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FULL PAPER

Satoshi Kanda · Tadanori Aimi · Seita Masumoto
Kaori Nakano · Yutaka Kitamoto · Tsutomu Morinaga

Photoregulated tyrosinase gene in *Polyporus arcularius*

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Abstract *tyr1*, the gene for tyrosinase, which is related to pigmentation of mycelia in dikaryotic strains, has been cloned and characterized from the basidiomycete *Polyporus arcularius*. The gene was present in a single copy in the genome. The putative amino acid sequence of Tyr1 was very similar to that of gLeTyr from *Lentinula edodes*. However, the carboxy-terminal region of the amino acid sequence of Tyr1 was variable among *L. edodes*, *Agaricus bisporus*, and this fungus. In the 5′-untranslated region near the initiation codon, a consensus sequence to the Dof1 binding site that is involved in light-regulated gene expression in maize was found. Transcription of *tyr1* was photoregulated; transcription of *tyr1* in *P. arcularius* was activated in light mycelia and inactivated in the dark mycelia. These results suggest that *tyr1* is a light-regulated gene regulated by a Dof-like transcription factor in *P. arcularius*. Although the enzyme activity was observed only in a dikaryon, *tyr1* was transcripted in both dikaryotic and monokaryotic strains. Thus, activation of the precursor of Tyr1 may require a posttranslational processing event that is developmentally regulated.

Introduction

Tyrosinase (*o*-diphenol: oxygen oxidoreductase, EC 1.14.18.1), which is one of the phenol oxidases, is a copper monooxygenase widely distributed in nature and involved in the formation of pigments such as melanin and other polyphenolic compounds (van Gelder et al. 1997). This enzyme catalyses two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity), and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity), which, in turn, are polymerized to brown, red, or black pigments. Pigmentation is one of the most obvious phenotypic characteristics in the natural world. Of the pigments, melanin is one of the most widely distributed and is found in bacteria, fungi, plants, and animals. Melanins are heterogeneous polyphenol-like biopolymers with a complex structure and color varying from yellow to black. The copper-containing enzyme tyrosinase is essential for melanization (Sanchez-Ferrer et al. 1995; Espin et al. 1999).

The light governs many developmental transitions and accompanying expression of genes in the life cycles of fungi (Kumagai 1988; Bell-Pedersen et al. 1996; Linden et al. 1997; Kues et al. 1998; Kues 2000; Corrochano 2002; Froehlich et al. 2002; He et al. 2002). The basidiomycete *Polyporus arcularius* is a polypore mushroom that needs light for two stages of formation of the fruiting body. The initiation of fruiting-body formation in *P. arcularius* is induced by blue light. Light is also required for the initiation of pileus formation in the fruiting body of *P. arcularius*. If a dark-grown vegetative mycelium is exposed to light, buff-colored mycelium is produced on the part of the colony at which the fruiting-body primordium subsequently forms. Mycelial color development during fruiting-body initiation suggests the involvement of activation of intracellular phenol oxidase to produce colored phenolic compound(s), and the regulation of tyrosinase is closely associated with the induction and developmental processes of fruiting-body formation in this mushroom. The activation of intracellular phenol oxidase was measured with 3,4-dihydroxyphenylalanine (DOPA) as the substrate, and it was associated with photoinduced fruiting-body initiation in *P. arcularius* (Kitamoto et al. 1999, 2000).

In this article, we describe cloning and characterization of the gene encoding tyrosinase, which is one of the phenol oxidases in *P. arcularius*, and its transcriptional regulation by light. The present study is part of our attempt to understand the mechanism and signaling pathway of photoin-

S. Kanda · S. Masumoto · K. Nakano · T. Morinaga (✉)
Department of Bioresource Development, Hiroshima Prefectural University, 562 Nanatsuka-cho, Shobara, Hiroshima 727-0023, Japan
Tel. +81-82-474-1777; Fax +81-82-474-1777
e-mail: tmorina@bio.hiroshima-pu.ac.jp

T. Aimi · Y. Kitamoto
Faculty of Agriculture, Tottori University, Tottori, Japan

S. Kanda
Izumo Technical High School, Shimane, Japan

duced fruiting-body development in *P. arcularius* using molecular biological techniques.

Materials and methods

Strains and culture conditions

Mycelia of the monokaryotic strains *Polyporus arcularius* BS4 (A1B1) and I (A3B3) were used, which had been isolated from the basidiospores of *P. arcularius* 69A (ATCC 24460) and 69B (ATCC 24461) strains, respectively. The strains were maintained on MYGA (1% malt extract, 0.4% yeast extract, 0.4% glucose, 2% agar). MYG liquid medium was used to culture fungi for DNA isolation. Fruiting-body formation medium [FBDM: 20 g maltose, 1.0 g polypeptone, 0.3 g K_2HPO_4, 0.3 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaCl_2{\cdot}2H_2O$, 0.3 mg $ZnSO_4{\cdot}7H_2O$, 0.15 mg $FeSO_4{\cdot}7H_2O$, 0.10 mg $CuSO_4{\cdot}7H_2O$, 0.10 mg $MnSO_4{\cdot}5H_2O$, 0.02 mg $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 0.50 mg thiamine hydrochloride, pH 5.6] (Kitamoto et al. 1999, 2000) was used for fungal culture to prepare protein and RNA samples.

