Compensatory expression of five chitin synthase genes, a response to stress stimuli, in *Wangiella (Exophiala) dermatitidis*, a melanized fungal pathogen of humans

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Numerous chitin synthase structural (*CHS*) genes have been identified in fungi, and usually there are several *CHS* genes per species. Compensatory expression of one *CHS* gene in response to defects in other *CHS* genes has not been reported. Five chitin synthase structural (*WdCHS*) genes have been identified in the melanized human pathogen *Wangiella dermatitidis*: *WdCHS1*, *WdCHS2*, *WdCHS3*, *WdCHS4* and *WdCHS5*. This study showed that increased *WdCHS* expression existed as a compensatory mechanism in response to stress induced by chitin synthase gene disruptions, or by exposure of the wild-type or two temperature-sensitive morphological mutants, for short or long periods, to 37 °C. In general, the compensatory responses varied with each *WdCHS* gene, and in accordance with the hypothesized functions of the chitin synthase (*WdChsp*) encoded. It is suggested that these compensatory responses indicate that *WdCHS* gene transcription in *W. dermatitidis* functions as part of a cell-wall integrity pathway in a manner similar to that recently described for *Saccharomyces cerevisiae*.

Keywords: *WdCHS*, virulence, cell wall, morphological mutants, semi-quantitative RT-PCR

INTRODUCTION

Fungal cell walls are mainly composed of manno-proteins, β-1,3-glucan, β-1,6-glucan and chitin. Chitin, a fibrous polymer of β-1,4-linked N-acetylglucosamine, is generally a minor component in yeast cell walls, accounting for only 1–2 % of the cell wall dry weight (Klis, 1994; Smits *et al.*, 1999), whereas in hyphae of filamentous fungi it constitutes a major fraction of the cell wall (Munro & Gow, 2001; Montijn *et al.*, 1997). Chitin synthases (*Chsp*) are responsible for the synthesis of chitin, and numerous fungal chitin synthase (*CHS*) genes have been identified and characterized. These genes encode different types of isozymes (*Chsp*), which are currently classified into six classes according to their derived amino acid sequences (Bowen *et al.*, 1992; Din *et al.*, 1996; Specht *et al.*, 1996). To our knowledge, no reports to date suggest that different *Chsp* make different chitins, even though most fungi have multiple *CHS* genes. Instead, it appears that the multiple chitin synthases are synthesized and/or activated to produce chitin either temporally at different times during normal vegetative growth, or spatially when induced by environmental conditions, e.g. during progression from one cell-cycle phase to another, when there are chitin insertions into new areas of the cell wall during stress, or during the transition from one growth form to another (Bulawa *et al.*, 1995; Cabib *et al.*, 1992; DeNobel *et al.*, 1991; Gow *et al.*, 1994; Mio *et al.*, 1996; Munro *et al.*, 2001). However, when numerous *Chsp* are present in the same fungus, it is logical to assume that some *Chsp* have overlapping functions and that one can compensate for a defect in the other. In *Saccharomyces cerevisiae*, chitin synthesis has been found to increase greatly as a compensatory stress mechanism in response to mutations that lead to a weakening of the cell wall, such as in manno-protein, β-1,3-glucan and β-1,6-glucan synthesis-disruption mutants (García-Rodríguez *et al.*, 2000; Popolo *et al.*, 1997; Valdivieso *et al.*, 2000), and in type II myosin-deficient strains (Cruz *et al.*, 2000). However, there have been no reports that suggest such compensations occur among different chitin synthases at the transcriptional level.
Wangiella dermatitidis is a model fungus for the more than 100 other melanized fungal pathogens of humans (Szaniszlo et al., 1993). In this agent of phaeohyphomycosis, five chitin synthase structural (WdCHS) genes have been identified, characterized to various degrees, and found to encode five different isozymes (WdChs) types: class II, WdChs1p (Mendoza, 1995); class I, WdChs2p (Wang et al., 2001); class III, WdChs3p (Wang et al., 2000); class IV, WdChs4p (Wang et al., 1999); and class V, WdChs5p (Liu et al., 2001). Each of these genes has been disrupted singly and in a variety of combinations. Our systematic analyses of the disruption mutants (wdchsa), which are still in progress, have shown that the products of most WdCHS genes in some way contribute indirectly or directly to virulence. This occurs either by the imparting of a temperature sensitivity to the disruption strains, which then cannot grow at the elevated temperatures of infection (e.g. at 37 °C; Wang et al., 1999; Liu et al., 2001), or by the lowering of virulence without affecting the strain’s ability to grow at the higher temperature (Wang et al., 2001; unpublished data). However, the comprehensive relationships among the expression of the five WdCHS genes at the transcriptional level have not been addressed. Here, we report that differential expression was detected among the WdCHS genes of W. dermatitidis. The patterns of differential expression observed suggest that compensatory mechanisms occur at the transcriptional level in response to a defective WdCHS gene, as well as in response to the exposure of the wild-type strains or two temperature-sensitive (ts) morphological mutants to the elevated temperature of an infected host.

