Lentinula edodes tlg1 Encodes a Thaumatin-Like Protein That Is Involved in Lentinan Degradation and Fruiting Body Senescence

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Lentinan is an antitumor product that is purified from fresh *Lentinula edodes* fruiting bodies. It is a cell wall component, comprising β-1,3-glucan with β-1,6-linked branches, which becomes degraded during postharvest preservation as a result of increased glucanase activity. In this study, we used N-terminal amino acid sequence to isolate *tlg1*, a gene encoding a thaumatin-like (TL) protein in *L. edodes*. The cDNA clone was approximately 1.0 kb whereas the genomic sequence was 2.1 kb, and comparison of the two indicated that *tlg1* contains 12 introns. The *tlg1* gene product (TLG1) was predicted to comprise 240 amino acids, with a molecular mass of 25 kD and isoelectric point value of 3.5. The putative amino acid sequence exhibits approximately 40% identity with plant TL proteins, and a fungal genome database search revealed that these TL proteins are conserved in many fungi including the basidiomycota and ascomycota. Transcription of *tlg1* was not detected in vegetative mycelium or young and fresh mushrooms. However, transcription increased following harvest. Western-blot analysis demonstrated a rise in TLG1 levels following harvest and spore diffusion. TLG1 expressed in *Escherichia coli* and *Aspergillus oryzae* exhibited β-1,3-glucanase activity and, when purified from the *L. edodes* fruiting body, demonstrated lentinan degrading activity. Thus, we suggest that TLG1 is involved in lentinan and cell wall degradation during senescence following harvest and spore diffusion.

The cell walls of filamentous fungi have been investigated in several species of basidiomycota, including *Schizophyllum commune* (Wessels et al., 1972), *Agaricus bisporus* (Mol and Wessels, 1990), *Coprinus cinereus* (Bottom and Siehr, 1979), and *Lentinula edodes* (Shida et al., 1981). These reports have indicated that the major components of the cell wall are chitin and β-1,3-glucan with β-1,6-linked branches. During the filamentous fungal life cycle, the cell walls are synthesized, reoriented, and lysed (Wessels, 1993; Moore, 1998). Cell wall lysis and changes in the constituent polysaccharides are essential processes during fruiting body development in basidiomycota (Kamada and Takemaru, 1977a, 1977b; Kamada et al., 1980), and in *C. cinereus* autolysis of pileus of fruiting bodies also occurs following basidiospore formation (Kües, 2000). Several β-1,3-glucans from basidiomycetous mushrooms display antitumor activity, for example, lentinan from *L. edodes* (Chihara et al., 1969) and schizophyllan from *S. commune* (Morikawa et al., 1985). The reported structure of lentinan comprises β-1,3-linked-D-glucan containing β-1,6 branches (Chihara et al., 1969). However, although it can be purified from fresh Shiitake mushrooms (*L. edodes*), its content decreases during storage as a result of increased glucanase activity (Minato et al., 1999, 2004). Previously, we reported that two exo-β-1,3-glucanase-encoding genes (*exg1* and *exg2*) are involved in morphogenesis of *L. edodes* (Sakamoto et al., 2005a, 2005b) and that the enzyme encoded by *exg2* is also involved in postharvest degradation of lentinan (Sakamoto et al., 2005b). In addition, *A. bisporus* mushrooms produce an endo-β-1,3-glucanase (Galán et al., 1999), and an endo-glucanase has also been reported for *L. edodes* (Grenier et al., 2000) that exhibits similarities to the antifungal thaumatin-like (TL) proteins that are highly conserved in plants.

Plants accumulate a large number of pathogenesis-related (PR) proteins, which are divided into five families (PR1–PR5). TL proteins share sequence homology with the thaumatin isoforms from *Thaumatococcus danielli* arils (Dudler et al., 1994) and members of the PR5 family (van Loon and van Strien, 1999), such as osmotin (Yun et al., 1998) and permatins (Roberts and Selitrennikoff, 1990). When analyzed using SDS-PAGE, plant TL proteins are monomeric soluble proteins of low molecular mass (15–39 kD). Activities attributed to these proteins include a sweet taste, antifreeze activity, and antifungal activity (Yun et al., 1998; Datta et al., 1999). In addition, some TL proteins exhibit both β-1,3-glucan binding (Trudel et al., 1998) and endo-β-1,3-glucanase activities (Grenier et al., 1999). Thus, since β-1,3-glucan is a common component

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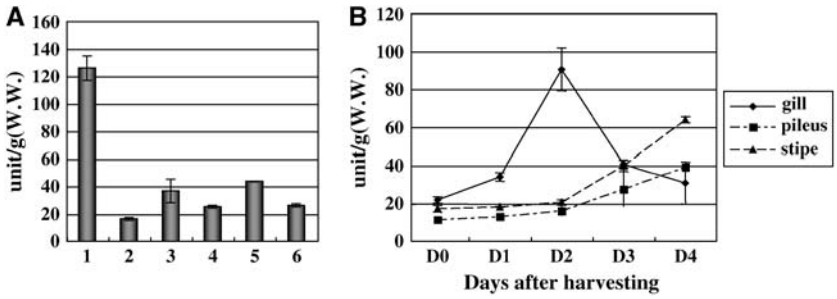

Figure 1. Endo-glucanase activity in *L. edodes* fruiting bodies. A, Endo-glucanase activities during fruiting body development of *L. edodes*, using AZCL-Pachyman as a substrate. Lane 1, Whole young fruiting bodies <1 cm; lane 2, whole young fruiting bodies 1 to 2 cm; lane 3, stipes from young fruiting bodies 2 to 3 cm; lane 4, pilei from young fruiting bodies 2 to 3 cm; lane 5, stipes from young fruiting bodies 3 to 5 cm; lane 6, pilei from young fruiting bodies 3 to 5 cm. Data represent the means and SD of three independent experiments. B, Endo-glucanase activities of *L. edodes* fruiting bodies during postharvest preservation, using AZCL-Pachyman as a substrate. Data represent the means and SD of three independent experiments.

of the fungal cell wall, it is possible that plant TL proteins play a role in host defense by destroying the cell walls of pathogenic fungi.

