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Cloning and expression analysis of a cDNA encoding an oxaloacetate acetylhydrolase from the brown-rot fungus *Fomitopsis palustris*

Hiromichi Hisamori¹, Tomoki Watanabe^{1,2}, Shiro Suzuki¹, Kumiko Okawa¹, Haruko Sakai³, Tsuyoshi Yoshimura⁴, Toshiaki Umezawa^{1,5} and Takefumi Hattori^{1,6,*}

¹ Laboratory of Metabolic Science of Forest Plants and Microorganisms, RISH, Kyoto University

² Present address: TAKARA BIO INC.

³ Nara Forest Research Institute

⁴ Laboratory of Innovative Humano-habitability, RISH, Kyoto University

⁵ Institute of Sustainability Science, Kyoto University

⁶ Present address: Institute of Socio-Arts and Sciences, The University of Tokushima

*Corresponding author. E-mail: thattori@ias.tokushima-u.ac.jp

ABSTRACT

The brown-rot fungus *Fomitopsis palustris* possess two oxalate-producing enzymes: oxaloacetate acetylhydrolase (*Fomitopsis palustris* oxaloacetate acetylhydrolase, FpOAH), which catalyzes hydrolysis of oxaloacetate, and cytochrome *c* dependent glyoxylate dehydrogenase (*Fomitopsis palustris* glyoxylate dehydrogenase, FpGLOXDH), which catalyzes dehydrogenation of glyoxylate. Oxaloacetate was regarded as the predominant precursor for oxalate, because greater FpOAH activity was detected than FpGLOXDH activity. In this study, a 1080-bp cDNA encoding FpOAH was cloned. Recombinant FpOAH showed oxaloacetate acetylhydrolase (OAH, EC 3.7.1.1) activity, which confirmed that the isolated cDNA encoded FpOAH. Expression of the gene encoding FpOAH was 22.0-140.8 times greater than that encoding FpGLOXDH, depending on culture times. The gene expression results support our proposed idea that FpOAH plays more significant role than FpGLOXDH in oxalate biosynthesis in *F. palustris*.

INTRODUCTION

Wood-rotting basidiomycetes cause severe damage to wooden structures. Oxalic acid produced from the wood-rotting fungi has several important roles in this degradation. For example, the acid hydrolyzes hemicelluloses, which increase the accessibility of decaying enzymes or low-molecular-weight decay agents to wood components [1]. During cellulose degradation by the Fenton reaction, a low concentration of oxalate promotes degradation [2] by increasing hydroxyl radical formation [3]; however, a higher concentration of the acid inhibits degradation [2] and radical formation [3]. Furthermore, oxalate forms Fe-oxalate complexes, which then diffuse into the wood cell wall, by which oxalate protects the hyphae of brown-rot fungi from attack by the Fenton reagent [4-6].

Therefore, to protect woody structures from the degradation caused by wood-rotting fungi, mechanisms of oxalate biosynthesis should be elucidated. We investigated oxalate biosynthesis in the brown-rot fungus *Fomitopsis palustris*, because the fungus accumulates large amounts of oxalic acid (33-78 mM) during liquid cultivation [7]. *F. palustris* has two metabolic pathways for oxalate biosynthesis. One is the hydrolysis of oxaloacetate catalyzed by oxaloacetate acetylhydrolase (*Fomitopsis palustris* oxaloacetate acetylhydrolase, FpOAH) [8]. The other is dehydrogenation of glyoxylate catalyzed by cytochrome *c* dependent glyoxylate dehydrogenase (*Fomitopsis palustris* glyoxylate dehydrogenase, FpGLOXDH) [9].

Oxaloacetate is regarded as the major precursor for oxalate, because greater FpOAH activity has been detected than FpGLOXDH activity [7]. However, oxalate production in *F. palustris* has not been investigated in terms of the expressions of genes involved in oxalate biosynthesis. Tang et al. [10] identified a putative gene encoding oxaloacetate acetylhydrolase (OAH, EC 3.7.1.1) in the genome of the brown-rot basidiomycetous fungus *Fibroporia radiculosa* (*Antrodia radiculosa*); however, the gene

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product has not been shown to be an OAH with activity. Thus, a gene encoding OAH (*OAH*) has not been identified from basidiomycetes, although several *OAHs* have been identified from ascomycetes, including *Aspergillus niger* [11], *Botrytis cinerea* [12] and *Penicillium chrysogenum* [13].

