Oxalate Efflux Transporter from the Brown Rot Fungus *Fomitopsis palustris* \(^\dagger\)†

Tomoki Watanabe, Nobukazu Shitan, \(^\ddagger\) Shiro Suzuki, Toshiaki Umezawa, Mikio Shimada, Kazufumi Yazaki, and Takefumi Hattori\(^*\)

Research Institute for Sustainable Humanosphere, Kyoto University, Uji, Kyoto 611-0011, Japan, and Institute of Sustainable Science, Kyoto University, Uji, Kyoto 611-0011, Japan

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An oxalate-fermenting brown rot fungus, *Fomitopsis palustris*, secretes large amounts of oxalic acid during wood decay. Secretion of oxalic acid is indispensable for the degradation of wood cell walls, but almost nothing is known about the transport mechanism by which oxalic acid is secreted from *F. palustris* hyphal cells. We characterized the mechanism for oxalate transport using membrane vesicles of *F. palustris*. Oxalate transport in *F. palustris* was ATP dependent and was strongly inhibited by several inhibitors, such as valinomycin and \(\text{NH}_4^+\), suggesting the presence of a secondary oxalate transporter in this fungus. We then isolated a cDNA, \(\text{FpOAR} \) (Efforts *Fomitopsis palustris* oxalic acid resistance), from *F. palustris* by functional screening of yeast transformants with cDNAs grown on oxalic acid-containing plates. \(\text{FpOAR} \) is predicted to be a membrane protein that possesses six transmembrane domains but shows no similarity with known oxalate transporters. The yeast transformant possessing \(\text{FpOAR} \) (\(\text{FpOAR} \)-transformant) acquired resistance to oxalic acid and contained less oxalate than the control transformant. Biochemical analyses using membrane vesicles of the \(\text{FpOAR} \)-transformant showed that the oxalate transport property of \(\text{FpOAR} \) was consistent with that observed in membrane vesicles of *F. palustris*. The quantity of \(\text{FpOAR} \) transcripts was correlated with increasing oxalic acid accumulation in the culture medium and was induced when exogenous oxalate was added to the medium. These results strongly suggest that \(\text{FpOAR} \) plays an important role in wood decay by acting as a secondary transporter responsible for secretion of oxalate by *F. palustris*.

Oxalate acid is produced by a wide variety of members of five kingdoms (Monera, Protista, Fungi, Plantae, and Animalia) (32) and plays multiple roles as a proton and electron source and strong metal chelator in ecosystem processes (11). With regard to wood decay by brown rot fungi, which cause severe damage to wooden structures, oxalic acid is secreted by the fungus in large amounts (8, 38). Several important roles of oxalic acid in the brown rot decay process are proposed. Oxalic acid hydrolyzes the side chains of hemicelluloses and amorphous cellulose, thus increasing the porosity of the wood structure to the hyphal sheath, decay enzymes, or other low-molecular-weight decay agents (12). In cellulose degradation by the Fenton reaction, a low concentration of oxalate promotes degradation (41) by facilitating hydroxyl radical formation (45), but a higher concentration of the acid inhibits the degradation (41) and radical formation (45). Furthermore, oxalate forms Fe-oxalate complexes, which can then diffuse into the wood cell wall for the Fenton reaction by which oxalate protects the hyphae of brown rot fungi from attack by the Fenton reagent (2, 16, 38). Therefore, from the viewpoint of the preservation of wooden buildings and cultural treasures, elucidation of the biochemical mechanisms for oxalate biosynthesis and its secretion in brown rot fungi is needed.

Recently, we clarified the mechanisms for oxalate biosynthesis in the brown rot basidiomycete *Fomitopsis palustris*, which is used as a standard fungus for Japanese Industrial Standards decay resistance tests. *F. palustris* secretes large amounts of oxalate (33 to 78 mM) into the culture fluid, which lowers the medium pH to ca. 2. The terminal enzymes for oxalate biosynthesis are cytosolic oxaloacetate acetylhydrolase (OAAH; EC 3.7.1.1) (1) and peroxisomal cytochrome \(c\)-dependent glyoxylate dehydrogenase (15, 43). Our biochemical analysis of oxalate fermentation by *F. palustris* led to the conclusion that the fungus acquires energy for growth by oxidizing glucose mainly to oxalate through the tricarboxylic acid (TCA) and glyoxylate (GLOX) cycles (28, 29, 35).