METHODS

Strains and culture preparation. The strains used in this study are listed in Table 1. In all cases, the strains were cultured in rich YPD (1% yeast extract, 2% peptone, 2% glucose) medium. For preparation of inocula for experimental cultures, a strain was first streaked on YPD plates and then cultured at 30 °C for 4 days, after which 5 ml YPD was inoculated with a single colony. The resulting broth culture was shaken at 25 °C for 3 days. Cells were then subcultured for 24 h three more times at 25 °C at shaking speeds of 200 r.p.m. in a New Brunswick water bath shaker. Each YPD subculture was inoculated with the previous subculture to an initial density of 10⁶ cells ml⁻¹ and the last subculture was used for experiments.

RNA extraction. RNA samples were always from cells in early exponential phase (approx. 10⁶ cells ml⁻¹) of growth, and approximately 10⁶ cells were harvested for each sample. Harvested cell pellets were immediately stored at −80 °C. RNA was extracted with acid phenol (Ausubel et al., 1997). RNA samples were then treated with RQ1 RNase-free DNase (Promega) at 37 °C for 1 h, followed by phenol/chloroform extraction, ethanol precipitation and washing. The DNase-treated RNA pellets were finally dissolved in 100 µl RNase-free water, serially diluted and aliquoted.

RT-PCR. The differential expression patterns of the five WdCHS genes were investigated by a semi-quantitative RT-PCR method, which was similar to those described by Noonan et al. (1990), Ogretmen et al. (1998) and Ogretmen et al. (2001). Primers for the RT-PCR were designed from non-conserved regions of each WdCHS gene, based on a multiple alignment of all five genes. The sequence of each primer pair and the expected size of each PCR product are listed in Table 2. Primers 18S for the 18S rRNA gene and the 18S PCR Competimer (Ambion) were used as an internal control to ensure that an equal amount of total RNA was present in each of the different samples. The RT-PCR of a sample was done in one tube using the Access RT-PCR system (Promega). A typical RT-PCR reaction for 25 µl consisted of 1 µl MgSO₄ (25 mM), 0.5 µl dNTP (10 mM), 2.5 µl buffer (10 ×), 1 µl forward and reverse primer (25 µM), 0.5 µl avian myeloblastosis virus RT, 0.5 µl Tfl DNA polymerase, 1–15 µl total RNA in appropriate dilution, and RNase-free water supplemented to 25 µl. The RT-PCR amplifications were carried out in a GeneAmp PCR System 9700 (Applied Biosystems) using the following cycling conditions: 48 °C for 1 h, 94 °C for 2 min, followed by different cycles of 94 °C for 30 s, 50–58 °C for 1 min and 68 °C for 1 min 20 s, then an extra step of elongation at 68 °C for 5 min. The annealing temperature was 50 °C for WdCHS1, WdCHS3 and WdCHS4 amplifications, 55 °C for 18S and WdCHS5, and 58 °C for WdCHS2. Cycle numbers varied from 25 to 29. Both cycle number and template amount were optimized for each primer pair.

