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Molecular and cellular biological studies on a RecQ helicase of the basidiomycete *Lentinula edodes*

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Contents

Chapter 1

General introduction

RecQ helicase family is a group of DNA helicases with a remarkable sequence conservation with all seven helicase motifs (I, Ia, II, III, IV, V, VI) (Wu and Hickson 2001); degree of the conservation of the amino acid sequence of the halicase domains between RecQ helicases are higher than that between RecQ and other non-RecQ helicases. An additional motif (named motif 0) that is adjacent to motif I by N-terminal side, is also conserved among RecQ helicase members (Bernstein and Keck, 2003). In most cases, the helicase domains of RecQ helicases are followed by conserved amino acid sequence called RecQ C-terminal domain and helicase and RNase D C-terminal (named HRDC) domain (Morozov *et al.*, 1997).

The members of the family have been widely found in organism from bacteria to human. The importance of these RecQ helicases is shown by phenotypes of the mutants. For example, by the mutations in the three of the five human *recQ* genes cause severe disorder characterized with premature aging or elevated predisposition of kinds of cancers; mutations in *WRN* cause Werner syndrome, mutations in *BLM* cause Bloom's syndrome, and mutations in *RecQ4* cause Rothmund and Thomson syndrome (Yu *et al.*, 1996; Ellis *et al*, 1995; Kitao *et al.*, 1999). Furthermore, *Saccharomyces cerevisiae SGS1* gene mutants cause hypersensitivity to DNA damaging agent, i. e., methyl methanesulfonate and hydroxylurea, and they show chromosome segregation abnormality and hyper recombination. (Kusano *et al.*, 1999; Yamagata *et al.*, 1998; Onoda *et al.*, 2000.; Watt *et al.*, 1995; Gangloff *et al.*, 1999). These observations indicate that RecQ helicases are involved in maintenance of genomic stability.

Biochemical assays revealed that common catalytic activity is unwinding of the DNA double helix in direction from 3' to 5' (Lu *et al.*, 1996). Interestingly, some

RecQ helicases are able to, or prefer to, unwind higher order DNA structure such as Holliday junction, D-loop structure, and G-quartet, as their substrate (Karow*et al.*, 2000; Sun *et al.* 1998; Brabant *et al.*, 2000). Taken together, RecQ helicases are involved in biological processes such as DNA replication, recombination and repair, and maintenance of genomic stability.

Concerning RecQ helicases (or their genes) in (from) filamentous fungi, the first report was ascomycete *Neurospora crassa QDE3* gene which is essential for the post transcriptional gene silencing, called quelling (Cogoni and Macino, 1999). *N. crassa* has another RecQ helicase gene *RecQ-2* may be active in different mechanisms because it is not involved in quelling (Pickford *et al.*, 2003). Plant pathogenic ascomycete *Magnaporthe grisea* and protobasidiomycete *Ustilago maydis* contain DNA sequences encoding RecQ helicase domain some of which are present in telomeric region. But it is not known whether the two fungi have intrinsically one sort of *recQ* gene (RecQ protein) or plural number of *recQ* genes (RecQ protein) (Gao *et al.*, 2002; Sanchez-Alonso and Guzman, 1998). So far *recQ* homologue gene has not been isolated from eubasidiomycetes. Eubasidiomycetes are unique filamentous fungi with dramatic morphological differentiation from dikaryotic vegetaive mycelia into a huge fruiting body (the so-called mushroom), in which a large number of basidia and basidiospores are produced. In the eubasidiomycetes a binucleate-celled dikaryon, which displays synchronized nuclear division, does form fruiting body. The molecular mechanisms of these phenomena, however, are not fully understood. As discrived above, RecQ helicases play important roles in other organisms. To understand the molecular mechanisms of biological phenomenon of basidiomycetous mushroom, the author has studied RecQ helicase in one of the most popular mushroom *Lentinula edodes*. One RecQ helicase gene (*recQ*) has been

cloned and analysed its structure and transcriptional expression. Property of the expressed product of the *recQ* gene has been also analyzed.

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Chapter 2

Cloning and sequence analysis of a *recQ* gene from basidiomycete *Lentinula edodes*

2-1. Introduction

RecQ helicases first reported in *Escherichia coli* RECQ (Nakayama *et al.*, 1984; Irino *et al.*, 1986) are widely found in organisms from bacteria to human and they are shown to be involved in biological processes such as DNA replication, recombination and repair, and maintenance genomic stability. Whereas in *E. coli* (Nakayama *et al.*, 1984; Irino *et al.*, 1986) and unicellular yeast (Gangloff *et al.*, 1994; Stewart *et al.*, 1997) only one RecQ protein is present, five and six different RecQ homologues have been found so far in human (Ellis *et al.*, 1995; Yu *et al.*, 1996; Puranam *et al.*, 1994; Kitao *et al.*, 1998) and plant *Arabidopsis thaliana* (Hartung *et al.*, 2000), respectively. The RecQ helicase family is generally composed of three different members that vary in size and in the presence or absence of specific functional domains: first, the small RecQ homologues such as *E. coli* RECQ (Irino *et al.* 1986) and *A. thaliana* RecQl1 (Hartung *et al.*, 2000), of 400 – 750 amino acids; secondly the longer RecQ homologues such as *A. thaliana* RecQl4A (Hartung *et al.*, 2000), *Schizosaccharomyces pombe* Rqh1 (Stewart *et al.*, 1997), *Saccharomyces cerevisiae* SGS1 (Gangloff *et al.*, 1994), human BLM (Ellis *et al.*, 1995), or *Neurospora crassa* QDE3 (Cogoni and Macino, 1999) of 1000 – 1900 amino acid; and thirdly, large RecQ homologues containing an additional exonuclease domain like human WRN (Yu *et al.*,1996) and *Xenopus laevis* FFA1 (Yan *et al.*, 1998). A new *recQ* gene was recently identified in the multicellular ascomycete fungus *N. crassa* and it was named *RecQ-2* (encoding 483 amino acids) (Pickford *et al.*, 2003). The genomic DNA fragment containing *recQ* sequence has been isolated from *Ustiligo maydis*, belonging to the protobasidiomycetes (Sanchez-Alonso *et al.*, 1998). There is no report on isolation of *recQ* homologue from the eubasidiomycetes forming fruiting body (so-called mushroom). In this chapter, the

author describes isolation of a *recQ* gene homologue from the eubasidiomycete *L. edodes* and discusses the problem whether or not *L. edodes*, a multicellular filamentous fungus, possesses a plural number of *recQ* genes. Hereafter *L.edodes recQ* gene is designated as *Le.recQ*.

2-2. Materials and Methods

2-2-1. PCR amplification of the DNA fragment corresponding to RecQ helicase domain

Genomic DNA was prepared from *Lentinula edodes* FMC2 (Katayose *et al.*, 1990) according to the method of Murray and Thompson (1980) and used for PCR as a template. The PCR-amplification was done by the DNA polymerase (rTaq (TOYOBO)) and the degenerated primer DNAs corresponding to the conserved amino acid sequence among RecQ helicases (see Fig.2-6): primer 1: 5'-MAITGGGGICAYGAYTTYMG-3' and primer 2: 5'-GCICKICCIGWYTCYTGRT ARTA-3' through 35 cycles of 95°C, 1 min; 37°C, 1min; 72°C, 1min.

2-2-2. Southern blot analysis

Southern-blot analysis of restriction endonuclease-digests of genomic DNA of *L. edodes* FMC2 (Katayose *et al*., 1990) was done by the method previously reported (Hori *et al*, 1991).

2-2-3. Screening of the L. edodes *genomic library*

A genomic DNA library was prepared by insertion of a size-fractioned *Eco*RI-digest of the *L. edodes* genomic DNA, into *Eco*RI-cleaved and dephosphorylated pBluescript II SK (+) vector (STRATAGENE), followed by transformation of *E. coli* DH5α (Hanahan, 1983). This genomic DNA library was screened by using ^{32}P-labelled probe of the PCR-amplified 0.7-kb *recQ* conserved sequence (Probe 1 of Fig. 2-2). Previous published conditions (Hori *et al.*, 1991) were used for the hybridization work. The genomic DNA clone (3.2-kb *Eco*RI−*Eco*RI fragment: Clone 1 in Fig. 2-2) was restriction mapped, and the restriction fragments were sequenced after subcloning into the pBluescript II SK (+) vector. Sequencing carried out by the dideoxy chain-termination method of Sanger *et al.* (1977)

2-2-4. inverse PCR

The *Sph*I-digested of the genomic DNA fragments were circularized by self-ligation and the resulting circular DNAs were used as a template for PCR. Two primers were designed based on the two nucleotide sequences of the aforementioned 3.2-kb *Eco*RI−*Eco*RI fragment: primer 3: 5'-CGTCTAGA(*Xba*I site)CCGTGACTTTCGATACTGTTC-3' and primer 4: 5'-CGTCTAGA(*Xba*I site)GTTGCATATTCTTATTACCTC-3' (see Figs. 2-2, 2-3 and 2-5). The amplified genomic fragment was digested with *Sph*I and *Eco*RI, yielding the 3.7-kb *Sph*I−*Eco*RI fragment (Clone 2 in Fig. 2-2). This fragment was restriction mapped and sequenced.

2-2-5. Reverse transcription (RT)-PCR for cloning of cDNA fragments

corresponding to Le.recQ

Total cellular RNA (1 μg) isolated from mature fruiting body of *L.edodes* FMC2 according to the method of Han *et al.* (1987) was subjected to RT-PCR. Two sets of primers were designed based on the nucleotide sequences of the genomic DNA clones (Clones 1 and 2 of Fig. 2-2): primer 5: 5'-GTTCACAGTGCGATCACAT GACGACTTC-3' and primer 6: 5'-TAGCTTGACTTTCTCTCGTCCACTG-3', and primer 7: 5'-GGATCC(*Bam*HI site)AGCAGATGAGATATCCAGTG-3' and $(dT)_{20}$ primer (see Fig. 2-5). Two cDNA fragments of *Le.recQ* gene were amplified by using the reverse transcriptase (ReverTraAce (TOYOBO)) and the DNA polymerse (KOD plus (TOYOBO)), and they were restriction mapped and sequenced.

2-2-6. Mapping of transcription start point (tsp) by primer extension method

Total cellular RNA (2 μg) isolated from mature fruiting body of *L. edodes* FMC2 was subjected to primer extension analysis. The analysis was done by using the reverse transcriptase and the fluorescent substance IRD800 (Nisshinbo)-labelled primer 8: 5'-TGGGATCAGAGATGCTACCTCG-3' (see Fig. 2-4, 2-5). The primer extension products were analyzed according to the method previously reported (Yamazaki *et al.*, 2000).

2-2-7. RT-PCR analysis for confirming the transcriptional starting point and translation start codon of Le.recQ

Two forward primers, primer 9: 5'-GGACTTGGAAACAGAGAAAGTTAAAC G-3' and primer 10: 5'-GATGCATATACTATCTTCCTCACCT-3', and a reverse primer, i.e., primer 11: 5'-GGTACC(*Bam*HI site)GTCAAGATTGTTCTTTGGTCCA

ATG-3', were used for the experiment (see Fig. 2-5). The reaction was done as previously described.

2-2-8. Computer analysis

BLAST searches were performed within BROAD institute *Coprinus cinereus* database (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/), DOE Joint Genome Institute *Phanerochaete chrysosporium* database (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html) and the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

2-3. Result

2-3-1. Cloning and nucleotide sequence analysis of Le.recQ *gene*

In order to clone the *Le.recQ* gene, *L. edodes* genomic DNA was digested with *Bam*HI, *Eco*RI or *Hind*III and the resulting digests were put through Southern-blot analysis at higher (65°C) and lower (58°C) temperatures using the probe of the PCR-amplified 0.7-kb *recQ* conserved sequence (Probe 1 of Fig. 2-2). As shown in Fig. 2-1, a single signal was detected in all three digests and at both higher (panel A) and lower (panel B) temperatures: 9.0 kb for *Bam*HI, 3.2 kb for *Eco*RI, and 8.0 kb for *Hin*dIII. The auther attempted to clone the 3.2-kb *Eco*RI–*Eco*RI fragment. Screening of the genomic DNA library using the ^{32}P-labelled 0.7-kb *recQ* conserved sequence (Probe 1 of Fig. 2-2) resulted in an isolation of the 3.2-kb *Eco*RI–*Eco*RI fragment (Clone 1 of Fig. 2-2). The nucleotide sequence analysis suggested that the cloned 3.2-kb fragment contains the

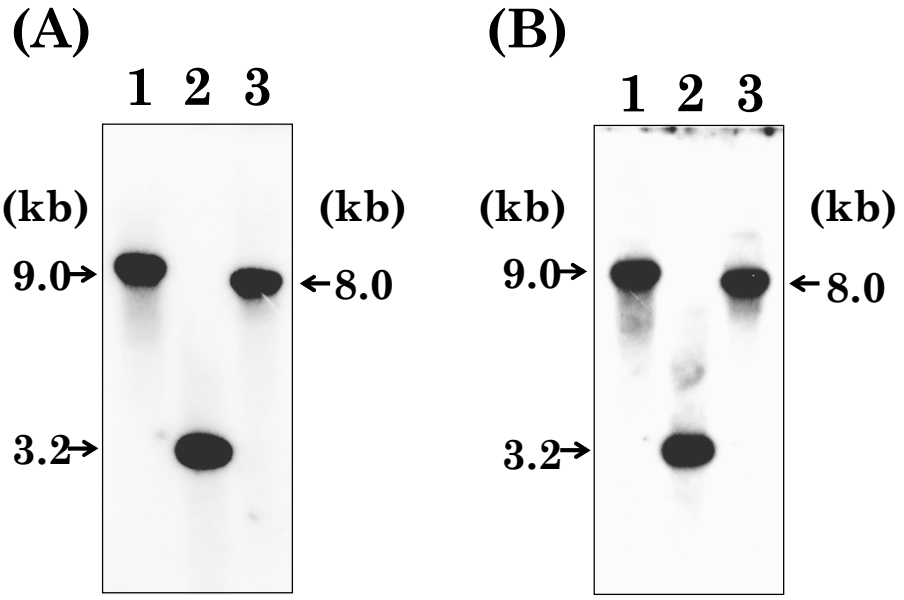

Fig. 2-1. Southern-blot hybridization of a ^{32}P-labelled probe of the 0.7 kb PCR-amplified fragment containing *recQ* conserved sequence and the *Bam*HI-, *Eco*RI- or *Hin*dIII-digested DNA of *L. edodes*.

Total cellular DNA (20 μg each) prepared from *L. edodes* dikaryotic strain FMC2 (Katayose *et al*., 1990) was digested with *Bam*HI (lane 1), *Eco*RI (lane 2) or *Hin*dIII (lane 3), size-fractionized by 1 % agarose-gel electrophoresis, and transferred to a nylon membrane. The membrane was hybridized with the probe at 65ºC (panel A) or at 58ºC (panel B) for 16 h and autoradiographed according to previous methods (Hori *et al*.,1991).

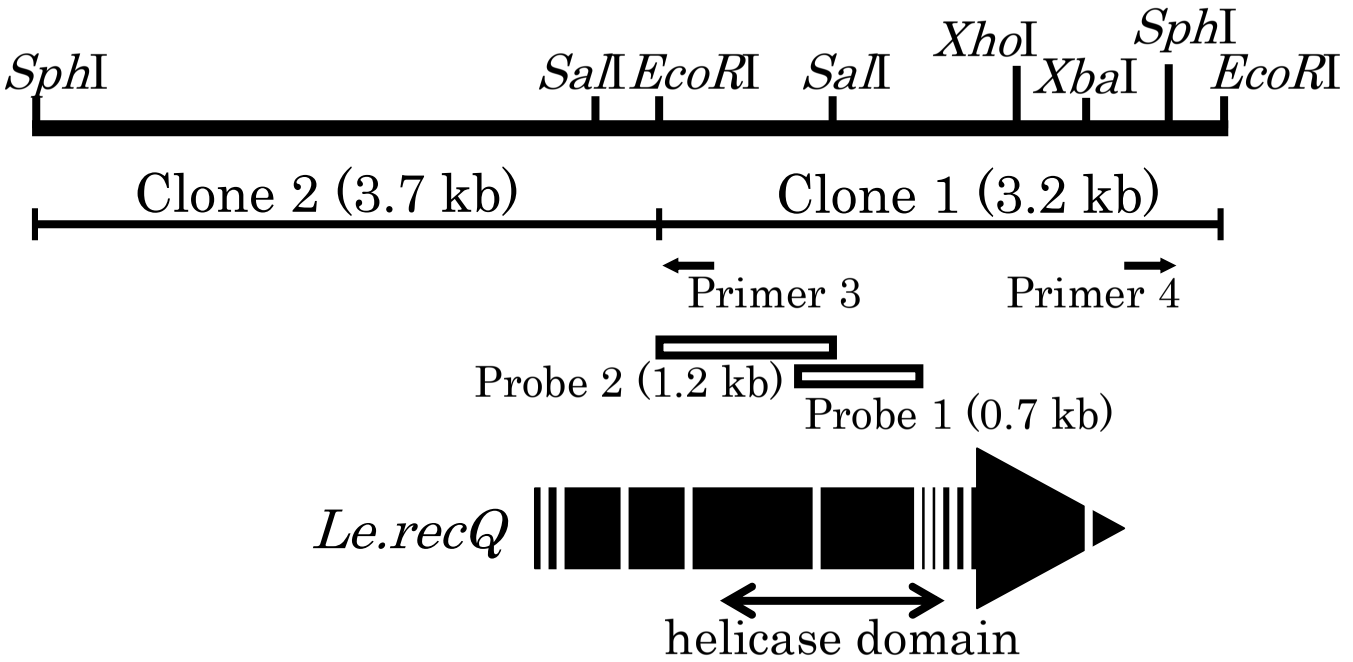

Fig. 2-2. Restriction and gene maps of the region containing *Le.recQ* gene on the chromosome of *L. edodes*.