TL protein-encoding genes have been found in organisms outside of the plant kingdom, such as in the nematode *Caenorhabditis elegans* (Kitajima and Sato, 1999) and in the locust *Schistocerca gregaria* (Brandazza et al., 2004). Moreover, Grenier et al. (2000) reported that several fungi, such as *Rhizoctonia solani* and *L. edodes*, have TL proteins. More recently, TL protein-encoding genes have been found in *Aspergillus nidulans* (Osherov et al., 2002; Greenstein et al., 2006). Homology between the N-terminal amino acid sequences of fungal and plant TL proteins includes three amino acid residues (Asn, Cys, and Trp) that are highly conserved (Grenier et al., 2000). Fungal TL proteins also exhibit both β-1,3-glucan-binding and β-1,3-endo-glucanase activities (Grenier et al., 2000). However, TL proteins in fungi have not yet been well characterized, and their role remains unclear.

We hypothesized that in *L. edodes* fruiting bodies, endo-β-1,3-glucanase might be involved in lentinan degradation during postharvest preservation. In this study, we isolated a gene encoding a TL protein from *L. edodes* and investigated its expression to examine the role it plays in the fungal life cycle.

RESULTS

Changes in Endo-Glucanase Activity in Fruiting Bodies

Endo-β-1,3-glucanase activity, using azurine-cross-linked (AZCL)-Pachyman as substrate, was highest in the youngest primordium and greater in the stipe than the pileus of young fruiting bodies in groups 2 to 3 cm and 3 to 5 cm (Fig. 1A). In tissue of the mature fruiting bodies, endo-glucanase activity was low but increased after harvesting (Fig. 1B). The most rapid increase in endo-glucanase activity occurred in the gills, where it

Table I. *Primers used in this study*

Primer Name	Sequence	Strategy
tlg-1U	TAYAAYGGNTGYCCNTTYAC(YNGCPFT)[a]	Degenerate PCR
tlg-2U	ATHTGGCCNGCNATGTTYAC (IWPAMFT)[a]	Degenerate PCR
tlg-474 L RACE	GCAGTTGGGGGAGTTTTGTTGGTCA	5′ RACE
tlg-172 L RACE	TGCATCCACCATCAAGACACGAGTT	5′ RACE
tlg-ATG	ATGAAGTTCACCACTGCTGCCTCTC	ORF sequence
tlg1-TAAL	TTAAGCAGGAGGACAGAATG	ORF sequence
tlg GW-238 L	TGAAAAAGGAGAAGAGGAGCAGGAG	Genome walking
tlg GW-316 L	AAAGGAAGAGGAGTGGGCGGAAAGG	Genome walking
tlg-391 L	CCTGGGGTGCCTAATGAGTG	Genome walking
tlg-259 L	CGTCGTATGCGTAGGCGTAG	Genome walking
tlg-302U	CTTTGTCGACGGATACAATC	Genome walking
tlg-541U	CTCAGAAGAACCCACGAACT	Genome walking
tlg-001U	ATTTGGCCTGCGATGTTCAC	Probe construction
tlg-391 L	CCTGGGGTGCCTAATGAGTG	Probe construction
tlg-Nt	GCTAGGACTTTCACGGTCTATAACG	Vector construction
tlg-Nt-*Bam*HI	CGCGGATCCGGCTAGGACTTTCACGGTCTATAACG	Vector construction
tlg1-TAAL-*Not*I	AAGGAAAAAAGCGGCCGCTTAAGCAGGAGGACAGAATG	Vector construction

[a]Amino acid sequence from which the degenerate primer was designed.

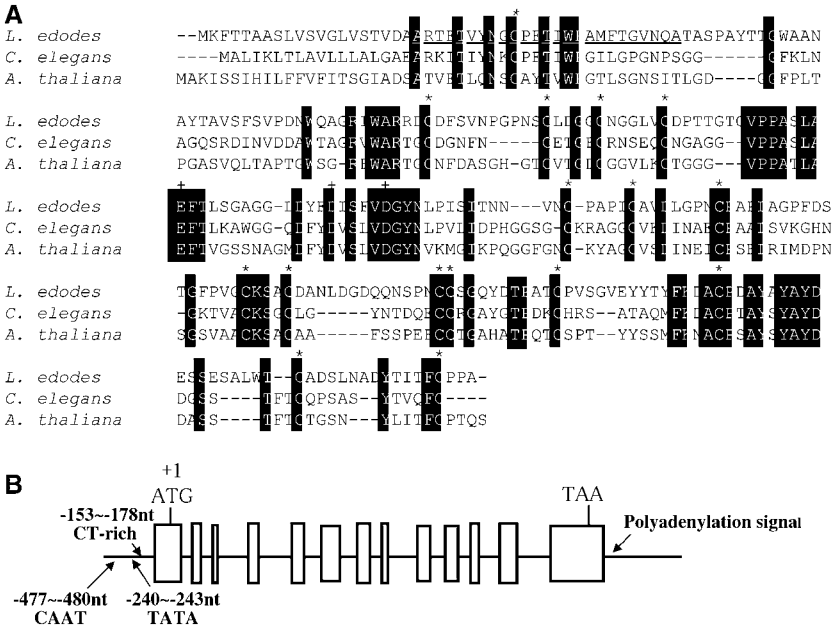

Figure 2. Structure of *tlg1*. A, Alignment of the amino acid sequences of TL proteins in *L. edodes*, Arabidopsis (DDBJ U83490), and *C. elegans* (DDBJ Z70684, protein ID CAA94598.1). The N-terminal amino acid sequence of purified TLG1 obtained by protein sequencing is underlined, and conserved amino acids are indicated by shadowing. Dashes in the sequence represent the introduction of a single amino acid gap. Conserved Cys are indicated with an asterisk, and conserved acidic amino acids in the acidic cleft are indicated with a +. B, Genomic structure of *tlg1* and its flanking regions. Schematic includes 12 introns and an upstream region containing a CT-rich region, as well as CAAT and TATA boxes.

reached a peak on day 2. On the other hand, higher activities were recovered from the stipe and the pileus than from the gills on day 4 (Fig. 1B).

Cloning of the *tlg1* Gene

tlg1 was cloned using the N-terminal amino acid sequence of a TL protein from *L. edodes* (Grenier et al., 2000). The approximately 400-bp fragment amplified by 3′ RACE-PCR using degenerate primers (Table I) was subcloned and sequenced, and its predicted amino acid sequence exhibited a strong similarity to TL proteins. Following 5′ RACE, a gene of approximately 1.0 kb (designated *tlg1*) was cloned and sequenced (DNA Data Bank of Japan [DDBJ] accession no. AB244759). The PSORT II program (http://psort.ims.u-tokyo.ac.jp/form2.html) predicted the mature protein to be an extracellular or cell wall protein, with a potential cleavage site between residues 21 and 22 (Fig. 2A). Thus, the putative mature protein comprises 240 amino acid residues with a molecular mass of 25 kD and a pI value of 3.48. The predicted N-terminal amino acid sequence was identical to the N terminus of the TL protein from *L. edodes* reported by Grenier et al. (2000; Fig. 2A). The complete translated sequence contained all 16 Cys residues (correctly positioned) that are conserved in TL proteins (Fig. 2A). Furthermore, it exhibited 43% identity to TL proteins of Arabidopsis (*Arabidopsis thaliana*) and approximately 40% identity to TL proteins from other species, including nematode and locust.

