Differential Stress-Induced Regulation of Two Quinone Reductases in the Brown Rot Basidiomycete *Gloeophyllum trabeum*

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Quinone reductases (QRDs) have two important functions in the basidiomycete *Gloeophyllum trabeum*, which causes brown rot of wood. First, a QRD is required to generate biodegradative hydroxyl radicals via redox cycling between two G. trabeum extracellular metabolites, 2,5-dimethoxyhydroquinone (2,5-DMHQ) and 2,5-dimethoxy-1,4-benzoquinone (2,5-DMBQ). Second, because 2,5-DMBQ is cytotoxic and 2,5-DMHQ is not, a QRD is needed to maintain the intracellular pool of these metabolites in the reduced form. Given their importance in *G. trabeum* metabolism, QRDs could prove useful targets for new wood preservatives. We have identified two *G. trabeum* genes, each existing in two closely related, perhaps allelic variants, that encode QRDs in the flavodoxin family. Past work with QRD1 and heterologous expression of QRD2 in this study confirmed that both genes encode NADH-dependent, flavin-containing QRDs. Real-time reverse transcription PCR analyses of liquid- and wood-grown cultures showed that *qrd1* expression was maximal during secondary metabolism, coincided with the production of 2,5-DMBQ, and was moderately up-regulated by chemical stressors such as quinones. By contrast, *qrd2* expression was maximal during fungal growth when 2,5-DMBQ levels were low, yet was markedly up-regulated by chemical stress or heat shock. The total QRD activity in lysates of *G. trabeum* mycelium was significantly enhanced by induction beforehand with a cytotoxic quinone. The promoter of *qrd2* contains likely antioxidant, xenobiotic, and heat shock elements, absent in *qrd1*, that probably explain the greater response of *qrd2* transcription to stress. We conclude from these results that QRD1 is the enzyme *G. trabeum* routinely uses to detoxify quinones during incipient wood decay and that it could also drive the biodegradative quinone redox cycle. However, QRD2 assumes a more important role when the mycelium is stressed.

Brown rot basidiomycetes cause a highly destructive type of wood decay and are important lignocellulose recyclers in forest ecosystems (7, 19, 32). During early brown rot, cellulose is rapidly oxidized and depolymerized, even though the porosity of sound wood is too low for enzymes such as cellulases to penetrate (6). This observation suggests that small oxidants rather than enzymes initiate brown rot. Numerous studies indicate that these oxidants include hydroxyl radicals produced via Fenton chemistry (H2O2/H2O → H+ + H2O2 → •OH) (8, 9, 29). It follows that brown rot fungi require extracellular mechanisms to reduce Fe3+ and O2, the forms of iron and oxygen they generally encounter, if they are to degrade wood by this mechanism.

Recent work has shown that two brown rot fungi, *Gloeophyllum trabeum* and *Postia placenta*, generate a hydroquinone-driven Fenton system. They produce extracellular 2,5-dimethoxyhydroquinone (2,5-DMHQ), which rapidly reduces both Fe3+ and O2 to give 2,5-dimethoxy-1,4-benzoquinone (2,5-DMBQ) (5, 11, 14, 21). Experiments with *G. trabeum* have shown that it drives this chemistry by continuously reducing extracellular 2,5-DMBQ to extracellular 2,5-DMHQ, thus assuring a steady supply of electrons for the reduction of Fe3+ and O2 (11, 14). Therefore, the extracellular Fenton system of *G. trabeum* must be catalyzed by a quinone reductase (QRD).

There is an additional reason why QRDs are important enzymes in *G. trabeum*. 2,5-DMBQ and many other quinones are cytotoxic because they readily accept one electron from intracellular reductants to form semiquinones, which rapidly reduce O2 to produce superoxide (22). Moreover, the electron-deficient carbonyl groups of quinones make them good electrophiles that can alkylate proteins and DNA (2). Hydroquinones, by contrast, do not have these properties, and therefore organisms generally reduce quinones to detoxify them. *G. trabeum* is likely to require an efficient reductive system to detoxify the large amounts of 2,5-DMBQ that it produces during biodegradation.