Importantly, it is widely recognized that oxalate is toxic to organisms. Exogenously added oxalate inhibits sporulation and growth of the filamentous fungi *Fusarium oxysporum* (7) and *Pythium vexans* (51). Therefore, oxalate-producing brown rot fungi must have a mechanism to prevent damage caused by intra- and extracellular oxalate. Several brown rot fungi show not only oxalate-producing but also oxalate-degrading activity. Among these oxalate-producing and -degrading brown rot fungi, *Postia placenta* produces oxalate decarboxylase to convert oxalate to formate and \(\text{CO}_2\) (25), which prevents overaccumulation of oxalic acid and forms a nontoxic, buffered, low-pH environment that facilitates the brown rot decay pro-
cess (26). Gloephyllym trabeum degrades extracellularly added oxalate to give rise to CO₂ (10). Similarly, Fomitopsis pinicola and Meruliporia incrassata have been reported to decrease the amount of extracellular oxalate (36).

In contrast to these oxalate-producing and -degrading brown rot fungi, oxalate-decomposing activity has not been reported for F. palustris. However, F. palustris grows vigorously in the presence of a high oxalate concentration (28, 29). The marked accumulation of oxalic acid in the culture fluid of F. palustris suggests that the fungus has an efficient system to transport oxalate out of the cells. Oxalate is continuously biosynthesized as a major end product of primary metabolism in the cytosol and peroxisome of F. palustris (15, 28, 35). Thus, while transporting oxalate from the peroxisome to the cytosol and eventually out of the cells, essential metabolic processes should be protected from oxalate toxicity. In this context, the oxalate transporter in the cytosolic membrane is expected to reduce the intracellular concentration of oxalate, which probably contributes to the oxalate resistance system in F. palustris. However, almost nothing is known about the transport systems of F. palustris.

Oxalate transporters are known to play several important roles in metabolizing or excreting oxalate by other organisms. For example, Oxalobacter formigenes, a Gram-negative anaerobe, possesses the oxalate:formate exchange protein OxtE, which is essential for O. formigenes to produce ATP (21). Humans and mice possess the SLC26 multifunctional anion exchangers, of which SLC26A6 (humans) and Slc26a6 (mice) are proposed to exchange Cl⁻ and SO₄²⁻ for oxalate or formate in the intestinal villi, renal proximal tubule, and cardiac myocytes (27). Furthermore, hepatopancreatic lysosomal membrane vesicles isolated from the lobster Homarus americanus exchange oxalate for Cl⁻ in relation to Zn²⁺ detoxification (40).

Recently, Mch5, a homolog of a putative oxalate:formate antiporter from Aspergillus fumigatus, was postulated to be a putative oxalate transporter in the yeast Saccharomyces cerevisiae. However, the oxalate-transporting activity of Mch5 and the possible roles of the gene product have not been demonstrated (5). Accordingly, an oxalate-transporting protein has not previously been characterized experimentally from any fungus.

Here we describe a cDNA encoding the protein involved in oxalate transport in F. palustris. To isolate a cDNA encoding the oxalate transporter, we previously isolated fungal cDNAs from yeast transformants with cDNA of F. palustris grown in the presence of a high oxalate concentration that is lethal to wild-type cells (48). Even if oxalate was incorporated into the cells, the transformants probably transported oxalate out of the cells or it was degraded by the gene products of the cDNA from F. palustris. Accordingly, these cDNAs were expected to encode transporters catalyzing oxalate transport out of the cells or oxalate-decomposing enzymes and other proteins possessing unidentified functions. By this strategy, we successfully isolated the cDNA FpTIP26 conferring oxalic acid resistance for F. palustris (48). We have further characterized the remaining transformants showing oxalic acid resistance (48) and obtained the gene product, FpOAR (Fomitopsis palustris oxalic acid resistance), which is a putative plasma membrane protein that showed distinct oxalate transport activity in yeast mem-

brane vesicles. The oxalate transport property of FpOAR was similar to that of P. italicus membrane vesicles. These results strongly suggest that FpOAR is involved in oxalate secretion in F. palustris hyphal cells.

MATERIALS AND METHODS

F. palustris culture conditions. Two mycelial plugs (5 mm in diameter) of F. palustris (Berkely et Curtis) Murill TY6p137 were grown as a stationary culture in a 200-ml Erlenmeyer flask containing 40 ml of liquid medium as previously reported (48).

