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Article

Comprehensive Analysis of *subtilase* Gene Family and their Responses to nematode in *Trichoderma harzianum*

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Abstract: The subtilase family is the second largest family of serine proteases. Some fungi including *Trichoderma* species can capture and kill nematodes by secreting hydrolytic enzymes or toxins, among which serine proteases are important enzymes that allow fungi to infect nematodes. Subtilase can degrade nematode and insect body walls. In this study, subtilase family genes were identified from the *Trichoderma harzianum* genome database, and bioinformatics analysis of the characteristics and evolutionary status of these genes, along with structural and functional analyses of their proteins, was performed. Gene structure analysis revealed that all the 41 subtilase genes contained introns, while some did not have upstream or downstream regions. Chromosome localisation showed that subtilase family members were unevenly distributed in 22 *Trichoderma* chromosomes, and 3 clusters were present, indicating that they may be hot spots of subtilase genes. Conserved motif analyses showed these proteins contained a commonly conserved motif, and motifs belonging to the same subfamily remained highly similar. The upstream region of the subtilase genes were enriched with different type and numbers of cis-elements, indicating that subtilase genes are likely to play a role in the response to diverse stresses. Transcription of 31 genes was increased after 5 days of infection with nematodes, whereas that of 10 genes decreased. In these subtilase genes, ThSBT4, ThSBT5, ThSBT12, ThSBT27, ThSBT34, ThSBT35, ThSBT38, and ThSBT40 showed significantly upregulated expression with a log2 fold change value of more than 4, and ThSBT35 showed the highest peak. These results laid a theoretical foundation for further research on the function of the subtilase genes and the mechanism of the resistance response.

Keywords: *Trichoderma harzianum*; Subtilase genes; Bioinformatics analysis; Nematode.

1. Introduction

Global agricultural losses caused by plant parasitic nematodes are as high as US$157 billion each year[1]. The use of chemical pesticides to control nematodes has been causing serious damage to the environment, whereas the use of fungi to control them is relatively less toxic and environmentally friendly, thus making fungi an effective means to achieve green prevention and control[2]. Microorganisms can attack and kill nematodes through diverse processes such as capturing, parasitising, and producing toxins and enzymes[3]. Extracellular enzymes

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including serine proteases, chitinases, and collagenases are important virulence factors that can degrade the main chemical constituents of the nematode cuticle and eggshell[4][5][6]. In 1990, Lopez-Llorca LV identified the first pathogenic-associated serine protease P32 from *Pochonia suchlasporia*[7]. Subsequently,serine proteases PII were isolated and identified from the nematode-trapping fungus *Arthrobotrys oligospora*[8]and the nematode-parasitic fungus *P. chlamydosporia*[9]. Currently, more than 20 pathogenic serine proteases (such as pSP-3[10], Ver112[11], PrC[12][13][14], Csp1[15], Hnsp[16]) have been isolated from different nematode fungi. RB Wang et al.[17] showed that the crude form of a protease obtained from the nematode fungus *Dactylella shizishanna* killed nematodes more efficiently than the purified form. This observation indicates the osmotic dissolution process requires the synergy of several different enzymes including collagenase, chitinase, and serine protease to act against the nematode epidermis and eggshell that are made up of complex components. M. K. Jashni reported that the synergistic action of a metalloprotease and a serine protease obtained from *Fusarium oxysporum* enhances fungal virulence[18].

Subtilases are a family of subtilisin-like serine proteases, which is the second-largest serine protease family characterised to date. Over 200 subtilases are presently known and the complete amino acid sequences of more than 170 have been documented[19]. The vast majority of these proteases are endopeptidases, exopeptidases, and tripeptidases[20][21]. The subtilase superfamily includes 3 families of MEROPS peptidase family S8 (subfamily S8A, subtilase), S8B (Kexin), and S53 (sedolisin). The subtilase family Peptidase_S8 (PF00082) is characterised by Asp/His/Ser catalytic triplets and α/β-sheet catalytic centres containing seven-chain parallel β-sheets[22]. Subtilase is an important virulence factor and pathogenic factor found in different host fungi involved in biological stimulation[23]. The wide distribution of the serine cuticle-degrading protease found in nematophagous fungi suggested that this enzyme exhibits considerable nematicidal activity for balancing nematode populations[24]. Subtilase can destroy nematode and insect body walls and also plays an important role in invading plant walls[25]. Guo Qiannan performed omics analysis to determine the infection mechanism of the nematode fungus *Drechmeria coniospora* and found that subtilase, metalloproteinase, and acid

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phosphatase played an important role in the process of degrading the nematode body wall and invading it. Mass spectrometry results revealed that merimycin is a secondary metabolite of non-ribosomal polypeptides containing unconventional amino acid analogues (AIB, AHMOD, and AMD) that exhibits nematicidal activity. *D. coniospora* has 26 subtilases exhibiting nematicidal activity, 13 of which belong to the subtilase Pr1C subfamily[26]. *Clonostachys rosea* can parasitise nematodes and insects[27][28]. The low-molecular-weight degradation products of the nematode cuticle could significantly induce the expression of a subtilase extracellular protease prC[12]. Z Hao et al. amplified the gene sequence of the *P. oligospora* serine protease P186 and successfully expressed it in *Pichia pastoris*. The purified protease reP186 exhibited nematicidal activity[29]. In addition, subtilase genes obtained from the nematode-parasitic fungus *Purpureocillium lilacinum* formed a unique cluster, putatively indicating that the fungus might have developed distinctive mechanisms for nematode pathogenesis[30]. *Bacillus* sp. B16 exhibits significant nematode activity against the nematode *Panagrellus redivivus*. The crude extracellular protein extract of the culture supernatant killed approximately 80% of the tested nematodes within 24 h, indicating the involvement of extracellular proteases. The deduced protein sequence shows significant similarity to the sequence of subtilase BPN', and the purified protease can hydrolyse collagen and nematode epidermis[31]. L.H. Lian reported that the *Bacillus* sp. strain RH219 exhibits remarkable nematicidal activity, and an extracellular cuticle-degrading protease termed Apr219 served as an important nematicidal factor[32]. By performing whole-genome sequencing of *Bacillus firmus* DS-1, Geng, Ce et al. reported that this species exhibited high toxicity to root nematode and soybean cyst nematode. Sep1, a novel serine protease isolated from *B. firmus*, exhibits nematicidal activity and capability of degrading intestinal tissues of nematodes[33].Yang, Jinkui performed genomic sequencing on the nematode predatory fungus *Aspergillus oligogenome* (ATCC24927) and found that this species expresses numerous genes similar to the ones encoding subtilase, cellulase, cellobiohydrolase and pectinesterase families. By establishing a nematode-trapping device model, various fungal signal transduction pathways were found to be activated and downstream genes related to various cellular processes, such as energy metabolism, cell wall production, adhesion protein biosynthesis, cell division, and glycerol accumulation, and biogenesis using peroxisomes, were

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upregulated[34]. Subsequently, the crystal structure of proteases (Ver112 and PL646) from the nematode fungus and the chitinase CrChi1 were extensively studied, the active site residues were identified, and the catalytic mechanism of these enzymes in causing host infection was elucidated[35]. Muszewska, A. et al.[36] analysed the phylogenetic distribution of more than 600 fungal protein groups, and their results showed that of the 54 serine protease families described in the MEROPS peptidase database, 21 are found in fungi and most eukaryotic organisms encode members of the 13–16 serine protease family. Among the analysed taxa, the number of serine proteases in each taxon was different, and the most abundant of them was S8 protease. Jinkui Yang studied a cuticle-degrading serine protease that was cloned from three isolates of *Lecanicillium psalliotae* (syn. *Verticillium psalliotae*) by using the 3′ and 5′ rapid amplification of cDNA ends method. The deduced protease sequence exhibited a high degree of similarity to other cuticle-degrading proteases isolated from other nematophagous fungi[37].

