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Cloning and characterization of ribonuclease T2 gene (*RNHe30*) from the basidiomycete, *Hericium erinaceum*

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ABSTRACT

A gene encoding a ribonuclease T2 (RNase T2) family enzyme, *RNHe30*, was cloned from *Hericium erinaceum* by PCR. The deduced amino acid sequence from the complimentary DNA (cDNA) (1074 bp) encodes a 302-aa protein (RNase He30) that has the consensus amino acid sequences of RNase T2 family enzymes including the putative signal peptide. The presence of five introns in the genomic DNA was confirmed by comparison of the cDNA and genomic DNA sequences. The promoter region contains a putative CAAT box and a consensus TATA box. Genes coding homologous enzymes were also identified in various other basidiomycetes. A phylogenetic tree of RNase T2s from these fungi was constructed from a multiple alignment of the deduced amino acid sequences. The tree showed that the enzymes were divided into two main groups.

1. Introduction

Ribonuclease T2 (RNase T2) family enzymes, represented by RNase T2 from *Aspergillus oryzae*, are 2′,3′-cyclic RNases that form 3′-nucleotides through 2′,3′-cyclizing (Sato and Egami 1957). Enzymes of this family have been widely detected in various groups including animals, plants, protozoa, fungi, bacteria, and viruses. This indicates that the enzyme is ubiquitous among various organisms (Irie 1997, 1999).

The primary structures of these RNases from various sources have been elucidated. The enzyme is constituted from a monomer protein with a molecular size of 20–30 kDa. The amino acid sequences around two His residues, which constitute the active center, are well conserved (**C**onserved **A**ctive **S**ite sequence 1 and CAS2) (Irie 1997, 1999). Although protein chemistry studies have been made, the functions of the enzyme *in vivo* have yet to be clarified. It has been reported that the enzyme activity increases under phosphate starvation in plants and fungi (Taylor et al. 1993; Tasaki et al. 2004), and during senescence in higher plants and algae (Bariola et al. 1994; Shimizu et al. 2001). The involvement in exogenous RNA digestion was stated as a common function in slime mold (Inokuchi et al. 1998). A housekeeping role was proposed for isozymes from an animal and a plant (Hillwig et al. 2009; MacIntosh et al. 2010). On the other hand, the enzyme from *Aspergillus niger* was reported to have antiangiogenic and anticarcinogenic function unrelated to its ribonuclease activity (Roiz et al. 2006). These properties of the fungal enzyme have attracted attention for medical applications (Schwartz et al. 2007).

Although RNase T2 family enzymes are regarded as ubiquitous (Irie 1997, 1999), the number of investigations on the enzyme in fungi has been limited. Enzymes from Zygomycota: *Rhizopus niveus* (Horiuchi et al. 1988), Ascomycota: *A. oryzae*

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(Sato and Egami 1957), *Trichoderma viride* (Inada et al. 1991), and *Saccharomyces cerevisiae* (MacIntosh et al. 2001), and Basidiomycota: *Lentinula edodes* (Kobayashi et al. 1992; Kobayashi et al. 1998), *Irpex lacteus* (Watanabe et al. 1995), and *Pholiota nameko* (Tasaki et al. 2004), have been identified. *Lentinula edodes*, and *I. lacteus* were reported to produce isozymes of the enzyme (Kobayashi et al. 1992; Watanabe et al. 1995; Kobayashi et al. 1998). The RNases Le2 and Le37 from *L. edodes* were isolated from the fruit body and the culture medium, respectively (Kobayashi et al. 1992; Kobayashi et al. 1998). They have a homologous primary structure in their active domain; however, the RNase Le37 has a unique structure on its C-terminal side (Inokuchi et al. 2000; Kobayashi et al. 2000; Kobayashi et al. 2003). The existence of these distinct enzymes might mean that the enzyme in the basidiomycetes could be richly varied.

Hericium erinaceum is an edible and medicinal mushroom in oriental countries including Japan and China and has attracted a great deal of attention because of its many biologically active compounds (Yang et al. 2003). Although proteomics on this fungus have been reported (Horie et al. 2008), biochemical investigations have only just started. We chose to investigate *H. erinaceum* to collect fundamental information on RNase T2 family enzymes from basidiomycetes. In this study we have identified two genes encoding RNase T2s from *H. erinaceum*, *RNHe38* and *RNHe30*. *RNHe30* was found to encode a new type of the enzyme, the details of which are reported in this paper.

2. Materials and methods

2.1. Materials

Hericium erinaceum was purchased from Nagano Kida Industries (Nagano, Japan). The mycelium was grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) for 30 days at 25 °C with agitation at 100 rpm. *Agaricus bisporus*, *Flammulina velutipes*, *Grifola frondosa*, *P. nameko*, and *Pleurotus ostreatus* were bought from a local market. *Ganoderma lucidum* was obtained from Mori & Company (Gunma, Japan). *Lenzites betulinus* was harvested at Komabano Park (Tokyo, Japan). Fruit bodies or mycelia were frozen in liquid nitrogen and pulverized with a mortar and pestle. The ground samples were kept frozen at −80 °C for later use in total RNA and genomic DNA extraction. PCR primers were obtained from Hokkaido System Science (Hokkaido, Japan). They are listed in Table 1.

Table 1 – Primer sequence used for RT-PCR, 3′ RACE, 5′ RACE, genomic DNA analysis including inverse PCR, and semi-quantitative RT-PCR.