The present study reports the isolation of a cDNA encoding FpOAH and a comparison of the transcript abundances of the FpOAH and FpGLOXDH genes. The results suggest that FpOAH has a greater role in oxalate biosynthesis in the brown-rot fungus *F. palustris*.

MATERIALS AND METHOD

Cloning of a cDNA encoding FpOAH

Partial cDNA fragments were amplified by PCR, which was performed in a 20- μ l reaction mixture containing 0.43 U Blend Taq polymerase (TOYOBO), 5 mM each of primers F1 and R1 (Fig. 1), and 10 ng of a cDNA library prepared previously using mRNA from *F. palustris* [14]. Primers F1 and R1 were designated using the nucleotide sequence of *oahA* (encoding oxaloacetate acetylhydrolase) from *A. niger* (AJ567910) and fgenes1_pg.C_scaffold_1200340 encoding a putative isocitrate lyase / phosphorylmutase (JGI protein ID 7156) from *Phanerochaete chrysosporium*. The PCR cycle protocol was as follows: 94°C for 2 min; 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and 72°C for 3 min. To determine both ends of the cDNA sequence, 5'- and 3'-rapid amplification of cDNA ends (RACE) was conducted with the primers shown in Fig. 1 (for 5'-RACE, F2 and Gene specific Primer R2; for 3'-RACE, R3 and Gene specific Primer F3) using the Gene Racer kit (Invitrogen), according to the manufacturer's instructions. The amplified cDNA fragments were subcloned into the TA cloning vector using the pCR2 TOPO TA cloning kit (Invitrogen). A clone containing an insert of the expected size was sequenced. The open reading frame (ORF) of the cDNA was cloned with the PCR primers F4 and R4, containing *Nde* I and *Not* I restriction enzyme sites, respectively (Fig. 1). Using primers F4 and R4, and a cDNA library as a template [14], PCR was performed in a 50- μ l reaction mixture containing 2.5 U Blend Taq polymerase, 10 μ M each primer and 10 ng the template. The PCR cycle protocol was as follows: 94°C for 2 min; 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and 72°C for 10 min.

Preparation and Purification of recombinant FpOAH

The ORF of the cDNA was subcloned into pET-23a vector (Novagen) at *Nde* I and *Not* I sites. The plasmid, hereafter named FpOAH-pET-23a, was transformed into *Escherichia coli* BL21 (DE3) competent cells (Novagen). The transformed cells were grown at 37°C with shaking at 250 rpm in LB broth

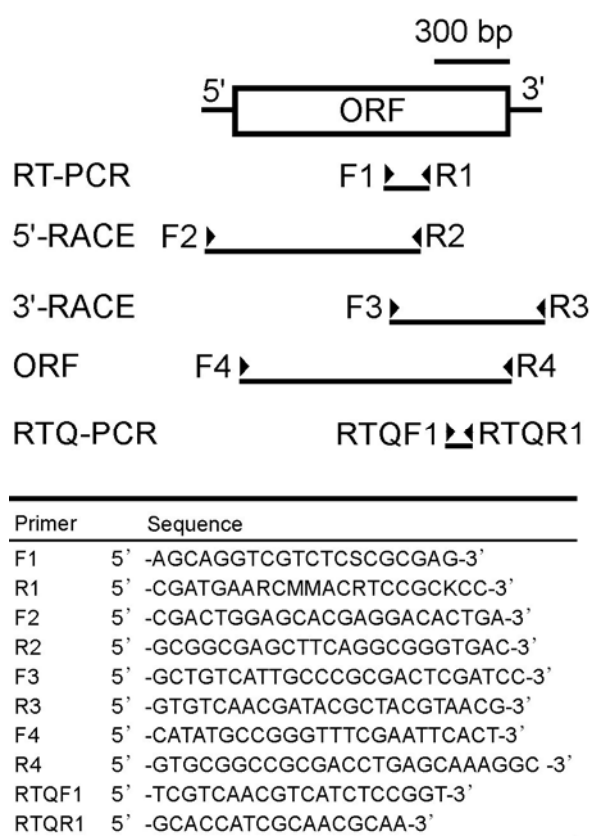


Fig. 1 Locations and nucleotide sequences of PCR primers used for cloning the cDNA encoding the oxaloacetate acetylhydrolase of *Fomitopsis palustris* (*Fomitopsis palustris* oxaloacetate acetylhydrolase, FpOAH). Arrowheads represent the primers, and underlines between them indicate the cDNA fragments amplified with these primers.

