

The relative mRNA expression levels of genes and the growth and development data were analyzed by one-way analysis of variance (ANOVA) using SPSS v. 19 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered significant for all treatments. All the figures were generated using GraphPad Prism 5.0.

3. Results

3.1. Effects of Different Concentrations of Glucosinolate on the Growth and Development of H. armigera

Different concentration gradients of glucosinolates (0–160 ppm) are added to the artificial diet to feed the second instar larvae of *H. armigera*. The biological parameters, including the developmental stages of larvae, pupae weight, and pupal rate, were observed. After feeding glucosinolate, the development duration of *H. armigera* was significantly delayed in the fifth instar and the pre-pupal stage. The development duration of *H. armigera* larvae fed with different glucosinolate concentrations significantly ($p < 0.05$) increased by 14.79–25.03% (22.66–24.68 d) as compared to the control group (19.74 d) (Figure 1). *H. armigera* pupae fed glucosinolate have deformed pupae and incomplete abdominal wrapping (Figure 2). Furthermore, the pupa weight results showed that low concentrations of glucosinolate (2 ppm) had a positive impact on *H. armigera* growth and development as compared to the control group ($p < 0.05$) (Figure 3A). The survival rate of the pupae of *H. armigera* fed with 4, 40, and 160 ppm glucosinolate was significantly decreased (32%–37%) as compared to the control group (56%) (Figure 3B).

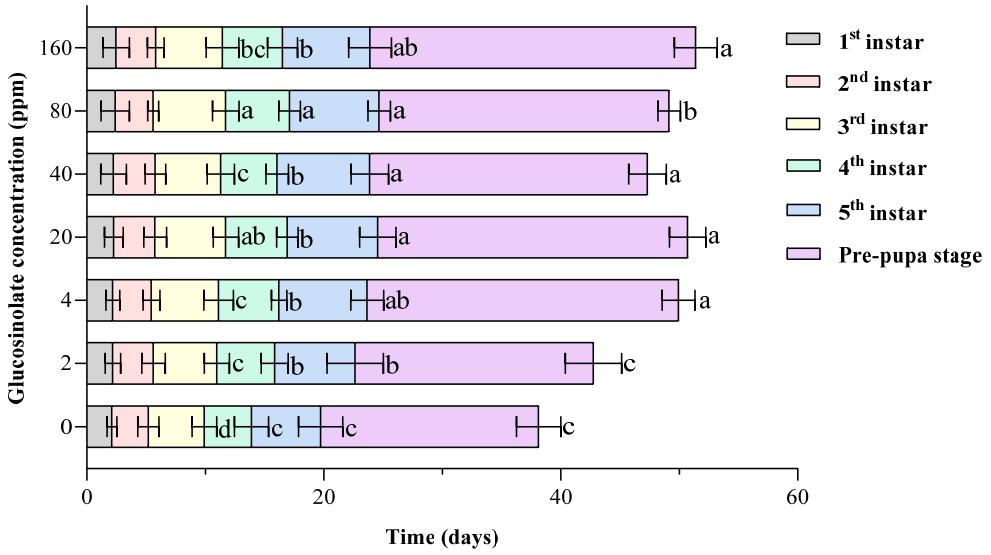

Figure 1. Growth and development of cotton bollworm larvae. The development period of the *H. armigera* at different instars under different concentrations of glucosinolate. Error bars indicate standard errors of the means (SE) ($n = 100$). Letters indicate values that are statistically different from the control group ($p < 0.05$).

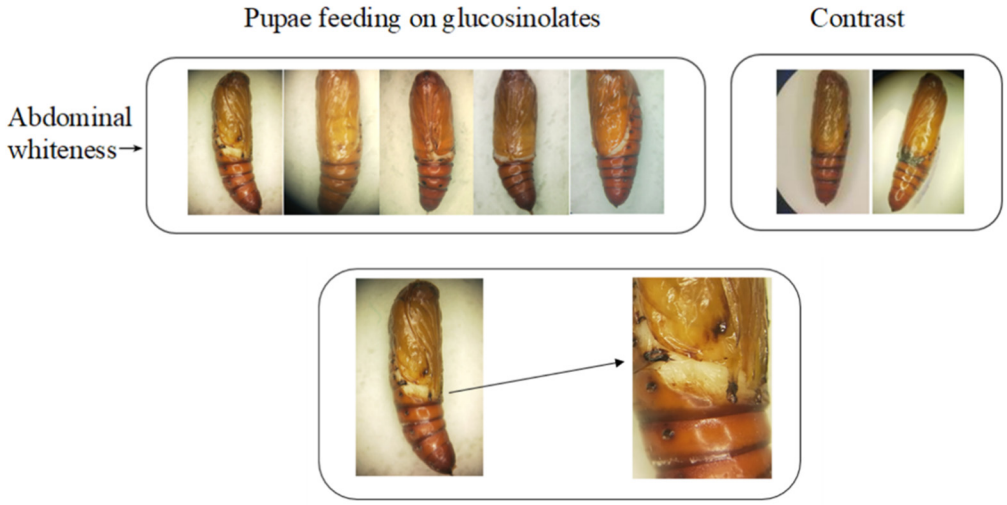

Figure 2. Phonotype of *H. armigera* pupa. The pupae of *H. armigera* fed with glucosinolate and the control group without glucosinolate.