In most organisms, flavin-containing QRDs have a major role in quinone detoxification. Animals produce DT diaphorase, an FAD-containing NAD(P)H:QRD that is part of an inducible set of enzymes, termed the phase 2 system, which protects the organism against chemical carcinogenesis (10, 26). In plants and fungi, quinone detoxification is evidently catalyzed by a different group of flavin mononucleotide-containing NAD(P)H:QRDs that are homologous to flavodoxin (1, 12, 16, 18). The genes that encode all of these QRDs are up-regulated by quinones and, in some cases, by other chemical stressors.

Since brown rot QRDs are required for both biodegradation and detoxification, it may eventually be possible to use QRD inhibitors to prevent the growth of these fungi on wood. There is a need for such relatively specific control agents because most of the wood preservatives in current use are highly toxic, general biocides. With this goal in mind, we recently characterized a flavodoxin NADH:QRD from *G. trabeum* that might...
detoxify 2,5-DMBQ, drive Fenton chemistry, or perhaps fill both roles (12). We have now identified an additional *G. trabeum* flavodoxin QRD. Both enzymes probably detoxify quinones, but they are expressed at different stages of growth and the genes that encode them are differentially regulated in response to chemical stress and heat shock.

**MATERIALS AND METHODS**

**Chemicals and organism.** DL-Methylouabain/butyl isothiocyanate (DL-sulforaphane) was purchased from ICN Biomedicals (Aurora, Ohio). All other chemicals were obtained from Sigma/Aldrich (St. Louis, Mo.), including 2,6-dimethoxybenzoquinone (2,6-DMBQ), 2-methyl-1,4-naphthoquinone (menadione), butylated hydroxyanisole (BHA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT).

Stock cultures of *G. trabeum* (ATCC 11539) were maintained on agar plates that contained (per liter) 10 g of glucose, 10 g of malt extract, 2 g of peptone, 2 g of yeast extract, 1 g of asparagine, 2 g of KH₂PO₄, 1 g of MgSO₄·7H₂O, and 0.001 g of thiamine.

**G. trabeum in liquid medium.** Solid liquid cultures of *G. trabeum* were grown at 31°C under anaerobic conditions in a 50-ml beaker at 120 rpm. One milliliter of culture medium, containing 50 mM MgCl₂, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, a 1 mM concentration of each deoxynucleoside triphosphate, 1 U of recombinant RNase inhibitor/μl, 15 U of reverse transcriptase/μl, and 0.5 μg of RNA/μg of random primers. The temperature program for the reaction was as follows: 25°C for 10 min, 42°C for 15 min, 95°C for 5 min, and 5°C for 5 min. Finally, the reaction mixture was diluted 1:1 with RNase-free ddH₂O and a 5-μl sample was used for real-time reverse transcription-PCR (RT-PCR) analyses as described below.

**Gene identification and characterization.** Oligonucleotide primers were obtained from the University of Wisconsin Biotechnology Center. To characterize the structure of a glyceraldehyde-3-phosphate dehydrogenase gene (gpd) in *G. trabeum*, we designed degenerate primers that were based on cDNA sequence comparisons between the gpd genes of *Phanerochaete chrysosporum* (GenBank accession no. M81754), *Schizophyllum commune* (M81724), *Laccaria edodes* (AB1136), and *Agaricus bisporus* (M81728). A forward primer (5'-GGTGCYATYGCCGAYTGTG-3') and reverse primer (5'-ATRACCCCTCAGCCCGC-3') were used to amplify *G. trabeum* cDNA (629 bp) and gDNA (750 bp) products by PCR. The PCR mixtures (50 μl; Promega) contained 60 pmol of degenerate primers, and the temperature program was as follows: a denaturation step at 94°C for 3 min followed by 35 cycles that consisted of 94°C for 30 s, 54°C for 1 min, and 72°C for 2 min and finally an extension step at 68°C for 7 min. The amplified cDNA and gDNA fragments were cloned and sequenced.

