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Characterization of Serpin Family Genes in Three Rice Planthopper Species and Their Expression Profiles in Response to *Metarhizium* Infection

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Abstract: Rice planthoppers, including *Nilaparvata lugens*, *Sogatella furcifera*, and *Laodelphax striatellus*, are major agricultural pests. Serpins, which function as serine protease inhibitors, play a pivotal role in the immune systems of these insects, especially within the Toll signaling pathway and the prophenoloxidase (PPO) cascade. This study presents a comparative analysis of *serpin* genes among these species, highlighting their roles in immunity and development. Utilizing genomic and bioinformatics approaches, we identified 11, 11, and 14 *serpin* genes in *N. lugens*, *S. furcifera*, and *L. striatellus*, respectively. Phylogenetic analysis revealed a close evolutionary relationship between these *serpin* genes and *Bombyx mori Bm*Serpins, emphasizing the functional diversity of the serpin family. Structural analysis confirmed the presence of the reactive center loop (RCL) in all serpin proteins, with the Serpin7 subfamily showing a unique dual RCL configuration. Expression profiling showed species-specific *serpin* expression patterns across different life stages and adult tissues. Moreover, transcriptional analysis of *serpin* genes in the three planthoppers following *Metarhizium* infection uncovered distinct immune regulatory patterns two days post-infection. Notably, the expression of *NlSerpin2-2/6*, *SfSerpin4/6/7-1, and LsSerpin4/5-2/6* was upregulated post-infection, potentially enhancing antifungal capabilities. In contrast, the expressions of *NlSerpin1/7-1/9* and *LsSerpin1/2/3/8/13* were downregulated, possibly suppressing immune responses. Moreover, *Serpin6s*, which share a conserved phylogenetic lineage, exhibited enhanced immune activity in response to fungal invasion. These insights into serpin-mediated immune regulation could contribute to the development of novel pest-control strategies.

Keywords: innate immunity; serpin genes; rice planthoppers; *Metarhizium*

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1. Introduction

The rice planthoppers, belonging to the family Delphacidae within the order Hemiptera, are significant agricultural pests in the major rice-producing regions of China [1]. Currently, three dominant species have been identified: *Nilaparvata lugens* Stål, *Sogatella furcifera* Horváth, and *Laodelphax striatellus* Fallén. These species are characterized by their wide distribution, extensive infestation areas, and frequent outbreaks. Moreover, their ability to migrate over long distances often leads to sudden and severe rice crop damage [2–4]. Planthoppers damage rice crops directly by feeding on the phloem and indirectly through

mechanical injury during feeding and oviposition, as well as contamination from their excretions. This damage can impede rice growth and, in severe cases, lead to large-scale crop loss [1]. Furthermore, planthoppers are vectors for several rice viruses, further compounding the harm to rice crops. *N. lugens* transmits Rice ragged stunt virus (RRSV) and Rice grassy stunt virus (RGSV) [5,6]; *S. furcifera* transmits Southern rice black-streaked dwarf virus (SRBSDV) [7]; and *L. striatellus* transmits Rice stripe virus (RSV) and Rice black-streaked dwarf virus (RBSDV) [4,8]. The spread of these viruses not only exacerbates crop damage but also makes controlling planthopper populations more challenging. Despite advancements in genome sequencing and annotation of these three planthopper species over the last decade, as well as improvements in transcriptomic data [9–13], our understanding of the interspecific homology and differences in immunity, development, and evolution among these rice planthopper species remains limited.

Serpins, a superfamily of serine protease inhibitors, are found in a variety of eukaryotic organisms, including animals, plants, and some viruses. They are also present in bacteria and archaea, with over 1500 serpins identified to date [14–16]. While most serpins inhibit serine proteases, others are capable of inhibiting cysteine proteases and papain-like cysteine proteases [17–19]. As suicidal protease inhibitors, serpins possess a unique structure comprising three β-sheets and 7–9 α-helices folded into a conserved tertiary structure. A reactive center loop (RCL) is located near the carboxy terminus and is exposed on the surface of the serpin, acting as bait for target proteases. When a protease cleaves the RCL between the P1 and P1′ amino acid residues, the serpin undergoes a significant conformational change, trapping the protease and distorting its active site, leading to the irreversible inactivation of the protease and thus inhibiting the proteolytic cascade and immune response [14,20]. Given their crucial role in immune regulation, insect serpins have garnered significant attention from researchers worldwide.

In insects, serpins primarily suppress the serine protease cascade, thereby negatively regulating the Toll signaling pathway and the prophenoloxidase (PPO) activation pathway, preventing the overexpression of antimicrobial peptides (AMPs) and excessive melanization, which could be harmful to the insect itself [21,22]. Early studies on the structure and function of serpins focused on model insects such as *Drosophila melanogaster* and non-model insects like *Manduca sexta* and *Tenebrio molitor* [23–25]. For instance, *Drosophila* Spn27Ac effectively inhibits PPO activation, thereby blocking the host's melanization process [23]. In *T. molitor*, SPN40, SPN55, and SPN48 bind to endogenous serine proteases MSP, SAE, and SPE, respectively, to negatively regulate the Toll signaling pathway and defend against pathogen invasion [24]. With the rapid advancements in omics and bioinformatics technologies, numerous *serpin* genes have been identified across various insect genomes [9,26,27]. However, there has been no systematic identification or functional research on serpins in rice planthoppers. Therefore, this study aims to investigate the evolutionary characteristics and functional roles of serpins in rice planthoppers by examining the homology and differences among *serpin* genes in three planthopper species.

2. Materials and Methods

2.1. Identification of Serpins in Three Rice Planthopper Species

This study utilized the complete genome data of *N. lugens* (Accession No.: GCF_014356525.2) from the National Center for Biotechnology Information (NCBI) database, and the complete genome data of *S. furcifera* (Accession No.: IBG_00709) and *L. striatellus* (Accession No.: IBG_00477) from the InsectBase 2.0 database [28]. The RCL domain of the serpin family (Pfam protein family database; Accession No.: PF00079) was used as a reference for HMMER 3.3 software to identify candidate serpin protein sequences from the proteome data of the three rice planthopper genomes. High-confidence sequences (E-value $< 1 \times 10^{-20}$) were selected as candidates. These sequences were further analyzed using the Pfam database to confirm conserved structures, and any sequences lacking complete *serpin* domains or exhibiting alternative splicing were manually curated to finalize the *serpin* gene members in the three planthopper species.

