Transcriptional regulation of laccase and cellulase in relation to fruit body formation in the mycelium of *Lentinula edodes* on a sawdust-based substrate

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Extracellular enzyme activities of laccase and cellulase and their transcriptional regulation were investigated at various growth stages in a sawdust-based substrate for *Lentinula edodes*. Changes of laccase and cellulase activities revealed a clear relationship with fruit body development stages. Laccase and cellulase activities were regulated at the level of gene transcription. The level of laccase mRNA was maximal at the fully colonized stage and declined during fruit body development. Cellulase mRNA began to accumulate at the pin (miniature fruit bodies) formation stage. Cellulase mRNA transcripts were maximally expressed at the veil-break stage of fruit body development. This tendency was clearer in the fruiting cultures with the wide-range-weather strains than in non-fruiting cold-weather strains. Transcription of laccase and cellulase genes was also affected by the water conditions of the sawdust-based substrate. Primordia initiation occurred when the water potential of the medium was high for rapid mRNA transcription by the mycelium.

Key Words——extracellular enzyme; fruit body; *Lentinula edodes*; mRNA; transcriptional regulation.

Lentinula edodes (Berk.) Pegler (shiitake) is the second most important cultivated mushroom in the world. Production in Japan in 1997 was estimated at 250,000 metric tons and valued 171 billion yen (US 1.6 billion). In Japan, indoor cultivation on a hardwood sawdust-based substrates is rapidly replacing traditional cultivation in bed-logs in the open air. The sawdust-based cultivation method for *L. edodes* has advantages such as a shorter cultivation period and better manipulation of flushing (fruit body formation) by control of environmental conditions. This method now accounts for about a half of all production and is expected to become the major cultivation method.

The major nutritional components of the growth medium comprise the various lignocellulose polymers from sawdust cells and some nutritional additives. Several extracellular enzyme activities of *L. edodes* have been detected in the sawdust-based substrate. These include laccase, peroxidases, cellulases, hemicellulases, proteases, and various glycosidases. Biodegradation is achieved by secretion of a set of extracellular enzymes. Laccase and cellulase especially have been studied in depth since their activities revealed rapid changes during fruit body development in *L. edodes* (Leatham and Stahmann, 1981; Leatham, 1985; Ohga, 1992) and in *Agaricus bisporus* (Turner, 1974; Turner et al., 1975;

Wood and Goodenough, 1977; Manning and Wood, 1983; Claydon et al. 1988). The growth of the mycelium and the production of fruit bodies are predominantly dependent on the efficient utilization by the extracellular enzymes of the lignocellulose polymers of the sawdust-based substrate. Increase in utilization of substrate is a target for strain manipulation, and this could be achieved by regulation of extracellular enzyme level, using strain selection methods including genetic engineering.

In *A. bisporus*, cloning of laccase and cellulase genes has provided probes for the analysis and quantitation of gene expression in both liquid and compost cultures. Laccase and cellulase activities are strongly regulated during fruit body production, and this regulation is associated with both transcriptional and post-transcriptional control (Raguz et al., 1992; Yague et al., 1994, 1996, 1997; Chow et al., 1994; Smith et al., 1998; Ohga et al., 1999). In the present report, we describe the regulation of laccase and cellulase gene expression in the sawdust-based substrate of *L. edodes* during culture of various strain types under different water conditions.

Materials and Methods

Fungal strains and culture conditions Twelve strains of *L. edodes* were evaluated, KS-3, -4, -12, -16, -23, -24, -46, -56, -58, -60, -67 and -76, all but two of which are wide-range-weather strains fruiting normally in a saw-

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dust-based substrate; SK-12 and -24 are cold-weather strains. All of them were originate from commercial sources. The colonies were maintained on potato dextrose agar medium at 4°C and subcultured on a sawdust-based substrate for 14 d at 25°C for spawn production.

A columnar ($\phi 11 \times 15$ cm, 1.2 kg) sawdust-based substrate culture system was used in this study. Culture components, sterilization, spawning, mycelial incubation, and fruit body formation procedures were as previously described (Ohga, 1998). Mycelial colony growth, culture surface whiteness, and fruit body yield were measured as described previously (Ohga, 1999). The whiteness was measured with a Minolta CR-200 colorimeter.

