TpBGL2 codes for a Tetrapisispora phaffii killer toxin active against wine spoilage yeasts

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Abstract

Tetrapisispora phaffii produces a killer toxin known as Kpkt that has extensive anti-Hanseniaspora/Kloeckera activity under winemaking conditions. Kpkt has a β-glucanase activity and induces ultrastructural modifications in the cell wall of sensitive strains, with a higher specific cytotoxic activity and a selective action towards target yeast cells. In this study, a two-step PCR-based approach was used to isolate the gene coding β-glucanase of T. phaffii. Initially, a fragment of the open reading frame was isolated by degenerate PCR, with primers designed on the NH₂-terminal sequence of the protein and on conserved motifs of Bgl2p of Saccharomyces cerevisiae and Candida albicans. Subsequently, the entire sequence of the gene was obtained by inverse PCR. BLAST analyses of TpBGL2 highlight high identity with homologous genes in other yeast species, in which TpBgl2p shows no killer activity. However, gene disruption resulted in complete loss of the glucanase activity and the killer phenotype, thus confirming that TpBgl2p has a killer activity.

Introduction

Tetrapisispora phaffii is known only from a single South African soil isolate (Van der Walt, 1963). Although it was originally assigned to the Fabospora genera on the basis of its ascospore morphology, this species was subsequently ascribed to the genus Kluyveromyces (Lodder, 1970). Successively, Ueda-Nishimura and Mikata (1999) redesignated the species as T. phaffii following the demonstration of its close relationships with Tetrapisispora iriomotensis, Tetrapisispora nanseiensis and Tetrapisispora arboricola, which were also isolated from soil and plant surfaces, in the Nansei Islands in Japan, together with the former Kluyveromyces phaffii.

The cells of T. phaffii are ellipsoid, ovoid to cylindrical. Reproduction occurs by bipolar budding, and no pseudomycelia are formed. The genus is homothallic and diploid. Asci that arise from diploid vegetative cells are ruptured or persistent on maturation, and contain 1–4 ovoid to reniform and smooth ascospores. Recently, the complete genome sequence of T. phaffii was deposited in the National Center for Biotechnology Information (NCBI) database at http://www.ncbi.nlm.nih.gov/genome/?term=Tetrapisispora+phaffii.

Tetrapisispora phaffii produces a killer toxin, or mycocin, known as Kpkt, which shows extensive anti-Hanseniaspora/Kloeckera activity under winemaking conditions. The biochemical characterization of Kpkt revealed its glycoprotein composition, with a molecular mass of 33 kDa and a β-glucanase activity. The NH₂-terminal sequence of Kpkt has 93% and 80% identity with 1,3-β-glucanase of Saccharomyces cerevisiae and β-1,3-glucanotransferase of Candida albicans, respectively (Comitini et al., 2004). In particular, the β-glucanase activity of T. phaffii is associated with its killer activity in vivo, as Kpkt induces ultrastructural modifications in the cell wall of sensitive strains, thus showing a strong specific cytotoxic activity and a selective action towards target yeast cells (Comitini et al., 2009).

The zymocidal activity of Kpkt against apiculate yeast is stable for at least 14 days in wine, and the killer toxin can control the proliferation, and limit the metabolic activity, of sensitive spoilage yeast (Comitini & Ciani,
Thus, Kpkt has great potential as a bioactive compound that can be used during the prefermentative stages of alcoholic fermentation.

To date, the genetic determinants of killer toxins produced by non-\textit{Saccharomyces} yeast have been poorly characterized. However, recent studies have reported on the identification of the genes encoding the killer toxins produced by \textit{Wickerhamomyces anomalous} and \textit{Williopsis saturnus}, which show exoglucanase activity (Xu et al., 2012; Muccilli et al., 2013).

As \textit{T. phaffii} is not a natural must fermenting yeast and therefore cannot be used as a wine starter, the objective of this study was the isolation of the gene that encodes Kpkt with a view to the potential heterologous production of the mycocin.