2.2. Prediction of Basic Information and Physicochemical Properties of Serpin Genes

The *serpin* genes from the genomes of *N. lugens*, *S. furcifera*, and *L. striatellus* were identified using the HMMER software (V3.3.2). A total of 11 *serpin* genes were identified in *N. lugens*, 11 in *S. furcifera*, and 14 in *L. striatellus* following the screening process (Tables 1–3). The online tool ExPASy was used to predict the amino acid composition, molecular weight, isoelectric point, and instability coefficient of the serpin proteins from the three rice planthopper species [29].

Table 1. Identification and characteristics of Serpin in *Nilaparvata lugens*.

Gene Name	Genome Identifier	Locus			CDS Length (bp)	Exon	Strand	Amino Acid (aa)	Molecular Weight (kDa)	Isoelectric Point	Instability Index
		Chromosome	Starting	Ending							
NlSerpin1	LOC111050206	Chr1	4722696	4757716	1272	8	-	400	44.94	9.12	28.64
NlSerpin2-1	LOC111058361	Chr1	71842308	71904797	1203	8	+	448	50.76	5.55	30.71
NlSerpin2-2	LOC111053438	Chr1	71931505	72030410	1347	8	+	465	52.89	5.81	32.15
NlSerpin3	LOC111053077	Chr1	90407916	90425172	1398	8	-	423	47.72	6.28	36.7
NlSerpin4	LOC111052989	Chr4	60771023	60876331	4533	6	-	1510	164.24	5.59	42.85
NlSerpin5	LOC111056094	Chr6	43941108	43955672	1518	5	-	505	56.56	8.11	44.73
NlSerpin6	LOC111054393	Chr7	34067080	34091623	1170	7	-	389	42.56	6.56	36.04
NlSerpin7-1	LOC111051915	Chr7	62939055	62961696	3141	8	-	548	61.77	4.91	43.19
NlSerpin7-2	LOC111058203	Chr7	62996589	63039442	1647	16	-	1046	116.86	7.2	43.49
NlSerpin8	LOC111056876	Chr13	7370951	7410196	2652	8	+	422	47.59	6.47	39.2
NlSerpin9	LOC111050465	Chr13	27064128	27131234	1269	8	-	883	95.50	4.58	48.6

Table 2. Identification and characteristics of Serpin in *Sogatella furcifera*.

Gene Name	Genome Identifier	Locus			CDS Length (bp)	Exon	Strand	Amino Acid (aa)	Molecular Weight (kDa)	Isoelectric Point	Instability Index
		Chromosome	Starting	Ending							
SfSerpin1	Sfur013897	chr1	2544303	2554594	1203	8	-	400	44.79	6.81	30.26
SfSerpin2-1	Sfur012619	chr1	8353090	8403086	2943	22	+	980	110.23	6.95	38.99
SfSerpin2-2	Sfur012518	chr1	8403987	8412046	420	4	+	139	15.61	9.79	52.16
SfSerpin3	Sfur013091	chr1	56817028	56830743	1272	8	-	423	47.93	6.38	38.22
SfSerpin4	Sfur011222	chr4	16278256	16412744	4536	6	+	1511	164.77	5.79	44.7
SfSerpin5	Sfur010406	chr6	30205975	30219874	1455	5	-	484	54.42	6.84	44.71
SfSerpin6	Sfur014604	chr7	5474082	5479646	846	5	+	281	31.06	8.67	35.27
SfSerpin7-1	Sfur014985	chr7	9996532	10003239	1623	7	-	540	61.17	4.81	40.07
SfSerpin7-2	Sfur015145	chr7	10018567	10048125	3204	16	-	1067	119.16	6.95	44.99
SfSerpin8	Sfur000384	chr13	11346342	11374632	1266	8	-	421	47.22	6.27	42.51
SfSerpin9	Sfur000122	chr13	15369699	15401964	2448	8	-	815	87.96	4.55	45.42

Table 3. Identification and characteristics of Serpin in *Laodelphax striatellus*.

Gene Name	Genome Identifier	Locus			CDS Length (bp)	Exon	Strand	Amino Acid (aa)	Molecular Weight (kDa)	Isoelectric Point	Instability Index
		Chromosome	Starting	Ending							
LsSerpin1	Lstr009525	Chr1	2369596	2381755	1203	8	-	400	45.10	6.34	37.35
LsSerpin2	Lstr010687	Chr1	12936772	12947697	1272	7	+	423	47.50	5.57	31.05
LsSerpin3	Lstr011347	Chr1	24993437	24999073	831	5	+	276	30.24	8.3	37.68
LsSerpin4	Lstr023739	Chr4	7984498	8058486	4548	6	+	1515	165.31	5.62	43.1
LsSerpin5-1	Lstr000153	Chr7	15780212	15809264	3120	16	+	1039	116.09	6.84	42
LsSerpin5-2	Lstr000801	Chr7	15825525	15835540	1614	8	+	537	60.63	4.85	39.22
LsSerpin6	Lstr001530	Chr7	27426245	27432731	519	4	+	172	18.74	8.48	44.17
LsSerpin7	Lstr016984	Chr13	6171816	6205344	1266	8	+	421	47.09	6.28	43.64
LsSerpin8	Lstr018277	Chr13	11210729	11230271	2373	6	+	790	84.94	4.54	50.92
LsSerpin9	Lstr017410	Chr13	12958370	12971973	1932	6	-	643	67.89	4.58	48.03
LsSerpin10	Lstr038401	WOVE01021936.1	1	1014	639	1	-	212	23.86	6.52	49.59
LsSerpin11	Lstr041904	WOVE01037137.1	92247	100048	1497	4	-	498	55.47	8.24	46.46
LsSerpin12	Lstr014327	WOVE01037432.1	4601	15631	1218	9	-	405	45.50	8.64	36.34
LsSerpin13	Lstr038579	WOVE01037535.1	18312	39817	1224	7	+	407	46.50	5.4	22

2.3. Construction of a Phylogenetic Tree for Serpins

Serpin protein sequences from *Aedes aegypti*, *Solenopsis invicta*, *Bemisia tabaci*, *Bombyx mori*, *D. melanogaster*, and *Tribolium castaneum* were sourced from the NCBI database. Sequence alignment was performed using the MAFFT software (V7.5.2) [30], followed

by phylogenetic analysis with the IQ-tree software (V2.2.2.9) [31] using the maximum likelihood method (ML), with 1000 bootstrap replicates and the genetic distance model Q.yeast+R5. The phylogenetic tree was refined and visualized using the Interactive Tree Of Life (iTOL) online tool [32].

2.4. Chromosome Localization and Synteny Analysis of the Serpin Family

Genome sequence annotation files (gff) were used in the "Gene Location" module of TBTools software (2.119) [33] to map the identified *serpin* genes onto chromosomes and to create chromosome localization maps for the *serpin* genes across the three rice planthopper species. The "One Step MCScanX" module of TBTools [34], integrated with the MCScanX (V1.0) and OrthoFinder software (V2.5.5) algorithms [35], was utilized to identify syntenic gene pairs and tandem repeat genes within the same chromosome at distances less than 100 kb. The "Multiple Synteny Plot" module of TBTools was employed to visualize these results.