Primer name	Sequence
RT-PCR	
HIK-18VN	5′-ATGGTAGCCTGCGATTAGACTCTCACG AdT_{18}VN-3′
HIK-17F	5′-TGGACIATICAYGGIYTITGGCCIGAYAA-3′
HTN-217R	5′-ICCRTCIATIATIGAICCYTTIARRTTRAA RTACCA-3′
RN30-89F	5′-TGGACIATICAYGGIYTITGGCCIGAYAAYT GYGAYGG-3′
RN30-166R	5′-CCRTARCAIGCIACRTCRAAIGTISWIGTRC AIGTIGCRTGYTT-3′
3′ RACE, and 5′ RACE	
HIK-ad1	5′-ATGGTAGCCTGCGATTAGACTCTCA-3′
cHe30-161F	5′-TGCACCTCGACCTTTGACGTCGCTTGCTAT-3′
cHe38-205F	5′-CCAGCCTTGAACTGTAACGGCAACACTGTC-3′
cHe30-161R	5′-ATAGCAAGCGACGTCAAAGGTCGAGGTGCA-3′
cHe30-181R	5′-AGCACGGACGACAGCATCGTAGAAATCAAC-3′
cHe38-169R	5′-CTTGACCACGGTCTGGTAGAAAGCGACCGC-3′
cHe38-205R	5′-GACAGTGTTGCCGTTACAGTTCAAGGCTGG-3′
Genomic DNA analysis and inverse PCR	
iHe30-1R	5′-ACAGGCGGCGATGTTCGGGAAAGTGTTGGG-3′
iHe30-34F	5′-CCAGGAGGGCTTGTTCTGCAGACTCAGTTC-3′
cHe30+68F	5′-CAGATCGCATGGTCCGAAAACTCACCCCGA-3′
cHe30+52F	5′-CGTCGTGGACGAAGCTTCTTCTCCCCTGGC-3′
cHe30-360R	5′-CATGATCACCGGTCGGGCTGTACGTGTAAA-3′
Semi-quantitative RT-PCR	
cHe30-176F	5′-CCCACCTGGACTATGCTCGCTGCCTCTGGC-3′
cHe30-270R	5′-GGCGAACGCTCATAGTAATGGATGCCAGTC-3′
cHe38-205F	5′-CCAGCCTTGAACTGTAACGGCAACACTGTC-3′
cHe38-330R	5′-GTTGCTACCAGACGTCACCGCAGAGAACGT-3′

N = A, C, G, T; R = A, G; S = C, G; V = A, C, G; W = A, T; Y = C, T.

2.2. Isolation of nucleic acids

Total RNA was extracted with TRIZOL (Invitrogen, Carlsbad, CA) reagent following the manufacturer's instructions. The RNA was used for first-strand complimentary DNA (cDNA) synthesis. Genomic DNA for PCR was isolated with the ISOPLANT kit from Nippon gene (Tokyo, Japan).

2.3. First-strand cDNA synthesis, RT-PCR, 3′ and 5′ RACE

First-strand cDNA for RT-PCR (reverse transcription polymerase chain reaction), 3′ RACE (rapid amplification of cDNA ends) and semi-quantitative RT-PCR was synthesized by Super Script II reverse transcriptase (Invitrogen) with an oligo dT primer, HIK-18VN (Chenchik et al. 1996). Consensus amino acid sequences (WTIHGLWPDN and WYFNLKGSIIDG) were obtained from previously characterized RNase T2-type enzymes from *L. edodes* and *I. lacteus* (Kobayashi et al. 2003). A pair of degenerate oligonucleotide primers (HIK-17F and HTN-217R) were designed for RT-PCR based on these sequences. Although this primer set was used as a starting point for our research, they had to be redesigned to obtain genes homologous to *RNHe30* from various other basidiomycetes. This second pair of degenerate oligonucleotide primers was named RN30-89F and RN30-166R. The details of the primer design are described in the Results and discussion section.

The sequences of the 3′- and 5′-ends of the cDNA were analyzed by RACE (Frohman et al. 1988). 3′ RACE was performed using cHe30-161F for *RNHe30* and cHe38-205F for *RNHe38* with HIK-ad1, an adapter primer for HIK-18VN. 5′ RACE was performed with the System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen) following the instructions provided. The 5′ RACE Abridged Anchor Primer (AAP), and the Abridged Universal Amplification Primer (AUAP), an adapter primer for AAP, are components of the kit. The gene-specific primers cHe30-360R for *RNHe30* and cHe38-205R for *RNHe38* were used to synthesize first-strand cDNA for 5′ RACE. Primary PCR was performed using cHe30-181R for *RNHe30* and cHe38-205R for *RNHe38* with the AAP anchor primer.

Table 2 – The list of the genes cited in this paper.

