Title: An oxalate efflux transporter from the brown-rot fungus *Fomitopsis palustris*

Running title: Oxalate transporter from *Fomitopsis palustris*

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Abstract

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 NH_4^+, suggesting the presence of a secondary oxalate transporter in this fungus. We then isolated a cDNA, *Fomitopsis palustris* Oxalic Acid Resistance (*FpOAR*), from *F. palustris* by functional screening of yeast transformants with cDNAs grown on oxalic acid-containing plates. 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 *FpOAR* (*FpOAR*-transformant) acquired resistance to oxalic acid and contained less oxalate than the control transformant. Biochemical analyses using membrane vesicles of the *FpOAR*-transformant showed that the oxalate transport property of FpOAR was consistent with that observed in membrane vesicles of *F. palustris*. The quantity of *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 FpOAR plays an important role in wood decay by acting as a secondary transporter responsible for secretion of oxalate by *F. palustris*.

Oxalic 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 side-chains of hemicelluloses, then depolymerizes the hemicellulose backbones 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 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. *Fomitopsis palustris* secretes large amounts of oxalate (33–78 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* (46). Therefore, oxalate-producing brown-rot fungi must have a mechanism to prevent damage caused by intra- and extra-cellular 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 CO_2 (25), which prevents overaccumulation of oxalic acid and forms a non-toxic, buffered, low-pH environment that facilitates the brown-rot decay process (26). *Gloephyllum trabeum* degrades extracellularly added oxalate to give rise to CO_2 (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 OxlT, 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 SO_4^{2-} 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 Zn^{2+} detoxification (40).

Recently, Mch5, a homolog of a putative oxalate:formate antiporter from *Aspergillus fumigatus*, was postulated to be a putative oxalate transporter in 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 (49). 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 *FpTRP26* conferring oxalic acid resistance for *F. palustris* (49). We have further characterized the remaining transformants showing oxalic acid resistance (49) 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 membrane vesicles. The oxalate transport property of FpOAR was similar to that of *F. palustris* membrane vesicles. These results strongly suggest that FpOAR is involved in oxalate secretion in *F. palustris* hyphal cells.

MATERIALS AND METHODS

***Fomitopsis palustris* culture conditions.** Two mycelial plugs (5 mm in diameter) of *F. palustris* (Berkely et Curtis) Murill TYP6137 were grown as a stationary culture in a 200 ml Erlenmeyer flask containing 40 ml liquid medium as previously reported (49).

Cloning of *F. palustris* cDNA conferring oxalic acid resistance. Functional screening of a *Saccharomyces cerevisiae* AD12345678 (6) transformant with a *F. palustris* cDNA library with vector pDR196 (51) was carried out to screen the transformants to grow with

12 mM oxalic acid (49). 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'-CCACGACCACCGCCGCAAGCATGAAGA-3'. The amplified cDNA fragments were subcloned into the TA cloning vector with 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 5'-ACTAGTATGACCGACCTGCATCGAAG-3' (sense) and 5'-GGATCCTCAGAGAAGATCTTCTTGCCG-3' (antisense), which were gene-specific primers containing *Spe* I and the *Bam*HI 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 (49). 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 (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, Germany) (48), while GC-MS analysis (14) was conducted for that in yeast cells. Yeast (OD_{600} 0.1) was cultured at 30°C in 50 ml SD (–Ura) liquid medium containing 2

150 mM oxalic acid until OD_{600} 1.0–2.0. The cells were harvested by centrifugation at 1000×*g*
151 for 10 min and washed twice with cold distilled water. The dry weight of cells was
152 determined after freeze-drying for 5 h. To the dried cells, 250 µl 1*N* HCl and 600 µl
153 ethylacetate were added and cells were homogenized with glass beads (Toshinriko, No. 04)
154 for 4 min. The oxalic acid extracted with ethylacetate was quantified as previously
155 described (14).

156 GC-MS (EI) was performed on a Shimadzu GC-MS QP-5050A. The column conditions
157 were as follows: CBP1-M25-025, 25 m × 0.22 mm (i.d.) (Shimadzu), column temperature
158 80–240°C (8°C/min), carrier gas He, and carrier gas flow rate 0.8 ml/min.

159 **Quantitative real-time PCR analysis of gene transcription.** Total RNA was isolated
160 from *F. palustris* mycelia with the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA
161 synthesis was performed with Superscript II reverse transcriptase (Invitrogen) using 0.2 µg
162 RNA. Real-time quantitative PCR was performed with a 7300 Real Time System (Applied
163 Biosystems) and amplicons were detected with SYBR Green (Applied Biosystems).
164 Quantifications of the amplicons were based on standard curves prepared for each target
165 cDNA. The gene-specific primers 5‘-CCTCGAACAAGCGAATTCTCTTT-3’ and
166 5’-AGTGTCCCGCCGAGGAA-3’ were used to generate an 85 bp amplicon for *FpOAR*
167 transcripts. The amount of transcripts was normalized by comparison with those of a 75 bp
168 amplicon derived from either total RNA or 28S rRNA (GenBank accession no. AY515333)
169 with the primers 5'-TGACACGGACTACCAGTGCTTT-3' (sense) and
170 5'-CACCCATTTTGAGCTGCATTC-3' (antisense).

171 *FpOAR* transcripts were quantified in mycelia from cultures to which either oxalate (50
172 mM final concentration) or H_2O was added on day 3. The cultures were incubated for 12 h
173 prior to RNA extraction.

174 **Preparation of *F. palustris* membrane vesicles.** The membrane vesicles were prepared
175 from *F. palustris* hyphal cells as described previously (33). Cells were collected and
176 homogenized in ice-cold homogenizing buffer (0.1 M Tris-HCl, pH 8.0, 10% [v/v] glycerol,
177 5 mM EDTA; 2 ml/g fresh weight of mycelia) using a Phoenix blender (USA). Prior to use,
178 KCl, DTT, and PMSF were added to the autoclaved buffer at the final concentrations 150
179 mM, 3.3 mM and 1 mM, respectively, and the mixture was stirred for 1 min. Unbroken
180 cells and debris were removed by centrifugation twice at 8,000×*g* for 10 min. The
181 supernatants were pooled and centrifuged at 100,000×*g* for 60 min. The microsome fraction
182 was resuspended with autoclaved resuspension buffer (10 mM Tris-HCl, pH 7.6, 10% [v/v]
183 glycerol, 1 mM EDTA). Isolated membrane vesicles were stored at –80°C in resuspension
184 buffer containing 1 mM DTT and 1 mM PMSF until use.

185 Yeast membrane vesicles for *in vitro* transport studies were isolated as described
186 previously (17, 44) with the following modifications. The *S. cerevisiae* AD12345678 strain
187 was grown overnight in SD (–Ura) liquid medium to OD_{600} 1–2. Cells were washed twice
188 with water and resuspended to OD_{600} 1–2 in spheroplast buffer (1.1 M sorbitol, 20 mM
189 Tris-HCl [pH 7.6], 1 mM DTT containing 57 units/ml Zymolyase 20T). After cell wall
190 digestion was completed, spheroplasts were collected by centrifugation at 1200×*g* for 10
191 min. Cell lysis was performed gently on ice in 25 ml breaking buffer (1.1 M glycerol, 50
192 mM Tris-ascorbate [pH 7.4], 5 mM EDTA, 1 mM DTT, 1.5% polyvinylpyrrolidone, 2

193 mg/ml BSA, 1 mM PMSF, 10 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin)
194 with a Dounce homogenizer and 40 strokes with a tight-fitting glass piston. Unbroken cells
195 and debris were removed by centrifugation twice at 3,000×*g* for 10 min. The supernatants
196 were pooled and centrifuged at 100,000×*g* for 60 min. Microsomal membrane proteins
197 were quantified by Bradford's method (3). The pellet was suspended to a protein
198 concentration of about 5 mg/ml in vesicle buffer (1.1 M glycerol, 50 mM Tris-MES [pH
199 7.4], 1 mM EDTA, 1 mM DTT, 2 mg/ml BSA, 1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml
200 aprotinin, and 2 μg/ml pepstatin). Small aliquots were immediately used for transport
201 assays or stored at –80°C until use. Membrane vesicles prepared with this method are a
202 mixture of inside-out and right-side-out orientation, whereas only inside-out vesicles can
203 hydrolyze ATP owing to the outside orientation of the ATPase catalytic sites that drive
204 membrane transport and because ATP cannot permeate the membrane. Therefore, in this
205 experiment the observation of oxalate uptake into the inside-out vesicle indicates oxalate
206 efflux activity of the hyphal cell membrane.

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

radioactivity of 100 µL filtrate was determined with a liquid scintillation counter. The yeast vesicles were absorbed onto nitrocellulose filters (3 cm in diameter, 0.45 µm pore size; GE Healthcare) by filtration with an aspirator. The vesicles on the membrane were washed three times with 1 ml transport buffer by filtration with an aspirator, dried on filter paper and subjected to liquid scintillation counting. Counts were corrected for background and quenching. Quantification and calculations were performed using a Liquid Scintillation Analyzer Tri-Carb 2800TR (Perkin Elmer, USA).

