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Lentinula edodes Genome Survey and Postharvest Transcriptome Analysis

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ABSTRACT *Lentinula edodes* is a popular, cultivated edible and medicinal mushroom. *Lentinula edodes* is susceptible to postharvest problems, such as gill browning, fruiting body softening, and lentinan degradation. We constructed a *de novo* assembly draft genome sequence and performed gene prediction for *Lentinula edodes*. *De novo* assembly was carried out using short reads from paired-end and mate-paired libraries and by using long reads by PacBio, resulting in a contig number of 1,951 and an N_{50} of 1 Mb. Furthermore, we predicted genes by Augustus using transcriptome sequencing (RNA-seq) data from the whole life cycle of *Lentinula edodes*, resulting in 12,959 predicted genes. This analysis revealed that *Lentinula edodes* lacks lignin peroxidase. To reveal genes involved in the loss of quality of *Lentinula edodes* postharvest fruiting bodies, transcriptome analysis was carried out using serial analysis of gene expression (SuperSAGE). This analysis revealed that many cell wall-related enzymes are upregulated after harvest, such as β-1,3-1,6-glucan-degrading enzymes in glycoside hydrolase (GH) families GH5, GH16, GH30, GH55, and GH128, and thaumatin-like proteins. In addition, we found that several chitin-related genes are upregulated, such as putative chitinases in GH family 18, exochitinases in GH20, and a putative chitosanase in GH family 75. The results suggest that cell wall-degrading enzymes synergistically cooperate for rapid fruiting body autolysis. Many putative transcription factor genes were upregulated postharvest, such as genes containing high-mobility-group (HMG) domains and zinc finger domains. Several cell death-related proteins were also upregulated postharvest.

IMPORTANCE Our data collectively suggest that there is a rapid fruiting body autolysis system in *Lentinula edodes*. The genes for the loss of postharvest quality newly found in this research will be targets for the future breeding of strains that keep fresh longer than present strains. *De novo Lentinula edodes* genome assembly data will be used for the construction of a complete *Lentinula edodes* chromosome map for future breeding.

KEYWORDS chitin, fruiting body, glucan, lentinan, senescence

Lentinula edodes, popularly known as the shiitake mushroom, is a widely cultivated edible and medicinal mushroom in Asia. The shiitake is the second most cultivated fungus, and over 1,321,000 tons of *Lentinula edodes* have been produced in China, Japan, Taiwan, and Korea (1, 2). *Lentinula edodes* is used as a medicinal mushroom; lentinan, a β-1,3-1,6-glucan with antitumor activity, is present in this mushroom (3). Recently, it was shown that lentinan can be effective for treating gut inflammation (4). It is also reported that polysaccharides extracted from *Lentinula edodes* can restore age-attenuated immune responses and reverse age-related alterations of gut microbiota compositions in mice (5). Furthermore, *Lentinula edodes* can be a source of ergosterol and ergothioneine (6). *Lentinula edodes* has the potential for use not only as a fresh food but also as a source of medicinal compounds. Therefore, it is necessary to

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breed new *Lentinula edodes* strains that contain these beneficial compounds. However, *Lentinula edodes* requires a longer cultivation time than other mushrooms, such as *Flammulina velutipes* and *Pleurotus ostreatus* (7), and thus it takes a very long time to breed new strains using typical breeding methods based on mating and evaluating fruiting body traits. Effective breeding methods based on gene function are needed.

Genome sequence data have been published for basidiomycetous fungi such as *Phanerochaete chrysosporium* (8), *Laccaria bicolor* (9), *Coprinopsis cinerea* (10), *Agaricus bisporus* (11), *Schizophyllum commune* (12), and *Flammulina velutipes* (13). Because of the popularity of next-generation sequencing, many genome sequence drafts of basidiomycetous fungi are publicly available, especially from the 1,000 Fungal Genomes Project (14). More recently, the constructions of *de novo* draft genome sequences of *Lentinula edodes* were reported (15, 16).

Some transcriptome analyses with genome sequence data have been reported in several mushrooms, such as *Agaricus bisporus* (11), *Schizophyllum commune* (12), and others. Transcriptome analyses for fruiting body development in *Lentinula edodes* were carried out using differential display (17) or serial analysis of gene expression (SAGE) analysis (18), and PCR subtraction was used for analyzing postharvest quality loss (19). More recently, transcriptome analysis was performed by transcriptome sequencing (RNA-seq) during browning film formation on mycelial bags (20) and during cellulose degradation (16). In *Lentinula edodes*, postharvest quality loss is a very significant problem from an economical viewpoint. In *Lentinula edodes*, significant quality loss is caused by gill browning and fruiting body softening, resulting in a foul odor 3 to 4 days after harvest if stored at room temperature (21). *Lentinula edodes* fruiting bodies turn brown owing to melanin synthesis and turn soft because of cell wall degradation. Melanin synthesis occurs from the increases in tyrosinase (22, 23) and laccase (24, 25) after harvest. Cell wall degradation is another significant postharvest issue in *Lentinula edodes*. Because lentinan, a β-1,3-1,6-glucan in cell walls with antitumor activity (3), is degraded by increased glucanase activity after harvest (26), we identified cell wall degradation-related genes that were upregulated after harvest by PCR subtraction (19). Genome sequence data of *Lentinula edodes* was not available for PCR subtraction, yet approximately 50 genes, such as putative chitinases (*chi1*, *chi2*, and *chi3*) and a chitosanase (*cho1*), were identified as upregulated after harvest (19, 21). Several β-1,3-glucanases were purified and characterized from *Lentinula edodes* fruiting bodies after harvest. EXG2, an exo-β-1,3-glucanase belonging to glycoside hydrolase (GH) family 55 (27), TLG1, an endo-β-1,3-glucanase with high similarity to thaumatin-like protein (28), GLU1, an endo-β-1,3-1,6-gluanase belonging to GH128 (29), and PUS30, an endo-β-1,6-glucanase belonging to GH30 (30), can degrade lentinan. Their expression increased after harvest; therefore, lentinan is degraded by multiple enzymes with synergic effects. The putative transcription factor *exp1*, which is a homolog of a gene that regulates cap autolysis in *Coprinopsis cinerea* (31), is also upregulated after harvest (19). This suggests that *exp1* in *Lentinula edodes* regulates genes involved in postharvest quality loss. However, it is highly possible that multiple transcription factors are involved in postharvest quality loss in *Lentinula edodes*. To establish methods for controlling postharvest quality loss, a more comprehensive transcriptome analysis of *Lentinula edodes* is needed.

