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Fujii, Mie

Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

Takahashi, Masateru

Division of Chemical & Life Sciences and Engineering, King Abdullah University of Science and Technology

Mon, Hiroaki

Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University

Tatsuke, Tsuneyuki

Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University : Research Fellow of the Japan Society for the Promotion of Science

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Molecular Cloning of the Silkworm p53R2-like Gene

Mie FUJII¹, Masateru TAKAHASHI², Hiroaki MON³, Tsuneyuki TATSUKE^{1,4},
Jae Man LEE³ and Takahiro KUSAKABE^{3*}

Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and
Pest Management, Faculty of Agriculture, Kyushu University Graduate School 6–10–1

Hakozaki, Higashi-ku, Fukuoka 812–8581, Japan

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The p53R2 is a homologue of the small subunit of mammalian ribonucleotide reductase. In human, the transcription of p53R2 is induced clearly by active form of p53 and required for DNA synthesis during cell division and DNA repair. In this study, we have isolated and determined cDNA sequence for *Bombyx mori* p53R2-like (p53R2-L) protein. The Bmp53R2-like protein had high homology to the Hsp53R2, but had histidine residue at a position corresponding to Y241 of Hsp53R2, which is a key residue in discriminating human p53R2 and R2. Bmp53R2-L expressed strongly in the silk gland, gonad and blood cell, in which cell division occurs actively and DNA synthesis is required. The knockdown of the *Bmp53R2-L* in BmN4-SID1 led to a substantial arrest in G1/S. This G1/S arrest may be caused by the retardation of DNA synthesis due to the depletion of dNTPs in nucleus under the conditions of the knockdown of *Bmp53R2-L*. Thus, *Bmp53R2-L* is a functional homolog of human R2 rather than p53R2, and considered to be indispensable for DNA synthesis and cell cycle progression in a DNA damage independent manner.

Key words: Cell cycle, DNA synthesis, DNA repair, G1/S phase, p53R2, RNAi, Silkworm

INTRODUCTION

Insects are known to be resistant to ionizing radiation (IR) compared with mammals (Shubhankar Suman *et al.*, 2009). In the previous research, it was showed that the silkworm cell line BmN4 did not undergo apoptosis after γ -ray irradiation at a dose of 100 Gy. In mammalian cells, 10 Gy of γ -irradiation is enough to activate G1/S or G2/M cell cycle arrest and induce apoptosis (Wilson *et al.*, 2004). In contrast, BmN4 cells could arrest the cell cycle at G2/M phase around 24h after 30 Gy of γ -irradiation, but then restarted the cell cycle progression gradually without inducing apoptosis (Takahashi *et al.*, 2006). Furthermore, UV-C irradiation at a dose of that cause G1 arrests and apoptosis in mammalian cells, could only induce the tentative G1 arrests. In mammalian cells, p53 is known to act as a master regulator to these responses against severe DNA damage (Levine *et al.*, 2006). For example, it has been reported that p53-deficient tumor cells could not arrest the cell cycle at the G1/S phase, when these cells encountered serious DNA damage (Kuerbitz *et al.*, 1992). From this point of view, *B. mori* p53 pathway is assumed to be different from that of mammal. The silkworm also has p53 homologous

gene in their genome, but its function and expression have been unrevealing. To elucidate the role of p53 in DNA damage response pathway in *B. mori*, we have focused on downstream factors of p53. In this study, we have cloned and characterized *B. mori* p53R2-like gene, named *Bmp53R2-L*. In human, p53R2 is ribonucleotide reductase induced by the activated p53 in response to DNA damage (Tanaka *et al.*, 2000). Low levels of the human p53R2 protein are also expressed constitutively in both non-proliferating and proliferating cells, even in the absence of DNA damage (Hakansson *et al.*, 2006). However, it has been reported that the overexpression of p53R2 in p53-deficient human cells causes a G2/M arrest (Tanaka *et al.*, 2000). Thus, basal levels of p53R2 are expressed regardless of p53 status and p53R2 expression is upregulated by p53 in response to DNA damage (Yanamoto *et al.*, 2005).

Human genome has the three ribonucleotide reductase genes, hRRM1 (R1), hRRM2 (R2), and p53R2. Ribonucleotide reductase functions a tetramer composed of two non-identical homodimers R1 and R2. The R1 dimer contains a catalytic site, binding sites for allosteric effectors, and redox active disulfide that participate in the reduction of substrates, while the R2 dimer contains a tyrosyl free radical generated by an iron center and essential for catalysis (Thelander *et al.*, 1994). The p53R2 (RRM2B) gene was first reported by Tanaka *et al.* (2000), in which it was initially found in a colon cancer cell line. The p53R2 has 80% sequence homology with the R2 (Tanaka *et al.*, 2000). There are two RR genes in *Drosophila melanogaster*, RnrL and RnrS, which are hRRM1(R1) and hRRM2(R2) homolog, respectively, and p53R2 homologous gene has not been reported.