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 NH_4Cl, 150 µM glibenclamide, or 5 µM cyclosporin A. 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 using ANOVA 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, [^{14}C]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 NH_4Cl, which abolish the $\Delta\Psi$ and ΔpH across the membrane, respectively, inhibited oxalate uptake by 86.3% and 90.1%, respectively. In contrast, glybencleamide and cyclosporin A, 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 $\Delta\Psi$ 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 (49). From the remaining transformants, we isolated one cDNA (1170 bp), named *FpOAR* (GenBank accession no. AB372882), which was found to encode a deduced 42,873 Da protein. A BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) 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 CC1G_06363 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

259 fungi and, in particular, *P. placenta* and *P. chrysosporium* are brown- and white-rot fungi,
260 respectively. However, no biochemical functions have been elucidated for these proteins to
261 date. A close similarity between FpOAR and oxalate-degrading enzymes, such as oxalate
262 decarboxylase (EC 4.1.1.2) and oxalate oxidase (EC 1.2.3.4), was not observed. However,
263 the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/) predicted that FpOAR possesses
264 six transmembrane domains (Supplemental Fig. S1).

265 **Oxalic acid resistance in yeast transformants with the cDNA FpOAR.** On plates
266 containing 8.5–10 mM oxalic acid, the yeast transformant possessing the cDNA *FpOAR*
267 (*FpOAR*-transformant) showed clear cell growth, whereas no growth was observed in the
268 control transformant with an empty vector (Figs. 3*A*, *B*). At an oxalic acid concentration
269 below 8.5 mM, the growth of the control and *FpOAR*-transformant did not differ
270 significantly (data not shown). In medium with the same pH containing malonate but not
271 oxalate, no difference between the control and *FpOAR*-transformant was observed, showing
272 that oxalate is more toxic than malonate (Fig. 3*C*). To eliminate the possibility that the
273 *FpOAR*-transformant was resistant to low pH (2.2–1.5), we investigated the growth of the
274 *FpOAR*-transformant on a low-pH plate (Fig. 3*D*), but no difference in growth between the
275 control and *FpOAR*-transformant was observed at pH 2.2-1.5 and pH 5. We then compared
276 the oxalic acid contents of the *FpOAR*-transformant and the empty vector control grown in
277 the presence of 2 mM oxalic acid. No difference in cell growth was observed between the
278 two cultures at this concentration, but the cellular content of oxalate in the
279 *FpOAR*-transformant strongly decreased to 25% compared with that of the control (Fig. 4).

280 **Oxalate transport in vesicles of the *FpOAR*-transformant.** In the presence of

281 MgATP, membrane vesicles of the *FpOAR*-transformant significantly accumulated
282 [^{14}C]oxalate, which was 1.4 times greater than that of the control lacking MgATP. When
283 the assay was conducted at 4 °C or with vesicles denatured at 95 °C, oxalate transport did
284 not differ from that of the empty vector control (Fig. 5).

285 Vanadate and gramicidin D, which are inhibitors of P-type ATPases and a
286 monovalent-selective ionophore that dissipates both the pH gradient and membrane
287 potential, inhibited oxalate transport by 28.5% and 55.8%, respectively (Fig. 6).
288 Valinomycin and NH_4Cl, which abolish $\Delta\Psi$ and ΔpH across the membrane, respectively,
289 inhibited oxalate transport by 34.6% and 34.9%, respectively. In contrast, the inhibitors of
290 ABC transporters, glybencleamide and verapamil, slightly inhibited and did not
291 significantly inhibit, respectively, oxalate transport in *FpOAR*-transformant vesicles. These
292 results are in agreement with the oxalate-transporting activity exhibited by membrane
293 vesicles of *F. palustris* (Fig. 1), and suggest that FpOAR is directly involved in oxalate
294 secretion.

295 **Quantitative real-time PCR analysis of *FpOAR* transcripts in *F. palustris*.** The
296 quantity of *FpOAR* transcripts increased concomitantly with accumulation of oxalate in the
297 culture medium (Figs. 7*A*, *B* and *C*). High levels were maintained even in the stationary
298 phase during days 9–13, while oxalic acid accumulation increased (day 13, 34 mM). The
299 amount of *FpOAR* transcripts increased three-fold compared with that of the control when
300 50 mM oxalic acid was added to the medium (Fig. 8).

301

302 **DISCUSSION**

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

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

319 **FpOAR is a plasma membrane-localized novel secondary oxalate transporter.**
320 FpOAR is proposed to be a novel oxalate secondary transporter based on three lines of
321 evidence: 1) MgATP was needed for oxalate transport activity; 2) FpOAR does not possess
322 an ATP-hydrolyzing domain (Walker motifs and ABC signature) as in ABC transporters,
323 which function as primary transporters (47); and 3) there is no similarity between FpOAR
324 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 compared with the control (Fig. 4). The probable cytosolic membrane localization of FpOAR is in agreement with the presence of six putative transmembrane domains in FpOAR (Supplemental Fig. S1). 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).

347 *Fomitopsis palustris* continuously secretes oxalate as the fungus grows. This oxalate
348 secretion is mainly due to continuous production of oxalate in primary carbon metabolism.
349 Furthermore, the acid secretion could be due to the absence of oxalate degradation activity
350 in *F. palustris*, in contrast to oxalate-decomposing white-rot fungi (9, 39, 48, 50) and
351 several brown-rot fungi such as *P. placenta* (25) and *G. trabeum* (10). Moreover, we have
352 suggested that *F. palustris* transports oxalate out of the cells to prevent possible inhibition
353 of intracellular metabolic reactions. For example, oxalate might inhibit the activities of
354 FPICL1 and malate synthase in peroxisomes of *F. palustris* (15, 35), because oxalate
355 competitively inhibits the activities of the two enzymes *in vitro* (30, 31). In addition,
356 oxalate produced from oxaloacetate by cytosolic OAAH in *F. palustris* (1, 35) possibly
357 shows product inhibition for OAAH activity, because oxalate is a competitive inhibitor (Ki
358 = 19 μM) for OAAH from *Botrytis cinerea* (13). Therefore, the oxalate production coupled
359 with energy metabolism and the efficient oxalate transport out of the cells are essential to
360 maintain carbon metabolism in *F. palustris*. Aided by the oxalate export system, including
361 FpOAR, and the oxalic acid resistance system, including FpTRP26 (49), carbon
362 metabolism through the TCA and GLOX cycles are probably prevented from inhibition by
363 oxalate *in vivo*. FpTRP26 was predicted to be a soluble protein and might help with oxalate
364 export from the hyphal cells (49). The yeast transformant with FpTRP26 showed similar
365 resistance to oxalic acid with regard to the acid concentration and pH (49). Further research
366 is needed to elucidate how FpTRP26 and FpOAR cooperatively work in the oxalate
367 resistance system. Whether the FpOAR homologous protein functions in oxalate-producing
368 and -degrading brown-rot fungi requires investigation. Furthermore, in the same fungi, a

369 possible role of oxalate degradation in relation to energy metabolism should be evaluated to
370 clarify the possible role of oxalate metabolism in brown-rot fungi. In this context, a
371 possible ATP-generation by oxalate degradation is hypothesized for *P. placenta* (20) based
372 on gene expression (24) as proposed for white-rot oxalate-degrading fungus *C.*
373 *subvermispora* in which oxalate degradation by oxalate decarboxylase with formate
374 dehydrogenase could produce NADH to be utilized for ATP synthesis (48).

375 **FpOAR homologous protein is a potential target for development of new wood**
376 **preservatives**. The FpOAR homologous protein may be distributed widely in wood-rotting
377 fungi, because the homologs showing significant identities with FpOAR were found in
378 genomes of the brown- and white-rot fungi *P. placenta* and *P. chrysosporium*, respectively
379 (Fig. 2). Oxalate production and secretion is commonly observed in wood-rotting fungi,
380 although brown-rot fungi accumulate greater amounts of oxalate in the culture fluid than do
381 white-rot fungi (8, 38). Therefore, the FpOAR homologous protein may generally function
382 as an oxalate transporter in wood-rotting fungi. From the viewpoint of wood preservation,
383 if the function of FpOAR is inhibited by wood preservatives, oxalate is expected to be
384 accumulated intracellularly in brown-rot fungi and growth would be seriously damaged. By
385 a similar inhibitory mechanism, oxalate excretion by white-rot fungi would decrease, which
386 may repress lignin degradation, because a certain amount of excreted oxalate is important
387 to stabilize Mn^{3+} oxidation for lignin degradation by manganese peroxidase (18).
388 Accordingly, the FpOAR homologous protein would be a possible target protein to develop
389 a new wood preservative to inactivate oxalate transport for extermination of wood-rotting
390 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: 1) oxalate efflux through FpOAR; and 2) 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.

