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Molecules and Cells

Molecular Cloning and Functional Expression of Chitinase-Encoding cDNA from the Cabbage Moth, *Mamestra brassicae*

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Chitinase is a rate-limiting and endo-splitting enzyme involved in the bio-degradation of chitin, an important component of the cuticular exoskeleton and peritrophic matrix in insects. We isolated a cDNA-encoding chitinase from the last larval integument of the cabbage moth, *Mamestra brassicae* (Lepidoptera; Noctuidae), cloned the ORF cDNA into *E. coli* to confirm its functionality, and analyzed the deduced amino acid sequence in comparison with previously described lepidopteran chitinases. *M. brassicae* chitinase expressed in the transformed *E. coli* cells with the chitinase-encoding cDNA enhanced cell proliferation to about 1.6 times of the untransformed wild type strain in a colloidal chitin-including medium with only a very limited amount of other nutrients. Compared with the wild type strain, the intracellular levels of chitin degradation derivatives, glucosamine and *N*-acetylglucosamine were about 7.2 and 2.3 times higher, respectively, while the extracellular chitinase activity was about 2.2 times higher in the transformed strain. The ORF of *M. brassicae* chitinase-encoding cDNA consisted of 1686 nucleotides (562 amino acid residues) except for the stop codon, and its deduced amino acid composition revealed a calculated molecular weight of 62.7 and theoretical pI of 5.3. The ORF was composed of *N*-terminal leading signal peptide (AA 1-20), catalytic domain (AA 21-392), linker region (AA 393-498), and *C*-terminal chitin-binding domain (AA 499-562) showing its characteristic structure as a molting fluid chitinase. In phylogenetic analysis, the enzymes from 6 noctuid species were grouped together, separately from a group of 3 bombycid and 1 tortricid enzymes, corresponding to their taxonomic relationships at both the family and genus levels.

INTRODUCTION

Chitin, a linear, water-insoluble homopolymer of *N*-acetylglucosamine, is the second most abundant polysaccharide biomass next to cellulose in the living world, occurring in arthropods, nematodes, mollusks, fungi and some algae. Chitinolytic enzymes are essential for chitin turnover and metabolism in chitin-containing organisms, but also exist in higher vertebrates, some plants, bacteria and baculoviruses containing no chitin compounds in relation to self-defense, nutrient assimilation and host invasion (Cohen-Kupiec and Chet, 1998; Collinge et al., 1993; Hawtin et al., 1997; Lyou et al., 2009).

In insects, chitin is an important component of the cuticular exoskeleton and peritrophic matrix of the midgut for protective function, and is degraded by two types of chtinolytic enzymes, endo-splitting chitinase (EC 3.2.1.14) and exo-splitting β-*N*-acetylglucosaminidase (EC 3.2.1.30) along their molting cycles in postembryonic development (Kramer and Muthukrishnan, 1997). The former is a rate-limiting enzyme in chitin biodegradation, because it acts on the first step of chitin compounds producing chito-oligomers that are subsequently converted to *N*-acetylglucosamine monomers (Filho et al., 2002).

Chitin degradation must be tightly regulated to maintain its role in insect development, and the transcription or enzyme activity of chitinase in integument is restricted to molting periods by hormonal regulation (Kramer et al., 1993; Zheng et al., 2002). Therefore, the chitin hydrolysis step has received significant attention as a potential target for pest management.

Two strategies to interfere with chitin degradation metabolism in insects have been attempted; Inhibitors of insect chitinases, such as allosamidin isolated from *Streptomyces* sp. were shown to prevent larval ecdysis (Cohen, 1993; Sakuda et al., 1986); Use of insect chitinase as a biopesticide itself by transforming host plants or entomopathogenic baculoviruses with insect chitinase-encoding cDNAs. A transgenic tobacco plant has been produced by introducing chitinase-encoding cDNA of the tobacco hornworm, *Manduca sexta* using *Agrobacterium*-mediated transformation. The expressed insect chitinase in the transformed plant exhibited considerable feeding damage to a lepidopteran pest, the tobacco budworm (*Heliothis virescenes*) (Ding et al., 1998) and even to a coleopteran pest, the merchant grain beetle (*Oryzaephilis mercator*) (Wang et al., 1996). In addition, introduction of an insect chitinase gene into a baculovirus, AcMNPV, improved its insecticidal activity (Krishnan et al., 1994), showing that the fall armyworm, *Spodoptera frugiperda* larvae injected with the transformed virus died faster (Gopalakrishnan et al., 1995).

Several molecular biological investigations on insect chitinases have focused mainly on the lepidopteran enzymes since

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the first isolation of mRNA transcript from *M. sexta* (Kramer et al., 1993), considering their potential use in biological pest control of the serious lepidopteran pest insects. The lepidopteran chitinases have a common multi-domain structure with a catalytic region, a PEST-like region enriched in proline, glutamate, serine and threonine, and a cysteine-rich chitin-binding region (Ahmad et al., 2003; Bolognesi et al., 2005; Fitches et al., 2004; Goo et al., 1999; Kim et al., 1998; Shinoda et al., 2001; Zheng et al., 2002), but there are still some controversies on the border positions between domains.

We isolated and cloned a cDNA encoding chitinase from the integument of the cabbage moth, *Mamestra brassicae* (Lepidoptera; Noctuidae), a serious polyphagous pest, and its ORF was functionally expressed in *E. coli* cells showing enhanced enzymatic activity to colloidal chitin. We also addressed its structural characteristics, phylogeny and hydropathy plot in comparison with previously reported lepidopteran chitinases.