Structure of *tlg1* in the Genome

The clone of genomic *tlg1* (DDBJ accession no. AB244760) was obtained by PCR using primers designed against the open reading frame (ORF) determined from the cDNA sequence, and the sequences of their coding regions were in agreement. Sequence comparisons between genomic and cDNA identified 12 introns in *tlg1* from *L. edodes* (Fig. 2B). These introns contain similarities to the 5′ and 3′ splice site consensus sequences (GTRNGT and YAG, respectively; Gurr et al., 1987). We sequenced approximately 1.3 kb upstream of the translational start site and, through comparison of genomic and cDNA sequences,

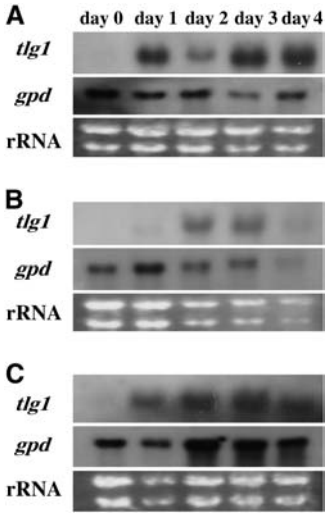

Figure 3. A to C, Northern-blot analysis of *tlg1* gene expression during postharvest preservation. RNAs were extracted from gill (A), pileus (B), and stipe (C) of fruiting bodies after harvesting. The 735-bp fragment from *tlg1* cDNA (top) and an 800-bp fragment from *gpd* cDNA (middle) were used as probes. The bottom sections show the rRNA loading control.

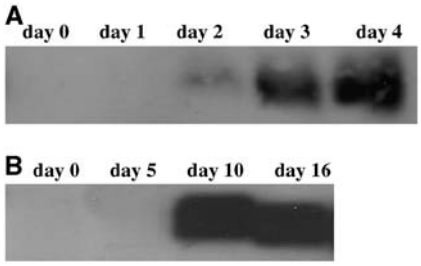

Figure 4. Western-blot analysis of TLG1. A and B, TLG1 expression in gills of fruiting bodies during postharvest preservation (A) and natural aging of fruiting bodies, i.e. without artificial harvesting (B).

identified the transcription start site at −91 nt. In filamentous fungi, a number of transcription start points may occur at, or immediately downstream, of a CT-rich sequence (Gurr et al., 1987). In the 5′ flanking region of *tlg1*, a CT-rich sequence was observed between −153 and −178 nt, a consensus TATA box was located at −240 to −243 nt, and a CAAT box was located at −477 to −480 nt from the translation start codon. We also sequenced approximately 2.3 kb downstream from the ORF and identified a polyadenylation signal (AATAAAA) at 25 nt and a polyadenylation site at 119 nt from the translational stop codon. Southern-blot analysis revealed that *tlg1* is present in multiple copies in the *L. edodes* genome (data not shown).

Transcription and Translation Patterns of *tlg1*

Although constitutive expression of *gpd* (glyceraldehyde-3-P-dehydrogenase) was found in all stages of *L. edodes* (Hirano et al., 1999), *tlg1* expression was not detected in the basidiospore, vegetative mycelium, or young fruiting bodies (data not shown). Therefore, we investigated *tlg1* transcription patterns in mature fruiting bodies as well as those undergoing postharvest preservation. Although transcription of *tlg1* was not observed in any tissue derived from fresh mature fruiting bodies, transcription levels increased in the gills and stipe following harvest. (Fig. 3, A and C). In contrast, *tlg1* transcription was observed in the pileus of fruiting bodies by day 2 and 3 of postharvest preservation, albeit at lower levels than observed for the gills and stipe (Fig. 3B).

TLG1 antibody (α-TLG1) was prepared from epitopes predicted for the amino acid sequence of TLG1. Western-blot analysis demonstrated that TLG1 was not present in the gills of mature fruiting bodies, but its expression increased during postharvest preservation (Fig. 4A), in correlation with the transcription pattern. However, in the gills of fruiting bodies following harvest, TLG1 levels did not match changes in endo-glucanse activity (Fig. 1B), and, thus, we suggest that there may be an additional endo-glucanase(s) in *L. edodes*. TLG1 levels also increased in naturally aging fruiting bodies (i.e. without postharvest preservation) following spore formation (Fig. 4B). Collectively, these data indicate that TLG1 is involved in lentinan degradation and cell wall lysis during postharvest preservation and senescence.

Heterologous Expression of *tlg1*

For heterologous expression of *tlg1* in *Escherichia coli*, we used the pET26 vector, and the recombinant TLG1 was extracted as a soluble protein from the periplasmic fraction (Fig. 5A). This TLG1 degraded carboxymethyl (CM)-Pachyman, laminarin, and AZCL-Pachyman, suggesting that *tlg1* encodes an endo-glucanase. The specific activity of purified TLG1 was lower than expected at 1 unit/mg when laminarin was used as a substrate.

In addition, *tlg1* was also heterologously expressed in wild-type *Aspergillus oryzae* RIB40. β-1,3-Glucanase activity in crude enzyme fractions from RIB40 vegetative mycelium was <10% of that observed in similar fractions from harvested *L. edodes* fruiting body. We randomly selected two transformants, F_2 and F_3, and investigated their TLG1 expression levels. In western-blot analysis, no endogenous protein from wild-type *A. oryzae* RIB40 cross-reacted with the α-TLG1. The F_2

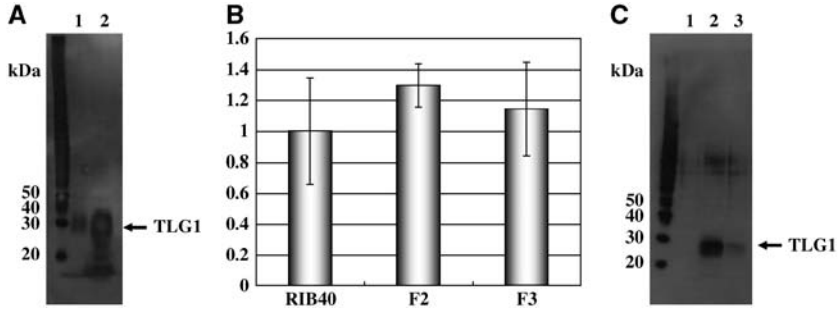

Figure 5. Heterologous expression of *tlg1*. A to C, Western blotting analysis of heterologously-expressed TLG1 (A and C) and glucanase activity (B). A, Expression of *tlg1* in *E. coli* using pET26. Proteins were extracted from periplasmic (lane 1) and cytoplasmic (lane 2) fractions. B, Relative glucanase activities of the wild-type strain *A. oryzae* RIB40 and transformed strains F_2 (lane 2) and F_3 (lane 3) using CM-Pachyman as a substrate. Data represent the means and SD of three independent experiments. C, Expression of the *tlg1* in *A. oryzae* using pPAN8142. Shown are proteins extracted from wild-type strain *A. oryzae* RIB40 (lane 1), and F_2 (lane 2) and F_3 (lane 3).