REFERENCES

1. **Akamatsu, Y., A. Ohta, M. Takahashi, and M. Shimada.** 1991. Enzymatic formation of oxalate from oxaloacetate with cell-free-extracts of the brown-rot fungus *Tyromyces palustris* in relation to the biodegradation of cellulose. Mokuzai Gakkaishi **37**:575-577.
2. **Arantes, V., Y. Qian, A. M. F. Milagres, J. Jellison, and B. Goodell.** 2009. Effect of pH and oxalic acid on the reduction of Fe^{3+} by a biomimetic chelator and on Fe^{3+} desorption/adsorption onto wood: Implications for brown-rot decay. International Biodeterioration & Biodegradation **63**:478-483.
3. **Bradford, M. M.** 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. **72**:248-254.
4. **Broad Institute of MIT and Harvard**. 2003. *Coprinus cinereus* sequencing project (http://www.broad.mit.edu.).
5. **Cheng, V., H. U. Stotz, K. Hippchen, and A. T. Bakalinsky.** 2007. Genome-wide screen for oxalate-sensitive mutants of *Saccharomyces cerevisia*e. Appl. Environ. Microbiol. **73**: 5919-5927.
6. **Decottignies, A., A. M. Grant, J. W. Nichols, H. de Wet, D. B. Mcintosh, and A. Goffeau.** 1998. ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J. Biol. Chem. **273**:12612-12622.
7. **Duchesne, L.C., B. E. Ellis, and R. L. Peterson.** 1989. Disease suppression by the ectomycorrhizal fungus *Paxillus involutus* –contribution of oxalic acid-. Can. J. Bot. **67**: 2726-2730.
8. **Dutton, M. V., and C. S. Evans.** 1996. Oxalate production by fungi: Its role in

pathogenicity and ecology in the soil environment. Can. J. Microbiol. **42**:881-895.

9. **Escutia, M. R., I. Bowater, A. Edwards, A. R. Bottrill, M. R. Burrnull, R. Polanco, R. Vicuña, and S. Bornemann.** 2005. Cloning and Sequencing of two *Ceriporiopsis subvermispora* bicupin oxalate oxidase allelic isoforms: Implications for the reaction specificity of oxalate oxidases and decarboxylases. Appl. Environ. Microbiol. **71**:3608-3616.

10. **Espejo, E., and E. Agosin.** 1991. Production and degradation of oxalic acid by brown-rot fungi. Appl. Environ. Microbiol. **57**: 1980-1986.

11. **Gadd, G. M.** 2007. Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. Mycol. Res. **111**:3-49.

12. **Green III, F., M. J. Larsen, J. E. Winandy, and T. L. Highley.** 1991. Role of oxalic acid in incipient brown-rot decay. Mater. Org. **26**:191-213.

13. **Han, Y., H. J. Joosten, W. Niu, Z. Zhao, P. S. Mariano, M. T. McCalman, J. A. L. van Kan, P. J. Schaap, and D. Dunaway-mariano.** 2007. Oxaloacetate hydrolase, the C-C bond lyase of oxalate secreting fungi. J. Biol. Chem. **282**:9581-9590.

14. **Hattori, T., N. Akitsu, G. S. Seo, A. Ohta, and M. Shimada.** 2000. A possible growth promoting effect of the organic acids produced in an axenic symbiotic culture of *Pinus densiflora* and *Lactarius hatsudake* on both of symbionts. Ann. Report. Interdiscipl. Res. Inst. Environ. Sci. **19**:59-65.

15. **Hattori, T., K. Okawa, M. Fujimura, M. Mizoguchi, T. Watanabe, T. Tokimatsu, H. Inui, K. Baba, S. Suzuki, T. Umezawa, and M. Shimada.** 2007

Subcellular localization of the oxalic acid-producing enzyme, cytochrome *c*-dependent glyoxylate dehydrogenase in grown-rot fungus *Fomitopsis palustris*. Cellulose Chemistry and Technol. **41**:545-553.

16. **Hyde, S.M., and P.M. Wood.** 1997. A mechanism for production of hydroxyl radicals by the brown-rot fungus *Coniophora puteana*: Fe(III) reduction by cellobiose dehydrogenase and Fe(II) oxidation at a distance from the hyphae. Microbiology **143**: 259-266.

17. **Klein, M., Y. M. Mamnun, T. Eggmann, C. Schüller, H. Wolfger, E. Martinoia, and K. Kuchler.** 2002. The ATP-binding cassette (ABC) transporter Bpt1p mediates vacuolar sequestration of glutathione conjugates in yeast. FEBS Letters **520:**63-67.

18. **Kuan, I. C., and M. Tien.** 1993. Stimulation of Mn-peroxidase activity - A possible role for oxalate in lignin biodegradation. Proc. Natl. Acad. Sci. USA **90**:1242-1246.

19. **Loftus, B.J., E. Fung, P. Roncaglia, D. Rowley, P. Amedeo, D. Bruno, J. Vamathevan, M. Miranda, I. J. Anderson, J. A. Fraser, J. E. Allen, I. E. Bosdet, M. R. Brent, R. Chiu, T. L. Doering, M. J. Donlin, C. A. D'Souza, D. S. Fox, V. Grinberg, J. Fu, M. Fukushima, B. J. Haas, J. C. Cuang, G. Janbon, S. J. M. Jones, H. L. Koo, M. I. Krzywinski, J. K. Kwon-Chung, K. B. Lengeler, R. Maiti, M.A. Marra, R. E. Marra, C. A. Mathewson, T. G. Mitchell, M. Pertea, F. R. Riggs, S. L. Salzberg, J. E. Schein, A. Shgvartsbeyn, H. Shin, M. Shumway, C. A. Specht, B. B. Suh, A. Tenney, T. R. Utterback, B. L. Wickes, J. R. Wortman, N. H. Wye, J. W. Kronstad, J. K. Lodge, J. Heitman, R. W. Davis, C. M. Fraser, R. W. Hyman.** 2005. The

genome of the basidiomycetous yeast and human pathogen *Cryptococcus neoformans*. Science **307**: 1321-1324.

20. **Mäkelä, M.R., K. Hildén, T. K. Lundell.** 2010. Oxalate decarboxylase: biotechnological update and prevalence of the enzyme in filamentous fungi. Appl. Microbiol. Biotechnol. **87**: 801-814.

21. **Malony, P.C.** 1994. Bacterial transporters. Current Opinion in Cell Biology **6**:571-582.

22. **Martin, F., A. Aerts, D. Ahréen, A. Brun, E. G. J. Danchin, F. Duchaussoy, J. Gibon, A. Kohler, E. Lindquist, V. Pereda, A. Salamov, H. J. Shapiro, J. Wuyts, D. Blaudez, M. Buée, P. Brokstein, B. Canbäck, D. Cohen, P. E. Courty, P. M. Coutinho, C. Delaruelle, J. C. Detter, A. Deveau, S. DiFazio, S. Duplessis, L. Fraissinet-Tachet, E. Lucic, P. Frey-Klett, C. Fourrey, I. Feussner, G. Gay, J. Grimwood, P. J. Hoegger, P. Jain, S. Kilaru, J. Labbé, Y. C. Lin, V. Legué, F. L. Tacon, R. Marmeisse, D. Melayah, B. Montanini, M. Muratet, U. Nehls, H. Niculita-Hirzel, M. P. Oudot-Le Secq, M. Peter, H. Quesneville, B. Rajashekar, M. Reich, N. Rouhier, J. Schmutz, T. Yin, M. Chalot, B. Henrissat, U. Kües, S. Lucas, V. de Peer, G. K. Podila, A. Polle, P. J. Pukkila, P. M. Richardson, P. Rouzé, I. R. Sanders, J. E. Stajich, A. Tunlid, G. Tuskan, I. V. Grigoriev.** 2008. The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. Nature **452**: 88-92.

23. **Martinez, D., L. F. Larrondo, N. Putnam, M. D. Gelpke, K. Huang, J. Chapman, K. G. Helfenbein, P. Ramaiya, J. C. Detter, F. Larimer, P. M. Coutinho, B. Henrissat, R. Berka, D. Cullen, D. Rokhsar.** 2004. Genome

sequence of the lignocelluloses degrading fungus *Phanerochaete chrysosporium* strain RP78. Nature Biotechnology **22**: 695-700.

24. **Martinez, D., J. Challacombe, I. Morgenstern, D. Hibbett, M. Schmoll, C. P. Kubicek, P. Ferreira, F. J. Ruiz-Duenas, A. T. Martinez, P. Kersten, K. E. Hammel, A. V. Wymelenberg, J. Gaskell, E. Lindquist, G. Sabat, S. S. BonDurant, L. F. Larrondo, P. Canessa, R. Vicuna, J. Yadav, H. Doddapaneni, V. Subramanian, A. G. Pisabarro, J. L. Lavı́, J. A. Oguiza, E. Master, B. Henrissat, P. M. Coutinho, P. Harris, J. K. Magnuson, S. E. Baker, K. Bruno, W. Kenealy, P. J. Hoegger, U. Kües, P. Ramaiya, S. Lucas, A. Salamov, H. Shapiro, H. Tu, L. C. Chee, M. Misra, G. Xie, S. Teter, D. Yaver, T. James, M. Mokrejs, M. Pospisek, I. V. Grigoriev, T. Brettin, D. Rokhsar, R. Berka, D. Cullen.** 2009. Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocelluloses conversion. Proc. Natl. Acad. Sci. USA **106**:1954-1959.

25. **Micales, J. A.** 1995. Oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta*. Mater. Org. **29**: 177-186.

26. **Micales, J. A.** 1997. Localization and induction of oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta*. International Biodeterioration & Biodegradation **39**: 125-132.