MATERIALS AND METHODS

Insects

Larvae of the cabbage moth, *Mamestra brassicae* (Noctuidae; Lepidoptera) were reared on Chinese cabbage at 25 ± 1°C and 60% relative humidity under a 16:8 h light:dark cycle. The integument was removed from the last instar larva under cold insect saline, and then immediately transferred into TRIzol (GIBCO/BRL).

Cloning of chitinase-encoding cDNA from *M. brassicae*

Total RNA was extracted from the integument immersed in TRIzol according to the manufacturer's instructions. Double-stranded cDNA was synthesized from total RNA using the Universal Riboclone cDNA Synthesis System (Promega) according to the manufacturer's instructions.

A PCR reaction was performed in PTC-100 Programmable Thermal Cycler (MJ Research) to amplify a conserved partial domain (190 amino acids), which is common to previously reported lepidopteran chitinase members, using a pair of gene-specific primers (Forward, 5′-GCCAACTTTAGGCTGATGGA-3′; Reverse, 5′-ACCTTTGAAGTCATCCATGT-3′) with the nucleotide sequence of chitinase cDNA isolated from a noctuid species, *S. litura* (Shinoda et al., 2001) to hybridize the very conserved amino acid motifs, ANFRLME and DMDDFKG, respectively. A total reaction volume of 50 ul containing 100 ng of cDNA template, 0.25 mM dNTPs, 0.2 mM $MgCl_2$, 10 uM 5′ and 20 uM 3′ primers was initiated by addition of 2 U Taq polymerase (Takara Ex-Taq) at 80°C following an initial 5 min denaturation at 95°C, followed by 39 PCR cycles (95°C for 1 min, 58°C for 1 min, and 72°C for 2 min). The amplified product (Fig. 1) was analyzed by agarose gel electrophoresis, and then eluted and ligated into *E. coli* plasmid pCR2.1 using a TA cloning kit (Invitrogen). The PCR product was sequenced next for verification.

In order to obtain the full-length coding region, the above primers were used to amplify the 5′ and 3′ cDNA ends by the RACE procedure (Frohman et al., 1988) using a Marathon cDNA amplification kit (Clontech) and Advantage cDNA polymerase (Clontech) according to the manufacturer's protocols. A reaction volume of 50 ul containing 100 ng of cDNA template, 0.25 mM dNTPs, 0.2 mM $MgCl_2$, 10 uM 5′ primer and 20 uM 3′ primer was initiated by the addition of 2U polymerase at 80°C following an initial 5 min denaturation at 94°C, followed by additional denaturation at 94°C for 30 s. Then, the amplification was carried out by three sequential denaturation-annealing cycles (5 cycles of 94°C for 30 s -67°C for 4 min; 5 cycles of 94°C for 30 s -65°C for 4 min; 35 cycles of 94°C for 20 s -65°C for 4 min) and one extension step (72°C for 5 min). Each of the amplified 5′- and 3′-RACE fragments (Fig. 1) were gel-purified, cloned into pCR2.1, and sequenced.

Based on the sequences of the two cDNA endings, a pair of gene-specific primers (Forward, 5′-ATGAGAGCTATACTAGC GACGTTG-3′; Reverse, 5′-CTAAGGCTCGCAGTCGGCGCG GTC-3′) was designed to amplify the full-length ORF cDNA. A reaction volume of 50 ul containing 100 ng of cDNA template, 0.25 mM dNTPs, 0.2 mM $MgCl_2$, 20 uM each of 5′ and 3′ primers was initiated by the addition of 2U *Taq* polymerase at 80°C following an initial 5 min denaturation at 95°C, followed by 39 PCR cycles (95°C for 1 min, 54°C for 1 min, and 72°C for 2 min). The PCR fragment of expected size was isolated and confirmed by DNA sequencing.

Culture of *M. brassicae* chitinase in *E. coli*

The full-length coding region of chitinase cDNA assembled in pCR2.1 using a TA cloning kit was introduced into *E. coli* XL1 cells. The selected transformant was grown in 10X-diluted NB liquid media (1% beef extract, 1% peptone, 0.5% NaCl) containing 1% colloidal chitin (Hsu and Lockwood, 1975), 1 mM IPTG and 0.01% ampicillin at 37°C, 225 rpm to evaluate its chitinolytic activity.

Real-time quantitative reverse transcription PCR (RT-PCR) analysis

Reverse transcription of total RNA isolated from the transformed *E. coli* was performed with Green PCR Master Mix (AB Applied Biosystems). A reaction volume of 20 ul of the reverse transcription mixture containing 100 ng template, and 10 pM each of 5′ and 3′ primers (Forward, 5′-CGTTGGCCGTCCTG GCGG-3′; Reverse, 5′-CGGGGATGTCTTCGATGC-3′) was used for quantitative PCR using Rotor-Gene 3000 (Corbett Research). The instrument settings were: initial enzyme activation at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 2 min. PCR specificity and product length were assessed by agarose gel electrophoresis and ethidium bromide staining to verify that the primer pairs amplified a single product of the predicted size.