Species	Gene name	Gene product name	Accession no. mRNA (gene)	Molecular mass[b]
Agaricus bisporus	*RNAb30*	RNase Ab30	AB200874[a] (AB200873)[a]	34.5 kDa[c]
	RNAb38	RNase Ab38	AB125057[a]	42.3 kDa
Flammulina velutipes	*RNFv30*	RNase Fv30	AB558529[a] (AB558530)[a]	34.7 kDa[c]
Ganoderma lucidum	*RNGl30*	RNase Gl30	AB330059[a] (AB330060)[a]	33.5 kDa[c]
Grifola frondosa	*RNGf30*	RNase Gf30	AB233350[a] (AB252503)[a]	33.8 kDa[c]
	RNGf29	RNase Gf29	AB164634[a]	42.2 kDa
Hericium erinaceum	*RNHe30*	RNase He30	AB093578[a] (AB120659)[a]	33.6 kDa[c]
	RNHe38	RNase He38	AB093579[a] (AB164395)[a]	42.0 kDa
Laccaria bicolor			LACBIDRAFT_235838	29.7 kDa
			LACBIDRAFT_186136	40.6 kDa
			LACBIDRAFT_187062	33.9 kDa[c]
Lentinula edodes	*RNLe37*	RNase Le37	AB101668[a] (AB669029)[a]	43.8 kDa
	RNLe2	RNase Le2	AB668999[a]	28.5 kDa
Lenzites betulinus	*RNLb32*	RNase Lb32	AB370193[a]	32.1 kDa
	RNLb30	RNase Lb30	AB438023[a] (AB438204)[a]	33.8 kDa[c]
Pholiota nameko	*RNPn30*	RNase Pn30	AB095076[a] (AB180667)[a]	34.0 kDa[c]
Pleurotus eryngii	*RNPe38*	RNase Pe38	AB124637[a]	41.4 kDa
Pleurotus ostreatus	*RNPo30*	RNase Po30	AB180239[a] (AB180238)[a]	34.0 kDa[c]
	RNPo28	RNase Po28	AB175277[a]	31.9 kDa
	RNPo38	RNase Po38	AB177387[a]	41.5 kDa

a Genes cloned by authors.
b Molecular mass calculated from the deduced amino acid sequence.
c RNase homologous to RNase H30.

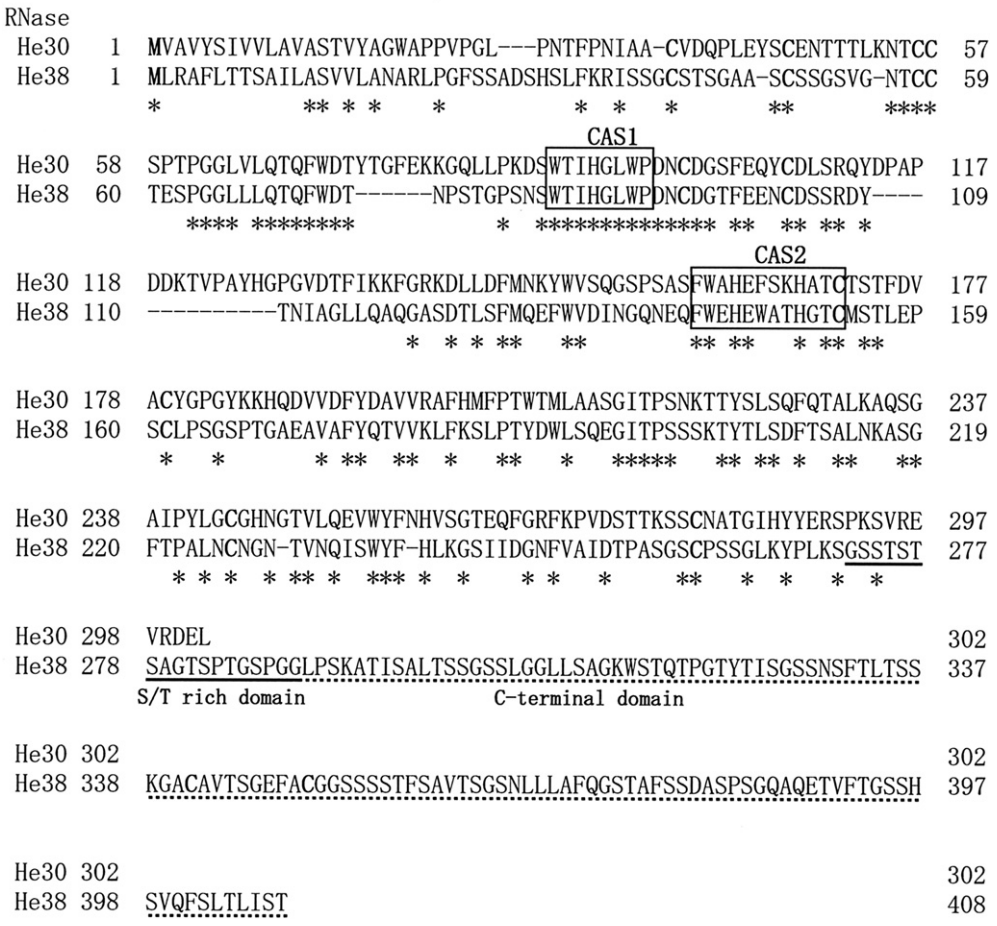

Fig. 1 – The deduced amino acid sequence comparison of RNase He30 and RNase He38. Amino acid sequences representing catalytic domains (CAS1 and CAS2) are shown in boxes. Conserved cysteine residues are indicated with bold letters. Dashed lines (–) indicate gaps introduced to maximize the identity. Small asterisks (*) indicate amino acid residues identical in both sequences. The sequences with an underline — and a dotted underline ····· indicate the positions of an S/T-rich domain and the C-terminal domain, respectively.