**Materials and methods**

**Yeast strains and media**

The yeast strains used were \textit{T. phaffii} CBS 4417, which is a killer strain deposited at Centraal Bureau voor Schimmel-cultures Fungal Biodiversity Centre, Utrecht, The Netherlands; and \textit{S. cerevisiae} DBVPG 6500 and \textit{Hanseniaspora uvarum} DBVPG 3037, which are deposited at the Industrial Yeast Collection of the University of Perugia, Italy, and which were both used as sensitive strains. The medium used was YEPD (1% yeast extract, 2% peptone, 2% dextrose) for short-term storage at 4 °C and for cultivation at 25 °C. The YEPD was supplemented with 100 mg L⁻¹ geneticin (G418; Sigma-Aldrich, Milan, Italy) and with 1 M sorbitol, to obtain YEPD + G418 and YEPD + sorbitol, respectively. Malt agar (Difco) containing 0.003% (w/v) methylene blue and buffered at pH 4.6 (with 0.1 M citrate phosphate buffer) was used for the well-test assay used to evaluate the killer phenotype, according to Rosini (1983).

**Total DNA extraction and degenerate PCR**

Yeast genomic DNA isolation was carried out according to Burke et al. (2000). The degenerate primers Kp-FW and Kp-RV (Table 1) were designed manually based on the NH₂-terminal sequence of purified Kpkt (Comitini et al., 2004) and on the conserved regions of the sequences of 1,3-β-glucanase of \textit{S. cerevisiae} and β-1,3-glucanotransferase of \textit{C. albicans}. The PCR amplification was carried out on a Gene AMP PCR System 9700 (Perkin-Elmer, Boston, MA). The reaction mixture was in a final volume of 25 μL, as follows: 0.8 μM each primer (Kp-FW, Kp-RV); 0.2 mM dNTPs; 3.0 mM MgCl₂; 50–100 ng genomic DNA; and 1 U \textit{Taq} DNA polymerase in \textit{Taq} buffer (Invitrogen, Life Science Technologies, Italy). The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 49 °C for 1 min, elongation at 72 °C for 1 min, and a final elongation at 72 °C for 10 min. The amplicon obtained was ethanol precipitated, phenol–chloroform extracted, ligated into the pYES 2.1/V5-HIS TOPO plasmid (Invitrogen), according to the manufacturer’s instructions, and sequenced using the GAL1 primer for V5C-rev on pYES 2.1/V5-HIS TOPO (BMR Genomics).

**Inverse PCR**

Inverse PCR (IPCR) was carried out according to Ochman et al. (1988) and Zara et al. (2002). Briefly, \textit{T. phaffii} genomic DNA was digested with different restriction enzymes that have no recognition sites in the sequence of the PCR product (BamHI, Clal, DraI, EcoRI, Hpal, NotI, PstI, PvuI, SacI, Scal, SmaI, XbaI, XhoI and Xmal). After a 2-h incubation at 37 °C, restriction mixtures were heated to 68–90 °C to inactivate the restriction enzymes, and the DNA was diluted and ligated. The ligation reactions contained 1–5 ng μL⁻¹ DNA, 1 μL (400 U) T4 DNA ligase (New England Biolabs), 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 25 μg mL⁻¹ bovine serum albumin, in 300 μL, and were performed for 2 h at 25 °C. Each ligation mixture was ethanol precipitated, resuspended in 30 μL H₂O, and subjected to IPCR with primers designed using the primer 3 software (Table 1). The inverse PCR mixtures had a final volume of 25 μL and contained 10 μM each of IPCRUp and IPCRLo primers; 10 mM dNTPs; 2.5 mM MgCl₂; 50–100 ng DNA template; and 1 U \textit{Taq} DNA polymerase in \textit{Taq} buffer (Invitrogen). The PCR cycling conditions were as follows: initial denaturation at 95 °C for 4 min, followed by 30 cycles of denaturation at 95 °C for 45 s, annealing at 47 °C for 45 s, elongation at 72 °C for 4 min, and final elongation at 72 °C for 10 min. The PCR products were visualized on agarose gels, cloned into the pYES 2.1/V5-HIS TOPO plasmid (Invitrogen), according to the manufacturer’s instructions, and sequenced using the GAL1-for and V5C-rev primers designed on pYES 2.1/V5-HIS TOPO (BMR Genomics; Table 1).