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containing 50 µg/ml ampicillin. When the OD₆₀₀ of the culture reached 0.4-0.6, isopropyl β-D-thiogalactopyranoside was added to the culture at 0.4 mM to induce recombinant protein expression. The culture was further incubated for 12.5 h at 20°C at 250 rpm. The cells were harvested by centrifugation (2000 ×g) for 10 min at 4°C. The cell pellet was suspended in lysis buffer [2 mM Tris-HCl (pH 7.5), 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride]. The suspension was sonicated at amplitude 20 for 5 seconds with a Sonicator S-4000 (Misonix), and centrifuged at 4°C for 10 min at 10000 ×g. The supernatant was loaded onto a His-Bind resin (Novagen) column equilibrated with a binding buffer, according to the manufacturer's protocol. The column was washed with 6 ml of binding buffer and 8 ml of wash buffer, and then eluted with 5 ml of elute buffer. The fraction containing the target recombinant protein was passed through a Sephadex G-25 column. The desalted recombinant protein was assayed immediately.

Enzyme assay for recombinant FpOAH

FpOAH activity of the recombinant protein was determined by the method of Akamatsu et al. [15], with a slight modification. Briefly, the enzyme reaction mixture (1.0 ml), containing 40 mM imidazole buffer (pH 7.6), 1.0 mM oxaloacetate, 1.0 mM manganese chloride, and 100 µl crude enzyme solution, was incubated at 30°C for 2 h. Oxalate was then extracted from the reaction mixture with ethyl acetate. To quantify oxalate, electron-impact mode GC-MS was performed on a Shimadzu GC-MS QP-2010, column: CBP1-M25-025, 25m×0.22mm, column temperature: 80-240°C (8°C /min), carrier gas: He, linear velocity: 50 cm/sec. Selected ion monitoring was carried out for quantification of oxalate at *m/z* 261.10.

Analysis of the FpOAH cDNA

A BLASTp search was carried out on the JGI website (<http://genome.jgi-psf.org/>) using the protein sequence deduced from the cDNA as the query. A neighbor-joining tree was generated using the CLC sequence viewer program. Identity and similarity were calculated using BioEdit, in which similarity matrix is BLOSUM62.

F. palustris growth conditions

The culture was initiated and grown as previously reported, with a slight modification [7]. Briefly, mycelia on a slant or plate culture maintained at 4°C on potato dextrose agar (PDA) medium were inoculated onto a PDA plate using an inoculating loop. Fungal inocula were cut from a colony grown on the agar plate with a cork borer (6 mm in diameter). Five plugs of mycelia were grown in 200-ml Erlenmeyer flasks with 40 ml of liquid medium containing 0.8 % (w/v) peptone, 0.05 % (w/v) KH₂PO₄, 0.05 % K₂HPO₄, 0.03 % MgSO₄·7H₂O, 5 ppm thiamine HCl and 2% (w/v) glucose.

Real-time quantitative PCR analysis of FpOAH and FpGLOXDH transcription

Total RNA was isolated at different time points from *F. palustris* mycelia using RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's protocol. After DNase treatment with RQ1 RNase-Free DNase (Promega), total RNA (0.3 µg) was applied to first-strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed using a 7300 Real Time system (Applied Biosystems). Power SYBR Green PCR Master Mix (Applied Biosystems) was used to detect amplicons. Quantification of the amplicons was based on standard curves prepared using pET-23a(+) plasmids harboring *FpOAH* and *FpGLOXDH* [16]. The gene specific primers, RTQF1 and RTQR1 (Fig. 1), were used to generate a 111bp amplicon for FpOAH transcripts. The gene specific primers, RTQF2 (5'-TGGTTCAAGAGCATCACGAAGAT-3') and RTQR2 (5'-CGAGAGAACGATTCCCTGCA-3'), were used to generate a 105bp amplicon for *FpGLOXDH* transcripts. The amount of transcripts was normalized by comparison with those of a 75-bp-amplicon derived from 28S rRNA (GenBank Accession number. AY 515333) using sense primer 5'-TGACACGGACTACCAGTGCTTT-3' and antisense primer 5'-CACCCATTTTGAGCTGCATTC-3' [17].

