Identification of fungal cell wall mutants using susceptibility assays based on Calcofluor white and Congo red

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The fungal cell wall is an essential organelle and represents a considerable metabolic investment. Its macromolecular composition, molecular organization and thickness can vary greatly depending on environmental conditions. Its construction is also tightly controlled in space and time. Many genes are therefore involved in building the fungal cell wall. Here we present a simple approach for detecting these genes. The method is based on the observation that cell wall mutants are generally more sensitive to two related anionic dyes, Calcofluor white (CFW) and Congo red (CR), both of which interfere with the construction and stress response of the cell wall. CFW-based and CR-based susceptibility assays identify cell wall mutants not only in ascomycetous yeasts (such as *Saccharomyces cerevisiae* and *Candida albicans*) but also in mycelial ascomycetes (such as *Aspergillus fumigatus* and *Aspergillus niger*), basidiomycetous species (*Cryptococcus neoformans*) and probably also zygomycetous fungi. The protocol can be completed in 4–6 h (excluding the incubation time required for fungal growth).

INTRODUCTION

The chemical structures of Calcofluor white (CFW) and Congo red (CR) are shown in **Figure 1**. Both compounds contain two sulfonic-acid groups. CFW and CR exert their antifungal activities only when they are solubilized — that is, under slightly acidic, neutral or basic conditions, when their sulfonic-acid groups are negatively charged[1]. For this reason, in CFW-based and CR-based susceptibility assays the pH of the medium should not be allowed to drop below 5.5. Importantly, because CFW and CR each possess two negative charges under the chosen test conditions, it is believed that they cannot pass through the plasma membrane and target components of the cell wall that are external to it[2].

In vitro, CFW and CR interact with various β-linked glucans[3], whereas *in vivo* in the alga *Poterioochromas stipitata* they interact strongly with nascent chitin chains[4]. As CFW preferentially stains chitin in the cell wall of fungi[5], it (and by analogy CR) is thought to interfere with cell wall assembly by binding to chitin. Conceivably, CFW and CR act by binding to nascent chitin chains, thereby inhibiting the assembly enzymes that connect chitin to β-1,3-glucan and β-1,6-glucan. As a result, the cell wall becomes weakened.

The addition of CFW or CR to growing fungal cells results in cell wall-related morphological changes, such as incomplete separation of mother and daughter cells in *Saccharomyces cerevisiae*[6,7], probably by inhibiting the action of chitinases. The presence of CFW or CR also results in swelling or lysis of hyphal tips in the filamentous fungi *Aspergillus niger* and *Geotrichum lactis*, as a result of the cell wall-weakening effect of these compounds and the internal turgor pressure[7–10]. The cell wall-weakening effect of CFW and CR activates the cell wall stress response (see ref. 11 for a review). This response includes transcriptional activation of many genes encoding proteins that have cell wall-reinforcing functions[12–14]. It also includes increased deposition of chitin in the cell wall[15–17]. As CFW and CR are believed to bind to chitin, these compounds also directly counteract the cell wall stress response itself.

Testing for altered susceptibility to CFW and CR is commonly used to identify cell wall mutants in fungi. Some mutants with lowered chitin levels in their walls become more resistant to CFW and CR. For example, *S. cerevisiae* mutants with a defective *chs3* gene, which encodes the major chitin synthase, and with defective *chs4–chs7* genes, which are required for proper Chs3p activity, are more resistant to CFW and CR[16,18]. Most cell wall mutants, however, have more chitin in their walls than wild-type cells, because of activation of the cell wall stress response, and become more sensitive to CFW and CR. For example, mutants disturbed in the synthesis of β-1,3-glucan (e.g., *fks1* and *gas1*) or β-1,6-glucan (e.g., *kre6* and *cwh41*), mannosylation of mannoproteins (*mnn9* and *vrg1*) and glycosylphosphatidylinositol (GPI)-anchor

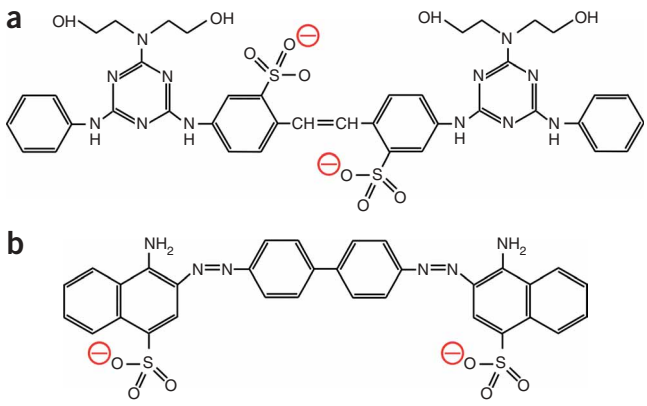

Figure 1 | The chemical structures of CFW (**a**) and CR (**b**). Note the presence of the negatively charged sulfonic-acid groups in both dyes at pH 5.5 or higher. As the negative charges of CFW and CR are essential for their antifungal activity, the pH of the medium should not drop below 5.5 to prevent protonation of the sulfonic-acid groups.