Cloning of tyrosinase gene from *P. arcularius*

To isolate the gene encoding the tyrosinase from *P. arcularius* BS4, genomic DNA was extracted from lyophilized mycelium of the *P. arcularius* BS4 strain using a hexadecyltrimethylammonium bromide (CTAB) procedure essentially as described previously (Zolan and Pukkila 1986). We amplified fragments of genomic DNA encoding the tyrosinase and actin using polymerase chain reaction (PCR) with the oligonucleotide primers CuA and CuB, and ActinF and ActinR, which are complementary to a conserved portion of fungal tyrosinase and actin genes, respectively (Table 1). To amplify a DNA fragment containing the tyrosinase gene, PCR was carried out in a 100-µl reaction mixture containing 1 × PCR buffer (Takara Biochemicals, Shiga, Japan), 100 ng extracted genomic DNA, 100 pmol each primer, 0.2 mM each dNTP, and 2.5 U rTaq polymerase (Takara Syuzo, Kyoto, Japan). The reaction was performed for 30 cycles with a 2400 DNA thermal cycler (Applied Biosystems Japan, Tokyo, Japan), and the following cycling profile was used. The first denaturing step was at 94°C for 2 min, and then the PCR cycles were 30 s denaturation at 94°C, 2 min annealing at 55°C, and 30 s extension at 72°C. Finally, the reaction mixture was kept at 72°C for 10 min. The resultant fragment containing a partial tyrosinase gene (650 bp) was cloned into the pT7Blue(R) T-vector (Novagen, Madison, WI, USA) to yield pTyr4. The clone was sequenced to confirm that it contained a partial DNA fragment of *tyr1*, encoding tyrosinase, The deduced amino acid sequence of the gene product was very similar to the sequence of tyrosinase from *Lentinula edodes* (Berk.) Singer and *Agaricus bisporus* (J.E. Lange) Pilat (their accession numbers in the DNA database are AB033993 and AJ223816, respectively) (data not shown). To amplify the DNA fragment containing the actin gene, PCR was carried out under the same conditions as already described, except for annealing temperature (50°C), primers (ActinF, ActinR), and extension time (1 min). The amplified partial DNA fragment (940 bp) containing the gene encoding actin was sequenced directly and confirmed to contain a portion of *act1*. An oligonucleotide primer for RT-PCR was then constructed from the partial nucleotide sequence of *act1* (see Table 1).

The genomic DNA library was constructed in the lambda DASH II vector (Stratagene, La Jolla, CA, USA) with DNA from the BS4 strain that was size fractionated after partial *Sau*3AI digestion. Lambda phage clones (lambda-PO5) corresponding to the amplified DNA fragments encoding *tyr1* were identified by plaque hybridization analysis from the DNA library with the DNA fragment containing a partial tyrosinase gene as probe, and several restriction fragments of the clone (lambda-PO5) were subcloned into pBluescript KS (+) (Toyobo, Osaka, Japan) and sequenced. Plaque lifting was carried out by standard protocols (Maniatis et al. 1982). Probe labeling and detection were performed using the ECL direct nucleic acid labeling and detection system (Amersham). Procedures for probe labeling, hybridization, and detection were carried out according to the manufacturer's recommendations.

Southern hybridization

Genomic DNA from the *P. arcularius* BS4 strain was isolated and singly digested with restriction enzymes such as *Sal*I and *Sac*I, and doubly digested with *Pst*I and *Xho*I, after which the fragments were separated by agarose gel electro-

Table 1. Polymerase chain reaction (PCR) primers used in this study

Primer	Sequence	Remark
CuA	5′-GTNYTNTTYCCNACN TGGCA-3′	Use for initial amplification of tyrosinase gene (*tyr*1)
CuB	5′-TGNARCCARAADATNGGRTC-3′	
ActinF	5′-ACATGGAGAAGATCTGGCAC-3′	Use for initial amplification of actin gene (*act1*)
ActinR	5′-CTCGTCGTACTCCTGCTT-3′	
TYR-RTF21	5′-TGGATAAGGATACTCCCCTG-3′	Use for RT-PCR of *tyr*1
TYR-RTR21	5′-CGCACTTCCATCCACCTTCT-3′	
Act-Bs4F	5′-GTAACGAGCGGTTCCGTGCT-3′	Use for RT-PCR of *act1*
Act-Bs4R	5′-CGCAACGATCTTGACCTTCA-3′	
T7primer	5′-TAATACGACTCACTATAGGG-3′	Used for 3′-RACE
Poly(T)-T7	5′-TAATACGACTCACTATAGGG(polyT)$_{17}$-3′	