In the present study, we revealed that the *Bmp53R2-L* had conserved important amino acid residues for its functions, and expressed predominantly in

¹ Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University

² Division of Chemical & Life Sciences and Engineering, King Abdullah University of Science and Technology, 4700 KAUST Thuwal 23955–6900 Jeddah, Kingdom of Saudi Arabia

³ Laboratory of Silkworm Sciences, Division of Genetics and Plant Breeding, Department of Applied Genetics and Pest Management, Faculty of Agriculture, Kyushu University

⁴ Research Fellow of the Japan Society for the Promotion of Science

* Corresponding author (E-mail: kusakabe@agr.kyushu-u.ac.jp)

silkgrand, gonad and blood cell. The silencing of *Bmp53R2-L* gene by RNAi caused G1/S arrest in BmN4-SID1 cultured cells, suggesting that Bmp53R2-L are constantly required for DNA synthesis in S phases in a DNA damage independent manner.

MATERIAL AND METHODS

Molecular cloning of *Bmp53R2-L*

The entry clones for *Bmp53R2-L* was constructed on pENTRTM11 from Invitrogen. To obtain the entry clone for these genes, the total RNA was isolated from the BmN4 cultured cells stocked in our university. This total RNA was reverse-transcribed with oligo (dT) primer and amplified with the two primer sets Bmp53R2-L-5'(GCTCCTACTGTTGATAAAGAGAATCTTCATAGC) and Bmp53R2-L-stop (GATGCAGACTTCTAGCACCTAAATCTCGAGGG) under the following conditions: 38 cycles of 94°C for 15 sec, 54°C for 30 sec, 68°C for 90 sec. The primer sets were designed from the registered *B. mori* sequence data. The amplified Bmp53R2 cDNA were digested with *Mse*I, blunt-ended, and subcloned into between the blunt-ended-*Nco*I site and the *Eco*RV site of the pENTRTM11. The resulting plasmids were named Bmp53R2-L-pENTR11, this nucleotide sequences was determined using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Cell culture and RNAi experiments

The BmN4-SID1 cells, which are transgenic cell line and keep *Caenorhabditis elegans sid-1* gene in their genome, stocked in our university were maintained in IPL-41 insect medium (Sigma Chemical) supplemented with 10% fetal bovine serum (GIBCO Invitrogen) at 23°C (Mon *et al.*, 2012). These cells were split at ration of 1:7 every 4–5 days. Double-strand RNA was prepared by in vitro transcription according to standard methods (Zamore *et al.*, 2000; Tomoyasu and Denell, 2004) with minor modification. T7 promoter sequence was attached to the both end of the fragments of the gene Bmp53R2-L. Primers 5'-GCGTAATACGACTCACTATAGGGCTTCGTTTTGGACCGTGAGGAAGTC-3' and 5'-GCGTAATACGACTCACTATAGGGCTGAATGTAAGTCCCGGCATAAGGC-3' were used. Obtained PCR product was phenol-extracted, precipitated and used as a template of transcription reaction by T7 RNA polymerase. Both strands were transcribed and double-strand RNA of about 500 bp in length was obtained. Cells were cultured, the next day, double-strand RNA were introduced into plate by 400 ng/ml. After 5 days, we were performed expression analysis and cell cycle analysis. In microscopy, all the images were collected using a KEYENCE BZ-8000.

Expression analysis of the *Bmp53R2-L* gene

Expression profile of Bmp53R2-L mRNA was analyzed in 28 cycles of RT-PCR against the cDNA reverse-transcribed from the testes, ovaries, fatbodies, midgut, silk gland, male and female blood cells, malpighian tubule, head and BmN4-SID1 cultured cells. The primers used

were 5'-GTTTAATGCGGTTGAGACTCTTCCC-3' and 5'-GTTTCACTAGATGCAGACTTCTAG-3' for Bmp53R2-L mRNAs. As a control, GAPDH mRNAs were amplified in 25 cycles of RT-PCR with the primers 5'-GGCCGCATTGGCCGTTTGGTGCTCCG-3' and 5'-GTGGGGCAAGACAGTTTGTGGTGCAAGAAG-3'. The amplified products were separated by electrophoresis through a 1% agarose gel in TAE buffer and stained with ethidium bromide.