Le.recQ gene is represented by arrow. Closed and open boxes show exons and introns, respectively. Clone 1 was isolated from the *Eco*RI-derived genomic library of *L. edodes*. Clone 2 was isolated by the inverse PCR using primers 3 and 4 (also see Fig. 2-3). Probe 1 and Probe 2 were used for cloning of Clone 1 and Clone 2, respectively.

sequences encoding all seven RecQ helicase motifs (I, Ia, II, III, IV, V, and VI) (Wu and Hickson 2001) (see Figs. 2-2), but it lacks 5'-terminal coding and promoter regions of the *Le.recQ* gene (see Fig. 2-2). To clone these missing sequences of *Le.recQ,* the following inverse PCR was carried out: the *L. edodes* genomic DNA was digested with *Sal*I, *Sph*I, *Xba*I, or *Xho*I, all of which cut the aforementioned 3.2-kb *Eco*RI−*Eco*RI fragment at a single site. The resulting digests were subjected to Southern-blot analysis using the ^{32}P-labelled 1.2-kb *Eco*RI−*Sal*I fragment (Probe 2 of Fig. 2-2) within the 3.2-kb *Eco*RI–*Eco*RI fragment. A single signal was detected in all four digests: 1.4 kb for *Sal*I, 6.4 kb for *Sph*I, 15 kb for *Xba*I, and 10 kb for *Xho*I (data not shown). Based on these data, a restriction map was constructed as shown in Fig. 2-2. The *Sph*I-digested *L. edodes* genomic DNA fragments were circularized by self-ligation and the resulting circular DNAs were subjected to inverse PCR using the primers 3 and 4, isolating the 3.7-kb *Sph*I−*Eco*RI fragment (see Fig. 2-3). This 3.7-kb fragment was restriction mapped and sequenced (Clone 2 of Fig. 2-2).

Based on the nucleotide sequences of the *Le.recQ* gene, the author attempted to synthesize its cDNA by RT-PCR method. First, the RT-PCR was done by using primer 5 and $(dT)_{20}$ (see Fig.2-5), but somehow a desirable size of the DNA fragment was not amplified. So the author used the two sets of primers: primers 5 and 6, and primer 7 and $(dT)_{20}$ (see Fig.2-5), resulted in an amplification of the 2.9-kb and 0.3-kb cDNA fragments of *Le.recQ*. The nucleotide sequence analysis suggested that the 2.9-kb fragment contains the sequences encoding all seven RecQ helicase motifs and the N-terminal region of the *Le.recQ* gene product, *Le*.RECQ protein (see Figs.2-5). The 0.3-kb cDNA fragment was suggested to contain the 0.2-kb sequence encoding the C-terminal region of *Le*.RecQ and the

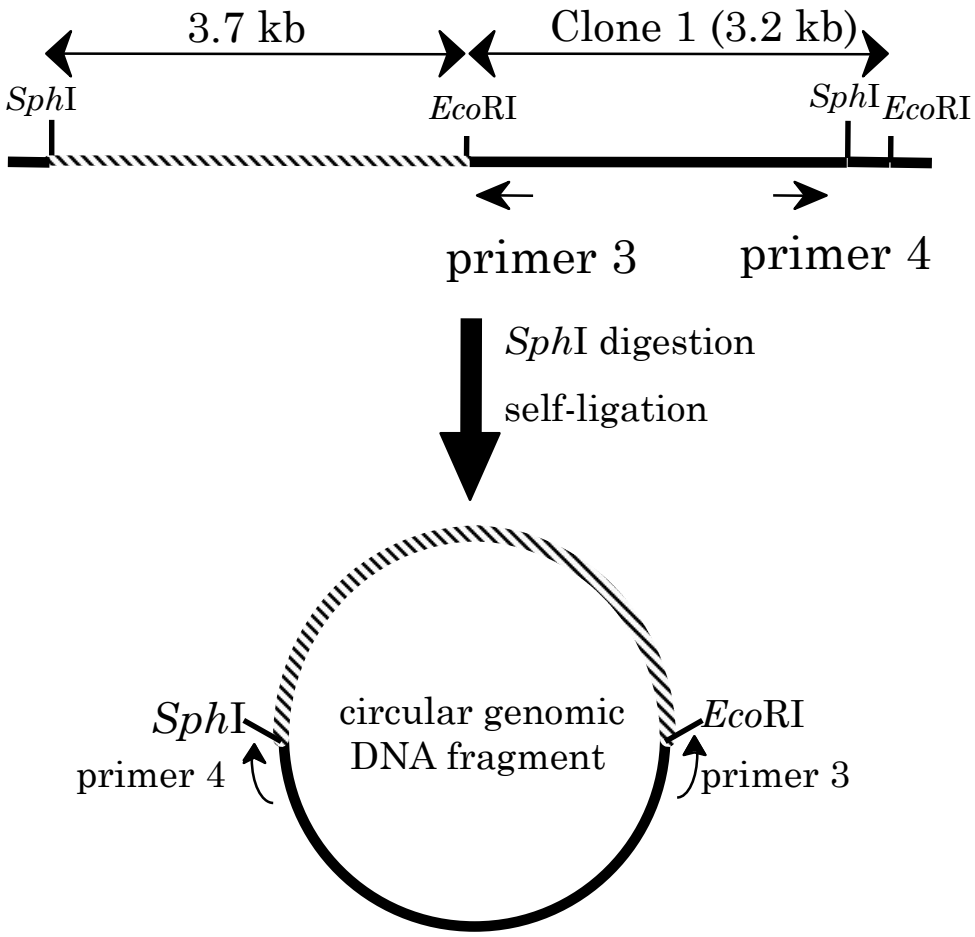

Fig. 2-3. Construction of substrate for inverse PCR.

Genomic DNA of *L.edodes* was digested with *Sph*I and self-ligated. The circular genomic fragment was used for PCR with primers designed according to the sequences of the *Eco*RI genomic fragmet (Clone 1). The region amplified by the inverse PCR is shown as striped line.

0.1-kb sequence of the 3'-untranslated region and the poly(A) addition site (see Fig. 2-5). The 6.9-kb genomic sequences (of the 3.2-kb *Eco*RI−*Eco*RI fragment plus 3.7-kb *Sph*I−*Eco*RI fragment) were compared with the 3.2-kb (2.9-kb plus 0.3-kb) cDNA sequences. Perhaps the *Le.recQ* gene contains 3396-bp coding region interrupted by eleven small (nucleotide 49−59) introns and encodes 945 amino acids (Fig. 2-5). A putative transcription termination signal of AATAAA and the signal-like sequence of AATACAA were found between translation stop codon (TAG) and poly(A)-addition site (Fig. 2-5).

2-3-2. The transcription start point (tsp) of the Le.recQ *gene.*

To confirm the translation start codon of the *Le.recQ* gene and analyze the structural features of the *Le.recQ* promoter, we determined the tsp of the *Le.recQ* gene by primer extension method. As shown in Fig. 2-4, the primer extension product of the *Le.recQ* transcript isolated from the *L. edodes* mature fruiting bodies gave a clear band at a position of 117-nucleotide upstream of the suggested translation start codon (see Fig. 2-5). Further, the author carried out a RT-PCR experiment to confirm the result of the primer extension analysis. In this experiment, two sets of primers were used: primer 9 (forward) and primer 11 (reverse), and primer 10 (forward) and primer 11 (see Fig. 2-5). The sequence of primer 9 lies downstream of the tsp and that of primer 10 upstream of it. The sequence of primer 11 lies in the 5'-terminus of the *Le.recQ* coding region. The RT-PCR using primers 9 and 11 resulted in an amplification of about 140-bp cDNA fragment, but the primers 10 and 11 gave no amplification of fragment (data not shown). These results confirm the tsp and the coding region of the *Le.recQ* gene as

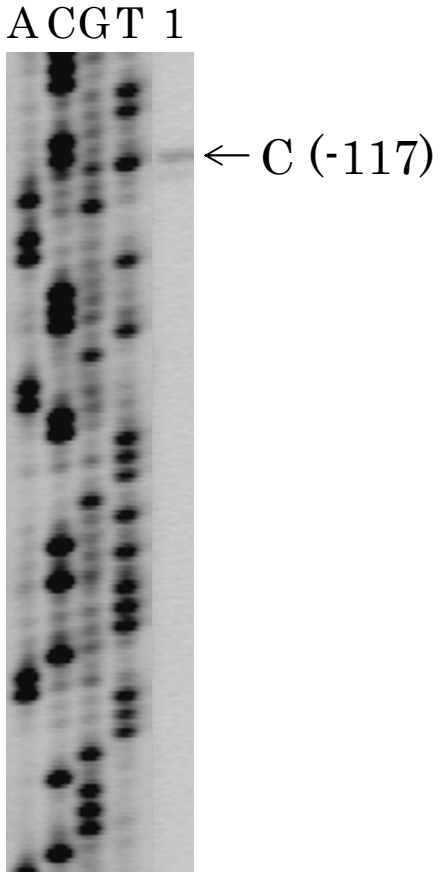

Fig. 2-4. Determination of the transcription starting point (tsp) of *Le.recQ* gene by the primer extension analysis.

The nucleotide residue corresponding to the end of extension product (marked by an arrow) was determined from the sequence ladders (ACGT), which were derived from Clone 2 (3.7 kb *Sph*I–*Eco*RI fragment of Fig. 2-2). The tsp shown is the nucleotide position when the strat codon is assigned the + 1 coordinate (see Fig. 2-5).

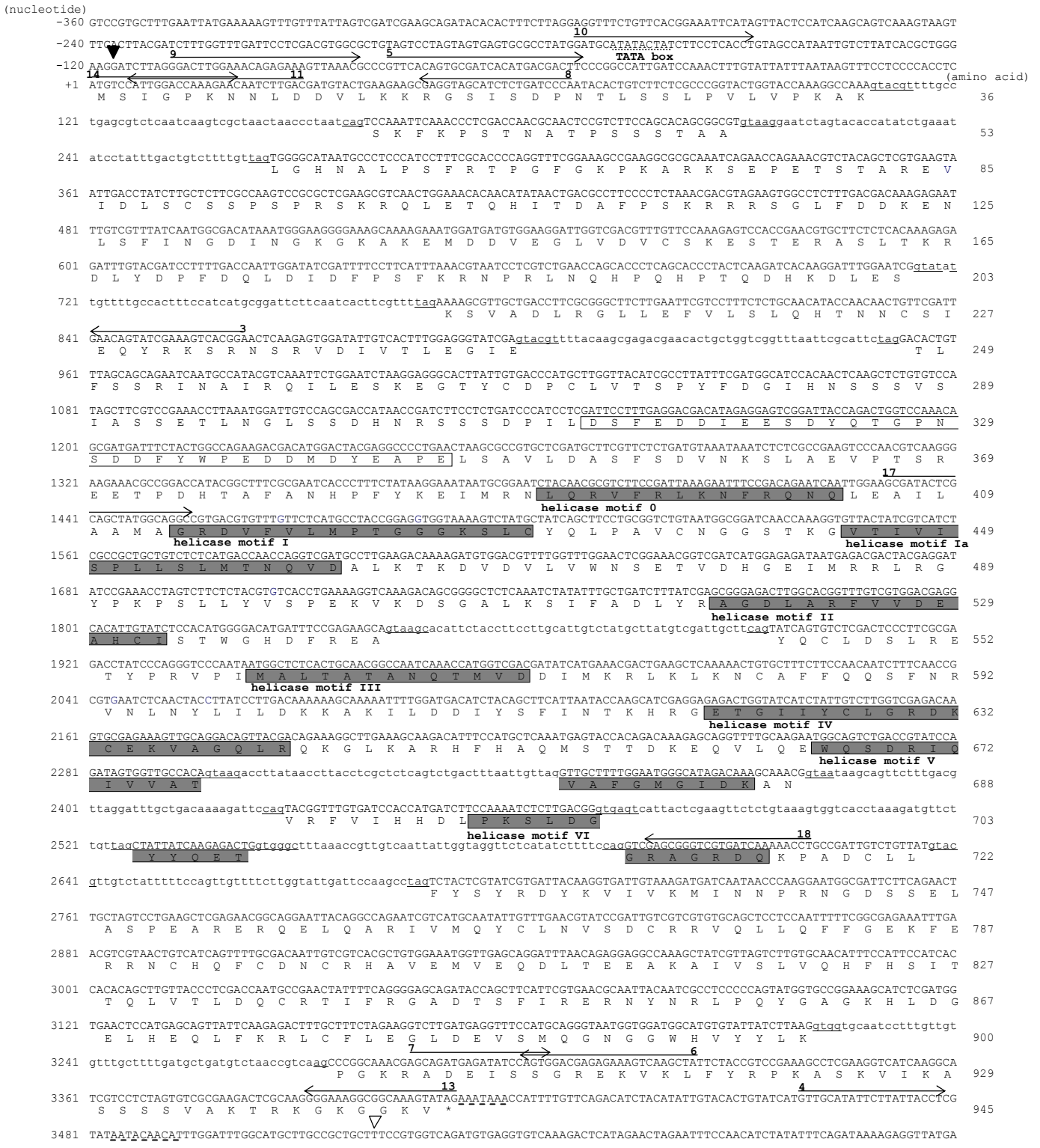

Fig. 2-5. The nucleotide sequences of *Le.recQ* gene and deduced amino-acid Sequences.

The presence of introns in lower-case letters was deduced from the comparison of the cDNA sequence. The nucleotide sequence coordinates and amino-acid sequence coordinates are presented on the left-hand side and right-hand side, respectively. The start codon is assigned the + 1 coordinate. The amino-acid residues responsible for the seven conserved helicase motifs (I, Ia, II~VI) and helicase motif 0 are shaded. The acidic amino-acid-rich sequence is boxed. Consensus sequences for splicing are underlined. Horizontal arrows with numerals are the primers used for isolation of *Le.recQ* cDNA fragments, primer extension analysis, and inverse PCR. The TATA-like sequence (TATACTAT) is shown by dotted underline. The tsp and poly(A)-addition site are shown by closed or open arrowhead, respectively. Putative transcription termination signals (AATAAA and AATACAA) are shown by broken underlines. The sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under Accession No. AB114857.

shown in Fig. 2-5. The promoter region of *Le.recQ* gene contained a TATA-like sequence (TATACTAT) 40-nucleotide upstream of the tsp, but not other eukaryotic (fungal) promoter consensus sequences such as GC-box, CAAT-box, and CT-stretch.