To find gpd genes, we used conserved regions in *G. trabeum* gQRD1 (GenBank accession no. AAL67660) and the previously reported *P. chrysosporum* QRD (AAD21025) to design a degenerate forward primer (5'-CARTGGGAARGNTTYTGGGAYDNCNATAC-3') and a degenerate reverse primer (5'-CNCKSNWCRGCTTACRAANGTGA-3'). PCR amplifications of *G. trabeum* gDNA, PCR conditions were as described above for gpd. We obtained a PCR product of 250 bp, which was cloned and sequenced to reveal the presence of both qrd1 and qrd2. Full-length qrd2 cDNA was amplified from the genomic sequence was obtained by using the Universal Genome Walker kit and Advantage GC Genomic Polymerase mix from Clontech (Palo Alto, Calif.) with the forward primer 5'-ATCCGGCGATGCGGCTGTG-3' and reverse primer 5'-CAGAATCTCCCGGCGGGATCAGTGA-3'. An additional agar plug inoculum of the fungus was placed on top of each plate (12). Isolation of nucleic acids and cDNA preparation. Genomic DNA (gDNA) was isolated from *G. trabeum* mycelium as described earlier (12). Northern hybridizations. PCR products from cDNAs of qrd2 and gpd2 were used to prepare 32P-labeled probes by using a Random Primed Strip-Able DNA Probe Synthesis kit (Ambion, Austin, Tex.) with [γ-32P]ATP (3,000 Ci/mmol; Amersham). Total RNA (10 μg per lane) was resolved on agarose gels that contained 2% formaldehyde. The RNA was then transferred to a positively charged nylon membrane (Roche, Indianapolis, Ind.). Northern hybridization was done with a Northern Max kit (Ambion) and 10 μl of [γ-32P]ATP per ml of hybridization solution overnight at 42°C. The membranes were washed under low-stringency (25°C) and high-stringency (45°C) conditions according to the manufacturer's instructions and then exposed to XAR film (Kodak, Rochester, N.Y.) for 4.5 h at ambient temperature.

**Real-time RT-PCR.** Relative quantitation of gene expression were done by using Taqman real-time RT-PCR on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, Calif.). This relatively new technique is highly sensitive, and the simultaneous use of two specific primers and one specific probe for each gene confers high specificity when closely related genes are analyzed (3). The primers and probes (Table 1) were designed with PrimerExpress software from Applied Biosystems and were purchased from that company. The probes spanned the junction of two exons to ensure discrimination between cDNA and gDNA. Each probe was labeled at the 5' end with the reporter dye TAMRA (6-carboxytetramethylrhodamine). PCRs contained an 800 nM concentration of each primer, a 250 nM concentra-
Gene Primer or probe Sequence (5′ → 3′) Length (bp)
gpd Forward CGTCGTGGTTGTCACATTGAC 22
Reverse GGGCAAGGAGTATTGGT 17
Probe ACCCAAGTACACTGCTGCTTCAAGTC 28
gpd1 Forward CCAGGCGTATCAAGGGTATTA 22
Reverse GCTCCTACGGTCCTAACTT 22
Probe CGCTTCCATCTTCCAGTGAGGACT 28
gpd2 Forward TACTCCTGTAAGCCACATC 21
Reverse MGGCTTTGCGTCGAGGAC 19
Probe CCAAGCTGGCGAGGCTGGA 21

Heterologous expression of QRD2. Recombinant QRD2 was expressed in Pichia pastoris by using Invitrogen’s Easy Select Pichia Expression Kit. P. pastoris strain X-33 and the media used to grow it are described in the Invitrogen manual. QRD2 was PCR amplified with PIs from a plasmid that carried the cDNA by using the forward primer 5′-GGAATTCCGAGGAGTGGT 5′-GACGTAATGGCCAGGACG GCC-3′ and the reverse primer 5′-GTCCCTTTTGCGCGAGAAT TTGGAAGC6G-3′. The amplified qrd2 coding region was then digested with EcoRI and NotI, gel purified, and cloned into plasmid pPICZ B (Invitrogen) as a translational fusion to a myc epitope and His6 tag. Positive clones were verified by PCR and DNA sequence analysis. Transformations into P. pastoris were carried out by electroporation according to the manufacturer’s instructions. Positive integrates were able to use methanol as the sole carbon source and were confirmed by PCR.

The transformants were grown on minimal glycerol medium plus histidine and then scaled up on minimal methanol medium plus histidine as described in the Invitrogen manual. Cells were grown for 54 h, frozen, suspended in breaking buffer (50 mM sodium phosphate [pH 7.4], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), placed in an ice-jacketed glass bead homogenizer (Bead-Beater; Biospec, Bartlesville, Okla.), and disrupted according to the manufacturer’s instructions. The suspension was centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant fraction was stored at 4°C until use.