Cell cycle analysis

The exposure of cultured cells to UV was performed using Spectrolinker XL-1500UV crosslinker (Spectronics, Lincoln, NE) and UV-radiation was carried out by UV-C (254 nm) light. Flow cytometric analysis was performed with a Guava PCA-96 Flow Cytometer (MILLIPORE), and the obtained data were analyzed using FlowJo software (Treestar). Cells were fixed by adding 70% ethanol and kept at 4°C until used. Fixed cells were washed with PBS and then treated with RNaseA. Cells were stained by propidium iodide (PI, SIGMA #P4170) and analyzed immediately using the flow cytometer.

RESULTS AND DISCUSSION

Molecular cloning of *B. mori p53R2-L* gene

p53R2 encodes a ribonucleotide reductase, an enzyme catalyzing the formation of deoxyribonucleotides from ribonucleotides to control DNA synthesis. In addition, p53R2 is thought to play a role in scavenging reactive oxygen species (ROS) and thereby protecting mitochondrial membrane potential from oxidative stress induced damage, indicating that p53R2 acts in preventing cancer by maintaining genomic integrity in the presence of oxidative stress. The Bmp53R2-L cDNA contained a complete ORF of 1128 bp encoding 375 amino acid residues and predicted molecular weight of 43 kDa. By amino acid sequence comparison with human p53R2, the residues to be important in its function were conserved in Bmp53R2-L amino acid sequences except Y241 in Hsp53R2. In human p53R2, residues E194, E228, H134, E131, H231 and D100 are responsible for coordination of the two iron atoms, and the two tyrosine residues that generate the tyrosyl radical are Y138 and Y124. Y49, Y285 and Y241 are tyrosine residues responsible for the antioxidant properties of p53R2 (Fig. 1, Zhou *et al.*, 2011, Xue *et al.*, 2006, Nordlund *et al.*, 2006). As mentioned above, amino acid residue corresponding to Y241 of Hsp53R2 was replaced with histidine at the position of a.a. 265 in the Bmp53R2-L. The similar substitution is reported in H279 of R2, another human ribonucleotide reductase that could not respond to p53 activation signal (Yamaguchi *et al.*, 2001).

Bmp53R2-L are expressed predominantly in silkgrand, gonad and blood cell

To examine the *Bmp53R2-L* expression patterns, we performed semi-quantitative RT-PCR analyses on various tissues from *B. mori* larvae on day 3 of the fifth instar. *Bmp53R2-L* are expressed strongly in the silkgrand,

Bmp53R2-L	1	MAPTVDKENLHSGINGENRVQTKSPMKNSVLATKNDNLTEEMDLKDNFEE	50
Hsp53R2	1	-----MGDPERPEAAGLDQD---ERSSSDTNESE	26
Bmp53R2-L	51	FDPQKEPLLRENPRRFVIFPIQYPDIIWQMYKAEASFWTVEEVDLSKDLS	100
Hsp53R2	27	IKSNEEPLLRSRRFVIFPIQYPDIIWQMYKQAQASFWTAEVVDLSKDLP	76
Bmp53R2-L	101	DWETLKDSERHFIKHVLAFFAASDGIIVNENLVERFSQEVQVTEARCHYGF	150
Hsp53R2	77	HWNKLEKADKFIKSHLAFFAASDGIIVNENLVERFSQEVQVPEARCHYGF	126
Bmp53R2-L	151	QIANENHSENYSLIDTYIRDPKERDFLFNAVETLPCVKKKADWALQWI	200
Hsp53R2	127	QILLENHSENYSLIDTYIRDPKKREFLFNAIETMPYVKKKADWALRWI	176
Bmp53R2-L	201	ASKTATFGERIVAFAAVEGIFFGSGSFASIFWLKKRGLMPGLTFSNELISS	250
Hsp53R2	177	ADRKSTFGERVVAFAAVEGIFFGSGSFAAIFWLKKRGLMPGLTFSNELISR	226
Bmp53R2-L	251	DEGLHDFACLMFCYLVQKPTKECVLHIIKDAVVIEQEFITDALPVRLLG	300
Hsp53R2	227	DEGLHDFACLMFCYLVNKPSEERVREIIVDAVKIEQEFITDALPVGLIG	276
Bmp53R2-L	301	MNCELMSCYIELVADRLLVDLMGEKHYNTKNPFDNMNLSLEGKTNFFEK	350
Hsp53R2	277	MNCILMKCYIEFVADRLLVELGFSKVFQAENPFDNMENISLEGKTNFFEK	326
Bmp53R2-L	351	KVGEYQKCGVMANRLDNVFTLDADF	375
Hsp53R2	327	RVSEYQRFAVMAETTDNVFTLDADF	351

Fig. 1. Alignment of the amino acid sequence of Bmp53R2-L and Hsp53R2.

gonad and blood cell, while *Bmp53R2-L* are expressed slightly in the fat body, midgut and malpighian tube (Fig. 2). In the silkgand, gonad and blood cell, cell division occurs actively and DNA synthesis is required. Thus, *Bmp53R2-L* is expressed strongly in these tissues.