2-3-3. Comparison of the amino-acid sequence of Le.*RECQ and other RecQ proteins*

To determine the relationship between *Le.recQ* and other *recQ* genes, their derived amino acid sequences were compared (Figs. 2-6, 2-7 and 2-8). *N. crassa* QDE3 (Cogoni and Macino, 1999), *S. pombe* Rqh1 (Stewart *et al*., 1997), *S. cerevisiae* SGS1 (Gangloff *et al*., 1994), *A. thaliana* RecQl4A (Hartung *et al*.,2000), *E. coli* RECQ (Irino *et al*., 1986), *Homo sapiens* BLM (Ellis *et al*., 1995), and *Homo sapiens* WRN (Yu *et al*., 1986) consist of 1955, 1328, 1447, 1182, 610, 1417, and 1432 amino acids, respectively. Among these RecQ-type helicases, *A. thaliana* RecQl4A was most homologous to the *Le*.RECQ helicase (945 amino acids) in size. The RecQ-type helicases are known to have a remarkably conserved is the helicase domain (motifs I, Ia, II~IV) (Wu and Hickson, 2001). So the amino acid sequences of the helicase domain of *Le*.RECQ were compared with those of other RecQ proteins. The *N. crassa* QDE3, *S. pombe* Rqh1, *S. cerevisiae* SGS1, *A. thaliana* RecQl4A, *E. coli* RECQ, *H. sapiens* BLM, and *H. sapiens* WRN showed 50%, 52%, 48%, 50%, 44%, 49%, and 36% identity to the *Le*.RECQ, respectively (Fig. 2-8).*S pombe* Rqh1 was most homologous to *Le*.RECQ in the amono-acid sequence of the helicase domain. An additional RecQ helicase motif (named motif 0) (Bernstein and Keck, 2003) showed a moderate homology between the *Le*.RECQ and other RecQ-type

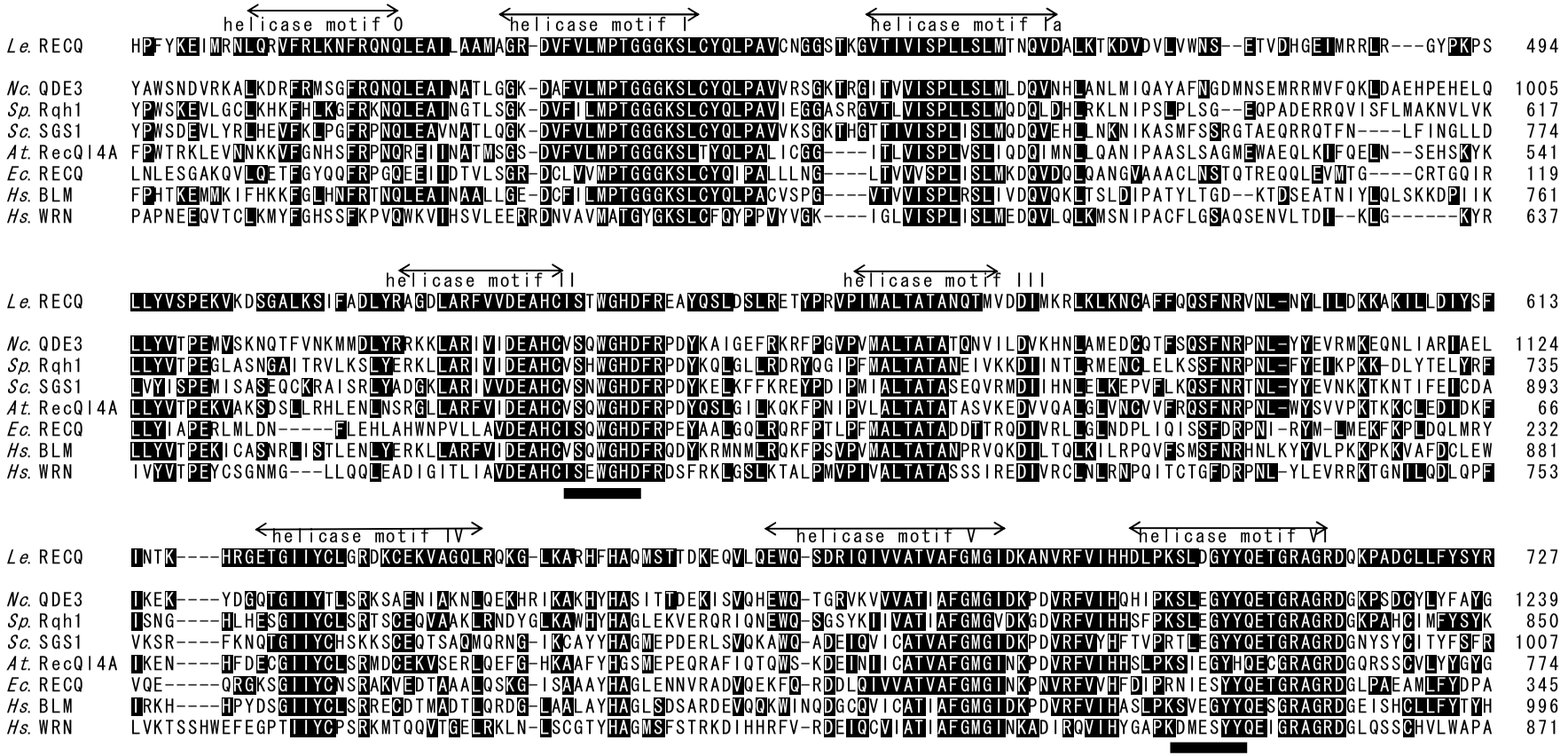

Fig. 2-6. Comparison of the amino-acid sequences of *L. edodes* RECQ (*Le*.RECQ) and other RecQ helicase proteins in their helicase domains.

The amino-acid sequences of *Le*.RECQ, *N. crassa* QDE3 (*Nc*.QDE3) (Cogoni and Macino, 1999), *S. pombe* Rqh1 (*Sp*.Rqh1) (Stewart *et al.*, 1997), *S. cerevisiae* SGS1 (*Sc*.SGS1) (Gangloff *et al.*, 1994), *A. thaliana* RecQl4A (*At*.RecQl4A) (Hartung *et al.*,2000), *E. coli* RECQ (*Ec*.RECQ) (Irino *et al.*, 1986), *Homo sapiens* BLM (*Hs*.BLM) (Ellis *et al.*, 1995)and WRN (*Hs*.WRN) (Yu *et a*l., 1986) are shown. The amino-acid sequences were aligned to optimize matches by using CLUSTAL W Program. The amino-acid residues identical to the *Le*.RECQ protein are dark-shaded. The numbers of the positions of amino-acid residues to the start codon are given at right-hand side. The seven helicase motifs(I, Ia, II~VI) and helicase motif 0 are shown. Two thick underlines are the amino-acid sequences used to design the PCR primers of amplification of the 0.7 kb fragment (probe 1) (see Fig. 2-2).

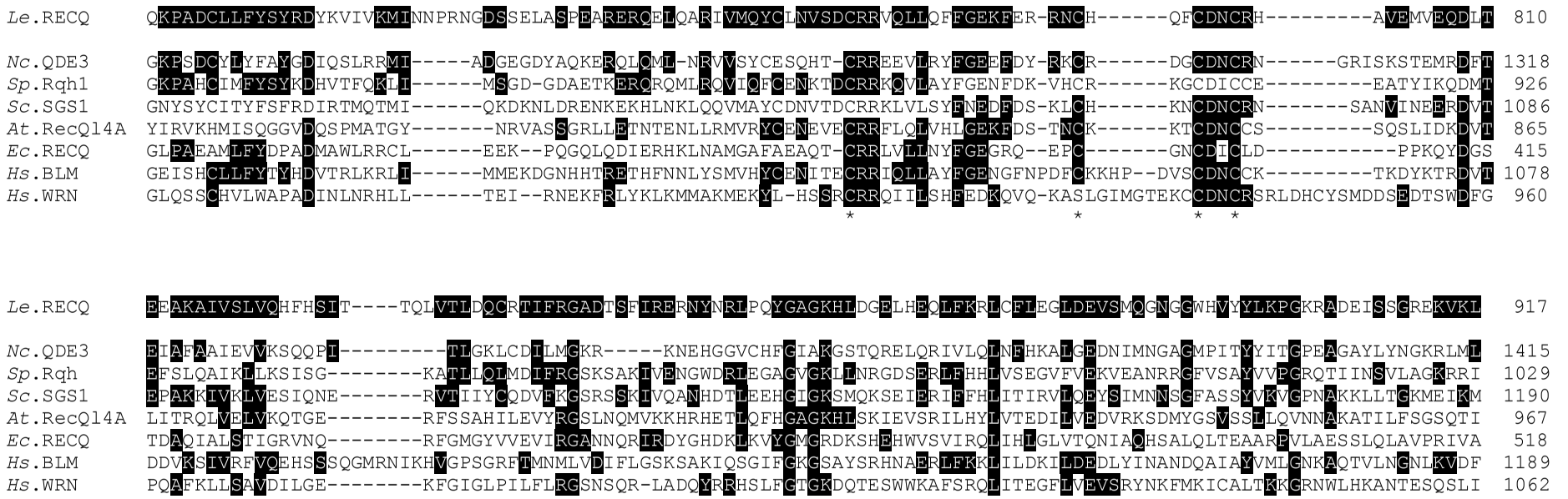

Fig. 2-7. Comparison of the amino-acid sequences of *L. edodes* RECQ (*Le*.RECQ) and other RecQ helicase proteins in Their RecQ helicase C-terminal Domains.

The amino-acid sequences of *Le*.RECQ, *N. crassa* QDE3 (*Nc*.QDE3) (Cogoni and Macino, 1999), *S. pombe* Rqh1 (Sp.Rqh1) (Stewart *et al*., 1997), *S. cerevisiae* SGS1 (*Sc*.SGS1) (Gangloff *et al*., 1994), *A. thaliana* RecQl4A (*At*.RecQl4A) (Hartung *et al*.,2000), *E. coli* RECQ (*Ec*.RECQ) (Irino *et al*., 1986), *Homo sapiens* BLM (*Hs*.BLM) (Ellis *et al*., 1995) and WRN *(Hs*.WRN) (Yu *et al*., 1986) are shown. The amino-acid sequences were aligned to optimize matches by using CLUSTAL W Program. The amino-acid residues identical to the *Le*.RECQ protein are dark-shaded. The numbers of the positions of amino-acid residues to the start codon are given at right-hand side. The asterisks show the conserved cysteine residues in the zinc-finger like motif.

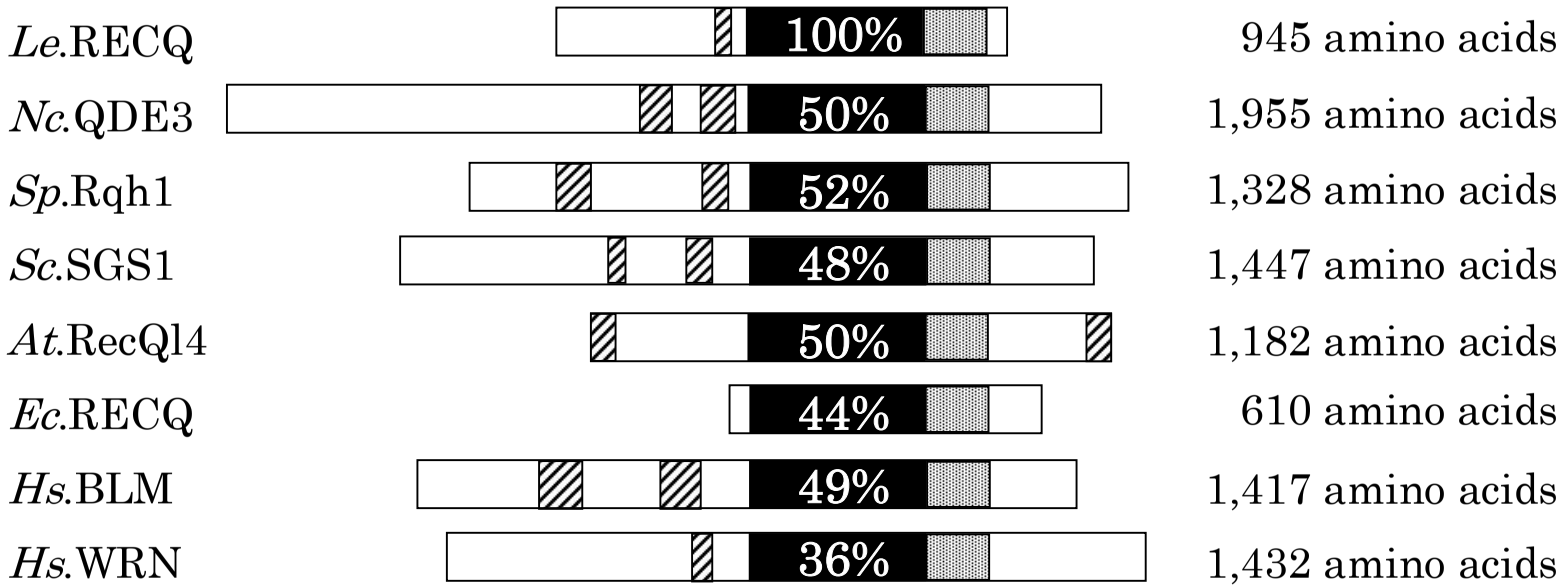

Fig. 2-8. Schematic representation of members of the RecQ helicase family of *Le*.RECQ, *Nc*.QDE3, *Sp*.Rqh1, *Sc*.SGS1, *At*.RecQl4A, *Ec*.RECQ, *Hs*.BLM and *Hs*.WRN.

The size of each protein is shown on the right. The regions corresponding to the helicase domains (motifs I, Ia, II~VI and motif 0) are shown by black bars, in which the percentages are the homologies to *Le*.RECQ of the other RecQ helicases. Acidic amino-acid-rich sequences and C-terminal conserved regions are shown by striped bars and light-shaded bars, respectively.

helicases (Fig. 2-6). In most RecQ-type proteins, the helicase domains are followed by RecQ C-terminal (named RecQ-Ct) domain which is concerned in DNA binding (Morozov *et al.*, 1997). Although the homology of amino acid sequence in the RecQ-Ct domain is lower than in the helicase domain, zinc finger-like motif (also called as zinc binding platform or zinc binding domain) including four cysteine residues are highly conserved (Bernstein *et al.*, 2003; Liu *et al.*, 2004). Amino-acid sequence comparison of the RecQ-Ct domain of the *Le*.RECQ and other RecQ-type proteins revealed that the *Le*.RECQ contains four cysteine and other residues suitable for formation of the RecQ-Ct domain (Fig. 2-7). RecQ helicases have been reported to frequently contain acidic amino-acid-rich sequence(s) usually in their N-terminal region (Bjergbaek *et al*., 2002) It was found that the *Le*.RECQ possesses both acidic amino-acid-rich sequence (amino acids approximately 313–347 in Fig. 2-5) and C-terminal conserved region (amino acids approximately 730–920 in Fig. 2-5) (see Fig.2-8).

Concerning the problem whether or not the eubasidiomycete *L. edodes*, a multicellular filamentous fungus, possesses a plural number of *recQ* genes, the 0.7-kb band in agarose gel of the PCR-amplified product (the sequence amplified by PCR with the degenerated primer DNAs corresponding to the conserved amino acid sequence among RecQ-type DNA helicases) was cut out from the gel and inserted into the pBluescript II vetor, followed by transformation of *E. coli*. Total 12 clones were selected and sequenced. The ten fragments have an identical nucleotidet sequence of *Le.recQ* and the two fragments have the nucleotide sequences unrelated to the *recQ* gene (data not shown). Southern hybridization at higher (65°C) and lower (58°C) temperatures of *Bam*HI-, *Eco*RI-, or *Hin*dIII-digested *L. edodes* genomic DNA using the PCR-amplified 0.7-kb *recQ*

conserved sequence gave a single signal (Fig. 2-1). These data seem likely to imply the presence of a single *recQ* gene homologue on *L. edodes* genome. To verify the presence of a single *recQ* gene in *L. edodes*, other approaches including a whole genome sequence analysis, are necessary.

2-4. Discussion

The author isolated a *recQ* gene homologue (*Le.recQ*) encoding RecQ-type DNA helicase from mushroom *L. edodes* as the first report on the *recQ* gene from eubasidiomycetous fungi. The *Le.recQ* gene product, *Le*.RECQ was shown to consist of 945 amino acids, clearly smaller than other fungal RecQ proteins such as *N. crassa* QDE3 (1955 amino acids), *S. pombe* Rqh1 (1328 amino acids), *S. cerevisiae* SGS1 (1447 amino acids). The construction of the *Le*.RECQ amino acid sequence is homologous to other RecQ proteins in its helicase domain (motifs I, Ia, II~VI,and motif 0) and RecQ-Ct domain containing the zinc finger-like motif. RecQ helicases frequently contain the third conserved domain: HRDC (helicase and RNase D C-terminal) domain that is proposed to be related to nucleic acid binding (Morozov *et al.*, 1997). The HRDC domain is the most variable domain of the three conserved domains such as the helicase domain, the RecQ-Ct domain, and is considered to be related to the preference for substrate DNA structures (Bernstein and Keck, 2005; Liu *et al.*, 1999; von Kobbe *et al.*, 2003). *Le*.RECQ lacks this domain. The substrate selection by *Le*.RECQ may be different from the HRDC-domain-containing RecQ helicases.