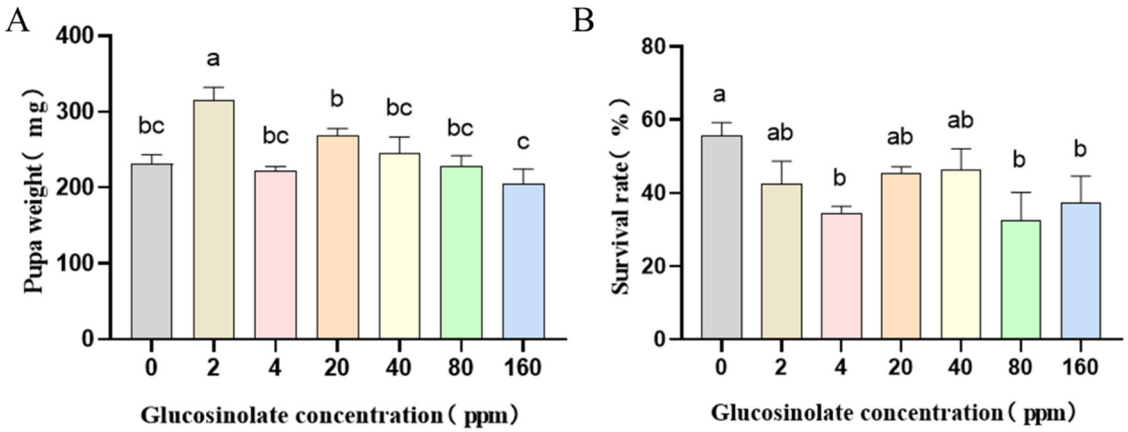

Figure 3. The weight and survival rate of *H. armigera* pupae under different concentrations of glucosinolate: (**A**) Weight of *H. armigera* pupae; (**B**) Survival rate of *H. armigera* pupae; Error bars indicate the standard error of the mean (SE) (n = 100). Lowercase letters indicate significant difference at $p < 0.05$ (One-way ANOVA).

The results of development duration, growth, and pupal weight showed that the response of the *H. armigera* to glucosinolate is not entirely linear with the glucosinolate concentrations. The pupa weight of *H. armigera* is concentration-dependent in the range of 20 to 160 ppm. The survival rate of *H. armigera* pupae is concentration-dependent in the range of 0 to 4 ppm (Figure 3). Furthermore, the pupa survival rate of *H. armigera* fed with 80 and 160 ppm glucosinolate was significantly lower than the control group ($p < 0.05$) (Figure 3B).

3.2. Identification of HaSulf Gene and Protein Prediction

Eight *HaSulfs* and one *HaSumf1* gene were identified, and a bioinformatics analysis of these sequences was carried out (Tables 2–4). The sulfatase gene family comprises four branches, namely, *SulfA, B, C*, and *D* [36]. In the evolutionary tree, *HaSulf1*, *HaSulf2*, and *HaSulf4* are on the C branch, *HaSulf5a* and *HaSulf5b* are on the A branch, *HaSulf6* is on the D branch, and *HaSulf7* and *HaSulf8* are on the B branch (Figure 4). The length of most predicted *HaSulfs* ranges from 290 to 615 amino acids (aa), of which 1 ultralong *HaSulf7* gene contains 1403 amino acids. The gene positions range from 3,168,620 to 3241586, showing a high degree of structural complexity (Table 2). It is predicted that there are big differences

in each protein's isoelectric point, extinction coefficient, instability coefficient, fat index, and hydrophilicity. The isoelectric points of most *HaSulfs* are 5.19 to 6.63, among which the isoelectric points of *HaSulf1*, *HaSulf6*, and *HaSulf7* are 8.86, 9.38, and 9.13, respectively, which are significantly higher than other proteins. The extinction coefficient of most *HaSulfs* ranges from 82,110 to 135595, while the extinction coefficient of *HaSulf5a* is 54680, which is substantially lower than other proteins. The extinction coefficient of *HaSulf7* is 180710, which is significantly higher than other proteins. The instability coefficients of most proteins are predicted to be 33.03 to 45.91, and the instability coefficient of *HaSulf7* is 52.71, which is significantly higher than other proteins (Table 3). It is predicted that the fat index of most proteins ranges from 76.85 to 91.04. However, the fat indices of *HaSumf1* and *HaSulf7* are 55.28 and 62.37, respectively. The hydropathic index of most *HaSulfs* is −0.163 to −0.690, and the hydropathic index of *HaSulf7* is −0.985, which is quite different from other proteins. By predicting the transcription binding site of the promoter region, it is interesting to find that *HaSumf1* has high similarity with the transcription factors of *HaSulf2*, *HaSulf4*, and *HaSulf5a* in the sulfatase gene family (Table 4), and we also found that *HaSulf2 and HaSulf4* are co-located on the *SulfC* evolutionary branch [36]. This phenomenon provides an important theoretical basis for subsequent studies on the regulation mechanism of sulfatase genes and their modifier genes.

Table 2. Identification of HaSumf1 and HaSulfs.

Protein Name	Gene ID	Gene Size (bp)	ORF (bp)	Protein (AA)	Location	Assembly
HaSumf1	110375229	2246	1020	339	NW_018395484.1 (276539..279127)	Harm_1.0 (GCF_002156985.1)
HaSulf1	110375086	3156	1848	615	NW_018395392.1 (3407398..3426461)	Harm_1.0 (GCF_002156985.1)
HaSulf2	110374783	2411	1608	535	NW_018395392.1 (3399889..3404850)	Harm_1.0 (GCF_002156985.1)
HaSulf4	110375159	2877	1659	552	NW_018395392.1 (3370118..3375945)	Harm_1.0 (GCF_002156985.1)
HaSulf5a	110381566	1327	873	290	NW_018395780.1 (177570..180958)	Harm_1.0 (GCF_002156985.1)
HaSulf5b	110374404	1644	1617	538	NW_018395467.1 (310504..316591)	Harm_1.0 (GCF_002156985.1)
HaSulf6	110370991	1806	1524	507	NW_018395415.1 (788596..790955)	Harm_1.0 (GCF_002156985.1)
HaSulf7	110373702	5627	4212	1403	NW_018395390.1 (3168620..3241586)	Harm_1.0 (GCF_002156985.1)
HaSulf8	110373657	1869	1491	496	NW_018395449.1 (312589..321088)	Harm_1.0 (GCF_002156985.1)

Table 3. Various physical and chemical parameters of HaSumf1 and HaSulfs.