To obtain a basis for constructing a reference sequence for *Lentinula edodes* transcriptome analysis, we constructed the genome sequence of *Lentinula edodes* G408PP-4 that was used for linkage mapping (32) using a combination of short-reads from Illumina sequencers and long reads from PacBio. Next, we predicted genes by combining assembled genome sequence data and RNA-seq data. Then, we analyzed transcriptomic data obtained from previously published PCR subtraction data (19) and SuperSAGE data for postharvest quality loss. The results suggest that multiple novel cell wall enzymes, such as putative β-1,3-gluanases, β-1,6-gluanases, and chitinases are upregulated after harvest. We also identified several transcription factors for postharvest quality loss. The results will provide insight for controlling postharvest freshness in *Lentinula edodes*.

RESULTS AND DISCUSSION

We constructed genome sequence data for G408PP-4, a wild strain isolated on Yakushima Island, Japan, which is crossed with strain D703PP-9 to construct a linkage map (32, 33). Short leads from the Illumina GAIIx and Illumina HiSeq2500 were assembled by the clc genomics workbench, and then we scaffolded the contigs using mate-paired libraries and a PacBio sequencing system. The resulting scaffold number was 1,951, the N_{50} was 103,018, and the total was 38,944,209 bp (see Table S1 in the supplemental material). The resulting scaffolds were comparable with those from other genome sequence data from mushroom-forming basidiomycetous fungal species, such as from *Coprinopsis cinerea* (37 Mb) (10), *Agaricus bisporus* (30 Mb) (11), and *Schizophyllum commune* (38.5 Mb) (12), but were smaller than that from the mycorrhizal basidiomycetous fungus *Laccaria bicolor* (65 Mb) (9). Previously, we carried out gene prediction by draft genome sequencing of *Lentinula edodes* strain D703PP-9 and found 8,271 putative genes (25). On the other hand, the number of predicted genes in *Coprinopsis cinerea* or *Schizophyllum commune* is around 15,000, much larger than our prediction for *Lentinula edodes*. Therefore, to more accurately predict genes, we combined RNA-seq data from whole life cycle and reference genome sequence data. Using RNA-seq data, the number of predicted genes was 12,959, and 100% of recorded genes in *Lentinula edodes* were predicted (except for pheromone precursors and mitochondrial and partial proteins) (see Table S2). *Lentinula edodes* belongs to *Marasmiaceae*, which includes white rot, saprobic, and plant-pathogenic fungi (34). Plant cell wall-degrading enzymes in *Lentinula edodes* G408PP-4 (see Table S3) were identical to those of strain W1-26 (16) and similar to other species of *Marasmiaceae* (Table S3) (35–37). Characteristically, no lignin peroxidase (Lip)-encoding genes were found in *Lentinula edodes* or in *Marasmiaceae* species whose genome sequences are publicly available (Table S3), suggesting that *Lentinula edodes* lacks Lip and depends on laccases and manganese peroxidases for lignin degradation.

Transcriptome analysis for fruiting bodies after harvest. Genes upregulated in the fruiting body after harvest were investigated by SuperSAGE (38). More than 200,000 tags were sequenced from fresh fruiting bodies and postharvest fruiting bodies. Tag counting data are summarized in Fig. S2, and totally, 62% of unique tags were matched with predicted genes. Approximately 95% of the genes that were true-positive upregulated genes after harvest by our previous PCR subtraction analysis (19) were expressed higher than 2-fold in the fruiting body after harvest compared with that in the fresh fruiting body (see Fig. S3, Table S4). This suggests that the results from SuperSAGE are reliable. To identify differentially expressed genes (DEGs), statistical and gene ontology analyses were performed. DEG analysis revealed that 66% of the genes that are true-positive genes upregulated after harvest by our previous PCR subtraction analysis were determined to be DEG in the present SuperSAGE analysis (Fig. S3). We identified an enriched gene ontology in the fruiting body after harvest compared with that in the fresh fruiting body using Blast2GO (Fig. 1). This revealed that catalytic activity and hydrolase activity on glycosyl bonds, transporters, and oxidoreductase activity were upregulated in the fruiting body after harvest (Fig. 1). We identified an enriched gene ontology in the fruiting body after harvest by DAVID and found that dehydrogenases, transporters, oxidoreductases, and transcription factors are found in the fruiting body (see Table S5). On the other hand, we compared the enriched gene ontologies in the fresh fruiting body by Blast2GO and DAVID, and the results suggested that many gene ontologies were enriched in the fresh fruiting body (see Fig. S4, Table S6). In particular, DNA, RNA, protein, and carbohydrate metabolic processes were enriched.