RT, reverse transcriptase; 3′-RACE, 3′-rapid amplification of cDNA end

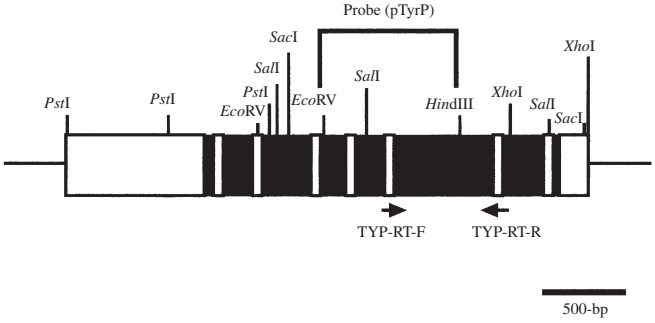

Fig. 1. Schematic diagram of the genes encoding the tyrosinase proteins (*tyr1*). *Solid boxes* indicate position of exons; *arrows* indicate the positions of the primers used for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Restriction fragment (*pTyrP*) used as probe for genomic Southern hybridization is shown as a *bracket*

phoresis and blotted onto nylon membranes. The DNA fragment from the clone (lambda-PO5) was digested with *Eco*RV and *Hin*dIII and then subcloned into pBluescript KS (+) and used as a probe (Fig. 1). The recombinant plasmid was assigned the name pTyrP. The blots were probed with the DNA fragment using the Gene Image random primed nucleic acid labeling and detection system (Amersham). Procedures for probe labeling, hybridization, and detection were carried out according to the manufacturer's recommendations.

RNA procedure

RNA isolation from *P. arcularius* dikaryotic (BS4 × I) and monokaryotic (BS4) strains grown in FBDM was performed as follows: mycelia were grown on MYGA at 25°C for 7 days, then a mycelial agar block (1 cm × 1 cm) was transferred into an Erlenmeyer flask containing 8 ml FBDM and incubated at 25°C for 7 days in the dark. The flask was then transferred to visible light (about 600 lux). After exposure to visible light for several hours, the mycelia were harvested and frozen in liquid nitrogen, and then ground in a mortar and pestle to a fine powder. Total RNA extraction buffer [20 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS), pH 5.2] was added. The RNA solution was extracted with phenol saturated with RNA extraction buffer, and then total RNA was recovered with ethanol precipitation. Formaldehyde agarose gel electrophoresis of total RNA was performed according to the procedure described by Sambrook et al. (Sambrook and Russell 2001). RT-PCR (reverse transcription-PCR) was carried out as follows: mRNA was reverse transcribed with reverse transcriptase using primers TYR-RTR21 and Act-Bs4R, respectively, then partial cDNA fragments of *tyr1* and *act1* were amplified using primer pairs TYR-RTF21 and TYR-RTR21, and Act-Bs4F and Act-Bs4R, respectively (see Table 1). These primers were designed on the basis of the putative cDNA sequences to overlap junctions between exons to avoid amplification of genomic DNA. Reverse transcription and PCR were performed using an Access Quick RT-PCR system (Promega) according to the manufacturer's instructions. Amplified cDNA fragments were directly sequenced.

3′-Rapid amplification of cDNA end (3′-RACE)

To detect *tyr1* transcripts in *P. arcularius* BS4 × I strain, mRNA was reverse transcribed with reverse transcriptase using a poly (T)-T7 primer. We then amplified partial cDNA fragments of *tyr1* using primer pairs TYR-RTF21 and T7 (see Table 1). Reverse transcription and PCR were performed as above. Amplified cDNA fragments were subcloned into the pT7Blue T-vector, and the resulting plasmids were sequenced.

Nucleotide sequence accession numbers

The nucleotide sequence of the *P. arcularius tyr1* gene has been deposited in the DDBJ (DNA Data Bank of Japan) database under accession no. AB033993.

Enzyme assay

To extract the intracellular tyrosinase of monokaryotic and dikaryotic *P. arcularius*, McIlvaine buffer (pH 7.0) containing 1 M NaCl was added to fine mycelial powder obtained as described previously for RNA extraction. The reaction mixture, containing 1 ml 5 mM DOPA solution as substrate, 1 ml McIlvaine buffer (pH 7.0) to which $CuSO_4$, adjusted to 1 mM before adding to the reaction mixture had been added, and 0.5 ml of the aforementioned extract as the enzyme solution, was incubated at 37°C for 30 min. Under these conditions, 1 unit of tyrosinase was defined as the amount of enzyme that caused an increase of 0.01 per minute in absorbance at 470 nm. The protein concentration was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as the standard.

Results

Characterization of tyr1 from *P. arcularius*

The gene encoding tyrosinase from *P. arcularius* is shown schematically in Fig. 1. The locations of the exons and introns of the gene were deduced from the interruptions in the amino acid sequence that was homologous to that of tyrosinase from gLeTyr from *L. edodes* (accession numbers in DNA database are AB033993), as well as from the sites of consensus sequences in conserved 5′- and 3′-splice sites within introns. All inferred introns started with the nucleotide pair GT and ended with AG.