27. **Mount, D. B., and M. F. Romero.** 2004 The SLC26 gene family of multifunctional anion exchangers. Pflügers Archive-Eur. J. Physiol. **447**:710-721.

28. **Munir, E., J. J. Yoon, T. Tokimatsu, T. Hattori, and M. Shimada.** 2001 A physiological role for oxalic acid biosynthesis in the wood-rotting basidiomycete *Fomitopsis palustri*s. Proc. Natl. Acad. Sci. USA **98**:11126-11130.

29. **Munir, E., J. J. Yoon, T. Tokimatsu, T. Hattori, and M. Shimada.** 2001. New role for glyoxylate cycle enzymes in wood-rotting basidiomycetes in relation to biosynthesis of oxalic acid. J. Wood Sci. **47**:368-373.

30. **Munir, E., T. Hattori, and M. Shimada.** 2002. Purification and characterization of isocitrate lyase from the wood-destroying basidiomycete *Fomitopsis palustris* grown on glucose. Arch. Biochem. Biophys. **399**:225-231.

31. **Munir, E., T. Hattori, and M. Shimada.** 2002. Purification and characterization of malate synthase from the glucose-grown wood-rotting basidiomycete *Fomitopsis palustris*. Biosci. Biotechnol. Biochem. **66**:576-581.

32. **Nakata, P. A.** 2003. Advances in our understanding of calcium oxalate crystal formation and function in plants. Plant Sci. **164**:901-909.

33. **Otani, M., N. Shitan, K. Sakai, E. M. Martinoia, F. Sato, and K. Yazaki.** 2005. Characterization of vacuolar transport of the endogenous alkaloid berberine in *Coptis japonica*. Plant Physiol. **138**:1939-1946.

34. **Page, R. D. M.** 1996. Tree View: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12**:357-358.

35. **Sakai, S., T. Nishide, E. Munir, K. Baba, H. Inui, Y. Nakano, T. Hattori, and M. Shimada.** 2006. Subcellular localization of glyoxylate cycle key enzymes involved in oxalate biosynthesis of wood-destroying basidiomycete *Fomitopsis palustris* grown on glucose. Microbiology **152**:1857-1866.

36. **Schilling, J. S., and J. Jellison.** 2005. Oxalate regulation by two brown rot fungi decaying oxalate-amended and non-amended wood. Holzforschung **59**: 681-688.

37. **Serrano, R.** 1988. Structure and function of proton translocation ATPase in plasma membranes of plants and fungi. Biochim. Biophys. Acta **947**:1-28.

38. **Shimada, M., Y. Akamatsu, T. Tokimatsu, K. Mii, and T. Hattori.** 1997. Possible biochemical roles of oxalic acid as a low molecular weight compound involved in brown-rot and white-rot wood decays. J. Biotechnol. **53**:103-113.

39. **Shimazono, H.** 1955. Oxalic acid decarboxylase, a new enzyme from the mycelium of wood destroying fungi. J. Biochem. **42**:321-340.

40. **Sterling, K. M., P. K. Mandal, B. A. Roggenbeck, S. E. Ahearn, G. A. Gerencser, and G. A. Ahearn.** 2007. Heavy metal detoxification in crustacean epithelial lysosomes: role of anions in the compartmentalization process. The Journal of Experimental Biology **210**:3484-3493.

41. **Tanaka, N., Y. Akamatsu, T. Hattori, and M. Shimada.** 1994. Effect of oxalic acid on the oxidative break down of cellulose by the Fenton reaction. Wood Res. **81**:8-10.

42. **Thomson, J. D., T. J. Gibson, F. Plewhiak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Res. **25**:4876-4882.

43. **Tokimatsu, T., Y. Nagai, T. Hattori, and M. Shimada.** 1998. Purification and characteristics of a novel cytochrome *c* dependent glyoxylate dehydrogenase from a wood-destroying fungus *Tyromyces palustris*. FEBS Letters **437**:117-121.

44. **Tommasini, R., R. Evers, E. E. Vogt, C. Mornet, G. J. R. Zaman, A. H. Schinkel, P. Borst, and E. Martinoia.** 1996. The human multidrug resistance-associated protein functionally complements the yeast cadmium resistance factor 1. Proc. Natl. Acad. Sci. USA **93**: 6743-6748.

45. **Varela, E. and M. Tien.** 2003. Effect of pH and oxalate on hydroquinone-derived hydroxyl radical formation during brown rot wood degradation. Appl. Environ. Microbiol. **69**: 6025-6031.

46. **Yamaji, K., H. Ishimoto, N. Usui, and S. Mori.** 2005. Organic acids and water-soluble phenolics produced by *Paxillus* sp 60/92 together show antifungal activity against *Pythium vexans* under acidic culture conditions. Mycorrhiza **15**:17-23.

47. **Walker, J.E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the alpha-subunits and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1**:945-951.

48. **Watanabe, T., S. Tengku, T. Hattori, and M. Shimada.** 2005. Purification and characterization of NAD-dependent formate dehydrogenase from the white-rot fungus *Ceriporiopsis subvermispora* and a possible role of the enzyme in oxalate metabolism. Enzyme Microb. Tech. **37**:68-75.

49. **Watanabe, T., N. Shitan, T. Umezawa, K. Yazaki, M. Shimada, and T. Hattori.** 2007. Involvement of FpTRP26, a thioredoxin-related protein, in oxalic acid-resistance of the brown-rot fungus *Fomitopsis palustri*s. FEBS Letters **581**:1788-1792.

50. **Watanabe, T., T. Fujiwara, T. Umezawa, M. Shimada, and T. Hattori.** 2008. Cloning of a cDNA encoding a NAD-dependent formate dehydrogenase involved in oxalic acid metabolism from the white-rot fungus *Ceriporiopsis subvermispora* and its gene expression analysis. FEMS Microbiol. Lett. **279**:64-70.

51. **Wipf, D., M. Benjdia, E. Rikirsch, S. Zimmermann, M. Tegeder, and W. B. Frommer.** 2003. An expression cDNA library for suppression cloning in yeast mutants, complementation of a yeast his4 mutant, and EST analysis from the symbiotic basidiomycete *Hebeloma cylindrosporum*. Genome **46**:177-181.

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The abbreviations used are: ABC, ATP-binding cassette; FpOAR, Fomitopsis palustris Oxalic Acid Resistance; GLOX, glyoxylate; TCA, tricarboxylic acid

Figure legends

FIG. 1. Oxalate transport and the effect of inhibitors using membrane vesicles from *F. palustris*. Mean value ±standard deviation.

* $p < 0.05$ ($n = 3$).

FIG. 2. Neighbor-joining tree for FpOAR and its homologs. The tree was generated using ClustalX (42) and visualized with TREEVIEW (34).

AAEY01000042, a transmembrane protein of *Cryptococcus neoformans* var. neoformans JEC21 (GenBank accession no. AAEY01000042); gwh2.7.329.1, predicted membrane proteins of *Phanerochaete chrysosporium* (JGI Protein ID 29288; gwh2.7.329.1); Plus.C_740045, predicted membrane proteins of *Postia placenta* (JGI Protein ID 111900; estExt_Genewise1Plus.C_740045); FpOAR (*Fomitopsis palustris* Oxalic Acid Resistance) (GenBank accession no. AB372882); Lbscf0011g04660, a major intrinsic protein of *Laccaria bicolor* (JGI Protein ID 297309; eu2.Lbscf0011g04660); AACS01000187, a hypothetical protein CC1G_06363 of *Coprinopsis cinerea* okayama 7#130 (GenBank accession no. AACS01000187). Numbers at forks are bootstrap values derived from 1000 replicates. The scale bar represents 0.05 substitutions per amino acid position.

FIG. 3. Growth of yeast transformants containing an empty vector (control) and *FpOAR* on SD (–Ura) plates containing (A) no additives (standard), (B) oxalic acid, (C) malonic acid and (D) HCl. Concentration of each additive and pH are shown above each image. The number of yeast cells inoculated on the plate are indicated by ▶ at the top of each figure (A-D).