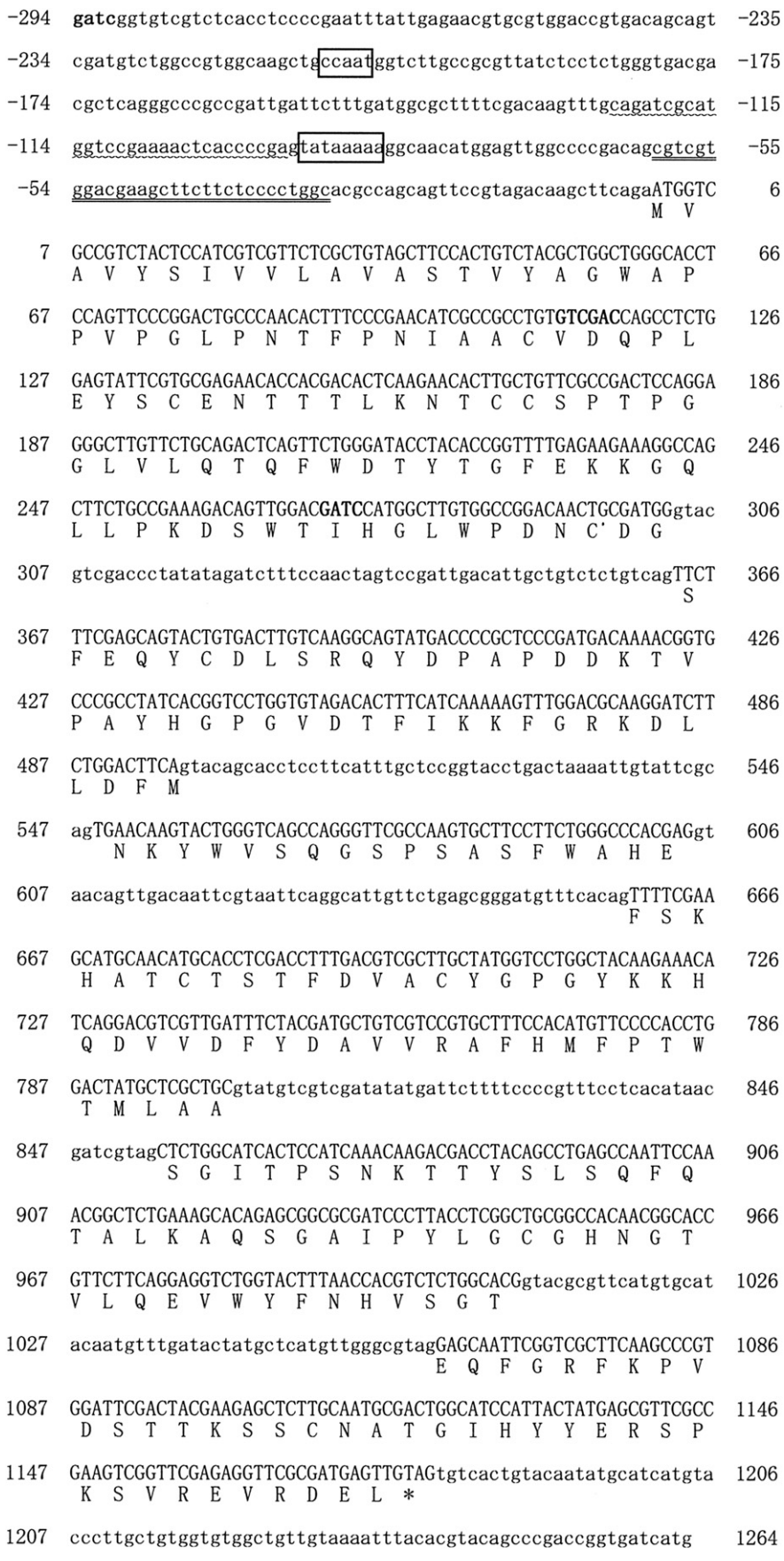

Fig. 2 — Nucleotide and deduced amino acid sequences of the *RNHe30* gene. Exons are indicated with capital letters. Introns are indicated with lowercase letters. The deduced amino acid sequence is shown below the respective codons. The deduced protein biosynthesis termination point is indicated by an asterisk (*). A putative CAAT box at −211 bp and a consensus TATA-like sequence at −93 bp are in boxes. The sequences highlighted a wavy underline ~ at −124 bp and a double underline = at −60 bp indicate the positions of the cHe30+68F and cHe30+52F primers, respectively, used to estimate the transcription start site. Restriction enzyme sites used for inverse PCR are indicated with bold letters (*Sal*I at 112 bp and *Sau*3AI at −294 and 270 bp).

Secondary PCR for further amplification was performed using cHe30-161R or cHe38-169R with the AUAP adapter primer.

2.4. PCR conditions and DNA sequencing analysis

Ex-Taq DNA polymerase for PCR was obtained from Takara Bio (Shiga, Japan). PCR conditions were as follows: an initial incubation of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 50 °C or 60 °C for 30 s, 72 °C for 30 s; and a final extension period of 72 °C for 3 min (Saiki et al. 1985; Mullis and Faloona 1987). The annealing temperature was 50 °C for RT-PCR, and 60 °C for 3′ RACE, 5′ RACE, and genomic DNA analysis including inverse PCR.

PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide (EtBr) staining. DNA was excised from the gel and purified with a GENECLEAN II Kit (BIO 101, Vista, CA). PCR product cloning was performed with a TOPO TA Cloning kit (Invitrogen). Plasmid DNA was isolated with a QIAprep spin miniprep kit (QIAGEN Sciences, Germantown, MD). DNA sequences were determined by a CEQ 8000 genetic analyzer (Beckmann Coulter, Fullerton, CA) using the Dye Terminator Cycle Sequencing Quick Start Kit (Beckmann Coulter) following the manufacturer's instructions.

2.5. Semi-quantitative RT-PCR

The first-strand cDNA synthesized with HIK-18VN was used for semi-quantitative RT-PCR. The primer sets for the RT-PCR were designed to produce different sized fragments, of 263 bp for *RNHe30* (cHe30-176F with cHe30-270R) and 435 bp for *RNHe38* (cHe38-205F with cHe38-330R), to distinguish them. Three sets of reaction mixtures containing first-strand cDNA with the primer sets for *RNHe30* and *RNHe38* were prepared to compare the degree of expression by applying different PCR cycle numbers (20, 25 or 30). PCR conditions were as follows: an initial incubation of 94 °C for 5 min; 20, 25 or 30 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. Mineral oil was loaded onto the mixture to avoid volume change by evaporation during the PCR procedure. The PCR products were analyzed by 2% agarose gel electrophoresis with EtBr staining. Densitometry was analyzed by Gel Doc 2000 with image analysis software Quantity One Version 4.3.0 (Bio-Rad Laboratories, Hercules, CA).