Nickel-affinity chromatography of recombinant QRD2 was done at 4°C under nondenaturing conditions on a Ni-CAM HC Resin column (Sigma) according to the manufacturer’s instructions. QRD2 was eluted with a buffer that consisted of 250 mM imidazole and 0.3 M NaCl in 50 mM NaHPO4 at pH 8.0. Fractions with QRD activity were then dialyzed against 20 mM NaHPO4 (pH 7.0) that contained 20% (vol/vol) glycerol.

Electrophoresis of recombinant QRD2. Recombinant QRD2 was subjected to polyacrylamide gel electrophoresis (PAGE) on 10% nondenaturing gels (Ready Gel Precast Gels; Bio-Rad) and stained for total protein with Coomassie blue R-250.

To detect the enzyme by immunoblotting, the proteins on the gel were transferred electrothermally to polyvinylidene difluoride membranes (Bio-Rad) at 100 V for 1 h with 25 mM Tris buffer (pH 8.3) that contained 192 mM glycine. The membranes were soaked in 10 ml of BLOTTO (13) for 1 h with shaking at 25°C and then washed and incubated with anti-myc antibody conjugated to alkaline phosphatase (Invitrogen) according to the manufacturer’s instructions. The QRD2 band was detected by immersing the membrane in 1-Step Nitro Blue Tetrazolium-bromochloroindolyl phosphate mixture (Pierce, Rockford, Ill.) until a blue-black color precipitated in 5 to 15 min, after which the membrane was rinsed with ddH2O.

The NADH:QRD reductase activity of recombinant QRD2 was detected in the gels by observing the NADH- and menadione-dependent reduction of MTT. After electrophoresis, the gels were immediately soaked in 50 mM Tris, pH 7.5, that contained 0.3 mg of MTT/ml and 1 mM NADH, with or without 30 μM menadione (30). The gels were shaken gently at 25°C in the dark until a blue band at the location of QRD2 developed in 30 to 60 min. The reaction was stopped with 5% acetic acid.

Nucleotide sequence accession numbers. The G. trabeum gDNA (including promoter region) and cDNA sequences have been deposited in the GenBank database under the following accession numbers: qrd1A promoter, AY286072; qrd1B gDNA, AY286073; qrd2A gDNA, AY286074; qrd2B gDNA, AY286075; qrd2A cDNA, AY286076; qrd2B cDNA, AY286077; gpd gDNA, AY286078; gpd cDNA, AY286079.

RESULTS

Identification of qrd genes in G. trabeum. We designed degenerate PCR primers that were based on two conserved regions in the previously reported QRD genes of G. trabeum (12) and P. chrysosporium (1), and we used these to amplify G. trabeum gDNA. When these sequences were extended, we obtained full-length genomic clones of two distinct G. trabeum genes, one of which matched the sequence we originally reported (12). Amplifications with gene-specific primers and a high-fidelity polymerase then showed that each gene exists in two closely related forms that may be allelic (see below). We named these qrd1A (the originally reported sequence), qrd1B, qrd2A, and qrd2B.

The sequences for qrd1A and qrd1B differ by seven nucleo-
tides and are 99.3% identical. Two of the changes occur in introns, of which qrd1 has four. One of the five changes in exons occurs in a putative leader sequence, whereas the other four result in amino acid changes in the predicted mature QRD1 proteins. The sequences for qrd2A and qrd2B differ by 14 nucleotides and are 98.6% identical. Three of the changes are in introns, of which qrd2 has three. One of the 11 changes in exons results in an amino acid change in a putative leader sequence. The remaining 10 nucleotide changes are located in the region that encodes the mature QRD2 protein, but none of them results in an amino acid change. Therefore, there are potentially two QRD1 proteins, but only one QRD2 protein.

We used qrd1- and qrd2-specific primers to PCR amplify cDNA from G. trabeum cultures that had been grown in liquid medium (2, 6, and 14 days) and on wood (8 days). Forty clones of each sample were sequenced, and the sequences were aligned with those of the qrd genes. The results showed that qrd1A mRNA was present in all samples, whereas qrd1B mRNA was not detected in any sample. By contrast, qrd2A and qrd2B clones were obtained with equal frequency from all samples. Therefore, qrd2A and qrd2B are very likely alleles. The relationship between qrd1A and qrd1B is more ambiguous, but the high similarity of their sequences suggests that they also may be alleles. So far, we have failed to induce G. trabeum to fruit and thus produce diagnostic qrd segregants.