Trichoderma species are used as biological control agents in many plant diseases and play an important role in plant protection by conferring resistance to nematodes and insects. They also maintain the dynamic balance of the microbial in the soil ecological environment. Several studies have shown that *Trichoderma* exerts a certain inhibitory effect on root-knot nematodes[38][39]. Studies conducted by Szabó et al. showed that genes encoding chitinase, serine protease, and aspartic protease in *Trichoderma harzianum* are expressed synergistically and play an important role in destroying nematode eggs[40]. *T. virens* has also been reported to produce growth-promoting agents and act as a biocontrol agent against *Meloidogyne incognita*[41]. The culture filtrate of *T. virens* G1-3 can inhibit the hatching of eggs of *M. incognita* and the activity of second-instar larvae (J2s)[42]. The crude extract from solid fermentation using the strain SMF2 (*T. pseudokoningii*) exhibits strong nematicidal activity against root-knot nematodes. The new serine protease SprT purified from this species can significantly shrink the epidermis of nematodes, kill nematode larvae, and inhibit egg hatching. Sequence analysis showed that SprT is a single-domain subtilase containing 284 amino acids[43]. Shuwu Zhang reported that *T. longibrachiatum* is a promising and highly effective antinematode biological control agent, and its conidia have a strong lethal and parasitic effect on *M. incognita*. It can

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attach to and parasitise the surface of second-instar larvae and penetrate the membrane to proliferate on the epidermis, leading to the partial dissolution of the epidermis by the metabolites of *T. longibranch*. In addition, *T. longibranch* can increase plant height, root length, and fresh weight of branches and roots of cucumber[44].

Recently, genome-wide identification and characterisation of the subtilase genes have been completed in some plants such as grapevine[45][46], tomato[47] and barley[48], which exhibit stress tolerance and disease resistance. However, the subtilase genes of *Trichoderma* that are involved in growth, development, and defence-related activities have not been extensively studied. Here we systematically characterised all 41 putative subtilase family genes of *Trichoderma* and performed an phylogenetic relationship analysis. In addition, gene structures, protein motifs, chromosomal location, and promoter were analysed. Furthermore, the expression patterns of the subtilase family genes in mycelial tissues were determined, whereas the differential expression of the subtilase genes was determined after nematode treatment by using RNA-seq analysis.

2. Results

2.1 Identification of the subtilase gene in T. harzianum

A total of 41 candidate subtilase genes were screened from the *T. harzianum* genome. On the basis of the conserved domain (Peptidase_S8peptidase-S8 domain), the genes were divided into 6 clusters and named ThSBT1 to ThSBT41. The parameter of the gene characteristics is summarised in Table 1, which includes chromosome position, exon number, protein length, protein molecular weight (MW), isoelectric point (pI), and predicted subcellular location information. The length of the protein encoded by these genes ranged from 708 to 1145 amino acids (aa), with an average aa length of 891. The MW ranged from 16974.26 to 45857.18, with an average MW of 23772.46. The pI values ranged from 4.2 to 6.66. According to the predicted subcellular data, ThSBT3–ThSBT9 and ThSBT35–ThSBT40 belonged to the category of secreted proteins. Those expressed in the cell wall included ThSBT10, ThSBT19, and ThSBT41. Those expressed in the cytoplasm included ThSBT1, ThSBT17–ThSBT21 (except ThSBT19), ThSBT26, and ThSBT33. Moreover, ThSBT14 and ThSBT22 were expressed in the chloroplast. ThSBT12

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and ThSBT15 were expressed in the mitochondria. ThSBT18 and ThSBT29 were expressed in peroxisomes. ThSBT23 and ThSBT34 were expressed in the chloroplast and peroxisomes. ThSBT2 and ThSBT28 were expressed on the cell membrane. ThSBT16, ThSBT25, and ThSBT27–ThSBT30 (except ThSBT28) were expressed in the nucleus. ThSBT24 was expressed in the membrane, cytoplasm, and nucleus.

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Table 1. Gene Message of subtilase gene in *Trichoderma harzianum*

Gene Name	Gene Identifier	Gene						Protein			
		Exon count	DNA LENGTH(bp)	Location Coordinates (5′–3′)	Scaffold	RNA LENGTH（bp）	CDS	Length(a.a.)	Mol.Wt.(kDa)	PI	Subcellular location
ThSBT1	M431DRAFT_494847	5	2001	605221..607221	6	1737	1..1737	578	62175	4.65	Cytoplasm
ThSBT2	M431DRAFT_98670	4	2023	247980..250002	21	1857	1..1857	618	66117	5.14	Membrane
ThSBT3	M431DRAFT_528472	5	2334	866124..868457	3	2036	141..2036	631	68517	4.93	Secreted protein
ThSBT4	M431DRAFT_154554	5	2226	254832..257057	21	1980	1..1914	637	67089	4.98	Secreted protein
ThSBT5	M431DRAFT_510269	1	1886	143846..145731	10	1886	37..1845	602	65372	5.34	Secreted protein
ThSBT6	M431DRAFT_110777	3	1586	3071034..3072619	3	1447	47..1276	409	42348	6.4	Secreted protein
ThSBT7	M431DRAFT_5561	4	1478	1492679..1494156	6	1302	1..1302	433	44778	5.92	Secreted protein
ThSBT8	M431DRAFT_522070	3	1370	1349423..1350792	9	1256	1..1227	408	43514	6.06	Secreted protein
ThSBT9	M431DRAFT_511032	3	1462	1032060..1033521	11	1332	19..1161	380	38901	5.65	Secreted protein
ThSBT10	M431DRAFT_149523	2	2131	1164822..1166952	11	2061	242..1861	539	57471	5.43	Cell wall
ThSBT11	M431DRAFT_497622	2	671	905662..906332	10	606	1..606	201	21614	6.83	Chloroplast; Peroxisome
ThSBT12	M431DRAFT_57616	2	818	1051682..1052499	1	750	1..750	250	27100	6.65	Mitochondrion
ThSBT13	M431DRAFT_61850	2	676	3368261..3368936	1	582	1..582	194	20675	5.14	Chloroplast; Peroxisome
ThSBT14	M431DRAFT_541862	2	921	605918..606838	15	846	1..846	281	77379	4.87	Chloroplast
ThSBT15	M431DRAFT_94513	5	3042	455050..458091	13	2772	1..2772	923	103132	5.9	Mitochondrion
ThSBT16	M431DRAFT_524534	7	3340	329636..332975	17	2584	86..2584	832	94265	5.59	Nucleus
ThSBT17	M431DRAFT_483258	4	3769	243309..247077	8	3561	1..3561	1186	132671	6.22	Cytoplasm
ThSBT18	M431DRAFT_501633	5	1197	114313..115509	31	930	1..930	309	34240	7.57	Cytoplasm
ThSBT19	M431DRAFT_129576	2	2826	10144..12969	35	2763	1..2763	920	97905	4.61	Cell wall
ThSBT20	M431DRAFT_494196	3	3094	1369677..1372770	5	2904	1..2904	967	109246	5.95	Cytoplasm
ThSBT21	M431DRAFT_71227	3	943	1060540..1061482	1	885	1..885	294	32514	6.21	Cytoplasm
ThSBT22	M431DRAFT_508157	3	3138	908782..911919	6	2925	98..2836	912	101078	6.61	Chloroplast
ThSBT23	M431DRAFT_99105	4	2863	187992..190854	22	2673	1..2673	890	100260	8.06	Peroxisome
ThSBT24	M431DRAFT_19399	7	6327	1008364..1014690	12	5468	1..5286	1761	199690	5.57	Cytoplasm; Nucleus; Membrane
ThSBT25	M431DRAFT_11340	4	2771	100648..103418	31	2586	1..2586	861	96449	5.14	Nucleus
ThSBT26	M431DRAFT_482677	6	2940	810930..813869	7	2505	1..2505	834	92427	6.32	Cytoplasm
ThSBT27	M431DRAFT_100793	3	2321	92388..94708	29	2187	1..2187	728	81830	7.64	Nucleus

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ThSBT28	M431DRAFT_93524	3	2307	546135..548441	12	2193	1..2193	730	82671	8.17	Membrane
ThSBT29	M431DRAFT_109420	4	3441	1321879..1325319	3	3233	61..3117	1018	113953	5.83	Nucleus
ThSBT30	M431DRAFT_98212	7	3276	215932..219207	20	2937	1..2937	978	110597	6.82	Nucleus
ThSBT31	M431DRAFT_546179	5	3226	204583..207808	22	2910	1..2910	969	109068	5.32	Nucleus
ThSBT32	M431DRAFT_513386	5	4119	237013..241131	22	3733	203..3640	1145	128477	6.43	Nucleus
ThSBT33	M431DRAFT_482039	3	2710	1213089..1215798	6	2562	1..2562	853	93894	5.15	Cytoplasm
ThSBT34	M431DRAFT_500340	11	2700	129882..132581	19	2127	1..2127	708	77249	4.87	Peroxisome
ThSBT35	M431DRAFT_116744	6	3176	77215..80390	7	2857	101..2857	918	97690	5.20	Secreted protein
ThSBT36	M431DRAFT_477752	3	2775	240477..243251	2	2637	1..2637	878	92443	6.84	Secreted protein
ThSBT37	M431DRAFT_133093	2	2859	370032..372890	2	2803	1..2523	840	88210	4.67	Secreted protein
ThSBT38	M431DRAFT_489009	2	2588	1029430..1032017	1	2526	1..2526	841	88218	4.88	Secreted protein
ThSBT39	M431DRAFT_89506	2	2939	1064723..1067661	8	2847	1..2847	948	101381	4.82	Secreted protein
ThSBT40	M431DRAFT_487795	2	2921	228375..231295	26	2856	1..2856	951	101346	4.75	Secreted protein
ThSBT41	M431DRAFT_520571	4	3104	245269..248372	7	2943	1..2739	912	97269	4.96	Cell wall