2.6. Genomic DNA analysis and inverse PCR

Primers for genomic DNA amplification of *RNHe30* were designed with the information obtained from 5′ and 3′ RACE (cHe30+52F with cHe30-360R). The sequence outside the boundaries of the known genomic upstream region was determined by inverse PCR (Ochman et al. 1988; Triglia et al. 1988; Silver and Keerikatte 1989). Genomic DNA was cleaved with *Sau*3AI, and then self-ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA). The ligated circular DNA was digested and linearized with *Sal*I. The resulting fragment was amplified by PCR with the iHe30-1R and iHe30-34F primers.

An estimation of the transcription start site on the *RNHe30* gene was made using RT-PCR. Two primer sets were used, cHe30+68F with cHe30-360R and cHe30+52F with cHe30-360R. PCR products were detected by 1.8% agarose gel electrophoresis with EtBr staining.

2.7. Sequence alignment and phylogenetic analysis

Phylogenetic analysis of RNase amino acid sequences was conducted using Maximum Likelihood. MAFFT ver. 6 (Katoh and Toh 2008) was used for preliminary multiple alignment of nucleotide sequences. The final alignment was manually adjusted using BioEdit (Hall 1999). Alignment gaps were treated as missing data, and ambiguous positions were excluded from the analysis. The Whelan and Goldman model (Whelan and Goldman 2001) was selected as the best-fit evolutionary model using MEGA5 (Tamura et al. 2011). The model was used to construct phylogenetic trees using the same software. In the Maximum Likelihood analysis, the percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows: when the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise the BIONJ method with a maximum composite likelihood distance matrix was used (Gascuel 1997). Node support was tested by bootstrap analysis (Felsenstein 1985) using 100 replications.

3. Results and discussion

3.1. Isolation of genes encoding RNase T2 enzymes from Hericium erinaceum

A DNA fragment approximately 500 bp in size was amplified by RT-PCR using a pair of degenerate oligonucleotide primers (HIK-17F and HTN-217R). DNA sequence analysis revealed the existence of two different genes encoding RNase T2 enzymes. Primers for analysis of the unknown sequences at the 3′- and 5′-ends of the mRNA by 3′ and 5′ RACE were designed from the sequence data. The full-length cDNA sequences obtained were designated *RNHe30* and *RNHe38* (Table 2). A putative signal peptide starting with methionine was deduced from the results of 5′ RACE. *RNHe30* and *RNHe38* encoded proteins of 302 (RNase He30) and 408 (RNase He38) amino acids, including the putative signal peptide (Fig. 1). Both had CAS1 and CAS2 sequences, which are characteristic of RNase T2 family enzymes (Irie 1997, 1999). Amino acid residues that are important for catalytic function were present in these segments, including His92, His163, His168, and Glu164 in RNase He30, and His88, His145, His150, and Glu146 in RNase He38. Both proteins also possessed 10 half-cystine residues, which are common in fungal RNases. Although RNase He30 and RNase He38 had homologous primary structures, RNase He38 had a distinctive structure on its C-terminal side (Fig. 1). Since the structure could be regarded as an S/T-rich domain and was similar to the C-terminal domain of RNase Le37 (Kobayashi et al. 2003), RNase He38 was classified as an RNase Le37-type enzyme. Because RNase He30 did not belong to any known type of the enzyme from basidiomycetes, we were interested to investigate *RHHe30* further.

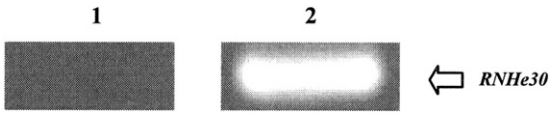

Fig. 3 – An estimation of the transcription start site. RT-PCR was performed with the primer combinations, 1: cHe30+68F and cHe30-360R, and 2: cHe30+52F and cHe30-360R.

3.2. *Structure of the* RNHe30 *gene*

The sequences of the 5′- and 3′-ends of the cDNA (*RNHe30*) were used to make primers for genomic DNA amplification (cHe30+52F and cHe30-360R) by PCR. Inverse PCR was used to amplify further upstream of the 5′-flanking region. The genomic DNA sequence (1558 bp) was determined (Fig. 2) by compiling the data. The presence of five introns in the gene encoding RNase He30 was confirmed by comparison of the cDNA and genomic DNA sequences. The sequences of the exons in the genomic DNA completely corresponded to the cDNA sequence. All introns obeyed the GT-AG rule (Breathnach et al. 1978; Mount 1982).

The 5′-untranslated region of the *RNHe30* gene showed several features that are typical of a lower eukaryotic promoter (Gurr et al. 1987). The predicted promoter region contained a putative CAAT box at −211 bp and a consensus TATA-like sequence at −93 bp. Translation of most fungal genes begins at the first ATG with a consensus A nucleotide at the −3 position occurring at an 83% frequency (Gurr et al. 1987). The nucleotide at the −3 position of the *RNHe30* open reading frame was found to be an A.

