Improvement of Cellulose Degradation via Highly Expressed LebglA in Radiation Induced Mutant of *Lentinula edodes*

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Abstract - Cellulose degradation improved mutants, LER120, LER121, LER277R80, LER277R88, and LER277R140 were induced from *Lentinula edodes* KACC42378 using gamma ray radiation (^{60}Co) at the dose of LD_{99} (0.94kGy). To understand the cellulase activity enhanced mechanism in these mutants, we examined the expression of cellulase genes, cellobiohydrolase (cel7A and cel6B), endoglucanase (Lecel5A and Lecel12A) and β-glucosidase (Lxyl-p1 and LebglA) using the qRT-PCR. The expression of LebglA was significantly up-regulated in all mutants (LER120; 6.5 fold, LER121; 10.2 fold, LER277R80; 2.0 fold, LER277R88; 17.6 fold, LER277R140; 18.5 fold) compared with that of the wild type (WT) in the 1% carboxymethylcellulose (CMC) media, while the expression of cellobiohydrolases and endoglucanases did not changed. To clarify the mutation on the sequences of the LebglA by radiation, we cloned LebglA coding DNA sequence (CDS). There was no sequence variations between LebglA CDSs of each mutant and WT. Based on commonly up-regulation of LebglA expression in all cellulose degradation improved mutants, LebglA could play a principal role in cellulose degradation of *L. edodes* and β-glucosidase specific regulator gene(s) might be turned on in mutants in this study.

Key words : Cellulase, β-glucosidase, *Lentinula edodes*, Mutant, Radiation

INTRODUCTION

L. edodes is the second most widely cultivated edible mushroom due to its flavor, nutritional, and medicinal value (Mizuno 1995). It has been grown on dead wood or its sawdust which mainly composed of cellulose, hemicellulose and lignin due to its lignocellulolytic ability. Overall, the bioconversion efficiency of cellulose is moderate in *Lentinula edodes* and its vegetative growth is longer than the other edible mushroom (Leatham 1985; Buswell *et al*. 1996; Ohga and Royse 2001). Therefore, the cellulase activity improvement is needed for the more efficient usage of cellulose and the shortening of lifecycle of mushroom cultivation.

The decomposition of cellulose to glucose was completed by synergistic action of enzyme complex including cellobiohydrolases (EC3.2.1.91), endoglucanases (E.C. 3.2.1.4), and β-glucosidases (EC3.2.1.21). Endoglucanases hydrolyze randomly the internal β-glycosidic bond of loosen amorphous region of cellulose and release the new end of cellulose for cellobiohydrolases (Teeri 1997). Cellobiohydrolases act processively on crystalline cellulose and cleave cellulose chains into cellobiose units. Endoglucanase and cellobiohydrolase cleaves cooperatively and synergistically the cellulose to cellobiose and oligosaccharides. β-glucosidases degrades finally cellobiose into glucose. The expression of these genes is induced by product (i.e. cellobiose) released from the degradation of plant cell wall and coordinately regulated by transcription factors (Amore *et al*. 2013; Tani *et al*. 2014). Also, carbon catabolite repression (CCR) triggered by glucose represses the expression of cellulases and hemicellulases (Sun and Glass 2011; Liu *et al*. 2013).

To date, owing to the absence of information on the full

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genomic sequence of *L. edodes*, only some cellulase genes have been characterized. cel7A (GH7) and cel6B (GH6) as genes encoding 1,4-β-D-cellobiohydrolases (EC3.2.1.91) were cloned (Lee *et al*. 2001). Lecel12A (GH12) encodes 1,4-β-D-endoglucanases (E.C. 3.2.1.4) (Takeda *et al*. 2013). Cheng *et al*. (2013) cloned and characterized the Lxyl-p1-1 and Lxyl-p1-2 which were bifunctional for β-D-glucosidase and β-D-xylosidase. Both Lxyl-p1-1 and Lxyl-p1-2 had higher β-D-glucosidase activity than β-D-xylosidase activity. Our recent RNA sequencing result showed that putative endoglucanase encoding gene, Lecel5A (GH5) and β-glucosidases encoding gene, LeblgA (GH3) were highly expressed in cellulase activity enhanced mutant LER277 under 1% CMC media (unpublished data).

In this study, we induced the cellulose degradation improved mutants, in *L. edodes* KACC42378 by using gamma ray radiation and analyzed the expression of cellulase genes to understand the mechanism of cellulase activity improvement.