In general, the number of *recQ* homologues present in an organism appears to be related directly to the organism's complexity, so that prokaryotes and unicellular eukaryotes, e.g., yeasts possess only a single *recQ* homologue

(Nakayama *et al.*,1984; Irino *et al.*, 1986; Gangloff *et al.*, 1994; Stewart *et al.*, 1997), while multicellular organisms, e.g., *H. sapiens*, *Mus musculus*, *D. melanogaster*, *C. elegans*, *A. thaliana* have multiple *recQ* homologues (Ellis *et al.*, 1995; Yu *et al.*,1996; Puranam *et al.*, 1994; Kitao *et al.*, 1998; Wang *et al.*, 1998; Kusano *et al.*, 1999; Jeong *et al.*, 2000; The *C. elegans* Sequencing Consortium, 1998; Hartung *et al.*, 2000; Wu and Hickson, 2000; Cobb *et al.*, 2002). The multicellular ascomycete fungus *N. crassa* are known to have two *recQ* homologues, (*QDE3* and *RecQ-2*) (Cogoni and Macino, 1999; Pickford *et al*, 2003). The genome of two plant pathogenic fungi, ascomycete *Magnaporte grisea* and protobasidiomycete *Ustolago maydis* contain a plural number of sequences encoding RecQ helicase domain in their telomeric regions (Gao *et al.*, 2002; Sanchez-Alonso and Guzman, 1998). But it is not clear how many *recQ* gene(s) are carried by the genomic of the two fungi. Southern blot analysis and PCR experiment suggested that eubasidiomycete *L. edodes* contains only one *recQ* gene. Recent genetic analysis indicates that the *Le.recQ* gene is present in the chromosome linkage group 6 (personal communication with Dr. Kazuhiro Miyazaki of Forestry and Forest Products Research Institute). The author carried out BLAST search for analyzing RecQ helicase sequence on the genomes of two basidiomycetous fungi *Coprinus cinereus* and *Phanerochaete chrysosporium*, of which whole genomic sequences were determined. One sequence of *C. cinereus* and two sequences of *P. chrysosporium* contain whole conserved RecQ catalytic core all seven helicase motifs (I,Ia,II~VI), motif 0, and the RecQ-Ct domain, although the BLAST research program found 18 sequences on the *Coprinus cinereus* genome and 9 sequences on the *Phanerochaete chrysosporium* genome, which encode amino acid sequences homologous to that of *Le.*RECQ. There is

possibility that *P. chrysosporium* (and *C. cinereus*) possesses the plural number of *recQ* genes.

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Chapter 3

In vitro and *in vivo* activity of *Le*.RECQ

3-1. Introduction

The catalytic activity of RecQ helicases is the unwinding of DNA double helix in the direction from 3' to 5' (Lu *et al.*, 1996). In *E. coli* RecQ, the helicase and RecQ-Ct domains have been reported to form a structural domain, named catalytic core (Bernstein *et al.*, 2003). The polypeptides containing the catalytic core derived from *E. coli* RecQ were shown to have DNA unwinding activity *in vitro* (Bernstein and Keck, 2003). To verify the helicase activity of the *Le.*RECQ protein, the author attempted to prepare the recombinant *Le.*RECQ protein in *Saccharomyces cerevisiae* and the insect Sf9 cells resulted in failure in the sufficient amount of soluble *Le.*RECQ protein. The *Le.*RECQ 565 amino-acids fragment of containing the catalytic core was successfully expressed in *E.coli* by using glutathione S-transferase (GST) fusion system. The expressed product partially purified exhibited 3'→5' helicase activity.

The author also attempted to express the *Le.recQ* cDNA in *S. cerevisiae* and study whether or not the *Le.recQ* cDNA complements defects associated with the loss of its homologue *S. cerevisiae SGS1* gene. The results showed a partially complementation by the *Le.recQ* cDNA.

3-2. Materials and Methods

3-2-1. Expression of the recombinant Le.*RECQ protein in* E. coli.

The *Le.recQ* cDNA fragment (nucleotide 1141–2835) encoding 565 amino-acids peptide of *Le.*RECQ catalytic core, named *Le.*RECQ(core), was

amplified with the primer 12: 5'-GCGGATCC(*Bam*HI)CACCCTTTCTATAAGGAA ATAA-3' and the primer 13: 5'-TTGGATCC(*Bam*HI)CTATACTTTGCCGCCTTTCC CC-3' using the *Le.recQ* cDNA fragment (refer to 3-2-5) as a template. The amplified fragment was digested with *Bam*HI and inserted into the *Bam*HI site of the glutathione-S-transferase (GST) fusion vector pGEX-2TK. The resulting plasmid named pGEX-2TK-Le.recQ (1141–2835) was sequenced and introduced into *E. coli* BL21 (F^-, *ompT*, *hsd*S (r_B^-, m_B^-), *gal*). The pGEX-2TK vector alone was also introduced into BL21. The transformant cells were grown at 37°C in the LB medium containing 100μg/ml of ampicillin to OD_{600} ~0.5. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM and incubated at 22°C for 3h. The cells were harvested by centrifugation, washed with Phosphate bufferd saline (PBS; 1.37 M NaCl, 80 mM Na_2HPO4, 15mM KH_2PO_4) and stored at -20°C.

3-2-2. Purification of the recombinant Le.RECQ protein

The cultured cells of *E. coli* BL21 containing pGEX-2TK-Le.recQ or pGEX-2TK were resuspended in the PBS containing 1mM dithiothreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF) and the protease inhibitor cocktail (NACALAI TESQUE) and were disrupted by sonication for 5×15sec. To remove cell debris, the lysate was centrifuged at 10,400×*g* for 45 min and the supernatant obtained was centrifuged at 40,000×*g* for 60 min. To the resulting supernatant, $MgCl_2$ and ATP were added to give a final concentrartion of 1mM or 2mM. The mixture was incubated at 37°C for 10min. The GST-*Le*.RECQ(core) and recombinant GST alone were purified by a batch method using Glutathione

Sepharose 4B (Amersham Bioscience) according to the following procedure. The protein-bounded Glutathione beads were washed five times with PBS and the proteins were eluted with the elution buffer (50mM Tris-HCl, pH 8.0, 10mM reduced glutathione). The eluted proteins were concentrated by ultrafilter membrane and stored in the storage buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 1mM EDTA, 1mM DTT, 50% glycerol). The indicated amount of protein samples were separated on 10%SDS-polyacrylamid gels and stained with Coomassie brilliant blue.

3-2-3. Helicase assay

The DNA substrate used for helicase assay was as follows. The 20-mer oligonucleotides, oligo1: 5'-ACTATGGTTGCTTTGACGAG-3' was labelled at the 5'-terminus with [γ-^{32}P] ATP and T4 polynucleotide kinase and boiled at 95°C for 5 min. Free [γ-^{32}P] ATP was removed by passage through ProbeQuant G-50 Micro Columns (Amarsham Bioscience). To prepare the partially double stranded substrate, the 5'-labelled oligo1 was mixed with 2-fold excess of unlabeled the 45-mer oligonucleotide, oligo2: 5'-CTCGTCAAAGCAACCATAGTTTTTTTTTTTTT TTTTTTTTTTTTT-3' (see Fig. 3-2A) This DNA mixture was incubated at 95°C for 5 min, and then cooled down to room temperature slowly.

Helicase activity was analyzed in the reaction mixture (20 μl) containing 50 mM Tris-Cl pH7.5, 5mM $MgCl_2$, 5mM ATP, 100μg/ml bovine serum albmin, 50mM NaCl, 1mM DTT 0.05μg of tRNA, and 80 fmol of the llabeled DNA substrate. To the mixture, the indicated amount of helicase protein was added and incubated at 37°C for 30 min. The reaction was stopped by addition of 5μl of the stop solution

(20% glycerol, 100 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue, 2.5% SDS and 0.8 pmol non-labelled oligo1. Ten μl each of reaction samples was electrophoresed on a 15% polyacrylamid gel in 0.5×TBE buffer at room temperature and visualized by IR Bass 2000 (Fuji Film).

3-2-4 . Construction of S.cerevisiae *strains for complementation tests of* Le.recQ

The *Le.recQ* cDNA fragment containing a complete ORF was amplified by RT-PCR using the primer 14: 5'-GCGGATCC(*Bam*HI site)ATGTCCATTGGACCA AAGAAC-3' and the aforementioned primer 13. The resulting product was cloned into pBluescript II vector and the sequence was confirmed to be error-free. The *Bam*HI−*Bam*HI fragment containing the complete *Le.recQ* ORF was excised from the recombinant plasmid and cloned into *S. cerevisiae* expression vector pYES2 (Invitrogen), yielding the recombinant plasmid pYES2-Le.recQ. The DNA fragment containing a complete *SGS1* ORF was amplified by PCR using the primer 15: 5'-GGATCC(*Bam*HI site)ATGGTGACGAAGCCGTCACATAAC-3' and primer 16: 5'-GGATCC(*Bam*HI site)TCACTTTCTTCCTCTGTAGTGACCTCGG-3' and the *SGS1*-carrying plasmid YCp1315 (kindly provided by Dr. Takemi Enomoto of Tohoku University) as a template. After confirming the sequence to be correct, the amplified *SGS1* ORF was cloned into pYES2, yielding pYES2-SGS1. The pYES2-Le.recQ, pYES2-SGS1 and pYES2 were introduced into *S. cerevisiae sgs1* strain 966NS-1 (*ura3-52 leu2-3,112 trp1-289 his 1-7 sgs1Δ::AUR-1C*) (kindly provided by Dr. Takemi Enomoto) according to the method of Gietz and Schiestl (1995). (Fig. 3-3). The transformants obtained were screened by growing on

complete minimal (CM) solid medium (Ausbel *et al.*, 1992) without uracil. The 966 NS-1 carrying pYES2-SGS1, the 966 NS-1 carrying pYES2-Le.recQ, and the 966 NS-1 carrying pYES2 were named 966NS-1[pYES2-SGS1], 966NS-1[pYES2-Le.recQ], and 966NS-1[pYES2] respectively (Fig. 3-3).

3-2-5. Northern blot analysis

Total cellular RNA was prepared from the exponentially growing cells of the 966NS-1 and the 966NS-1[pYES2-Le.recQ] cultured in CM liquid medium containing 2% raffinose and 0.2% galactose with or without uracil at 30°C, according to the methods of Burke *et al.* (2000). The ^{32}P-labelled probes of the 1.2kb fragment of *Le.recQ* genomic fragment (Probe 2 in Fig. 2-2) and 1.2 kb *S. cerevisiae-ACT1* gene (Kajiwara *et al*, 2000) were used. Concerning the detail of the analysis, refer to 4-2-1.

3-2-6. Analysis of the methyl methanesulfonate (MMS)- and hydroxyurea (HU)-sensitivities of the S .cerevisiae *strains.*

The 966NS-1[pYES2-Le.recQ], and 966NS-1[pYES2] were precultured in CM (without uracil) liquid medium containing 2% glucose. After washing with water, the three transformants were cultivated at 30 °C in CM (without uracil) liquid medium containing 2% raffinose. The 966NS-1 and its parental wild type MR966 were also cultured in the CM medium. To analyze methyl methanesulfonate (MMS)- and hydroxylurea (HU)-sensitivity, the exponentially growing cells were washed and serially diluted with water by 5-fold starting at the same $OD_{600,}$ and then spotted on the CM solid medium containing 2% agar, 2%

raffinose, 0.2% galactose and 0.01% MMS or 75mM HU. The agar plates were incubated at 30 °C for 3 days.

3-2-7. Analysis of the growth rate of the S. cerevisiae *strains*

The 966NS-1[pYES2-Le.recQ], the 966NS-1[pYES2-SGS1], and the 966NS-1[pYES2], were precultured in CM (without uracil) liquid medium containing 2% glucose and then, cultivated at 30°C in the CM (without uracil) liquid medium containing 2% raffinose and 0.2% galactose. If necessary, 0.001% (MMS) was added into the culture medium. The growth rate was analyzed by measurement of OD_{600} at intervals of 3 h. The cultivation started at OD_{600} 0.1.

3-3. Results

3-3-1. In vitro *helicase activity of* Le.*RECQ protein*

To examine the *in vitro* helicase activity of *Le*.RECQ, the recombinant GST and *Le*.RECQ catalytic core fusion protein (named GST-*Le*.RECQ(core)) was expressed in *E. coli* BL21 and purified by glutahion sepharose beads (Fig. 3-1). Helicase activity was measured by the dissociation of a ^{32}P-labelled 20-mer from the partially double stranded substrate, the hybrid of the 20-mer and non labeled 45-mer (Fig. 3-2A). As shown in Fig. 3-2B1, the GST-*Le*.RECQ(core) actually dissociated the ^{32}P-labelled 20-mer from the substrate, indicating 3'→5 helicase activity.

3-3-2. Functional complementation of Le.recQ *cDNA in the* Saccharomyces

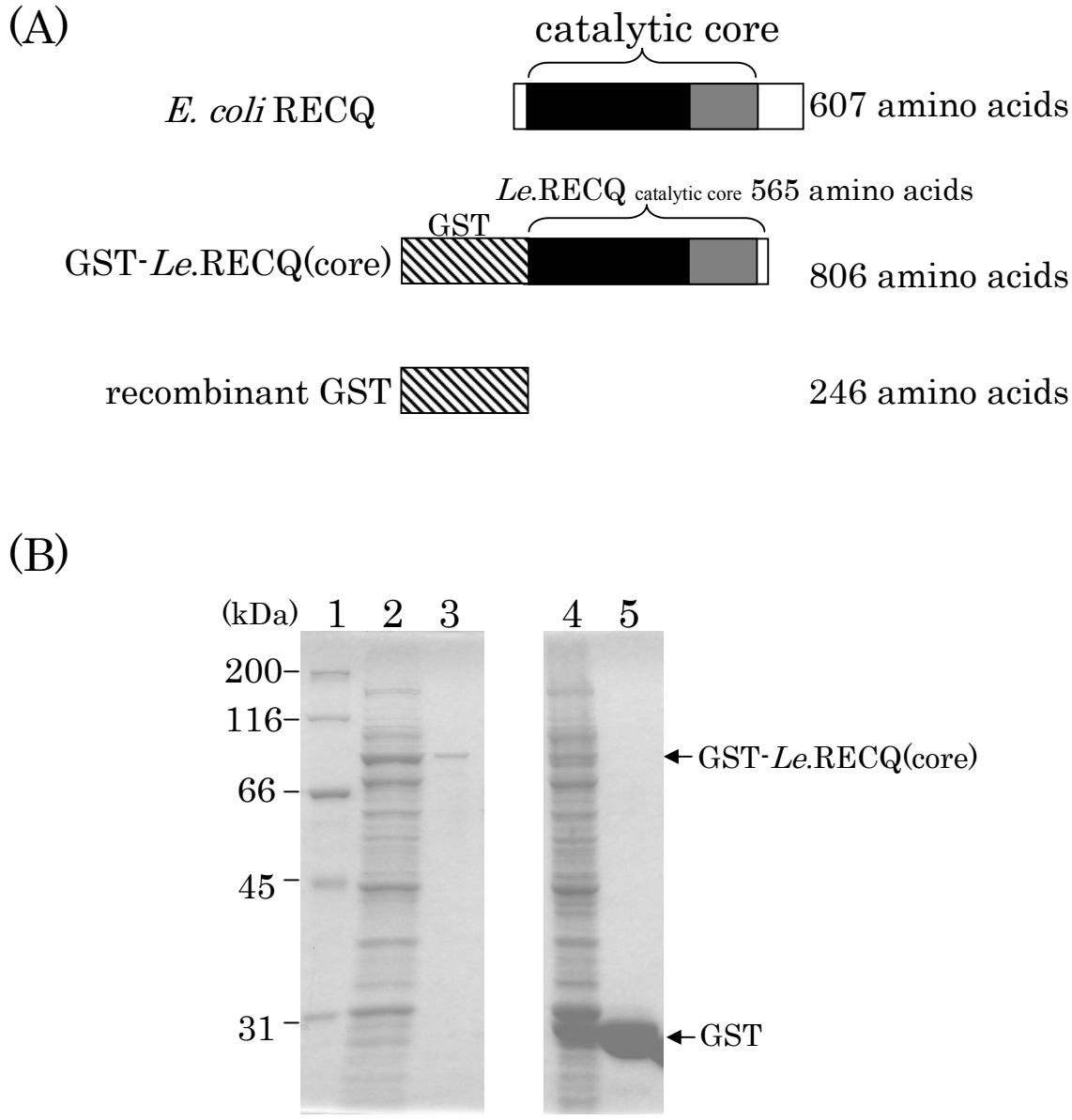

Fig. 3-1. Expression of the fused protein GST and *Le*.RECQ catalytic core (named GST-Le.RECQ(core)).