Cloning of F. palustris cDNA conferring oxalic acid resistance. Functional screening of a Saccharomyces cerevisiae AD12345678 (6) transformant with an F. palustris cDNA library with vector pDR196 (50) was carried out to screen the transformants to grow with 12 mM oxalic acid (48). Because the cDNA fragment recovered from oxalic acid-resistant yeast showing a strong phenotype lacked the 5' region, the 5' end of the cDNA was determined with the Gene Racer kit (Invitrogen) according to the manufacturer's instructions. The 5' end of the cDNA was cloned with the gene-specific antisense PCR primer 5'-CCACGCACCAAGGCGAATGAGA-3' (sense) and 5'-GGATCTTCGTAGAGAAGAATCTTCTTGGC-3' (antisense), which were gene-specific primers containing SpeI and the BamHI restriction site, respectively. The coding region thus obtained was named FpOAR (Fomitopsis palustris oxalic acid resistance).

Characterization of oxalic acid resistance activity of FpOAR. The plasmid containing FpOAR was reintroduced into the S. cerevisiae AD12345678 strain to characterize its oxalic acid resistance according to our method reported previously (48). Oxalic acid resistance was determined by growth of the yeast transformant. Similarly, the transformants were cultivated separately on SD (~Ura) plates containing different HCl concentrations (~Ura) plates containing different HCl concentrations (pH 1.5, 1.6, and 2.2).

Quantification of oxalic acid. Oxalic acid in the F. palustris culture medium was quantified with a commercial kit after the pH of the medium was adjusted for the assay (Roche) (47), whereas gas chromatography–mass spectrometry (GC-MS) analysis (14) was conducted for that in yeast cells. Yeast (optical density at 600 nm [OD₆₀₀] of 0.1) was cultured at 37°C in 30 ml of liquid medium containing 2 mM oxalic acid until OD₆₀₀ of 1.0 to 2.0. The cells were harvested by centrifugation at 1,000 × g for 10 min and washed twice with cold distilled water. The dry weight of cells was determined after freeze-drying for 5 h. To the dried cells, 250 µl of 1 N HCl and 600 µl of ethyl acetate were added, and the cells were homogenized with glass beads (Toshiba, no. 04) for 4 min. The oxalic acid extracted with ethyl acetate was quantified as previously described (14).

GC-MS (EI) was performed on a Shimadzu GC-MS QP-5050A. The column conditions were as follows: CBP1-M25-025, 25 m by 0.22 mm (inner diameter; Shimadzu); column temperature, 80 to 240°C (8°C/min); carrier gas, He, and carrier gas flow rate, 0.8 ml/min.

Quantitative real-time PCR analysis of gene transcription. Total RNA was isolated from F. palustris mycelia with the RNaseasy plant mini-kit (Qiagen). First-strand cDNA synthesis was performed with Superscript II reverse transcriptase (Invitrogen) using 0.2 µg of RNA. Real-time quantitative PCR was performed with a 7300 real-time system (Applied Biosystems), and amplicons were detected with SYBR green (Applied Biosystems). Quantifications of the amplicons were based on standard curves prepared for each target cDNA. The gene-specific primers 5'-CCTCGAACAAACGCGAGACTTCTT-3' and 5'-AGTGCTCCGCC GAGAA-3' were used to generate an 85-bp amplicon for FpOAR transcripts. The amount of transcripts was normalized by comparison with those of a 75-bp amplicon derived from either total RNA or 28S rRNA (GenBank accession no. AY515333) with the primers 5'-TGACCGGCTACACCTGCTTCTT-3' and 5'-CACCATTTTGAGCTTGACATC-3' (antisense). FpOAR transcripts were quantified in mycelia from cultures to which either oxalate (50 mM final concentration) or H₂O was added on day 3. The cultures were incubated for 12 h prior to RNA extraction.