Analysis of the qrd promoters. We sequenced the qrd promoters by gene walking with reverse primers that were specific for each of the four qrd genes. Amplifications with gene-specific primers and a high-fidelity polymerase yielded sequences that extended about 800 bp upstream from the ATG codons. Both qrd1 genes contain a TATA box, whereas neither qrd2 gene does (Table 2). All of the genes contain CAAT boxes, and in the two qrd2 genes this element is very close to the ATG codon, as is generally the case for genes without TATA boxes (17). The core promoters (~40 bp to +40 bp) for qrd1A and qrd1B differ by eight nucleotides, whereas those of qrd2A and qrd2B are identical. In addition, qrd1B lacks a palindrome that is present upstream of qrd1A, qrd2A, and qrd2B (Table 2).

The promoters of the three transcribed genes (qrd1A, qrd2A, and qrd2B) all contain putative stress response elements (STRE) and metal response elements (MRE). No other response elements are evident in the qrd1A promoter, but both of the qrd2 promoters contain consensus sequences for the xenobiotic element (XRE) and heat shock element (HSE), as well as sequences that differ by one nucleotide from the consensus for the antioxidant response element (ARE) and AP1-binding element (TRE). The qrd2A and qrd2B promoters differ by seven nucleotides, but none of these changes occurs in a response element.

Properties of QRD2. The deduced polypeptide sequence of G. trabeum QRD2 contains 243 amino acids, including a putative leader sequence of 40 amino acids similar to one found in G. trabeum QRD1, P. chrysosporium QRD, and some related sequences (1, 12). The mature QRD2 protein (without the leader) is 69% identical to the previously described P. chrysosporium QRD (AAD21025) (1) and 67% identical to G. trabeum QRD1 (AAL67860) (12). It is also similar to plant quinone reductases in this flavodoxin family, e.g., 56% identical to an Arabidopsis thaliana QRD (BAA97523) (16) and 54% identical to a Triphysaria versicolor QRD (AAG53945) (18) (Fig. 1).

We expressed QRD2 with a C-terminal myc epitope and His6 tag in P. pastoris and purified it by nickel-affinity chromatography. Most of the QRD activity was lost on the column, but sufficient enzyme was recovered to assess its basic properties. Sodium dodecyl sulfate-PAGE showed that the enzyme was apparently pure and that it had a subunit molecular mass of 25 kDa, in agreement with the predicted value of 24.9 kDa (data not shown). Nondenaturing PAGE revealed a single band by Coomassie blue staining or immunoblotting against an anti-myc antibody. Activity staining of the native gel with NADH, menadione, and the tetrazolium dye MTT established that QRD2 is an NADH:quinone reductase (Fig. 2). Additional experiments showed that QRD2, like QRD1, shows low specificity for quinones but exhibits much higher activity with NADPH than with NADH (12). The visible absorption spectrum of recombinant QRD2 was that of a typical flavoprotein, with maxima at 450 and 380 nm (data not shown).

Expression of qrd1 and qrd2 by G. trabeum. We used real-time RT-PCR to compare qrd transcript levels in G. trabeum cultures (Fig. 3B). This method is quantitative when transcripts that arise from a single qrd gene are compared. However, it is only semiquantitative for comparisons between qrd1 and qrd2 transcripts. The problem is that two criteria must be met for a quantitative comparison: the RT efficiencies for the qrd1 and qrd2 mRNAs must be the same, and the PCR efficiencies for the resulting qrd1 and qrd2 cDNAs must be the same. It is evident that the PCR efficiencies were near 100% for both cDNAs because plots of cycle threshold versus log dilution of cDNA gave slopes of −log2 (Applied Biosystems User Bulletin no. 2). However, it is possible that the RT efficiencies were not the same.