**Construction of the TpBGL2 deletion allele in \textit{T. phaffii}**

The TpBGL2 gene was subjected to disruption using the loxP-kanMX-loxP cassette (Güldener et al., 1996), modified using the PRODIGE protocol (Edlind et al., 2005). Briefly, pUG6 containing the loxP-kanMX-loxP cassette was used as the template in PCRs carried out with the Upprodkp and Loprodkp primers, which contain flanking 2010.21. Thus, Kpkt has great potential as a bioactive compound that can be used during the prefermentative stages of alcoholic fermentation.
sequences that align to the TpBGL2 gene (Table 1). The PCR mixture (volume, 50 µL) contained 1 mM dNTPs; 2 mM MgCl2; 10 µM each primer; 12–15 ng DNA template (pUG6); and 1 U Taq DNA polymerase (Eppendorf) in Taq buffer. The PCR cycling conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 3 min, and final elongation at 72 °C for 10 min. The PCR product was purified using ChargeSwitch PCR Clean-Up kits (Life Science Technologies, Italy), according to the manufacturer’s instructions.

### Yeast transformation

The yeast transformation was performed by electroporation. Briefly, *T. phaffii* CBS 4417 were inoculated into 10 mL YPD and incubated overnight at 30 °C in an orbital shaker at 150 r.p.m. (MaxQ® 4450 Benchtop Orbital Shakers; Thermo Scientific). The cells were then diluted into 50 mL YPD in a 250-mL sterile flask, grown for 2 h at 30 °C with shaking, collected by centrifugation (ROTOMIC 3, Hettich Zentrifugen) at 2500 g for 5 min, washed twice in 2 mL ice-cold 1 M sorbitol. Subsequently, the cells were resuspended in 1 mL 25 mM dithiothreitol and kept for 10 min at room temperature, then washed with 2 mL ice-cold 1 M sorbitol and resuspended in 250 µL ice-cold 1 M sorbitol. Fifty microlitres of the cell suspension was mixed with 5 µL DNA (1–5 ng DNA, in low ionic strength buffer). The cell/DNA suspension was added to the bottom of a 0.2-cm cuvette and electroporated under the following conditions: pulsed at 1.5 kV, 200 Ω, 25 µF (pulse time, 5 ms A). After electroporation, 1 mL 1 M sorbitol was added, and aliquots of 250 µL of the transformed cells were directly plated on YEPD + G418 and incubated at 25 °C for 7–10 days.

### Detection of gene disruption

To confirm the integration of the loxP-kanMX-loxP cassette into the *T. phaffii* genome, the transformed yeast cells were sampled directly from the YEPD + G418 plates and their genomic DNA was isolated and used as the template in PCRs, using the KanIntUp and KpBGL2Lo primers. The PCR mixture (volume, 50 µL) contained 1 µM each primer; 1 mM dNTPs; 2 mM MgCl2; 12–15 ng DNA; and 1 U Taq DNA polymerase (Eppendorf) in Taq buffer. The PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of 1 min at 95 °C, 1 min at 55 °C, 3 min at 72 °C, and final elongation for 10 min at 72 °C.

### Killer test phenotype

The frequency of the killer phenotype of the wild-type and disrupted strains was determined using the streak plate agar diffusion assay (Rosini, 1983). Briefly, final concentrations of 10⁵ cells mL⁻¹ for the DBVPG 6500 and DBVPG 3037 strains were uniformly suspended in 20 mL malt agar buffer at pH 4.6. The putative disrupted strains were streaked in duplicate on the agar surface of plates containing both the *S. cerevisiae* and *H. uvarum* sensitive strains and incubated at 25 °C for 72 h. Killer activity was scored positive when the killer strain was surrounded by a clear zone of growth inhibition.