Sampling procedures Samples were obtained from triplicate cultures at various selected growth stages, frozen in liquid nitrogen, and stored at -70°C until use for RNA extraction. Samples were taken at the seven stages of fruit body development, d 90, colonization (C); d 94, aggregate (A); d 98, pin (P); d 100, button (B); d 102, veil-break (V); d 104, senescence (S); d 140, second flush (2F).

Moisture condition as ϕ A 130-d-culture (KS-58) was soaked in water at 18°C for 8 or 24 h. Water potential (ϕ) was measured as an indicator of the moisture condition at various culture stages by use of a Wescor HR-33T psychrometer equipped with a CF-52 sample chamber, as described previously (Ohga, 1999).

Enzyme assays Laccase and cellulase activities were measured by similar methods to those described previously (Ohga, 1992). Briefly, the samples were homogenized in 10 mM acetate-acetic buffer (pH 4.2), then the resulting supernatants were concentrated by ammonium sulfate precipitation. Crude enzyme was obtained after dialysis.

Laccase (EC 1.10.3.2) activity was determined using p-phenylendiamine as a substrate. Cellulase (endoglucanase, EC 3.2.1.4) was assayed by measuring reducing sugar liberated from carboxymethyl cellulose (CMC). One unit of laccase activity was defined as a change in absorbance of 0.001 at 525 nm per min. One unit of cellulase activity was defined as the amount of enzyme required to produce 1 μmol of glucose per min.

RNA isolation Total RNA was isolated following Raguz et al. (1992) using 5-g samples of colonized and fruiting sawdust-based substrate. Samples from at least three separate substrates were used for each RNA isolation. The frozen colonized sawdust-based substrates were blended to a fine powder with dry CO_2 pellets in an electric coffee grinder. RNA was extracted using triisopropylnaphthalene sulphonate and phenol/cresol, followed by ethanol precipitation.

Competitive RT-PCR The conditions for reverse transcription, amplification, and electrophoresis conditions were similar to those described by Smith et al. (1998). Primers used were 5'-GATCAGAGCTCCTATGACTG-3' and 5'-AGTTGAGGGTGATGTGCTTAT-3' for laccase, and 5'-TCAAGCTCCTCCTCCCAC-3' and 5'-GGGCAGATCGTAGACAAC-3' for cellulase (Chow et al., 1994; Perry et al., 1993). Total RNA (2 μg) was used as

the template to generate first-strand cDNA. Competitive PCRs were performed with 0.1 to 100 pg of competitor genomic DNA. Amplification was performed under the conditions described previously (Ohga et al., 1999).

Results

Mycelial growth and fruit body formation Mycelial growth and fruit body yield are shown in Fig. 1. Rapid expansion of the mycelial colony was observed in the sawdust-based substrate inoculated with wide range-weather strains. Whiteness of the substrate surface showed a positive relation to mycelial density in the sawdust-based substrate of *L. edodes* (Ohga, 1995). Mycelial density judged from the surface whiteness was superior in the wide-range-weather strains to that in the cold-weather strains.

Strains of the wide-range-weather group normally formed 10 or more complete fruit bodies on sawdust-based substrate within 100 d of incubation (first flush). Fruit body yield ranged from 230 g (KS-4) to 305 g (KS-58) in the first flush period. Cold-weather strains KS-12 and -24 remained at the mycelial stage until 100 d, producing no fruit bodies under the same substrate conditions.

Water potential (ϕ) of culture for 2nd flush The ϕ of the substrates varied with the period of soaking for second flush fruit body formation, being higher after soaking for 24 h compared with 8 h (Fig. 2). Substrates just after 24-h treatment revealed a suitable ϕ (-0.4 MPa) for second flush newly primordia initiation. Submergence for 8 h was not sufficient to provide the best water conditions, giving a ϕ of -2.1 MPa, which is too low for
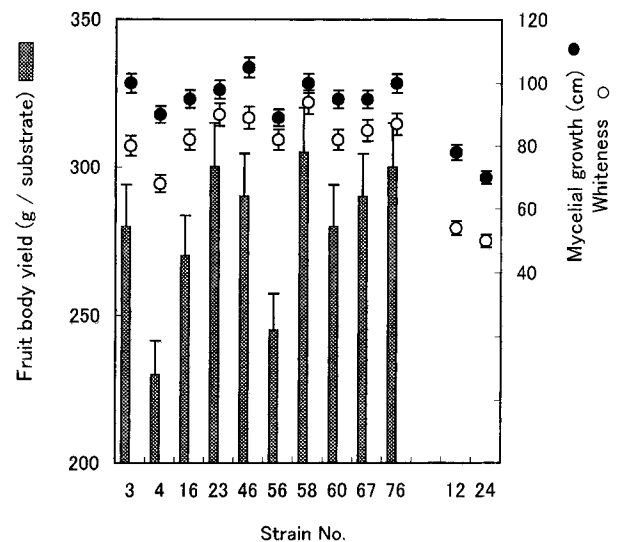