Protein Name	Molecular Weight	Theoretical pI	Extinction Coefficients (M^{-1} cm^{-1}) *	Estimated Half-Life (hours) **	Instability Index	Aliphatic Index	Grand Average of Hydropathicity (GRAVY)
HaSumf1	38,590.95	5.64	82,110	30	38.17(stable)	55.28	−0.690
HaSulf1	68,881.90	8.86	135,595	30	38.58(stable)	83.85	−0.338
HaSulf2	59,732.77	5.19	104,670	30	36.61(stable)	89.29	−0.163
HaSulf4	67,365.48	6.07	104,990	30	35.13(stable)	90.15	−0.204
HaSulf5a	32,028.26	6.45	54,680	30	41.02(unstable)	87.83	−0.358
HaSulf5b	59,969.67	6.63	99,280	30	33.03(stable)	91.04	−0.214
HaSulf6	58,420.09	9.38	89,075	30	41.02(unstable)	87.28	−0.360
HaSulf7	162,832.01	9.13	180,710	30	52.71(unstable)	62.37	−0.985
HaSulf8	55,756.01	5.74	85,440	30	45.91(unstable)	76.85	−0.347

* Abs 0.1% (=1 g/L), assuming all pairs of Cys residues form cystines. ** The N-terminal of the sequence considered is M (Met). The estimated half-life is 30 h (mammalian reticulocytes, in vitro); >20 h (yeast, in vivo); >10 h (Escherichia coli, in vivo). https://web.expasy.org/protparam/ (accessed on 3 January 2022).

Table 4. Promoter transcription binding site prediction.

Matrix ID	Name	Score	Relative Score	Sequence ID	Start	End	Strand	Predicted Sequence
MA0049.1	MA0049.1.hb	11.975525	0.9738739070180799	HaSumf1	574	583	+	GCAAAAAAAA
MA0015.1	MA0015.1.Cf2	10.631421	0.9114732446482743	HaSulf1	346	355	+	GTATATGTTT
MA0049.1	MA0049.1.hb	10.612954	0.9318941952719153	HaSulf2	78	87	+	GAGAAAAAA
MA0049.1	MA0049.1.hb	11.291026	0.9527850486969089	HaSulf4	510	519	+	GAACAAAAA
MA0049.1	MA0049.1.hb	11.139023	0.9481019493665909	HaSulf5a	1072	1081	+	GAATAAAAAT
MA0015.1	MA0015.1.Cf2	11.832657	0.9412119301944221	HaSulf5b	63	72	+	ATATATGTGT
MA0010.1	MA0010.1.br	12.489317	0.9206608060709957	HaSulf6	399	412	+	AAAATAAACAAAAG
MA0012.1	MA0012.1.br	8.726485	0.8802668436514	HaSulf7	978	988	+	TCAACTATAAC
MA0015.1	MA0015.1.Cf2	8.444539	0.857333168027844	HaSulf8	33	42	+	ATAAATGTAC

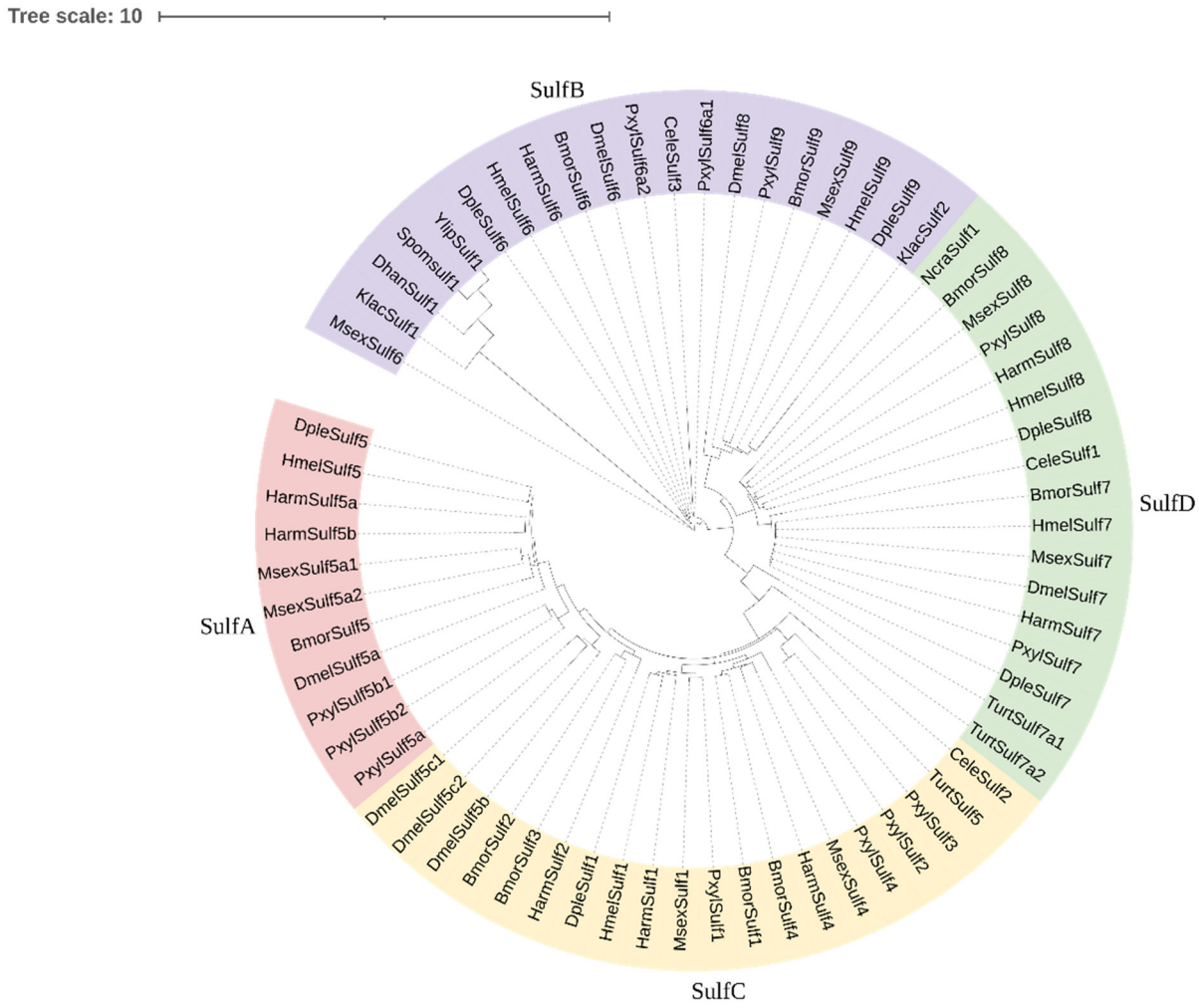

Figure 4. Phylogenetic tree of HaSulf amino acid sequence.