Expression of putative β-1,3-glucanases after harvest. β-1,3-1,6-Glucans are the most abundant cell wall components in the *Lentinula edodes* fruiting body; therefore, we first analyzed β-1,3-1,6-glucan-degrading enzymes expressed abundantly after harvest (Table 1). *Lentinula edodes* has three GH55 family exo-β-1,3-glucanases that are mostly conserved in filamentous fungi and also exist in several bacteria (39). EXG2, a

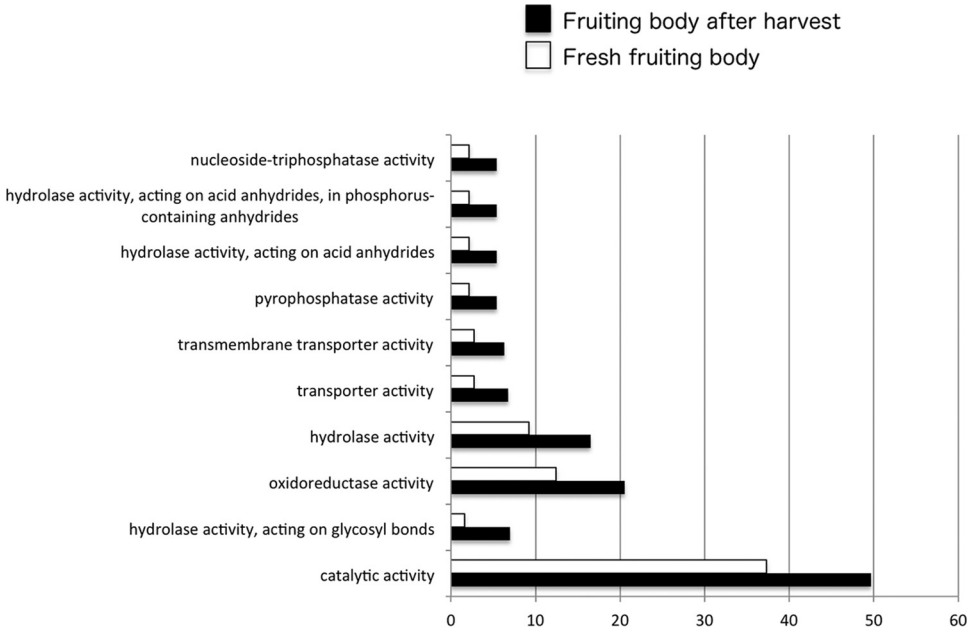

FIG 1 Comparison of enriched gene ontologies between fruiting bodies after harvest and fresh fruiting bodies (just after harvest) by using Blast2GO enrichment analysis (Fisher's exact test). Black and white bars indicate enriched gene ontologies in the fruiting body after harvest and the fresh fruiting body, respectively. The *x* axis indicates the number of genes enriched.

member of the GH55 family which is involved in cell wall degradation after harvest (27), has a strong effect on lentinan degradation after harvest (40). The enzyme encoded by *exg2* in the GH55 family of exo-β-1,3-glucanases was not determined to be a DEG, but its expression was upregulated more than 15-fold in the fruiting body after harvest (Table 2). Two other genes encoding putative GH55 family enzymes (g7383.t1 and g7404.t2) (Table 1) were slightly increased after harvest (Table 2). We previously characterized another exo-β-1,3-glucanase, EXG1 (g4132.t1), an enzyme of the GH5 family (41). Twenty-one putative GH5 members were found in *Lentinula edodes* (Table 1). The expression of *exg1* decreased after harvest (Table 2), but another GH5 gene (g4131.t1) located in the *Lentinula edodes* genome in tandem with *exg1*, similar to the one found in *Agaricus bisporus* (42), increased after harvest (Table 2). We further identified 3 DEGs that increased after harvest encoding GH5 family members (Table 2). One endo-β-1,3-glucanase in GH128 involved in lentinan degradation in *Lentinula edodes*, GLU1, was purified and characterized (29). We found 4 putative GH128 family enzymes in *Lentinula edodes* (Table 1). The expression of *glu1* increased significantly after harvest and was deemed a DEG (Table 2), but the other three GH128 family genes were not identified or decreased after harvest (see Table S4). Thaumatin-like protein, TLG1, which also has endo-β-1,3-glucanase activity, was purified from the *Lentinula edodes* fruiting body after harvest (28). The gene *tlg1* was identified as a DEG, and one other thaumatin-like protein was judged to be a DEG as well (g10798.t1) (Table 2). We found four other putative thaumatin-like proteins that were not DEGs (Table 1; see also Table S4), but the expression of one gene (g10969.t1) increased 18.5 times after harvest (Table 2). We also found 6 glycopeptide proteins which are weakly similar to thaumatin-like protein (43), and the expression of three of them (g6764.t1, g6761.t1, and g707.t1) increased significantly after harvest (Table 2). Additionally, we observed that the β-1,6-glucanase LePUS30 that belongs to GH30 is involved in cell wall degradation after harvest (30). Expression of *pus30* and *ghf30* (19), encoding other GH30 family members, increased after harvest. The other gene encoding a GH30 member enzyme (g1578.t1) was not identified as a DEG (Table 2). Some enzymes belonging to the GH16 family can degrade β-1,3-1,6-glucans, such as laminarin (44), and *Lentinula edodes* has 28 genes encoding putative GH16 family members (Table 1). We found that 4 of them (including

TABLE 1 Number of CAZy enzymes among cell wall-degrading enzymes in basidiomycetous fungi[a]

