Functional screening for resistance genes against trichothecenes in the library of *Saccharomyces cerevisiae* deletion mutants

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**Abstract**

Trichothecenes are mycotoxins produced by various species of fungi, including *Fusarium* species. They contaminate important crops such as wheat and corn, and cause toxicity in animals and humans mainly by inhibition of protein synthesis. In this study, we performed genome-wide screening of genes that play roles in protection of *Saccharomyces cerevisiae* against A-type trichothecene, T-2 toxin, and D-type trichothecene, verrucarin A. Although a distinct difference exists in the structures of these two trichothecenes, these toxins inhibited the growth of the mutant strains of *S. cerevisiae* with common disrupted genes. The genes whose deletion conferred high sensitivity to these trichothecenes were considered as trichothecene resistance genes, and they included the ones encoding ABC transporter protein, enzymes for ergosterol biosynthesis, vacuolar H⁺-ATPase, and others. These results possibly lead to the construction of a yeast detection system with improved sensitivity to trichothecenes.

**Introduction**

Trichothecenes are fungal secondary metabolites characterized by a tetracyclic 12, 13-epoxy-trichothec-9-ene skeleton, and they have been implicated in adverse effects on the health of humans and livestock that are exposed to them¹). Trichothecenes are a large family of mycotoxins that contains more than 200 compounds²), and are produced by various fungal genera, including *Fusarium, Myrothecium,* and *Trichoderma.*

Based on chemical structures, trichothecenes are divided into 4 groups, Type A, B, C, and D³). Type A trichothecenes have a hydroxyl group, an ester side chain, or no side chain at C-8, and T-2 toxin (Fig. 1), produced by *Fusarium sporotrichioides* and other *Fusarium* species, belongs to this group. Type B trichothecenes were distinguished from Type A by the presence of a keto group at C-8. They include deoxynivalenol.

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and nivalenol that are produced by *Fusarium* species such as *F. graminearum* and *F. culmorum*, and are known for being the main contaminants in agriculture products. Type C trichothecenes have a second epoxide ring at C-7, 8. Type D trichothecenes are exemplified by macrocyclic trichothecene and verrucarin A, produced by *Myrothecium* and others, belongs to this group. Trichothecene toxicity follows the order Type D > Type A > Type B⁹, while Type C are hardly toxic.

Trichothecenes are well-known translation inhibitors, which bind to the eukaryotic peptidyltransferase, and result in the induction of ribotoxic stress response followed by cell death in eukaryotic cells⁹. However, the molecular mechanisms of trichothecene-induced toxicities are more complicated than a simple function of inhibition of translation, and a critical role for the mitochondrial translation and membrane maintenance has been suggested⁹.

Among eukaryotic microorganisms, *Saccharomyces cerevisiae* is relatively resistant to trichothecenes, compared to other sensitive yeasts, such as *Kluyveromyces marxianus*⁷; thus, *S. cerevisiae* may have genes that play a role in resistance to trichothecenes. Since a collection of deletion mutants of *S. cerevisiae*, in which each gene was knocked out via homologous recombination, was available, we performed genome-wide screening for resistance genes against trichothecenes. Elucidation of trichothecene resistance genes could contribute to the construction of genetically engineered yeast mutants for sensitive detection of trichothecenes and/or to the development of techniques that can be applied to control *Fusarium* species and their mycotoxins.

**Materials and Methods**

**Reagents**  Verrucarin A was purchased from Sigma-Aldrich Co. (St Louis, MO). T-2 toxin was obtained as briefly described; *F. sporotrichioides* (NBRC9955) was incubated in liquid yeast-glucose-peptone medium with shaking at 28 °C for 3-5 days, and extracted with ethyl acetate. The extract was applied to a gel column (Sephadex LH-20; GE Healthcare, Pittsburgh, PA) and eluted with methanol. The fractions containing T-2 toxin were developed on a TLC (Merck F₂₅₃ silica TLC, Whitehouse Station, NJ) by using ethyl acetate/
toluene (3:1), and the T-2 toxin on a plate was scraped and extracted with ethyl acetate. The purity of T-2 toxin used for assays was higher than 97% as confirmed by HPLC with UV detection at 195 nm.