SDS-PAGE and western blotting of *M. brassicae* chitinase expressed in *E. coli*

The *E. coli* cells were cultured for 6 h at 37°C in NB liquid media with 0.2 or 1 mM IPTG, and collected by centrifugation (3,000 × *g*, 4°C for 10 min). The cell pellet was extracted by ultrasonification (Branson Sonifier 450) with PBS buffer (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7.4) of 1/10 volume of culture media. After centrifugation (16,000 × *g*, 4°C for 10 min), the precipitant was resuspended and ultrasonified in the same volume of PBS again. The cell lysate and precipitant were loaded on SDS-PAGE (4-20% gradient), respectively.

The separated proteins were transferred to PVDF (polyvinylidene fluoride) membrane for 1 h. The membrane was washed with TBS (50 mM Tris base, 0.9% NaCl, pH 8.4), reacted with a blocking solution (5% skim milk in TBS) for 2 h, and washed twice with TBST (0.05% Tween-20 in TBS) for 30 s. The proteins were treated with the first antibody (His-antibody mouse monoclonal; Santa Cruz Biotechnology #53073, 1:5000) for 3 h, and then with second antibody (Goat anti-mouse IgG-HRP; Santa Cruz Biotechnology #2005, 1:5000) for 30 min. An X-ray film was exposed to the fully washed membrane with TBST.

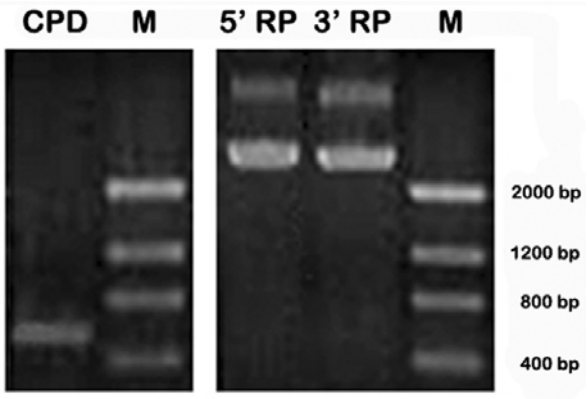

Fig. 1. Amplified conserved partial domain and RACE products of chitinase-encoding cDNA isolated from *M. brassicae.* CPD, Conserved partial domain; 5′ and 3′ RP, 5′ and 3′ RACE products; M, Marker DNAs.

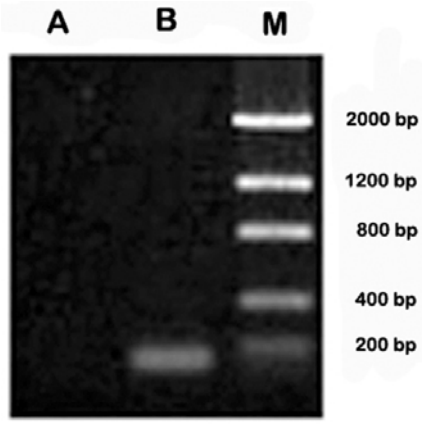

Fig. 2. Expression of *M. brassicae* chitinase-encoding cDNA in the *E. coli* transformant. The amount of insect chitinase transcript was measured by real-time quantitative RT-PCR. Line A, wild *E. coli* strain; line B, transformed *E. coli* with *M. brassicae* chitinase-encoding cDNA.

Assays of expressed *M. brassicae* chitinase activity

The transformed *E. coli* cells were inoculated (37°C) at 10^4 cells / ml in the 10X-diluted NB liquid media with 1 mM IPTG. After incubation for 72 h, the culture was centrifuged (7,000 × *g*, 4°C for 10 min) to remove bacterial cells and chitin precipitants. 500 ul of supernatant was mixed with the same volume of CC-RBB (colloidal chitin dyed with Remazol Brilliant Blue, Gomez et al., 2004). Reaction of the mixture at 50°C for 1 h released the dye, and was then stopped by boiling for 5 min. The re-maining chitin was removed by centrifugation (5,000 × *g*, for 5 min). OD of the supernatant containing the released dye was measured at 595 nm using the Spectronic Genesys 5 spectrophotometer (Milton Roy).

The cultured *E. coli* cells as the above were harvested by centrifugation (7,000 × *g*, 4°C for 10 min), resuspended in 1 ml distilled water, and sonicated on ice using Sonifier 450 (Branson). After removing the cell debris by centrifugation (10,000 × *g*, 4°C for 10 min), the lysate was subjected to HPLC analysis for D-glucosamine and *N*-acetyl-D-glucosamine quantification. A total of 50 ul lysate was injected onto an Altex Ultrasphere ODS column (5 um, 4.6 mm × 15 cm) and eluted with acetonitrile (A) and distilled water (B) using a gradient program (100% B for 0-2 min, linear gradient to 40% A and 60% B for 2-10 min, and linear ramp to initial condition for 10-12 min).

Sequence alignment, phylogenetic analysis, and hydropathy plotting

All the analyses were performed using the tools contained in the MacVector 7.0 software suite (Oxford Molecular, USA) together with previously reported lepidopteran chitinases; the common cutworm (*Spodoptera litura*) from Shinoda et al. (2001), the fall armyworm (*Spodoptera frugiperda*) from Bolognesi et al. (2005), the cotton bollworm (*Helicoverpa almigera*) from Ahmad et al. (2003), the tomato moth (*Lacanobia oleracea*) from Fitches et al. (2004), the fall webworm (*Hyphantria cunea*) from Kim et al. (1998), the tobacco hornworm (*Manduca sexta*) from Kramer et al. (1993), the wild silkmoth (*Bombyx mandarina*) from Goo et al. (1999), the silkworm (*Bombyx mori*) from Kim et al. (1998), and the spruce budworm (*Choristoneura fumiferana*) from Zheng et al. (2002).