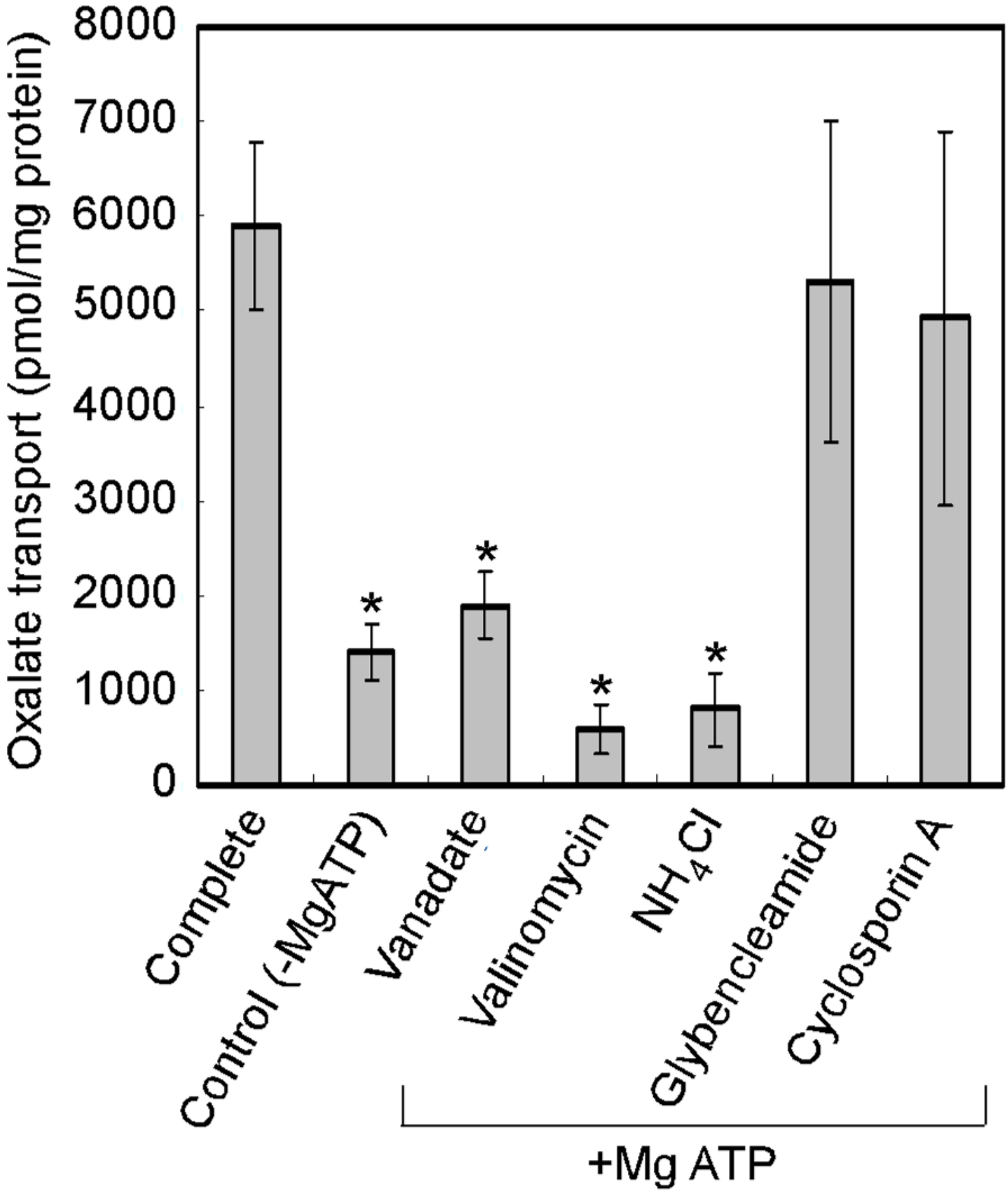
FIG. 4. Amount of oxalic acid in yeast transformants containing an empty vector (control) and *FpOAR*. Mean value ±standard deviation.** $p < 0.01$ ($n = 4$).

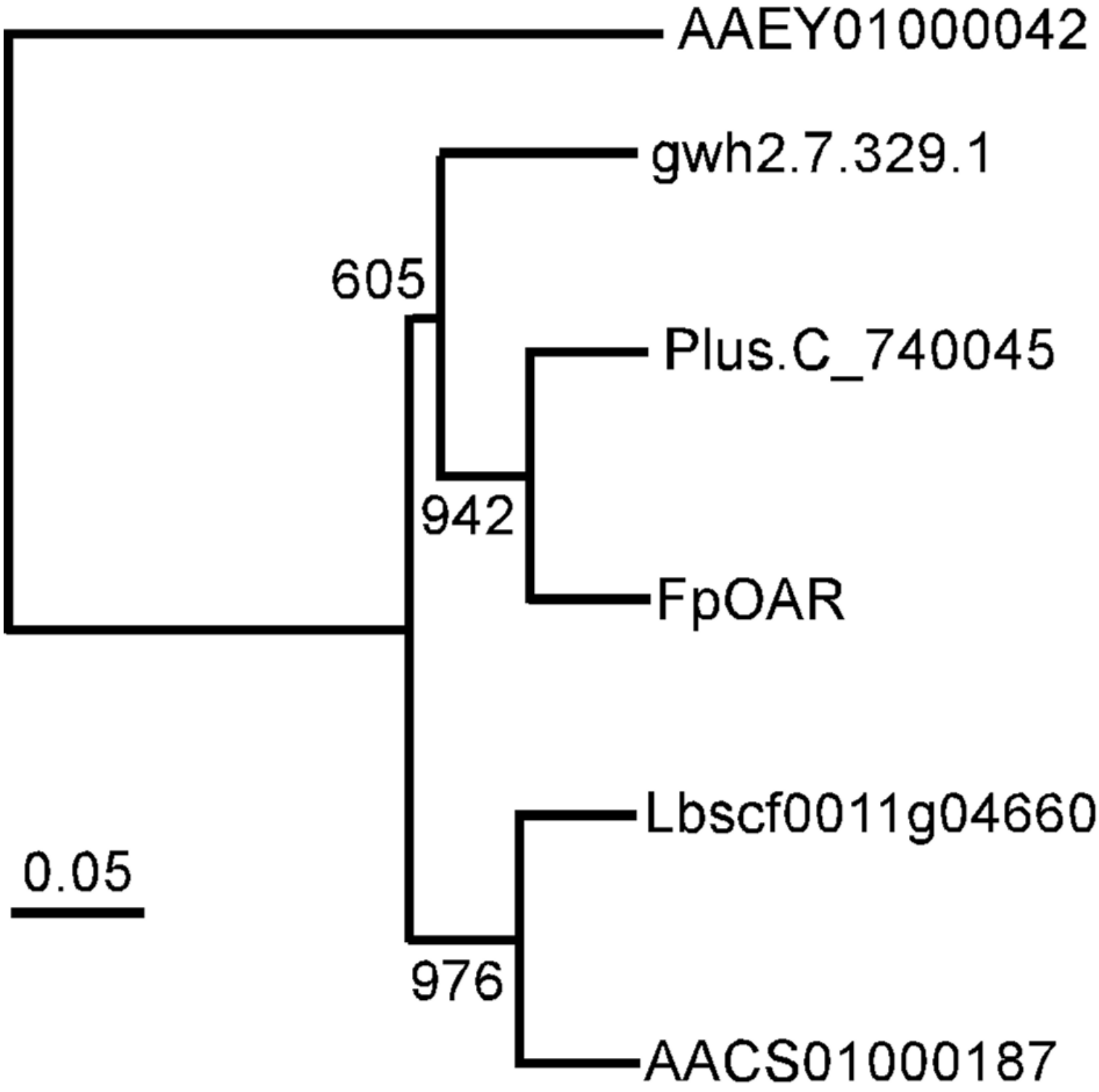
FIG. 5. Oxalate export in yeast transformants containing *FpOAR* (FpOAR-transformant) and an empty vector (control). **$p < 0.01$ ($n = 4$). The complete system, FpOAR-transformant + MgATP, showed significantly greater oxalate transport with ** than any other system. Denatured: the assay using the vesicles denatured at 95°C; 4°C: the assay conducted at 4°C. Mean value ±standard deviation.

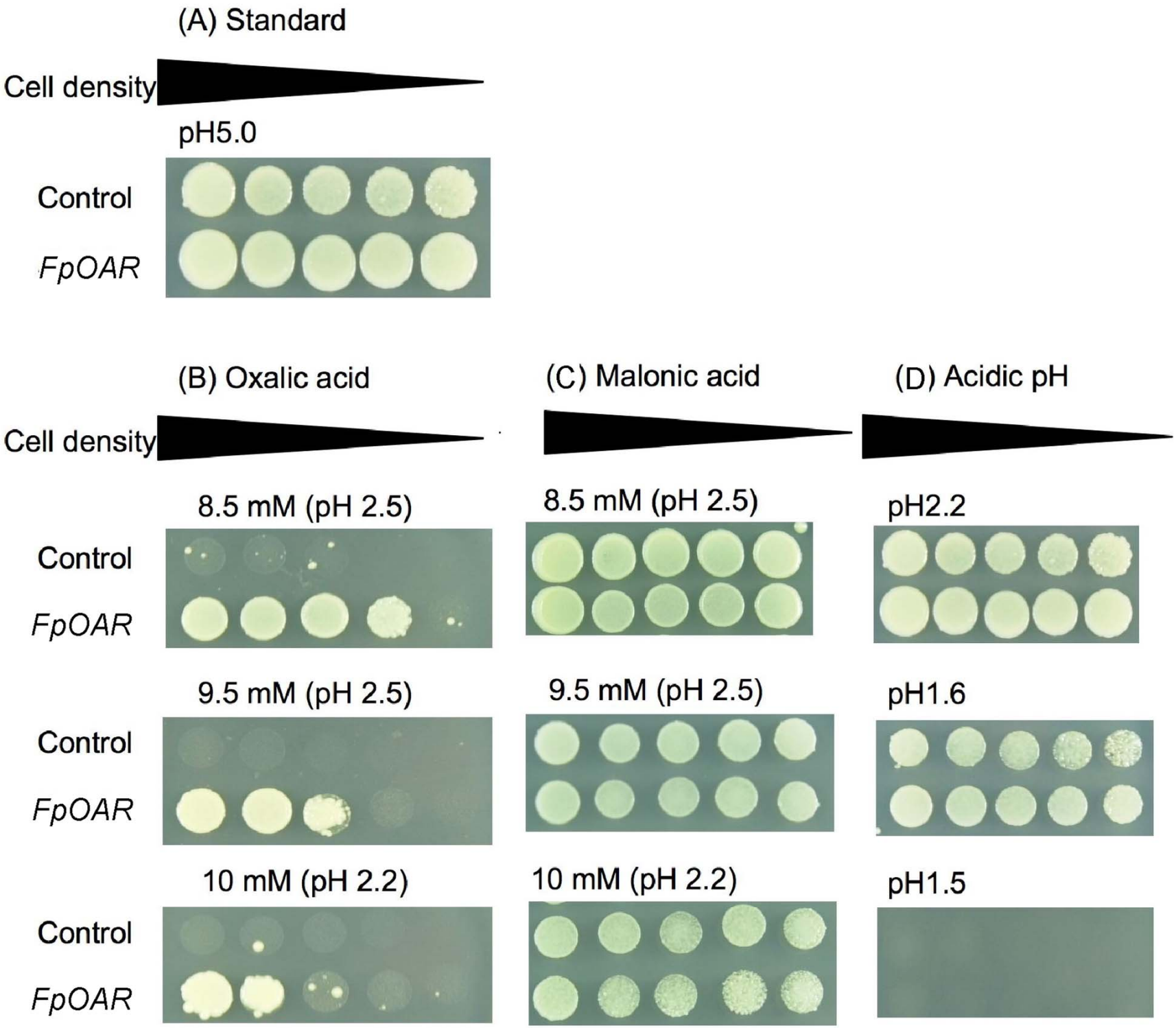
FIG. 6. Oxalate transport and effect of inhibitors using vesicles prepared from the *FpOAR*-transformant. The reaction was carried out for 20 min. Mean value ±standard deviation. * $p < 0.05$ ($n = 3$).