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2.2 Phylogenetic tree construction of subtilase family genes

Phylogenetic analysis was performed based on the relationship and length of all branches of the family members in the tree, wherein the proximity of different sequence associations showed the evolution origin process. We constructed the phylogenetic tree using 287 protein sequences of subtilase family members. The accession number of the gene identifier is listed in Table S1. The gene subfamily was divided by the phylogenetic tree branches and the annotation of the Peptidase_S8 domain in Pfam, NCBI, and SMART databases. By using the maximum likelihood algorithm, the bootstrap value of the evolutionary tree branch was found to be relatively high (95%). According to the phylogenetic tree shown in Figure 1, these sequences belong to 6 clusters. The annotation database information showed that they belong to the Peptidases_S8_S53 superfamily (cl10459), S8_S53 family (cd00306), and 5 subfamilies. ThSBT11–ThSBT19 belonged to the Peptidases_S8_S53 superfamily (cl10459). ThSBT20–ThSBT28 belonged to the S8_S53 family (cd00306). ThSBT1–ThSBT5 belonged to the Peptidases_S53 subfamily (cd04056). ThSBT6–ThSBT10 belonged to the Peptidases_S8_PCSK9_ProteinaseK_like subfamily (cd04077). ThSBT29–ThSBT32 belonged to the Peptidases_S8_7 subfamily (cd07491). ThSBT33 belonged to the subfamily Peptidases_S8_Protein_convertases_Kexi subfamily (cd04059). ThSBT41 belonged to the Peptidase S8 family, subfamily 5 (cd07489). Figure 1 specifically shows that the subtilase genes had different evolutionary origins, and members of the subfamily had highly conserved regions with similar functions. The number of members in the Peptidases S8_S53 subfamily was relatively large, whereas that in the Peptidases_S53 subfamily was relatively small. Compared with *Trichoderma* subspecies, most sequences were more closely related to the sequences found in *T. reesei*, *T. viride*, *T. guizhouense*, *T. virens*, *T. atroviride*, and *T. lentiforme*, while ThSBT9, ThSBT20, ThSBT22, ThSBT23, ThSBT26, ThSBT30–ThSBT32, and ThSBT35 were closely related to nematicidal fungal proteins.

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Figure 1. Molecular phylogenetic tree of 287 sequences from the subtilase family. The tree is constructed by MegaX 7 software with the maximum likelihood method.

2.3 Gene structure analysis of T. harzianum subtilase family genes

GSDS was used to display the intron/exon structure of the subtilase family genes in *T. harzianum* (Figure 2). Supplementary Table 2 includes the information on the sequence length of each gene. The results showed that the CDS sequence length of these genes ranged between 582 and 5286 kb, with an average sequence length of 2217 kb, of which ThSBT13 was the shortest and ThSBT24 was the longest. The number of CDS regions in these genes was between 2 and 11, and most genes had 3 to 4 CDS regions. Those with upstream regions included ThSBT3, ThSBT6, ThSBT9, ThSBT10, ThSBT16, ThSBT22, ThSBT29, ThSBT32, and ThSBT35. On the other hand, ThSBT4–ThSBT6, ThSBT8–ThSBT10, ThSBT22, ThSBT24, ThSBT29, ThSBT32, ThSBT37,

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and ThSBT41 had downstream regions. The remaining genes did not have upstream and downstream regions. Moreover, the pattern of regions, numbers, and location of the same subfamily with strong homology had a certain degree of similarity. ThSBT11–ThSBT14 and ThSBT37–ThSBT41 showed the sa*me pa*ttern in the figure 2. Furthermore, the genetic structure of ThSBT27 and ThSBT28, and ThSBT39 and ThSBT40 were almost identical.

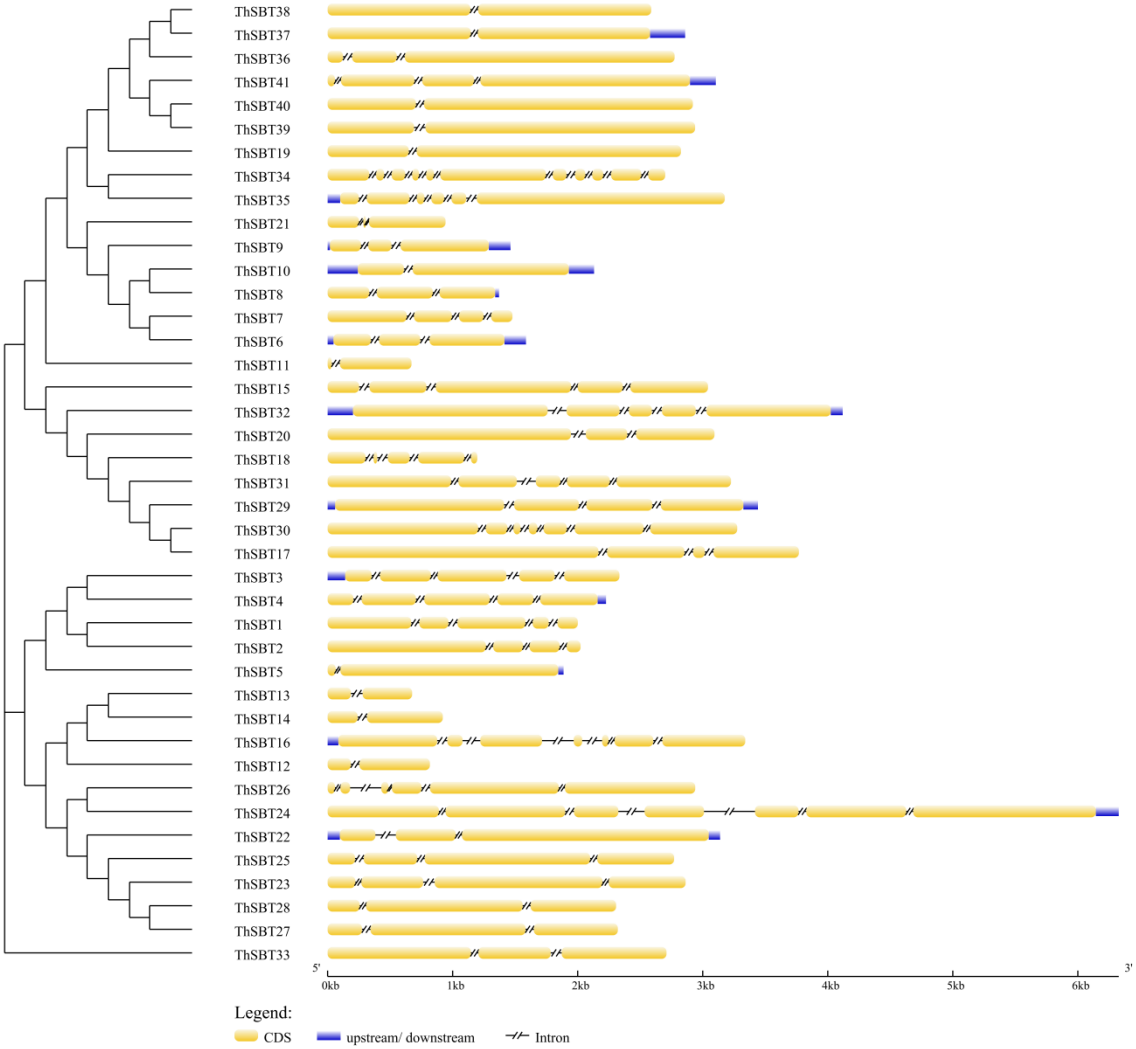

Figure 2. Phylogenetic relationship and gene structure of subtilase genes. The figure on the left shows the subtilase phylogenetic tree constructed by the maximum likelihood method. The image on the right shows the gene structure of subtilase, and the exons are represented by yellow rounded rectangles. Introns are indicated by gray double diagonal lines, and the upstream and downstream regions are indicated by blue rectangles.