Multiple sequence alignments of deduced amino acid sequences were obtained by using the CLUSTALW (1.4) algorithm (Higgins et al., 1996; Thompson et al., 1994). Phylogenetic trees were generated by the neighbor-joining method of Saitou and Nei (1987), and hydropathy plots were prepared according to Kyte and Doolitle (1982). The two borders between the domains and the linker region were annotated according to the consensus inferred from selected tentative positions described in previous reports (Fig. 6).

RESULTS AND DISCUSSION

In order to screen the chitinase-encoding cDNA, we first amplified a conservative partial core domain (Fig. 1) from the cDNA library constructed from the integumental total RNA of the last instar larvae using a primer pair designed to hybridize the following amino acid motifs, ANFRLME (amino acids 191-197) and DMDDFKG (amino acids 374-380), respectively (Fig. 6). PCR amplification produced an exactly anticipated size of DNA (570 bp) with the correct amino acid sequence to lepidopteran chitinase members, typically characterized by four conservative cystein motifs and one *N*-glycosylation site (NAT) (Shinoda et al., 2001) in the catalytic domain (Fig. 6), although two and three nucleotides (bold) in the primers (Forward, 5′-GCCAA**C**(t)TTT**T**(a)AGGCTGATGGA-3′; Reverse, 5′-**A**(t)CC**T**(c)TTGAAGTC**A**(g)TCCATGT-3′) mismatched with those (lower case) of *M. brassicae* cDNA, respectively. Because the position of the partial core domain is very central in the chitinase ORF, we used the same primers for the RACE PCR, and amplified each reliable size 5′ and 3′ RACE products with corresponding nucleotide sequences, respectively (Fig. 1). After confirming the nucleotide sequence of the 5′ and 3′ endings, we successfully amplified the full-length ORF cDNA using a primer pair starting with the initiation and termination codons to hybridize the very endings of the ORF, respectively.

Insect chitinases have a 40-85 kDa molecular mass range (Kramer and Muthukrishnan, 1997), and previously described lepidopteran chitinases are in a size range of 52-63.4 kDa (Goo et al., 1999; Kim et al., 1998; Kramer et al., 1993; Shinoda et al., 2001). The ORF of chitinase-encoding cDNA isolated from *M. brassicae* was consisted of 1686 nucleotides (562 amino acid residues) (GenBank accession No. JN558350) except for the stop codon, and its deduced amino acid composition revealed a calculated molecular mass of 62.7 kDa and theoretical pI of 5.3. The aligned amino acid sequence (Fig. 6) revealed its characteristic structure as a molting fluid chitinase.

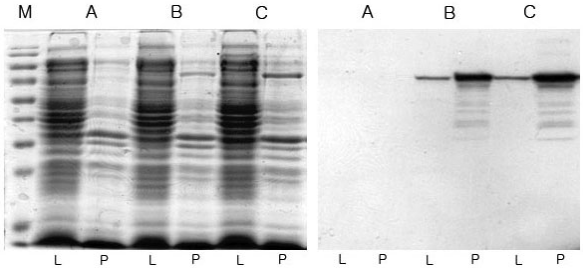

Fig. 3. SDS-PAGE and Western blotting of *M. brassicae* chitinase expressed in *E. coli* grown in media with IPTG. Enzyme protein was isolated from cell lysate (L) and precipitant (P). Line A, negative control with no IPTG; line B, 0.2 mM IPTG; line C, 1 mM IPTG.

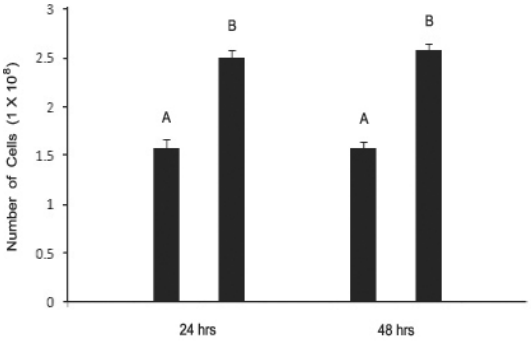

Fig. 4. Growth rate of *E. coli* transformed with *M. brassicae* chitinase-encoding cDNA (B) and wild type *E. coli* strain (A).

We transformed the XL1 *E. coli* strain with the *M. brassicae* chitinase-encoding ORF cDNA inserted in pCR2.1, and confirmed its correct transcription by real-time quantitative PCR (Fig. 2). The reaction for 40 cycles amplified 915 ng/ul of a single PCR product (129 bp) from the total cDNA of *E. coli* transformed with *M. brassicae* chitinase-encoding cDNA, while no amplification was observed in the wild type *E. coli* strain as a negative control. As seen in the results from SDS-PAGE and western blotting (Fig. 3), the expressed *M. brassicae* chitinase was confirmed as a distinct band on the SDS gel in both the cell lysate and precipitant.