Classification	Fungal polysaccharide degraded	*Lentinula edodes*	*Gymnopus luxurians*	*Omphalotus olearius*	*Moniliophthora perniciosa*	*Coprinopsis cinerea*[b]	*Schizophyllum commune*[b]	*Laccaria bicolor*[b]	*Phanerochaete chrysosporium*[b]	*Auricularia subglabra*[b]	*Cryptococcus neoformans*[b]	*Ustilago maydis*[b]	Reported gene(s) in *Lentinula edodes*
GH5	β-1,3-Glucosidase	21	21	14	5	26	16	23	19	43	10	12	*exg1*
GH16	Endo-1,3-β-glucanase	28	8	6	2	32	35	31	23	44	14	20	*mlg1*
GH17	Endo-1,3-β-glucosidase	5	1	2	0	4	3	4	2	7	1	2	
GH18	Chitinase	14	17	11	12	9	16	11	11	26	4	4	*chi1*, *chi2*, *chi3*
GH20	β-Hexosaminidase	3	4	3	2	2	3	2	3	4	1	2	*hex20A*, *hex20B*
GH30	β-1,6-Glucanase	3	6	2	1	3	5	9	2	1	0	2	*pus30*
GH55	Exo-β-1,3-glucanase	3	2	3	12	1	2	2	2	5	0	1	*exg2*
GH75	Chitosanase	1	3	1	1	0	0	0	0	3	0	0	*Cho*
GH128	Endo-1,3-β-glucanase	4	2	0	0	4	5	5	5	9	6	5	*glu1*
Thaumatin-like protein	Endo-1,3-β-glucanase	6	11	3	13	6	7	2	6	10	1	0	*tlg1*

[a]CAZy, Carbohydrate-Active Enzymes database.

[b]Data were based on genome sequences in MycoCosm (http://genome.jgi.doe.gov/programs/fungi/index.jsf).

TABLE 2 β-Glucan-degrading enzymes involved in fruiting body softening after harvest

Classification	ID[a]	Day 0 mean[b]	Day 4 mean[c]	Fold change (day 4/day 0)	*P* value	DEG[d]
GH55	g7384.t1 (*exg2*)	1.67	25.33	15.2	9.82E−03	0
GH55	g7383.t1	9	12.67	1.41	9.40E−01	0
GH55	g7404.t2	10.67	18	1.69	1.00E+00	0
GH5	g4132.t1 (*exg1*)	28.67	21.67	0.76	1.14E−01	0
GH5	g4131.t1	3	17.33	5.78	7.62E−02	0
GH5	g3633.t1	0	19	∞	1.98E−05	1
GH5	g1981.t1	0	10.33	∞	6.98E−04	1
GH5	g1273.t1	17	152	8.94	4.26E−04	1
GH128	g8562.t1 (*glu1*)	9	269.33	29.93	1.33E−08	1
Thaumatin-like protein	g9371.t1 (*tlg1*)	27.33	560	20.49	5.15E−08	1
Thaumatin-like protein	g10798.t1	4.67	2328.33	498.93	1.27E−07	1
Thaumatin-like protein	g10969.t1	0.67	12.33	18.5	1.44E−02	0
Glycopeptide	g707.t1	0	8.33	∞	3.18E−03	1
Glycopeptide	g6764.t1	3	3716.33	1238.78	1.23E−24	1
Glycopeptide	g6761.t1	0.67	239.67	359.5	1.18E−10	1
GH30	g10719.t2 (*ghf30*)	0	37.67	∞	4.37E−08	1
GH30	g1693.t1 (*pus30*)	0	30	∞	3.50E−06	1
GH30	g1578.t1	251	333	1.46	8.56E−01	0
GH16	g1113.t1	0	297	∞	1.49E−09	1
GH16	g6709.t1	0	120.33	∞	5.59E−13	1
GH16	g1786.t1 (*mlg1*)	159.67	6197	38.81	8.96E−12	1
GH16	g3753.t1	9	123.33	13.7	5.67E−06	1

[a]ID, identification number. The gene names are shown in parentheses.
[b]Mean number of Super-SAGE tag counts ($n = 3$) of fresh fruiting body (day 0).
[c]Mean number of Super-SAGE tag counts ($n = 3$) of fruiting body after harvest (day 4).
[d]1, estimated as DEG; 0, not estimated as DEG.

mlg1) are differentially expressed after harvest (Table 2). We expressed *mlg1* in *Aspergillus oryzae* and showed stable expression and secretion. The recombinant protein encoded by *mlg1* was purified using ammonium sulfate fractionation, metal affinity resin, and anion-exchange chromatography (see Fig. S5). Purified MLG1 could degrade laminarin (β-1,3-1,6-glucan) and barley glucan (β-1,3-1,4-glucan), a typical feature of mixed-linked glucanases in the GH16 family (Table 3). However, recombinant MLG1 hardly degraded lentinan, cellulose, pustulan, or lichenan. This suggests that MLG1 degrades oligosaccharides degraded from lentinan by endo-β-1,3-glucanases, such as TLG1 and GLU1, and that rapid lentinan degradation after harvest was caused by a synergetic effect between the exo-β-1,3-1,6-glucanase EXG2, endo-β-1,3-glucanases TLG1 and GLU1, β-1,3-glucanase MLG1, and the endo-β-1,6-glucanase PUS30.

Expression of chitin-related genes after harvest. Chitin is a major cell wall component in *Lentinula edodes*, and chitin-degrading-enzyme-encoding genes (*chi1*, *chi2*, and *chi3*) and chitinase activity increased after harvest (19, 21). *chi3* is a homolog of the chitinase-encoding gene in *Coprinopsis cinerea*, *chiII*, involved in cap autolysis (45). Therefore, the chitin degradation process is important for postharvest cell wall degradation in *Lentinula edodes*; however, there is little information on chitin degra-

TABLE 3 Substrate specificity of MLG1

Substrate	Glycosidic bond	Relative activity (%)[a]
Laminarin	β-1,3-1,6	100
Pachyman	β-1,3-1,6	40.2
Cardran	β-1,3	32.0
Lentinan	β-1,3-1,6	ND[b]
Barley glucan	β-1,3-1,4	29.2
Lichenan	β-1,3-1,4	ND
Cellulose	β-1,4	ND
Pustulan	β-1,6	ND

[a]Released reducing sugar after incubation of MLG1 and each substrate was matured by the PAHBAH (4-hydroxybenzoic acid hydrazide) method. Relative activity of each substrate was indicated as a ratio (%) of absorbance of each substrate toward that of laminarin.
[b]ND, not detected.