2.4 Multiple sequence alignment and domain site analysis of T. harzianum subtilase family proteins

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To explore the structural characteristics of the subtilase family, DNAMAN 8 was used to perform multiple sequence alignments of the subtilase proteins, and HMMER was used to analyse the domain and active site of the amino acid sequences. The results showed that all 41 subtilase protein sequences had Peptidase-S8 domains. Moreover, according to different subfamilies, the domains contained 5 different patterns(Figure 3). These proteins had a specific domain (Peptidase_S8), with active sites on this domain being D, H, and S[49], and some of them had extra specific domains of different types. The number of domains and sites is shown in Supplementary Figure 1. The site location is shown in Supplementary Table 3. ThSBT1–ThSBT5, which belongs to the subfamily 5 of the Peptidase_S8 family, had a specific and active domain prokumamolisin. This domain was located at the N-terminus of the peptidase of SOP (sedolisin, clan SB) of the MEROPS peptidase family and contains ferredoxin-like folds with alpha+beta folds. Cleavage of this domain promoted peptidase activation[50]. ThSBT6 and ThSBT8–ThSBT10 had a specific domain, peptidase inhibitor I9, which belongs to the N-terminal propeptide domain of peptidase of subtilisin belonging to the S8A of the MEROPS family. The propeptide in subtilisin acts as a molecular chaperone to help in the folding of mature peptidases. Removal of the propeptide by proteolytic cleavage led to enzyme deactivation[51][52]. ThSBT19 and ThSBT39–ThSBT41 had a specific domain fn3_5, which is similar to an fn3-like domain, and is one the first of 3 domains on the streptococcus C5a peptidase (SCP). SCP is a highly specific protease and an adhesin/invasin[53]. Moreover, ThSBT33 had a specific domain P_proprotein, which is a highly conserved sequence in the eukaryotic subtilisin-like proprotein convertase and located downstream of the catalytic domain with approximately 150 residues (P domain)[54][55].

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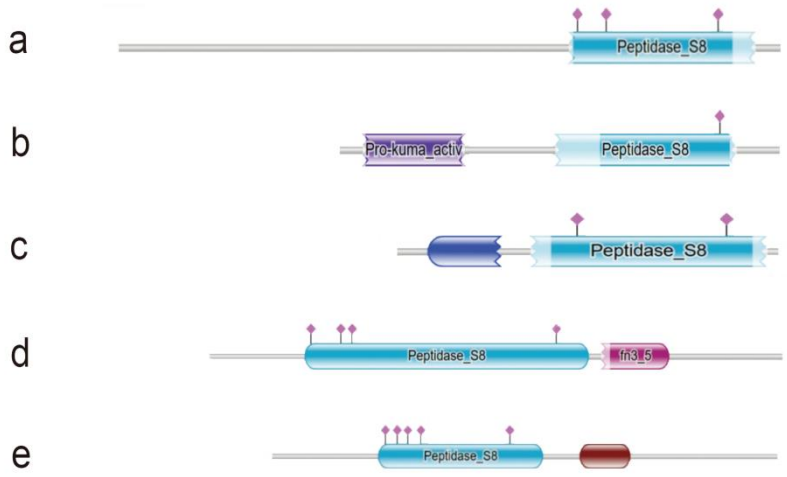

Figure 3. Five different domain and site patterns of 41 subtilase proteins

Multiple sequence alignment revealed that the amino acid sequence identity among members of the same subfamily was high(Figure 4). The amino acid sequence identity alignment of ThSBT1–ThSBT5 was 44.56%, that of ThSBT6–ThSBT10 was 40.18%, that of ThSBT11–ThSBT19 was 12.44%, that of ThSBT20–ThSBT28 was 15.95%, and that of ThSBT29–ThSBT33 was 28.08%. The ThSBT34–ThSBT41 alignment was 43.64%.

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Figure 4. Multiple sequence alignment of subtilase genes in Trichoderma harzianum. Sequences were aligned by DNAMAN 8 software. The domain was marked by a horizontal line, and the red box showed the active site.

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2.5 Identification of protein motifs of T. harzianum subtilase family genes

The MEME programme was used to analyse the conserved motifs in the Peptidase_S8 domain of *T. harzianum* subtilase genes, and the result is shown in Figure 5. There were 19 different motifs obtained from all the sequences, and the motif sequence and length information are shown in Supplementary Table S3. By using the Pfam database, these motifs were annotated and related functional information was queried. The figure shows the motif distribution of the conserved domains in different colours and positions. The distribution of motifs among subfamilies varied, and interestingly, the motifs belonging to the same subfamily were highly similar. Except for ThSBT18 and ThSBT28, all the proteins contained the commonly conserved motif1 and motif6, which proved that these two are highly conserved motifs. Moreover, motif3 and motif5 existed in ThSBT6–ThSBT41, which are also highly conserved motifs. ThSBT1–ThSBT5 belonging to the Peptidases_S53 subfamily had 3 common specific motifs of motif 10, motif11, and motif18. ThSBT6–ThSBT10 belonging to the Peptidases_S8_PCSK9_ProteinaseK_like subfamily had a common specific motif, motif19. Moreover, ThSBT15–ThSBT41 had unique motif4, while ThSBT19 and ThSBT34–ThSBT41 contained motif8 and motif9. According to the Pfam annotation query, motif1 and motif4 are encoded by the subtilase family, whereas the other motifs have no specific description.

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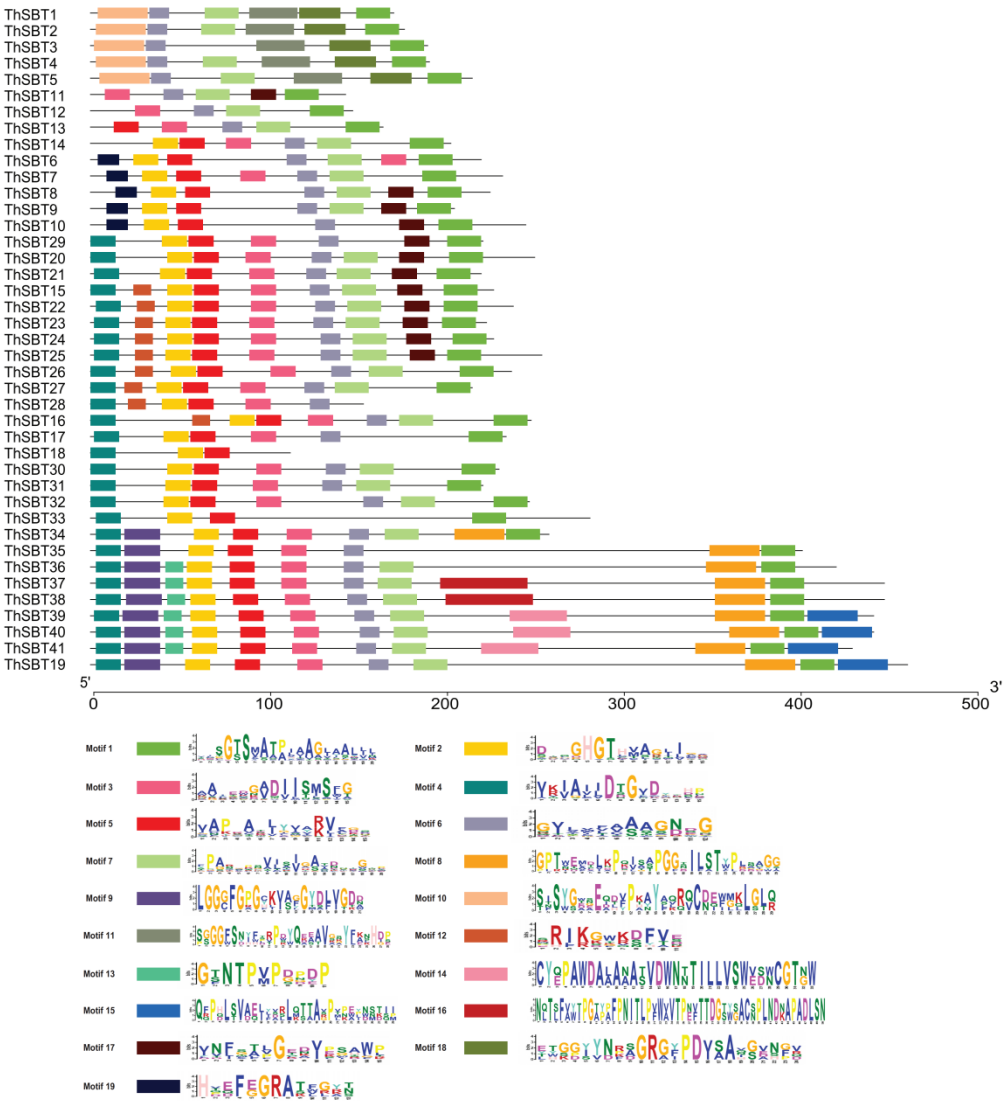

Figure 5. Conserved motif of subtilase genes in Trichoderma harzianum. The MEME analysis obtained the distribution of 19 conserved motifs. The colored boxes represented different motifs and their position in each sequence.