2.5. Analysis of Conserved Domains and Motifs

The serpin family gene sequences from the three rice planthopper species were analyzed for the conserved RCL domain using the Pfam database. MEME software (V5.5.7) was utilized to identify conserved motifs within the protein sequences of the serpin family, with parameters set to discover up to 10 motifs without specifying the number of repetitions [36]. The results were synthesized and visualized using the "Gene Structure View" module of TBTools.

2.6. Expression Analysis of the Serpin Family Genes

RNA-seq data relevant to the developmental stages and tissues of the three rice planthopper species as of 1 August 2024 were collected from the NCBI SRA database. The datasets include 15 developmental stage samples for *N. lugens* (BioProject PRJNA714229); 17 tissue samples for *N. lugens* (BioProjects PRJNA714229 and PRJNA838417); 26 developmental stage samples for *S. furcifera* (BioProjects PRJNA681674 and PRJNA835226); 12 tissue samples for *S. furcifera* (BioProject PRJNA987195); three developmental stage samples for *L. striatellus* (BioProject PRJNA393384); 12 tissue samples for *L. striatellus* (BioProject PRJNA841412).

Kallisto software (V0.46.2) was used to quantify mRNA expression levels [37], and the expression profiles of the serpin family genes were visualized based on gene TPM (transcripts per million) values. Expression patterns across different developmental stages and tissues were analyzed, with TPM values log2 transformed and normalized by gene. The results were presented as a heatmap using the "Heatmap" module of TBTools.

2.7. Fungal Culture and Infection

M. anisopliae (MaqS1902) were obtained from the Laboratory of Insect Microbiology and Biotechnology, Bahauddin Zakariya University, Multan, Pakistan. These fungi were cultivated on Potato Dextrose Agar (PDA) medium (200 g potatoes, 20 g dextrose, and 20 g agar diluted in 1 L distilled water) at a temperature of 25 ± 1 °C and a relative humidity (RH) of 75–80%.

Third-instar nymphs of three rice planthopper species were infected with *Metarhizium* at a sublethal concentration of 1×10^4 spores using an immersion method. A control group was immersed in spore germination liquid. Following the treatment, the planthoppers were cultured on rice plants for 2 days, after which the surviving planthoppers were collected. Total RNA was extracted from the collected planthoppers using the Trizol method, and cDNA was synthesized through reverse transcription. The relative expression levels of the serpin gene family were then quantified using qRT-PCR. The qRT-PCR was conducted on a Bio-Rad CFX96 real-time System (Bio-Rad, Hercules, CA, USA). The 20 µL reaction mixture consisted of 2 µL of cDNA template, 0.5 µL of each primer (10 µM), 10 µL of SYBR®Green Mix (Yeasen, Shanghai, China), and 7 µL of ddH_2O. The PCR was performed

using the following program: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s, concluding with a dissociation curve. The β-actin gene served as an internal control for the normalization of cDNA templates. The primers utilized for qRT-PCR are detailed in Supplementary Materials Table S1. Data were analyzed using the Ct method. Transcript abundances for each gene were measured in three independent samples, which were then compared by the *t*-test.

3. Results

3.1. Phylogenetic Analysis of the Serpins in Three Rice Planthopper Species

A phylogenetic tree was constructed to compare serpin sequences from *N. lugens*, *S. furcifera*, *L. striatellus*, and six other insects (Figure 1). Firstly, *N. lugens Nl*Serpin2-1/2-2, *L. striatellus Ls*Serpin2/13, and *S. furcifera Sf*Serpin2-1/2-2 clustered with most serpin genes of *D. melanogaster* (Dmel), forming an evolutionary branch (indicated by purple lines), particularly closely related to *Dmel*Serpin55B. Secondly, in the second evolutionary branch (indicated by black lines), *Ls*Serpin7, *Sf*Serpin8, and *Nl*Serpin8 gathered into one cluster, showing higher homology with *B. mori Bmor*Serpin6, while *Ls*Serpin10/11, *Sf*Serpin5, and *Nl*Serpin5 formed another branch, with higher homology to *Dmel*Serpin28Dc. Again, *Nl*Serpin1/3, *Sf*Serpin1/3, and *Ls*Serpin1/12 clustered with most serpin genes of *B. mori*, forming another evolutionary branch (indicated by yellow lines), with the highest homology to *Bmor*Serpin26. Additionally, *Ls*Serpin5-2, 5-3, 6, 4, 9, and 8, *Nl*Serpin7-1, 6, 4, and 9, and *Sf*Serpin7-1, 6, 4, and 9 formed another branch (indicated by green lines), among which *Nl*Serpin7-1, *Sf*Serpin7-1, and *Ls*Serpin5-2 showed higher homology with *Bmor*Serpin10. The *Serpin9s* of rice planthoppers showed higher homology with *Bmor*Serpin34 and *Dmel*Serpin85F. Lastly, *Ls*Serpin5-1, *Sf*Serpin7-2, and *Nl*Serpin7-2 gathered into a cluster, forming another evolutionary branch (indicated by red lines), clustering with some serpins of *Drosophila* and *B. mori*, and showing higher homology with *Bm*Serpin11/12. In general, the Serpins in rice planthoppers are evolutionarily closer to those of classical model insects such as *Drosophila* and *B. mori*, while they are more distantly related to the Serpins of non-model insects like *A. aegypti*, *S. invicta*, *B. tabaci*, and *T. castaneum*.

3.2. Chromosome Localization and Genomic Collinearity Analysis of Serpin Genes

After an in-depth analysis of the gene structure annotation files, the distribution of the serpin gene family members on the chromosomes of the three rice planthopper species was mapped using the TBTools software (V2.119). The results indicated that the *Serpin1*, *Serpin2*, and *Serpin3* gene families are clustered on the first chromosome, while the *Serpin4* family is uniquely located on the fourth chromosome. The *Serpin6* family members are evenly distributed on the seventh chromosome, and the *Serpin8* and *Serpin9* families are co-located on the thirteenth chromosome. Intriguingly, the *Serpin5* and *Serpin7* families in *N. lugens* and *S. furcifera* are located on the sixth and seventh chromosomes, respectively, whereas their homologs in *L. striatellus* are found on the seventh and thirteenth chromosomes (Figure 2A). The analysis of syntenic gene pairs among the three planthopper species, performed using MCScanX (V1.0) and TBTools (V2.119), revealed seven pairs of orthologous genes, consistent with the results of the phylogenetic analysis (Figure 2B).