Nevertheless, it is evident that any error thus introduced is small, because comparisons between qrd1 and qrd2 transcript levels by Northern blotting (Fig. 3C), which does not involve an RT step, gave results similar to those obtained by real-time RT-PCR (Fig. 3B). Since the real-time RT-PCR method was evidently no less quantitative than Northern blotting for our purposes and since it is by far the more sensitive technique (3), we used it to compare qrd1 and qrd2 transcript levels.

Analyses of G. trabeum grown on nitrogen-limited liquid medium showed that the peak level of qrd2 transcripts (i.e.,

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### TABLE 2. Putative transcription elements in the qrd1A and qrd2AB promoters

<table>
<thead>
<tr>
<th>Element</th>
<th>Sequence</th>
<th>Positiona in:</th>
<th>qrd1</th>
<th>qrd2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TATA box</td>
<td>TATAAA</td>
<td>58</td>
<td>—d</td>
<td>—d</td>
</tr>
<tr>
<td>CAAT box</td>
<td>CCAT</td>
<td>43, 273</td>
<td>17</td>
<td>—</td>
</tr>
<tr>
<td>Palindrome</td>
<td>ACTAGT</td>
<td>698</td>
<td>796</td>
<td>—</td>
</tr>
<tr>
<td>HSE</td>
<td>CGAACGTTCGAAA</td>
<td>—</td>
<td>300</td>
<td>—</td>
</tr>
<tr>
<td>XRE</td>
<td>GCGTG</td>
<td>—</td>
<td>116, 336, 465</td>
<td>—</td>
</tr>
<tr>
<td>ARE</td>
<td>GCCACGGTCG</td>
<td>—</td>
<td>155</td>
<td>—</td>
</tr>
<tr>
<td>STRE</td>
<td>CCCCT</td>
<td>634, 643</td>
<td>307, 443</td>
<td>—</td>
</tr>
<tr>
<td>MRE</td>
<td>TGCAAGC</td>
<td>286</td>
<td>338, 341, 693</td>
<td>—</td>
</tr>
<tr>
<td>TRE</td>
<td>TCACCTAA</td>
<td>—</td>
<td>754</td>
<td>—</td>
</tr>
</tbody>
</table>

a The elements were identified by using the FindPatterns program of GCG Wisconsin Package software.

b Position upstream from the start codon (ATG).

d Reverse orientation of the element.
mRNAs made from either qrd2A or qrd2B was relatively low and occurred during the growth phase (Fig. 3A and B). By contrast, qrd1 transcripts (i.e., mRNAs made from qrd1A) were more abundant and peaked after mycelial growth ceased, i.e., during secondary metabolism. The rate at which the mycelium reduced quinones and the extracellular concentration of 2,5-DMHQ plus 2,5-DMBQ also peaked during secondary metabolism, in agreement with previous work (28). That is, the properties that are associated with extracellular Fenton chemistry by G. trabeum (11, 14) coincided with a high level of qrd1 transcripts and a low level of qrd2 transcripts.

When G. trabeum was grown on spruce wood under conditions that lead to significant decay (9), qrd1 transcript levels after 8 days were much higher than qrd2 transcript levels, which they exceeded by a factor of about 20 (Fig. 3B). 2,5-DMBQ was present in the colonized wood at a concentration of 0.9 μmol/g (dry weight). These results provide the first evidence that G. trabeum expresses its hydroquinone-driven Fenton system not only on glucose (11, 14) and cellulose (5) but also on wood.

Induction of qrd1 and qrd2 in G. trabeum. Since G. trabeum’s principal natural quinone, 2,5-DMBO, is too insoluble to prepare concentrated stock solutions, we used a structurally related quinone, 2,6-DMBO, to test whether the qrd genes were induced by quinones. Previous work has shown that G. trabeum reduces 2,5- and 2,6-DMBO at the same rate and that the two resulting hydroquinones drive Fenton chemistry equally well (11, 14).

Both qrd genes were induced when 2,6-DMBO was added to 4-day-old nitrogen-limited liquid cultures of G. trabeum, with greater induction occurring at a higher quinone concentration (Fig. 4). Transcript levels were already elevated 15 min after administration of the quinone, peaked at 1 h, and declined to...
near baseline levels by 5 h (data not shown). The response of qrd2 to the quinone was much stronger than that of qrd1, with transcript levels transiently exceeding 100 times those found in uninduced mycelia. Similar results were obtained when the experiment was repeated with 7-day-old cultures (data not shown).