### β-Glucanase and killer enzymatic activity

The β-glucanase activities of the wild-type and the three transformant strains were assayed as described by Comitini *et al.* (2004), using laminarin and glucon as the enzyme substrates. The units of β-glucanase activity were defined as micromole glucose liberated per milligram

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**Table 1. PCR primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kp-FW</td>
<td>5′-AA(AG)AA(C/T)AA(C/T)G(A/T)G-3′</td>
<td>Amplification of a portion of the gene ORF</td>
</tr>
<tr>
<td>Kp-RV</td>
<td>5′-ATCCCCTAAGTG(C/T)TCTTC-3′</td>
<td>Sequencing</td>
</tr>
<tr>
<td>GAL 1 for</td>
<td>5′-AATATACCTATATACCTAC-3′</td>
<td>Gene isolation by inverse PCR</td>
</tr>
<tr>
<td>V5C-rev</td>
<td>5′-ACCGAGGAGGTAGGAT-3′</td>
<td></td>
</tr>
<tr>
<td>IPCRU</td>
<td>5′-GTGACGTGTTTGGAGC-3′</td>
<td></td>
</tr>
<tr>
<td>IPCRLo</td>
<td>5′-TTTACGCTGCTGTA-3′</td>
<td></td>
</tr>
<tr>
<td>Upprodkp</td>
<td>5′-ATATTCCCTACTCTAGGCTATCAGG-3′</td>
<td></td>
</tr>
<tr>
<td>Loprodkp</td>
<td>5′-ACTCTCTGATATTTATATATATGCTGTTGATGTC-3′</td>
<td></td>
</tr>
<tr>
<td>KanIntUp</td>
<td>5′-ACGGACATTACAAAAACCAT-3′</td>
<td>Sequencing and assessment of gene disruption</td>
</tr>
<tr>
<td>KpBGL2Lo</td>
<td>5′-CTGCTCTCAGTTCACCTACCA-3′</td>
<td></td>
</tr>
<tr>
<td>KpBGL2Up</td>
<td>5′-GCATGGTGAAGCTGATGA-3′</td>
<td></td>
</tr>
</tbody>
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protein per minute. Glucose was determined using the 716251 enzyme kits (Boehringer Mannheim). Laminarinase (Sigma-Aldrich) was used as the positive control of enzyme activity.

Results

Isolation of the T. phaffii BGL2 gene

The use of degenerate PCR primers on the total DNA of T. phaffii yielded a single amplicon of about 800 bp (Fig. 1a and b). This was cloned into pYES 2.1/V5- HIS TOPO (Invitrogen) and sequenced. To obtain the complete sequence of the gene with the upstream and downstream flanking sequences, an IPCR approach was used. Thus, the total DNA of T. phaffii was enzymatically restricted, circularized by ligation, and used as the template DNA in PCRs using the IPCRUp and IPCRLo primers. The PCRs of the DNA restricted with Psf1 produced an amplicon of 1.8 kbp that was cloned into pYES 2.1/V5- HIS TOPO and sequenced (Fig. 1a and c). Sequence analyses with the Open Reading Frame (ORF) Finder software available in the NCBI database (http://www.ncbi.nlm.nih.gov/projects/orff/) revealed that TpBGL2 spans 948 bp. The sequence was submitted to Genbank (accession number, EF514211).

The alignment of this sequence against the T. phaffii genome (available online at http://www.ncbi.nlm.nih.gov/genome/?term=Tetrapisispora%2Bphaffii) indicated that the gene is located on chromosome 1. BLASTN analysis of the sequence obtained revealed more than 75% identity with the corresponding genes of other yeast species (Table 2).

Using the Expasy Translate tool (available at http://ca.expasy.org), a predicted protein of 316 amino acids was obtained. BLASTP of this sequence showed high identity with the corresponding proteins of several Saccharomyces species.