Preparation of F. palustris membrane vesicles. The membrane vesicles were prepared from F. palustris hyphal cells as described previously (33). Cells were collected and homogenized in ice-cold homogenizing buffer (0.1 M Tris-Cl pH 8.0, 10% [vol/vol] glycerol, 5 mM EDTA; 2.5 ml/g fresh weight of mycelia) using a Phoxin blender (Oster). Prior to use, KCl, dithiorthioleth (DTT), and phenyl- methylsulfonyl fluoride (PMSF) were added to the autoclaved buffer at the final concentrations 150, 3.3, and 1 mM, respectively, and the mixture was stirred.
for 1 min. Unbroken cells and debris were removed by centrifugation twice at 8,000 × g for 10 min. The supernatants were pooled and centrifuged at 100,000 × g for 60 min. The microsome fraction was resuspended with a 1 M Tris-Cl buffer (pH 7.6), 10% (vol/vol) glycerol, 1 mM EDTA. Isolated membrane vesicles were stored at −80°C in resuspension buffer containing 1 mM DTT and 1 mM PMSF until use.

Yeast membrane vesicles for in vitro transport studies were isolated as described previously (17, 44) with the following modifications. The S. cerevisiae ADJ12345678 strain was grown overnight in SD (−Ura) liquid medium to an OD₆₀₀ of 1 to 2. Cells were washed twice with water and resuspended to an OD₆₀₀ of 1 to 2 in a spheroplast buffer (1.1 M sorbitol, 20 mM Tris-HCl [pH 7.6], 1 mM DTT containing 57 U of Zymolyase 20T/ml). After cell wall digestion was completed, spheroplasts were collected by centrifugation at 1,200 × g for 10 min. Cell lysis was performed gently on ice in 25 ml of breaking buffer (1.1 M glycerol, 50 mM Tris-acetate [pH 7.4], 5 mM EDTA, 1 mM DTT, 1.5% polyvinylpyrrolidone, 2 mg of bovine serum albumin [BSA]/ml, 1 mM PMSF, 10 µg of leupeptin/ml, 2 µg of aproigin/ml, and 2 µg of pepstatin/ml) with a Dounce homogenizer and 40 strokes with a tight-fitting glass piston. Unbroken cells and debris were removed by centrifugation twice at 3,000 × g for 10 min. The supernatants were pooled and centrifuged at 100,000 × g for 60 min. Microsomal membrane proteins were quantified by the Bradford method (3).

The pellet was suspended to a protein concentration of about 5 mg/ml in vesicle buffer (1.1 M glycerol, 50 mM Tris-MES [pH 7.4], 1 mM EDTA, 1 mM DTT, 2 mg of BSA/ml, 1 mM PMSF, 1 µg of leupeptin/ml, 2 µg of aprogin/ml, and 2 µg of pepstatin/ml). Small aliquots were immediately used for transport assays or stored at −80°C until use. Membrane vesicles prepared with this method are a mixture of inside-out and right-side-out orientation, whereas only inside-out vesicles can hydrolyze ATP owing to the outside orientation of the ABC proteins which drive membrane transport and because ATP cannot permeate the membrane. Therefore, in this experiment the observation of oxalate uptake into the inside-out vesicle indicates oxalate efflux activity of the hyphal cell membrane.

In vitro oxalate transport assay. Vesicles (100 µg of protein) were mixed with transport buffer (0.4 M glycerol, 100 mM KCl, 20 mM Tris-MES [pH 7.4], 1 mM DTT) and incubated with 0.2 mM [14C]oxalic acid at 25°C for 10 min in the absence or presence of 5 mM MgATP with a final volume of 125 µl, unless stated otherwise. In the assay using F. palustris vesicles, the vesicles (500 µg of protein) were suspended in a final volume of 500 µl. Uptake of [14C]oxalic acid (185 MBq/ml) into membrane vesicles was measured by two methods. For F. palustris membrane vesicles, a 130-µl reaction mixture was loaded on a Sephadex G-50 fine spin column and centrifuged at 2,000 rpm for 2 min. The radioactivity of 100 µl of filtrate was determined with a liquid scintillation analyzer (Perkin-Elmer).

Effects of inhibitors on oxalate transport. Using F. palustris vesicles, the following inhibitors were added separately to assay solutions: 1 mM vanadate, 2 µM valinomycin, 1 mM NH₄Cl, 150 µM glibenclamide, or 5 µM cyclosporine. Oxalate transport was recorded as described above.

To assess the inhibition using yeast vesicles from the FpOAR-transformant, we used 5 µM verapamil and 5 µM gramicidin D in addition to the reagents used for the F. palustris vesicle assay. Oxalate transport was recorded as described above.

Statistical analysis. Analyses were carried out by using analysis of variance, followed by the Dunnett test with a level of significance of P = 0.05.