Fig. 1. Mycelial growth and fruit body yield of various strains of *L. edodes* on the sawdust-based substrate. Mycelial growth was measured as the diameter of colony at d 14, and whiteness of the colony surface was measured at the same time by use of a colorimeter. Fruit body yield means the total quantity during 1st flushing period (90–110 d). Error bars indicate standard deviations (n=30).
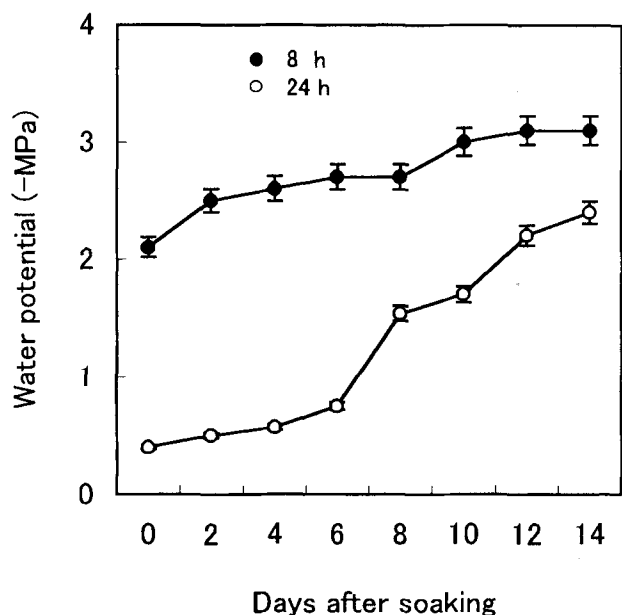
Fig. 2. Influence of soaking times on the water potential of the sawdust-based substrate of *L. edodes* (KS-58) at the 2nd flushing (140 d). Error bars indicate standard deviations (n=30).
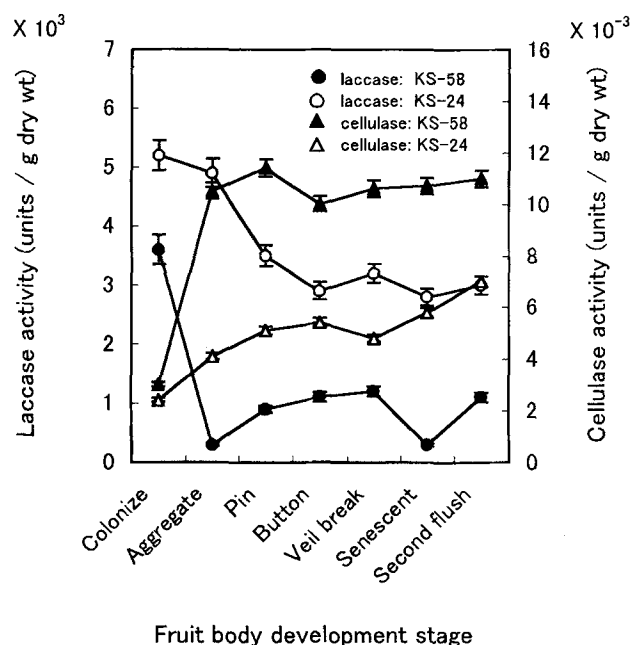

Fruit body development stage

Fig. 3. Activities of laccase and cellulase in two different types of *L. edodes* strains (KS-58 and KS-24) in the sawdust-based substrate. Error bars indicate standard deviations (n=5).

primordia initiation.

Laccase and cellulase activities The enzyme activities and mRNA transcript levels described below are for two typical strains, KS-58 (wide-range-weather) and KS-24 (cold-weather).

