pZHK2, a bi-functional transformation vector, suitable for two step gene targeting

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Homologous recombination is a prerequisite for the generation of knock out strains by means of DNA-mediated transformation. In filamentous fungi however, the frequency of ectopic integration events is rather high and the actual efficiency of homologous recombination depends upon the length of homologous DNA flanking the transformation marker. Recently, d´Enfert and coworkers (Chaveroche et al., 2000) presented a two-step technology for the integration of a bi-functional zeocin-*pyrG* cassette into a target sequence of interest using an *Escherichia coli* strain expressing the phage lambda Red functions. In the resulting recombinant cosmids, the selection marker is flanked by fungal DNA sequences longer than 1 kb, which can be used to transform appropriate fungal recipient strains. For selection of fungal transformants, those workers used the *A. nidulans pyrG* gene encoding orotidine-5´-monophosphate decarboxylase, which confers prototrophy in appropriate uridine/uracil auxotrophic recipient strains.
Here, we describe the novel bi-functional transformation vector pZHK2, which carries in addition to the zeocin resistance gene the hygromycin B phosphotransferase gene often used as a dominant selectable marker gene in fungal recipient strains. The applicability of the vector is demonstrated by generating a ura3⁻ knock out strain from *Sordaria macrospora* showing auxotrophy.

The construction of plasmid pZHK2 was achieved as follows: Initially, we generated plasmid pEM7ZH2 by ligating the 1.4 kb *Eco*RI fragment from plasmid pCB1003 (Carroll et al., 1994), carrying the *hph* gene under the control of the *A. nidulans trpC* promoter, into the single *Eco*RI site of vector pEM7/Zeo (Invitrogen, Carlsbad, CA). This plasmid harbors the intact ampicillin resistance gene and, consequently, this plasmid can appear as a contamination in subsequent selection steps in which amplicillin containing media are used for selection of recombinant clones. Hence, a 1.035 kb *Bgl*II-*Xmn*I fragment carrying the kanamycin resistance gene from vector pCR2.1 (Invitrogen, Carlsbad, CA) was inserted into the *Xmn*I site of pZEM7ZH2. For blunt end ligation, the 5´ protruding *Bgl*II site was filled up in a Klenow reaction. This construct has a size of 5301 bp and was further shortened by deleting a 415 bp *Bsa*I-*Sca*I fragment. The resulting vector pZHK2 has a size of 4886 bp and is shown in Figure 1.

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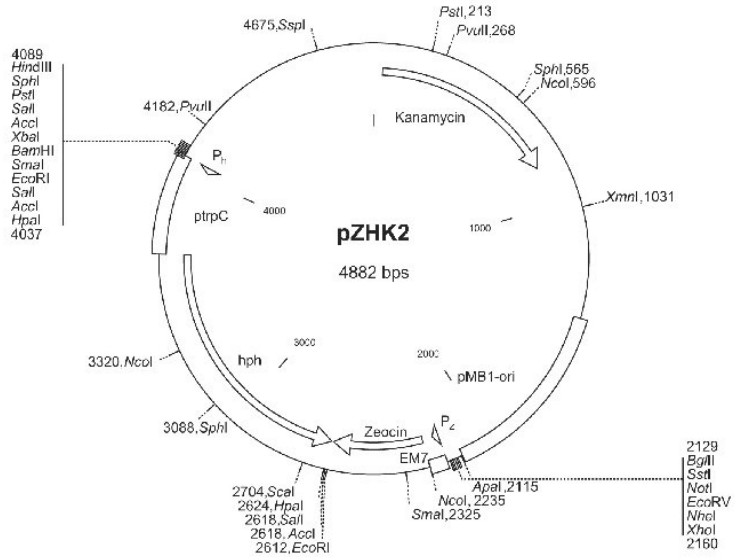