MATERIALS AND METHODS

Strains and culture condition

Lentinula edodes KACC 42378 from the Korea Agriculture Collection Center (Korea) were cultured on potato dextrose agar (PDA) plates at 25°C and maintained at 4°C.

Mutation induction

Mycelia of *L. edodes* KACC42378 were irradiated on potato dextrose agar (PDA) plates by gamma ray radiation by the already mentioned method (Chung *et al*. 2017). Irradiated mycelia were punched out using 5 mm sterile cork borer and placed on new PDA plates. The plates were incubated for 9 days at 25°C and then were placed in the middle of 1% CMC (CMSM II) agar plates (Sathesh-Prabu and Lee 2012) to screen the cellulase activity improved mutants. The plates were incubated at 25°C for 20 days. Mycelial lengths were measured by the distance from the center to the periphery of agar plug for 3 days and 20 days.

qRT-PCR

Mycelia of the wild type and LER120, LER121, LER277 R80, LER277R88 and LER277R140 were cultured on 1% CMC agar plate for 20 days. Mycelia were collected and immediately frozen with liquid nitrogen (LN_2). Mycelia were fine grounded with mortar and blue pestle in LN_2. Total RNA was extracted using PureLink RNA mini kit coupled with Trizol (Ambion, Life Technology) according to the manufacturer's protocol. cDNA was reverse transcribed using SuperScript III First-strand Synthesis Supermix (Invitrogen). Total RNA (0.5 μg) and oligo-dT (50 μM) primer were used in 40 μl reaction. qRT-PCR was performed triplicates for each sample using SYBR green on the CFX96 Real-Time PCR Detection System (Bio-rad). The cellulase genes and their primers used in qRT-PCR were listed in Table 1. Actin was used as reference gene to normalize the expression of target genes.

Cloning of LebglA

cDNA from mycelia of WT and LER120, LER121, LER 277R80, LER277R88 and LER277R140 were used to amplify the full length cDNA of LebglA. The primer sets used for PCR were listed in Table 1. PCR reaction was carried out in a final volume of 50 μl (2 μl of the above synthesized cDNA as a template, 10 μl of 5X Phusion HF Buffer, 1 μl of 10 mM dNTPs, 2.5 μl of 10 μM each primer, and 0.5 μl of Phusion DNA Polymerase) in initial denaturation at 98°C for 3 min, 35 cycling (98°C for 10 sec, 69°C for 10 sec, 72°C for 1 min 30 sec), and final extension at 72°C for 10 min. Each amplified LebglA cDNA was cloned into pCR®8/GW/TOPO vector (Invitrogen). Each cloned LebglA from WT, LER120, LER121, LER277R80, LER277R88 and LER277 R140 was sequenced by Cosmo Genetech (Seoul, Korea). The nucleotide sequence alignment analysis was done with Clustal W.

RESULTS

Mutant induction

To induce the cellulose degradation improved mutants, the dose of LD_{99} (0.94 kGy) of gamma ray (^{60}Co) was irradiated on mycelia of *L. edodes* KACC42378 and LER277. The cellulose degradation improved mutants, LER120, LER121, LER277R80, LER277R88 and LER277R140 were selected on 1% CMC agar plates. CMC was used as the only carbon source. Mycelial lengths of WT, LER120, LER121, LER 277R80, LER277R88 and LER277R140 were 7.3, 8.1, 7.4, 7.8, 8.8, and 8.8 mm at 3 days and 18.2, 28.4, 23.1, 21.2,

Table 1. Primer lists used in this study

Gene name		Sequence	For use
Reference	Actin	Forward 5′-GTGCTCCTGAGGCCCTCTT-3′ Reverse 5′-GGATTCCGGCAGCTTCTAAA-3′	qRT-PCR
β-glucosidase	LebglA	Forward 5′-GGATATTTGAGGACGGAAGGC-3′ Reverse 5′-CCAATAAGTATCCAGTCGCCAG-3′	
Endoglucanase	Lecel5A	Forward 5′-GTGATTTGATCTGGCAAGCTG-3′ Reverse 5′-ACACATATACATCACTTCCAGGG-3′	
	Lecel12A	Forward 5′-CACTGCAAAGGGCGTACAGCTC Reverse 5′-GACAGGTTGGATACCTCCTTGGC	
Cellobiohydrolase	cel6B	Forward 5′-TCCTTCCCTGCTCATTTCATC Reverse 5′-TTACACCAGTCACCCCATTG	
	cel7A	Forward 5′-CTACACCAACTGCTACACCG-3′ Reverse 5′-TTCGTAATCAGCCCCATCAAG-3′	
β-glucosidase	LebglA	Forward 5′-GTGCTCCTGAGGCCCTCTT-3′ Reverse 5′-GGATTCCGGCAGCTTCTAAA-3′	cDNA