3.3. Prediction of Conserved Domains of HaSulfs and Construction of Phylogenetic Tree

Twenty different conserved motifs were identified from the eight *HaSulfs* of the *H. armigera* (Figure 5). Previous research has shown that C/SXPXRXTG is the shortest catalytic active center sequence that can maintain activity following *HaSulf* translation modification [37,38]. Different *HaSulf* sequences show substantial similarity at this site. We constructed an evolutionary tree to better understand the evolutionary relationship of *H. armigera HaSulf*. The *HaSulf* of *H. armigera* has homology with other insects, but there are some differences between different *HaSulfs* (Figures 4 and 5).

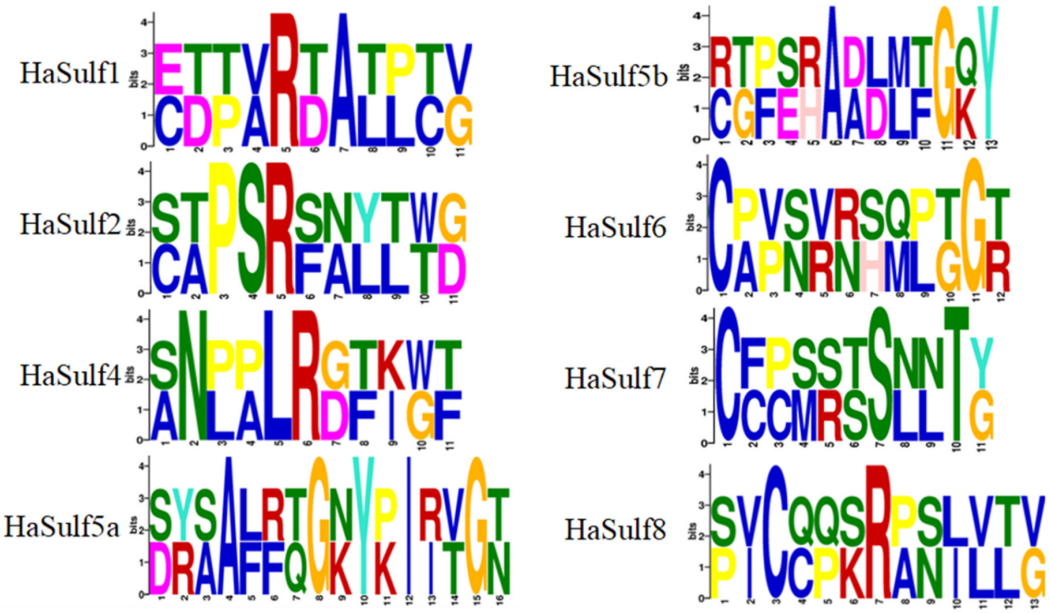

Figure 5. The core catalytic sequence of *HaSulfs*.

3.4. RT-qPCR Analysis of the Effects of Different Concentrations of Glucosinolate on the HaSulf Genes of H. armigera

Different concentrations of glucosinolate (0–160 ppm) were fed to the second instar larvae of *H. armigera*. At the fifth instar (24–48 h after molting), RT-qPCR was used to analyze the expression levels of the *HaSulfs* gene family in the midgut of *H. armigera*. The results showed that, except for *HaSulf7*, glucosinolate significantly induced other *HaSulf* genes' expression (Figures 6 and 7). The *HaSumf1* gene fed with 40 ppm glucosinolate had no difference compared with the control group. Furthermore, the *HaSumf1* gene was up-regulated to varying degrees when fed different concentrations of glucosinolate (Figure 6). The expression of the *HaSulf1* gene was highly upregulated (48.1 times) when fed 2 ppm glucosinolate (Figure 6). The expression of the *HaSulf2* gene was significantly increased (3.6 times) when fed 4 ppm glucosinolate ($p < 0.05$). The expression of *HaSulf4* gene fed with 80 ppm glucosinolate was significantly upregulated (34.9 times) compared to the control. The *HaSulf5a* gene fed with 2 ppm glucosinolate was upregulated 4.7 times more compared to the control (Figure 6). However, the *HaSulf5a* gene fed with 40 and 160 ppm glucosinolate was downregulated to a certain extent. A significant difference was observed in the expression level of the HaSulf5a gene when *H. armigera* was fed with 2, 40, and 160 ppm glucosinolate ($p < 0.05$). The expression of the *HaSulf5b* gene was highly upregulated (3.4 times) at a 20 ppm concentration of glucosinolate (Figure 7). Compared with the control group, the HaSulf8 gene fed with 80 ppm glucosinolate has a 3.3 times greater expression. *HaSulf8* fed with 160 ppm glucosinolate had a certain degree of downregulation compared to the control group. A significant difference was observed when the *HaSulf8* gene was fed with 80 and 160 ppm glucosinolate ($p < 0.05$). There was no significant difference in the expression of *HaSulf7* gene under different concentrations of glucosinolate (Figure 7).

3.5. Western Blot Analysis of HaSulf Production at the Protein Level

The production of HaSulf was also observed at the protein level using the Western blot technology. The midguts of *H. armigera* (fifth instar) were dissected, and the proteins were extracted. The *HaSulf* antibody was used to detect the protein production level of *HaSulf*. In vitro induced and purified *HaSulf* protein was used as a positive control. The results showed that the prokaryotic expressed and purified *HaSulf* had a clear target band, with an Mr size of about 79,700 (Figure 8). However, the midgut protein of the *H. armigera* did not detect a visible target band (Figure 8).