(A) Schematic structures of GST-Le.RECQ(core) and *E.coli* RECQ. The recombinant GST is the expressed product of pGEX2TK vector. Black boxes and grey boxes indicate the helicase domain and the RecQ C-terminal domein,respectively the recombinant GST is shown by striped boxes. (B) Coomassie brilliant blue-staind 10% SDS-polyacrylamide gel of size marker (lane 1), the crude extract from IPTG-induced BL21containing pGEX-2TK-Le.RECQ(core) (~20μg) (lane 2), the glutathion sepharose 4B-purified of GST-Le.RECQ(core) (~0.2μg) (lane 3), the crude extract from IPTG-induced BL21containing pGEX -2TK (~30μg) (lane 4), and the glutathion sepharose recombinant GST (~10μg) (lane 5).

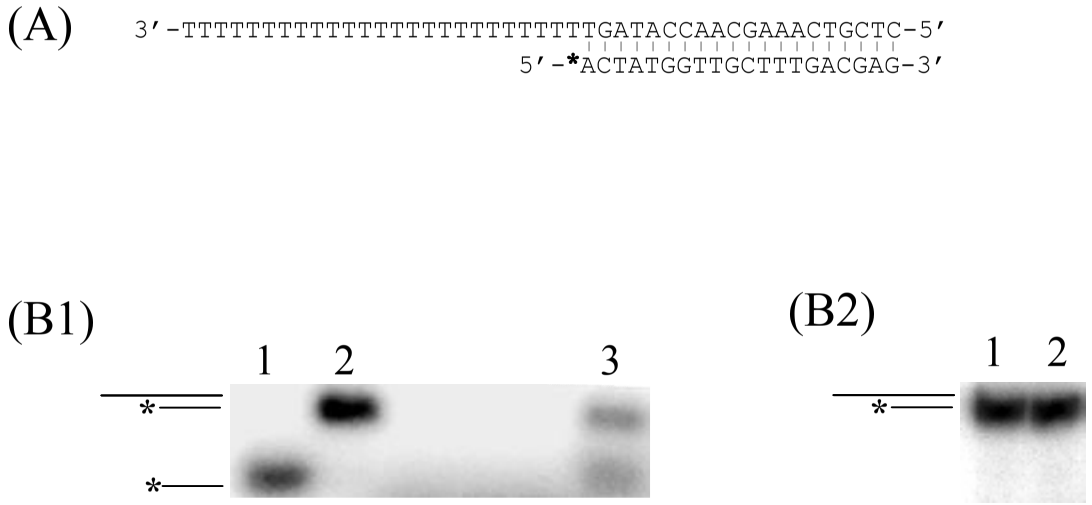

Fig 3-2. In vitro helicase activity of *Le*.RECQ(core) protein.

(A) The double-stranded DNA substrate used for experiment. An asterisk denotes a radioactive ^{32}P-labelle. (B1, B2) Ten μl each of the reaction samples was electrophoresed on a 15% polyacrylamid gel in 0.5×TBE buffer. (B1) the ^{32}P-labelled 20-mer alone (lane1), the partially double-stranded substrate (lane2), the reaction mixture with 50nM GST-Le.recQ(core) (lane3). (B2) The partially double-stranded substrate (lane1), the reaction mixture with 50nM recombinant GST (lane 2).

cerevisiae sgs1 *mutant*

S. cerevisiae possesses one *recQ* gene, *SGS1*. The *sgs1* defective mutation causes *S. cerevisiae* a slight delay in growth and hypersensitivity to DNA-damaging compound MMS and the DNA synthesis inhibitor HU (Miyajima *et al.*, 2000). The author examined whether *Le.recQ* gene is able to complement these phenotypes of the *sgs1* mutant strain, 966NS-1. *S. cerevisiae* 966NS-1 carrying the *Le.recQ* cDNA expression plasmid pYES2-Le.recQ, i.e., the 966NS-1[pYES2-Le.recQ] was used in the experiment (see Fig. 3-3). Northern-blot analysis showed that the *Le.recQ* gene is actually expressed (see Fig. 3-4). The 966NS-1 carrying the vector pYES2, i.e., the 966NS-1[pYES2], 966NS-1, and its parental wild type MR966 were used as controls. The MMS- and HU-sensitivities of the transformants were analyzed by spotting the cells on the CM solid medium containing 0.01% MMS or 75mM HU. As shown in the Fig. 3-5, no significant difference was observed between the 966NS-1[pYES2-Le.recQ] and, the 966NS-1[pYES2].

Next, the growth rates of the 966NS-1[pYES2-Le.recQ], the 966NS-1[pYES2], and the 966NS-1[pYES2-SGS1] were analyzed in the CM (without uracil) liquid medium containing 2% raffinose and 0.2% galactose under the absence (Fig. 3-6A) and presence of 0.001% MMS (Fig. 3-6B). The concentration (0.001%) of MMS was determined judging from the data of viability of the aforementioned three strains on agar plates containing 0.01%, 0.005%, and 0.001% MMS. At concentrations of 0.01% and 0.005%, 966NS-1[pYES2-SGS1] grew, but 966NS-1[pYES2-Le.recQ] and 966NS-1[pYES2] did not. At a concentration 0.001% all three strains grew, though 966NS-1[pYES2-SGS1] grew

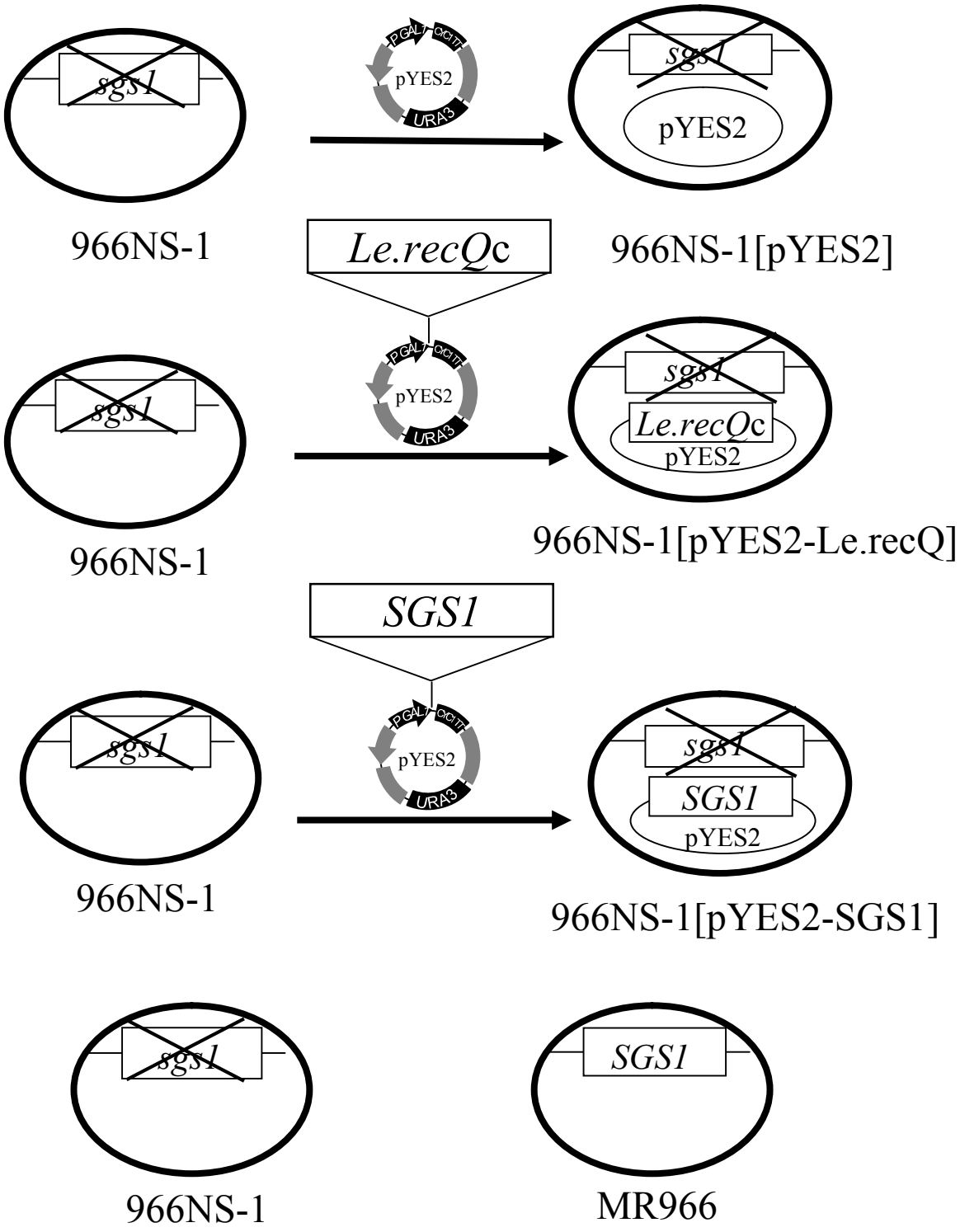

Fig3-3. Construction of the yeast transformants.

The *sgs1* mutant strain 966NS-1(shown at the bottom) was transformed with pYES2 vector alone or pYES2 containing *Le.recQ* cDNA or *SGS1* gene. The transformants obtained are named as 966NS-1[pYES2], 966NS-1[pYES2-Le.recQ], and 966NS-1[pYES2-SGS1], respectively. The parental wild type MR966 of 966NS-1is also shown at the bottom.

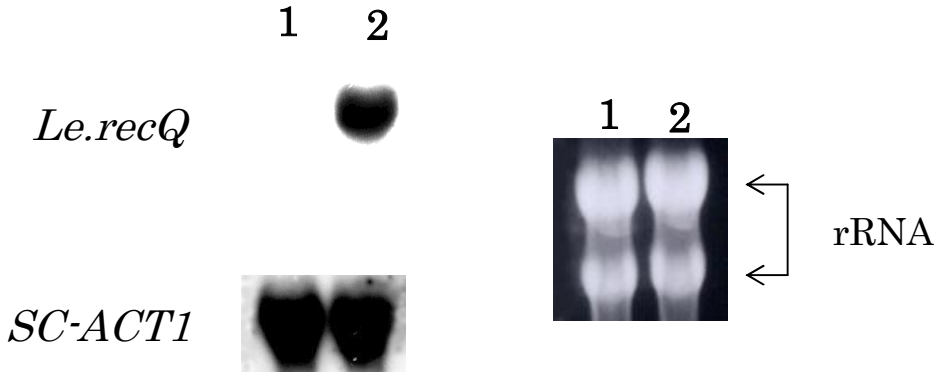

Fig. 3-4. Tranascriptional expression of *Le.recQ* gene in 966NS-1[pYES2-Le.recQ].

Total cellular RNA isolated from 966NS-1 (lane 1) and 966NS-1[pYES2-*Le.recQ*] (lane2) were hybridized with ^{32}P-labelled probes of the *Le.recQ* fragment (Probe 2, in Fig.2-2) and *S. cerevisiae-ACT1* gene.

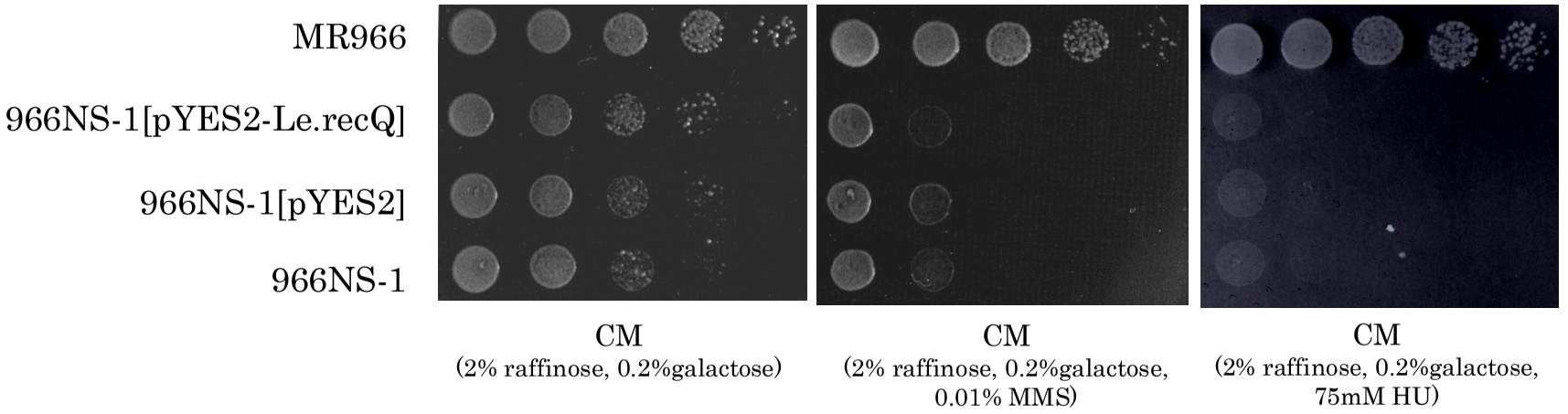

Fig. 3-5. Growth of the *S. cerevisiae* transformants on CM plate containing MMS or HU.

The exponentially growing cells of were serially diluted and spotted on the CM plates. They were incubated at 30°C for three days.

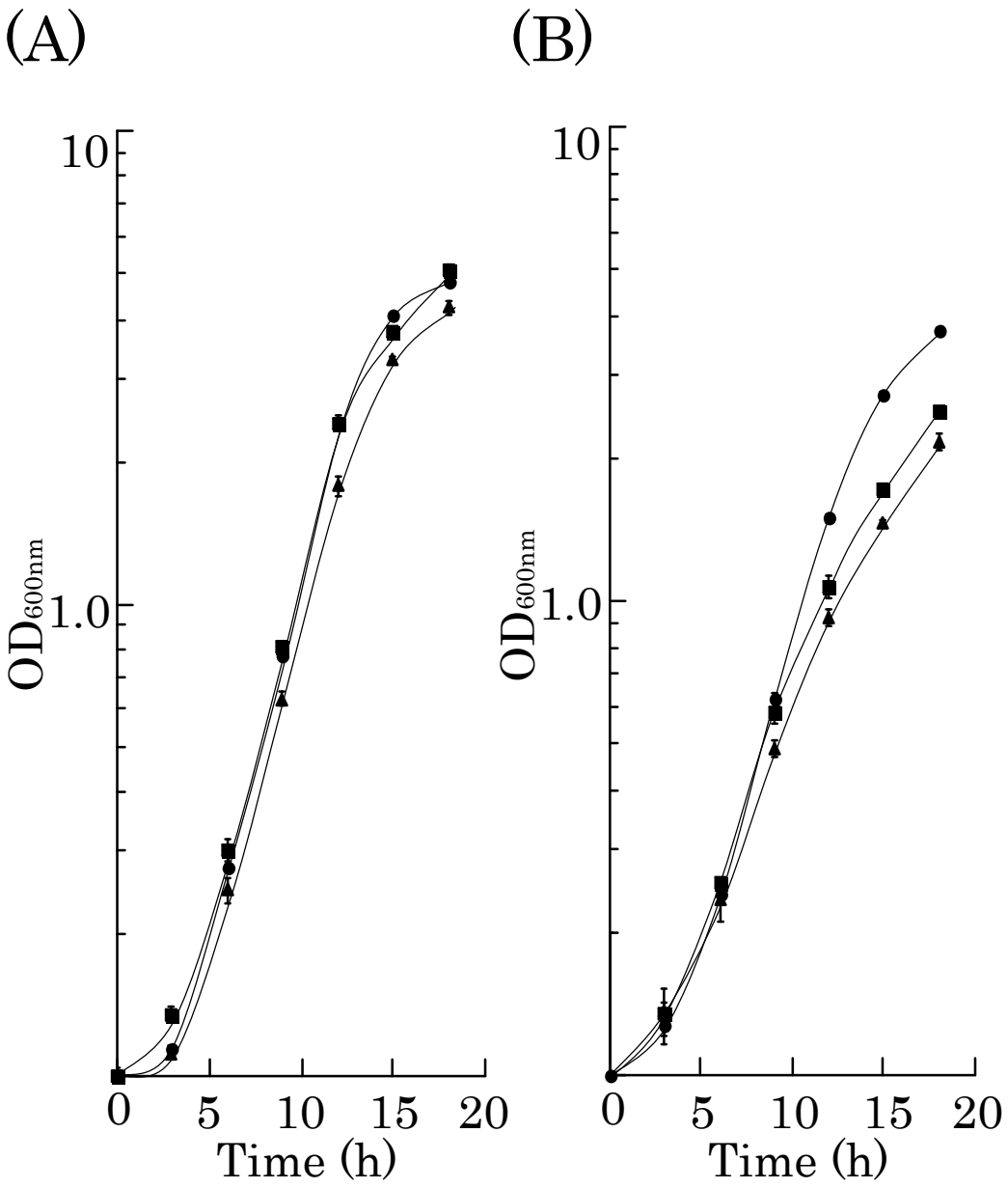

Fig. 3-6. Growth curves of the 966NS-1[pYES2-Le.recQ] (■), the 966NS-1[pYES2-SGS1] (●), and the 966NS-1[pYES2] (▲).