biosynthesis (*gpi1* and *gpi3*) have increased chitin levels, and are hypersensitive to CFW and CR[15,16]. Intriguingly, not all mutants with a CFW-hypersensitive phenotype display increased chitin levels[15,19], indicating that the chitin level in the cell wall is not the only factor determining CFW sensitivity.

Increased susceptibility to CFW or CR is indicative of cell wall defects not only in *S. cerevisiae*, but also in other yeasts (such as *Candida albicans*[20] and *Yarrowia lipolytica*[21]) as well as mycelial fungi (such as *A. niger*[9,10], *Aspergillus nidulans*[22] and *Aspergillus awamori*[23]) and the basidiomycetous fungus *Cryptococcus neoformans*[24]. As CFW also inhibits the growth of the basidiomycetous fungus *Rhodotorula rubra* and the zygomycetous fungus *Mucor rouxii*[7], it seems likely that CFW-based and CR-based susceptibility assays could also be used in these and related organisms. Interestingly, growth of the ascomycetous fission yeast *Schizosaccharomyces pombe*, which has no chitin in its wall, is resistant to CFW[7], consistent with the notion that CFW preferentially interacts with chitin.

The concentration of CFW or CR to be used in susceptibility assays depends on several parameters. Both the size of the inoculum and the fungal species to be tested might affect the inhibitory effect of CFW[7]. Test concentrations of CFW and CR vary not only between different fungi, but also depending on the genetic background within the same species. The preferred way to determine CFW or CR susceptibility is to inoculate a concentration series of cells (in the case of yeasts) or asexually derived conidiospores (in the case of mycelial fungi) in the form of spots on plates containing CFW and CR. For the reasons mentioned above, an initial experiment is required to determine the appropriate CFW or CR concentration. Preferably, the selected concentration should be sublethal for the wild-type strain. In the spot assay, the concentrations of CFW or CR used for *S. cerevisiae* are between 10 and 100 μg ml^{-1}, respectively[13,19,25]. The concentrations used for *A. niger* are higher and vary between 50 and 1,000 μg ml^{-1} (ref. 26). *C. neoformans* is relatively insensitive to CFW and CR, and high concentrations of 1.5 mg ml^{-1} CFW and 5 mg ml^{-1} CR have been used to test its susceptibility[24].

Testing for increased susceptibility to other drugs (such as SDS, caffeine and hygromycin) has also been used to identify (additional) cell wall-defective mutants[19,25]. To characterize cell wall-defective mutants in more detail, further phenotypic tests might be carried out, which include measuring changes in sensitivity to the β1,3-glucanase zymolyase, killer toxins, β1,3-glucan synthase inhibitors (caspofungin or papulacandin B) and vanadate, or by determining the sugar composition of the cell wall[15,19,25].

MATERIALS

REAGENTS

- CFW fluorescent brightener (F-3543, Sigma)
- CR (860956, Sigma)
- 10% potassium hydroxide (wt/vol; KOH; Sigma)
- 87% glycerol (vol/vol; Sigma)
- 0.5 M 2-(*N*-morpholine)-ethane sulfonic acid-sodium hydroxide (MES-NaOH) pH 6.0 (Sigma)

EQUIPMENT

- Agar plates with fungal growth medium (the composition of the growth medium is dependent on the species tested)
- Hemocytometer (Bürker-Türk, Marienfeld)

REAGENT SETUP

1% (wt/vol) CFW or CR stock solutions Timing is ~1 h. Make sure that the compounds are well dissolved in sterile water. Sterilization of the stock solutions by filtration is optional, and we routinely omit this step without encountering infections by other microorganisms. CFW M2R, which is the di-sodium salt form, is readily soluble in water; this was a product of the American Cyanamid Company, but is no longer available. The free di-acid form of CFW (fluorescent brightener F-3543) does not directly dissolve in water. We prepare a 1% (wt/vol) stock solution by dissolving CFW in 0.5% (wt/vol) KOH and 83% (vol/vol) glycerol. CR is in the di-sodium form and is readily dissolved in water. ! **CAUTION** CFW and CR are hazardous and potentially carcinogenic. Wear protective gloves and other protective clothing. Wear a face mask to prevent inhalation. ▲ **CRITICAL** Prepare fresh on the day of the experiment. During handling, CFW and CR solutions should be protected from light by wrapping the containers in aluminum foil.