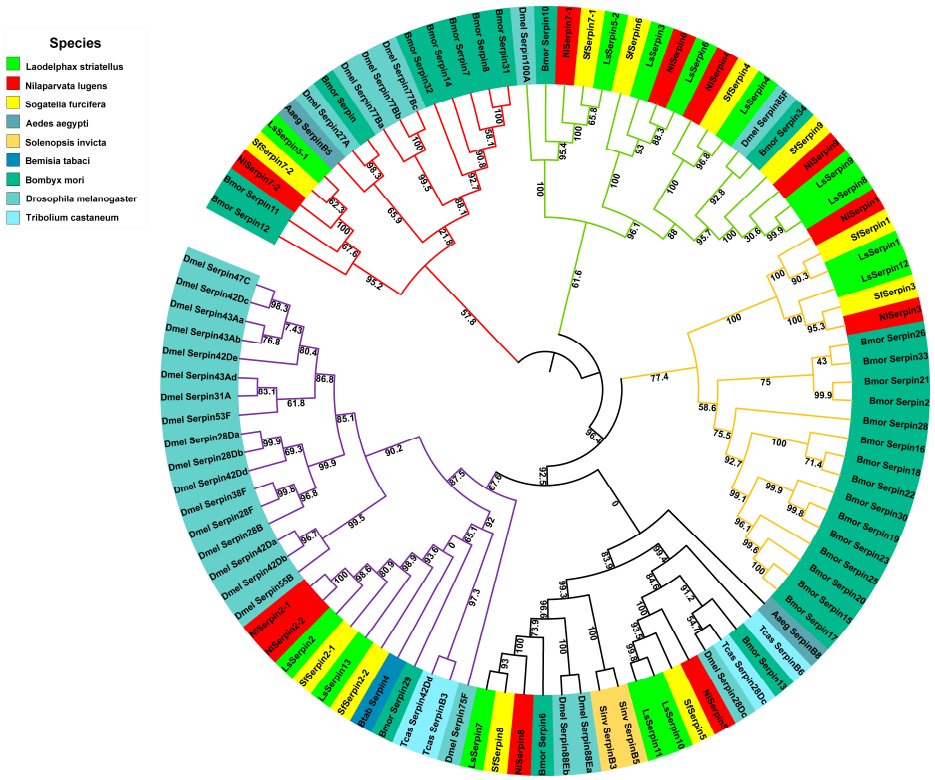

Figure 1. Phylogenetic tree of the Serpin family genes. Label of green markers represent *L. striatellus*, the red markers represent *N. lugens*, and the yellow markers represent *S. furcifera.* Different colored branches indicate different evolutionary branches.

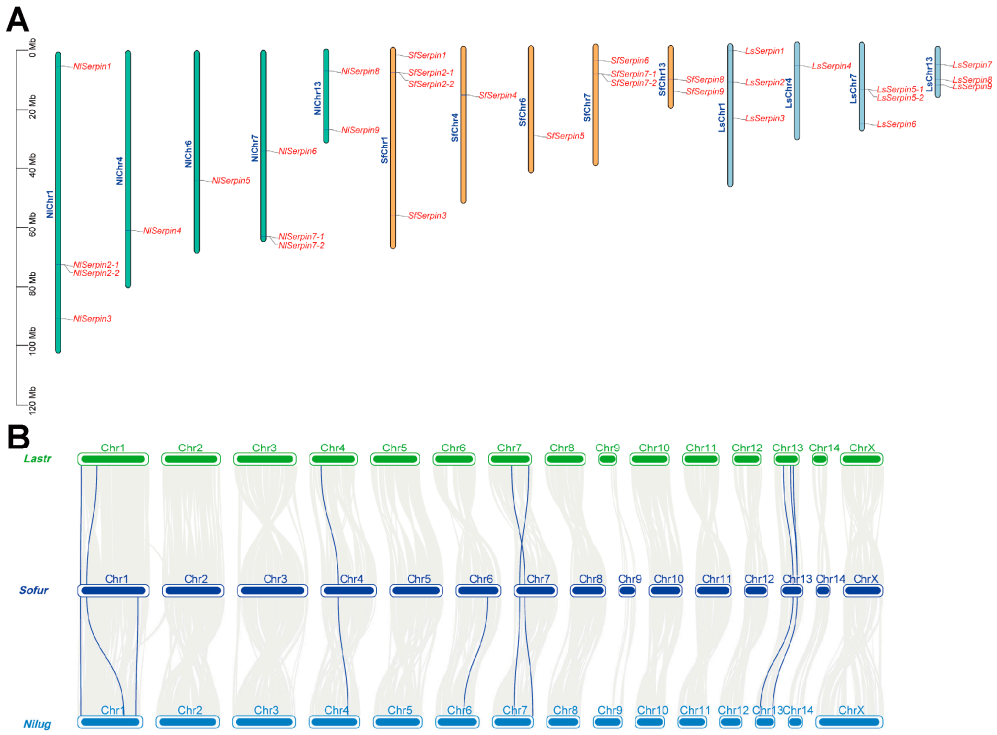

Figure 2. Chromosome mapping and genomic collinearity of the Serpin family genes in the three rice planthopper species. (**A**) Distribution of the Serpin family genes on the chromosomes, with green representing *N. lugens*, orange *S. furcifera*, and blue *L. striatellus*. (**B**) Genomic collinearity analysis among the three rice planthopper species, highlighting the homologous *serpin* genes across species.

3.3. Analysis of Conserved Domains and Motifs in the Serpin Gene Family

The serpin protein family is characterized by its RCL, which is essential for serpin protein activity and their interaction with target proteins. Most serpin proteins in the three rice planthopper species were found to contain at least one RCL. However, variations were observed in certain serpins from *L. striatellus*, which lack the RCL. Particularly, the Serpin7 subfamily members in all three planthopper species contain two RCLs and serpin domains, while the *L. striatellus Ls*Serpin2-2 has two very short serpin domains and RCLs (Figure 3).