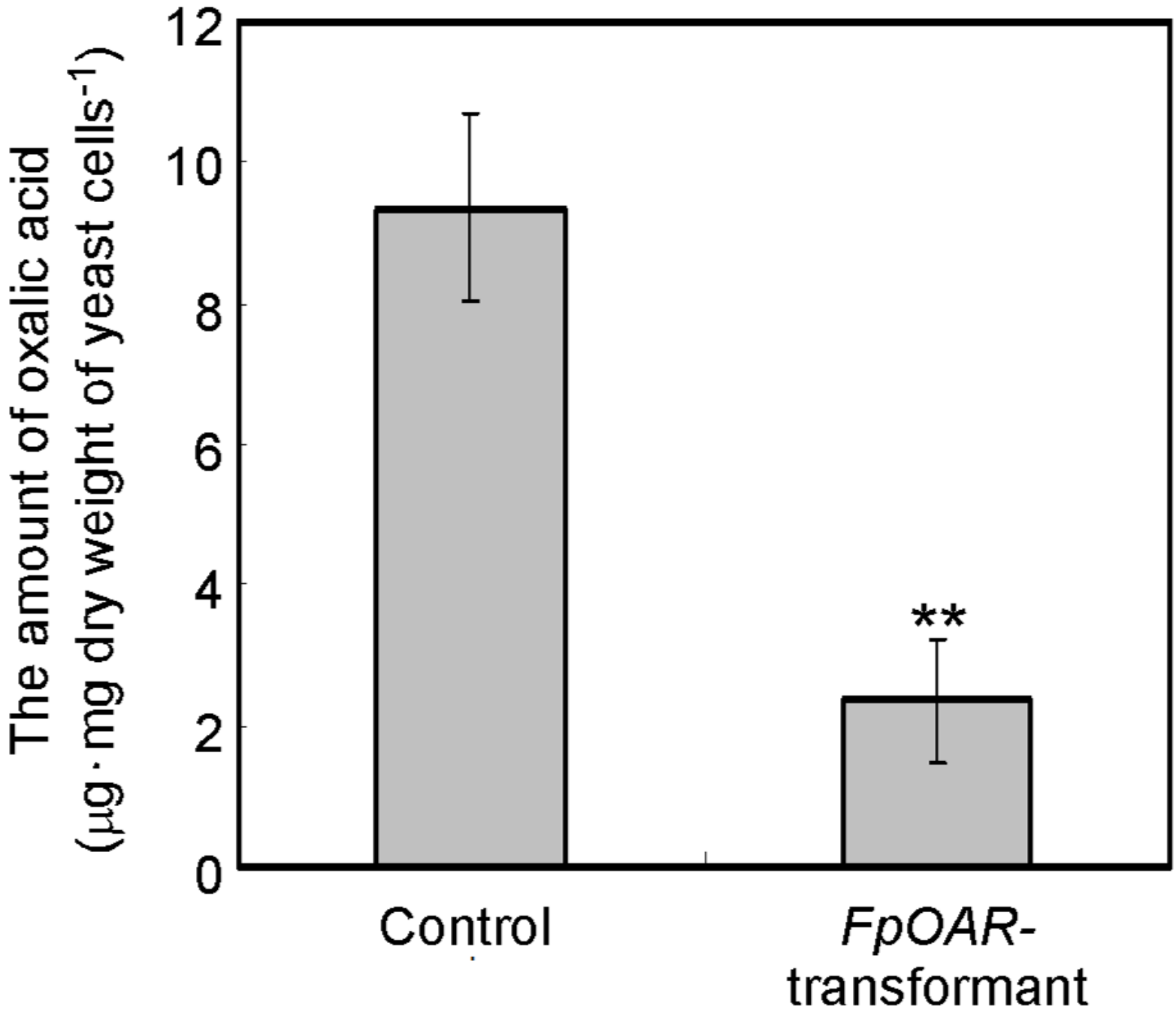
FIG. 7. Changes in (A) dry weight of mycelia, (B) oxalic acid accumulated and pH in the medium during culture of *F. palustris*. In (C) the copy number is shown based on 0.2 μg total RNA, where the copy number is defined as the number of molecules of mRNA coding FpOAR. The mean values and standard deviations were determined based on triplicate samples. Mean value ±standard deviation.

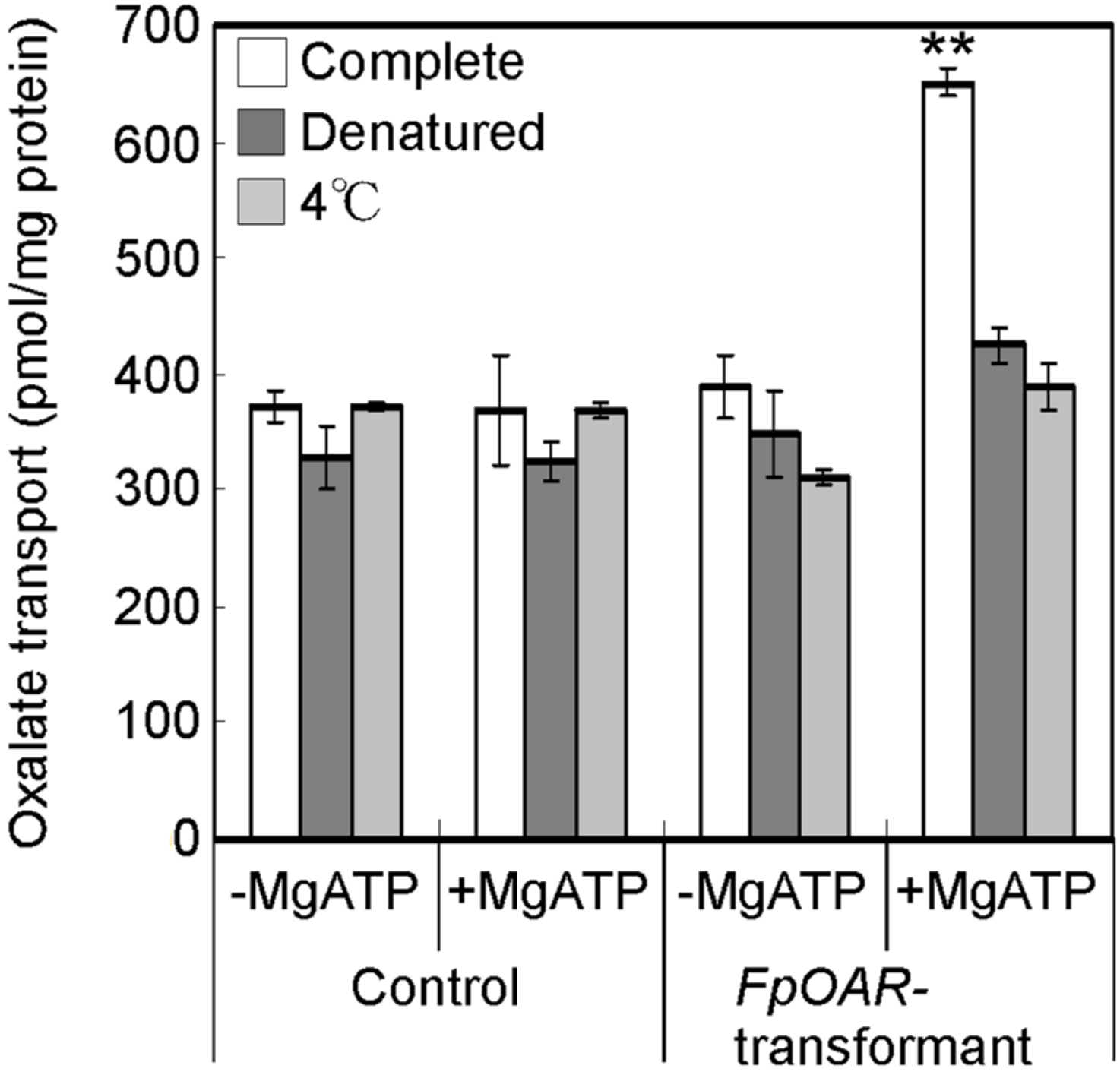
FIG. 8. Effect of exogenous addition of oxalic acid to the *F. palustris* culture on *FpOAR* transcription. Mean value ±standard deviation. ** $p < 0.01$ ($n = 4$).