BHA and sulforaphane, two known inducers of mammalian phase 2 genes (10, 23, 26), gave moderate elevations of G. trabeum qrd transcript levels when they were added to 4-day-old cultures (Fig. 4) or 7-day-old cultures (data not shown) on liquid medium. As with 2,6-DMBQ, these inducers had a greater effect on qrd2 transcripts than they did on qrd1 transcripts. A variety of other phase 2 inducers, including H2O2, paraquat, 1-nitrocyclohexene, and β-naphthoflavone, failed to induce either of the G. trabeum genes.

Heat shock (45°C for 1 h) induced qrd2 markedly but had no effect on qrd1 transcription in 4-day-old cultures (Fig. 4) or 7-day-old cultures (data not shown) on liquid medium. The effect on qrd2 was so large that we repeated the experiment with wood-grown cultures, in which qrd1 transcripts were normally dominant (Fig. 3B), to see whether qrd2 transcripts would become quantitatively important. On wood, qrd2 transcripts were increased nearly 80-fold by heat shock, making them significantly more abundant than qrd1 transcripts under this type of stress. When the colonized wood was returned to 31°C, transcripts reverted to near their preinduced levels after 2 h (Fig. 4).

Induction of QRD activity in G. trabeum. To determine whether greater transcription of the qrd genes would have an observable effect on translation, we assayed NADH:2,6-DMBQ oxidoreductase activity in lysates of 4-day-old mycelia from liquid cultures that had been induced with 2,6-DMBQ or treated with an equivalent volume of water. Since the activities of the two enzymes, QRD1 and QRD2, are indistinguishable, this approach was only able to yield a composite value of QRD activities from all sources in the mycelium. An immunochromatography approach based on non-cross-reacting monoclonal antibodies to QRD1 and QRD2 would have been better, but so far we have been unable to produce enough recombinant QRD2 to obtain antibodies.

We found that the QRD specific activity in lysates of G. trabeum cultures was elevated more than threefold by pretreatment with 500 μM 2,6-DMBQ (Table 3). The QRD activity per volume of extract was also elevated by the same factor, thus showing that none of the specific activity increase was attributable merely to lower total protein levels in induced samples. Induction was first observable at 2 h after induction, after transcript levels had peaked. It is not clear why QRD induction at the enzyme level was considerably less than qrd induction at the RNA level in these experiments, but similar discrepancies have been reported in other systems and are thought to reflect posttranscriptional regulation (27).

DISCUSSION

QRD1. Our results provide three indications that QRD1 rather than QRD2 is the enzyme that catalyzes routine intracellular quinone reductions by G. trabeum: (i) qrd1 transcripts predominated in G. trabeum cultures that were grown in a liquid medium that elicits high levels of quinone biosynthesis
and biodegradative Fenton chemistry; (ii) levels of qrd1 transcripts rather than qrd2 transcripts paralleled levels of 2,5-DMBQ plus 2,5-DHQ in these liquid-grown cultures; and (iii) qrd1 transcripts predominated during early wood decay by G. trabeum, when holocellulose cleavage is most rapid (15) and the need for 2,5-DMBQ redox cycling therefore greatest.

These expression patterns may indicate that QRD1 catalyzes the extracellular 2,5-DHQ-driven Fenton system of G. trabeum, while simultaneously ensuring that no toxic 2,5-DMBQ accumulates intracellularly. Alternatively, the cycle may be driven by an uncharacterized reductase located on the mycelial surface, and the sole function of QRD1 may be in surveillance against any quinones that happen to penetrate the mycelium while the cycle is operating. On the face of it, a quinone redox cycle that avoids any intracellular steps seems a safer and more efficient way to drive extracellular Fenton chemistry. However, so far we have not found significant membrane-bound quinone reductase activity in G. trabeum (12), and QRD1, despite its intracellular location, remains a reasonable candidate for the enzyme that catalyzes the cycle.