Fig. 1. (a) Two-step PCR-based approach for the isolation of TpBGL2. Degenerate primer PCR resulted in an 800-bp amplicon of the gene ORF. IPCR primers designed on the sequence of the amplicon obtained were used on the Tetrapisispora phaffii genomic DNA, and the resulting 1.8-kbp amplicon containing the entire TpBGL2 gene was cloned into pYES 2.1/V5- HIS TOPO and sequenced. (b and c) Electrophoresis gels of the 800-bp amplicon using the degenerated primer pair Kp-FW/Kp-RV (b) and the 1.8-kb amplicon cloned after IPCR (c). M, 1-kb molecular weight marker (Invitrogen, Life Science Technologies, Italy).
species, thus confirming the data already obtained by Comitini et al. (2004).

**Involvement of TpBGL2 in the killer phenotype**

To confirm that TpBGL2 is essential for the killer phenotype, a null allele of TpBGL2 was constructed by replacing the entire ORF with theloxP-kanMX-loxP cassette (Wach, 1996), as modified by Güldener et al. (1996) and using the PRODIGE protocol (promoter-dependent disruption of genes; Edlind et al., 2005; Fig. 2a). Using this procedure, 149 transformants were obtained, as isolated from the YEPD + G418. All of these were subjected to killer assays against the S. cerevisiae and H. uvarum sensitive strains, to individuate mutant strains that lacked killer activity, as shown in Fig. 3. The results showed that all of the transformant strains lost their killer activity, and among these, three strains were further analysed: TpBGL2Δ1, TpBGL2Δ25 and TpBGL2Δ27.

To confirm and strengthen the effects of TpBGL2 gene disruption, the glucanase activities of the three mutants were also evaluated. These results showed that the β-glucanase activity of the wild-type strain is comparable to that of the commercial laminarinase used as the positive control (specific activity 1.62 ± 0.015 and 1.80 ± 0.019 μmol mg⁻¹ protein min⁻¹, respectively), while the TpBGL2Δ1, TpBGL2Δ25 and TpBGL2Δ27 strains did not show glucanase activity.

Afterwards, the KanIntUp and KpBGL2Lo primers were used in PCRs on the total DNA of these three strains, to confirm the homologous gene recombination. The three transformants generated amplicons of 1500 bp that included theloxP-kanMX-loxP cassette and a fragment of the TpBGL2 gene, while the amplicon was lacking in the wild type (Fig. 2b). To determine the correct integration of theloxP-kanMX-loxP deletion cassette, the TpBGL2A1, TpBGL2Δ25 and TpBGL2Δ27 strains were sequenced. Sequence alignment with CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) confirmed that gene disruption occurred at the homologous position.

Moreover, the KpBGL2up and KpBGL2Lo primers (which were designed within the TpBgl2 gene) were used to PCR amplify the total DNA of the wild type and TpBGL2Δ1, TpBGL2Δ25 and TpBGL2Δ27 transformants. In contrast to wild-type strain, which showed an amplicon of 1300 bp, the disruptant strains did not show any amplicon, which suggested the disruption of both of the alleles (Fig. 4).

**Discussion**

Tetrapispora phaffii secretes a killer toxin, known as Kpkt, that is of biotechnological interest due to its wide spectrum of action against several spoilage yeast species. In particular, Kpkt is active towards wine spoilage yeast that belong to the species Kloeckera apiculata and H. uvarum (Ciani & Fatichenti, 2001), and it is stable under winemaking conditions. This indicates its promising features for use as a novel bioactive antiseptic agent in the wine and beverage industries.

In the present study, the isolation of the gene encoding Kpkt was followed by the knockout of the isolated gene to confirm its involvement in the killer phenotype.