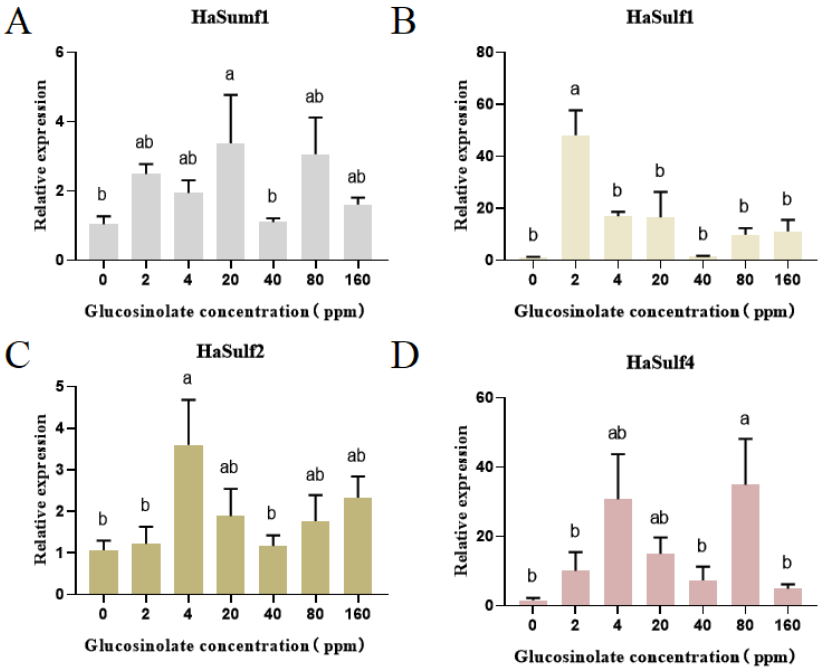

Figure 6. The effect of different concentrations of glucosinolate on the expression of HaSulfs gene family. The picture shows the expression level of HaSulfs gene family after feeding different concentrations of glucosinolate. (**A**) The relative gene expression of *HaSumf1*. (**B**) The relative gene expression of *HaSulf1*. (**C**) Relative gene expression of *HaSulf2*. (**D**) The relative gene expression of *HaSulf4*. Error bars indicate the standard error of the mean (SEM) (n = 5). Lowercase letters indicate significant difference at $p < 0.05$ (One-way ANOVA).

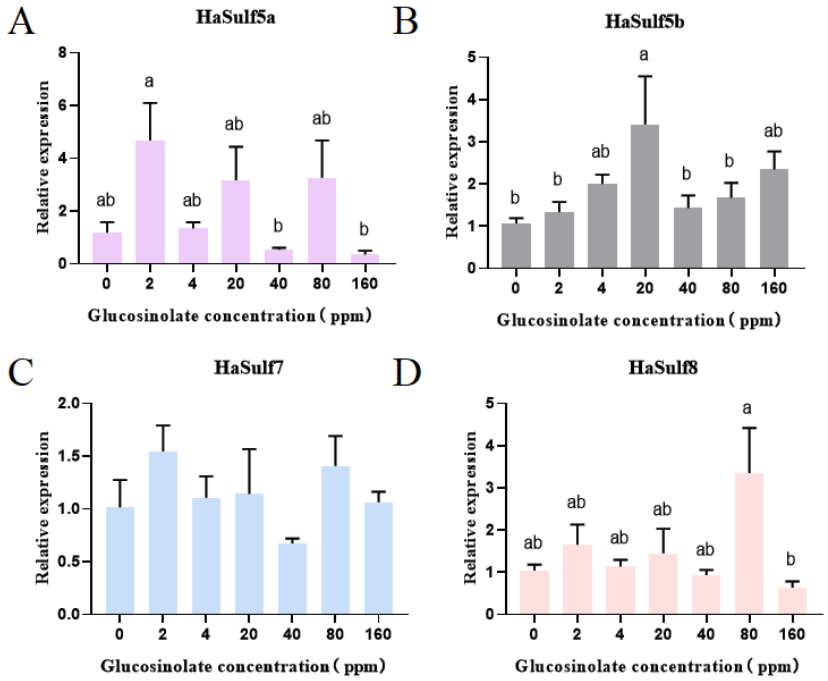

Figure 7. The effect of different concentrations of glucosinolate on the expression of HaSulfs gene family. The picture shows the expression level of *HaSulfs* gene family after feeding different concentrations of glucosinolate. (**A**) The relative gene expression of *HaSulf5a*. (**B**) The relative gene expression of *HaSulf5b*. (**C**) The relative gene expression of *HaSulf7*. (**D**) The relative gene expression of *HaSulf8*. Error bars indicate the standard error of the mean (SEM) (n = 5). Lowercase letters indicate significant difference at $p < 0.05$ (One-way ANOVA).

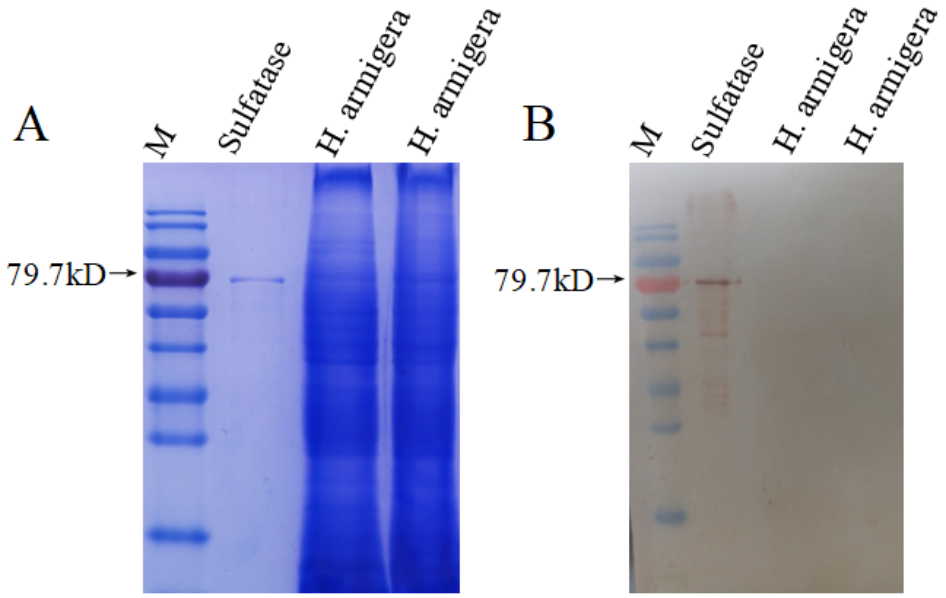

Figure 8. SDS-PAGE and Western blot analysis of sulfatase protein expressed in vitro and cotton bollworm protein. (**A**) SDS-PAGE analysis of sulfatase protein expressed in vitro and cotton bollworm protein; (**B**) Detection of sulfatase and cotton bollworm protein in vitro by Western blot analysis using anti-sulfatase antiserum. M: Protein marker.