The three *S. cerevisiae* transformants were cultured at 30°C in the CM (without uracil) medium containing 2% raffinose and 0.2% galactose under the absence (**A**) or the presence of 0.001% methylmethanesulfonate (MMS) (**B**). Three independent experiment were done. Average values and SDs are presented.

better than other two strains. As shown in Fig. 3-6A, the growth rate in the absence of MMS of 966NS-1[pYES2-Le.recQ] was similar to that of 966NS-1[pYES2-SGS1], and was higher than that of 966NS-1[pYES2]. In the presence of MMS (Fig. 3-6B), on the other hand, 966NS-1[pYES2-Le.recQ] grew faster than 966NS-1[pYES2], but slower than 966NS-1[pYES2-SGS1]. These results suggest that the *Le.recQ* cDNA can complement *sgs1* mutation of *S. cerevisiae*, but that the complementation is not as efficient as that given by *S. cerevisiae SGS1* gene.

3-4. Discussion

The *Le.*RECQ(core) protein was confirmed to have helicase activity *in vitro*. The functional complementation tests indicated that *Le.recQ* cDNA does complement the slow growth phenotype of the *S. cerevisiae sgs1* mutant, implying that *Le.recQ* play a role in DNA synthesize and cell divisions of the yeast. On the other hand, *Le.recQ* only partially complements the MMS- or HU-sensitivities of the *sgs1* mutant. It has been reported that the N-terminal region of SGS1, which interacts with type I topoisomerase (Top3) is required for complementation of MMS-sensitivity of *sgs1* mutant (Mullen *et al.*, 2000; Ui *et al*, 2001). Since *Le.*RECQ protein is noticeably smaller than SGS1 protein, there is the possibility that *Le.*RECQ protein lacks the domain required for the interaction with Top 3 in *S. cerevisiae*. The biological significance of *Le.recQ* gene in *L. edodes* totally remains to be determined.

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Chapter 4

Transcriptional expression of *Le.recQ* in *Lentinula edodes*

4-1. Introduction

As described in the chapter 3, the functional complementation tests indicated that *Le.recQ* cDNA complements the slow growth phenotype of *sgs1* mutant, implying that *Le.recQ* play a role in DNA synthesize and cell divisions of the yeast. The author studied, in this chapter, the expression of the *Le.recQ* to estimate the biological function of it.

Expression of the five *recQ* genes has been analyzed in different tissues in *A. thaliana* by RT-PCR method. The result showed that the expression of the *RecQl1*, *RecQl2*, *RecQl4A*, and *RecQl4B* genes is higher in shoots and flowers than in leaves and seedlings, but the expression of the *RecQl3* gene is similar among all tissues examined (Hartung *et al.*, 2000). Using Northern-blot analysis, the expression of the *D. melanogaster Recqe* gene during *Drosophila* development has been studied. The *Recqe* mRNA was shown to accumulate in oocytes, and is expressed at high levels in early embryos (Joeng *et al.*, 2000). Except for the *Drosophila* experiment, there has been no report on analysis of the expression of a *recQ* gene during development and morphological differentiation of organisms, as far as I know.

In the typical eubasidiomycetes, a binucleate-celled dikaryon forms a fruiting body. Mycelial development is as follows (see Fig, 4-1). Mycelial aggregation on sawdust-rice (or -corn) bran medium (Takagi *et al.*, 1998) results in the formation of thick membrane-like mycelia (designated preprimordial aggregated mycelia). Primordia are formed on the preprimordial aggregated mycelia. They grow to immature fruiting bodies and progressively up to mature fruiting bodies. Basidia in the fruiting body produce basidiospores and they

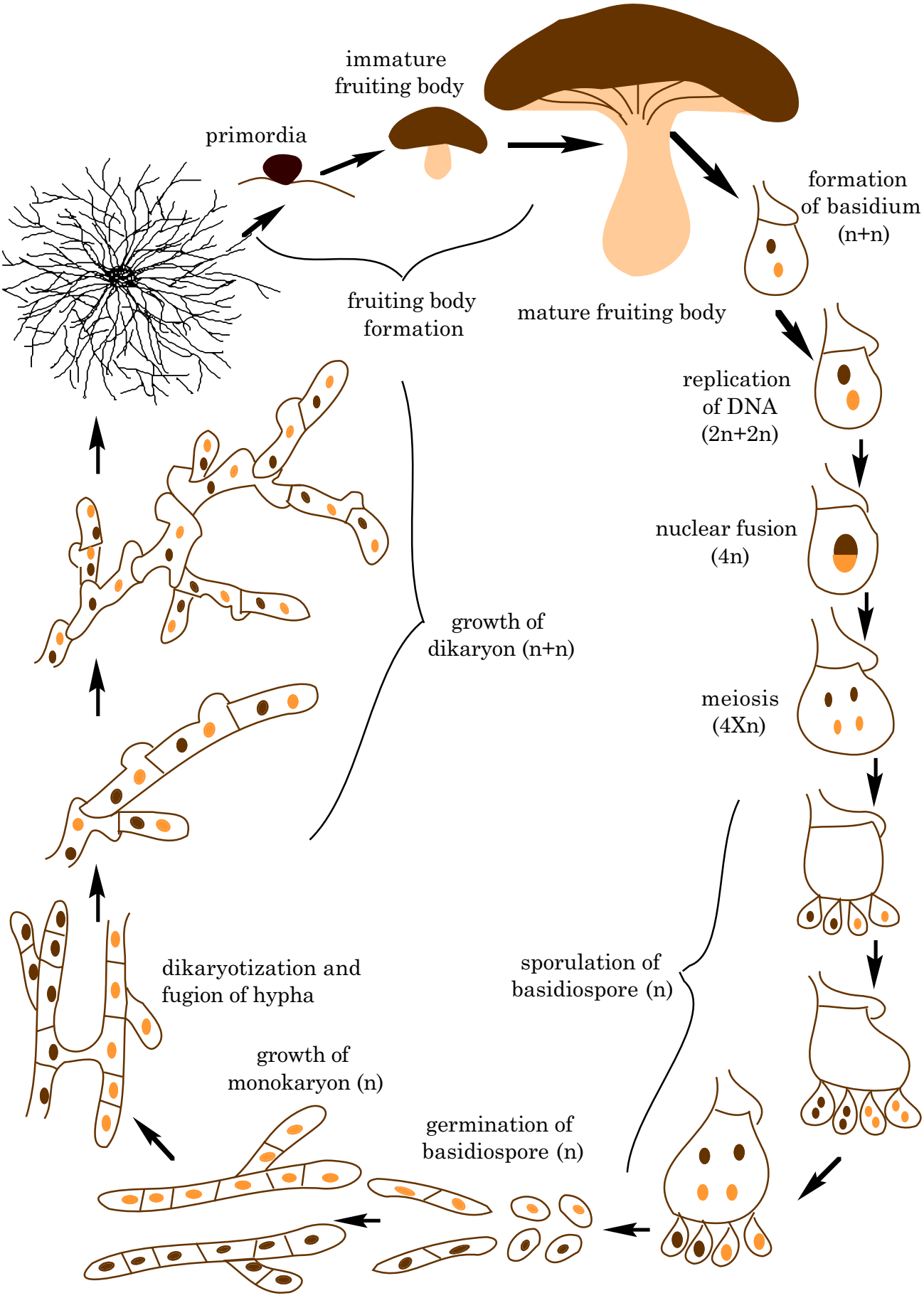

Fig. 4-1. The life cycle of basidiomycete

germinate into uninucleate-celled monokaryon. Crossing of a pair of compatible monokaryon results in a binucleate-celled dikaryon which displays synchronized nuclear division. The author analyzed, in this chapter, the *Le.recQ* transcript in various stages of mycelial development by Northern-blot hybridization, that in parts of fruiting bodies by quantitative reverse transcription (RT)-PCR method, and that in hymenophore (gill tissue) by *in situ* hybridization.

4-2. Materials and Methods

4-2-1. Northern-blot analysis

Total cellular RNA was isolated from preprimordial aggregated mycelia, primordia, immature fruiting bodies, and mature fruiting bodies of *L. edodes* FMC2. Fruiting bodies were formed by growing dikaryotic (binucleate-celled) mycelia on the sawdust-corn bran medium at 17°C (Takagi *et al.*, 1998). Total cellular RNA was also isolated from vegetatively growing mycelial cells of two compatible monokaryotic strains of FMC2, i.e., FMC2-1.1 and FMC2-1.2 (Yasuda and Shishido, 1999) and dikaryotic strain FMC2-2 obtained by crossing FMC2-1.1 and FMC2-1.2. These three strains were cultured in liquid SMY medium (1% sucrose, 1% malt extract, 0.4% yeast extract, pH 5.6) at 25°C for two weeks with shaking. The RNA samples (20 μg each) were size-fractioned on 2.2 M formaldehyde-1.2% agarose gel (Lehrach *et al.*, 1977), transferred to a nylon filter (Reed and Mann, 1985), hybridized with ^{32}P-labelled probes of the PCR-amplified 0.7-kb fragment containing *Le.recQ* conserved sequence and the 1.2 kb *Le.ras* cDNA (Hori *et al.*, 1991) (see Fig. 4-2) under stringent conditions according to the

method of Hori *et al* (1991) and autoradiographed.

4-2-2. Quantitative RT-PCR

This was done for the same amount (1 μg each) of the total cellular RNA isolated from gill-depleted pileus (P), gill tissue (G), and stipe (S) of the fruiting bodies of fruiting-body maturation stage I of *L. edodes* FMC2 (Kaneko and Shishido, 2001) using the specific primers corresponding to *Le*.RECQ conserved amino-acid sequence among RecQ-type helicases, i.e., primer 17 (forward): 5'-GCGATACTCGCAGCTATGGCAGGCC-3' and primer 18 (reverse): 5'-AGGTTTTTGATCACGACCCGCTCG-3' (see Fig. 2-5). For the RT-PCR experiments, the specific of the 5'- and 3'- coding region of the *uck1* gene (Kaneko *et al.*, 1998) were also used and they were primer 19 (forward): 5'-ATTGTCTGATGCTCTCCATCTTCACTCC-3' and primer 20 (reverse): 5'-GCGGTAGAGCCAGAAAGAAGTTTAG-3' . The PCR reaction was under the condition previously reported (Akiyama *et al.*, 2002).

4-2-3. Preparation of ultrathin sections of Lentinula edodes

The ultrathin sections of fruiting bodies were prepared according to the method of Bochenek and Hirsch (1990). The samples were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) (137mM NaCl, 2.68mM KCl, 10mM NaH_2PO_4, 1.67mM KH_2PO_4, pH 7.4) at 4°C for 4 h and washed twice (30 min×2) with 0.5M mannitol/0.02% DEPC solution at 4°C. The resulting samples were embedded in OCT compound (Tissue-Teck) and frozen in liquid nitrogen. The frozen samples were cut longitudinally or laterally into 10-μm thin

cryosection by a cryostat at -20°C and attached to silanized slides (DACO). The slides were dried up at 45°C for 16 h and stored at -80°C until required.

4-2-4. In situ RNA staining of the ultrathin sections of Lentinula edodes

The ultrathin sections of fruiting bodies were stained shocking pink with methylgreen pyronin stain solution (NACALAI TESQUE). The samples were incubated in the stain for 30 min at room temperature and then washed with TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0) (Nishizawa *et al.*, 2002).

4-2-5. In situ RNA-RNA hybridization

Sense and antisense RNA probes were prepared by the *in vitro* transcription of recombinant plasmid, pSPT19-Le.recQc(1712−2256), with T7 or SP6 RNA polymerase and digoxigenin-UTP (Roche Diagnostics GmbH) according to the manufacturer's instructions. The pSPT19-Le.recQc(1712−2256) was constructed as follows. The full length cDNA of *Le.recQ* was digested with *Sal*I and *Xho*I, and the resulting 545-bp fragment was inserted into the *Sal*I site of pSPT19 vector (Roche Diagnostics GmbH). Five μg of pSPT19-Le.recQc(1712−2256) linearized by *Eco*RI was used as a template for the synthesis of sense digoxigenin-UTP labeled RNA probe by T7 RNA polymerase, and 5 μg of pSPT19-Le.recQc(1712−2256) linearized by *Sal*I was used for synthesis of antisense probe by SP6 RNA polymerase. To remove the template DNAs the reaction mixture were treated with DNaseI (0.2U/ml) for 15 min at 37°C, followed by precipitation of the RNA products by addition of 400mM LiCl and 3 volumes of ethanol. The RNA probes obtained were dissolved in 25 μl of DEPC-treated H_2O and stored.

Aforementioned ultrathin sections on slides were immersed in 0.2 M HCl for 10 min and in 0.2% Triton X-100/PBS for 10min at room temperature, followed by washing them with DEPC-treated H_2O. The resulting sections were treated with 1mg/ml Proteinase K/PBS for 20 min at 37°C. After washing with DEPC-treated H_2O, the sections on slides were equilibrated with 0.1M triethanolamine, pH 8.0, for 5 min, and acetylated with 0.25% acetic anhydride in 0.1M triethnolamine, pH 8.0. The acetylated sections were rinsed with DEPC-treated H_2O, and dehydrated with a series of increasing concentrations (30%, 50%, 75%, 95%, 99.5%) ethanol and dried up at 45°C.

The hybridization with the RNA probes was carried out basically according to the procedures reported by Bochenek and Hirsch (1990). The heat-denatured RNAprobes were diluted to give a final 2 μg/ml with hybridization buffer (50% formanide, 300mM NaCl, 10mM Tris-HCl (pH 7.4), 1mM EDTA, 1×Denhart, 0.25%SDS, 10% dextran sulfate, 100μg of salmon sperm DNA and 80U/ml RNase inhibitor). The aforementioned pretreated sections on slides were hybridized with each of RNA probes at 45°C over night. The sections were washed twice with 2×SSC each for 30min at 45°C, and treated with 20μg/ml RNase A in RNase buffer (10mM Tris-Cl, pH 7.5, 1mM EDTA, 500mM NaCl) for 30 min at 37°C. The sections were washed again twice with 2×SSC and twice with 0.1×SSC each for 30 min at 45°C. The sections washed were dehydrated in a series of increasing concentrations (30%, 50%, 75%, 95%, 99.5%) of ethanol and immersed twice in acetone, and then dried up at 45°C.

Immunological detection of the hybridized probe was done using the digoxigenin-nucleic acid detection kit (Roche Diagnostics GmbH) basically according to the manufacturer's instructions. The section hybridized with the probes were briefly washed with maleic acid buffer (100mM maleic acid, 150mM NaCl, pH7.5) and incubated for 2 h in blocking buffer (Roche Diagnostics GmbH).

The resulting sections were incubated with the antibody-conjugate diluted by 5000-fold with the blocking buffer (Roche Diagnostics GmbH), washed twice with the washing buffer (100mM maleic acid, 150mM NaCl, 0.3% Tween20, pH7.5), rinsed briefly in the detection buffer (100mM Tris-HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5), and finally they incubated from 6-16 h with the color substrate solution (NBT/BICP solution) (Roche Diagnostics GmbH) prepared by 50-fold dilution with the detection buffer containing 5% polyvinylalcohol (average molecular weigh 70,000- 100,000) (SIGMA). The coloring reaction was stopped sufficiently washing with TE buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0).

4-3. Result

4-3-1. Transcriptional expression of the Le.recQ *gene in the course of fruiting-body formation of* L. edodes

A Northern-blot analysis was carried out to investigate the expression of *Le.recQ* during mycelial development in *L. edodes* fruiting-body formation on sawdust-corn bran medium. Total cellular RNA was isolated from preprimordial aggregated mycelia, primordia, immature fruiting bodies, and mature fruiting bodies (fruiting bodies of fruiting-body maturation stage I) (Kaneko and Shishido, 2001), and subjected to blot analysis using ^{32}P-labelled probes of the PCR-amplified 0.7-kb *Le.recQ* conserved sequence (Probe 1 in Fig. 2-2) and the cDNA (1.2 kb) of *Le.ras*, which has been shown to be transcribed at similar levels during mycelial development in the fruiting-body formation of *L. edodes* (Hori *et al.*, 1991) . The specific radioactivities of the two probes were almost the same. A

single signal of 3 kb, corresponding to the size of *Le.recQ* cDNA, was detected in all RNA blots and the signal intensities were similar, though they were significantly weaker than those of the 1.2-kb *Le.ras* signals (Fig. 4-2A). The intensities of the *Le.ras* signals were similar in all RNA blots, ensuring an equal loading and transfer of RNA preparations. These results indicate that *Le.recQ* is constitutively transcribed during the fruiting-body formation of *L. edodes*, but that the transcript levels are relatively low.

