**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-β-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...
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 a 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 1. Primers used in this study

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<td>Degenerate PCR</td>
</tr>
<tr>
<td>tlg-2U</td>
<td>ATHTGGCCNGCNATGTTYAC (IWPAMFT) 6</td>
<td>Degenerate PCR</td>
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<td>5’ RACE</td>
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<td>5’ RACE</td>
</tr>
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<td>tlg-ATG</td>
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</tbody>
</table>

* Amino acid sequence from which the degenerate primer was designed.
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,
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 pleuro 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-glucanase 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, F2 and F3, 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 F2

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).

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 F2 (lane 2) and F1 (lane 3) using CM-Pachymann as a substrate. Data represent the means and so of three independent experiments. C, Expression of the tlg1 in A. oryzae using pPAN81142. Shown are proteins extracted from wild-type strain A. oryzae RIB40 (lane 1), and F2 (lane 2) and F3 (lane 3).
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 F2 than in F3 (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 after harvest 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, lane 1), 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 lentilin, 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 phylogenetic 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

<p>| 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. |</p>
<table>
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<th>Total Protein</th>
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<th>Specific Activity</th>
<th>Purification</th>
<th>Yield</th>
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<td>416.5</td>
<td>45.53</td>
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<td>50% ammonium sulfate precipitate</td>
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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.
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 specificity 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 unreplied 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 (exo-glucanase) 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.

### Table III. Substrate specificity of TLG1

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<tr>
<td><em>L. edodes</em> cell wall</td>
<td>50</td>
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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*, AB006609; *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.

Measurement of Glucanase Activity

For the measurement of endo-glucanase activity, 1 g 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-glucanase (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 (Megazyme) as a substrate. One unit of enzyme activity was defined as the amount required to liberate 1 μmol/h of reducing sugar.

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 (tlg1-1U and tlg1-2U; Table I) designed against the N-terminal amino acid sequence from a TL protein of L. edodes (Krenier 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.fungi.net/ outlink.html). ClustalW (http://www.ddbj.nig.ac.jp/search/clustalw.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 phylogenetic tree was drawn using

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 desicator 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.

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TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treевiew.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 1D5, 1TWH, 1IQW, 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 Bed 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 235-bp DNA fragment, using primers tlg1-01U and tlg1-391 L (Table I), and gly3 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 (RIDDVFSPGFIKN, KSADANLDDQGPN, and KDACPDA-YAYDIES) 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
Heterologous expression of tlg1 construct (pET26-tlg1) was transformed into E. coli BL21 (DE3) and expressed to a polyvinylidene difluoride membrane as described previously (Sakamoto et al., 2005a). The resulting expression construct (pET26-tlg1) was amplified using the PCR method and fragment vector pET26 (Novagen) with BamHI and NotI, 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. PCR amplification of tlg1 was performed using the primers tlg1-Ni-BamHI and tlg1-TAAL-NotI (Table I). The PCR fragment and expression vector pET26 (Novagen) were ligated with BamHI and NotI, 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. PCR-amplified tlg1 DNA fragment, which was amplified using the primers tlg1-ATG and tlg1-TAAL (Table I). The resulting expression construct (pPAN8142-tlg1) was transformed into A. oryzae as described previously (Gomi et al., 1987). Transformants were cultured for 3 d in DPF 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 and resuspended 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) in 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, 1945), using CM-PhyAman 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 (0–0.35M) gradient at a flow rate of 0.5 mL min⁻¹. Purified enzymes were analyzed by SDS-PAGE and subsequent Coomasie 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).

LITERATURE CITED
Thaumatin-Like Protein in *Lentinula edodes*


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