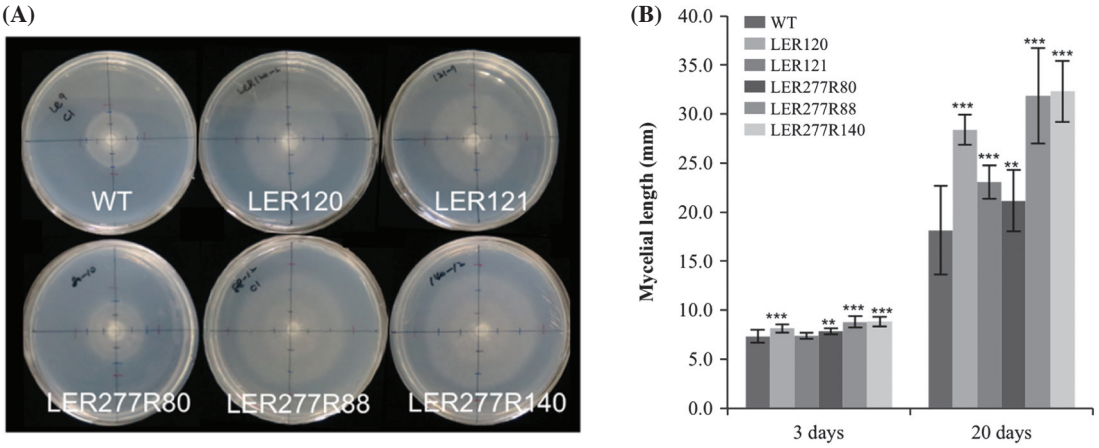

Fig. 1. Mycelial growth of the wild type and mutants induced by using gamma ray radiation. A. Mycelia of the wild type and mutants grown on 1% CMC agar plates for 20 days. B. Mycelial lengths of the wild type and mutants grown on 1% CMC agar plates for 20 days. Data indicate means ± SD of triplicates (n = 20, *t*-test: **; $p < 0.01$, ***; $p < 0.001$).

31.9, and 32.3 mm at 20 days on 1% CMC agar plates, respectively (Fig. 1). Mycelial length of LER120, LER121, LER277R80, LER277R88 and LER140 increased 57%, 27%, 17%, 75%, and 78% compared with that of WT at 20 days, respectively.

The expression of cellulase genes in cellulose degradation improved mutants

To analyze the expression of cellulase genes in cellulose degradation improved mutants, we carried out the qRT-PCR with mycelia of WT, LER120, LER121, LER277R80, LER 277R88 and LER277R140 grown on 1% CMC media for 20 days. The expression of cel7A in LER120 and LER277R80 was upregulated 1.7 and 1.9 fold, respectively, while that of LER121 and LER277R140 was downregulated 2.4 fold and LER277R80 did not changed. cel6B, Lecel5, Lecel12A and Lxy-P1 was not changed or decreased in all mutants. Whereas the expression of LebglA of LER120, LER121, LER277R80, LER277R88 and LER140 was significantly upregulated 6.5, 10.2, 2.0, 17.6 and 18.5 fold, respectively, compared with that of WT at 20 days (Fig. 2). These data suggest that specific transcriptional regulation of LebglA play an important role in cellulose degradation improved mutants of *L. edodes* KACC42378.