Consistent differences in laccase and cellulase activities were found between the two different strain types (Fig. 3). Laccase activity was quite high during colonization, then declined rapidly as fruit bodies developed in strain KS-58. The non-fruiting strain KS-24 showed high laccase activity at the colonized stage, but the subsequent marked decline in the late growth stages seen in strain KS-58 was not observed. While laccase activity decreased to a low level at the aggregate stage in strain KS-58, cellulase activity rose sharply. The cellulase activities remained high during fruit body development. No clear increase in cellulase activity was found in samples where no fruit body formed in strain KS-24.

mRNA transcript levels RNA was obtained from the sawdust-based substrate of *L. edodes*. The yield of total RNA was 27–59 μgg^{-1} wet weight.

The mRNA transcription in strain KS-58 coincided with individual changes of enzyme activities (Fig. 4). Transcript levels of laccase mRNA were highest at the fully colonized stage, and rapidly declined at the aggregate and primordium formation stages. In contrast, cellulase mRNA began to accumulate at the pin formation stage and was maximally expressed at the veil-break stage during fruit body development. Strain KS-24, which remained mycelial and thus produced none of the stages of fruiting, showed different mRNA transcriptional regulation. Transcript levels of laccase and cellulase mRNA showed no marked changes at any growth stages,

in contrast with KS-58.

Transcription was affected by the ψ of the sawdust-based substrate in the 2nd flush period (Figs. 2, 5). Primordium initiation occurred when the ψ of the medium

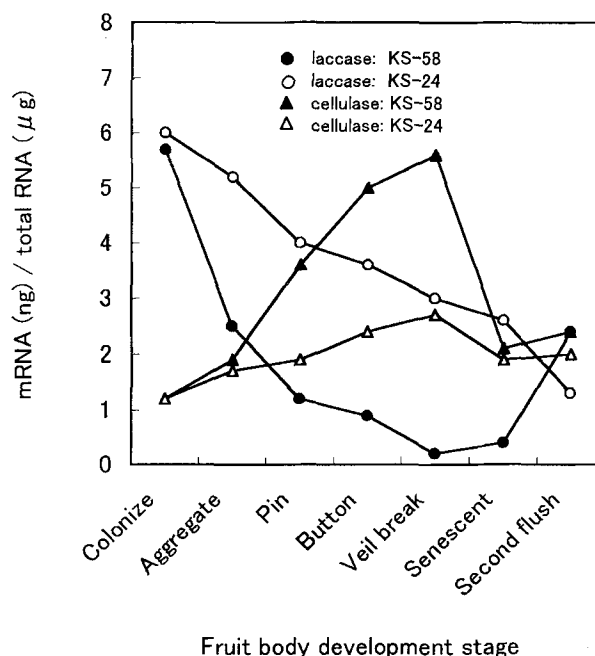Fruit body development stage

Fig. 4. Regulation of laccase and cellulase transcripts in two different types of *L. edodes* strains (KS-58 and KS-24) in the sawdust-based substrate.
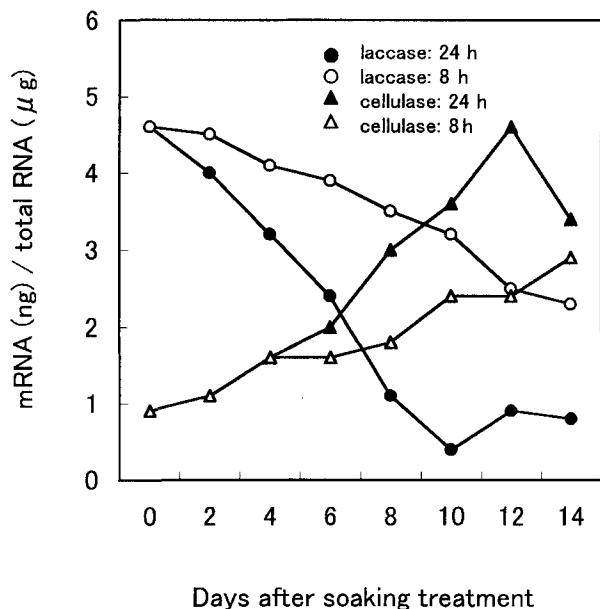
Fig. 5. Regulation of laccase and cellulase transcripts of *L. edodes* (KS-58) in the sawdust-based substrates having different water potentials.

was sufficient for rapid metabolism by the mycelium. A similar transcriptional regulation pattern to the 1st flush period appeared in the 2nd flushing stage, that is, laccase mRNA transcript levels rapidly decreased, and then cellulase mRNA increased to fruit body development. No large changes in mRNA transcription were recognized in the non-fruiting cultures.