Table II. *Purification of TLG1 from L. edodes fruiting bodies at day 4 after harvest*
Glucanase activity was measured by the method of Somogyi-Nelson using CM Pachyman as a substrate.

	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	*unit*	*unit/mg*		
Extract from fruiting body	9.148	416.5	45.53	–	1
50% ammonium sulfate precipitate	2.22	28.36	12.77	1	0.24
Superdex 75 10/30	0.296	16.44	55.54	4.35	0.03
Mono Q 5/50 GL	0.006	0.69	115	9	0.01

transformant expressed TLG1 in the cytoplasm more abundantly than the F3 transformant (Fig. 5C), and TLG1 was not secreted into the medium. Glucanase activity was greater in the two *tlg1* transformants than in the wild-type strain, and activity was higher in F_2 than in F_3 (Fig. 5B). However, the activities of the transformants were not significantly higher than that of the wild-type strain.

Purification of TLG1

Western-blot analysis revealed that TLG1 was present in the 50% ammonium sulfate fraction of extracts from fruiting bodies at day 4 of postharvest preservation, and little TLG1 was detected in the 50% to 80% fraction (data not shown). However, endo-glucanase activity was observed in the latter fraction (data not shown), indicating the presence of another endo-glucanase(s). Proteins in the 50% fraction were separated by gel filtration, followed by anion-exchange column chromatography (Table II), and one peak of glucanase activity was identified using CM-Pachyman as a substrate. SDS-PAGE and Coomassie Brilliant Blue staining showed that the activity peak corresponded to a single major band (Fig. 6, lane1), which had an identical N-terminal amino acid sequence to the deduced sequence of *tlg1*. In addition, α-TLG1 cross-reacted with purified TLG1 (Fig. 6, lane 2), suggesting that translated *tlg1* results in a functional enzyme. Finally, purified TLG1 degraded laminarin, Pachyman, and lentinan, as well as the alkali-insoluble cell walls of *Saccharomyces cerevisiae* and *L. edodes* (Table III).

DISCUSSION

TL proteins are known as PR-5 proteins in plants, and some are known to exhibit endo-β-1,3-glucanase activity (Trudel et al., 1998). They are considered to exhibit antifungal activity, lysing the β-1,3-glucan in the cell walls of pathogenic fungi. Recent studies have revealed that TL proteins also occur outside of the plant kingdom, for example, in animals (the nematode *C. elegans*; Kitajima and Sato, 1999) and in fungi (*L. edodes*: Grenier et al., 2000; *A. nidulans*: Greenstein et al., 2006). However, the gene encoding the TL protein in fungi has not yet been well characterized, and its biological role in the mushroom remains unclear. In this study, we cloned a TL protein-encoding gene (*tlg1*) from *L. edodes* and revealed that its product exhibited endo-β-1,3-glucanase activity.

The predicted amino acid sequence of TLG1 from *L. edodes* shared 43% and 38% identity with Arabidopsis and *C. elegans* TL proteins, respectively. Recently, the genome sequences from several fungi were determined, and predicted amino acid sequences similar to TL proteins were identified in both basidiomycota and ascomycota. Therefore, we compared the TL protein sequences from fungi, plants, and animals. The phylogenic tree indicates three major clades representing the animal, plant, and fungal kingdoms (Fig. 7). In fungi, basidiomycota and ascomycota were clearly separated, and the basidiomycetous yeast *Cryptococcus neoformans* was separated from the filamentous basidiomycota (Fig. 7). However, we were unable to identify sequences similar to TL proteins in the ascomycetous yeast genomes (*S. cerevisiae* and *Candida albicans*). These data

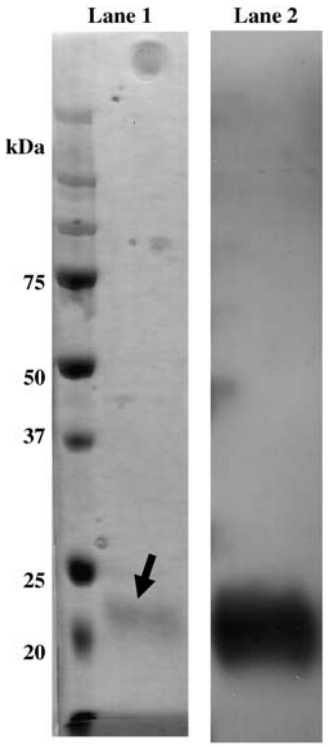

Figure 6. SDS-PAGE and western-blot analysis of purified TLG1. Lane 1, SDS-PAGE and Coomassie Brilliant Blue staining of TLG1; lane 2, western-blot analysis of TLG1.

Table III. *Substrate specificity of TLG1*

Substrate	Protein
	unit/mg
Lentinan	115
Pachyman	104
Laminarin	650
Yeast cell wall	40
L. edodes cell wall	50

indicate that TL proteins are not unique to plants but are spread throughout eukaryotes.

All TL proteins share 16 conserved Cys that are required for eight disulfide bonds (Fig. 2A) and that play important roles in maintaining structure and activity (Koiwa et al., 1999). In *E. coli*, TLG1 was expressed using pET26, which has signal sequence that results in the periplasmic localization of the recombinant protein. The periplasm represents a favorable environment for folding and disulfide bond formation (Rietsch et al., 1996). Thus, recombinant TLG1 extracted from the periplasmic fraction exhibited glucanase activity, whereas the abundant TLG1 in the cytoplasmic fraction (Fig. 6, lane 2) did not (data not shown). This might suggest that disulfide bond formation is crucial for TLG1 activity. However, the specific activity of TLG1 expressed heterologously in *E. coli* was lower than wild-type TLG1 expressed in the *L. edodes* fruiting body following harvest. This suggests that the heterologously expressed TLG1 had unrequired disulfide bonds. Three-dimensional analysis of TL proteins has revealed that the acidic cleft is important for β-1,3-glucan-binding activity (Koiwa et al., 1999), and computer modeling of TLG1 from *L. edodes* indicated that the acidic amino acids Glu (E99), Asp (D112), and Asp (D117) were conserved in the appropriate positions of the acidic cleft (Fig. 8B). These analyses suggest that TL proteins have been strongly conserved throughout the evolutionary process.