Subsequently, the *H. armigera* larvae (fifth instar) fed with 4 ppm glucosinolate were collected. After dissection, the midgut and midgut contents were collected. The proteins were extracted from all samples. The target band was visible on the midgut wall, with an Mr size of around 59700, which was consistent with the size of the target band. No corresponding band was found in the contents, as detected by the HaSulf antibody (Figure 9). Furthermore, the fifth instar *H. armigera* was fed with 4 ppm gossypol, glucosinolate, and methyl jasmonate. After 24 h, the midgut was dissected, and the protein was extracted. Then, the protein production level of *HaSulf* was detected by *HaSulf* antibody. The results showed that only *H. armigera* larvae had clear bands after feeding glucosinolate. It was also hypothesized that plant glucosinolate induced *HaSulf* in *H. armigera* (Figure 10).

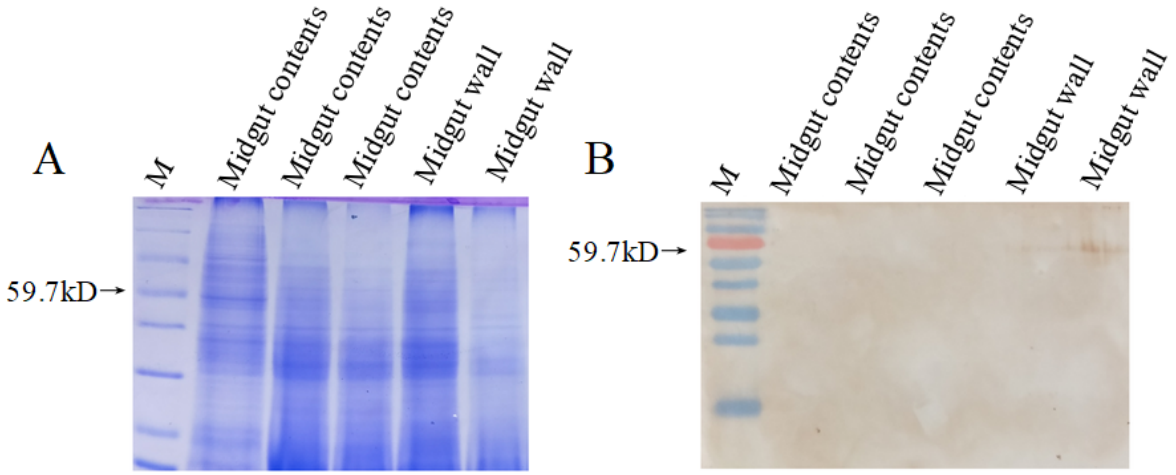

Figure 9. SDS-PAGE and Western blot analysis of cotton bollworm midgut wall and midgut contents. (**A**) SDS-PAGE analysis of cotton bollworm midgut wall and midgut contents; (**B**) Detection of cotton bollworm midgut wall and midgut contents in vitro by Western blot analysis using anti-sulfatase antiserum. M: Protein marker.

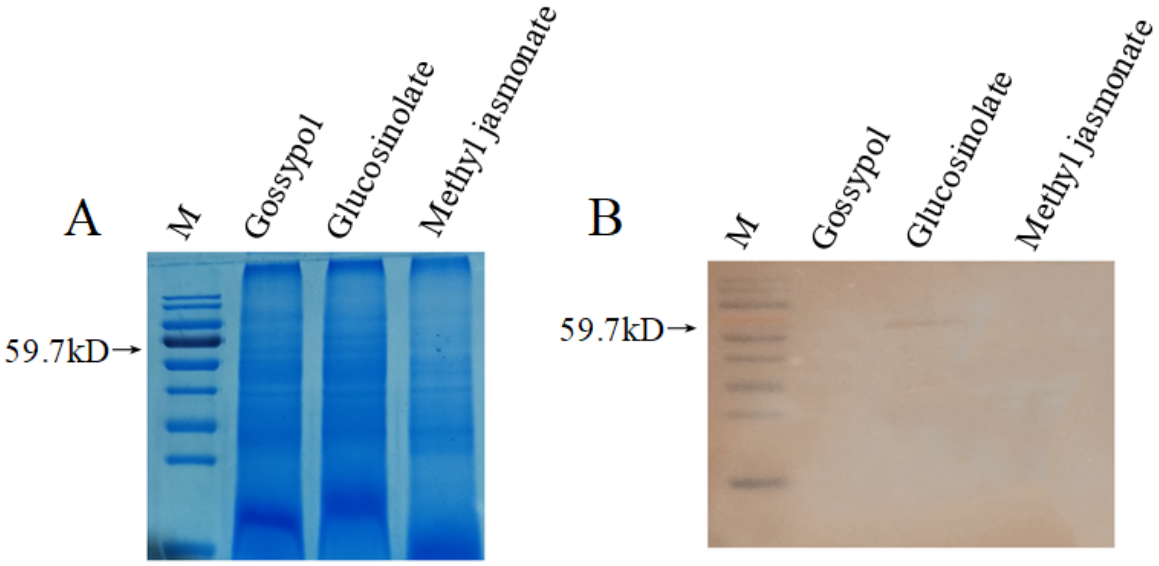

Figure 10. SDS-PAGE and Western blot analysis of cotton bollworm fed with gossypol, glucosinolate, and methyl jasmonate. (**A**) SDS-PAGE analysis of cotton bollworm fed with gossypol, glucosinolate, and methyl jasmonate; (**B**) Detection of cotton bollworm fed with gossypol, glucosinolate, and methyl jasmonate by Western blot. M: Protein marker.

4. Discussion

HaSulf genes play multiple roles in insects, including growth, development, and detoxification metabolism. The *HaSulf* family genes are closely linked with the anti-plant defense system. Understanding the expression level of the *HaSulf* gene family in *H. armigera* is crucial to elucidate the relationship between *HaSulf* and glucosinolate. Therefore, simulating the difference in the glucosinolate content of insects feeding on the same plant with different glucosinolate concentrations might reduce the complexity of insects feeding on different plants.