The author also investigated the transcript levels of the *Le.recQ* gene in vegetatively growing mycelial cells of two compatible monokaryotic strains of FMC2-1.1 and FMC2-1.2 (Yasuda and Shishido, 1999) and dikaryotic strain FMC2-2, obtained by crossing FMC2-1.1 and FMC2-1.2. These strains were cultured in liquid SMY medium at 25°C for two weeks with shaking. As shown in Fig. 4-2B, FMC2-2 (lane 3) contained a several times larger amount of *Le.recQ* transcript than FMC2-1.1 (lane 1) or FMC2-1.2 (lane 2). It was also shown that FMC2-2 grown in liquid medium with shaking (lane 3 of Fig. 4-2B) contains a clearly larger amount of *Le.recQ* transcript than FMC2 (the parental strain of FMC2-2) grown on solid medium (lane 1 of Fig. 4-2A). These results suggest that the *Le.recQ* gene might function much more actively in the binucleate-celled dikaryon of which growth is faster than the uninucleate-celled monokaryons (FMC2-2 grows approximately 1.3 times faster than FMC2-1.1 and 2 times faster than FMC2-1.2).

4-3-2. Transcriptional expression of the Le.recQ *gene in part of the friuiting body.*

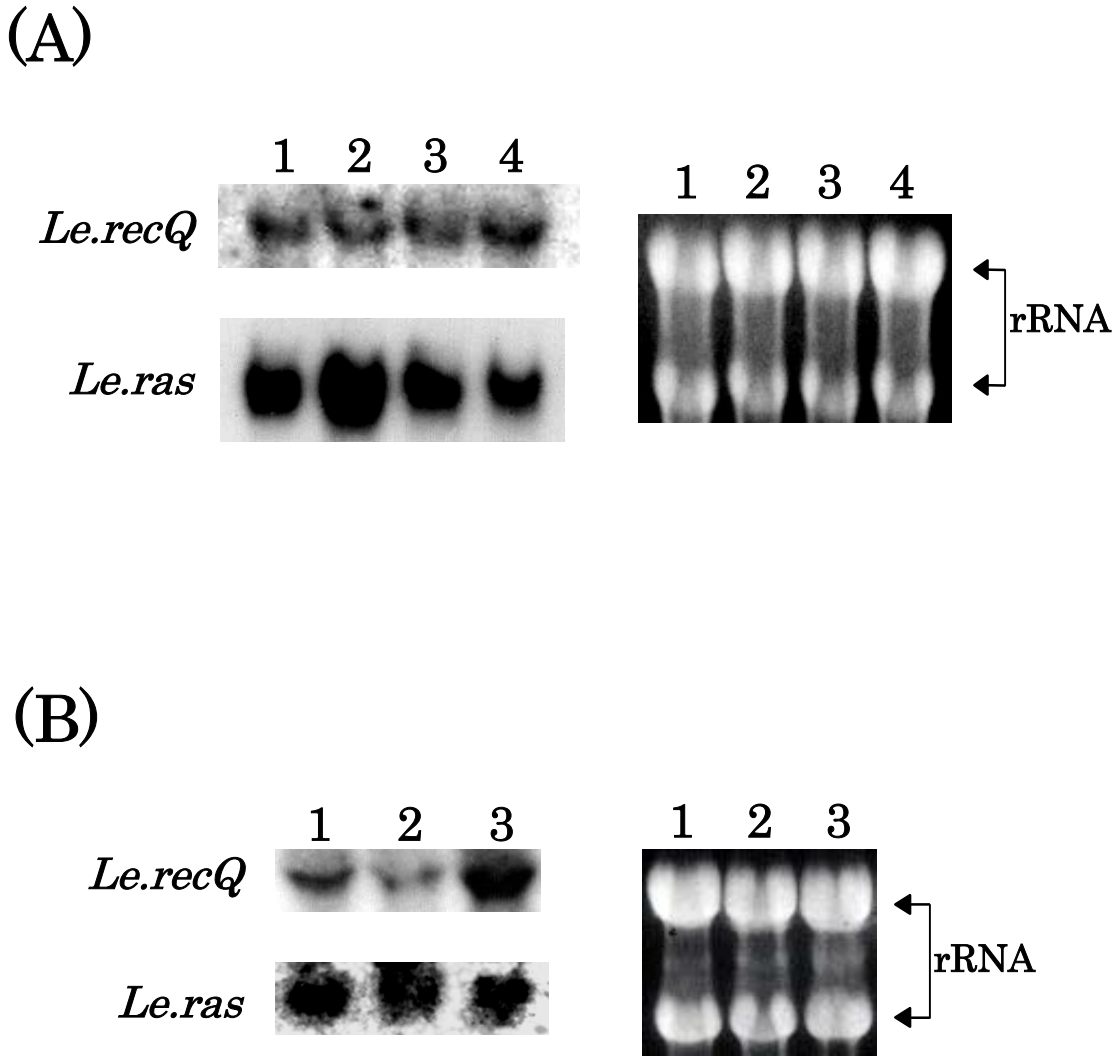

Fig. 4-2. Transcriptional expression of the *Le.recQ* gene in the course of fruiting-body formation (A) and in vegetatively growing dikaryotic and monokaryotic strains of *L. edodes* (B).

(**A**) Total cellular RNA samples (20 μg each) isolated from the preprimordial aggregated mycelia (lane 1), primordia (lane 2), immature fruiting bodies (lane 3) and mature fruiting bodies (fruiting bodies of fruiting-body maturation stage I) (lane 4) were analyzed by Northern-blot hybridization using ^{32}P-labelled mixed probes of the 0.7 kb fragment carrying the conserved helicase domain of *Le.recQ,* and the *L. edodes ras* (*Le.ras*). (**B**) Total cellular RNA samples (20 μg each) isolated from the monokaryotic strains FMC2-1.1 (lane 1) and FMC2-1.2 (lane 2) and the dikaryotic strain FMC2-2 (lane 3) were analyzed by Northern-blot hybridization using the mixed probes described in (**A**).

Quantitative RT-PCR was done for the same amount (1 μg each) of the total cellular RNAs isolated from stipe (S), hymenophore (gill tissue) (G), and hymenophore-depleted pileus (P) of fruiting bodies of of fruiting-body maturation stage I of *L. edodes* FMC2 (Kaneko and Shishido, 2001) using the specific primer DNAs corresponding to the *Le*.RECQ conserved amino acid sequence among RecQ helicases (primers 17 and 18 of Fig. 2-5). For the RT-PCR experiments, the specific primers of the 5'- and 3'-coding region for the *uck1* gene (Kaneko *et al.* 1998; Kaneko and Shishido, 2001) were also used. The DNA fragments amplified by RT-PCR were analyzed by agarose gel electrophoresis (Fig. 4-3). RT-PCR products of *Le.recQ* were detected at 25 cycles and 30 cycles of the reaction. The amounts of the PCR products amplified for the RNAs from stipe (S), hymenophore (gill tissue) (G), and hymenophore-depleted pileus (P) were similar. Kaneko *et al.* have reported that the *uck1* gene is most actively transcribed in the hymenophore (Kaneko *et al.* 1998; Kaneko and Shishido, 2001). As shown in Fig. 4-3, the results expected were obtained. This shows that quantitative RT-PCR does work. Then, the distribution of total RNA in fruiting body was investigated. The fixed 10-μm thin longitudinal cryosection of fruiting body was stained shocking pink with methylgreen pyronin stain (Nishizawa *et al.*, 2002). As shown in Fig. 4-4, total RNA is not evenly distributed in the hymenophore (gill tissue), stipe, or hymenophore-depleted pileus. The order of density of total RNA was as follows: hymenophore > stipe >> hymenophore-depleted pileus. The microscopic observation showed that this order reflects that of the cell density itself (Fig. 4-4B–D). The data in Figs. 4-3 and 4-4, taken together, indicate that *Le.recQ* transcript is present in all the parts of the fruiting body, but in hymenophore at the highest density. The transcript is present also in the stipe at higher density.

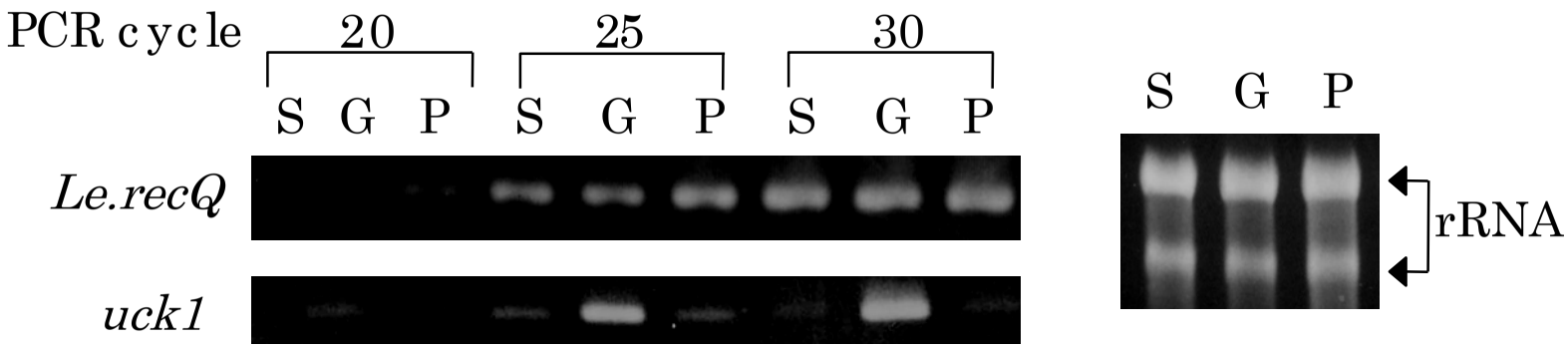

Fig. 4-3. Quantitative RT-PCR of the transcripts of *Le.recQ* and *uck1* genes of *L. edodes*.

The same amount (1 μg each) of total cellular RNAs was used for the experiments. Total cellular RNA samples were isolated from the stipe (S), hymenophore (gill tissue) (G), and hymenophore-depleted pileus (P) of fruiting bodies of fruiting-body maturation stage I. The numbers of PCR cycles are shown at the top. One tenth of the RT-PCR products was put on agarose gels and stained with ethidium bromide. The ethidium bromide-stained ribosomal RNA (rRNA) bands are shown as an internal control on the right side.

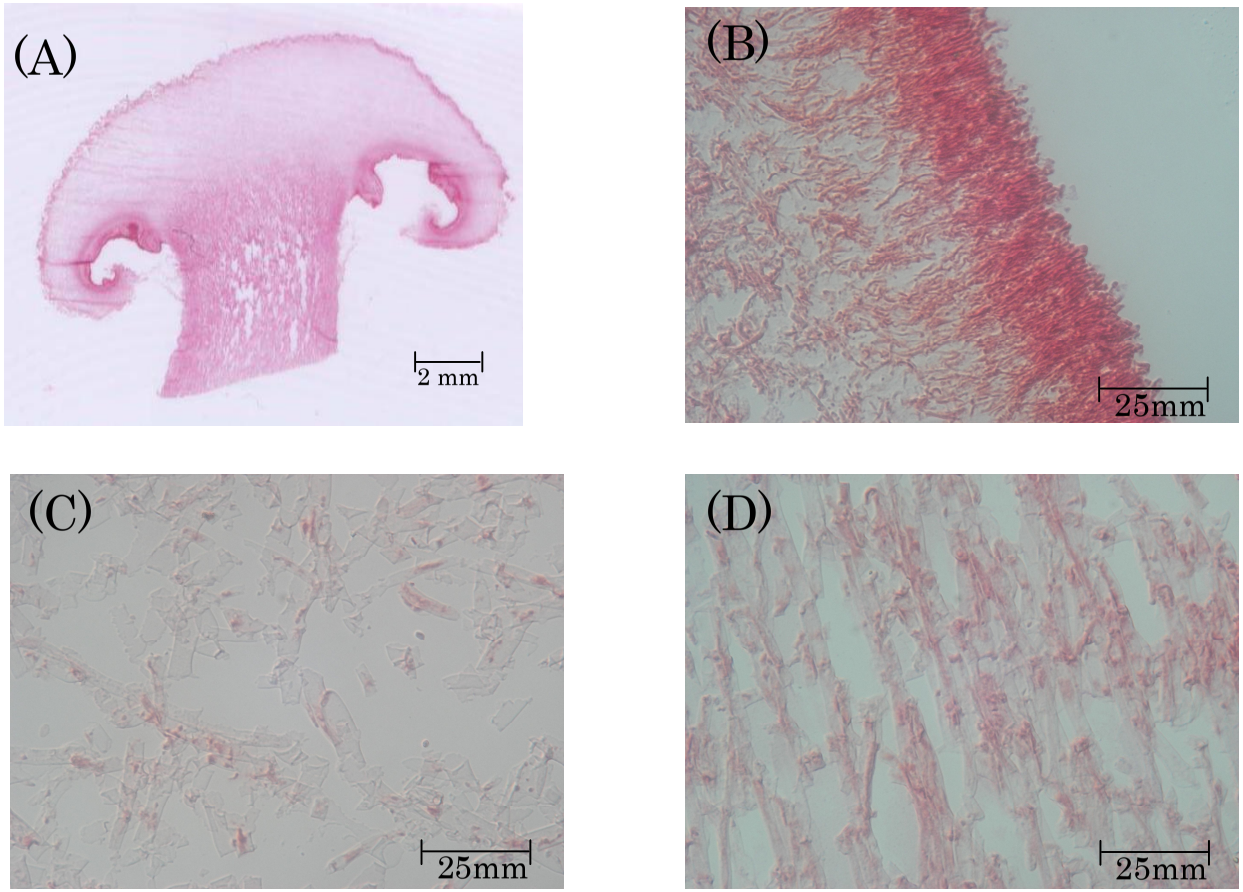

Fig. 4-4. Staining of the total cellular RNA molecules in whole fruiting body and in parts of fruiting body of *L. edodes*.

The longitudinal ultrathin section of the *L. edodes* fruiting body of fruiting-body maturation stage I was stained shocking pink with methylgreen pyronin stain. Panel A is fruiting body and Panels B, C, and D are hymenophore, pileus, stipe, respectively.

The density of the transcript was relatively low in the hymenophore-depleted pileus. The cell density in hymenophore is remarkably high.

4-3-3. Distribution of the Le.recQ *transcripts in the hymenophores of fruiting bodies of* L. edodes

The longitudinal and lateral 10-μm thin sections of hymenophores of fruiting bodies of fruiting-body maturation stage I were stained with methylgreen pyronin stain to investigate the distribution of the RNA molecules. The results showed that total RNA molecles distribute in hymenium at the higher density than subhymenium and trama in the hymenophore (Fig. 4-5).