To predict the regulation of *tyr1* transcription, consensus promoter sequences in the 5′-untranslated region near the initiation codon of *tyr1* were searched with the MOTIF program for searching protein and nucleic acid sequence motifs on the World Wide Web server (http://motif.genome.ad.jp/). An interesting consensus sequence was

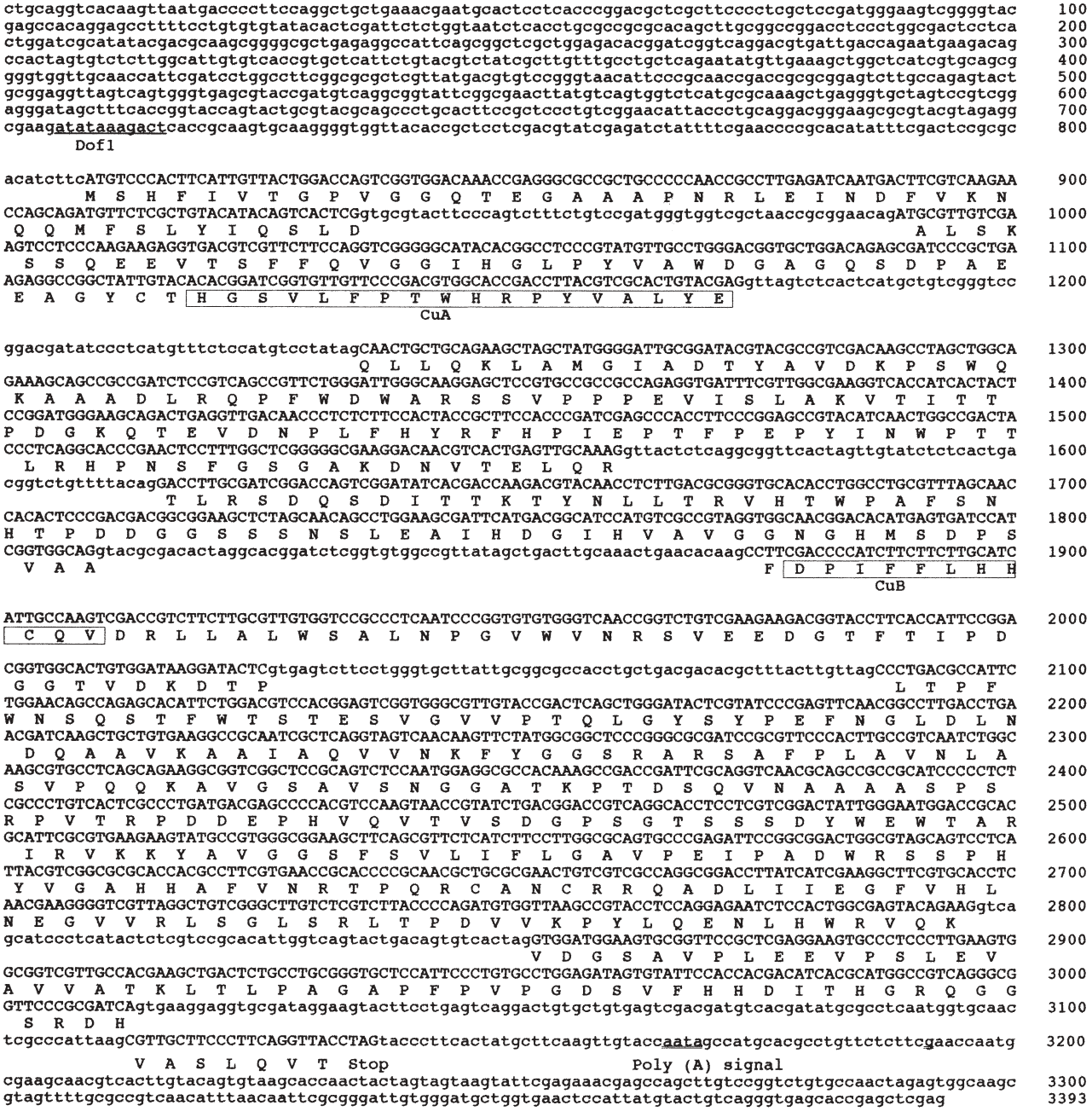

Fig. 2. Nucleotide sequences of gene for the tyrosinase from *Polyporus arcularius*. Intron, 5′-upstream, and 3′-downstream sequences are given in *lowercase letters*. The deduced amino acid sequence of tyrosinase is given *below* the nucleotide sequence. The consensus sequence of transcription factor Dof1 binding site is *underlined*. Conserved amino acid sequences in tyrosinase, which is the copper-binding domain signature (CuA and CuB), are *boxed*. Putative polyadenylation signal and poly (A) start site are *double underlined*

found (Fig. 2). The sequence was 5′-ATATAAAGACT-3′ at nucleotides 706–716, which is a consensus with the Dof1 binding site motif (5′-ANNWAAAGNNN-3′). Dof1 is a single zinc finger transcription factor, and maize Dof1 is one of the plant-specific Dof transcription factors involved in light-regulated gene expression (Yanagisawa and Sheen 1998).