RESULTS

Biochemical analysis of oxalate transport using F. palustris membrane vesicles. To characterize oxalate transport of F. palustris, we prepared membrane vesicles from hyphae of the fungus and investigated whether MgATP is required for the transport activity. In the presence of MgATP, [14C]oxalate uptake into the vesicles was 4.27 times that in the control lacking MgATP (Fig. 1). This result indicated that MgATP is needed for oxalate transport activity in F. palustris.

We then investigated the effects of a variety of inhibitors on oxalate transport. Vanadate, which is an inhibitor of P-type ATPases and ATP-binding cassette (ABC) transporters, reduced oxalate uptake into the membrane vesicle by 68.0%. The addition of valinomycin or NH₄Cl, which abolish the Δψ and ΔpH across the membrane, respectively, inhibited oxalate uptake by 86.3 and 90.1%, respectively. In contrast, glibenclamide and cyclosporine, which are typical inhibitors of ABC transporters, did not influence oxalate transport. These results indicate that ABC transporters are not primarily responsible for oxalate transport. Collectively, the results strongly suggest that a secondary oxalate transporter functions in F. palustris, in which Δψ and ΔpH are involved.

Cloning of a cDNA conferring oxalic acid resistance on the yeast transformant. Previously, we isolated the cDNA FpTRP26 from one of eight S. cerevisiae transformants (48). From the remaining transformants, we isolated one cDNA (1,170 bp), named FpOAR (GenBank accession no. AB372882), which was found to encode a deduced 42,873-Da protein. A BLASTp search revealed that the deduced FpOAR showed 86, 82, 73, 66, and 43% identities with predicted membrane proteins of Postia placenta (24) and Phanerochaete chrysosporium (23), a major intrinsic protein of Laccaria bicolor (22), a hypothetical protein CCG1_G0636 of Coprinopsis cinerea Okayama 7#130 (4), and a transmembrane protein of Cryptococcus neoformans var. neoformans JEC21 (19), respectively (Fig. 2). These are all basidiomycete fungi and, in particular, P. placenta and P. chrysosporium are brown rot and white rot fungi, respectively. However, no biochemical functions have been elucidated for these proteins to date. A close similarity between FpOAR and oxalate-degrading enzymes, such as oxalate decarboxylase (EC 4.1.1.2) and oxalate oxidase (EC 1.2.3.4), was not observed. However, the SOSU1 program (http://bp.nuap.nago.oy-u.ac.jp/sosui/) predicted that FpOAR possesses six transmembrane domains (see Fig. S1 in the supplemental material).

Oxalic acid resistance in yeast transformants with the cDNA FpOAR. On plates containing 8.5 to 10 mM oxalic acid, the yeast transformant possessing the cDNA FpOAR (FpOAR-transformant) showed clear cell growth, whereas no growth...
was observed in the control transformant with an empty vector (Fig. 3A and B). At an oxalic acid concentration below 8.5 mM, the growth of the control and FpOAR-transformant did not differ significantly (data not shown). In medium with the same pH containing malonate but not oxalate, no difference between the control and FpOAR-transformant was observed, showing that oxalate is more toxic than malonate (Fig. 3C). To eliminate the possibility that the FpOAR-transformant was resistant to low pH (1.5 to 2.2), we investigated the growth of the FpOAR-transformant on a low-pH plate (Fig. 3D), but no difference in growth between the control and FpOAR-transformant was observed at pH 1.5 to 2.2 and pH 5. We then compared the oxalic acid contents of the FpOAR-transformant and the empty vector control grown in the presence of 2 mM oxalic acid. No difference in cell growth was observed between the two cultures at this concentration, but the cellular content of oxalate in the FpOAR-transformant strongly decreased to 25% compared to that of the control (Fig. 4).
Oxalate transport in vesicles of the FpOAR-transformant. In the presence of MgATP, membrane vesicles of the FpOAR-transformant significantly accumulated [14C]oxalate, which was 1.4 times greater than that of the control lacking MgATP. When the assay was conducted at 4°C or with vesicles denatured at 95°C, oxalate transport did not differ from that of the empty vector control (Fig. 5).