**Yeast deletion library screen** The *S. cerevisiae* strain BY 4742 (MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and its gene knockout library containing nearly 5,000 viable strains were obtained from Open Biosystems (Huntsville, AL).

In the first screen, deletion strains in the BY4742 background were screened for their sensitivity to T-2 toxin (5.0 µg/ml) or verrucarin A (0.5 µg/ml). The pre-culture of each gene-disrupted mutant strain was grown in YPD liquid medium in 96-well plates with shaking (Invitro shaker, TAI-TEC Co., Saitama, JAPAN) overnight at 30 °C. Subsequently, 10 µl of each inoculate was transferred to 96-well plates containing 190 µl of YPD liquid medium with T-2 toxin (5.0 µg/ml), verrucarin A (0.5 µg/ml), or the vehicle only (untreated control), and was incubated for 18 h with shaking at 30 °C. The OD₆₂₀ for treated and untreated strains was obtained with a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA), and the value was subtracted by the corresponding OD₆₂₀ at zero time-point. The relative growth rate was calculated as the OD₆₂₀ ratio of the toxin-treated samples to the untreated controls. The experiment was performed in duplicate. When the growth inhibition rate of a toxin-treated sample was more than 40% compared to untreated control, the disrupted gene in the strain was defined as a candidate resistance gene against the toxin. In the second screen, the selected candidate strains were screened again with lower concentrations of trichothecenes, T-2 toxin (2.5 µg/ml) and verrucarin A (0.15 µg/ml). The strains (except pseudogene deletion strains) that showed 60% growth inhibition by both of these trichothecenes were subjected to a dose-response assay.

In a dose-response assay, the selected strains were pre-incubated in test tubes overnight with shaking at 30 °C, and the OD₆₂₀ of each strain was adjusted to 0.1. Thereafter, the strains were incubated with various concentrations of T-2 toxin (0, 0.05, 0.15, 0.5, 1.5, 5.0, 15.0, and 50.0 µg/ml) for 20 h at 30 °C and the growth inhibition rate was calculated; these experiments were performed in triplicate. We also added several deletion mutant strains that might be sensitive to trichothecenes according to the previous studies⁵,⁸ but could have been missed in our screening.

We also classified all the selected genes in the second screening into distinct functional classes according to Saccharomyces Genome Database (http://www.yeastgenome.org/).

**Results and Discussion**

In the first screening with T-2 toxin (5.0 µg/ml) or verrucarin A (0.5 µg/ml), 123 and 109 deletion strains were selected as being sensitive to T-2 toxin and verrucarin A, respectively. In total, 194 strains were selected, while 38 strains were common between the 2 groups. Subsequently, the 194 selected strains in the first screening were passed through a second screening with T-2 toxin (2.5 µg/ml) or verrucarin A (0.15 µg/ml), and 45 and 43 deletion strains were selected as sensitive, respectively. In total, 65 deletion strains were selected, while 23 deletion strains were selected in common between the 2 groups; vma5Δ, vma7Δ, vma10Δ, vma13Δ, vph2Δ, erg4Δ, erg6Δ, gal11Δ, srb5Δ, rpb4Δ, spt10Δ, swi3Δ, taf14Δ, hfi16Δ, gon7Δ, atp2Δ, fzo1Δ, chc1Δ, pdr5Δ, arg82Δ, ref2Δ, sac1Δ, and YKL118WΔ (a pseudogene deletion mutant, which partly lacks VPH2). These results suggest that although these 2 trichothecenes have distinct differences in their structures, they inhibited the growth of strains through a common disrupted gene.
Next, we performed a dose-response assay of yeast growth with T-2 toxin as an inhibitor, and examined 22 gene deletion mutants (Table 1). The IC₅₀ value of the wild-type strain BY4742 was approximately 50 µg/ml, and this strain was less sensitive than any selected deletion mutants. Nine strains, including erg6Δ and pdr5Δ, were highly sensitive to T-2 toxin (IC₅₀ < 5 µg T-2 toxin/ml). In this dose-response study, the inhibition rates of these strains were relatively low compared with the inhibition rates in the screening, probably because of a difference in incubation conditions between these 2 experiments.