Transcriptional termination of the *tyr1* gene occurred at a G nucleotide, 56th nucleotide from the TAG termination codon of the *tyr1* gene. In the 3′-untranslated region, an A-rich element that is a consensus polyadenylation signal-like sequence, most often the sequence 5′-AAUAAA-3′, is 10 to 30 nucleotides upstream from the poly (A) site (Gross and Moore 2001). In *tyr1*, the sequence 5′-AAUA-3′ was found in the 3′-untranslated region of the *tyr1* gene 24 to 27 nucleotides upstream from the poly (A) site.

The length of the coding region was 2329 bp. The deduced amino acid sequence of the protein is shown and aligned with homologous proteins from other basidiomycetes in Fig. 3. *Polyporus arcularius tyr1* encodes a polypep-

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                                                    CuA
P. arcularius     1 MSHFIVTGPVGGQTEGAAAPNRLEINDFVKNQQMFSLYIQSLDAL-SKSSQEEVTSFFQVGGIHGLPYVAWDGA-GQSDP    78
L. edodes         1 ...YL...AT..S.S..............QEDQ......A.QYIY.SK..DDID....I.........P....-.NKPV    79
A. bisporus       1 ...LL.S-.L..----GVQ.-.....N....DRQ....V.A..RMYAT-P.N.TA.Y...A.V..Y.LIPF.D.V.PTEF    73

P. arcularius    79 AE-EA--GYCTHGSVLFPTWHRPYVALYEQLLQKLAMGIADTYAVDKPSWQKAAADLRQPFWDWARSSVPPPEVISLAKV   155
L. edodes        80 DT-D.WE.............F.....L.I..AI.AA.VD..A..I..RARY.D..LN....Y.....NP.........DE.   158
A. bisporus      74 SPFDQWT.......T..........LIL..I.SGH.QQ.....T.N.SE.K...TEF.H.Y....SN..........P..   153

P. arcularius   156 TITTPDGKQTEVDNPLFHYRFHPI-EPTFPEPYINWPTTLRHPNSFGSGAKDNVTELQRTLRSDQSDITTKTYNLLTRVH   234
L. edodes       159 ..VN.S.EKIS.P...RR.T....-D.S.....QS.S......L.DDAN.S...P..KA....AGPQLK...........   237
A. bisporus     154 .....N.QK.S.A...MR.T.NSVNDGG.YG..NQ.D....Q.D.T.VN.....NR.KSV.KNA.ASL.RA..DMFN..T   233

                                                    CuB
P. arcularius   235 TWPAFSNHTPDDGGSSSNSLEAIHDGIHVAVGGNGHMSDPSVAAFDPIFFLHHCQVDRLLALWSALNPGVWVNRSVEEDG   314
L. edodes       238 ..............T.....G...SV..D.....Q.......G......M..A......S.......R..ITDGPSG..   317
A. bisporus     234 ...H..S...AS...T...I.....N...L.............P.........AN....I.....IRYD..TSPGDAQF.   313

P. arcularius   315 TFTIPDGGTVDKDTPLTPFWNSQSTFWTSTESVGVVPTQLGYSYPEFNGLDLNDQAAVKAAIAQVVNKFYGGSRARSAFP   394
L. edodes       318 .W...PDTV.G...D......T..SY.I.AN--VTDTSKM..T.....N..MGNEV..RS...AQ...L...PFTK--.A   393
A. bisporus     314 .Y.LRYKQS..ES.D.A.W.KT.NEY.K.N.--LRSTES...T....V...MYNKD..NKT.SRK.AQL..PQ.GGQRSL   391

P. arcularius   395 LAVNLAS---VPQQKAVGSAVSNGGATKPTDSQVNAAAASPSRPVTRPDDEPHVQVTVSDGPSGTSSSDYWEWTARIRVK   471
L. edodes       394 A.IQQP.SQTTADASTI.N-.TSDASSHLV..KI.PT---.N.SI----..A.Q.KIA--STLRNNEQKEF......VQ..   464
A. bisporus     392 VEDLSN.HARRS.RP.KR.RLGQLLKGLFS.WSAQIKFNRHEVGQSFSVCLFLGN.PEDPREWLV.PNLVGARH.FV.SV   471

P. arcularius   472 KYAVGGSFSVLIFLGAVPEIPADWRSSPHYVGAHHAFVNRTPQRCANCRRQADLIIEGFVHLNEGVVRLSGLSRLTPDVV   551
L. edodes       465 ..EI....K..F...S..SD.KE.ATD..F...F.G...SSAE........Q.VVL.........IANI.N.NSFD.I..   544
A. bisporus     472 .TDHVAEEIGF.PINQWIAEHTGLP.FAVDLVKPLLAQGLQWRVLLADGTP.E.DSLEVTI.EVPSELTDDEPNPRSRPP   551