### Table 1. W. dermatitidis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Properties</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>8656</td>
<td>Wild-type</td>
<td>ATCC 34100; Cooper et al. (1993)</td>
</tr>
<tr>
<td>Mc3</td>
<td>ts mutant; grows isotropically at 37 °C</td>
<td>ATCC 38716; Roberts &amp; Szaniszlo (1978)</td>
</tr>
<tr>
<td>Hf1</td>
<td>ts mutant; grows as hyphae at 37 °C</td>
<td>Ye et al. (2000)</td>
</tr>
<tr>
<td>WdCHS1A-1</td>
<td>WdCHS1 insertion mutant, wdchs1::hph</td>
<td>Zheng (1997)</td>
</tr>
<tr>
<td>WdCHS2A-1</td>
<td>WdCHS2 replacement mutant, wdchs2::hph</td>
<td>Wang et al. (2001)</td>
</tr>
<tr>
<td>WdCHS3A-1</td>
<td>WdCHS3 insertion mutant, wdchs3::hph</td>
<td>ATCC MYA886; Wang &amp; Szaniszlo (2000)</td>
</tr>
<tr>
<td>WdCHS4A-1</td>
<td>WdCHS4 insertion mutant, wdchs4::hph</td>
<td>ATCC MYA888; Wang et al. (1999)</td>
</tr>
<tr>
<td>WdCHS5A-11</td>
<td>WdCHS5 replacement mutant, wdchs5::hph</td>
<td>Liu et al. (2001)</td>
</tr>
</tbody>
</table>

* All mutants used in this study were derived from the wild-type parental strain 8656.
Compensatory expression among \textit{WdCHS} genes

Table 2. Primers and products

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Product size (bp)</th>
</tr>
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<tr>
<td>Wdchs1-F</td>
<td>GCTCCATCGATACCACCTTTCTTCAT</td>
<td>405</td>
</tr>
<tr>
<td>Wdchs1-R</td>
<td>CTTCGCACGGTGGCCATATGTTCG</td>
<td></td>
</tr>
<tr>
<td>Wdchs2-F</td>
<td>GCCTCATCGACTAGTGCTGACCATG</td>
<td></td>
</tr>
<tr>
<td>Wdchs2-R</td>
<td>GAGATTTTCCGGAGAACTTGCAG</td>
<td>402</td>
</tr>
<tr>
<td>Wdchs3-F</td>
<td>CTAATTCAACAAAAACTTCCAG</td>
<td></td>
</tr>
<tr>
<td>Wdchs3-R</td>
<td>CCACTATGTTAGCAAGACTCTCC</td>
<td>410</td>
</tr>
<tr>
<td>Wdchs4-F</td>
<td>GAGCCCGATCATGTCTAATCCCAATCC</td>
<td></td>
</tr>
<tr>
<td>Wdchs4-R</td>
<td>GTAGATTTCCAAATACCTTGGAGGC</td>
<td>416</td>
</tr>
<tr>
<td>Wdchs5-F</td>
<td>CCACTATGTTAGCAAGACTCTCC</td>
<td></td>
</tr>
<tr>
<td>Wdchs5-R</td>
<td>CAGAGTTCACTATAACCTGCCATG</td>
<td>452</td>
</tr>
</tbody>
</table>

were carefully calibrated for each experiment to ensure that the RT-PCR was done within the exponential phase of amplification. For each sample, a parallel negative control having the same components, except avian myeloblastosis virus RT, as those used in the standard RT-PCR was subjected to amplification to ensure the absence of trace DNA contamination. The RT-PCR products were examined using 1.2% ethidium bromide/agarose gel electrophoresis, viewed under UV and finally analysed densitometrically with the AlphaImager 1220 documentation and analysis system (Carnock).

RNA dot-blots. To investigate the relative transcript abundance of the five \textit{WdCHS} genes, the same samples prepared for RT-PCR were also used for RNA dot-blot analysis. For this, 500 ng total RNA from wild-type cells cultured at 37 °C for 24 h was heat-denatured at 75 °C for 10 min and then blotted onto five pieces of nylon membranes in parallel, so that the same amount of total RNA would be detected separately by the five probes. After UV cross-linking, blotted membranes were pre-hybridized for 0.5 h and then hybridized for 2 h at 68 °C by using PerfectHyb Plus hybridization buffer (Sigma). The same primers used for RT-PCR (Table 2) were used to amplify \textit{W. dermatisidis} genomic DNA to generate the \textit{WdCHS} DNA fragment required for probes, which were labelled with \textsuperscript{32}P using the DECAprime random priming DNA labelling kit (Ambion). The relative radioactive signal was measured with a PhosphorImager (445 S1).