Do similarities in the conserved structures provide insights into the biological roles played by TL proteins? Some plant TL proteins show endo-β-1,3-glucanase activity (Grenier et al.,1999) and help defend against pathogenic fungi (Datta et al., 1999). Nematodes and locusts also have TL proteins (Kitajima and Sato, 1999; Brandazza et al., 2004), and they are also subject to predation and pathogenic infection by nematophagous (Thorn and Barron, 1984) and entomopathogenic fungi (Clarkson and Charnley, 1996), respectively. Thus, it is possible that TL proteins represent an antifungal strategy in animals (Brandazza et al., 2004). Several fungi are pathogenic to other filamentous fungi and produce a cell wall lytic enzyme (exoglucanase) to degrade the host cell wall (Rotem et al., 1999). Thus, there might be fungal TL proteins that play roles in fungal-fungal interactions, such as those involved in the invasion of, or the protection from, other fungi. However, *L. edodes tlg1* was not transcribed in vegetative mycelium grown in liquid culture and sawdust culture (data not shown), suggesting that it is induced under stress conditions or that it might not act as an antifungal agent. On the other hand, it was reported that the TL protein-encoding gene *cetA* from *A. nidulans* is significantly enriched in mature conidia and involved in germination (Greenstein et al., 2006). This suggests that fungal TL proteins play roles that are different from those of their plant TL protein

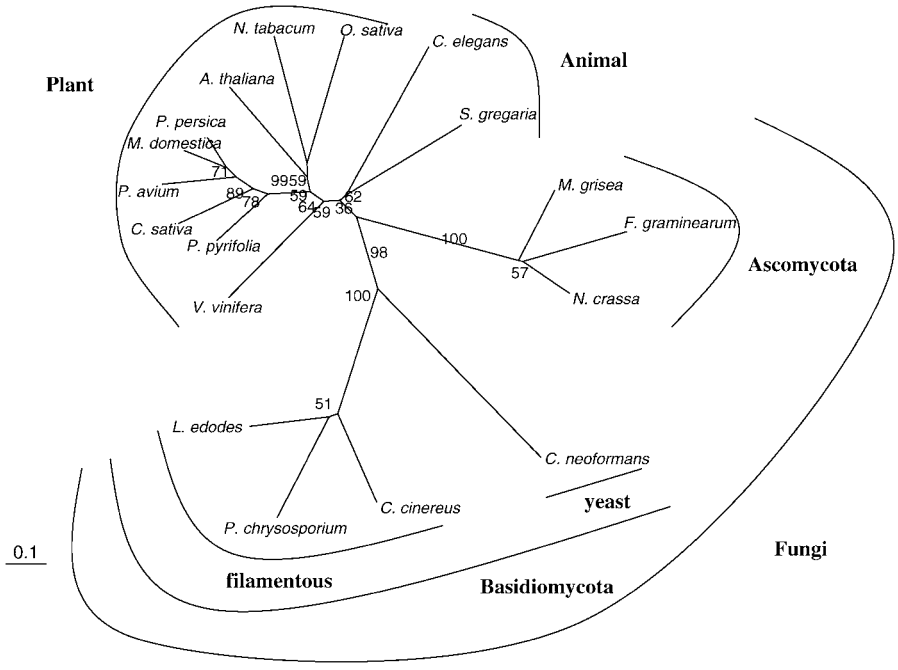

Figure 7. Phylogram of TL proteins in fungi, plant, and animal kingdoms. Amino acid sequences of TL protein were obtained from the fungal genome database (http://www.fgsc.net/outlink.html) and DNA data bank (accession nos. *Pyrus pyrifolia*, AB006009; *Oryza sativa*, AP003935, protein ID, BAD45633; *Nicotiana tabacum*, AB000834; *Malus domestica*, AF090143; *Vitis vinifera*, AF195653; *Castanea sativa*, AJ242828; *Prunus avium*, U32440; *Prunus persica*, AF362988; *Brassica campestris*, U71244). The phylogram was constructed using the neighbor-joining method. The scale bar indicates a distance of 0.1, and the numbers on branches indicate percentage bootstrap support values (based on 1,000 replications).

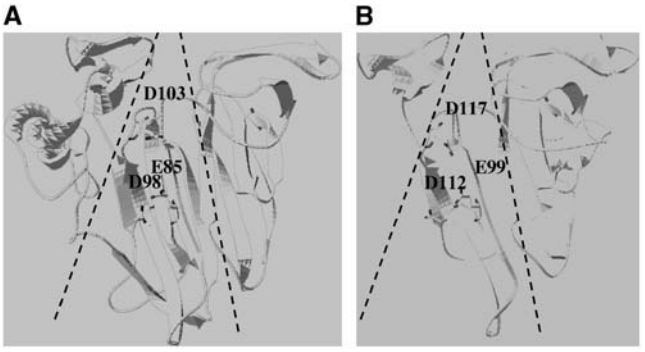

Figure 8. Three-dimensional homology model of the TL protein from *N. tabacum* (A) and TLG1 from *L. edodes* (B). Dashed lines indicated the acidic cleft; acidic amino acids in the cleft are shown.

counterparts. One of these roles is perhaps their propensity to function as antifungal agents.

L. edodes TLG1 degrades lentinan, a β-1,3-glucan found in its own cell wall (Shida et al., 1981) and thus may play a role in cell wall degradation. Increasing endo-glucanase activity (Fig. 1B) and TLG1 expression (Figs. 3 and 4) were observed in fruiting body after harvest and aging fruiting bodies following spore diffusion. Collectively, these data suggest that *L. edodes* TLG1 is responsible for cell wall lysis during the fruiting body senescence that occurs following artificial harvesting or spore diffusion. TLG1 expression was not observed in growing fruiting bodies; however, endo-glucanase activity was observed in young fruiting bodies, especially in the stipe (Fig. 1A). This suggests that there is another endo-glucanase(s) that is different from TLG1 in the *L. edodes* fruiting body. Cell wall lytic enzyme activities (β-1,3 glucanase, β-1,6 glucanase, and chitinase) were involved in stipe elongation in *C. cinereus* (Kamada and Takemaru, 1977b; Kamada, et al., 1980, 1982); therefore, endo-glucanase activity in the stipe of growing fruiting bodies may also contribute to stipe elongation in *L. edodes*.