Figure 1: (Facing page) Physical map of the vector pZHK2. P_z and P_h refer to priming sites on the bi-functional *zeo-hph* cassette. Size and location of the kanamycin, zeocin and hygromycin B (*hph*) resistance genes are indicated. The *hph* gene is fused with the fungal promoter sequence (ptrpC) from the *Aspergillus nidulans trpC* gene

In order to show the applicability of pZHK2, we inserted the *zeo-hph* cassette in the *ura3* gene to generate a uracil auxotrophic strain from *Sordaria macrospora*. For this purpose, two 70 bp primers (P_z-ura3:
GACGATGTCGAGCATGCGCGAGAGCTCCTCGCCCTTGCCGACAAGATTGGTGTTGACAATTAATCATCGGCATAG
and P_h-ura3:
CCCACCCTGTGATCAGGTCATAGTGGGTCTTGAGGACGACAATCGAGGGGGGGCTTGGCTGGAGCTAGTGGAGG)
were synthesized.

The primers have a 50 nt homology to the *S. macrospora ura3* gene (Nowrousian and Kück, 1998), followed by 20 nt with homology to the *trpC* promoter (P_h) or the zeocin resistance gene (P_z). Amplification with these primers using pZHK2 as a template resulted in a 2 kb fragment containing the *zeo-hph* cassette that is flanked by 50 bp of the *ura3* sequence.
The PCR amplicon was used to transform the *E. coli* recipient strain KS272 carrying plasmid pKOBEG and plasmid p20.26 (Chaveroche et al., 2000). The latter contains a 6.6 kb *Pst*I-*Cla*I restriction fragment with the *S. macrospora ura3* coding region (Fig. 2).