The cloning of LebglA of cellulose degradation improved mutants

Because the sequence mutation by radiation can lead to

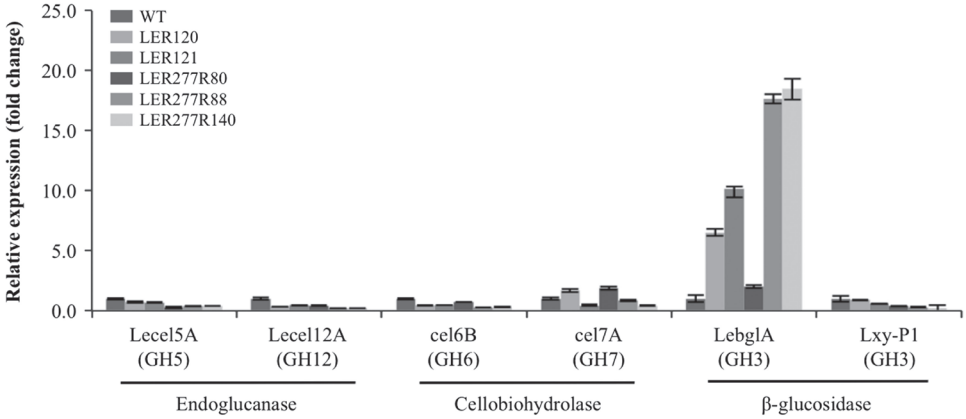

Fig. 2. Expression of cellulase genes using qRT-PCR. Mycelia were cultured in 1% CMC agar media for 20 days. The expression of actin gene was used as reference gene for normalization. Error bars indicate standard deviation of three replicates. Expression level of each gene in WT was set to 1.

enhance the cellulase activity, LebglA CDS of WT and all mutants were amplified by PCR and cloned. LebglA in *L. edodes* KACC42378 had two variants, LebglA-1 and LebglA-2. Both variants consisted of 2250 base pairs (bp) and had 98.75% (2222 bp/2250 bp) identity. Deduced amino acid sequences of LebglA-1 and LebglA-2 had 99.3% (744 aa/749 aa) identity. The sequence variation of deduced amino acid of these variants appeared only in Fn3 like domain, C-terminal of LebglA (Fig. 3). Fn3 like domain was mainly found in gylcoside hydrolase 3 (GH3) which have a β-glucosidase activity and its function has not been known. Only LebglA-1 was cloned in all mutants and there has not identified any mutation on the sequences of LebglA-1 CDSs among WT and all mutants (data not shown). Although the expression level of two variants was not verified due to high sequence homology identity, the result indicated that LebglA-1form might be dominantly regulated for enhanced cellulase activity in all mutants.

DISCUSSION

Because edible mushrooms were grown on lignocellulosic material such as woods, its sawdust, and agricultural byproducts, their lignocellulolytic ability is very important to improve the productivity and quality of mushroom. Thus, we induced the cellulase acitivity enhanced mutants and analyzed the expression pattern of cellulase genes to understand the cellulose degradation improved mechanism. We induced successfully the cellulose degradation improved mutants, LER120, LER121, LER277R80, LER277R88 and LER277R140 by using gamma ray radiation.

When the mycelia of WT and mutants were cultivated on CMC media for 20 days, LebglA significantly upregulated in all mutants compared with WT. The expression of cellobiohydrolase and endoglucanases was overally downregulated in cellulose degradation improved mutants except the expression of cel7A was slightly upregulated in LER120 and LER277R80. Generally, cellulose was completely degraded by synergistic mechanism by cellobiohydrolases, endoglucanases and β-glucosidase. The expression of these encoding genes was highly coordinated by transcriptional activation and repression mechanism (Amore *et al*. 2013). When the cellulose was used as the only carbon source for cellulolytic fungi, it was degraded into cellobiose by basal expressed cellobiohydrolase and endoglucanse. Accumulated cellobiose induced β-glucosidase. High glucose increased by β-glucosidase acts as repressor of cellulases by carbon catabolite repression (CCR) and β-glucosidase gene expression. Consistent with this, it has been reported that the fine tune regulation between cellulase gene expression and β-glucosidase gene expression is important for cellulose degradation in filamentous fungus (Li *et al*. 2015). These support that the high concentration of glucose by an increase of LebglA expression might result in CCR and in turn might result in a reduction of cellulase gene expression. Another possibility is the low concentration of cellobiose by an increase of LebglA expression might result in repression of cellobiohydrolase and endoglucanase genes expression.

Upregulation of LebglA gene expression in cellulose degradation improved mutants was supported by many studies showing that the improvement of β-glucosidase activity