At present, the sulfatase gene has not been studied deeply in insects. The increasing number of insect genome sequencing provides an opportunity to analyze the insect genes involved in the detoxification of plant glucosinolates from the whole genome. Sulfatase is a hydrolase that can decompose sulfate from various substrates, including glycosaminoglycans, sulfolipids, and steroid sulfates [38]. The function of insect sulfatase is related to the gradual adaptation of the host plant's defense system in the co-evolutionary arms race of cruciferous plants. The specialist pest *P. xylostella* can use its glucosinolate sulfatase (GSSs) to competitively combine with the glucosinolates of the host plant to quickly desulfurize glucosinolates, so that the host plant myrosinase cannot recognize and hydrolyze the substrate glucosinolates and cannot produce toxic secondary metabolic defense substances [39,40]. Generalist pests also contain sulfatases, however, the function of sulfatase genes and the molecular mechanism of how to detoxify host plant glucosinolates in generalist pests are still unclear. Our previous study identified eight *HaSulf* genes and one modifier gene *HaSumf1*. The *HaSulf* has a certain degree of homology with other insect *HaSulf* enzymes (Figure 4). The *HaSUMF1* is similar to the *SUMF2* of *Tetranychus urticae* and *Drosophila melanogaster* through evolutionary trees (Figure 11). Different *HaSulf* sequences show substantial similarity at the C/SXPXRXTG site (Figure 5). Each amino acid column has a distinct height, showing variances in the amount of amino acid information and conservation of each *HaSulf*. Differences in amino acids and conserved motifs may affect the biological functions of sulfatase and the binding and hydrolytic activity of substrates to a certain extent [40]. Moreover, *HaSulfs* have different isoelectric points, extinction coefficients, instability coefficients, fat indices, hydrophilicity, and conservative motifs with different effects. The length of most predicted *HaSulfs* ranges from 290 to 615 amino acids (aa), of which one ultralong HaSulf7 gene contains 1403 amino acids. The gene positions

range from 3,168,620 to 3,241,586, showing a high degree of structural complexity. From the similarity of the sulfatase genes between *H. armigera* and other insects, we speculate that *H. armigera* sulfatase also detoxifies host plant glucosinolates [41]. The sequence expansion and differences in the *H. armigera* sulfatase family members may be related to their feeding habits. The sulfatase family members have a certain degree of synergy and new functionalization to adapt to the secondary metabolites of different host plants [42].

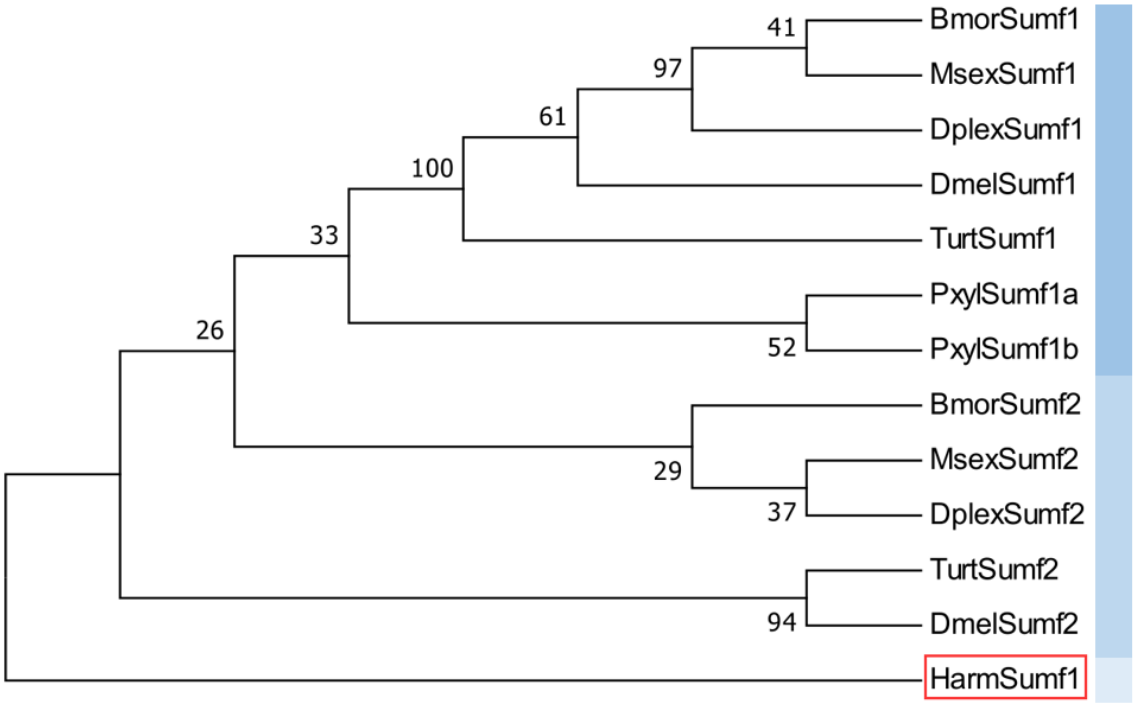

Figure 11. Phylogenetic tree of the amino acid sequence of HaSulf modifying factor (HaSumf1).