Growth medium for agar plates Timing is ~30 min. The medium should be buffered to pH 5.5–7.0 to prevent acidification, and therefore protonation and precipitation of CFW and CR. Buffering the medium with 50 or 100 mM MES-NaOH to pH 6.0 works well for *S. cerevisiae* and *A. niger*, respectively. Alternative buffers are 50 mM phthalate-NaOH (pH 6.0), 50 mM succinate-NaOH (pH 6.0) and 50 mM HEPES-NaOH (pH 7.0). The plates can be stored overnight to be used the next day. We avoid storing CFW and CR plates for longer. As a precaution, CFW and CR plates are kept in the dark during overnight storage at room temperature (between 15 and 25 °C).

PROCEDURE

Preparation of agar medium ● TIMING ~1–2 h

1| Autoclave the agar medium. Add CFW or CR from the stock solution to melted and cooled agar medium (60–70 °C) to reach the desired concentration.

Preparation of a dilution series of cells or spores ● TIMING ~1–2 h

2| Prepare a 10-fold dilution series of cells or spores. Spot 5 μl containing 5×10^4 to 5×10^0 cells or spores on plates containing CFW or CR and a control plate. Yeast cell or spore concentrations should be determined using a hemocytometer.

Incubation of plates ● TIMING ~2–4 d

3| Incubate the plates at 30 °C or, if desired, at a higher temperature for 2–4 d. As a precaution to prevent possible inactivation of CFW and CR by light, the plates should be incubated in the dark. Note that the sensitivity to CFW and CR tends to increase strongly at higher temperatures (≥37 °C depending on the species tested).

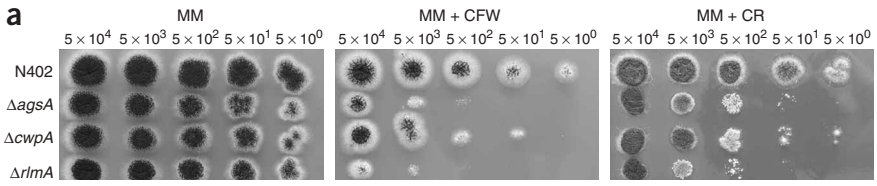

Figure 2 | Susceptibility of *A. niger* cell wall mutants to CFW (200 μg ml^{-1}) or CR (150 μg ml^{-1}). Ten-fold dilutions, starting at 5 × 10^4 spores, were spotted on minimal medium plates buffered with 100 mM MES-NaOH (pH 6.0; **a**), or similar plates containing CFW (200 μg ml^{-1}; **b**) or CR (150 μg ml^{-1}; **c**). Pictures were taken after 3 d of growth at 30 °C. The strains used are the N402 wild-type parental strain, the *ΔagsA* knockout strain lacking α1,3-glucan synthase A[9], the *ΔcwpA* knockout strain lacking cell wall protein A and the *ΔrlmA* knockout strain lacking the MADS-box transcription factor required for transcriptional activation of cell wall stress-responsive genes[10].

Interpreting and monitoring results

4| Sensitivity to CFW and CR is determined by comparing the extent of colony formation (in the case of filamentous fungi) or colony density (in the case of yeasts) between parental and mutant strains on the control plate and the plates containing CFW or CR. As an example, the increased sensitivity to CFW and CR of some *A. niger* cell wall mutants is shown in **Figure 2**.

? TROUBLESHOOTING

● TIMING

Preparation of 1% (wt/vol) CFW or CR stock solutions: ~1 h
Preparation of growth medium for agar plates: ~30 min
Preparation of agar medium (Step 1): ~1–2 h
Preparation of a dilution series of cells or spores (Step 2): ~1–2 h
Incubation of plates (Step 3): 2–4 d

? TROUBLESHOOTING

Precipitation of CFW (and therefore insensitivity to it) or of CR indicates that the pH of the medium has dropped below 5.5. A stronger buffer or another buffer system (see REAGENT SETUP) might be tried as an alternative.

CFW-based and CR-based susceptibility assays are simple and powerful methods for the identification of fungal cell wall mutants. Sensitivity to CFW and CR depends on several parameters, including the fungal species or strain background, the inoculum size and the growth medium. Therefore, one should start by determining a sub-lethal concentration of CFW or CR that affects the growth of the wild-type strain under the selected growth conditions. Small differences in the susceptibility of the wild-type and mutant strains might require the use of a series of CFW or CR concentrations to identify that which gives the largest growth difference.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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