TABLE 4 Chitin- and chitosan-degrading enzymes involved in fruiting body softening after harvest

Classification	ID[a]	Day 0 mean[b]	Day 4 mean[c]	Fold change (day 4/day 0)	*P* value	DEG[d]
GH18	g976.t2 (*chi5*)	0	5.67	∞	1.41E−02	0
GH18	g10886.t1 (*chi7*)	1	116	116	4.64E−05	1
GH18	g1744.t1 (*chi4*)	1.33	10.67	8	3.56E−01	0
GH18	g10746.t4 (*chi6*)	3	9.33	3.11	5.54E−01	0
GH18	g2804.t1 (*chi8*)	104	146	1.4	7.87E−01	0
GH18	g5859.t1 (*chi3*)	0	5	∞	1.65E−01	0
GH18	g8924.t1 (*chi2*)	1	1110	1110	8.91E−17	1
GH18	g8040.t1 (*chi1*)	8.33	405.67	48.68	3.40E−06	1
GH20	g10209.t2 (*Hex20A*)	0	2.67	∞	2.24E−01	0
GH20	g10237.t1 (*Hex20B*)	19	11.33	0.6	5.24E−02	0
GH20	g9894.t1	1.33	9	6.75	8.10E−02	0
CBM50	g6653.t1	0	18.33	∞	8.30E−05	1
CBM50	g8231.t1	0.67	104.67	157	3.74E−05	1
CBM50	g6766.t1	205.67	1024.67	4.98	1.04E−02	0
CBM50	g6268.t1	43.33	136	3.14	1.31E−01	0
CBM50	g11628.t1	89.33	270	3.02	2.58E−01	0
Expansin family protein	g6097.t1	2	60	23.67	4.70E−04	1
Expansin family protein	g7005.t1 (*baw1*)	27.67	3210.5	122.76	9.33E−17	1
GH75	g3293.t2 (*cho1*)	0	39	∞	8.52E−09	1
CE4	g7070.t1 (*chd1*)	242.33	613	3.59	5.28E−02	0

[a]ID, identification number. The gene names are shown in parentheses.
[b]Mean number of Super-SAGE tag counts ($n = 3$) of fresh fruiting body (day 0).
[c]Mean number of Super-SAGE tag counts ($n = 3$) of fruiting body after harvest (day 4).
[d]1, estimated as DEG; 0, not estimated as DEG.

dation processes in basidiomycetous fungi. We identified 14 putative chitinases with GH18 domains in the *Lentinula edodes* genome (Table 1). Three of them (*chi1*, *chi2*, and *chi7*) showed significantly increased expression after harvest (Table 4). The *chi7* gene is a newly identified putative endochitinase that has a chitin-binding module (CBM5). Other putative chitinases were not determined to be DEGs, but the expression of three other chitinases (*chi4*, *chi5*, *chi6*) increased after harvest (Table 4). These putative chitinases do not have CBM5, but several chitinases without CBM5 have chitin-degrading activity in basidiomycetous fungi (46). In particular, the *Coprinellus congregatus* chitinase Chi2 purified from droplets during cap autolysis, which has a high similarity to that encoded by *chi5*, can degrade chitin oligosaccharides (47). These results collectively suggest that multiple proteins with GH18 domains are coordinately involved in chitin degradation in the fruiting body after harvest. One chitin-degrading enzyme family is the hexosaminidases in GH20, namely, Hex20A (48) and Hex20B (49). These two hexosaminidases can degrade crystalized chitin in an exo-manner. Hex20A increased after harvest, and Hex20B decreased after harvest (Table 4). We also identified another GH20 family gene (g9894.t1), and it was not judged as a DEG but its expression increased 6.75-fold after harvest (Table 4). We identified other putative chitin-related proteins that have carbohydrate-binding module family 50 domains (CBM50, known as the LysM domain). Several LysM domains have chitin-binding ability, and several chitinases contain LysM domains (50). We found 7 proteins containing CMB50 domains; 5 of 7 such proteins were increased after harvest, and 2 of 5 were identified as DEGs in the fruiting body after harvest (g6653.t1 and g8231.t1) (Table 4). Expansin is involved in loosening cellulose crystal structures for cellulose degradation (51), and an expansin-like protein in *Schizophyllum commune* enhances chitin degradation (52). The expression of several expansin-like proteins, such as those encoded by *baw28* (g7005.t1) and g6097.t1, increased after harvest. Chitosan is found in *Lentinula edodes* in addition to chitin (53, 54). Previous PCR subtraction data suggested the chitosanase *cho1* is upregulated after harvest (19), and the SuperSAGE data were in agreement (see g3293.t2 in Table 4). The gene *cho1* has high similarity to chitosanases in the GH75 family in ascomycetous fungi, but no similar sequence was found in basidiomycetous genomes except for that in *Marasmiaceae* (Table 1) and *Auriculariales* (55) species. We also found that the putative chitin deacetylase *chd1* (g7070.t1) that encodes a protein

TABLE 5 Transcription factor genes that were upregulated in the fruiting body after harvest