P. arcularius   552 KPYLQENLHWRVQKVDGSAVPLEEVPSLEVAVVATKLTLPAGAPFPVPGDSVFHHDITHGRQGGSRDHVASLQVT        626
L. edodes       545 E...K..........S.EV.N.DAAT....V....R.E..P.EI....AETHH..H.....P....HS...SSS-        618
A. bisporus     552 RYHKDITHGK.GGCREA----------------------------------------------------------        568
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Fig. 3. Comparison of the deduced amino acid sequences of the *Polyporus arcularius* Tyr1 protein with those from mushrooms. Amino acid residues identical to those of the Tyr1 protein are indicated by *dots*. Conserved amino acid sequences in tyrosinase, which is the copper-binding domain signature (CuA and CuB), are *boxed*. Protein sequence of tyrosinase protein from *Lentinula edodes* and *Agaricus bisporus* appeared in the DNA database under accession numbers AB033993 and X85113, respectively

tide of 626 amino acids, contains seven introns, and has 58% amino acid identity and 71% similarity with gLeTyr of *L. edodes*, and 44% identity and 58% similarity with PPO1 of *A. bisporus* (accession numbers in DNA database are AB033993 and X85113, respectively). Two copper binding site motifs (CuA and CuB) (Jackman et al. 1992), which are conserved in tyrosinases and were in the PROSITE database (Falquet et al. 2002), were present. The positions of CuA and CuB in the Tyr1 protein of *P. arcularius* were between amino acids 87 and 102, and 280 and 290, respectively (Figs. 2, 3).

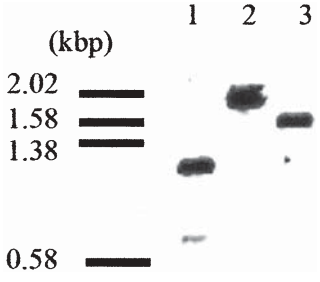

Fig. 4. Hybridization of *Eco*RV-*Hin*dIII fragments (pTyrP) of the gene for tyrosinase from *P. arcularius* to genomic DNA: 1 µg genomic DNA from *P. arcularius* was digested with restriction enzymes *Sal*I (*lane 1*); *Sac*I (*lane 2*); and *Pst*I and *Xho*I (*lane 3*). The size markers correspond to lambda DNA *Eco*RI- and *Hin*dIII digested

Copy number of tyr1 in the genome of the monokaryotic strain *P. arcularius* BS4

We performed Southern hybridization to estimate the copy number of the gene encoding tyrosinase in the genome (Fig. 4). To accomplish this, genomic DNA from the *P. arcularius* BS4 strain was singly digested with restriction enzymes such as *Sal*I and *Sac*I, and doubly digested with *Pst* I and *Xho*I; after which the fragments were separated by agarose gel electrophoresis and blotted onto nylon membranes. The blots were probed with the *Eco*RV-*Hin*dIII fragment of the lambda phage clone containing a sole *Sal*I site (Fig. 1). The hybridization signals corresponded with restriction fragments of the lambda phage clone. Fragments of chromosomal DNA about 1200 and 600 bp appeared with *Sal*I, and only a single fragment was detected by single digestion with *Sac*I and by double digestion with *Pst*I and *Xho*I. Thus, only a single copy of *tyr1*, the putative tyrosinase-encoding gene, was present in the haploid genome (Fig. 4).

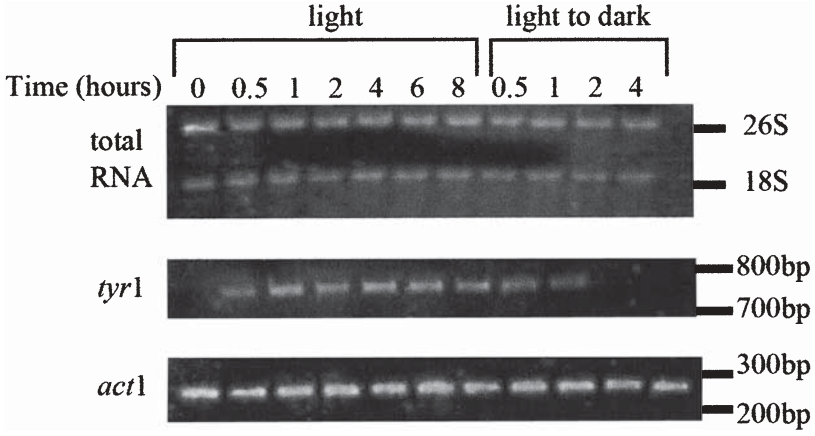

Fig. 5. Effect of visible light on transcription of *tyr1* in dikaryon strain (BS4 × I). *Light:* Dikaryon strain was grown in the dark at 25°C for 7 days and then exposed to visible light for 0, 0.5, 1, 2, 4, 6, and 8 h. *Light to dark:* Dikaryon strain was grown in the dark at 25°C for 7 days, and then after exposing to visible light for 4 h, the dikaryon strain was transferred to the dark and incubated for 0, 0.5, 1, 2, and 4 h. The *upper part* of the figure shows formaldehyde gel electrophoresis of total RNA; the *middle part* shows the RT-PCR products for *tyr1*; and the *lower part* displays the same experiment with *act1*. The size markers correspond to a 100-bp DNA ladder