An estimation of the transcription start site was made using two different PCR primers (cHe30+68F and cHe30+52F), as indicated in Fig. 2. The 5′-end of cHe30+52F at −60 bp corresponded to the 5′ RACE extended end and cHe30+68F was located before the consensus TATA-like sequence. Both cHe30+68F and cHe30+52F were used with cHe30-360R for RT-PCR to attempt to amplify a full-length cDNA using the single-stranded cDNA (Fig. 3). A full-length cDNA band (1053 bp) was observed on the gel with the combination of cHe30+52F and cHe30-360R, but amplification was not detected with cHe30+68F and cHe30-360R. This result implies that the transcriptional start site of *RNHe30* is likely to be located around the putative TATA-like sequence.

3.3. *Expression ratio of* RNHe30 *to* RNHe38

The transcription ratio of *RNHe30* to *RNHe38* was estimated by semi-quantitative RT-PCR. Total RNA was isolated from fruit bodies and mycelia cultured in YPD medium. A comparison was made by varying the cycle numbers of the PCR. The density of the *RNHe30* band versus *RNHe38* was approximately 1:3 using RNA from the fruit body at 25 cycles (Fig. 4). Conversely, *RNHe38* was transcribed at extremely low levels compared to *RNHe30* in the mycelia; only a trace of band was detected for *RNHe38* even at 30 cycles. These data show that the two genes do not share a common induction pathway and that the enzymes encoded by these genes should be involved in different metabolic functions.

Although these results are limited, the involvement of the enzyme coded by *RNHe30* in a housekeeping role is a strong possibility. Detailed studies on *RNHe30*, including the 5′-flanking region, are required to elucidate the function of the enzyme.

3.4. *Cloning of* RNHe30 *homologs from various basidiomycetes*

Pleurotus ostreatus was used to obtain an *RNHe30*-type gene following *H. erinaceum*. The primer set HIK-17F and HTN-217R was used for RT-PCR as in the *RNHe30* cloning. The full-length cDNA and genomic DNA sequences were also determined by applying the same procedures used for *RNHe30* cloning. The nucleotide sequence obtained was designated *RNPo30* (Table 2). Consensus amino acid sequences (WTIHGLWPDNCDG and KHATCTSTFDVACYG) were obtained from the deduced amino acid alignment of *RNHe30* and *RNPo30*. A pair of degenerate oligonucleotide primers, RN30-89F and RN30-166R, were designed from the above mentioned consensus sequences, respectively. This primer set was used to obtain *RNHe30*-type genes by RT-PCR from other basidiomycetes.

Genes homologous to *RNHe30* were isolated from *A. bisporus*, *F. velutipes*, *G. lucidum*, *G. frondosa*, *L. betulinus*, and *P. nameko*, and *P. ostreatus*. They were named *RNAb30*, *RNFv30*, *RNGl30*, *RNGf30*, *RNLb30*, *RNPn30*, and *RNPo30*, respectively (Table 2). Their deduced amino acid sequences and intron positions were well conserved (Fig. 5). They were all composed of about 300 amino acids (34–35 kDa), and had the CAS1 and CAS2 sequences and 10 half-cystine residues common to fungal RNase T2 family enzymes (Irie 1997, 1999). The eminent difference was a deletion of six amino acids at the position of Thr121–Val122 in RNase He30.

Since the deduced amino acid sequences are the only information available, the position of the signal peptide processing point remains unclear. This point is assumed to be located between Met1 and Cys37 in RNase He30, since Cys37 is the first well conserved Cys found in mature ribonuclease T2

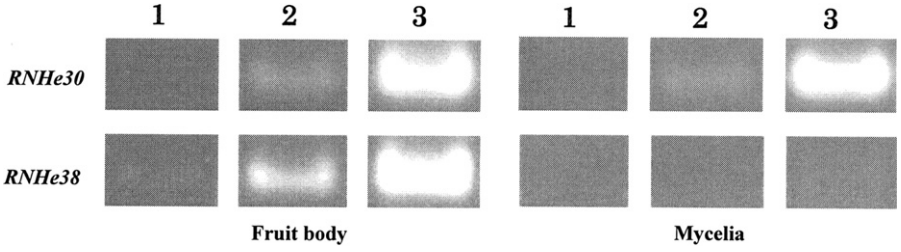

Fig. 4 – Determination of the expression ratio of *RNHe30* to *RNHe38* in fruit bodies and mycelia by semi-quantitative RT-PCR. The comparison was made by altering the PCR cycle numbers (Lane 1, 20 cycles; Lane 2, 25 cycles; Lane 3, 30 cycles).

family enzymes from fungi (Irie and Ohgi 1998; Kobayashi et al. 2003). Since the sequence Pro28-Asn-Thr-Phe-Pro32 is the cluster that aligned well before Cys37, the signal peptide processing point might be right before or around Pro28, assuming that they have similar N-terminal sequence lengths (30–40 amino acids) as in the case of RNase Le37, RNase Le2, and RNase Irp3 (Kobayashi et al. 2003). Sequence homology was not observed in the Met1–Leu27 region (the prospective