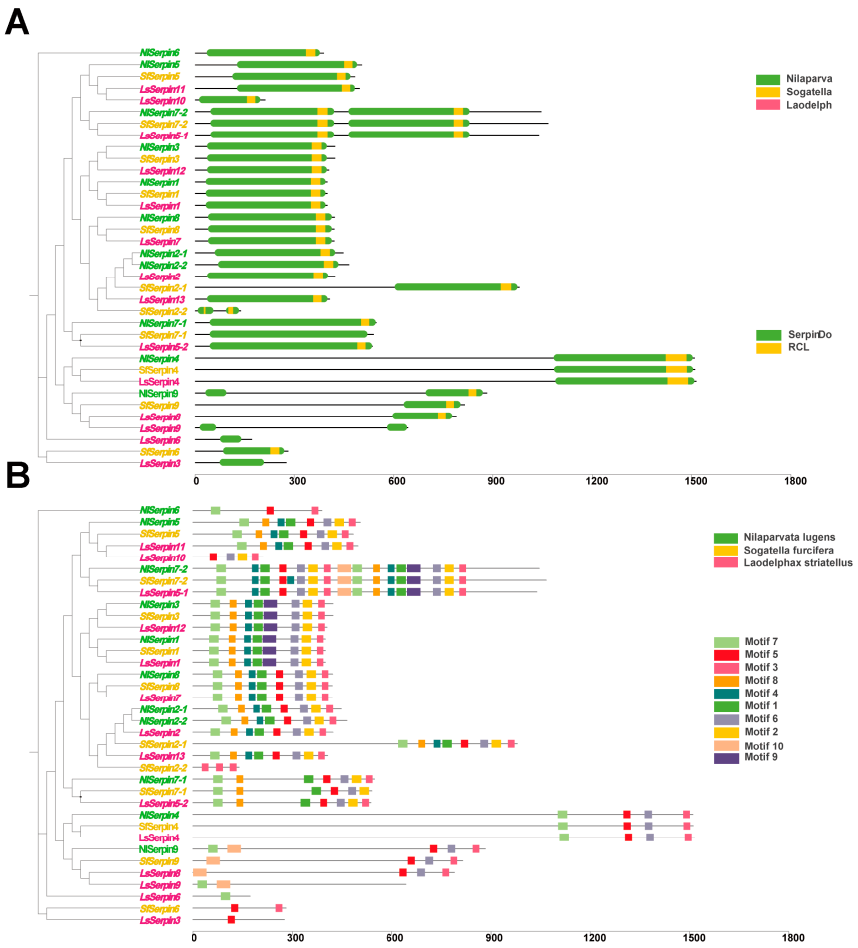

Figure 3. Motif patterns and conserved domains of the Serpin family genes in three rice planthopper species. (**A**) The conserved domains of the Serpin family genes. (**B**) The motif patterns of the Serpin family genes.

Motifs, which are short, functionally conserved sequences, may include specific binding sites or be involved in particular biological processes. In this study, motif analysis of the serpins from the three rice planthopper species revealed a diverse composition of motifs among the identified proteins. Notably, serpins from the same evolutionary branch share almost identical motif sequences (Figure 3). This diversity in motifs not only reflects the structural complexity of serpin proteins but also suggests a wide range of functions and adaptability.

3.4. Expression Analysis of Serpin Genes at Different Developmental Stages in Three Rice Planthopper Species

A comprehensive transcriptomic analysis across various developmental stages of the three rice planthopper species was performed, and the resulting gene expression dynamics were visualized using heatmaps. Notably, the distinctive expression profile of the *NlSerpin7-1* gene in *N. lugens*, which demonstrates significantly elevated expression levels during the

late embryonic phase, persists through all nymphal stages (Figure 4A). This high expression is striking compared to the relatively subdued levels observed for other *serpin* genes within the same developmental timeframe.

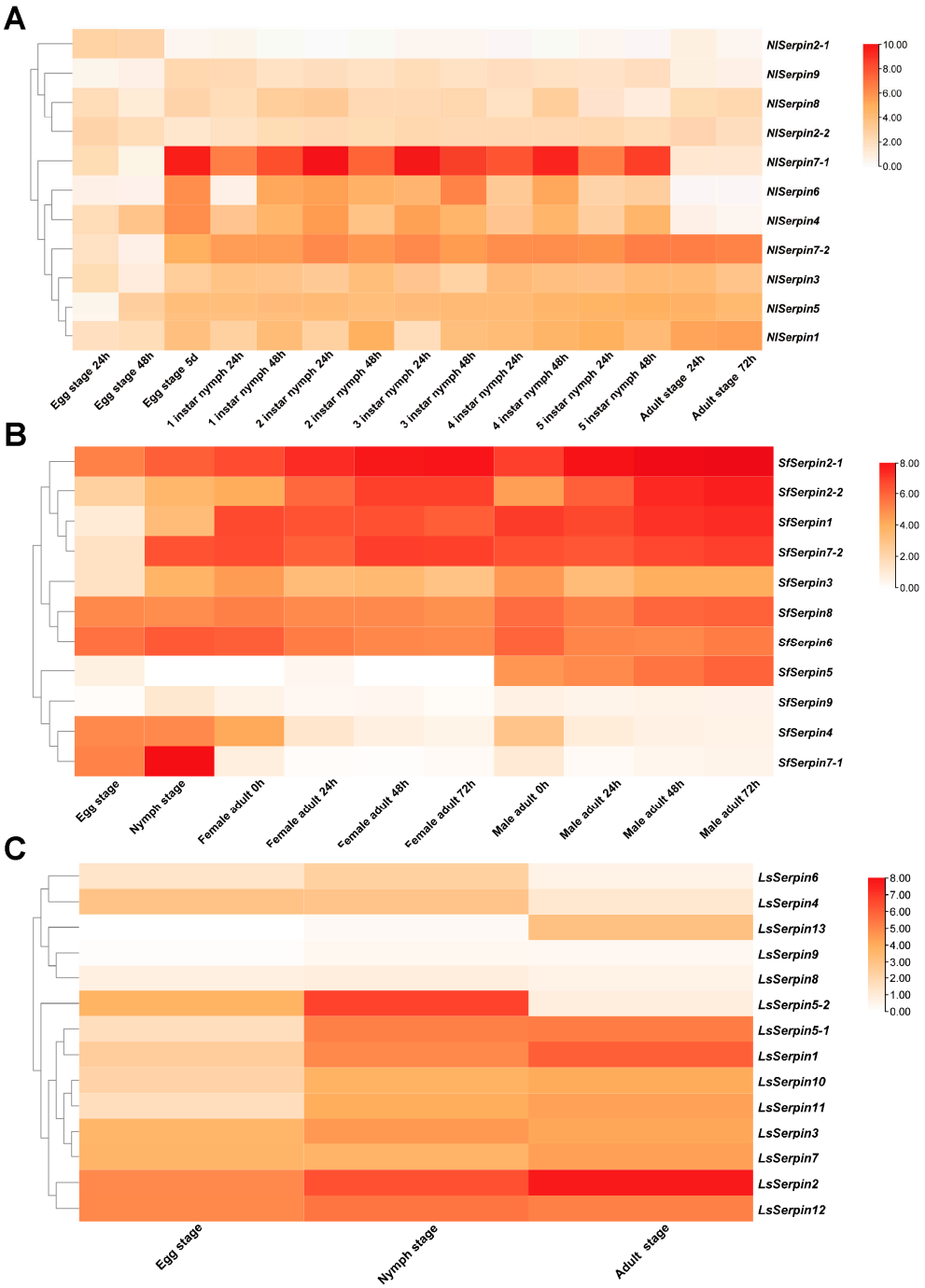

Figure 4. Heatmaps of Serpin family gene expression across different developmental stages in three rice planthopper species. (**A**) Heatmap of *serpin* genes expression in *N. lugens*; (**B**) Heatmap of *serpin* genes expression in *S. furcifera*; (**C**) Heatmap of *serpin* genes expression in *L. striatellus*.