Discussion

Over one hundred strains of *L. edodes* are now listed for use as commercial spawn in Japan. It is known that strain selection is one of the most important factors for obtaining good fruiting. The wide-range-weather strains are usually used in the sawdust-based substrate cultivation because of their sensitive response in fruiting. We prepared fruiting and non-fruiting cultures for laccase and cellulase mRNA transcriptional regulation studies by manipulating the strain variety and soaking treatment.

Mycelial growth in the substrate was much faster in the wide-range-weather strains than the cold-weather strains. This fast mycelial growth gave rise to dense mycelial formation as judged by surface whiteness. The wide-range-weather strains easily fruited, but the cold-weather strains continued mycelial growth with no fruit body formation under the conditions employed in this study. Primordia formation was higher in the culture inoculated with KS-58 (wide range-weather strain) than that of KS-24 (cold-weather strain) (data not shown).

Various compounds in the substrate are decomposed by a set of extracellular enzymes. A wide range of enzymes is produced and identification of their activity indicates that most of the potential nutrient sources in substrate are available for fungal growth and fruit body

formation. Extracellular enzyme activities of different strain types revealed different patterns on the sawdust-based substrate. Interestingly, decrease of laccase activity was recognized in association with primordium formation, and a complementary increase was recognized in the cellulase activity (Ohga, 1992). These dramatic changes of the two enzymes were recognized only in the fruiting substrate.

Transcriptional regulation of laccase and cellulase mRNA coincided with the enzyme activity changes. Laccase gene expression was maximal during colonization and declined during the fruiting stages. Cellulase mRNA was maximally expressed in the sawdust-based substrate at the veil-break stage of fruit body development. These regulation patterns were similar to that of *A. bisporus* on compost (Ohga et al., 1999). This regulation corresponded to the developmental stage before maximal activity. Gene transcription of extracellular enzyme activities has been studied for mannitol dehydrogenase of *A. bisporus* (Stoop and Mooibroek, 1998), laccase of *Trametes versicolor* (Collins and Dobson, 1997), and manganese peroxidase (Gettemy et al., 1998) and cellobiose dehydrogenase (Vallim et al., 1998) of *Phanerochaete chrysosporium*. Expression of genes in *A. bisporus*, *T. versicolor*, and *P. chrysosporium* was specifically regulated by nutrient levels and environmental conditions. Several factors affect fruit body production efficiency on sawdust-based substrates, including the genotype (Diehle and Royse, 1986; Ohga, 1998), spawn run time (Royse and Bahler, 1986; Ohga et al., 1992), and substrate formulation (Leatham and Stahmann, 1984; Royse, 1985). These environmental factors may affect gene transcription for fruiting. Transcriptional regulation of enzymes under various culture conditions will be studied in more detail.

The present study examined the influence of strain variety and culture water conditions on transcriptional regulation. Mycelial density of the sawdust-based substrate was dense with wide-range-weather strains and resulted in high yields of fruit bodies. Transcriptional regulation coincided with individual enzyme activities at various fungal growth phases. Water condition is one of the most important factors in primordium formation and development of fruit bodies. Measurement of ϕ was useful for judging the actual water content for the growth and fruiting of *L. edodes*. KS-58 cultures with different degrees of ϕ were prepared by soaking treatment. The longer soaking period resulted in good water conditions with high ϕ, -0.4 MPa. This value is coincided with the previous report, which concluded that a suitable ϕ for fruiting initiation is around -0.7 MPa (Ohga, 1999). As mentioned before, transcription of laccase and cellulase show a complementary relationship in the primordium formation stage. This trend was found in the culture with suitable water condition for fruiting. Regulation of laccase and cellulase gene transcription in the 2nd flushing was the same as in the 1st flushing period.

In conclusion, laccase and cellulase activity changes in *L. edodes* sawdust-based substrate are controlled at the level of gene transcription. Transcription of extracel-

lular enzyme genes was affected by strain types (KS-24 and KS-58) and water conditions (-0.4 and -2.1 MPa) of the culture. Genetic manipulation of gene expression has the potential for improving cultivation processes such as fruit body yield and flush timing.

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