The transformed bacterial cells cultured in a 10X-diluted NB liquid medium including colloidal chitin, in which there were only $1/10^{th}$ the amount of available nutrients other than colloidal chitin, compared with regular NB medium, showed about 1.6 times higher growth rate than the untransformed strain through 24 and 48 h of cultivation (Fig. 4). This reflected that the transformants were able to actively consume the included chitin compound using the introduced insect enzyme. The functionality of the expressed chitinase was also demonstrated by the enhanced extracellular and intracellular enzyme activities. Compared with the wild strain, the intracellular levels of glucosamine (GlcN) and *N*-acetylglucosamine (GlcNAc), the chitin degradation derivatives, were about 7.2 and 2.3 times higher, respectively, while the extracellular chitinase secreted by the transformants into the media was about 2.2 times higher (Fig. 5).

Insect chitinases belong to family 18 of the glycohydrolase superfamily, and are characterized by a multidomain structure composed of two major functional domains, *N*-terminal catalytic domain led by a signal peptide and *C*-terminal chitin-binding domain, as well as a linker region connecting the two domains

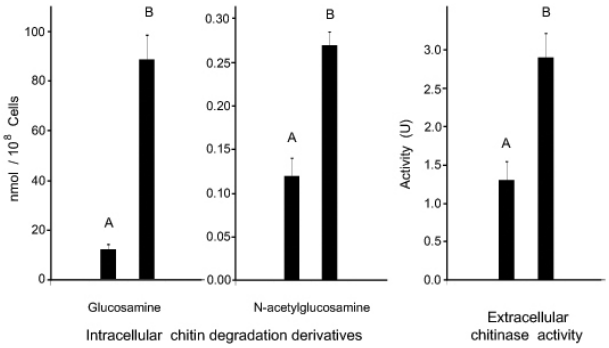

Fig. 5. Intracellular chitin degradation derivative contents and extracellular chitinase activity of *E. coli* transformed with *M. brassicae* chitinase-encoding cDNA (B) and wild type *E. coli* strain (A).

(Kramer and Muthukrishnan, 1997). *N*-terminal of the catalytic domain has a very hydrophobic leading signal peptide consisting of 20 amino acid residues (Figs. 6 and 7), which mediates protein secretion into the endoplasmic reticulum (Müller, 1992; von Heijne, 1990), consistently with the other reported lepidopteran chitinases except for one from *M. sexta,* in which the signal peptide is 19 amino acids long. The final 6 residues (TTAIEA) in the signal peptide are particularly common to all the six noctuid species (*M. brassicae*, *S. litura*, *S. frugiperda*, *H. amigera*, *L. oleracea* and *H. cunea*), while the peptides of three bombycid (*M. sexta*, *B. mandarina* and *B. mori*) and one tortricid (*C. fumiferana*) species ends with only three common residues (VQS).

Compared with the leading signal peptide, the residue composition of the catalytic domain (AA 21-392) is much more highly conserved (Fig. 6). Despite a few variations in the catalytic domain, no significant alterations are observed in the hydropathy plots except for slight reversions in two regions caused by an occurrence of large apolar residues; L (AA 121) in *C. fumiferana*; V (AA 178) in *H. cunea* and *M. sexta* (Fig. 7). Homology in amino acid sequence of *M. brassicae* chitinase to the other lepidopteran enzymes is the highest in the catalytic domain as 92.7% mean identity and 96.1% mean sum of identity and similarity, while all the other parts show lower scores than the homology in the full-length ORF, 83.2% mean identity and 88.0% mean sum of identity and similarity (Table 1). The catalytic domain of eukaryotic chitinases is known to be glycosylated with *N*-linkage. Although some reports have suggested one or two more putative *N*-glycosylation sites in the linker region (Ahmad et al., 2003; Kim et al., 1998; Zheng et al., 2002) and the substrate binding domain (Kramer et al., 1993), the sites did not appear to be common to all the confirmed lepidopteran chitinases. Only the two consensus sites in the catalytic domain (NFT, AA 87-89; NAT, AA 304-306) are confident (Fig. 6).

Carbohydrate conjugation in lepidopteran chitinase is remarkable as approximately 25% of the mass of mature chitinase (85 kDa) in *M. sexta* (Gopalakrishnan et al., 1995; Wang et al., 1996), but it seems that *N*-glycosylation in the catalytic domain contributes only a very small portion, since the linker region, the so-called "PEST-like" because of enriched proline, glutamate, serine and threonine, is extensively *O*-glycosylated on the highly rich serine and threonine residues while the catalytic domain is moderately *N*-glycosylated (Arakane et al., 2003). In *M. brassicae*, the content of the two residues was about 22.6% in the linker region (AA 393-498), and almost all of them were positioned from AA 397 to AA 462, except for two threonines (AA 495 and AA 497), showing high conservatism in the lepi-