Sequence ID[a]	Domain	Day 0 mean[b]	Day 4 mean[c]	Fold change (day 4/day 0)	*P* value
g3094.t1	Zn2Cys6 (GAL4:ftf2)	5.67	64	11.29	9.88E−04
g9959.t1	Zn2Cys6 (GAL4)	2.33	27.67	11.86	6.51E−04
g5145.t1	Zn2Cys6 (GAL4)	5	661	132.2	1.24E−08
g4572.t3	Zn2Cys6 (GAL4)	0	31.67	∞	2.89E−07
g5092.t1	Zn2Cys6 (GAL4)	0	30.67	∞	1.30E−06
g250.t1	Zn2Cys6 (GAL4)	0	22.33	∞	8.63E−05
g5558.t1	Zn2Cys6 (GAL4)	0	10.67	∞	3.94E−04
g7227.t1	Zn2Cys6	1	19	19	4.47E−03
g9056.t2	Zn2Cys6	0	16	∞	2.02E−05
g721.t1	Zn2Cys6	0	13.67	∞	1.21E−03
g5340.t1	Zn2Cys6	0	11.33	∞	9.82E−04
g2567.t1	Zn2Cys6	0	10.33	∞	2.40E−03
g8104.t1	Znf-CCHC	0.67	17.67	26.5	1.37E−03
g9472.t1	Znf-CCHC	0	40	∞	9.53E−08
g7860.t1	Znf-C2H2	3	34	11.33	7.75E−04
g9975.t1	Znf-C2H2	0	38.33	∞	4.86E−08
g10687.t1	Znf-C2H2	0	25	∞	4.32E−05
g10687.t1	Znf-C2H2	0	25	∞	4.32E−05
g2176.t3	Znf-C2H2	0	18.33	∞	1.35E−04
g9593.t1	HMG (*exp1*)	1.33	90.67	68	7.23E−06
g10925.t1	HMG	9.67	147	15.21	7.38E−04
g429.t2	HMG	1	42.33	42.33	2.66E−03
g6148.t1	bZIP	6.67	53	7.95	3.43E−03
g5086.t1	bZIP	2.67	137.33	51.5	2.03E−06
g271.t1	bHLH (*hlh*)	3.67	49.33	13.45	9.48E−04
g5556.t1	SWI	1.67	52	31.2	1.03E−03
g872.t1	SNF5	2.67	74.33	27.88	5.52E−06
g11151.t1	SNF5	0.67	31	46.5	4.77E−03
g2328.t1	SNF2	1.33	106.33	79.75	1.85E−08
g143.t1	Homeodomain-like	0.67	80.67	121	5.77E−05

[a]ID, identification number.
[b]Mean number of Super-SAGE tag counts ($n = 3$) of fresh fruiting body (day 0).
[c]Mean number of Super-SAGE tag counts ($n = 3$) of fruiting body after harvest (day 4).

that can catalyze chitin to chitosan increases after harvest (19) (Table 4). These data collectively suggest that there are rapid chitin and chitosan degradation systems for cell wall lysis in *Lentinula edodes* fruiting bodies after harvest.

Other genes upregulated in the fruiting body after harvest. Many transcription factor (TF) genes were found in the fruiting bodies after harvest using DAVID (see Table S5); especially, TF genes containing zinc finger domains were upregulated after harvest (Table 5). Some genes containing Zn_2Cy_6 in their N termini, designated GAL4 domains, are involved in fruiting body development, such as *priB* (56) and *ftf1*, *ftf2*, and *ftf3* (19, 21), and are upregulated during fruiting body formation. We found 7 genes (including *ftf2*) that have GAL4 domains in their N termini that were upregulated after harvest. The transcription factor *exp1* regulates cap autolysis during spore diffusion (31), and its homolog in *Lentinula edodes* is upregulated after harvest (19). Two novel genes that contain high-mobility-group (HMG) domains were upregulated after harvest (g11706.t1 and g429.t2) (Table 5). Some of the genes with HMG domains have a wide gene regulation ability (57, 58) by interacting with other transcription factors to regulate transcription in a variety of genes (59). Therefore, these genes might be more effective in controlling fruiting body quality after harvest. We found other putative transcription factor genes that contain basic leucine zipper (bzip [g5086.t1 and g6148.t1]) and helix-loop-helix (bHLH [g271.t1]) domains (Table 5) that were upregulated after harvest. We found chromatin remodeling-related genes, such as the SWI/SNF complex genes (g872.t1, g5556.t1, g11151.t1, and g2328.t1), and histone deacetylases containing Sin3 domains (g143.t1) in fruiting bodies after harvest (Table 5). These results collectively suggest that several transcription factor genes and chromatin remodeling are coordinated to transcribe genes related to quality loss after harvest.