Vanadate and gramicidin D, which are inhibitors of P-type ATPases and a monovalent-selective ionophore that dissipates both the pH gradient and membrane potential, inhibited oxalate transport by 28.5 and 55.8%, respectively (Fig. 6). Valinomycin and NH₄Cl, which abolish ΔΨ and ΔpH across the membrane, respectively, inhibited oxalate transport by 34.6 and 34.9%, respectively. In contrast, the inhibitors of the ABC transporters, glybenclamide and verapamil, slightly inhibited and did not significantly inhibit, respectively, oxalate transport in FpOAR-transformant vesicles. These results are in agreement with the oxalate-transporting activity exhibited by membrane vesicles of F. palustris (Fig. 1) and suggest that FpOAR is directly involved in oxalate secretion.

Oxalate transport in vesicles of the FpOAR-transformant. In the presence of MgATP, membrane vesicles of the FpOAR-transformant significantly accumulated [14C]oxalate, which was 1.4 times greater than that of the control lacking MgATP. When the assay was conducted at 4°C or with vesicles denatured at 95°C, oxalate transport did not differ from that of the empty vector control (Fig. 5).

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Quantitative real-time PCR analysis of FpOAR transcripts in F. palustris. The quantity of FpOAR transcripts increased concomitantly with accumulation of oxalate in the culture medium (Fig. 7). High levels were maintained even in the stationary phase during days 9 to 13, whereas oxalic acid accumulation increased (day 13, 34 mM). The amount of FpOAR transcripts increased 3-fold compared to that of the control when 50 mM oxalic acid was added to the medium (Fig. 8).

DISCUSSION

FpOAR functions as a secondary oxalate transporter conferring oxalic acid resistance in F. palustris. In the present study, we characterized oxalate transport using membrane vesicles of F. palustris. We isolated a cDNA, FpOAR, encoding a
novel membrane oxalate transporter protein specifically conserved among basidiomycete fungi.

The isolated cDNA FpOAR conferred resistance to oxalic acid on the FpOAR-transformant (Fig. 3). The oxalate transport activity in the membrane vesicles of the yeast (Fig. 5) indicated that FpOAR was responsible for oxalate transport. The effects of inhibitors on oxalate transport in membrane vesicles of the FpOAR-transformant (Fig. 6) suggest that FpOAR transports oxalate by a secondary transport system, in which ΔpH and ΔΨ are the driving force. The effects of the inhibitors on yeast vesicles (Fig. 6) were similar to those on vesicles from F. palustris (Fig. 1). Furthermore, expression of FpOAR and oxalic acid accumulation in the medium were positively correlated (Fig. 7). Expression of FpOAR was induced by addition of oxalic acid to the culture (Fig. 8). Collectively, these results strongly suggest the involvement of FpOAR as a secondary oxalate transporter to confer oxalic acid resistance in F. palustris.

**FpOAR is a plasma membrane-localized novel secondary oxalate transporter.** FpOAR is proposed to be a novel oxalate secondary transporter based on three lines of evidence: (i) MgATP was needed for oxalate transport activity; (ii) FpOAR does not possess an ATP-hydrolyzing domain (Walker motifs and ABC signature) as in ABC transporters, which function as primary transporters (46); and (iii) there is no similarity between FpOAR and known oxalate transporters, e.g., SLC26 family proteins (27), the oxalate:formate antiport protein, OxlT (21), and the putative yeast monocarboxylate transporter, Mch5 (5).

FpOAR is suggested to be a plasma membrane oxalate efflux transporter based on the lower oxalate content in the FpOAR-transformant than that in the control (Fig. 4). The probable cytosolic membrane localization of FpOAR is in agreement with the presence of six putative transmembrane domains in FpOAR (see Fig. S1 in the supplemental material). Furthermore, the PSORT program (http://psort.hgc.jp/) predicted that FpOAR is localized to the plasma membrane. The fungal plasma membrane H^-ATPase generates a proton gradient through the cytosolic membrane (37), which is likely to drive oxalate transport through FpOAR.

**FpOAR probably plays a crucial role to maintain carbon metabolism by F. palustris.** During vegetative growth, F. palustris produces oxalate from a glucose carbon source with an 80% theoretical yield based on the amount of carbon in glucose (28). Two intrinsic features of F. palustris metabolism facilitate such a high yield of oxalate. First, the precursors of oxalate, glyoxylate and oxaloacetate, are constantly supplied through the GLOX cycle in the peroxisome, in which isocitrate lyase (FPICL1; EC 4.1.3.1) and malate synthase (EC 2.3.3.9) play indispensable roles as key enzymes (28, 29, 35). The FPICL1 and malate synthase of F. palustris show high activity even in glucose-grown mycelia (28, 29), which is in sharp contrast to the general GLOX cycle in other microorganisms, in which the functioning of the GLOX cycle is repressed by a glucose carbon source (35). Second, it is proposed that the GLOX cycle of F. palustris supplies succinate constitutively to the TCA cycle lacking 2-oxoglutarate dehydrogenase activity (28, 29, 35), by which oxaloacetate can be supplied for cytosolic oxalate production through the TCA cycle, as well as the GLOX cycle (28, 29, 35).