RESULTS AND DISCUSSION

Compensatory expression of the five \textit{WdCHS} genes at the temperature of infection (37 °C)

Although \textit{W. dermatisidis} grows at temperatures up to 40 °C or slightly higher (Kwon-Chung & Bennett, 1992), 37 °C is most often associated with growth in its human host, and 25 °C is considered representative of a saprophytic environment. Therefore, we hypothesized that studies of \textit{WdCHS} gene expression in the wild-type strain grown under different temperature regimens might reflect the different behaviour of these genes under one selected condition of infection, and serve as a first step toward discovering insights into how their encoded proteins might contribute to virulence. The three temperature regimens used in these experiments were as follows: (1) cells were cultured at 25 °C until harvest, i.e. at 25 °C for 24 h; (2) cells were shifted from 25 to 37 °C a short time before harvest, i.e. at 25 °C for 21 h plus 37 °C for 3 h; (3) cells were cultured at 37 °C until harvest, i.e. at 37 °C for 24 h. Based on the RT-PCR results (Fig. 1a) and the densitometry data (Fig. 2a), the responses of the five \textit{WdCHS} genes to the three culture regimens turned out to be quite varied. For example, the expression levels of \textit{WdCHS1} and \textit{WdCHS2} changed little with temperature change, regardless of whether the exposure to the higher temperature was for 3 h or 24 h. The modest increase in transcription detected for these two genes possibly explains why no differential expression was detected previously by Northern analysis of total RNA (unpublished data), and is in agreement with a recent result derived by the method used in this study for \textit{WdCHS2} (Wang et al., 2001). In contrast, the \textit{WdCHS5} transcription level increased quickly and almost doubled when cells were shifted to 37 °C, compared to cells maintained at 25 °C, and remained high when the cells were kept at this temperature for an additional 21 h. \textit{WdCHS3} transcription, like that of \textit{WdCHS5}, also responded quickly to the temperature increase. However, unlike the increased expression of \textit{WdCHS5}, that of \textit{WdCHS3} continued to increase to levels 3-5 times higher than those observed at 25 °C. Consistent with these data were the results of the relative abundances of the five \textit{WdCHS} genes as determined by RNA dot-bolts, which also showed that of the five \textit{WdCHS} genes of \textit{W. dermatisidis}, the \textit{WdCHS3} transcript was most abundant and the \textit{WdCHS5} transcript was the second most abundant when cells were grown at 37 °C (Fig. 3). Increased expression of \textit{WdCHS4} in cells shifted to 37 °C for 24 h was also detected. This was unexpected because a prior Northern result with total RNA suggested there was little, if any, increased transcription associated with this temperature shift (Wang et al., 1999). Possibly the more sensitive semi-quantitative RT-PCR used in the present study allowed for the detection of the increased levels of transcription of \textit{WdCHS4} at 37 °C above that of cells grown at 25 °C (Fig. 2a).

The observed expression behaviours of \textit{WdCHS3} and \textit{WdCHS5} suggest that their products may serve in a
stress-response capacity, which could have relevance to virulence. This is consistent with results from previous reports that showed the following: (1) that the single chitin synthase disruption mutant *wdchs*Δ (Liu et al., 2001) and the double mutant *wdchs*2Δ*wdchs*3Δ (Wang et al., 2001) were both significantly less virulent in a mouse model of acute infection; (2) that the *WdCHS3* transcript could be detected in Northern blots of total RNA only from cells subjected to a variety of stress conditions, including culture at 37 °C (Wang & Szaniszlo, 2000); (3) that the *WdChs3p* detected by Western blots accumulated linearly with time from 0 to 24 h when cells were shifted from 25 to 37 °C, and with a temperature increase from 18 to 42 °C (Wang & Szaniszlo, 2000); (4) that at least one negative regulatory element exists in the 5′ upstream sequence of *WdCHS3* and that many putative *cis*-regulatory elements also exist in that sequence, as well as in the 5′ regulatory sequence of *WdCHS5* (Wang & Szaniszlo, 2000; unpublished data). In this respect, it is interesting to note here that *WdCHS3* and possibly *WdCHS5*, which encode class III and class V *WdChsp* isozymes, respectively, seem to be more responsive to stress conditions than are the other *WdCHS* genes. This is in marked contrast to the situation in *S. cerevisiae* and *Candida albicans*, which have no genes that encode class III and class V orthologues and where the main stress-regulated genes (*CHS3*) encode class IV enzymes. This suggests that the stress-induced regulation of *CHS* genes has changed significantly among fungi over time.