We previously identified tyrosinase and laccase (22–25) as involved in melanin synthesis in the *Lentinula edodes* fruiting body after harvest. The putative phenylalanine ammonia-lyase (PAL) that produces several phenolic compounds (60) for pigment synthesis was upregulated after harvest. This suggests that PAL is coordinately involved in pigment formation with tyrosinase and laccase after harvest (see Table S7). Oxidative stress affects postharvest quality in horticultural crops (61, 62). We found that 7 glutathione *S*-transferase genes were DEGs in the fruiting body after harvest (Table S7), and glutathione reductase (g3721.t1) and catalase (g2239.t2) were not DEGs but were expressed at 3-fold higher levels in the fruiting body after harvest compared with those in the fresh fruiting body (Table S4). These proteins are involved in antioxidant defense (63). Furthermore, the expression of ascorbate oxidase (g9281.t1) also increased after harvest (Table S7). The ascorbate glutathione pathway has an antioxidant role in oxidative stress (64). These genes are involved in reducing oxidation stress. Thioredoxin and cytochromes that are also involved in the reduction of oxidation stress (63) were upregulated in the fruiting body postharvest (Table S7). These antioxidant enzyme activities increased after harvest (65) in *Lentinula edodes*, collectively suggesting that the antioxidant defense system is induced after harvest. Protein kinases have a critical role for signal transduction in stress responses (66), and many protein kinases were upregulated after harvest (Table S7). This suggests that some signal transduction systems are newly functioning after harvest. We found that several programmed cell death-related genes were upregulated after harvest (Table S7). The autophagy-related genes encoding inositol hexakisphosphate kinase 1 (InsP6K1 [g615.t1]) and a cullin domain-containing protein (g11374.t1) were upregulated after harvest (Table S7). InsP6K forms diphosphoinositol pentakisphosphate [InsP(7)], which induces autophagy (67). Furthermore, InsP6K mediates the assembly/disassembly of the cullin-RING ubiquitin ligases (CRL)-signalosome complex to regulate cell death (68). The vacuolar assembling sorting protein VPS16 (g9687.t2) (Table S7), which is involved in vacuolar assembling for biosynthetic, endocytic, and autophagic pathways (69), was also upregulated after harvest. Typical autophagy-related genes, such as those encoding ATG8 and APG6 (g3164.t2 and g3678.t1, respectively) (70), were expressed in the fruiting body after harvest (Table S4), suggesting that autophagy occurs in fruiting bodies postharvest. We also found that genes encoding proteases, such as subtilisin-like protease and metacaspase (g9815.t1 and g485.t1, respectively), involved in autophagy or programmed cell death (71, 72), were upregulated after harvest (Table S7). These results collectively suggest that programmed cell death occurs after harvest in the *Lentinula edodes* fruiting body. Nutrient limitation by harvesting will induce postharvest autophagy or programmed cell death.

Conclusion. We constructed a *de novo* assembly of genome sequence data of *Lentinula edodes* strain G408PP-4 and predicted 12,959 genes from the genome sequence. We also constructed a predicted gene set of *Lentinula edodes* as a reference sequence for transcriptome analysis. This analysis provided significant insight into freshness control in the *Lentinula edodes* fruiting body after harvest. We identified novel cell wall-associated genes, putative transcription factors, and putative cell death-related genes (Fig. 2). RNA interference tools are available in *Lentinula edodes* (40, 73), and TILLING, a method for screening mutants that have a mutation in a target gene (74), will be applicable for breeding. Gene expression for wood decay, biofilm formation in sawdust, medium cultivation, and fruiting body primordia in *Lentinula edodes* has been reported. Therefore, to carefully identify postharvest-specific genes and mutate the genes by the above techniques, we will construct strains that retain freshness long after harvest. These results collectively suggest that genome sequence data, predicted gene sets, and transcriptome data will be applicable for breeding *Lentinula edodes* in the near future.

MATERIALS AND METHODS

Strains and culture conditions. *Lentinula edodes* monokaryotic strain G408PP-4 (NBRC 111202), which was a mating partner of D703PP-9 (ICMP 20921) for linkage map construction (32), was used for

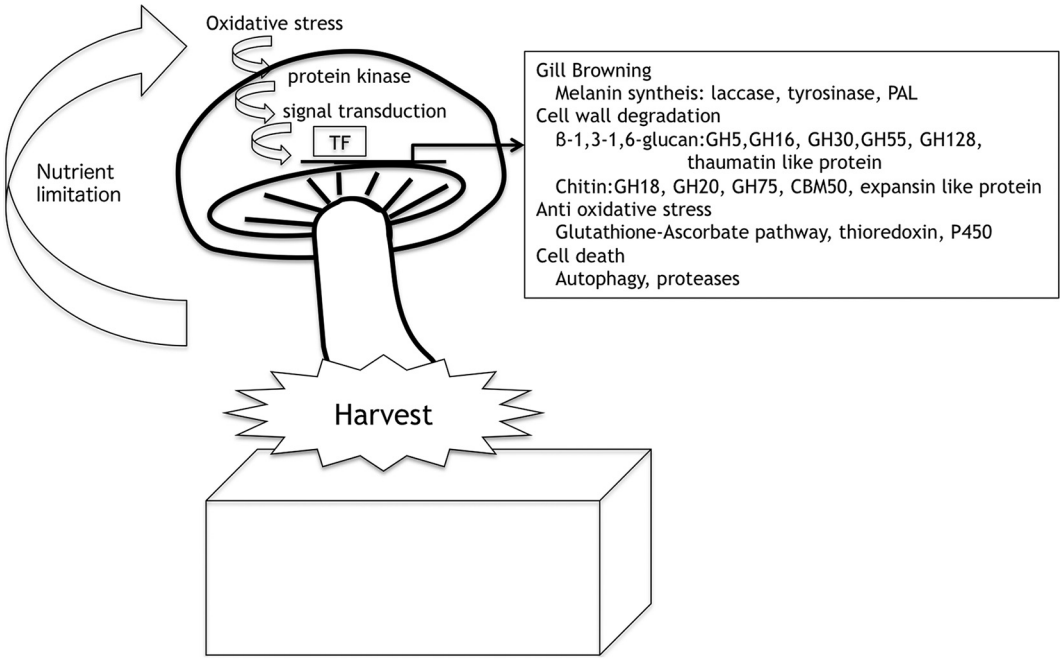

FIG 2 Working model illustrating the modification of the postharvest quality loss of a *Lentinula edodes* fruiting body. Oxidative stress will occur after harvest, as will sequential signal transduction via protein kinases and *de novo* gene expression via transcription factors. *De novo* gene expression includes genes for gill browning, cell wall degradation, antioxidative stress, and cell death.

genome sequence analysis. The dikaryotic commercially cultivated strain *Lentinula edodes* KRCF1660 (NBRC 111652, designated H600 [19, 75]) was used for transcriptome analysis. Mycelial cultures on sawdust medium were prepared as described previously (41). Fruiting body growth was performed as described previously (41), and for postharvest analysis, harvested mature fruiting bodies were immediately transferred to a desiccator at 25°C (41) and sampled daily from day 0 (fresh) to day 4. Upon sampling, mushrooms were separated into the pileus, gill, and stipe, and were immediately frozen in liquid nitrogen.