Furthermore, RT-qPCR results showed that the *HaSulf1* and *HaSulf5a* genes were up-regulated in the midgut of *H. armigera* fed with 2 ppm glucosinolate, the *HaSumf1* and *HaSulf5b* genes were upregulated at 20 ppm, while the *HaSulf4* and *HaSulf8* genes at 80 ppm glucosinolate (Figures 6 and 7). Based on these results, it is hypothesized that these genes may have a cooperative expression [36]. The *HaSulf7* gene may be relatively conservative, therefore, no significant difference was observed. The *HaSumf1*, *HaSulf2*, *HaSulf4*, *HaSulf5a*, and *HaSulf8* genes in the *H. armigera* fed 80 ppm glucosinolate were up-regulated to varying degrees compared with 40 ppm glucosinolate (Figures 6 and 7). It is hypothesized that the *H. armigera* may activate other defense mechanisms when fed 40 and 80 ppm glucosinolate. RT-qPCR results showed that the expression level of *HaSulf* was not consistent when fed with different glucosinolate concentrations (Figures 6 and 7). Therefore, it is speculated that different *HaSulf* genes play a crucial role in various concentrations. Ren J et al. studied the expression levels of different sulfatase genes in *Bemisia tabaci* and found that the same sulfatase gene is differentially expressed in different subspecies. This different differential expression phenomenon may be related to the feeding habits of insects [42]. Manivannan A and others also measured the expression of the sulfatase gene. In vivo silencing of the *B. tabaci* GSS gene expression via RNA interference led to lower levels of desulfoglucosinolates in honeydew [41]. The biological function of *H. armigera* sulfatase requires *HaSumf1* to activate and *HaSumf1* also has different responses to different concentrations of glucosinolates (Figure 7), which may also be one of the reasons for the different expression of sulfatase. The cooperative expression of sulfatase genes seems to be consistent with the results in the evolutionary tree (Figures 6 and 7). The sulfatase gene family comprises four branches, namely, *SulfA, B, C,* and *D* [36]. In the evolutionary tree, *HaSulf1*, *HaSulf2*, and *HaSulf4* are on the C branch, *HaSulf5a* and *HaSulf5b* are on the A

branch, *HaSulf6* is on the D branch, and *HaSulf7* and *HaSulf8* are on the B branch (Figure 4). The differential expression of sulfatase genes in *H. armigera* may be related to the hydrolysis and binding activity of the substrate [40].

The HaSulf antibody was used to detect the expression level of sulfatase protein. In vitro induced and purified HaSulfs were used as a positive control (Figure 8). It is found that the production of sulfatase protein was only detectable in the midgut wall of *H. armigera* after glucosinolate feeding (Figure 9). Subsequently, we used different secondary metabolites gossypol and methyl jasmonate to demonstrate that the production of sulfatase protein detected in the midgut wall of *H. armigera* is not due to biotic stress (Figure 10). Fabian et al. reported that the sulfatases of the generalist moth *H. virescens* were induced by glucosinolates, and further biochemical experiments are needed to confirm [30]. This study reported that *Hasulfs* genes and protein levels are both induced by glucosinolates. We found that feeding low concentrations of glucosinolates can increase the production of *H. armigera* sulfatase, but we have not yet found a regulatory relationship between glucosinolate concentration and sulfatase production. Therefore, it is speculated that glucosinolates can induce the production of sulfatase protein in the midgut wall of *H. armigera*, but this induction phenomenon is not a concentration-dependent regulatory relationship, which requires further biochemical experiments to verify. Chen et al. found that after knocking out the sulfatase gene of *P. xylostella* through CRISPR/Cas9 technology, the egg hatching rate and larval survival rate of the mutant *P. xylostella* were significantly reduced, and the survival time of the larval stage was prolonged [40]. Agnihotri et al. showed that the developmental duration, survival rate, body length, and weight were significantly decreased when *H. armigera* was fed with 0–500 ppm glucosinolate. Moreover, the trypsin, chymotrypsin, cathepsin, and total protease activities were also reduced considerably [43]. The inhibition of digestive proteases by glucosinolate may cause insects to starve, leading to nutritional deficiencies, reduced free amino acids, and decreased metabolic energy [38]. It indicates that glucosinolate intake may affect the sulfatase content of the *H. armigera*, which in turn affects the growth and development of the *H. armigera* [40]. In our research, we also found that the glucosinolate-fed *H. armigera* had varying degrees of developmental delay compared to the control group (Figure 1). Moreover, the pupa weight was decreased, and the mortality rate increased (Figure 3). It is hypothesized that when the *H. armigera* is fed glucosinolate, it consumes energy to produce *HaSulf* to detoxify glucosinolate, indicating that *HaSulf* may affect the growth and development of insects. These combined effects of glucosinolate consumption result in growth retardation, developmental defects, and, ultimately, death of insects. The results showed whiteness in the abdomens of *H. armigera* fed glucosinolate, confirming that glucosinolate influenced *H. armigera* growth and development (Figure 2).

Low concentrations of glucosinolate (2 ppm) have a stimulating effect on the growth and development of *H. armigera* (Figure 1). However, the response of *H. armigera* to glucosinolate and its concentrations is not linear. The pupa weight of *H. armigera* is concentration-dependent in the range of 20–160 ppm, while the survival rate of pupae is in the range of 0 to 4 ppm (Figure 3B). The pupa weight of *H. armigera* fed with 80 and 160 ppm glucosinolate was not substantially different from the control group (Figure 3A). However, the pupa survival rate was considerably lower than the control group ($p < 0.05$). Therefore, it is speculated that glucosinolates may have affected the energy storage during the metamorphosis and development of the *H. armigera* pupa or another set of anti-defense mechanisms [13,15]. *P. chrysocephala* is a specialist pest that feeds on cruciferous plants. It can detoxify the glucosinolate-myrosinase defense system by desulfurization. However, testing found that desulfurized glucosinolates do not account for a high proportion of glucosinolates in *P. chrysocephala*, and it can use a variety of anti-defense mechanisms to resist the host plant's glucosinolate-myrosinase defense system [44]. Combined with the results of qPCR experiments, it is found that different sulfatase genes have different response patterns to glucosinolates. Therefore, it is speculated that *H. armigera* sulfatase is not the specific detoxification mechanism of glucosinolates.

5. Conclusions

Taken together, the glucosinolate damaged the physiological functions, growth, and development process of *H. armigera* when fed with different concentrations. Our results showed that *HaSulf* gene family is involved in glucosinolate detoxification and may be linked to *H. armigera* growth and development. Further research on this phenomenon could show how the generalist pest *H. armigera* adapts its physiological activities to feed on plants containing various plant defense compounds. Therefore, an in-depth functional study of *HaSulf* is essential to determine the underlying molecular mechanism in *H. armigera*.

Author Contributions: X.M. and L.J. planned and designed this study; X.L., F.C., C.L., G.K. and S.Z. performed calculation analysis, performed experiments, and generated numbers; X.M., X.L. and L.J. contributed to the development and/or revision of the manuscript; C.L. and Y.Z. contributed to sample preparation; X.L. conducted qRT-PCR experiments and data analysis; X.M., X.L., L.J., C.L. and Y.Z. participated in the discussion. All authors have read and agreed to the published version of the manuscript.

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