2.6 Chromosomal distribution of subtilase genes

Overall, 41 subtilase genes were unevenly distributed on 22 *Trichoderma* chromosomes. (Figure 6). Chromosomes 1 and 6 contained most subtilase genes (4) and chromosomes 3, 7, and 22 had 3 subtilase genes each. Other chromosomes contained relatively few subtilase genes (1–2). Subsequently, tandem repeat and fragment repeat events of 41 subtilase genes were analysed. There were 3 genes clustered on chromosome 1 (ThSBT12, ThSBT21, and ThSBT38), 2

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genes clustered on chromosome 21 (ThSBT2 and ThSBT4), and 3 genes clustered on chromosome 22 (ThSBT23, ThSBT31, and ThSBT32). There were 2 genes on chromosome 31 clustered together (ThSBT18 and ThSBT25). The distribution of these gene clusters showed that they may be hot spots of subtilase genes and implicated that tandem duplication events were the main reason for gene expansion.

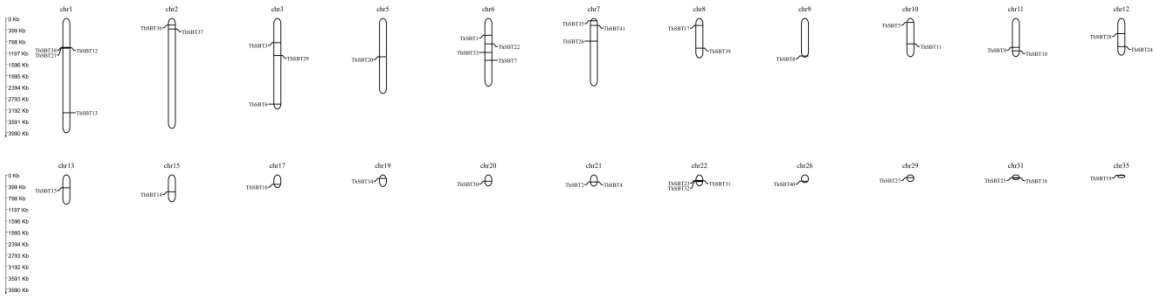

Figure 6. Chromosomal location of subtilase genes. Chromosome numbers were indicated above each chromosome. The size of a chromosome was indicated by its relative length. Gene positions and chromosome sizes were estimated in kb to the left of the figure.

2.7 Promoter cis-elements analysis of T. harzianum subtilase genes

To understand the function and regulatory mechanism of the subtilase genes, the PlantCARE programme was used to analyse the upstream sequence of all the genes, and a series of cis-elements related to a stress response were identified（Table 2）. The results showed that the subtilase gene contained various types of cis-acting elements, and the same element had multiple action sites in the promoter region of a gene(Figure 7). This study identified elements closely related to the stress response, namely abscisic acid-responsive element (ABRE), ethylene-responsive element (ERE), gibberellin response element (GARE), low-temperature response element (LTR), participating in drought-induced MYB-binding site (MBS), defence and stress reactive elements (TC-rich repeats), wound- and pathogen-reactive elements (WUN-motif), salicylic acid-reactive elements (TCA-element), and methyl jasmonate-responsive element (TGACG motif). All these elements play an important role in biological stress regulation pathways. Table 2 presents the number of various cis-acting elements on different genes, and Figure 6 presents their position in the sequence upstream of the promoter sequences. Among all the subtilase members, ThSBT30 had the most cis-acting

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elements (14), while ThSBT2 and ThSBT6 had the lowest number of elements (4). The TGACG element had the widest distribution (ranging between 1 and 9) in all the gene promoter sequences, except ThSBT1. The second most was the ABRE, except for that in ThSBT7, ThSBT19, ThSBT20, and ThSBT39, and it was found in all the remaining sequences (ranging between 1 and 5). The distribution of MBS in LTR was relatively even, with most of them having 0 or 1 and a few having 2 or 3 MBS elements. The GARE motif and TCA-element were less distributed; 1 or 2 of these elements were present in a few sequences, whereas most sequences did not have any of these 2 elements. TC-rich repeats, WUN-motif, and ERE had the least distribution, with only 1 element existing in a few sequences. These results indicated that the subtilase genes may play a role in stress tolerance.

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Table 2. Promoter type and numbers of subtilase genes upstream 2000bp

	ABRE	LTR	MBS	TCA-element	TGACG-motif	TC-richrepeats	GARE-motif	WUN-motif	ERE
ThSBT1	2	3		1					
ThSBT2	1	1	1		1				
ThSBT3	3	3	1		3				1
ThSBT4	4				1		1	1	
ThSBT5	3	2	1	1	2				
ThSBT6	1				2			1	
ThSBT7			2	1	8		1		
ThSBT8	2	2		1	5		1		
ThSBT9	4	2			4				
ThSBT10	3	1			9				
ThSBT11	3	1		1	2		1		
ThSBT12	3				1		1	1	1
ThSBT13	1	1	1	1	2			1	
ThSBT14	2	1	3	2	1				
ThSBT15	3		1		1				

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ThSBT16	1	1	1		4					
ThSBT17	2	2		1	3		1	2		
ThSBT18	1		1	1	3					
ThSBT19		2	2		4					
ThSBT20		2			3					1
ThSBT21	4		1	1	1					
ThSBT22	1	1			3		1			
ThSBT23	4			1	6	1				
ThSBT24	3		1		6	1	1			
ThSBT25	2	2	1	1	2					
ThSBT26	1	2	1		1					
ThSBT27	2	1	1	1	3					
ThSBT28	1	1	1	1	4					
ThSBT29	3		1		3	1	1			
ThSBT30	3	1	2	1	5	1	1			
ThSBT31	1		1		2		1			
ThSBT32	1		3	2	3					
ThSBT33	5	1	1	1	3					
ThSBT34	1		1		4					

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ThSBT35	2		1		2					
ThSBT36	2		1	1	2	1				
ThSBT37	1		1		3					
ThSBT38	2	3			1					
ThSBT39			1	1	1		2	1	1	
ThSBT40	3	1			3					
ThSBT41	2				1		1		1	

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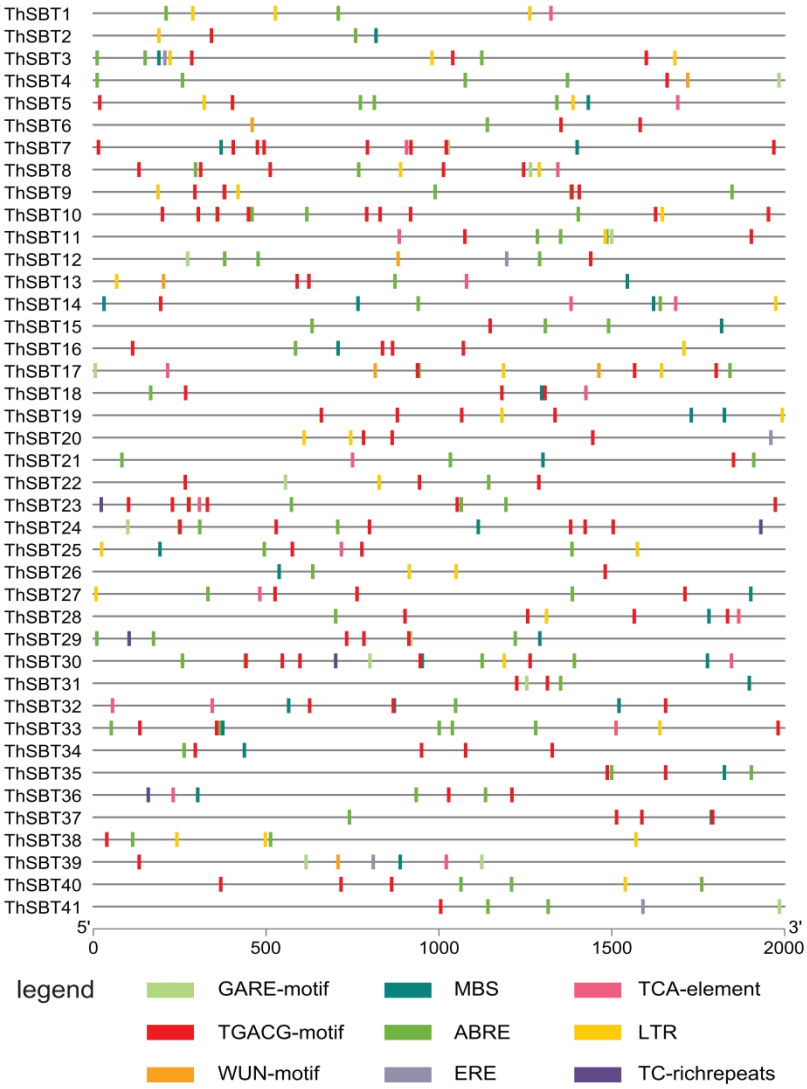