We categorized the 65 selected genes in the second screening, based on the functions of the proteins encoded (Table 2). In our study, one of the most sensitive deletion mutants was pdr5Δ, a strain with a disrupted copy of the gene encoding ATP-binding cassette (ABC) transporter. The pdr phenotype has shown a loss of “pleiotropic drug resistance”⁹, and S. cerevisiae strains with a disrupted PDR5 gene were reported to be hypersensitive to deoxynivalenol and other trichothecenes¹⁰,¹¹.

It was also clearly shown that the deletion mutants that lack the genes encoding proteins related to ergosterol biosynthesis were highly sensitive to these trichothecenes: erg2Δ, erg3Δ, erg4Δ, erg6Δ, and erg24Δ. Ergosterol is an important and major sterol constituent of membranes of yeasts and fungi, and it has been

<p>| Table 1: The deletion mutants classified based on IC₅₀ values of T-2 toxin |
|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>IC₅₀ values</th>
<th>Deletion mutants of BY4742</th>
</tr>
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<tbody>
<tr>
<td>1.5 – 5.0 µg/ml</td>
<td>chc1 Δ, erg6 Δ, fzo1 Δ, hfl1 Δ, pdr5 Δ, spt10 Δ, taf14 Δ, vma7 Δ, vph2 Δ</td>
</tr>
<tr>
<td>5.0 – 15 µg/ml</td>
<td>atp2 Δ, erg4 Δ, gal11 Δ, gon7 Δ, ref2 Δ, rpb4 Δ, sac1 Δ, srb3 Δ, vma5 Δ, vma10 Δ, vma13 Δ</td>
</tr>
<tr>
<td>15 – 50 µg/ml</td>
<td>arg82 Δ, swi3 Δ</td>
</tr>
</tbody>
</table>

<p>| Table 2: Functional categories of the genes whose deletion conferred sensitivity to T-2 toxin and/or verrucarin A |
|-----------------------------|-----------------------------|</p>
<table>
<thead>
<tr>
<th>Standard names of the genes</th>
<th>Functional Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR1, FEN2, PDR5</td>
<td>Membrane transporter protein</td>
</tr>
<tr>
<td>ERG2, ERG3, ERG4, ERG6, ERG24</td>
<td>Ergosterol biosynthesis</td>
</tr>
<tr>
<td>PPA1, TFP3, VMA4, VMA5, VMA7, VMA10, VMA13, VMA22, VP2</td>
<td>Subunit of vacuolar H⁺-ATPase, or required for V-ATPase</td>
</tr>
<tr>
<td>PEP3, PEP5 / VPS15, VPS16, VPS51</td>
<td>Vacuole biogenesis/vacuolar protein sorting</td>
</tr>
<tr>
<td>AKR1, CHC1, SHE4</td>
<td>Involved in endocytosis</td>
</tr>
<tr>
<td>PHO23, RPD3, SIN3, SPT10, SWI3</td>
<td>Chromatin remodeling</td>
</tr>
<tr>
<td>GAL11, PGD1, SRB2, SRB5</td>
<td>Subunit of the RNA polymerase II mediator complex</td>
</tr>
<tr>
<td>RPB4 / PAF1</td>
<td>RNA polymerase II subunit/RNA Polymerase II Associated Factor</td>
</tr>
<tr>
<td>BUD22, BUD23, EFG1</td>
<td>Protein required for maturation of 18S rRNA</td>
</tr>
<tr>
<td>BUD32, GON7</td>
<td>Proteins required for tRNA modification</td>
</tr>
<tr>
<td>RPS16B, RPS17A</td>
<td>Ribosomal protein</td>
</tr>
<tr>
<td>ATP2, FZO1, GRX5</td>
<td>Mitochondrial protein</td>
</tr>
<tr>
<td>ANP1, ARG82, BDF1, CAX4, DEP1, HFI1, LEA1, MOT2, PHO85, REF2, SAC1, SPT20, TIF1, TAF14, TPD3, YDJ1</td>
<td>Others</td>
</tr>
<tr>
<td>YKL118W, HXT12, YCL007C</td>
<td>Pseudogenes</td>
</tr>
</tbody>
</table>

Genes whose deletion conferred sensitivity to both T-2 toxin and verrucarin A in the second screening are double-underlined, those only to T-2 toxin are underlined, and those only to verrucarin A are not underlined.
reported that mutation or deletion of the genes participating in ergosterol biosynthesis results in increased sensitivity to various drugs\textsuperscript{12-14}. Emter et al. showed that \textit{ERG6} limited the rate of passive drug diffusion across the membrane, and did not affect Pdr5p-mediated drug export, which means that the regulatory mechanisms of \textit{PDR5} and \textit{ERG6} on drug accumulation are distinct\textsuperscript{15}.