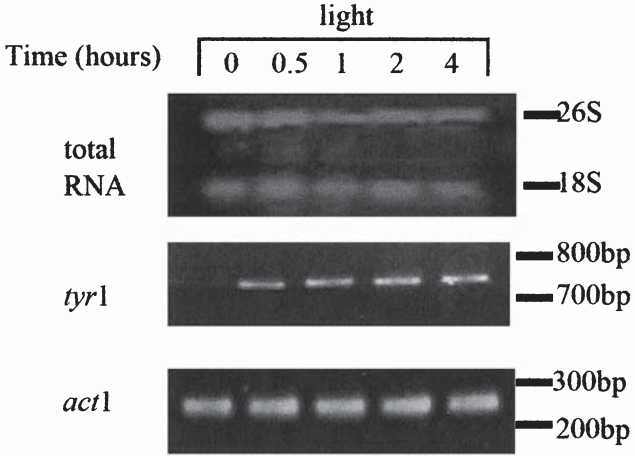

Fig. 6. Effect of visible light on transcription of *tyr1* in monokaryon strain BS4. The monokaryon strain was grown in the dark at 25°C for 7 days and then exposed to visible light for 0, 0.5, 1, 2, and 4 h. The *upper part* of the figure shows formaldehyde gel electrophoresis of total RNA; the *middle part* shows the RT-PCR products of *tyr1*; and the *lower part* displays the same experiment with *act1*. The size markers correspond to a 100-bp DNA ladder

Photoregulation of tyr1 in *P. arcularius* dikaryotic and monokaryotic strains

To analyze the expression profiles of *tyr1* in *P. arcularius*, we performed RT-PCR. We isolated total RNA of a *P. arcularius* dikaryotic strain (BS4 × I) and a monokaryotic strain (BS4) and amplified the partial cDNA fragments of *tyr1* and *act1* using primer pairs TYR-RTF21 and TYR-RTR21, and Act-Bs4F and Act-Bs4R, respectively (Figs. 5, 6). The expression profile of the *P. arcularius act1* gene, was also examined as an internal standard in the RT-PCR. Actin is one of the housekeeping genes in filamentous fungi (Schlosser et al. 2001). The RT-PCR product of *act1* was equally detectable throughout the examined period (Figs. 5, 6). On the other hand, RT-PCR products of *tyr1* in the dikaryon and monokaryon were not detected without exposing the strains to visible light and disappeared when they were transferred back to the dark. The induction of *tyr1* rapidly occurred, and transcription of *tyr1* was maintained during exposure to visible light. These results indicate that light is essential for transcription of *tyr1*. The *tyr1* was transcribed in both dikaryotic and monokaryotic mycelia after exposure to visible light, although pigmentation of mycelia occurred only in the dikaryon (Fig. 7).

Table 2. Effect of visible light on intracellular tyrosinase activity in monokaryon and dikaryon strain

Exposing time (h)	Specific activity (U/mg protein)	
	Monokaryon (BS4)	Dikaryon (BS4 × I)
0	0	0
0.5	0	0
1	0	3.42
2	0	5.11
4	0	8.83

Intracellular tyrosinase activity

Tyrosinase production of both the monokaryon and dikaryon is shown in Table 2. The time of exposure to visible light was longer, and higher enzyme activity was detected in the dikaryon. However, no enzyme activity was detected in the monokaryon.

Discussion

Southern blot analysis (see Fig. 4) indicated that *tyr1* is the sole tyrosinase gene in *P. arcularius*, which strongly suggests that the gene is not pseudosequence and has a role for pigmentation. The amino acid length of the *P. arcularius* Tyr1 polypeptide was similar to that of *L. edodes* gLeTyr; however, both were longer than that of the *A. bisporus* PPO1 polypeptide. In addition, the amino acid sequence of the carboxy terminal region of the protein from *A. bisporus* was different than that of *P. arcularius* and *L. edodes*. However, the amino-terminal region and the region around the copper-binding sites were similar. Therefore, the carboxy-terminal region of tyrosinase may not be important for its activity.