Figure 7. Cis-acting elements analysis of subtilase genes in the promoter region. Gray lines represent promoter regions. Different color boxes represent different cis-acting elements.

2.8 Expression of subtilase genes in the nematode resistance response

The current understanding of the function of the subtilase genes in *Trichoderma* is limited. We performed an expression analysis of the nematode resistance subtilase genes to determine its possible role. Many genes showed substantial expression differences between the treatment and control groups, which indicated that they may be involved in resistance response.

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Moreover, the subtilase genes have been previously reported to be differentially expressed in nematocidal fungi in response to nematode infection, indicating its potential role in nematode resistance interactions. Therefore, these genes are functional candidates for future research. The development and improvement of next-generation sequencing technology have allowed the wide use of RNA-seq in various studies of non-model organisms. In this study, we used the transcriptome data from a previous study to verify gene expression(Figure 9). Transcription of 31 genes was increased after 5 days of infection with nematodes, whereas that of 10 genes decreased. ThSBT1–ThSBT5 belonged to Peptidases_S53 in which ThSBT1 was downregulated, while ThSBT2–ThSBT5 were significantly upregulated. Protein K-type gene Peptidases_S8_PCSK9_ProteinaseK_like (5 out of 41 genes), which showed protein resistance expression, had 1 upregulated and 4 downregulated genes. Among ThSBT11–ThSBT19, ThSBT11 and ThSBT17 were downregulated, while the rest were upregulated. Among ThSBT29–ThSBT32 belonging to Peptidases_S8_7, ThSBT 29 and ThSBT 30 were upregulated, while ThSBT31 and ThSBT32 were downregulated. ThSBT33, which belonged to Peptidases_S8_ProteinKivertases, was upregulated. Among ThSBT34–ThSBT41, which belonged to subfamily 5 of the peptidase S8 family, ThSBT39 was downregulated and the rest were upregulated. The cluster heat map showed the overall changes in the trend of these genes. About 75% of the expression of subtilase genes during the resistance period of mycelia showed an upward trend, and 25% showed a downward trend. These identified genes showed differential expression under the given nematode resistance, which provided a reference for similar studies and further functional analysis.

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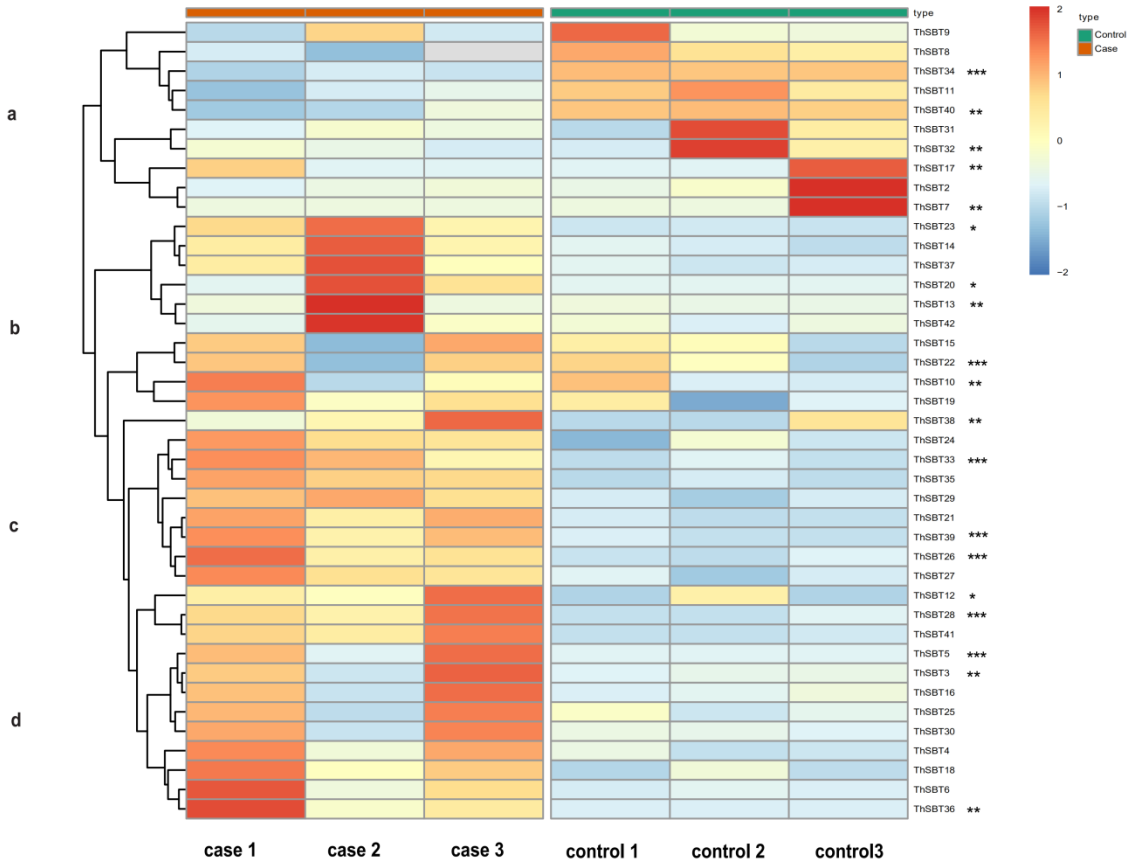

Figure 8. The heatmap of 41 subtilase genes expression pattern of Trichoderma in nematode resistance response. Note: * 0.01<P≤0.05,** 0.001<P≤0.01,*** P≤0.001.

To test the possibility that the subtilase genes are transcriptionally induced by the nematode, the mycelia of *Trichoderma* were inoculated with nematodes for 5 days. The heat map showed the expression pattern of subtilase family genes in treatment and control groups. These genes were divided into four clusters (designated a, b, c, and d) based on patterns of their responses to nematode infection (Figure 8). Cluster a included 10 subtilase genes in the treatment group whose expression was slightly decreased by nematode treatment compared with the control group. Transcription of the other 31 genes increased to a certain extent after 5 days of nematode treatment, especially in cluster d in which 12 genes were relatively strongly expressed. Cluster c included 13 genes that showed similar expression patterns, indicating that they may be performing related functions involved in nematode resistance. All the

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aforementioned results suggest that the 41 subtilase genes were slightly or strongly induced at 5 days by interaction with the nematodes.