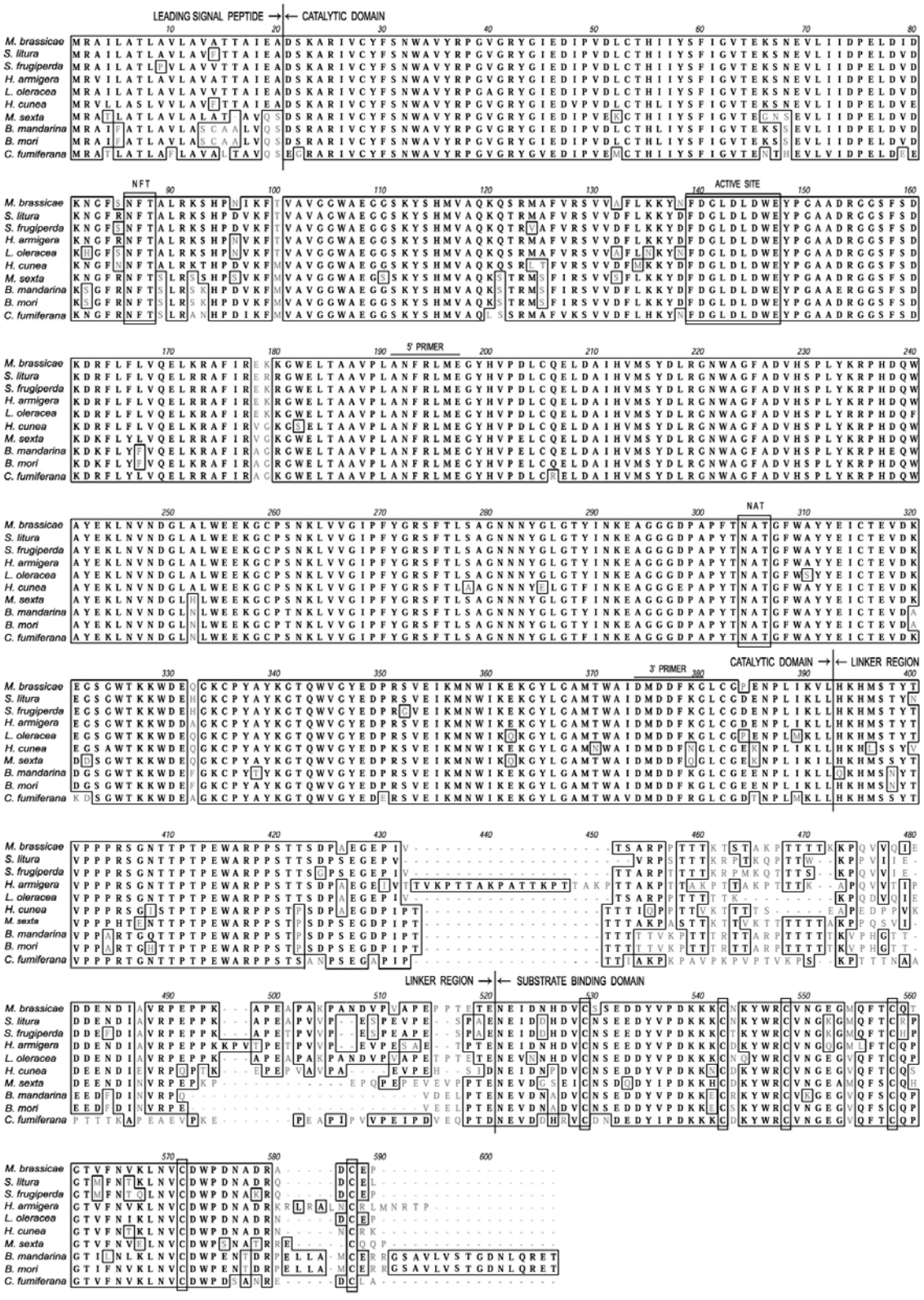

Fig. 6. Alignment of deduced amino acid sequence of *M. brassicae* chitinase-encoding cDNA with previously reported lepidopteran chitinase sequences. The tentative borders between the structural elements are marked with vertical lines and arrows. The active site and two *N*-glycosylation sites in the catalytic domain and 6 cysteine motifs in the chitin-binding domain are boxed. The positions for primers that amplified the partial domain (see in text) are indicated.