Genome assembling using pyrosequence data. Genomic DNA was extracted from 2-week-old liquid cultures after crushing the mycelia in liquid nitrogen and using a MasterPure yeast DNA extraction kit (Epicentre Biotechnologies, WI, USA) in accordance wtih the manufacturer's instructions. Libraries for genome sequencing were prepared using a TruSeq DNA sample Prep kit v2 (Illumina, CA, USA), and 76-bp paired-end sequencing was performed with an Illumina Genome Analyzer IIx system. Furthermore, 76-bp paired-end sequencing was performed with a HiSeq 2500 through a custom service provided by Genaris, Inc. (Kanagawa, Japan). The mate pair library was constructed using a Mate Pair library prep kit v2 (Illumina, CA USA) and was sequenced using an Illumina Genome Analyzer IIx system. PacBio sequencing was performed using the custom service provided by Filgen, Inc. (Aichi, Japan). *De novo* sequences were assembled using Velvet assembler version 0.7.34 (http://www.ebi.ac.uk/~zerbino/velvet/) by varying several parameters and by the clc genomics workbench 8.5.1 (Filgen Inc., Aichi, Japan). We chose a set of contigs created under the conditions that generated the longest N_{50} for further analyses. Scaffolding of assembled contigs was performed using SSPACE (76) (BaseClear B.V., Netherlands) with sequence reads from the mate pair library. Further scaffolding was carried out using sequence data from PacBio sequence with Pbjelly (77) (http://sourceforge.net/p/pb-jelly/wiki/Home/).

Gene prediction with RNA-seq data. For RNA-seq analysis, we used RNA from mycelia cultivated for 2 weeks in liquid medium, mycelia grown for 3 weeks on sawdust medium, mycelia grown for 3 months just before fruiting body production, primodium, stipe, and pileus of a young fruiting body, stipe, pileus, and gills of a mature fruiting body, and gills at 4 days after harvest of the fruiting body. For RNA extraction, mycelia were cultured in malt extract-yeast extract-peptone-glucose (MYPG) liquid medium at 25°C with shaking as described previously (41). To extract RNA from mycelia grown on sawdust medium, a membrane filter was placed on the sawdust and covered with 1.5% agar. Mycelia from sawdust cultures were harvested from the surface of the filter membrane 2 weeks after inoculation (19). To extract RNA from fruiting bodies, primordia and fruiting bodies were prepared after harvest, as described previously (27, 78). Equal amounts of RNA were mixed and used for RNA-seq analysis. The construction of a library for RNA-seq and subsequent sequencing by a HiSeq 2500 instrument (Illumina, CA, USA) were carried out using a custom service (Genaris, Inc., Kanagawa, Japan). Gene prediction was performed using Augustus 3.0.1 (79) (http://augustus.gobics.de) using training files from *Coprinopsis cinerea* and a mixture of RNA from mycelia (grown in liquid medium and sawdust medium), from young fruiting bodies, from mature fruiting bodies, and from fruiting bodies after harvest. Library construction and sequencing using the HiSeq 2500 (Illumina) were performed through a custom service from Genaris, Inc. (Kanagawa,

Japan). RNA-seq reads were mapped onto the reference genome using Bowtie2 (80) (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) and TopHat2 (81) (https://ccb.jhu.edu/software/tophat/index.shtml). Intron regions were extracted by filterBam in Augustus; hint files for gene prediction were constructed, and genes were predicted by Augustus (3.0.1) with the hint file. The annotation of predicted genes was performed using Blast2GO Pro (82) in the clc genomics workbench plug-in.

SuperSAGE analysis. RNA from the gills of fresh fruiting bodies and fruiting bodies 4 days after harvest was used for SuperSAGE according to the method previously described (38). Purified and mixed PCR products for SuperSAGE were applied to cluster formation on the flow cell of the Illumina Genome Analyzer IIx (illumina, CA, USA) and then were sequenced. Matching of tag sequences to *Lentinula edodes* genes was performed with the blastn algorithm (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/), and tag counts were calculated following a scheme described in Fig. S1 in the supplemental material. Statistical analysis was performed using TCC in the R package (83). Gene ontology analysis was performed by Blast2GO (BioBam Bioinformatics S.L., Valencia, Spain) and DAVID (84). Transcription factor genes were analyzed using the Fungal Transcription Factor Database (85) (http://ftfd.snu.ac.kr/index.php?a=view).

Expression of putative cell wall-related enzymes in *A. oryzae*. Several putative glucanases containing GH16 domains were heterologously expressed by using the vector pPPAMYBsp in *Aspergillus oryzae* according to the methods described previously (86). Transformants were cultured for 3 days in dextrin-polypeptone-yeast extract (DPY) medium with shaking, and proteins were expressed and secreted according to the methods described previously (28). Recombinant proteins secreted in the medium were concentrated by precipitation with 80% saturated sulfur ammonium. Recombinant proteins were detected by Western blot analysis and visualized by a penta-His horseradish peroxidase (HRP) conjugate kit (Qiagen GmbH, Germany). Recombinant proteins were purified by a Talon metal affinity resin (TaKaRa Bio, Inc., Tokyo, Japan) and further purified by anion-exchange chromatography using Mono Q (GE Healthcare, UK). Glucanase activity was measured using the methods described previously (87).

Accession number(s). Scaffold data were deposited in the DDBJ (accession no. BDGU01000001 to BDGU01001951).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02990-16.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

SUPPLEMENTAL FILE 2, XLSX file, 1.1 MB.

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