In conclusion, we have isolated a novel TL protein-encoding gene (*tlg1*) from *L. edodes* and demonstrated that this gene is involved in the degradation of lentinan during postharvest preservation. TLG1 exhibits cell wall lytic activity and is responsible for cell wall degradation, specifically in fruiting body senescence, following artificial harvesting and spore diffusion. In addition to providing insights into the biological functions of TL proteins in fungi, this study represents the gene encoding a fungal TL protein to be isolated. Further investigation of *tlg1* will improve our understanding of the evolutionary development of this TL protein-encoding gene.

MATERIALS AND METHODS

Strain and Culture Conditions

Lentinula edodes cultivation strain H600 was used in all experiments. Mycelia were cultured in MYPG liquid medium for 2 weeks for northern-blot analysis and 4 weeks for genomic DNA extraction, at 25°C with shaking as described previously (Sakamoto et al., 2005a). For RNA and protein extraction, fruiting bodies were prepared as described previously (Sakamoto et al., 2005a). Young fruiting bodies were grouped (<1 cm, 1–2 cm, 2–3 cm, and 3–5 cm in height of fruiting bodies), and the latter two groups were separated into pileus and stipe. Mature fruiting bodies (in which the pileus veil had disappeared) were separated into pileus, gill, and stipe. *Aspergillus oryzae* RIB40 was grown for 3 d in DPY at 30°C with shaking, as described previously (Gomi et al., 1987).

For postharvest preservation, harvested mature fruiting bodies were immediately transferred to a desiccator at 25°C (Sakamoto et al., 2005a), and fruiting bodies during preservation were sampled daily day 0 (fresh) through day 4. Following the postharvest preservation, all samples were separated into pileus, gill (lamellae), and stipe, and then frozen immediately in liquid nitrogen. Aging fruiting bodies were sampled at days 0 (designated as the day at which the pileus veil had just disappeared), 5, 10, and 16 following pileus veil disappearance (basidiospore diffusion was observed from days 5–10). All aging fruiting bodies were separated into pileus, gill, and stipe, and then frozen immediately in liquid nitrogen. All samples frozen in liquid nitrogen were stored at −80°C.

Measurement of Glucanase Activity

For the measurement of endo-glucanase activity, 1 gm of fruiting bodies was frozen in liquid nitrogen, suspended in 5 mL of extraction buffer (200 mM sodium acetate, pH 4.2), incubated with rotation for 15 min at room temperature, and then centrifuged as described previously (Sakamoto et al., 2005a). To assay for endo-β-1,3-glucanase activity, 1 mL of extract supernatant was first incubated for 5 min at 30°C before adding a tablet of 1,3-β-glucazyme (Megazyme) containing AZCL-Pachyman (a β-1,3, 1,6-glucan) and incubating for an additional 1 h at 37°C, as described in the manufacturer's instructions. Standard endo-glucanase activity was determined in extracts obtained from samples at 3 d postharvest preservation and measured by the Somogyi-Nelson method (Nelson, 1944) using CM-Pachyman (Megazyme) as a substrate. One unit of enzyme activity was defined as the amount required to liberate 1 μmol/h of reducing sugar.

The substrate specificity of TLG1 was determined using laminarin (β-1,3-glucan; Sigma-Aldrich), lentinan (β-1,3, 1,6-glucan; Minato et al., 1999), and CM-Pachyman, as well as yeast (*Saccharomyces cerevisiae*) and *L. edodes* fruiting body cell walls as substrates, which were prepared as reported previously (Grenier et al., 1993). The glucanase activity of TLG1 for the substrates was measured by the Smonogyi-Nelson method as described above.

Cloning and Sequencing of the *tlg1* Gene

cDNA was synthesized from total RNA extracted from the gills of fruiting bodies at day 4 postharvest preservation (day 4 RNA) using the SMART PCR RACE kit (BD Bioscience) according to the manufacturer's protocol. 3′ RACE was performed using degenerate primers (tlg-1U and tlg-2U; Table I) designed against the N-terminal amino acid sequence from a TL protein of *L. edodes* (Grenier et al., 2000), as described previously (Sakamoto et al., 2005a). cDNA for the 5′ RACE PCR template was synthesized from the day 4 RNA using the GeneRacer kit (Invitrogen), and PCR was performed as described previously (Sakamoto et al., 2005a) using *tlg1*-specific and GeneRacer primers (Invitrogen).

Vegetative mycelium that had been cultured for 4 weeks was collected and crushed in liquid nitrogen, and genomic DNA was isolated using Isoplant (Nippon Gene) following the manufacturer's protocol. Genomic DNA fragments corresponding to the ORF of *tlg1* were PCR amplified using the primers tlg-ATG U and tlg-TAA L (Table I), then subcloned into pCR2.1-TOPO (Invitrogen) and sequenced as described previously (Sakamoto et al., 2005a). Genome walking was performed with *tlg1* gene-specific primers (Table I) and adaptor primers (BD Bioscience), using a genome-walking library (Sakamoto et al., 2005a), following the PCR conditions described previously (Sakamoto et al., 2005a).

Comparative Analysis of *tlg1*

Amino acid sequences with similarity to TL protein from filamentous fungi were obtained from the fungal genome database (http://www.fgsc.net/outlink.html). ClustalW (http://www.ddbj.nig.ac.jp/search/clustalw-j.html; Thompson et al., 1994) was used to align the TL proteins (with some manual arrangements). Phylogenetic analysis of amino acid sequence of TL proteins was performed using ClustalW, and the phylogenic tree was drawn using

TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). A three-dimensional model was predicted using Swiss-model (http://swissmodel.expasy.org//SWISS-MODEL.html), for residues 1 to 156 (of 240 amino acids) of TLG1, and the TL proteins 1DU5, 1THW, 1RQW, and 1KWN were used as templates. This structure was drawn using Swiss-pdb Viewer (http://ca.expasy.org/spdbv/mainpage.htm).