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RESULTS AND DISCUSSION

Characterization of the FpOAH cDNA

We isolated a cDNA (1080 bp, GenBank accession no. AB690578) encoding a deduced 38212-Da protein. To determine whether the cDNA encodes FpOAH, the OAH activity of the recombinant protein was assayed. GC-MS analyses showed that a significant amount of oxalate (9.25 $\mu\text{mol}/\text{mg}$ recombinant protein) was produced during a 2-h reaction. By contrast, no oxalate was detected from the control cultures (Table 1). The results clearly showed that the isolated cDNA encodes FpOAH.

Table 1. Quantification of oxalate produced in enzymatic reaction.

Assay system	Oxalate produced ($\mu\text{mol}/\text{mg}$ recombinant enzyme $\cdot$ 2h)
Complete	9.25
Control 1 (denatured enzyme) * ¹	0
Control 2 (without substrate)	0
Control 3 (without enzyme)	0

*¹ The denatured enzyme was prepared by heating the sample at 100°C for 30 min.

Blastp analysis showed that the deduced FpOAH belonged to the isocitrate lyase/PEP mutase enzyme superfamily. Joosten et al. [17] proposed that the active site serine in isocitrate lyase/PEP mutase enzyme superfamily is conserved. As in the case of other enzymes of this family, the deduced FpOAH was found to have an active site serine (data not shown).

We characterized the deduced FpOAH in comparison with deduced OAHs from other microorganisms. OAHs have been cloned from ascomycetes: *oahA* (accession number AJ567910), *OAHA* (accession number AY590264), and PC22g28430 (accession number XM_002566325) from *A. niger* [11], *B. cinerea* [12] and *P. chrysogenum* [13], respectively. The deduced FpOAH shared 40%, 39%, and 39% identities and 55%, 54%, and 55% similarities with *oahA*, *OAHA*, and PC22g28430, respectively.

To determine whether homologous proteins of FpOAH are encoded in the genomes of other wood-rotting fungi that have been reported to produce oxalate [19-25], a BLASTp search was conducted against the genomes of these fungi listed in the JGI website. Deduced homologous proteins with the lowest e-values for each wood-rotting fungus were chosen. It is important to note that there had been no reports showing OAH activities of these proteins and they were not annotated as OAHs. All e-values of chosen proteins were 0.0. A neighbor-joining tree for these homologous deduced proteins from the wood-rotting fungi and *oahA*, *OAHA* and PC22g28430 is shown in Fig. 2. The results show that FpOAH and estExt_fgenes1_pg.C_230033 from *Fomitopsis pinicola* are the most closely related proteins, which is not surprising as they both belong to the same genus. The deduced proteins from white- and brown-rot fungi listed in Fig. 2 were not strictly separated into different clades by their decay type, i.e. brown- or white-rot. Further discussion of the phylogenetic relationships of these FpOAH homologs should await determination of their OAH activities.

Changes in expressions of genes encoding oxalate-biosynthesis enzymes of F. palustris during cultivation

We compared the expressions of the two genes, *FpOAH* and *FpGLOXDH*. The amounts of *FpOAH* transcript were 22.0 times greater on day 4 (minimal magnitude) and 140.8 times greater on day 9 (maximal magnitude) compared with those of *FpGLOXDH* (Table 2). Munir et al. [7] strongly suggested that FpOAH is the major enzyme responsible for the production of oxalate, based on the greater activity of FpOAH compared with that of FpGLOXDH. The greater expression of *FpOAH* compared with FpGLOXDH supports this proposed idea [7].