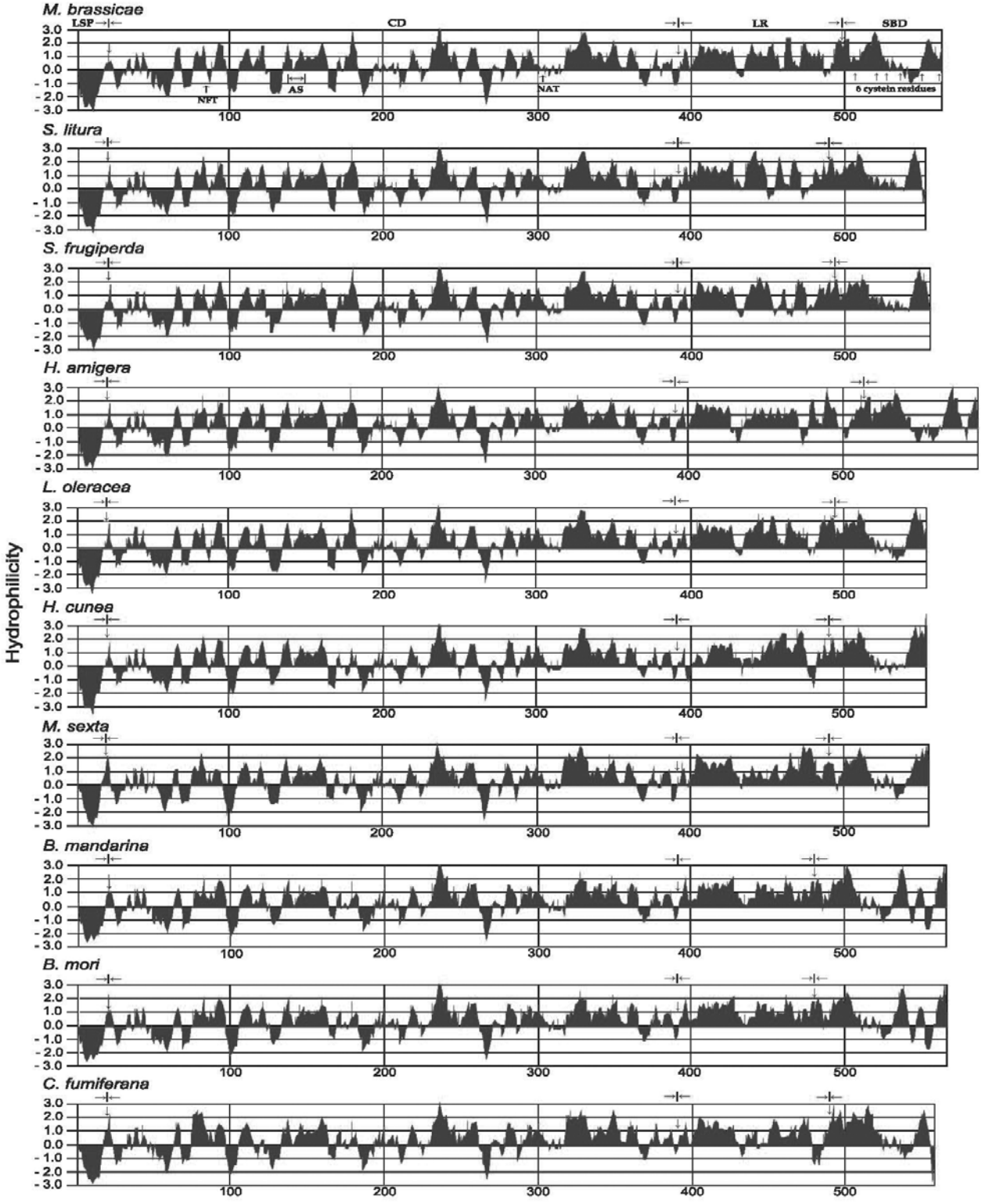

Fig. 7. Kyte-Doolitle hydropathy plots of *M. brassicae* chitinase from this study and previously described lepidopteran chitinases. The tentative borders between the structural elements are marked with vertical lines and arrows; (LSP) leading signal peptide, (CD) cata-lytic domain, (LR) linker region, and (SBD) substrate-binding domain. The active site (AS) and two *N*-glycosylation sites (NFT and NAT) in catalytic domain and 6 cysteine motifs in chitin-binding domain are also indicated.

Table 1. Percent identities (sum of identities and similarities) in deduced amino acid sequence of chitinase-encoding cDNA isolated from *Mamestra brassicae* to previously described lepidopteran chitinases

Species	ORF	Leading signal peptide	Catalytic domain	Linker region	Substrate binding domain
S. litura	88 (91)	90 (90)	95 (97)	68 (70)	84 (88)
S. frugiperda	89 (92)	85 (85)	96 (98)	74 (77)	81 (88)
H. armigera	85 (87)	100 (100)	96 (98)	62 (69)	72 (74)
L. oleracea	93 (95)	90 (90)	96 (98)	89 (89)	87 (93)
H. cunea	82 (87)	80 (90)	92 (96)	50 (60)	82 (85)
M. sexta	79 (86)	65 (85)	90 (95)	51 (63)	66 (81)
B. mandarina	77 (84)	50 (80)	90 (95)	48 (59)	55 (63)
B. mori	78 (85)	50 (80)	91 (95)	49 (59)	58 (65)
C. fumiferana	78 (85)	65 (80)	88 (93)	43 (53)	75 (82)
Mean	83.2 (88.0)	75.0 (86.7)	92.7 (96.1)	59.3 (66.6)	73.3 (79.9)