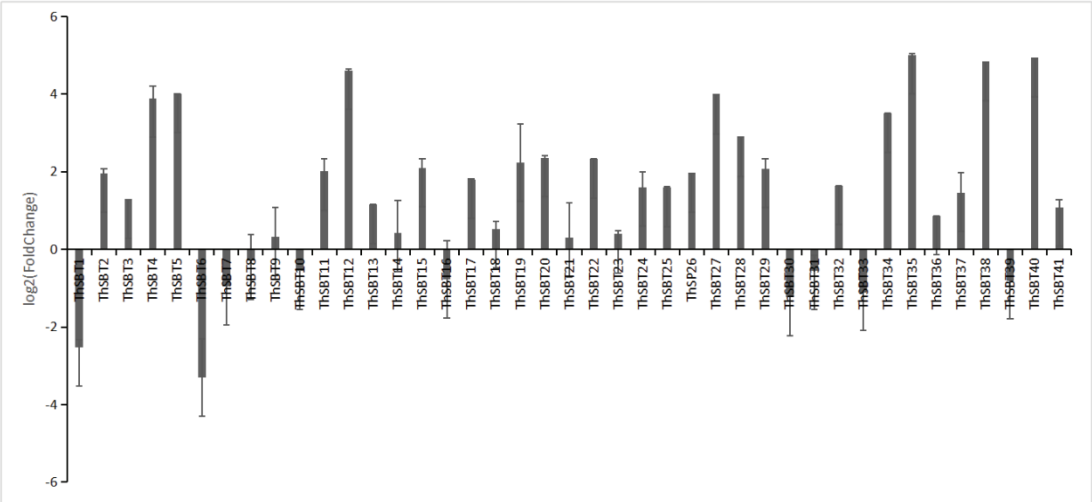

Figure 9. Gene expression of log2foldchange data of subtilase genes expression in *Trichoderma* within nematode resistance response in 5 days .

3. Discussion

Nematodes cause considerable destruction of agricultural produce worldwide. At present, although many natural nematophagous fungi or bacteria have been used in biocontrol methods, disadvantages such as culture technologies and host limitations still exist[56][57]. In the recent years, the omics sequencing technology of fungi has been rapidly developed, and the publication of genome and transcriptome data has allowed researchers to conduct an in-depth analysis of nematodes[1][53][59]. Serine proteases are widely found in fungi, and their nematicidal effects have been studied[60][61]. Most of these proteases are substrate enzymes with similar biochemical properties and similar sequences. They are one of the important enzymes involved in the recognition of nematodes[34]. Among them, subtilase is essential in the evolution of fungal pathogenicity to nematodes. Subtilase was initially discovered as an extracellular secreted alkaline protease from *B. subtilis*[62]. Subsequently, researchers globally

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conducted more in-depth and extensive research using various species and isolated similar proteases. The present study mainly focused on parasitic insects and pathogenic fungi. The subtilase proteases of pathogenic fungi play the role of virulence factors, mainly involved in the processing and assembly of various proteins such as membrane surface glycoproteins, growth factors, cytotoxins, complement system-related enzymes, and blood coagulation-related enzymes[63].

Subtilase is a member of the serine protease family and has evolved independently to form the polymerised Asp/Ser/His catalytic triad. The structure is an alpha/beta sheet containing a 7-chain parallel beta plate fold, and the order is 2314567[20]. All subtilase family members have a Peptidase-S8 domain with high homology as determined by Pfam and HMMER analysis, indicating that the domain is relatively conservative and is also the premise for constructing the evolutionary tree. Based on the phylogenetic analysis, these subtilase genes were grouped into 6 clusters. The tree showed that proteases in the same subfamily have higher homology. According to the predicted subcellular localisation data (Table 1), some genes are expressed in the cytoplasm, whereas most genes encode secreted proteins that produce toxins that act outside the cell. The pI value of these proteins ranged between 4.61 and 8.17. Some studies have found that a higher pI value is conducive to the hydrolytic activity of the protease itself and plays an important role in the binding process of the enzyme and the nematode epidermis[5]. We constructed the phylogenetic tree with 287 protein sequences of subtilase family members. ThSBT9 belonged to S8_PCSK9_ProteinaseK_like, was clustered with proteinase T-like protein of *Purpureocillium lilacinum*[64] and alkaline serine protease P32 of *Metapochonia rubescens*[65], both of which belong to nematicidal fungi, and subtilisin-like protease Tapr1a[66] of *T. arundinaceum*, which is related with trichothecenes, a family of terpenoid toxins. Multiple sequence comparisons showed high homology and their catalytic motifs were highly conserved. We may assume that they may have a similar function. Moreover, ThSBT20 was clustered with PlSBT21[67], PlSBT22, and PlSBT23[64], and the last of two belonged to the subtilase family domain-containing protein. ThSBT30 was clustered with MerSBT30 of *Metarhizium guizhouense*, MerSBT31 of *Metarhizium robertsii*, and MeaSBT30 *Metarhizium*

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anisopliae. Apivit Thongkaewyuan reported the use of *Metarhizium* spores in combination with its protease-inhibited hatching of the root-knot nematode *M. incognita*[68]. Furthermore, IC Paterson reported that *Metarhizium anisopliae* produces several extracellular cuticle-degrading proteases and this evidence is consistent with the finding of a previous study reporting that PR1, a chymoelastase, is a determinant of pathogenicity[69].

The diversification of exons and introns plays an important role in the evolution and function of gene families. In this study, 41 subtilase genes of *T. harzianum* were obtained, and their lengths and number differed significantly. The number of exons and introns, as well as the structure of the genes, were similar in the members of the subfamily, while they showed great difference between each subfamily. As shown in Figure 2, ThSBT4 and ThSBT5, which had a close homology, shared the same type of exon and intron in terms of position and number. Moreover, for ThSBT1 and ThSBT11, which had a relatively distant homology, the sequence length, exon and intron position, and number varied greatly. Gene chromosome location and synteny analysis within the *Trichoderma* genome demonstrated the expansion and evolution of subtilase genes. We found 3 gene clusters on chromosomes 1, 21, and 22, respectively. The distribution of these gene clusters showed that they may be hot spots of subtilase genes in *Trichoderma*, and thus, it was inferred that they may have similar functions.

The multiple sequence alignment of subtilase proteins showed that subtilase belonged to a large family. The family members differed from each other, and the similarity of some protein sequences was even lower than 20%. However, the active domain of the protein was highly conserved. Their small changes in structure lead to their functional evolution. The number and types of conserved motifs in all subtilase genes were determined. We obtained the most conserved motif in the subtilase genes according to the Pfam annotation (Figure 4). The MEME results showed that among genes with similar homology, motifs in the subgroups with the same type and number are the same, indicating that the evolutionary relationship in the subgroups was reasonable and corresponded to similar structures. The conserved domain of the same subfamily had similar lengths and similar physical and chemical properties. Subtilase may have unique functions because of changes in the protein residues of the catalytic motif.

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Previous studies have shown that parasitic fungi can compensate for the loss of protease activity because of the production of serine protease inhibitors by the host by expressing other proteases[34]. Some proteases were not very similar but they all had the same domain and conservative catalytic motifs, which indicated that their presence could compensate for the enzymatic activity of the invasive protease.

When the strain is stimulated by external stress factors, it transduces transcription factors through a series of signals. These activated transcription factors combined with the cis-acting elements of the target gene promoters then activate the transcriptional expression of stress resistance genes and respond to external regulatory stress signals[70]. The cis-acting element in the promoter region of the gene plays a key role in the biological stress response[71]. To explore the function of the subtilase gene regulatory region in the process of resistance to the stress, we collected the sequence information of the 2000-bp region upstream of the promoter of the subtilase gene, and the element name and site information were also obtained. We focused on the cis-acting elements related to antiadversity, defence response, and detoxification effects. All these motifs were randomly distributed in the positive and negative chains of the promoter sequence. Analysis of the cis-acting element genes in the promoter region showed that each subtilase gene carried multiple elements related to stress response in its promoter region, indicating that the genes may be involved in stress resistance and defence response. First, the cis-acting element TGACG motif, which is involved in the jasmonic acid response, had the highest distribution in all the genes, and ThSBT10 had the most number of TGACG motifs. We assumed that this gene is strongly associated with the defence response. Second, the ABRE, which is involved in the abscisic acid reaction, had high distribution in most genes, especially in ThSBT4, ThSBT9, ThSBT21, ThSBT23, and ThSBT33; they had approximately 4–5 elements in the sequences. Moreover, 0 or 1 MBS element was involved in drought induction in most sequences, while ThSBT14 and ThSBT32 had 3 MBS elements. The distribution of LTR, involved in the low-temperature response, was relatively even; most of them had 1, but ThSBT1, ThSBT3, and ThSBT38 had 3 LTR elements. In addition, the TCA-element, involved in the salicylic acid reaction, and the gibberellin response element GARE had relatively low distribution. The

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defence and stress response element (TC-rich repeats) had lower distribution and was only observed in ThSBT23, ThSBT24, ThSBT29, ThSBT30, and ThSBT36. The wound- and pathogen-reactive element WUN motif and the ethylene-responsive element ERE had the least distribution in these sequences, with only 1 element in few sequences.