In the 5′-untranslated region near the initiation codon, a sequence that fits the consensus sequence for the Dof1

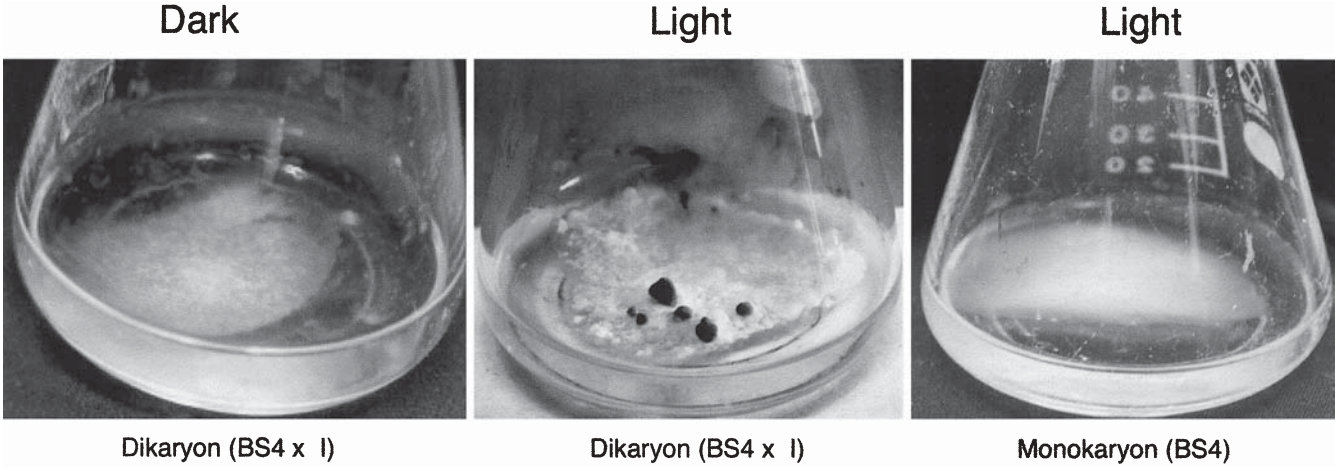

Fig. 7. Pigmentation of mycelium in *P. arcularius*. *Dark:* Dikaryon (BS4 × I) strain was grown in the dark at 25°C for 7 days, and then continued to incubate at 25°C in the dark for 7 days without exposing to visible light. *Light:* Monokaryon (BS4) and dikaryon (BS4 × I) strain were grown in the dark at 25°C for 7 days and then exposed to visible light for 7 days

binding site, which is involved in a light-regulated gene expression in maize (Yanagisawa and Sheen 1998; Yanagisawa 2002), was found. Maize Dof1 has been reported as one of the plant-specific Dof transcription factors; however, the activation domain of maize Dof1 was also found to be functional in both human cells and yeast, implying that Dof1 may stimulate transcription through a mechanism evolutionarily conserved among eukaryotes (Yanagisawa 2001). Transcription of *tyr1* in *P. arcularius* was photoinduced, and the transcription of *tyr1* was inactivated in dark-grown mycelium. These results suggest that *tyr1* might be a light-regulated gene with a Dof-like transcription factor in *P. arcularius* cells.

The copper-containing enzyme tyrosinase is essential and is a key enzyme for melanization (Sanchez-Ferrer et al. 1995; Espin et al. 1999). Brown pigmentation of mycelia in *P. arcularius* occurred only in a dikaryotic strain that was grown under visible light before development of primordia and did not occur in a monokaryon even when grown under visible light. Moreover, pigmentation of mycelia in dark-grown dikaryons did not occur (see Fig. 7). Tyrosinase activity in the dikaryon was low in dark-grown mycelia and became higher under visible light; the highest tyrosinase activity was found in stipes that developed from mycelia under visible light (Kitamoto et al. 2000). Therefore, photoinduction of pigmentation and activation of tyrosinase in *P. arcularius* seem to be a specific event in dikaryons during development of the fruiting body. Although quantity of RT-PCR products was not measured in this study, *tyr1* was transcribed in both dikaryotic and monokaryotic mycelia after exposure to visible light. Therefore, activation of tyrosinase activity for melanization may be regulated by a posttranslational event such as processing of protein. Moreover, although mRNA of *tyr1* was transcribed in mycelia under visible light, tyrosinase activity in the monokaryon was not detected and pigmentation did not occur. From this result, in the case of a dikaryon, a protease that specifically processes protyrosinase to mature tyrosinase might be active under visible light.

However, the relationship between tyrosinase activity and pigmentation is experimentally not clear. In further study, mutational analysis such as disruption of *tyr1* should be performed. In the ascomycete *Neurospora crassa* Shear et B.O. Dodge, tyrosinase is synthesized as a precursor; the molecular weight of protyrosinase exceeds that of mature tyrosinase by about 50% and the amino acid length of protyrosinase exceeds that of mature tyrosinase by 213 amino acids at its carboxy terminus (Kupper et al. 1989). This phenomenon could be applicable to the phenomenon in *P. arcularius*. Carboxy-terminal processing in enzyme activation may be a general event in filamentous fungi, because the amino acid sequence of the carboxy terminus is variable and that of the amino terminus is conserved. Moreover, the processing of the carboxy terminus of protyrosinase may be developmentally regulated in *P. arcularius*. To reveal the role of tyrosinase during fruiting-body formation, further study to identify the enzyme related to maturation of protyrosinase is needed.

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