In *S. furcifera*, the *SfSerpin2-1* and *SfSerpin2-2* genes exhibit a gradual increase in expression as the insect matures. However, *SfSerpin2-2* displays an abrupt decline in expression precisely at the 0-h mark in adult males, a fluctuation likely attributed to sample-to-sample variability. Moreover, *SfSerpin1* and *SfSerpin7-2* are characterized by significantly

heightened expression during the nymphal and adult phases compared to the embryonic phase, with *SfSerpin7-2* reaching peak expression during the nymphal phase (Figure 4B).

In *L. striatellus*, constraints in data acquisition have resulted in a more generalized collection of expression data across the embryonic, nymphal, and adult stages. Despite these limitations, discernible differences were observed in the expression profiles. Notably, the *SfSerpin7* gene does not show the elevated expression seen in the Serpin7 homologs of *N. lugens* and *S. furcifera*. Instead, *LsSerpin5-2* and *LsSerpin2* exhibit unique expression patterns—*LsSerpin5-2* shows a phase-specific surge during the nymphal stage, while *LsSerpin2* maintains high expression throughout both nymphal and adult stages (Figure 4C).

3.5. Expression Analysis of Serpin Genes in Different Tissues of Adult Rice Planthoppers

Transcriptome data from different tissues of adult rice planthopper species were analyzed, and heatmaps were generated to illustrate the expression patterns of *serpin* genes (Figure 5). In *N. lugens*, the *NlSerpin7-2* gene displayed notably high expression in the antennae and ovipositor (Figure 5A), suggesting a role in the sensory and reproductive functions of these tissues. In *S. furcifera*, a cohort of *serpin* genes—*SfSerpin3*, *SfSerpin8*, *SfSerpin7-2*, *SfSerpin2-2*, *SfSerpin2-1*, and *SfSerpin1*—exhibited a uniformly high expression across tissues, including the abdomen, antennae, legs, mouthparts, and thorax (Figure 5B). This pervasive expression signature implies a fundamental role for these genes in the broad physiological machinery of the white-backed planthopper. In contrast, *L. striatellus* displayed more varied expression patterns. For instance, *LsSerpin1* was highly expressed in the salivary glands, possibly linked to specialized functions in the feeding process, underscoring a potential role in digestion or interaction with plant defenses. Meanwhile, *LsSerpin2* showed robust expression in the gut and testes, and *LsSerpin13* had focused high expression in the testes, suggesting that these genes could be pivotal to the planthopper's reproductive health and digestive efficiency (Figure 5C). These tissue-specific expression profiles highlight the potential roles of *serpin* genes in sensory perception, reproductive development, and digestive processes of the three rice planthopper species.

3.6. Expression of Serpin Genes in Rice Planthopper Species Post-Infection with Metarhizium

To examine the response of rice planthopper to fungal infections, we analyzed the expression of *serpin* genes in *N. lugens* and *S. furcifera* two days after infection with the fungus *Metarhizium* (Figure 6). The analysis revealed that specific *serpin* genes in both planthopper species showed a significant upregulation in response to infection. Specifically, the *NlSerpin2-2* and *NlSerpin6* genes in *N. lugens*, along with the *SfSerpin4, SfSerpin6* and *SfSerpin7-1* genes in *S. furcifera*, as well as *LsSerpin4*, *LsSerpin5-2*, and *LsSerpin6* in *L. striatellus* exhibited significantly higher expression levels 2 days post-infection (dpi) compared to the control group, suggesting the potential roles of these genes in antifungal defense, particularly the *Serpin6*, which may have a defensive effect against a variety of fungi within the rice planthopper family. Conversely, a subset of genes, including the *NlSerpin1/7-1/9* in *N. lugens* and the *LsSerpin1/2/3/8/13* in *L. striatellus*, displayed a significant downregulation in expression levels at the two-day mark post-infection. This reduction suggests that these genes might play an inhibitory role in modulating the planthoppers' immune responses to fungal invasion.