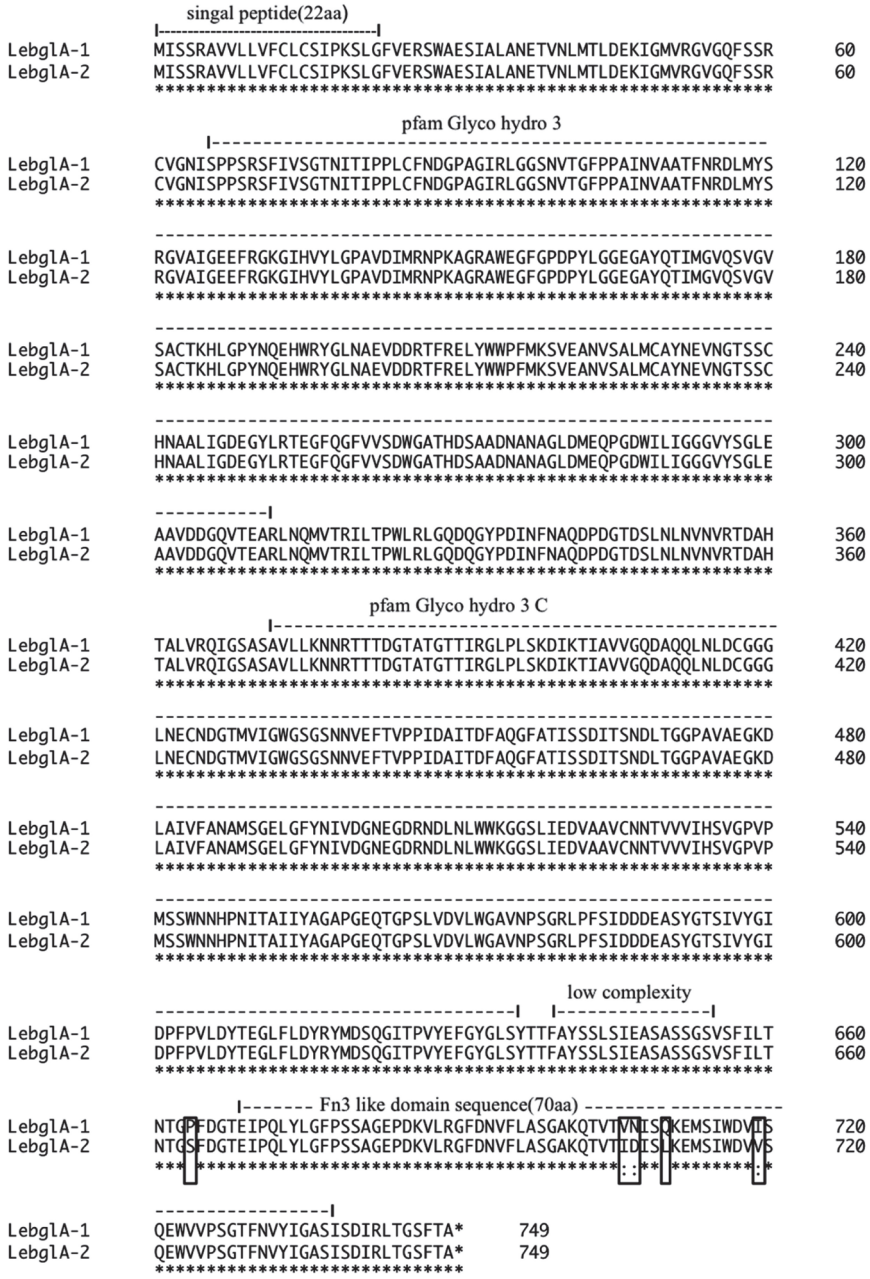

Fig. 3. Amino acid sequence of LebglA variants, LebglA-1 and LebglA-2.

increased the cellulase activity. *Penicillium oxalicum* has been studied as harmonized cellulases producer with high β-glucosidase activity compared with *Trichodema reesei* which have a poor β-glucosidase activity (Yao *et al*. 2016). Constitutively or conditionally overexpressed β-glucosidase genes of *P. oxalicum* prominently enhanced cellulase activity (Yao *et al*. 2016). Zhao *et al*. (2016) reported that β-glucosidase gene expression was antagonistically regulated with cellulase genes and xylanase genes in cellulase and xylanase high activity mutant of *P. oxalicum* HP7-1. Nitta *et al*. (2012) revealed that the expression of β-glucosidase was regulated by β-glucosidase specific regulator BglR in *Trichodema reesei*. Thus, β-glucosidase specific regulator gene(s) might be turned on in mutants.

LebglA CDS presented two variants LebglA-1 and LebglA-2 in WT. Both LebglA-1 and LebglA-2 are highly conserved (99.3% amino acid identity) and showed only 5 substitution of Fn3 like domain of C-terminus, indicating two variants were evolved by duplications of ancestral gene. Of two variants, because only LebglA-1 was cloned in all mutants,

LebglA-1 form might be dominantly regulated for the enhanced cellulase activity in all mutants. Fn3 like domain might affect the efficiency of the β-glucosidase activity. There was no sequence variation between LebglA-1 CDSs of WT and each mutant.

In summary, we suggest that β-glucosidase specific regulator gene(s) might be turned on in mutants in this study and LebglA plays a significant role in processing cellulose degradation.

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