Northern-Blot Analysis

Total RNA was isolated using the FastRNA Pro Red kit (Q-BIOgene), according to the manufacturer's instructions and RNA concentration was measured using RiboGreen (Molecular Probes), and RNA samples (10 μg) were used for northern-blot analysis as described previously (Hirano et al., 1999). Labeling of the DNA probes, hybridization, and signal detection were performed using the Alkphos Direct DNA/RNA Labeling and Detection system (Amersham Bioscience) following manufacturer's instructions. The *tlg1*-specific probe was PCR amplified from a 735-bp DNA fragment, using primers tlg-001U and tlg-391 L (Table I), and *gpd* probe was prepared as described previously (Hirano et al., 1999).

Western-Blot Analysis

Rabbit anti-TLG1 was prepared (custom service of Takara Bio) using three peptides (RDCDFSVNPGPNS, KSACDANLDGQQNSPN, and KDACPDA-YAYAYDES) that were identified as potential TLG1 epitopes using Epitope Adviser 2.1 (FQS). Protein samples were prepared from the gills of fruiting bodies by crushing in liquid nitrogen, followed by suspension in extraction buffer (200 mM sodium acetate, pH 4.2). Protein concentration was determined using the Bio-Rad Protein Assay kit (Bio-Rad Laboratory) with a bovine serum albumin standard. Samples were separated by electrophoresis and transferred to a polyvinylidene difluoride membrane as described previously (Sakamoto et al., 2005a). Western-blot analysis was carried out as described previously (Sakamoto et al., 2005a); rabbit anti-TLG1 was used as the first antibody, followed by horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Bioscience) as the secondary antibody. Hybridization was visualized using the ECL detection kit (Amersham Bioscience) following the manufacturer's instructions.

Heterologous Expression of *tlg1*

PCR amplification of *tlg1* was performed using the primers tlg1-Nt-*Bam*HI and tlg1-TAAL-*Not*I (Table I). The PCR fragment and expression vector pET26 (Novagen) were digested with *Bam*HI and *Not*I, then ligated. The resulting construct (pET26-tlg1) was transformed into *E. coli* BL21(DE3) and expressed in the periplasmic fraction, following the manufacturer's instructions.

tlg1 was also expressed in *A. oryzae* using the vector pPAN8142 constructed by ligating a *Pst*I/*Sma*I fragment from pNAN8142, which contains promoter and terminator regions (Ozeki et al., 1996), and a *Pst*I/*Sma*I-digested fragment of pPTR I (Takara Bio; Kubodera et al., 2000), which contains the pyrithiamine selectable marker (ptrA). pPAN8142 was digested with *Pma*CI and ligated to the PCR-amplified *tlg1* DNA fragment, which was amplified using the primers tlg1-ATG and tlg1-TAAL (Table I). The resulting expression construct (pPAN8142-tlg) was transformed into *A. oryzae* RIB40 as described previously (Gomi et al., 1987). Transformants were cultured for 3 d in DPY medium with shaking and proteins extracted as described above.

Purification of β-1,3-Glucanase

Proteins were extracted from 20 g of fruiting body 4 d following harvest. Samples were crushed in liquid nitrogen, suspended in 50 mL of 200 mM sodium acetate, pH 4.2, and incubated with rotation for 15 min at room temperature. Extracts were precipitated with 50% ammonium sulfate (50% fraction) followed by resuspension of the precipitate in 10 mM sodium phosphate buffer, pH 7.0. The resuspended 50% fraction was applied to a Superdex 75 10/30 gel filtration column (Amersham Biosciences) equilibrated in 10 mM sodium phosphate buffer, pH 7.0, and proteins eluted in the same buffer at a flow rate of 0.25 mL min^{-1}. Glucanase activity was measured by the method of Somogyi-Nelson (Nelson, 1944), using CM-Pachyman as substrate, and fractions containing β-1,3-glucanase activity were collected and concentrated using an Amicon Ultra 10,000 NMWL (Millipore) filter. The concentrate was then applied to a Mono Q 5/50 GL anion-exchange column (Amersham Biosciences) equilibrated and washed in 10 mM sodium phosphate buffer, pH 7.0. Adsorbed proteins were eluted using a linear NaCl (0–0.35 M) gradient at a flow rate of 0.5 mL min^{-1}. Purified enzymes were analyzed by SDS-PAGE and subsequent Coomassie Brilliant Blue staining of the gels according to the method of Sakamoto et al. (2005a). Otherwise, the gels were subjected to western-blot analysis using α-TLG1. The N-terminal amino acid sequence of purified TLG1 was analyzed by the method of Sakamoto et al. (2005a).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AB244759 (*tlg1* cDNA) and AB244760 (*tlg1* genome).

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LITERATURE CITED

Bottom CB, Siehr DJ (1979) Structure of an alkali-soluble polysaccharide from the hyphal wall of the basidiomycete *Coprinus macrorhizus* var. *microsporus*. Carbohydr Res **77:** 169–181

Brandazza A, Angeli S, Tegoni M, Cambillau C, Pelosi P (2004) Plant stress proteins of the thaumatin-like family discovered in animals. FEBS Lett **572:** 3–7

Chihara G, Maeda Y, Hamuro J, Sasaki T, Fukuoka F (1969) Inhibition of mouse sarcoma 180 by polysaccharides from *Lentinus edodes* (Berk.) Sing. Nature **222:** 687–688

Clarkson JM, Charnley AK (1996) New insights into the mechanisms of fungal pathogenesis in insects. Trends Microbiol **4:** 197–203

Datta K, Velashahn R, Oliva N, Ona I, Mew T, Khush GS, Muthukrishnan S, Datta SK (1999) Over-expression of the cloned rice thaumatin-like protein (PR5) gene in transgenic rice plants enhances environmental friendly resistance to *Rhizoctonia solani* causing sheath blight disease. Theor Appl Genet **98:** 1138–1145

Dudler R, Mauch F, Reimmann C (1994) Thaumatin-like protein. *In* M Witty, JD Higginbotham, eds, Thaumatin. CRC Press, Boca Raton, FL, pp 193–199

Galán B, Mendoza CG, Calonje M, Novaes-Ledieu M (1999) Production, purification, and properties of an endo-1,3-β-glucanase from the basidiomycete *Agaricus bisporus*. Curr Microbiol **38:** 190–193

Gomi K, Iimura Y, Hara S (1987) Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene. Agric Biol Chem **51:** 2549–2555

Greenstein S, Shadkchan Y, Jadoun J, Sharon C, Markovich S, Osherov N (2006) Analysis of the *Aspergillus nidulans* thaumatin-like *cetA* gene and evidence for transcriptional repression of *pyr4* expression in the *cetA*-disrupted strain. Fungal Genet Biol **43:** 42–53

Grenier J, Potvin C, Asselin A (1993) Barley pathogenesis-related proteins with fungal cell wall lytic activity inhibit the growth of yeasts. Plant Physiol **103:** 1277–1283