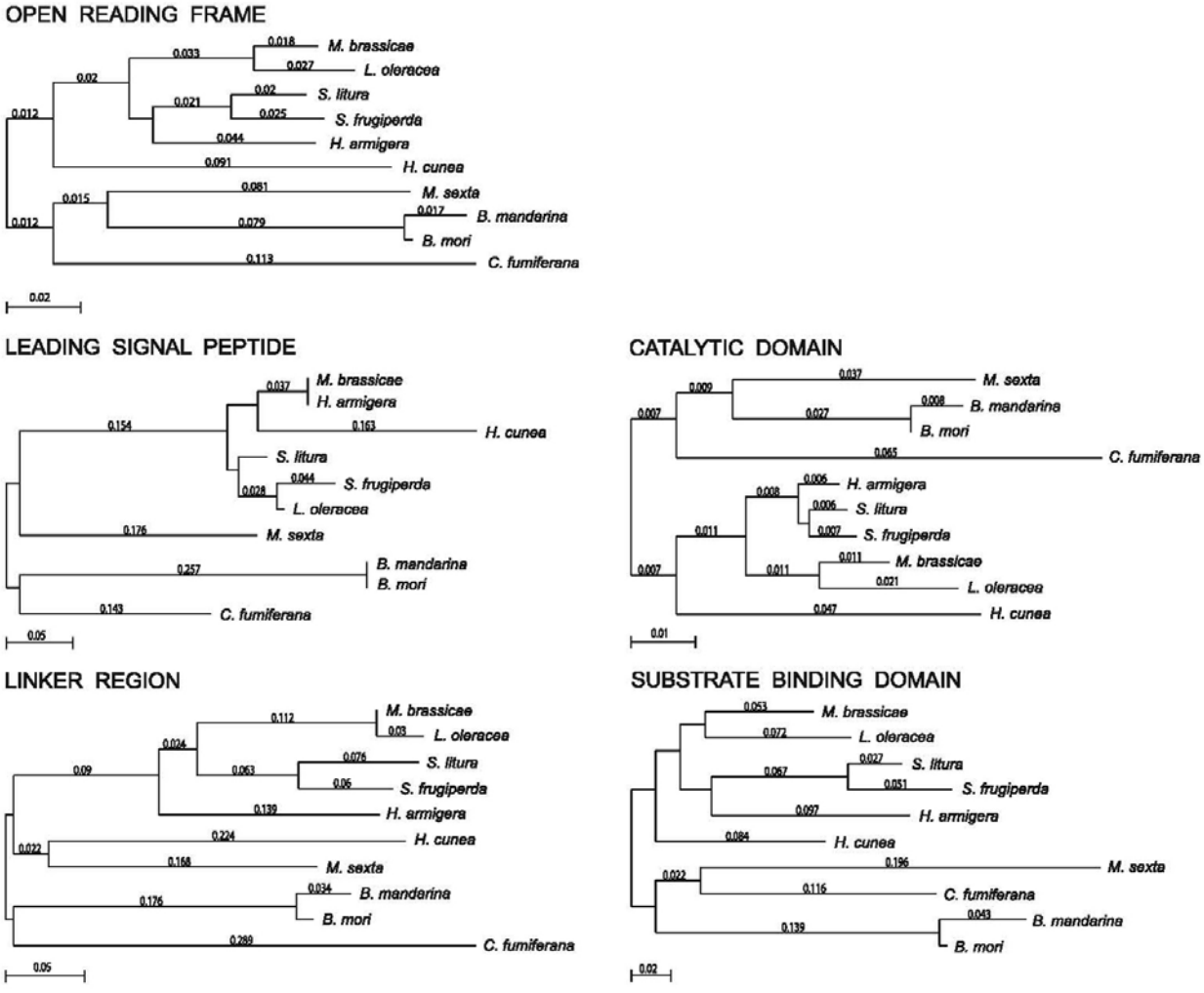

Fig. 8. Consensus bootstrap trees showing homology relationships in amino acid sequence between lepidopteran chitinases. The numbers above the lines are branch lengths.

dopteran chitinase group located on the very *N*-terminal ending of the linker region (Fig. 6). The linker region not only connects the two domains, but also plays a role in the facilitation of enzyme secretion with a high amount of conjugated carbohydrate

(Gopalakrishnan et al., 1995; Zheng et al., 2002). Although the linker region was the most divergent in amino acid sequence among the lepidopteran chitinases, 59.3% mean identity and 66.6% mean sum of identity and similarity (Table 1), the *O*-glycosylation-bearing serine and threonine residues were in consensus between the aligned sequences (Fig. 6).

We indicated borders between the catalytic domain, the linker region (AA 393-498) and the chitin-binding domain (AA 499-562) according to the consensus from selected tentative positions described in previous reports (see in "Materials and Methods", Figs. 6 and 7). The one between the catalytic domain and the linker region was suitably recognized by the significant hydrophobic nature of the *N*-terminal of the catalytic domain with a high apolar residue content and by comparatively less hydrophobic or basic residues on the *C*-terminal of the linker region, while the border between the linker region and the chitin binding domain was not distinguishable in the hydropathy plots because of no strike change in polarity through the end of the linker region and the beginning of the substrate binding domain. This provisional catalytic domain-linker region border, however, was not definitive because its location was inside an exon (Exon 7), while the linker region-substrate binding domain border was more reliable because it located just one amino acid after an intron (Intron 8) position (Choi et al., 1997; Shinoda et al., 2001). The chitin-binding domain has a typical 6-cysteine motif structure to the chitin-binding type-2 peptides extensively found in animal proteins such as insect chitinase, peritrophin and mucin, and in mammalian chitinases (Gains et al., 2003; Tjoelker et al., 2000). It has been observed that the catalytic domain can degrade the substrate alone, but the activity dramatically decreased to just one-fourth of the full-length enzyme equipped with its chitin-binding domain (Wang et al., 1996).

We analyzed the homology relationships in the deduced amino acid sequence of each of the structural elements as well as the ORF between the 10 lepidopteran chitinases including one from this work (Fig. 8). In the ORF sequence, the 6 enzymes from noctuid species (*M. brassicae, L. oleracea, S. litura, S. frugiperda, H. armigera* and *H. cunea*) were grouped together, separately from a group of 4 enzymes from 3 bombycid (*M. sexta, B. mandarina* and *B. mori*) and 1 tortricid species (*C. fumiferana*), corresponding to their taxonomic phylogenetic relationships at both the family and genus levels. The catalytic domain showed exactly the same relationship as the ORF, while for the other three fractions the 6 noctuids mapped together similar to the ORF and the catalytic domain, but *M. sexta* mapped together with *C. fumiferana* onto a lineage apart from the two *Bombyx* species with dramatically longer *C*-terminals in the chitin-binding domain (Fig. 6), and joined the noctuid group in the signal peptide and the linker region.

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