**Compensatory expression of the five *WdCHS* genes in single *wdchs* disruption mutants**

Five chitin synthase (*WdCHS*) genes have been cloned, characterized to different degrees, and disrupted singly and in various combinations. The absence of a dramatic decrease in chitin contents in any single mutant makes us assume that general compensation by at least one chitin synthase exists when one of the other *WdCHS* genes is defective. This suggested that study of the relative transcription levels of each of the remaining four *WdCHS* genes in the absence of one other might help confirm this hypothesis. For these experiments, cells of each *wdchs*Δ mutant and of the wild-type were grown at 37 °C for 24 h. The RT-PCR results (Fig. 1b) were evaluated after the densitometry data of each *WdCHS* mutant were normalized against those of the wild-type. The results (Fig. 2b) showed that the expression levels detected again varied differently depending upon which *WdCHS* gene was defective. These results also supported the notion that the product of some *WdCHS* genes might have a special role in cell proliferation and/or in maintaining the structural integrity of the cell wall. For example, *WdCHS1* and *WdCHS2* both appear to compensate somewhat for each of the other chitin synthase genes in their respective mutants. Furthermore, *WdCHS1* disruption was compensated for only by higher expression of *WdCHS2* (Fig. 2b; compare data in vertical row 1), whereas *WdCHS2* disruption appears to be compensated for by increased expression of *WdCHS1*, together with *WdCHS3* and *WdCHS4* (compare data in vertical row 2). These particular two datasets provide additional supporting evidence for the idea that *WdCHS1* and *WdCHS2* are functionally overlapping in *vivo* (Wang et al., 2001). They may also help explain why *WdCHS1* and *WdCHS2* cannot both be defective in the same background for cell viability at 37 °C, in spite of the fact that they can grow weakly, albeit abnormally, at 25 °C (Zheng et al., 1997). Taken together with the above observation that *WdCHS1* and *WdCHS2* responded only weakly to a temperature increase (Fig. 2a), these data indicate that *WdCHS1* and *WdCHS2* play basic roles in cell proliferation. This hypothesis is further supported by our finding that the transcription levels for both *WdCHS1* and *WdCHS2* are the least abundant among those of the five *WdCHS* genes assayed, based on a relative abundance analysis by
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Fig. 2. Densitometry analysis of the RT-PCR products depicted in Fig. 1(a–c), to estimate the relative transcriptional level of each WdCHS gene when the wild-type and mutant cells were cultured as indicated for Fig. 1. The black, hatched and white bars in (a) indicate that the strains involved were cultured at 25 °C for 24 h, at 25 °C for 3 h at 37 °C, or at 37 °C for 24 h, respectively; in (c), the black, hatched and white bars indicate that the data were derived from cultures of the wild-type, Mc3 or Hf1, respectively. The mean range of variation measured in the two independent experiments is shown.

Fig. 3. Relative abundance of each of the five WdCHS transcripts assayed by RNA dot-blotting (see Methods). (a) The PhosphorImager pictures. (b) Densitometry analysis of the PhosphorImager pictures by use of the ImageQuant 5.1 program. The relative abundance data for the other four WdCHS transcripts were obtained by normalizing against WdCHS1. The results shown are representative of two independent experiments. The mean range of variation measured in the two independent experiments is shown.

Compensatory expression of the five WdCHS genes in two ts morphological mutants

M3c and Hf1 are two ts morphological mutants of W. dermatitidis. These mutants have special importance because whereas the wild-type produces mainly yeast cells at both 25 and 37 °C in rich media, the two ts mutants form multicellular (sclerotic) forms and hyphae, respectively, from yeast cells when shifted from the lower to the higher temperature in the same media (Roberts & Szaniszlo, 1978; Cooper & Szaniszlo, 1993; Ye & Szaniszlo, 2000). Thus, results with these ts mutants and the wild-type grown identically can be compared in such ways that any differences detected among them can be ascribed to changes in specific morphology and not to differences in environments. Previous results have shown that total chitin content and chitin synthase activity increased in the mutants compared to the wild-type when W. dermatitidis was grown in its multicellular form or as hyphae (Szaniszlo et al., 1983; Wang & Szaniszlo, 2000). The results here suggest that the higher chitin synthase levels and chitin increases noted previously may have resulted, in part, from the

dot-blotting of RNA from cells grown at the restrictive temperature (Fig. 3).