To investigate the role of subtilase genes in response to nematode resistance, the expression levels of 41 subtilase genes were examined in the mycelia of *Trichoderma*. In these subtilase genes, ThSBT4, ThSBT5, ThSBT12, ThSBT27, ThSBT34, ThSBT35, ThSBT38, and ThSBT40 showed significantly upregulated expression with a log2 fold change value of more than 4, and ThSBT35 showed the highest peak. The ThSBT6 had the highest level of downregulated expression among all of the genes. The proteinase K family is an important protein family in the subtilase serine protease family. The biocontrol activity test was performed with the subtilisin serine protease PSP-3 produced by the endoparasitic nematode fungus *Paecilomyces lilacinus*, and the results showed that PSP-3 can degrade insect and nematode body walls[24]. ThSBT9, which belongs to the ProteinaseK_like family, showed upregulated expression during the resistance process. This is consistent with the phylogenetic results, indicating a similar function potentiality in the response of killing nematodes with the proteinase of *Purpureocillium lilacinum* and alkaline serine protease P32 of *Metapochonia rubescens*. This study showed that an alkaline serine protease is produced by the *P. lilacinus* strain in the presence of a nematode eggshell and other substrates such as vitellin and chitin[72]. Furthermore, ThSBT20 showed upregulated expression with a log2 fold change of more than 2, and it was clustered with genes from *Purpureocillium lilacinum*. In addition, ThSBT32 was clustered with FoSBT32 in *Fusarium oxysporum*. A previous study reported that the extracts of *Fusarium oxysporum* exhibit inhibitory activities against *M. incognita* and *Radopholus similis*[73]. This study provides the required data for selecting candidate genes in the subtilase gene family and for further research.

4. Materials and Methods

4.1 Gene identification and sequence retrieval

T. harzianum whole-genome sequence data were downloaded from the NCBI database. Bioedit software was used to extract the gene sequence of the subtilase family of *T. harzianum*

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from the genome file, after which the full-length amino acid sequence was queried and downloaded from the NCBI website. By using the Hidden Markov Model (HMMER) (http://www.ebi.ac.uk/Tools/hmmer/) in the Pfam database[74], a LocalBlast search (Evalue=5) was performed to obtain the basis of the conserved domain of the family sequence and conservative sites. Then, the NCBI conserved domain data website[75] (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the SMART website[76] (http://smart.embl-heidelberg. de/) were used to filter and verify the conserved domain. We obtained the basic information of gene sequences including gene number, gene position coordinates (5'-3'), number of exons, and length of the open reading frame. The Expasy website (https://web.expasy.org/protparam/) was used to obtain the theoretical isoelectric point (PI) and molecular weight (MW) information of protein sequences. The WoLPPSORT (https://wolfpsort.hgc.jp/) was used to predict the subcellular localisation of the genes. NCBI Blast was used to screen and obtain the submember sequences of closely related subtilase family genes.

4.2 Phylogenetic tree construction of subtilase genes

The Muscle software programme was used to perform multisequence alignment on the 287 protein-encoding genes of the subtilase family. Evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model[77] in Mega7 software[78]. The test parameter (Bootstrap) was set to 100 to generate the molecular phylogenetic evolution tree. iTOL was used to draw the evolutionary tree[79].

4.3 Gene structure analysis of T. harzianum subtilase family genes

The web server Gene Structure Display Server (GSDS) [80](http://gsds.cbi.pku.edu.cn) was used to analyse the DNA sequences of *T. harzianum* subtilase genes. We got the corresponding CDS sequences and the exon-intron organization of these genes. The phylogenetic evolutionary tree was related to the gene structure within different subfamilies.

4.4 Identification of the conserved domain, functional site information, and protein motif

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DNAMAN8 software was used to perform multiple sequence alignments on the full length of 41 subtilase proteins of *T. harzianum*. By using the HUMMER programme (https://www.ebi.ac.uk/Tools/hmmer/) and SMART (http://smart.embl-heidelberg.de), the conserved domain and functional site information were obtained. The MOTIF search (https://www.genome.jp/tools/motif/) was used to extract the conserved domain information from each protein sequence. MEME[81] (http://meme.sdsc.edu/meme/itro.html) and TBtools[82] was used to identify the conserved motifs of the 41 protein domain sequences from *T. harzianum* subtilase genes, and the parameter settings were as follows: number of repetitions = zoops, maximum number of patterns = 20, and optimal motif length = 6–200 residues. Then, we obtained the different motif recognition regions, and the corresponding function and sequence message were analyzed using the Pfam database.

4.5 Chromosome location and cis-acting elements analysis of T. harzianum subtilase genes

The chromosomal location information of 41 subtilase genes was identified from the NCBI database, and their distribution on the chromosome was analysed using MapGene2Chrom web v2 software (http://mg2c.iask.in/mg2c_v2.1/). TBtools was used to extract and screen the upstream sequence (2 kb) of subtilase genes from the whole genome sequence of *T. harzianum*. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to predict and analyse the cis-acting element distribution of the promoter region[83].

4.6 Fungal materials and RNA extraction

Trichoderma mycelium was cultured at 25°C for 5 days in the Potato Dextrose Agar medium. The nematode was cultured in the presence of Botrytis cinerea using the corn kernel medium. The mature nematodes were extracted by using the Bellman funnel method[84]. The volume of the extracted liquid containing pinewood nematodes was adjusted to 6000/mL, and then added to the mycelia and covered uniformly. The mycelium was collected on the fifth day and considered the treatment group, while the mycelium collected at 0 h was considered the control group. The samples were sent to Novogene company for RNA extraction and detection, cDNA library establishment, and quality inspection. Total extracted RNA was used to synthesise

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cDNA using the RNALAPCR kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions.

4.7 Expression analysis of subtilase genes in Trichoderma in response to nematodes

To determine the expression profiles of subtilase genes in *Trichoderma* in response to nematodes, RNA-seq reads were retrieved from previous experiments performed using *Trichoderma* mycelia. RNA purity and integrity were tested by agarose gel electrophoresis and using the NanoDrop 2000 spectrophotometer (Thermo) and Agilent 2100 Bioanalyzer. Reverse transcription of the cleaved RNA fragments was performed to prepare the final cDNA library in accordance with the NEB general library preparation kit (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®). HISAT2 software was used to rapidly and accurately compare clean reads with the reference genome *Trichoderma_harzianum*_CBS_226.95 to obtain the location information of the reads on the reference genome. Differential gene expression was analysed by expectation maximisation. The expression estimations of the genes were normalised and represented in the form of fragments per kilobase of transcript per million mapped reads (FPKM), and fold change (log2) values were calculated using the ratio of gene expression from the treatment group compared to the control group. Genes with absolute log2 values >2 and t-test values ($p < 0.05$) after Bonferroni correction served as significantly differential expressed genes. On the basis of the FPKM values, a heat map was generated using the R studio[85].

5. Conclusions

In the recent years, the research on protease genes and metabolites has increased considerably, with a focus on hydrolytic proteases and their metabolites. Identifying effective methods for controlling the populations of various insects, pathogenic bacteria, and nematodes; reducing the crisis of chemical control; and promoting the development of biological control are the outstanding points of the new generation of biological agents. The subtilase family plays an important physiological role in biological organisms and has extensive research and application value. In this study, 41 subtilase family genes were screened based on the *T. harzianum* genome database, and bioinformatics analysis was performed to determine gene characteristics, protein structure, evolutionary status, and function. According to their expression profile, we

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speculated their role in the process of resisting stress. The roles of subtilase gene expression differences in response to nematode stresses provide insight into the roles of defence and resistance in the mycelia of *Trichoderma*. These results lay the theoretical foundation for further research on the function of the subtilase gene and nematicidal mechanism.

Supplementary Materials: Table S1. Details of subtilse genes from *Trichoderma guizhouense, Trichoderma virens, Trichoderma reesei, Trichoderma atroviride, Trichoderma asperellum, Trichoderma lentiforme, Trichoderma parareesei, Metapochonia rubescens, Purpureocillium lilacinum, Fusarium austroafricanum, Lasiodiplodia theobromae, Colletotrichum simmondsii, Metapochonia rubescens, Zopfia rhizophila*. Table S2. Details of CDS message of subtilase genes in *Trichoderma harzianum*. Table S3. Details of protein site message of subtilase genes in *Trichoderma harzianum*. Table S4. Detailed information of the 19 motifs in the subtilase proteins of *Trichoderma harzianum*.Figure S1. Detailed information of subtilase conserved domains.

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