Effects of Bmp53R2-L on cell cycle in BmN4-SID1

To investigate the role of *Bmp53R2* in BmN4-SID1 cultured cells (Mon *et al.*, 2012), we tried to decrease the expression of *Bmp53R2-L* gene by RNAi. As expected, the expression of *Bmp53R2-L* was decreased drastically (Fig. 3). Silencing of *Bmp53R2-L* led to a substantial arrest in G1/S (Fig. 4), probably due to the retardation of DNA synthesis by the depletion of dNTPs in the nucleus. We also noted distinctive morphological alterations in the Bmp53R2-L knocked down cells. The shape of Bmp53R2-L knocked down cells strikingly stretched relative to that of control cells (Fig. 5).

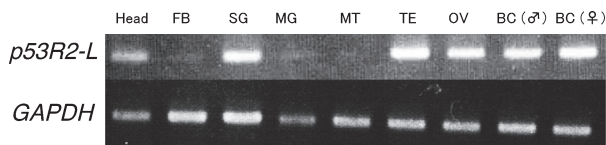


Fig. 2. The expression pattern of Bmp53R2-L in various tissues of *B. mori* day 3 fifth instar larvae. Semi-quantitative RT-PCR analyses were performed cDNA library from the head, fat body (FB), silk gland (SG), midgut (MG), malpighian tube (MT), testis (TE), ovary (OV) and blood cell (BC ♂ and BC ♀).

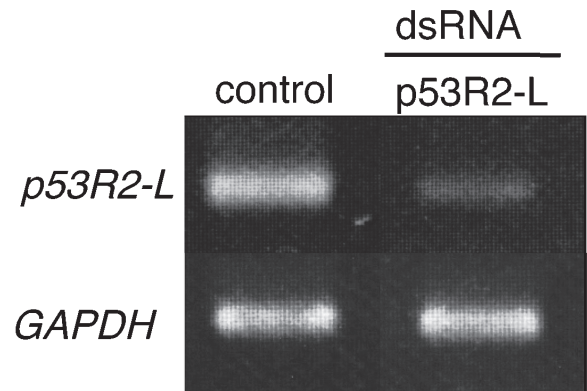


Fig. 3. Semi-quantitative RT-PCR analyses were performed using the cDNA library from Bmp53R2-L knocked-down BmN4-SID-1 cells.

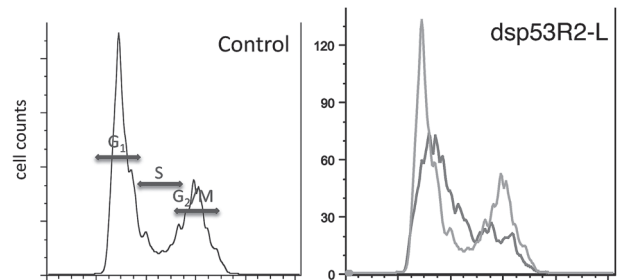


Fig. 4. Cell cycle analysis of BmN4-SID1 cells under the conditions of knockdown of Bmp53R2 (dsBmp53R2-L) and without dsRNA (Control).

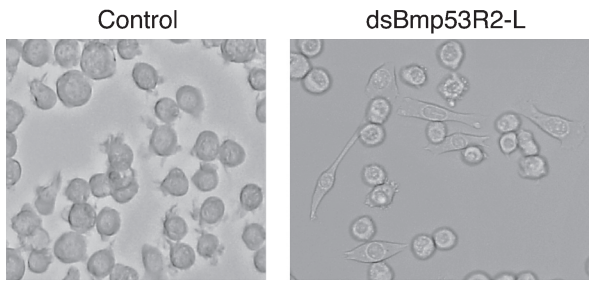


Fig. 5. Morphological change of BmN4-SID1 cells, dsBmp53R2-L and no dsRNA (Control) introduced.

Furthermore, the overexpression of silkworm p53 could not up-regulate the transcription of *Bmp53R2-L* mRNA (Shimada *et al.*, manuscript in preparation). Taken together, mRNA expression profile and the presence of H265 strongly suggested that Bmp53R2-L is a functional homolog of R2, and p53-responsible ribonucleotide reductase gene is absent from the silkworm genome.

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