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With regard to changes in the amounts of *FpOAH* transcripts during cultivation, significant amounts of *FpOAH* transcripts were detected on day 2 (37.2 copy number/pg total RNA). The amount of the transcript decreased gradually to 19.1 copy number/pg total RNA until day 6. This change was likely to be consistent with changes in the specific activity for *FpOAH* during a similar cultivation period [7]. However, the greatest amount of transcript, 103.7 copy number/pg total RNA, was observed on day 9, which was not consistent with the changes in specific activity of *FpOAH* [7]. Further research is needed to elucidate reasons underlying this contradiction.

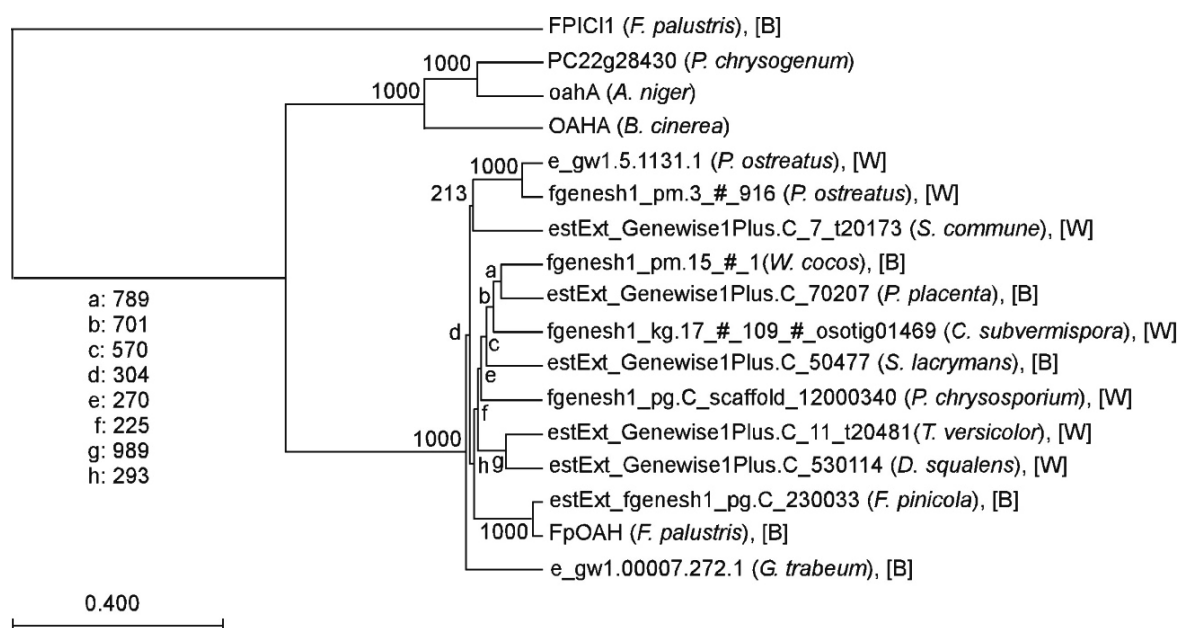


Fig. 2. Phylogenetic tree of *FpOAH* with identified OAHs from *A. niger*, *B. cinerea* and *P. chrysogenum*, and putative *FpOAH* homologs from wood-rotting fungi.

Numbers at the branch nodes are bootstrap values (per 1000 trials). The scale bar represents 0.400 substitutions per amino acid position. Deduced proteins from brown- and white-rot fungi are marked with [B] and [W], respectively. PC22g28430, an oxaloacetate acetylhydrolase of *P. chrysogenum* (NCBI accession no. XM_002566325); oahA, an oxaloacetate acetylhydrolase of *A. niger* (NCBI accession no. AJ567910); OAHA, an oxaloacetate acetylhydrolase of *B. cinerea* (NCBI accession no. AY590264); e_gw1.5.131.1, an *FpOAH* homolog from *Pleurotus ostreatus* v2.0 (JGI protein ID 29565; e_gw1.5.131.1); fgenes1_pm.3_#_916, an *FpOAH* homolog from *P. ostreatus* v1.0 (JGI protein ID 95096; fgenes1_pm.3_#_916); estExt_Genewise1Plus.C_7_t20173, an *FpOAH* homolog from *Schizophyllum commune* (JGI protein ID 105238; estExt_Genewise1Plus.C_7_t20173); fgenes1_pm.15_#_1, an *FpOAH* homolog from *Wolfiporia cocos* (JGI protein ID 92053; fgenes1_pm.15_#_1); estExt_Genewise1Plus.C_70207, an *FpOAH* homolog from *Postia placenta* (JGI protein ID 112832; estExt_Genewise1Plus.C_70207); fgenes1_kg.17_#_109_#_isotig01469, an *FpOAH* homolog from *Ceriporiopsis subvermispora* (JGI protein ID 87490; fgenes1_kg.17_#_109_#_isotig01469); estExt_Genewise1Plus.C_50477, an *FpOAH* homolog from *Serpula lacrymans* (JGI protein ID 105238; estExt_Genewise1Plus.C_50477); fgenes1_pg.C_scaffold_12000340, an *FpOAH* homolog from *Phanerochaete chrysosporium* (JGI protein ID 7156; fgenes1_pg.C_scaffold_12000340); estExt_Genewise1Plus.C_11_t20481, an *FpOAH* homolog from *Trametes versicolor* (JGI protein ID