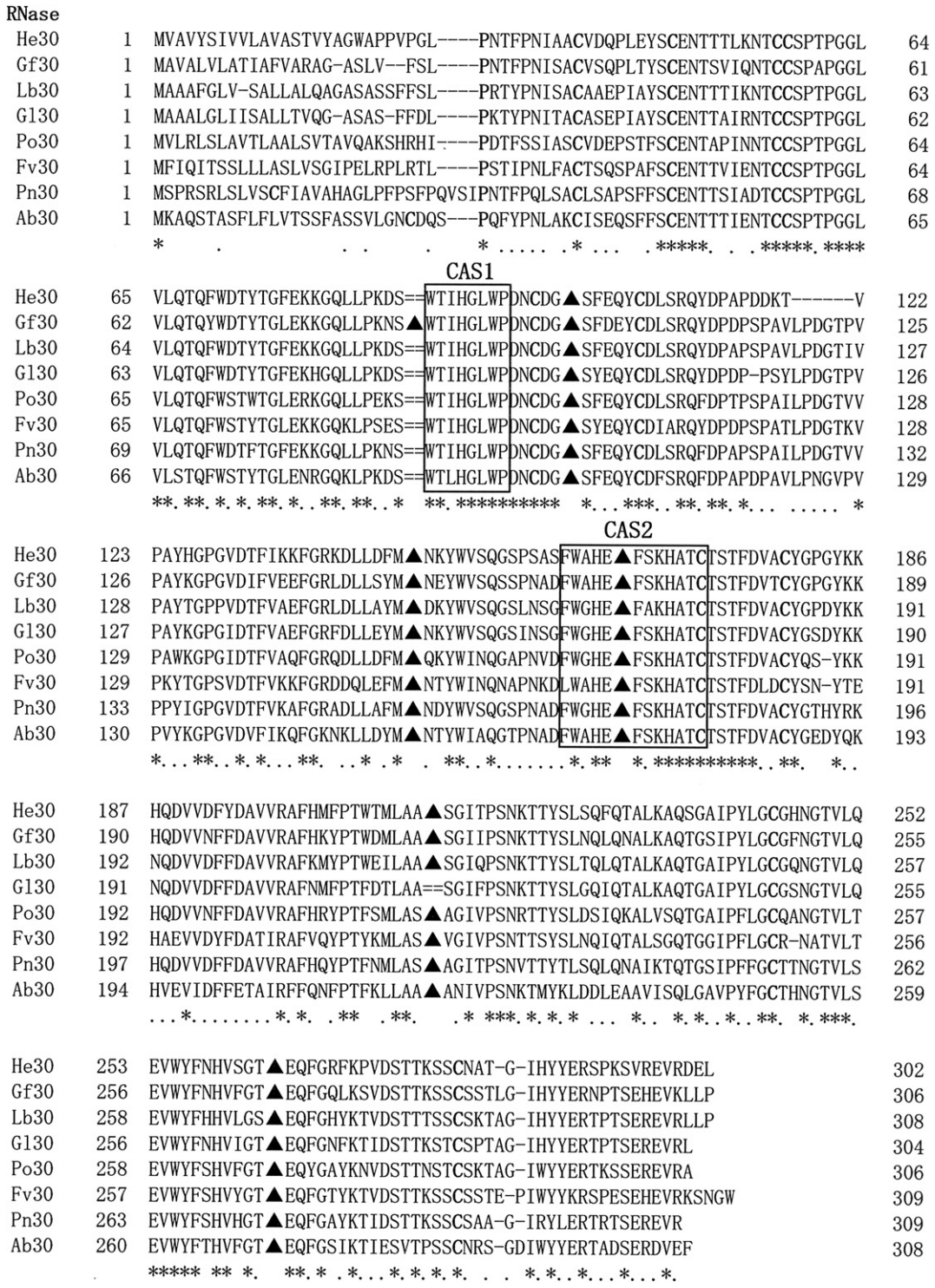

Fig. 5 – The deduced amino acid sequence alignment from full-length cDNAs homologous to *RNHe30* isolated from various basidiomycetes. The sequences representing catalytic domains are shown in boxes. A proline residue of the predicted signal peptide processing point and conserved cysteine residues are indicated with bold letters. Dashed lines (–) indicate gaps introduced to maximize the identity. Small asterisks (*) and dots (.) indicate amino acid residues 100% and more than 50% identical among the sequences, respectively. The positions of introns are indicated by triangles (▲). Equal signs (= =) are placed in cases where no intron exists.