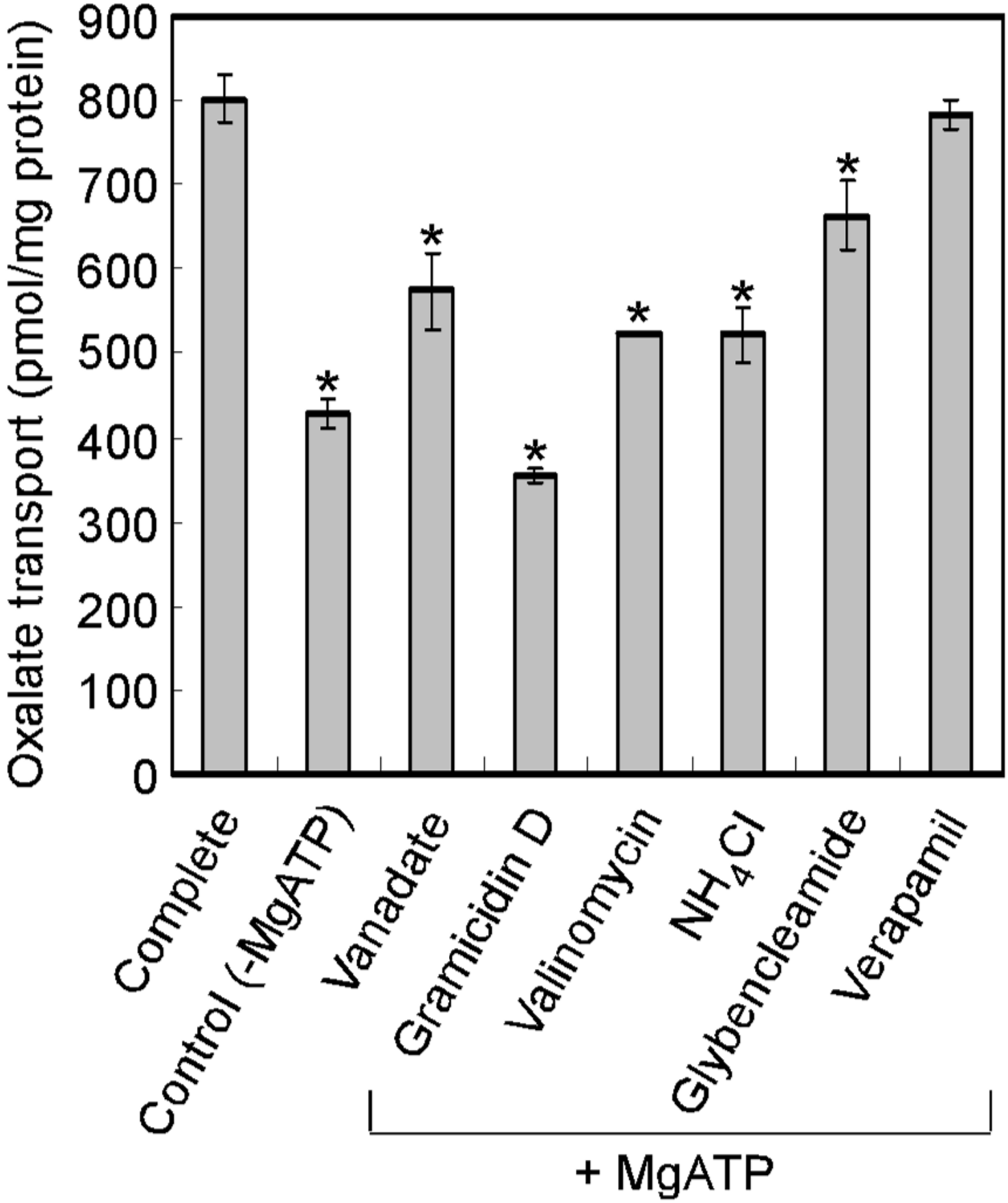

Oxalate transport (pmol/mg protein)
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
*
*
Complete
Control (-MgATP)
Vanadate
Valinomycin
NH4Cl
Glybencleamide
Cyclosporin A
+Mg ATP

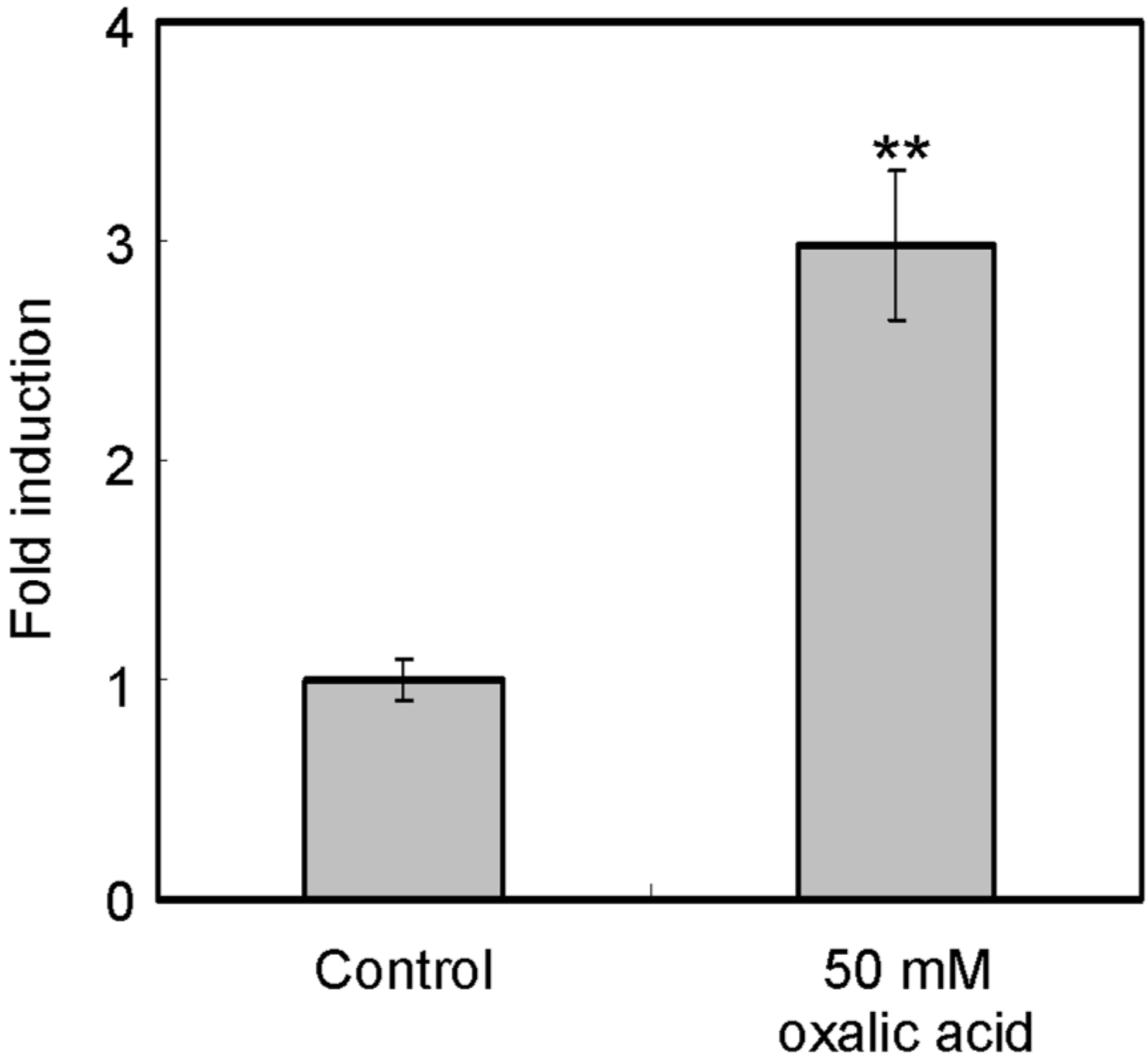

AAEY01000042
gwh2.7.329.1
605
Plus.C_740045
942
FpOAR
Lbscf0011g04660
0.05
976
AACS01000187