Grenier J, Potvin C, Asselin A (2000) Some fungi express β-1,3-glucanases similar to thaumatin-like proteins. Mycologia **92:** 841–848

Grenier J, Potvin C, Trudel J, Asselin A (1999) Some thaumatin-like proteins hydrolyse polymeric β-1,3-glucans. Plant J **19:** 473–480

Gurr SJ, Unkles SEU, Kinghorn JR (1987) The structure and organization of nuclear genes of filamentous fungi. *In* JR Kinghorn, ed, Gene Structure in Eukaryotic Microbes. IRL Press, London, pp 93–139

Hirano T, Sato T, Okawa K, Kanda K, Yaegashi K, Enei H (1999) Isolation and characterization of the glyceraldehyde-3-phosphate dehydrogenase gene of *Lentinus edodes*. Biosci Biotechnol Biochem **63:** 1223–1227

Kamada T, Fjii T, Takemaru T (1980) Stipe elongation during basidiocarp maturation in *Coprinus macrorhizus*: changes in activity of cell wall lytic enzymes. Trans Mycol Soc Jpn **21:** 359–367

Kamada T, Hamada Y, Takamaru T (1982) Autolysis in vitro of the stipe cell wall in *Coprinus macrorhizus*. J Gen Microbiol **128:** 1041–1046

Kamada T, Takemaru T (1977a) Stipe elongation during basidiocarp maturation in *Coprinus macrorhizus*: mechanical properties of stipe cell wall. Plant Cell Physiol **18:** 831–840

Kamada T, Takemaru T (1977b) Stipe elongation during basidiocarp maturation in *Coprinus macrorhizus*: changes in polysaccharide composition of stipe cell wall during elongation. Plant Cell Physiol **18:** 1291–1300

Kitajima S, Sato F (1999) Plant pathogenesis-related proteins: molecular mechanisms of gene expression and protein function. J Biochem (Tokyo) **125:** 1–8

Koiwa H, Kato H, Nakatsu T, Oda J, Yamada Y, Sato F (1999) Crystal structure of tobacco PR-5d protein at 1.8 Å resolution reveals a conserved acidic cleft structure in antifungal thaumatin-like proteins. J Mol Biol **286:** 1137–1145

Kubodera T, Yamashita N, Nishimura A (2000) Pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. Biosci Biotechnol Biochem **64:** 1416–1421

Kües U (2000) Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol Mol Biol Rev **64:** 316–353

Minato K, Kakawami S, Nomura K, Tsuchida H, Mizuno M (2004) An exo β-1,3 glucanase synthesized de novo degrades lentinan during storage of *Lentinula edodes* and diminishes immunomodulating activity of the mushroom. Carbohydr Polym **56:** 279–286

Minato K, Mizuno M, Terai H, Tsuchida H (1999) Autolysis of lentinan, an antitumor polysaccharide, during storage of *Lentinus edodes*, Shiitake mushroom. J Agric Food Chem **47:** 1530–1532

Mol PC, Wessels JGH (1990) Differences in wall structure between substrate hyphae and hyphae of fruit body stipes in *Agarucus bisporus*. Mycol Res **94:** 472–479

Moore D (1998) Fungal morphogenesis. Cambridge University Press, Cambridge, UK

Morikawa K, Takeda R, Yamazaki M, Mizuno D (1985) Induction of tumoricidal activity of polymorphonuclear leukocytes by a linear β-1,3-D-glucan and other immunomodulators in murine cells. Cancer Res **45:** 1496–1501

Nelson N (1944) A photometric adaptation of the somogyi method for the determination of glucose. J Biol Chem **153:** 375–380

Osherov N, Mathew J, Romans A, May GS (2002) Identification of conidial-enriched transcripts in *Aspergillus nidulans* using suppression subtractive hybridization. Fungal Genet Biol **37:** 197–204

Ozeki K, Kanda A, Hamachi M, Nunokawa Y (1996) Construction of a promoter probe vector autonomously maintained in *Aspergillus* and characterization of promoter region derived from *A. niger* and *A. oryzae* genome. Biosci Biotechnol Biochem **60:** 383–389

Rietsch A, Belin D, Martin N, Beckwith J (1996) An in vivo pathway for disulfide bond isomerization in *Escherichia coli*. Proc Natl Acad Sci USA **93:** 13048–13053

Roberts WJ, Selitrennikoff CP (1990) Zeamatin, an antifungal protein from maize with membrane-permeabilizing activity. J Gen Microbiol **136:** 1771–1778

Rotem Y, Yarden O, Sztejnberg A (1999) The mycoparasite *Ampelomyces quisqualis* expresses *exgA* encoding and exo-β-1,3-glucanase in culture and during mycoparasitism. Phytopathology **89:** 631–638

Sakamoto Y, Irie T, Sato T (2005a) Isolation and characterization of a fruiting body-specific exo-β-1,3-glucanase-encoding gene, *exg1*, from *Lentinula edodes*. Curr Genet **47:** 244–252

Sakamoto Y, Minato K, Nagai M, Kawakami S, Mizuno M, Sato T (2005b) Characterization of the *Lentinula edodes exg2* gene encoding a lentinan-degrading exo-β-1,3-glucanase. Curr Genet **48:** 195–203

Shida M, Ushioda Y, Nakajima T, Matsuda K (1981) Structure of the alkali-insoluble skeletal glucan of *Lentinus edodes*. J Biochem (Tokyo) **90:** 1093–1100

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res **22:** 4673–4680

Thorn RG, Barron GL (1984) Carnivorous mushrooms. Science **224:** 76–78

Trudel J, Grenier J, Potvin C, Asselin A (1998) Several thaumatin like proteins bind to β-1,3-glucans. Plant Physiol **118:** 1431–1438

van Loon LC, van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis PR-1 type proteins. Physiol Mol Plant Pathol **55:** 85–97

Wessels JGH (1993) Fruiting in the higher fungi. Adv Microb Physiol **34:** 147–202

Wessels JGH, Kreger DR, Marchant R, Regensburg BA, De Vries OM (1972) Chemical and morphological characterization of the hyphal wall surface of the basidiomycete *Schizophyllum commune*. Biochim Biophys Acta **273:** 346–358

Yun DJ, Ibeas JI, Lee H, Coca MA, Narasimhan ML, Uesono Y, Hasegawa PM, Pardo JM, Bressan RA (1998) Osmotin, a plant antifungal protein, subverts signal transduction to enhance fungal cell susceptibility. Mol Cell **1:** 807–817