In situ RNA-RNA hybridization was done to investigate the expression of the *Le.recQ* gene in parts of the hymenophore of fruiting bodies of fruiting-body maturation stage I. The aforementioned 10-μm thin sections were used for the experiment. The degoxigenin-labelled sense and antisense RNA probes of *Le.recQ* cDNA, were hybridized to the ultrathin section of the hymenophores. Since the transcription level of the *Le. recQ* gene is relatively low, the hybridization signals given by the antisense probe were not so intense. The results of *in situ* RNA-RNA hybridization (Fig. 4-6), however, showed that the *Le.recQ* transcript level within the hymenophore is higher in the hymenium (in/on which a large number of basidia and basidiospores are formed), the subhymenium (on top of which the hymenium is formed), and the outer region of the trama (the region branching out into the suybhymenium). Trama cells themselves were found to contain a lower level of the transcript than other parts of the hymenophore. Since the subhymenium is a branching tissue with low cell density, the thin sections

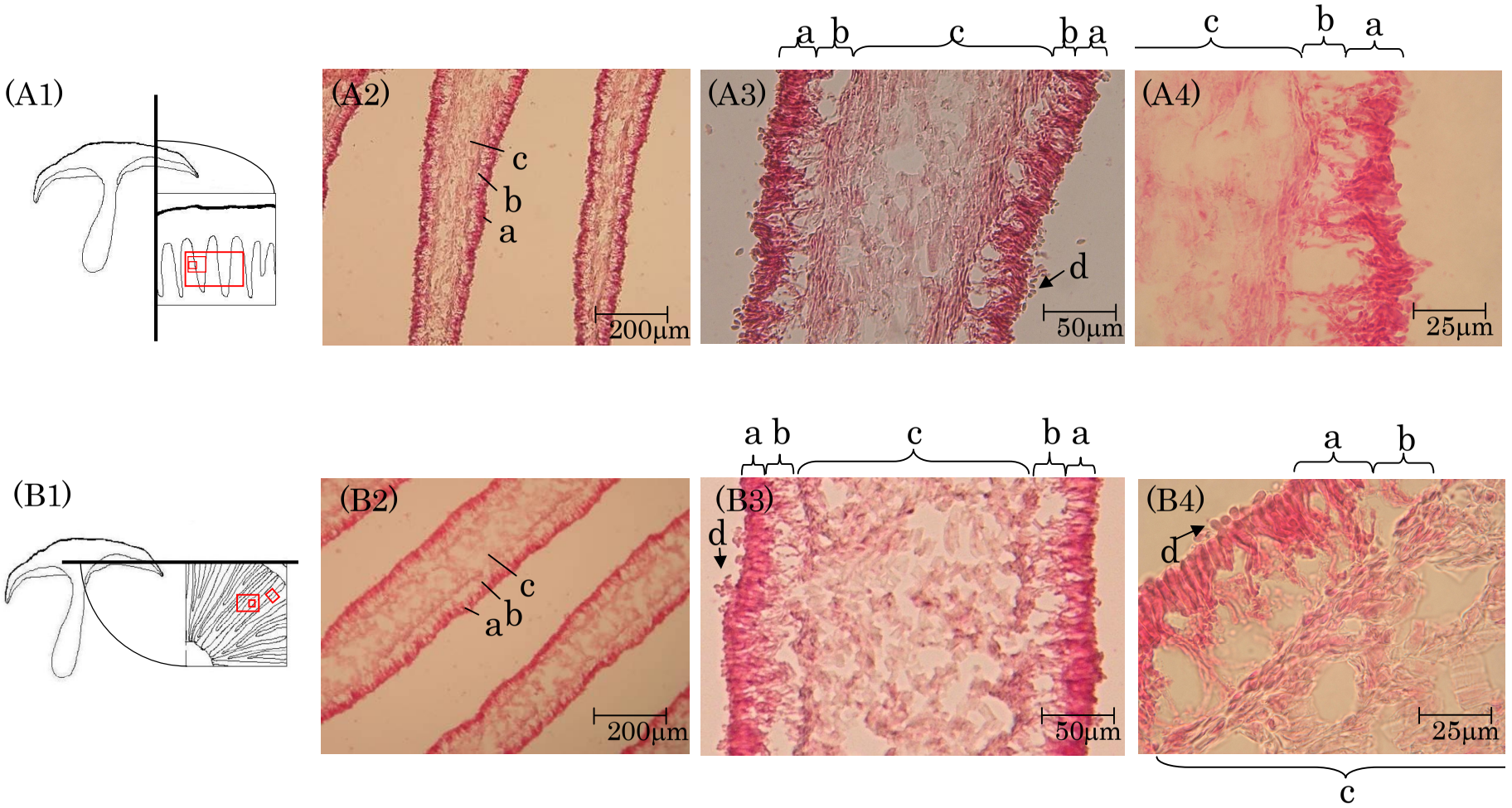

Fig. 4-5.Staining of the total cellular RNA molecules in the hymenophore of fruiting body of *L. edodes*.

The longitudinal (A2–A4) and lateral (B2 –B4) ultrathin section of hymenophores of the fruiting body of fruiting-body maturation stage I were stained shoking pink with methylgreen-pyronyn stain. (a) hymenium: the basidiospores-producing layer. (b) Subhymenium: on the top of which hymenium is formed. (c) trama: the inner part of hymenophore (the outer region of trama is the region branching out into subhymenium). (d) basidiospore. The figures on the left side are the schematic representation of the parts (red boxed) of the hymenophore used for staining (A1, B1).

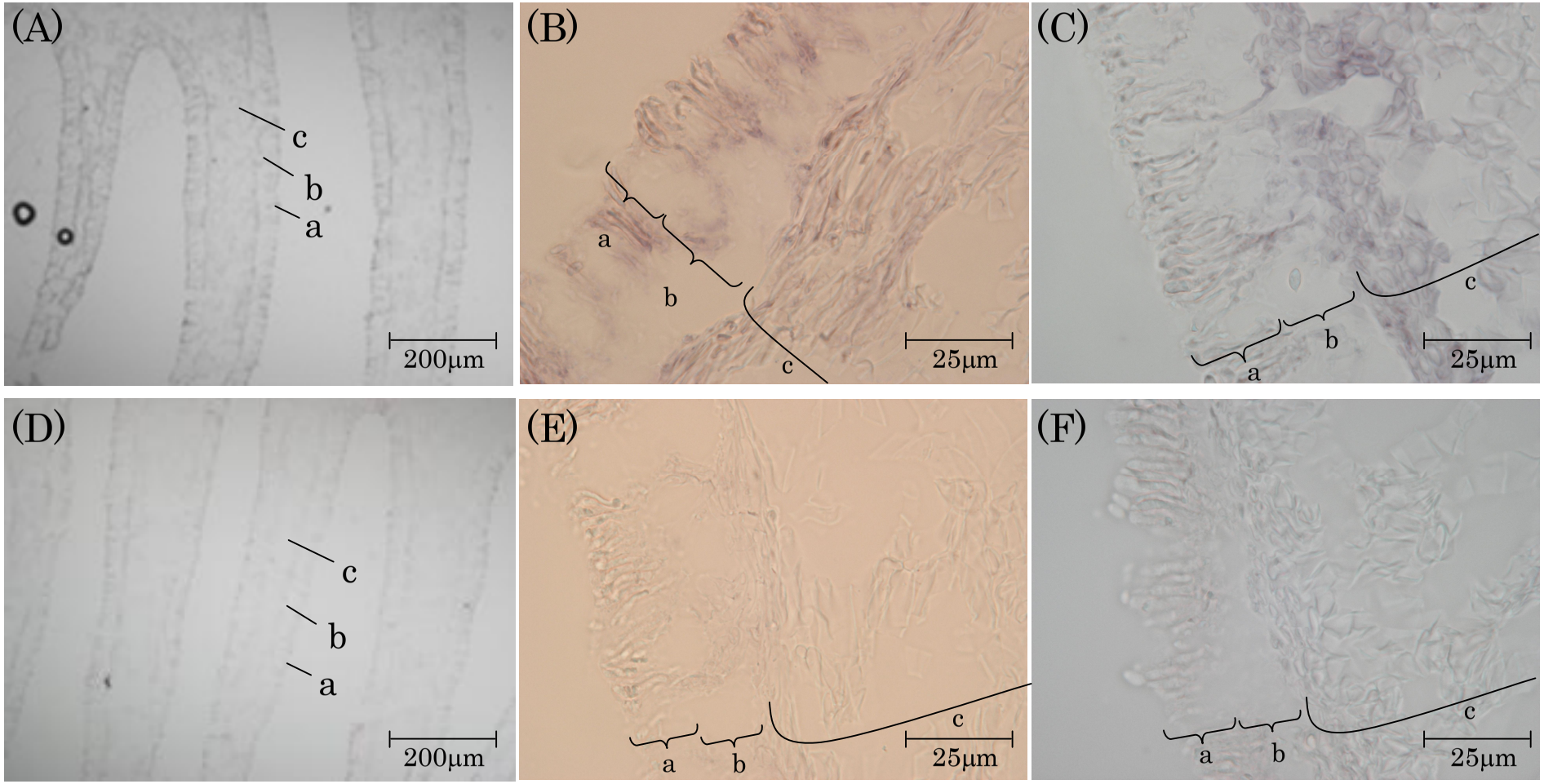

Fig. 4-6. Distribution of the *Le.recQ* transcript in hymenophores of fruiting bodies of *L. edodes*.

Fixed longitudinal (panels A, B, D, and E) and laternal (panels C and F) thin sections of the hymenophores of the fruiting body of fruiting-body maturation stage I were hybridized with digoxigenin-labeled *Le.recQ* antisense (panels A, B, and C) or sense (panels D, E, and F). In panels A–F: (a) hymenium: the basidiospores-producing layer. (b) Subhymenium: on the top of which hymenium is formed. (c) trama: the inner part of hymenophorethe (the outer region of trama is the region branching out into subhymenium).

prepared had holes in the subhymenium region (see Fig. 4-5A4 and B4). Owing to this, the hybridization signal of the subhymenium region was sparse. But the intensity of signal was similar to those of the hymenium and the outer region of the trama. These results suggest that the *Le.recQ* gene may play a role in the growth of mycelial cells in divergence regions of the hymenophore and may be involved in basidiospore formation.

4-4. Discussion

The *Le.recQ* gene is expressed at similar levels at all stages of the fruiting-body formation of *L. edodes* (Fig. 4-2), suggesting that it always functions in all dikaryotic mycelial cells of *L. edodes*. *Le.recQ* transcript levels are low as compared with the *Le.ras* transcript levels (Fig.4-2). One of the possible explanations for this difference is as follows: Whereas the *Le.ras* promoter contains a CAAT-box, two consecutive TATA-boxes, and a CT-rich stretch (Kajiwara and Shishido, 1992) the promoter region (nucleotide (-)117 (transcription start point) — nucleotide (-)500) of the *Le.recQ* gene contained only a TATA-like sequence (Fig. 2-5). The promoter of the *L. edodes priA* gene, of which the expression level is relatively high, contains a GC-box, a CAAT-box, a TATA-box, and a CT-rich stretch (Yamazaki *et al.*, 2000; Kajiwara and Shishido, 1992). The CT-stretch is known frequently to be found near/in the transcription start point of fungal strong promoters (Kajiwara and Shishido, 1992).

Northern-blot analysis indicated that the binucleate-celled dikaryotic strain FMC2-2 contains an amount of *Le.recQ* transcript several times larger than uninucleate-celled monokaryotic strains of FMC2-1.1 or FMC2-1.2. The growth

rate of FMC2-2 is approximately 1.3 times higher than that of FMC2-1.1 and 2 times higher than FMC2-1.2. FMC2-1.1, which grows more rapidly than FMC2-1.2, contains a larger amount of *Le.recQ* transcript. The dikaryotic strain FMC2-2, which grew more rapidly in SMY liquid medium (with shaking), contains a significantly larger amount of *Le.recQ* transcript than the dikaryotic strain of FMC2 ,which grew slowly on sawdust-corn bran (solid) medium. So there exists a correlation between *Le.recQ* transcription level and growth rate of the mycelial cells. Efficient expression of the *Le.recQ* gene is considered to be required for good growth of mycelial cells, implying a role in DNA replication.

Quantitative RT-PCR analysis and total RNA staining showed that *Le.recQ* transcript is present at highest dencity in the hymenophre, where a large number of spores are produced. So the author investigated the distribution of *Le.recQ* transcript in the hymenophore. Results of *in situ* RNA-RNA hybridization showed that the hymenium, the spore-producing place, contains relatively large amount of *Le.recQ* transcript, though the hybridization signals were not intense. The subhymenium, on top of which hymenium is formed, and outer region of trama, the region branching to subhymenium, also contained relatively large amount of *Le.recQ* transcripts. The results of Northern-blot analysis, quantitative RT-PCR, and in situ hybridization, taken together, showed that the levels of *Le.recQ* transcription are relatively high in actively growing mycelia, branching tissues, and reproductive tissue, suggesting the function of *Le.recQ* in DNA replication. However, to clarify the biological significance of the *Le. recQ* gene in *L. edodes*, a genetical approach is necessary.

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Chapter 5

Conclusion

RecQ helicases have been reported to be related to the fundamental genetic processes such as replication, recombination, and repair, and maintenance of genomic stability in various organisms. In this thesis, the author cloned and sequenced a *recQ* gene homologue (*Le.recQ*) from mushroom *Lentinula edodes*, as the first report of RecQ helicase in eubasidiomycetous fungi. Molecular and cellular biological studies were performed to characterize the *Le.recQ* gene and its expressed product. The following new evidences are obtained from the experiments.

1. The *Le.recQ* gene was found to have a coding capacity of 945 amino acids and be interrupted by 11 small introns. The promoter region of the *Le.recQ* gene contains only a TATA-like sequence at the 40 nucleotide upstream of the transcription starting point.

2. The comparison of the amino-acid sequences of the *Le.recQ* gene product (945 amino acids), *Le.*RECQ and other RecQ proteins such as *Neurospora crassa* QDE3 (1955 amino acids), *Schizosaccharomyces pombe* Rqh1 (1328 amino acids), *Saccharomyces cerevisiae* SGS1 (1447 amino acids), *Arabidopsis thaliana* RecQl4A (1182 amino acids), *Escherichia coli* RECQ (610 amino acids), *Homo sapiens* BLM (1417amino acids), and *Homo sapiens* WRN (1432 amino acids) revealed that the helicase domain (motifs I, Ia, II~VI,and motif 0) and RecQ C-termonal domain containing the zinc finger-like motif are highly conservrd between *Le.*RECQ and other RecQ proteins The *Le.RECQ* exhibited the highest homology to the *A. thaliana RecQl4A* in its size and to *S. pombe*

Rqh1 in its amino acid sequence of the helicase domain.

3. The *in vitro* helicase assay using partially double-stranded DNA substrate confirmed that GST-*Le*.RECQ catalytic core fusion protein produced in *E. coli*, possesses 3'→5' helicase activity.

4. The functional complementation tests indicated that *Le.recQ* cDNA complemens the slow growth phenotype of the *S. cerevisiae sgs1* mutant, implying that *Le.recQ* play a role in DNA synthesize and cell divisions of the yeast. On the other hand, *Le.recQ* only partially complements methyl methanesulfonate-sensitivity of the *sgs1*mutant.

5. The Northern-blot analysis showed that the *Le.recQ* gene is transcribed at similar levels during mycelial development in *L. edodes* fruiting-body formation, suggesting that it always function in all dikaryotic mycelial cells of *L. edodes*. On the other hand, the vegetative dikaryotic mycelial cells were found to contain a larger amount of *Le.recQ* transcript than the two compatible monokaryotic mycelial cells. The result suggested that the *Le.recQ* gene might function much more actively in the binucleate-celled dikaryon of which growth is faster than the uninucleate-celled monokaryons.

6. The reverse transcription PCR experiment and the total RNA staining indicated that the *Le.recQ* transcript is present in hymenophore at the highest density in the fruiting body. The *in situ* RNA-RNA hybridization analysis showed the presence of larger amount of *Le.recQ* transcripts in the hymenium (in/on which basidia and basidiospores are formed), subhymenium (on top of

which the hymenium is formed), and outer region of trama (the region branching out into the subhymenium), in the hymonophore. The trama cells themselves contain a lower amount of transcript.

7. The evidences described in 4–6 suggest that *Le.recQ* is involved in DNA replication in cell growth and basidiospore formation of *L. edodes*. The author hopes that this study contributes to the understanding of the molecular mechanisms of genomic maintenance in eubasidiomycetous fungi.

List of papers

Papers for dissertation

1. Katsukawa, S., Yamazaki, T., Kajiwara, S., and Shishido, K., "A *recQ* gene homolog from the basidiomycetous mushroom *Lentinula edodes*: sequence analysis and expression", *Biosci. Biotechnol. Biochem.*, **68**, 2588-2597, (2004)
2. Katsukawa, S, and Shishido, K., "Analysis of *recQ* gene transcript in fruiting bodies of basidiomycetous mushroom *Lentinula edodes*", *Biosci. Biotechnol. Biochem.*, **69**, 2247-2249, (2005).

Other papers

1. Tanaka, Y., Kaneko, S., Katsukawa, S., Yamazaki, T., Ohta, A., Yasumasa, M., Shishido, K., "Specific distribution in homobasidiomycete hymenophores of the transcripts of Ras protein and G-protein α-subunit genes", *FEMS Microbiol. Lett.*, **242**, 169-175 (2005).

2. Katsukawa, S., Yamazaki, T., Kajiwara, S., and Shishido, K., "A New Member of RecQ Helicase Family from Mushroom", Proceedings of 7th European Conference on Fungal Genetics (April 17-20, 2004, Copenhagen, Denmark) P.97 (2005).

3. Katsukawa, S., Kaneko, S., Tanaka, Y., Sakuragi, Y., Yamazaki, T., Miyazaki, Y., and Shishido, K., "Specific distridution in basidiomycete *Lentinula edodes* hymenophore of the transcripts of various *L. edodes* genes", Proceedings of 23rd Fungal Genetics Conference(March 15-20, 2005, Asilomar Conference Ground, America) p.159 (2005).

4. Katsukawa, S, and Shishido, K., "Sequence analysis and expression of *recQ* gene homologue from *Lentinula edodes*", Proceedings of Genetics and Cellular Biology of Basidiomycetes VI (June 3-6, 2005, Pamplona, Spain) p.64 (2005).

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