F. palustris continuously secretes oxalate as the fungus grows. This oxalate secretion is mainly due to continuous production of oxalate in primary carbon metabolism. Furthermore, the acid secretion could be due to the absence of oxalate degradation activity in F. palustris, in contrast to oxalate-decomposing white rot fungi (9, 39, 47, 49) and several brown rot fungi such as P. placenta (25) and G. trabeum (10). Moreover, we have suggested that F. palustris transports oxalate out of the cells to prevent possible inhibition of intracellular metabolic reactions. For example, oxalate might inhibit the activities of FPICL1 and malate synthase in peroxisomes of F. palustris (15, 35), because oxalate competitively inhibits the activities of the two enzymes in vitro (30, 31). In addition, oxalate produced from oxaloacetate by cytosolic OAAH in F. palustris (1, 35) possibly shows product inhibition for OAAH activity because oxalate is a competitive inhibitor (K_i = 19 μM) for OAAH from Botrytis cinerea (13). Therefore, the oxalate production coupled with energy metabolism and the efficient oxalate transport out of the cells are essential to maintain carbon metabolism in F. palustris. Aided by the oxalate export system, including FpOAR, and the oxalic acid resistance system, including FpTRP26 (48), carbon metabolism through the TCA and GLOX cycles are probably prevented from inhibition by oxalate in vivo. FpTRP26 was predicted to be a soluble protein and might help with oxalate export from the hyphal cells (48). The yeast transformant with FpTRP26 showed similar resistance to oxalic acid with regard to the acid concentration and pH (48). Further research is needed to elucidate how FpTRP26 and FpOAR cooperatively work in the oxalate resistance system. Whether the FpOAR homologous protein functions in oxalate-producing and -degrading brown rot fungi requires investigation. Furthermore, in the same fungi, a possible role of oxalate degradation in relation to energy metabolism should be evaluated to clarify the possible role of oxalate metabolism in brown rot fungi. In this context, a possible ATP generation by oxalate degradation is hypothesized for P. placenta (20) based on gene expression (24) as proposed for the oxalate-degrading white rot fungus C. subvermispora in which oxalate degradation by oxalate decarboxylase with formate dehydrogenase could produce NADH to be utilized for ATP synthesis (47).

FpOAR homologous protein is a potential target for development of new wood preservatives. The FpOAR homologous
protein may be distributed widely in wood-rotting fungi, because the homologs showing significant identities with FpOAR were found in genomes of the brown rot and white rot fungi Phanerochaete chrysosporium, respectively (Fig. 2). Oxalate production and secretion is commonly observed in wood-rotting fungi, although brown rot fungi accumulate greater amounts of oxalate in the culture fluid than do white rot fungi (8, 38). Therefore, the FpOAR homologous protein may generally function as an oxalate transporter in wood-rotting fungi. From the viewpoint of wood preservation, if the function of FpOAR is inhibited by wood preservatives, oxalate in wood-protein may be distributed widely in wood-rotting fungi, because a certain amount of oxalate is important to stabilize Mn3+ oxidation for lignin degradation by manganese peroxidase (18). Accordingly, the FpOAR homologous protein would be a possible target protein to develop a new wood preservative to inactivate oxalate transport for extermination of wood-rotting fungi.

In summary, we have isolated a cDNA, FpOAR, encoding a novel oxalate transporter from F. palustris. The deduced FpOAR protein is suggested to play an important role in wood decay by acting as a secondary transporter responsible for secretion of oxalate from F. palustris hyphal cells. Two strategies for prevention of oxalate toxicity have been found for brown rot fungi: (i) oxalate efflux through FpOAR and (ii), in addition to oxalate efflux, oxalate degradation in oxalate-producing and -degrading brown rot fungi. Further characterization of oxalate transport by FpOAR is needed to elucidate the underlying mechanisms. Oxalate transport from the peroxisome to the cytosol remains to be investigated.

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