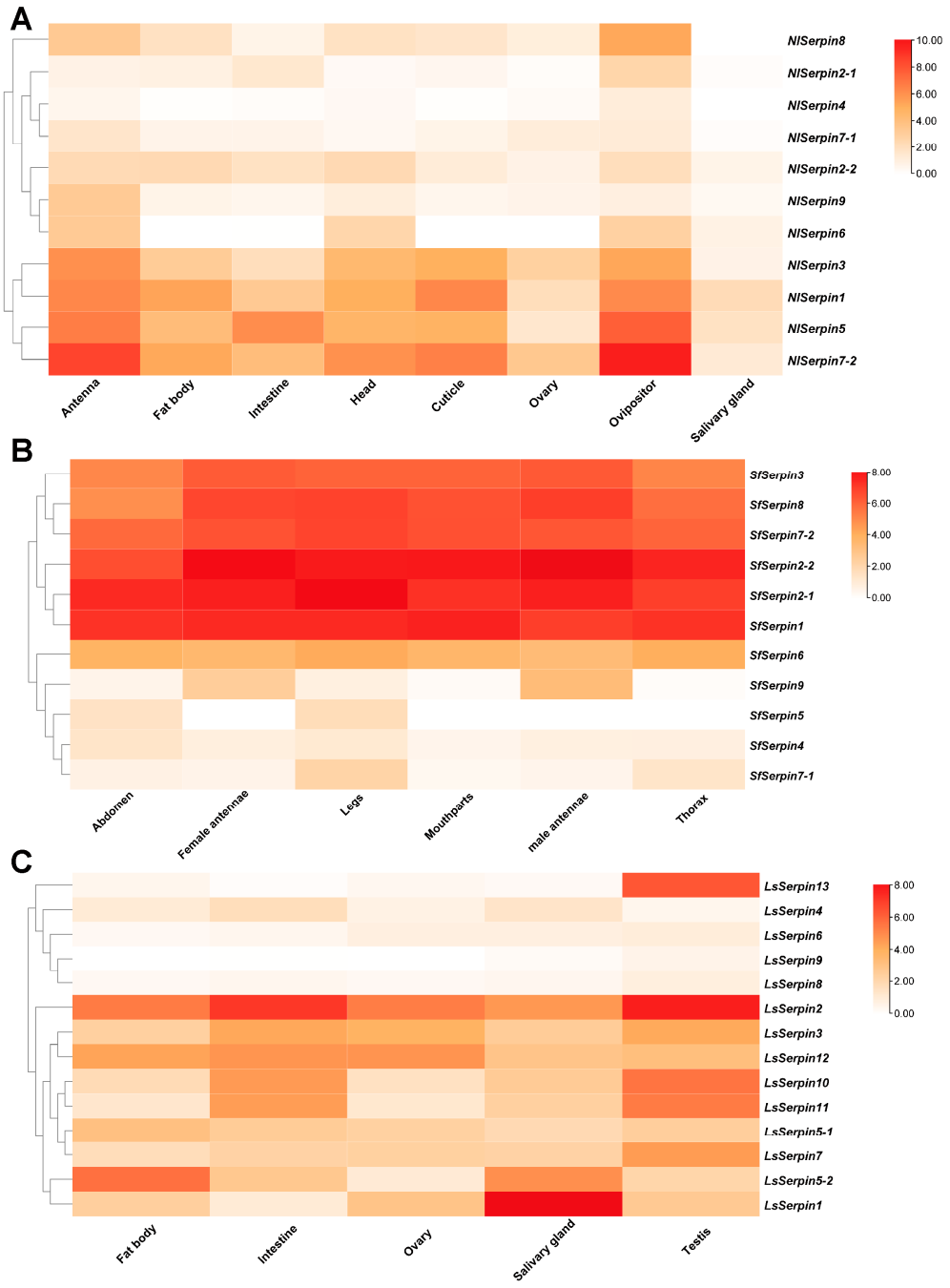

Figure 5. Heatmaps of *serpin* gene expression in various adult tissues of the three rice planthopper species. (**A**) Expression heatmap of *serpin* genes in *N. lugens*; (**B**) Expression heatmap of *serpin* genes in *S. furcifera*; (**C**) Expression heatmap of *serpin* genes in *L. striatellus*.

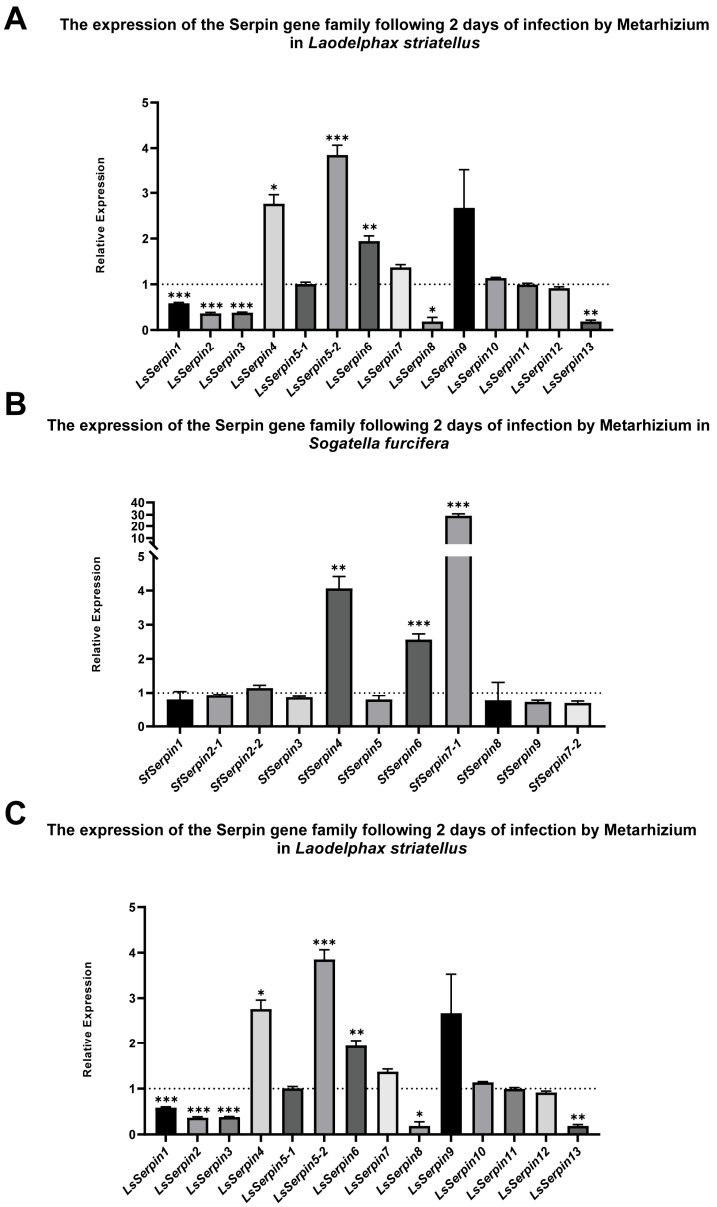

Figure 6. Expression of *serpin* genes in rice planthoppers post-infection with *Metarhizium*. (**A**) Expression of *serpin* genes 2 days post-infection in *N. lugens*; (**B**) Expression of *serpin* genes 2 days post-infection in *S. furcifera*; (**C**) Expression of *serpin* genes 2 days post-infection in *L. striatellus*. Transcript abundances for each *Serpin* family member were measured in three independent samples, which were then compared by the *t*-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4. Discussion

Serpins constitute a relatively large superfamily of proteins, typically ranging from 350–500 amino acids in length and with molecular weights of approximately 45–50 kDa, and are renowned for their role as protease inhibitors [14]. In this study, we conducted an in-depth statistical and analytical survey of the genomes of three rice planthopper species—*N. lugens*, *S. furcifera*, and *L. striatellus*—identifying 11, 11, and 14 *serpin* genes, respectively. Phylogenetic analysis revealed that the *serpin* genes from these rice planthoppers are more closely related to those of the silkworm (*B. mori*) across various branches. This close relationship may be due to similar ecological pressures and selective environments faced by these insects during their evolutionary process, leading to the conservation of similar *serpin* genes for essential biological functions [38].

The isolation and identification of serpins from invertebrates, particularly insects, began with the silkworm *B. mori* [39] and the tobacco hornworm *M. sexta* [40]. To date, a significant number of serpins have been identified in the genomes and transcriptomes of invertebrates, especially in *Lepidopteran* insects. For instance, 34 *serpin* genes have been reported in the silkworm [41] and 32 in the tobacco hornworm [27]. Additionally, our previous research in *Plutella xylostella* identified 28 *serpins* [42]. Although *Dipteran* insects also contain a considerable number of *serpin* genes, there is a significant variation in the number of genes among different species, with 31 identified in *Culex quinquefasciatus* and 18 in *Anopheles gambiae* mosquitoes [43]. The functions of many serpin homologs in the silkworm remain largely undefined, indicating the need for further research into the roles of serpins in rice planthoppers.