Because the expression level of WdCHS5 changed little in response to the disruption of other WdCHS genes, it seems that the product of WdCHS5 is unique and may play roles very distinct from those of the other WdCHS genes. This possibility is enhanced by the knowledge that WdCHS5 has a nucleotide sequence that is divergent from those of the other four WdCHS genes when they are multiply aligned (data not shown), that its derived protein has a myosin motor-like domain, and, most importantly, by the observation that among the five different single wdcbsΔ mutants, only wdcbsΔ mutants showed loss of viability in stationary phase at 37 °C, and loss of virulence in a mouse model of acute infection (Liu et al., 2001). On the other hand, WdCHS expression in a wdcbsΔ mutant was compensated for by each of the other four remaining WdCHS genes (Fig. 2b; compare data in vertical row 5). These data support the idea that even though the other four WdCHS genes were induced to higher levels of transcription in the wdcbsΔ mutant, they are either not translated or their encoded proteins do not function in ways that can compensate for the loss of WdChs5p function and prevent eventual cell death at 37 °C.
higher levels of expression of WdCHS1, WdCHS3 and WdCHS4 in the case of the ts multicellular-form mutant, Mc3, and of WdCHS1 and WdCHS3 in the case of the ts hyphae-producing mutant, Hf1. Although the results with WdCHS3 were not unexpected, because they were indicated previously by Northern analyses (Wang & Szaniszlo, 2000), those with WdCHS4 and particularly WdCHS1 were not anticipated. Possibly they indicate special roles for the products of these genes in multicellular forms and hyphae. However, as yet, attempts to derive mutants with these genes disrupted in the Mc3 and Hf1 backgrounds have all failed, possibly because some WdChsp defects in these backgrounds impart synthetic lethality. The results with WdCHS5 were also somewhat unexpected, at least for those in the Hf1 background. In this case, we had suspected that WdCHS5 (class V) would be the most highly expressed WdCHS gene in Hf1 at 37 °C, because the first class V chitin synthase was first isolated from A. nidulans (Fujiiwara et al., 1997), and then was found subsequently only among other filamentous fungi (Park et al., 1999; Zhang & Gurr, 2000; Liu et al., 2001). However, WdCHS5 expression in Hf1 did not increase. Instead, it occurred at a level equal to those of the wild-type and Mc3, a result consistent with the recent finding that a wdchs5Δ mutant did not show any apparent morphological difference from the wild-type when both were cultured identically in a medium that induced hyphae.

Conclusions

The primary aim of this study was to investigate the hypothesis that compensatory expression of individual chitin synthase genes of W. dermatitidis occurs in response to stress stimuli. The secondary aim was to elucidate further the possible functional relationships among the WdCHS-encoded proteins. The results presented here showed that each of the five WdCHS genes exhibited a somewhat different expression pattern in response to the different growth situations tested, which supports the hypothesis that the expression of each one may have special, and sometimes distinct, roles in cell growth, viability and virulence. On the other hand, a general compensatory expression was evident in each single chitin synthase disruption mutant, which may explain the previous observations that chitin contents were not dramatically decreased in any single wdchsΔ mutant, and that no single wdchsΔ mutant lost viability at 25 °C (Zheng, 1997; Wang et al., 1999, 2001; Wang & Szaniszlo, 2000; Liu et al., 2001). We postulate that the general compensatory expression detected in W. dermatitidis in response to chitin synthase gene disruption and to exposure to other stresses might function as part of the cell wall integrity pathway in a manner similar to that recently described in S. cerevisiae (Smits et al., 1999). Support for this possibility is provided by very recent results that show that the transcripts of all five WdCHS genes are up-regulated in mutants defective in melanin biosynthesis (unpublished data). It is suspected that similar up-regulatory patterns of WdCHS transcription will be found that correlate with other cell wall defects.

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