The two-step PCR-based approach and the gene disruption strategy used proved to be useful for genetic manipulation of T. phaffii. In particular, the gene disruption protocol that was applied was based on a promoterless marker gene (Edlind et al., 2005) and reduced...
**Fig. 2.** Construction of the null allele of TpBGL2. (a) The TpBGL2 ORF was replaced with the loxP-kanMX-loxP cassette. (b) Electrophoresis gels following amplification of the total DNA of the three mutant strains lacking killer activity with the KanInUp and KpBGL2Lo primers. M, 1-kb molecular weight marker (Invitrogen, Life Science Technologies, Italy); lane 1, Tetrapisispora phaffii wild type; lanes 2–4, strains TpBGL2Δ1, TpBGL2Δ25 and TpBGL2Δ27, respectively.

**Fig. 3.** Killer plate assay of Tetrapisispora phaffii wild type (A), surrounded by an inhibition halo, and the three deleted strains TpBGL2Δ1, TpBGL2Δ25 and TpBGL2Δ27 (B–D, respectively), showing loss of their killer phenotype. The sensitive strain used was DBVPG 6500.

**Fig. 4.** Electrophoresis gel following amplification of the total DNA of the Tetrapisispora phaffii wild type (lanes 1, 2, two copies) and the three mutant strains TpBGL2Δ1, TpBGL2Δ25 and TpBGL2Δ27 (lanes 3–5, respectively) lacking the killer activity, using the KpBGL2Up and KpBGL2Lo primers. M, 1-kb molecular weight marker (Invitrogen, Life Science Technologies, Italy).
nonhomologous recombination events that are typical of a great number of yeast, and led to the obtaining of three mutants lacking killer activity. Considering that T. phaffii is a diploid yeast, the complete absence of the killer phenotype and the absence of the β-glucanase activity demonstrate the loss of a functional allele in TpBGL2Δ21, TpBGL2Δ25 and TpBGL2Δ27, which suggests that the cassette was inserted into the TpBGL2 gene.

The comparison of the entire ORF with the Saccharomyces Genome Database (www.yeastgenome.org) and the NCBI database (http://www.ncbi.nlm.nih.gov/) confirmed what was previously reported by Comitini et al. (2004). The predicted protein shows high identity with the BGL2 gene products of several yeast species. Thus, the gene coding this protein was named TpBGL2.

In S. cerevisiae, the BGL2 gene codes for an endo-β-1,3-glucanase that was not previously identified as a killer toxin. Bgl2p is a highly conserved cell wall protein with an apparent molecular mass of 29 kDa and a single N-linked oligosaccharide of 2.3 kDa. Bgl2p has also been described as a glucanosyltransferase (Mrsa et al., 1993; Goldman et al., 1995; Sarthy et al., 1997). Although the involvement of Bgl2p in cell wall modelling and maintenance has been reported in several studies, its physiological role is still not entirely clear. BGL2 gene disruption in S. cerevisiae results in an increase in cell wall chitin, in comparison with the wild type (Laurinaviciute et al., 1995; Sarthy et al., 1997). This increased chitin deposition in the cell walls is suggested to be part of the general repair mechanism that helps these cells to counteract cell wall damage (Kapteyn et al., 1999). In agreement with this, in T. phaffii, the BGL2 gene codes for Kpkt, an analogue of endo-β-1,3-glucanase of S. cerevisiae, and Kpkt has killer activity that causes the disruption of the cell wall in sensitive cells.

Recent studies have highlighted the glucanase nature of some of the killer toxins produced by yeast. In particular, Xu et al. (2012) and Wang et al. (2012) isolated the genes coding for two analogous killer toxins that are produced by the WC91-2 and IFO 0895 W. saturnus strains, respectively. The killer toxins produced by these two yeast are very similar, and they show exoglucanase activity. Muccilli et al. (2013) investigated an exoglucanase gene that encodes a killer toxin of W. anomalus isolated from olive brine, and reported that two genes, PAEXG1 and PAEXG2, are responsible for the toxic exo-β-1,3-glucanase activity. However, TpBGL2p still represents the first case in which killer activity is due to the expression of an endoglucanase-coding gene.

On the basis of these data, further experimental studies will be aimed at heterologous production of the Kpkt protein, for its future application during the prefermentative stage, to avoid or greatly reduce SO2 addition in grape juice, thus limiting the SO2 content in the final product.

References


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