Structural analysis further confirmed that serpin proteins from these three rice planthoppers all contain the RCL, a key region for their interaction with and regulation of target proteins [44]. Notably, members of the Serpin7 subfamily in all three planthoppers contain two RCLs and two serpin domains, similar to the dual-domain serpin protein SPN93 in *D. melanogaster*, which interacts with two proteins in the Toll signaling pathway [45]. This structural feature may have evolved to enhance the versatility of these serpins in regulating multiple proteolytic events, which could be crucial for the complex life cycles and diverse ecological adaptations observed in these insects [27].

Gene structure analysis shows that all *serpin* genes in rice planthoppers possess unique motifs, with members on the same evolutionary branch sharing nearly identical motif sequences. Differences in gene structure may arise from evolutionary events such as exon and intron loss or insertion, which play a significant role in adaptive processes [46]. The presence of multiple RCLs in certain *serpin* subfamilies and differences in gene structure may provide important clues to the functional diversity and evolutionary adaptation of *serpins* in rice planthoppers, further emphasizing the need for in-depth functional characterization of these genes.

The diversity and structural differences in *serpin* genes in rice planthoppers may be due to a variety of factors. First, different ecological niches and environmental pressures may have driven the rapid evolution and diversification of these genes to adapt to changing environmental conditions and defend against various predators or pathogens [47]. For example, rice planthoppers may require different serpins to modulate their immune response when facing attacks from predators and pathogens, thereby enhancing their survival capabilities. Second, gene duplication events may provide the raw material for the emergence of new functions, which may involve the inhibition of different proteases, thereby enhancing the adaptive capacity of rice planthoppers to specific ecological challenges [48]. In addition, gene recombination and mutation may also be important factors leading to structural and functional differences among members of this gene family [49]. These factors work together to shape the diversity and complexity of *serpin* genes in rice planthoppers, enabling them to cope with complex ecological challenges.

In insects, serpin proteins are extensively involved in the regulation of immune responses, including the melanization reaction controlled by the important immune pathways Toll and PPO [50]. The Toll signaling pathway, primarily activated by Gram-positive bacteria and fungi, induces the production of AMPs and other immune-active substances that target and eliminate pathogens [50]. The PPO pathway, which drives melanization, plays a crucial role in isolating and killing pathogens, with some overlaps between the Toll and PPO pathways [51]. The functions of serpin proteins in insect immune regulation include: (1) inhibiting the serine protease cascade in the PPO pathway to regulate AMP production in innate immunity; for example, *Bm*Serpin4 in *B. mori* and *Ms*Serpin1J in *M. sexta* inhibit hemolymph proteinase, affecting the activation of proSpaztle and AMP expression [44,52]; (2) regulating melanization in insect hemolymph by inhibiting the serine protease cascade in the PPO pathway; for instance, *BmSerpin6* in *B. mori* and *Dm*Serpin27A in *D. melanogaster* negatively regulate melanization by inhibiting the activity of phenoloxidase-activating enzyme [53,54]; (3) serving as acute-phase proteins, which are rapidly upregulated in

response to pathogen infection or tissue damage; for example, the SRPN10 protein is highly expressed in midgut epithelial cells of *A. gambiae* upon parasite infection [55]. Although serpin genes typically show low expression in insect hemolymph under normal conditions, they are notably upregulated in response to immune stimulation. For instance, *Serpin-2*, *Serpin-3*, and *Serpin-6* in *M. sexta* are expressed at low levels in larval hemolymph but exhibit significant upregulation following pathogen exposure [24,56]. Here, we observed the overexpression of *NlSerpin2-2* and *NlSerpin6* genes in *N. lugens*, *SfSerpin4*, *SfSerpin6* and *SfSerpin7-1* genes in *S. furcifera*, and *LsSerpin4*, *LsSerpin5-2*, and *LsSerpin6* in *L. striatellus* post-infection with *Metarhizium* (Figure 6). The upregulation of these genes may help to enhance the immune response of rice planthoppers by inhibiting the protease activity of pathogens, thereby limiting pathogen growth and spread. Conversely, the downregulation of some other Sepins (*NlSerpin1/7-1/9* and *LsSerpin1/2/3/8/13*) may suggest that these genes play an inhibitory role in regulating the immune response, or in some cases, they may also be involved in the regulation of excessive immune responses to avoid damage to the host. This dual regulation likely represents a strategy for rice planthoppers to maintain immune balance in the face of fungal infections [57]. Notably, the *Serpin6* subfamily members, which are evolutionarily conserved, exhibited enhanced immune activity in response to fungal invasion, further highlighting the complex role of Serpins in regulating the antifungal immune responses in rice planthoppers and emphasizing their significance in host–pathogen interactions. Moreover, in *B. mori*, Serpin6 has been shown to regulate the activity of prophenoloxidase and the expression of antimicrobial proteins [58], indicating that Serpin6 may play a broad and important role in insect immune defense. Our research results not only enhance the understanding of the regulation of immune responses in rice planthoppers but also provide potential molecular targets for the development of new pest management strategies based on RNA interference.

5. Conclusions

This study provides a comprehensive analysis of *serpin* genes in rice planthoppers, revealing their crucial roles in immune responses and development. Through genomic and bioinformatics approaches, we identified a varying number of *serpin* genes across *N. lugens*, *S. furcifera*, and *L. striatellus*, with a close evolutionary relationship to *B. mori* BmSerpins. Structural analysis confirmed the presence of the RCL in all serpin proteins, with unique configurations observed in the Serpin7 subfamily. Expression profiling revealed serpin-specific expression patterns in rice planthoppers that vary across developmental stages and tissues. After infection with *Metarhizium*, experimental validation further uncovered the immune regulatory roles of these serpins. Some serpin genes are upregulated to enhance antifungal capabilities, while others are downregulated, potentially suppressing immune responses. The findings underscore the serpin-mediated immune regulation as a complex and species-specific mechanism, offering insights into host–pathogen interactions. This knowledge could pave the way for developing novel pest control strategies, such as utilizing *Metarhizium* as a biopesticide, thereby reducing reliance on chemical pesticides and promoting sustainable agriculture. Future research should focus on the specific functions of these serpin genes and their interactions with other immune-related genes to further strengthen the defense mechanisms of rice planthoppers.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14112630/s1; Table S1: All qRT-PCR primers.

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