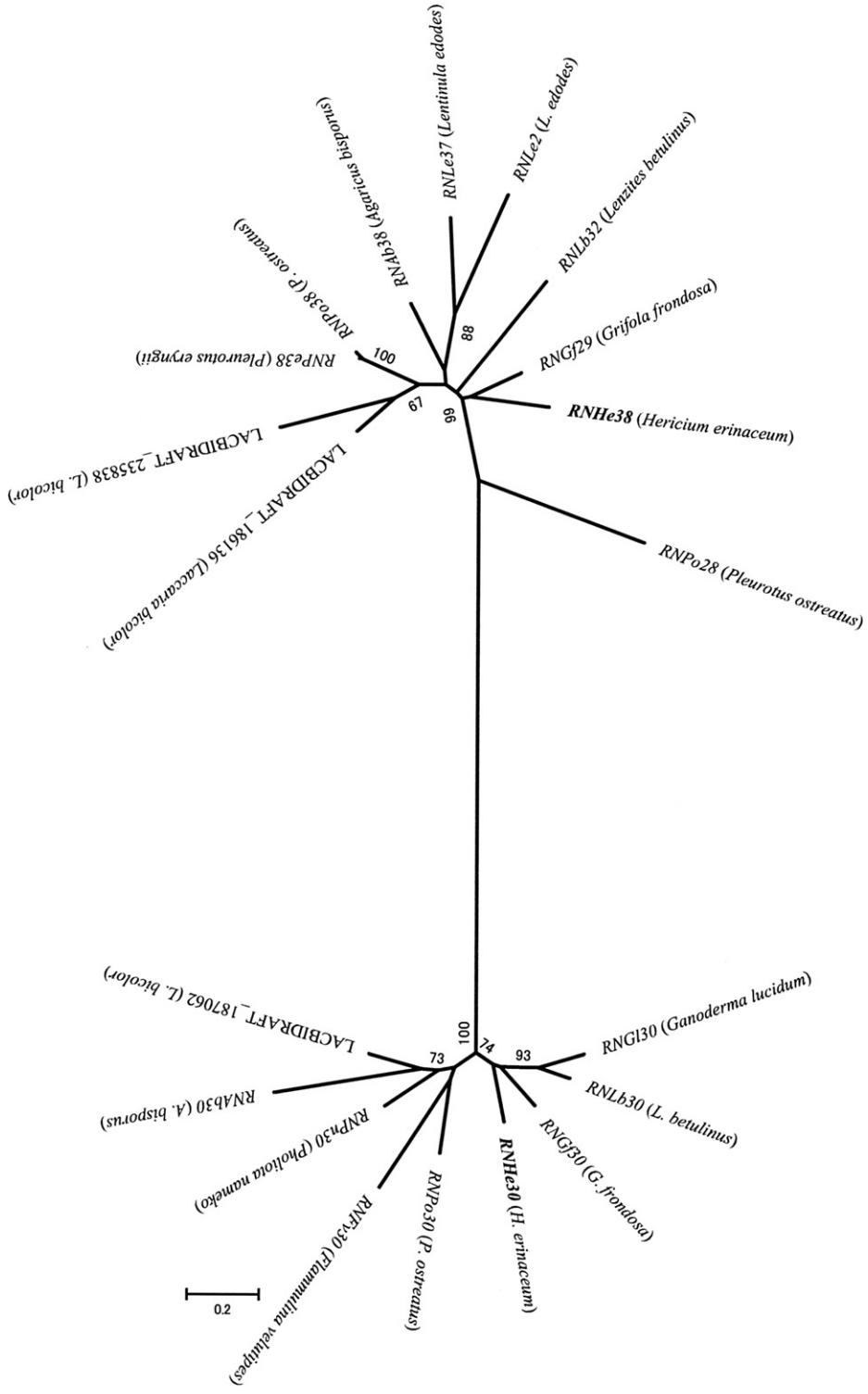

Fig. 6 – Maximum likelihood phylogeny of RNase T2s from eleven basidiomycete species. The individual RNases are indicated with genes as listed in Table 2. The tree was constructed from the deduced amino acid sequences using the Whelan and Goldman evolutionary model (Whelan and Goldman 2001). The tree with the highest log likelihood (−5968.4119) is shown. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.2702)). The rate variation model allowed some sites to be evolutionarily invariable (+I, 12.0992% of sites). Numbers on/under nodes indicate support above 50% from 100 bootstrap replicates.

signal peptide amino acid sequence), contrary to the region following Pro28. This prospect was supported by the results of a sequence motif search on the SOSUI signal computation service (Classification and Secondary Structure Prediction of Membrane Proteins, Mitaku Group, Department of Applied Physics, Nagoya University: http://bp.nuap.nagoya-u.ac.jp/sosui/).

It has been reported that *L. edodes* produces isoforms of RNase T2-type enzymes (Kobayashi et al. 1998). *Hericium erinaceum* was confirmed to have at least two independent genes encoding these enzymes in this investigation. *Lenzites betulinus* was also confirmed to have at least two different genes (*RNLb30* and *RNLb32*). Their deduced amino acid sequences were well correlated to RNase Le2 and RNase He30, respectively. *Pleurotus ostreatus* was confirmed to have three genes encoding RNase Le37-type (*RNPo38*), RNase Le2-type (*RNPo28*), and RNase He30-type (*RNPo30*) enzymes (Table 2). These results imply that at least three kinds of genes coding RNase T2-type enzymes exist in basidiomycetes. This result was well supported by the data of the *Laccaria bicolor* genome project (Martin et al. 2008), which reported three genes encoding RNase T2-type enzymes. The deduced amino acid sequences of LACBIDRAFT_235838, LACBIDRAFT_186136, and LACBIDRAFT_187062 showed strong homology to the sequences of RNase Le2, RNase Le37, and RNase He30, respectively (data not shown).

3.5. *Phylogenetic analysis*

The phylogenetic relationships of RNase T2 sequences from basidiomycetes were analyzed using the maximum likelihood method. A tree was constructed using a multiple alignment of the complete predicted amino acid sequences from *A. bisporus*, *F. velutipes*, *G. lucidum*, *G. frondosa*, *H. erinaceum*, *L. bicolor*, *L. edodes*, *L. betulinus*, *P. nameko*, *Pleurotus eryngii*, and *P. ostreatus* (Fig. 6). *Ganoderma lucidum*, *G. frondosa*, and *L. betulinus* are closely related taxa to *H. erinaceum* according to phylogenetic analysis using internal transcribed spacer (ITS) sequence data among the basidiomycetes used in this study (data not shown). The tree clearly shows that the RNases encoded by *RNHe30* and *RNHe38* from *H. erinaceum* belong to independent groups. Each of them was closely grouped with homologous RNases from *G. lucidum*, *G. frondosa*, and *L. betulinus* in distinct clusters. The RNase Po28 from *P. ostreatus* was located just outside of these clusters. Since we were unable to obtain genes encoding RNase Le2-type enzymes like RNPo28 from the other taxa under the PCR conditions we used here, it is difficult to draw any conclusions from this tree.

Further attempts to clone RNase Le2-type genes from these taxa will be required in the future. It will also be necessary to investigate various other phylogenetically-related fungal species to elucidate the relationship between fungal evolutionary history and the evolution of the genes encoding RNase T2 family enzymes.

Disclosure

The authors declare no conflict of interest. All the experiments undertaken in this study comply with the current laws of Japan.

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