**TABLE 3. NADH:QRD activity in G. trabeum mycelial lysates after induction of cultures with 500 μM 2,6-DMBQ**

<table>
<thead>
<tr>
<th>Length of induction (h)</th>
<th>Treatment</th>
<th>Activity/μl of extract</th>
<th>Sp act</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>U/ml ± SD</td>
<td>Fold change</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>H₂O</td>
<td>4.91 ± 0.68</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>2,6-DMBQ</td>
<td>4.19 ± 0.91</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>H₂O</td>
<td>4.56 ± 0.87</td>
<td>1.0</td>
</tr>
<tr>
<td>2.5</td>
<td>2,6-DMBQ</td>
<td>7.93 ± 0.39</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>H₂O</td>
<td>3.42 ± 1.21</td>
<td>1.0</td>
</tr>
<tr>
<td>2.5</td>
<td>2,6-DMBQ</td>
<td>10.06 ± 1.33</td>
<td>2.9</td>
</tr>
<tr>
<td>3.5</td>
<td>H₂O</td>
<td>3.52 ± 0.23</td>
<td>1.0</td>
</tr>
<tr>
<td>3.5</td>
<td>2,6-DMBQ</td>
<td>11.08 ± 0.18</td>
<td>3.1</td>
</tr>
</tbody>
</table>

- a Standard deviation for triplicate samples.
- b Experiment 1.
- c Experiment 2.

`qrd1A` transcription was transiently enhanced by 2,6-DMBQ, an analogue of 2,5-DMBQ, the principal quinone that G. trabeum produces. This result suggested that QRD1 might be regulated similarly to mammalian phase 2 detoxification enzymes, which are also up-regulated by quinones. There are many phase 2 inducers, all of which share the common property of being good electrophiles that react readily with sulfhydryl groups. This feature may provide a mechanism for interaction between these inducers and regulatory proteins (10, 23, 24, 26). We found that some phase 2 inducers enhanced `qrd1A` transcription, but most did not. The `qrd1A` promoter’s lack of ARE or XRE, which are required for induction of the mammalian phase 2 genes (10, 24), likely contributed to this result. The basis for `qrd1A` induction by 2,6-DMBQ remains unclear, but the putative STRE that we found may play a role.

It is not yet clear why `qrd1B` was not transcribed in liquid medium or on wood, but it may be significant that its core promoter, a region required for RNA polymerase to bind (4), differs considerably from that of `qrd1A`. Another possible contributor is the palindromic sequence absent in the `qrd1B` promoter region. This palindrome was earlier shown to be required for transcription of a phase 2 metalloproteinase in mouse fibroblasts (25). It is present not only upstream of G. trabeum `qrd1A`, `qrd2A`, and `qrd2B` but also upstream of a related *A. thaliana* quinone reductase gene that is induced by auxin (16). It remains to be determined whether `qrd1B` is a functional gene that is regulated differently from `qrd1A` or whether it is simply a `qrd1` allele that has been rendered non-functional by deleterious mutations.

**QRD2.** G. trabeum apparently does not express QRD2 routinely at high levels during lignocellulose degradation but can deploy it to detoxify quinones when the mycelium is under stress. The marked induction of `qrd2` by heat shock supports a role for QRD2 in the general stress response of *G. trabeum* (20). The very strong induction of `qrd2` by 2,6-DMBQ, a compound of known cytotoxicity (22), also supports this conclu-
The transient nature of qrd induction by 2,6-DMBQ is probably attributable to the fact that this quinone does not persist in cultures, because G. trabeum rapidly reduces it to nontoxic 2,6-dimethoxyhydroquinone. Similarly, G. trabeum maintains its natural quinone, 2,5-DMBQ, predominantly in its nontoxic reduced form (11).

The qrd2 promoter contains an HSE sequence that probably accounts for the induction of qrd2 by heat shock. The auxin-responsive A. thaliana quinone reductase gene (16) also contains this HSE sequence upstream of its coding region, but has apparently not been tested for its response to heat shock. In addition, the G. trabeum qrd2 promoter contains several likely XRE sequences and a possible ARE sequence that probably explain why qrd2 responded much more strongly to 2,6-DMBQ than did qrd1. Mammalian phase 2 gene promoters also contain some of these response elements (10, 24), but since qrd2, like qrd1, failed to respond to most phase 2 inducers, it appears that the regulatory mechanisms differ somewhat in fungal and animal systems. Further progress in our understanding of the G. trabeum qrd promoters will require the development of a transformation system with which one can manipulate their response elements.

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