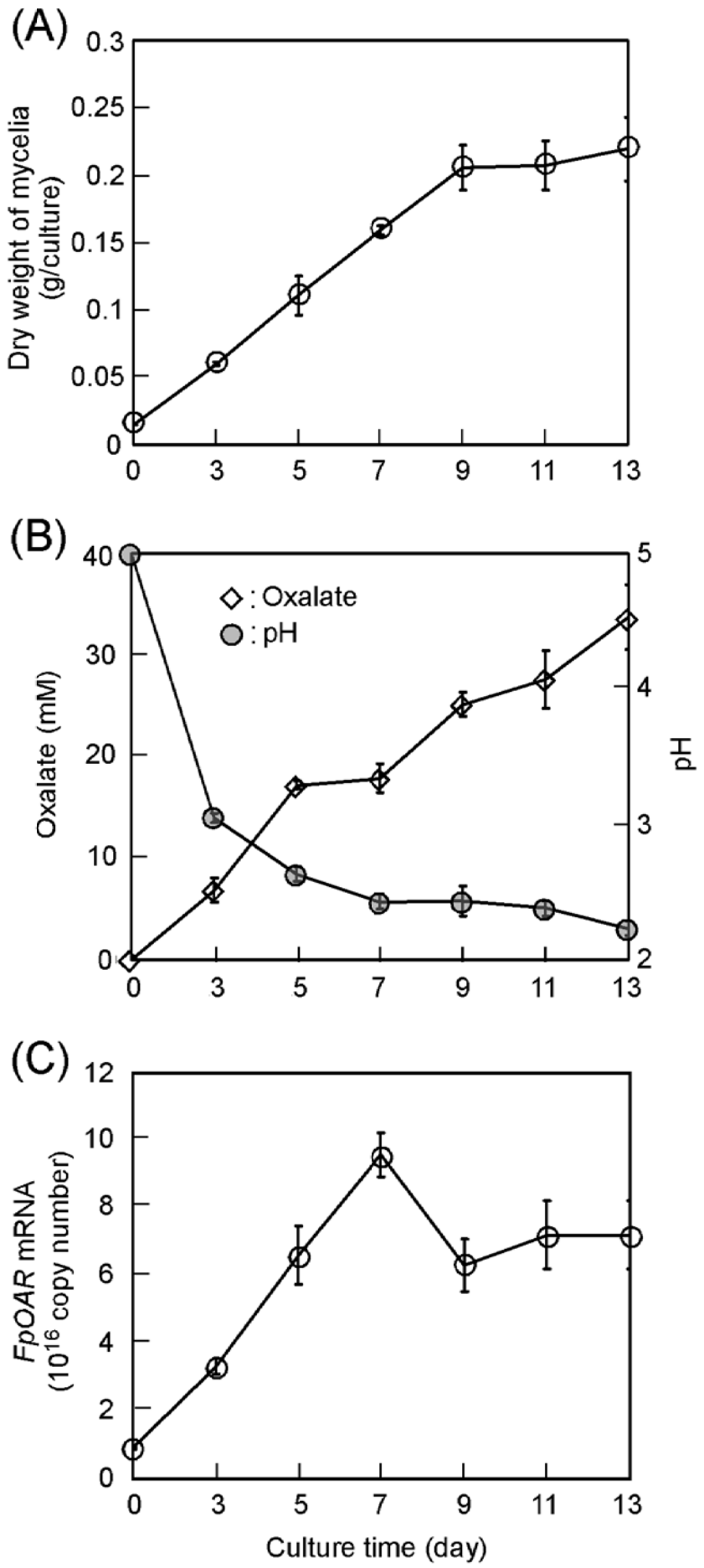

(A) Standard
Cell density
pH5.0
Control
FpOAR
(B) Oxalic acid
Cell density
8.5 mM (pH 2.5)
Control
FpOAR
9.5 mM (pH 2.5)
Control
FpOAR
10 mM (pH 2.2)
Control
FpOAR
(C) Malonic acid
8.5 mM (pH 2.5)
9.5 mM (pH 2.5)
10 mM (pH 2.2)
(D) Acidic pH
pH2.2
pH1.6
pH1.5


The amount of oxalic acid
(μg · mg dry weight of yeast cells-1)
12
10
8
6
4
2
0
**
Control
FpOAR-
transformant


Oxalate transport (pmol/mg protein)
700
600
500
400
300
200
100
0
Complete
Denatured
4℃
**
-MgATP
+MgATP
-MgATP
+MgATP
Control
FpOAR-
transformant


Oxalate transport (pmol/mg protein)
900
800
700
600
500
400
300
200
100
0
*
*
*
*
*
*
Complete
Control (-MgATP)
Vanadate
Gramicidin D
Valinomycin
NH4Cl
Glybencleamide
Verapamil
+ MgATP


4
3
2
1
0
Fold induction
**
Control
50 mM
oxalic acid


(A)
Dry weight of mycelia (g/culture)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
3
5
7
9
11
13
(B)
Oxalate (mM)
pH
◇ : Oxalate
● : pH
40
30
20
10
0
5
4
3
2
0
3
5
7
9
11
13
(C)
FpOAR mRNA
(10^16 copy number)
12
10
8
6
4
2
0
0
3
5
7
9
11
13
Culture time (day)

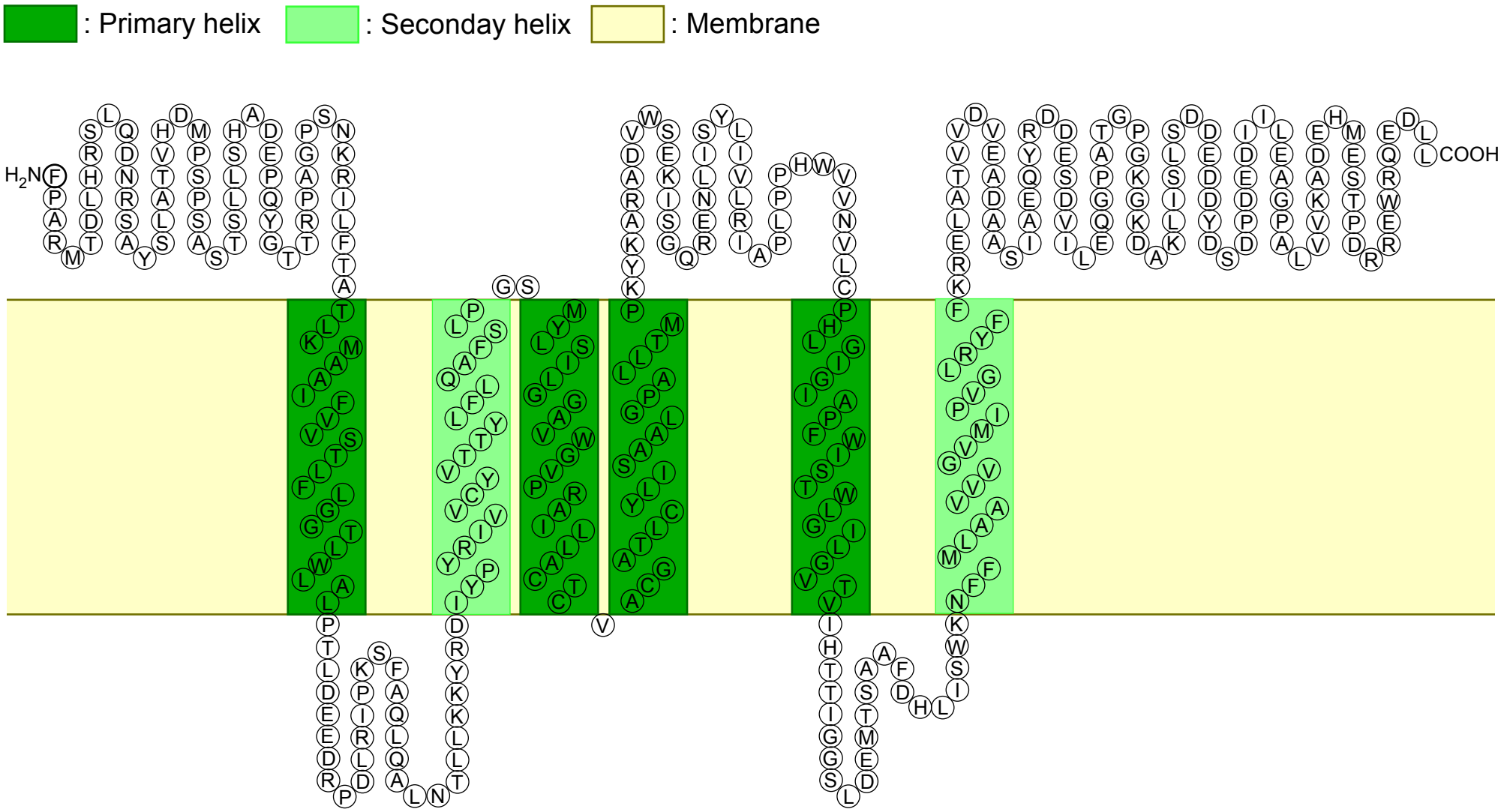


Fig. S1. Predicted transmembrane domains of FpOAR. The prediction was conducted with the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/). The predicted transmembrane domains are shown as primary and secondary helixes.

Supplemental Figure S1