The largest group of genes whose deletions conferred sensitivity to these toxins was associated with vacuolar H\textsuperscript{+}-ATPase (V-ATPase): \textit{PPA1, TFP4, VMA4, VMA5, VMA7, VMA10, VMA13, VMA22, and VPH2}. In addition, 5 more genes that encode proteins related to vacuole, \textit{PEP3, PEP5, VPS15, VPS16, and VPS51}, were selected. This is the first study that shows that deletion of genes related to V-ATPase and vacuole conferred sensitivity to these trichothecenes.

V-ATPase is a large multi-subunit complex that plays a role in vacuolar acidification and maintenance of intracellular pH homeostasis\textsuperscript{16}. In budding yeasts, subunits of V-ATPase are encoded by a set of \textit{VMA} genes, and the mutation of these genes results in display of the so-called \textit{vma} phenotype, including multidrug sensitivity and hypersensitivity to high pH\textsuperscript{19}. It at least partly explains high sensitivity of \textit{vma} strains to T-2 toxin and verrucarin A. Interestingly, \textit{erg} phenotype and \textit{vma} phenotype showed striking similarity, such as disturbed cellular Ca\textsuperscript{2+} and H\textsuperscript{+} homeostasis\textsuperscript{17-19}.

We also found some other potential resistance genes in these screens. Although their mechanisms of action remained unknown, deletion of some of these genes was reported to confer sensitivity to other toxins, and therefore, these genes could be pleiotropic drug resistance genes\textsuperscript{14, 17, 20}. However, some genes listed here might be responsible specifically for trichothecene resistance, since some of the deletion mutants showed no hypersensitivity to cycloheximide, a potent inhibitor of protein synthesis. Further studies using other protein synthesis inhibitors and antifungal drugs will be required for elucidation of the roles for these genes.

In addition to the deletion mutants selected in these screenings, \textit{ltv1\Delta, yar1\Delta} and \textit{stm1\Delta} were examined because these mutants and \textit{rpb4\Delta} were hypersensitive to anisomycin\textsuperscript{21-23}, which is a potent inhibitor of peptidyltransferase, similar to trichothecenes\textsuperscript{5}. However, sensitivity of these gene deletion mutants to trichothecenes did not increase compared to that of the wild-type, possibly because anisomycin and T-2 toxin have somewhat different mechanisms of inhibition of peptidyltransferase. We also examined deletion mutants of \textit{PDR10, PDR15} (encoding ABC transporters), \textit{AYT1} (encoding a trichothecene-3-O-acetyltransferase, a detoxifying enzyme of deoxynivalenol), \textit{UBI4} (encoding ubiquitin), and \textit{UBP6} (encoding ubiquitin-specific protease), since deletion of these genes was previously reported to confer hypersensitivity to deoxynivalenol in \textit{S. cerevisiae} S 288 C\textsuperscript{8}. However, with an exception of the \textit{ubi4\Delta} strain, the IC\textsubscript{50} of these gene deletion strains in response to trichothecenes were similar when compared with the IC\textsubscript{50} of the wild-type strain BY 4742; this may be because of a difference in the genetic backgrounds of the yeast and/or of incubation protocol used for the assay (data not shown).

In this study, we found a group of potential resistance genes whose deletion conferred increased sensitivity to T-2 toxin and verrucarin A. These results suggest that deletion of multiple resistance genes with different mechanisms confers sufficient sensitivity to B-type trichothecenes, which are less toxic than the trichothecenes examined in this study. Possible application of genetically engineered yeasts for trichothecene detections contributes to more appropriate regulation of trichothecenes, since this detection method is different from other detection methods such as ELISA, HPLC, and GC-MS, and is easy, inexpensive, and based on the toxicity of trichothecenes.
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