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173629; estExt_Genewise1Plus.C_11_t20481); estExt_Genewise1Plus.C_530114, an FpOAH homolog from *Dichomitus squalens* (JGI protein ID 111644; estExt_Genewise1Plus.C_530114); estExt_fgenesh1_pg.C_230033, an FpOAH homolog from *F. pinicola* (JGI protein ID 155899; estExt_fgenesh1_pg.C_230033); FpOAH, an oxaloacetate acetylhydrolase of *F. palustris* (DDBJ, accession no. AB690578); e_gw1.00007.272.1, an FpOAH homolog from *Gloeophyllum trabeum* (JGI protein ID 42369; e_gw1.00007.272.1); FPICL1, one of the members of the ICL/PERM_KPHMT enzyme superfamily in *F. palustris* (DDBJ, accession no. AB079254 [27]) is shown as an outgroup.

F. palustris acquires energy for growth by oxidizing glucose, mainly to oxalate, through the tricarboxylic acid (TCA) and glyoxylate (GLOX) cycles [7, 26, 27]. The greater expression of *FpOAH* compared with *FpGLOXDH* supports the proposed idea that oxalate is biosynthesized mainly in the cytosol by FpOAH but not in the peroxisome by FpGLOXDH [7, 27]. To protect FpOAH from possible inhibition by oxalate, an oxalic acid resistance system, including FpTRP26 [17], and oxalate transport out of the cell, involving FpOAR [14], probably have important roles in maintaining carbon metabolism in *F. palustris*.

Table 1. Expressions of *FpOAH* and *FpGLOXDH* during growth.

Culture time (day)	2	4	6	9	12	15
FpOAH mRNA	37.2	28.8	19.1	103.7	26.7	48.4
(copy number/pg total RNA)	± 7.2	± 4.4	± 6.4	± 2.5	± 6.3	± 11.2
FpGLOXDH mRNA	0.7	1.3	0.3	0.7	0.4	1.6
(copy number/pg total RNA)	± 0.2	± 0.2	± 0.1	± 0.2	± 0.1	± 0.3

Values are means ± standard error, $n=5$, technical replicates=2

The insignificant expression of FpGLOXDH compared to FpOAH suggests that 1) leaking of glyoxylate from the GLOX cycle for oxalate production is not significant; and 2) inhibition of isocitrate lyase and malate synthase by oxalate [28, 29], two key enzymes GLOX cycle, might not be essential. Accordingly, the results support the proposed idea that the metabolic flow of the GLOX cycle is probably sufficient to support the TCA cycle.

By contrast, Tang et al. [10] recently suggested that the brown-rot fungi *F. radiculosa* and *P. placenta* produce oxalate mainly from glyoxylate by glyoxylate dehydrogenase (GLOXDH), based on the greater number of genes encoding GLOXDH than OAH in the two fungi. Therefore, there may be fungal species-dependent variation in oxalate biosynthesis in terms of which precursor, oxaloacetate or glyoxylate, is dominant for oxalate production in basidiomycetes. The characterization of the isolated cDNA encoding FpOAH contributes to the determination of oxalate biosynthesis in wood-rotting fungi.

Acknowledgments

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