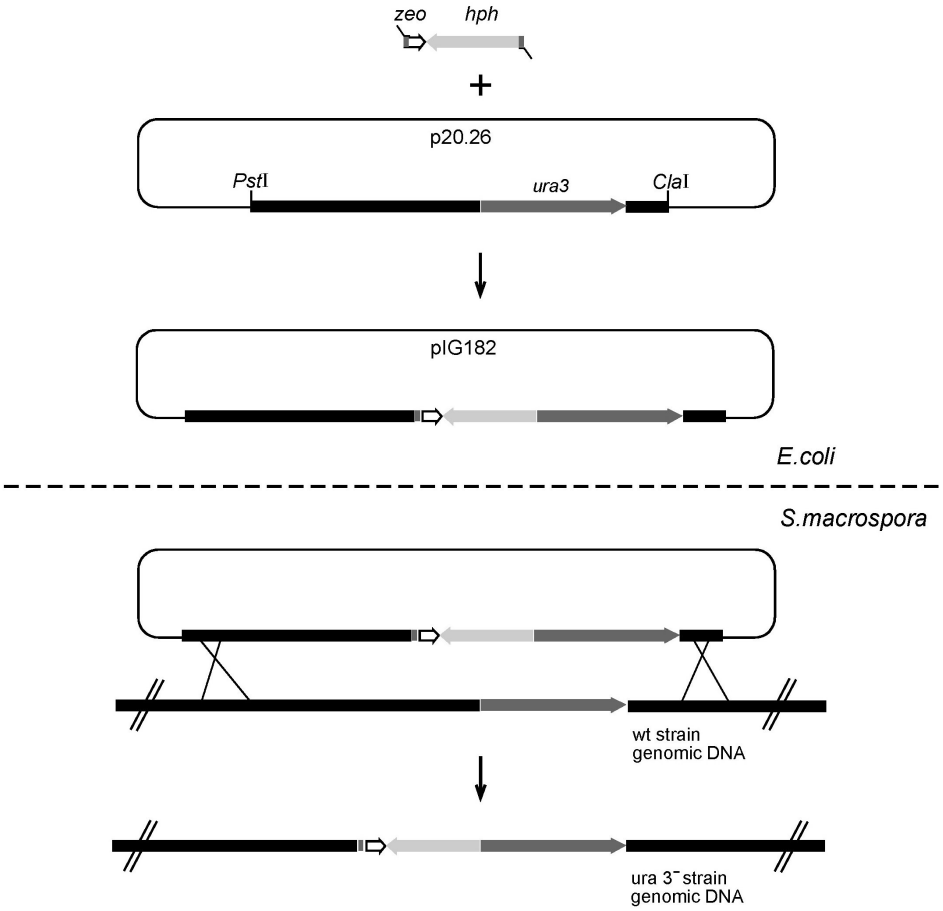

Figure 2: Gene disruption of the *ura3* gene in *E.coli* and in *Sordaria macrospora*. Schematic drawing of the disruption construct using pZHK2. The PCR primers shown by arrows were designed to amplify the region across the *zeo-hph* cassette and used to screen the mutants carrying the disrupted *ura3* gene. Plasmid p20.26 carries a 6.6 kb *Pst*I-*Cla*I fragment from the *S. macrospora ura3* gene region, and pIG182 is a derivative, with the *zeo-hph* cassette inserted into the *ura3* coding region (adapted from Chaveroche et al., 2000).

After selection of transformed bacteria on LB-medium containing ampicillin and zeocin, we identified recombinant plasmids in which the *zeo-hph* cassette was inserted into the *ura3* gene. The resulting plasmid pIG182 (Fig. 2) was used to transform the wild type strain of *S. macrospora* as described earlier (Nowrousian et al., 1999). For transformation, plasmid pIG182 was linearized, leaving 5.1 and 1.5 kb of homologous sequence at each end of the resistance cassette. Fungal transformants were selected on BMM medium supplemented with 100 µg/ml hygromycin B. A total of 30 fungal transformants were then tested on minimal medium devoid of any supplements. One transformant, named T7, showed hygromycin B resistance, but was unable to grow on minimal medium without uracil. Using genomic DNA, a 6.6 kb *Pst*I-*Cla*I probe detected in a Southern blot a 2.3 kb *Sac*I restriction fragment derived from the wild type *ura3* gene. This fragment can easily be distinguished from the 4.2 kb SacI fragment in T7, which carries the *zeo-hph* cassette and is inserted into the *ura3* coding sequence. In order to verify the *ura3* disruption in strain T7, plasmid p20.26 was used for DNA-mediated transformation of the auxotrophic recipient strain. The analysis of DNA from two representative prototrophic fungal transformants is shown in Fig. 3.

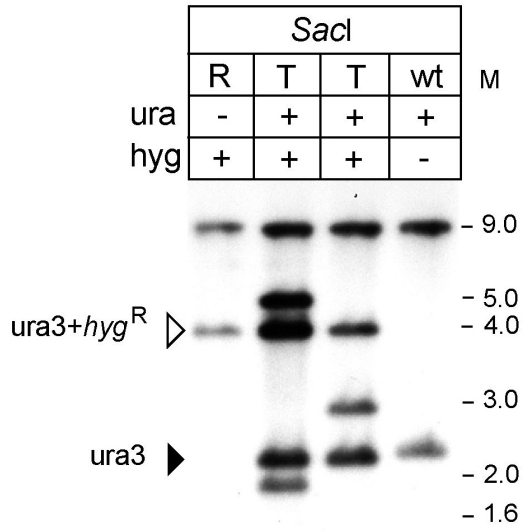

Figure 3: Autoradiograph of a Southern hybridization with genomic DNA from a *S. macrospora* wild type (wt) strain, the ura⁻ recipient strain T7 (R) strain, and prototrophic transformants (T). DNA was treated as indicated and probed with the radiolabeled *Pst*I-*Cla*I fragment, shown in Fig. 2.

When compared with the recipient strain, the transformants showed a complex hybridization pattern, which can be explained by ectopic integration of plasmid p20.26 into genomic DNA. Using vector pZHK2, we were able to disrupt the *ura3* gene, thus generating an auxotrophic strain, which offers the opportunity to transform *S. macrospora* with a second marker gene.

In summary, the new bi-functional transformation vector pZHK2 presented here carries a kanamycin resistance marker and a zeocin-hygromycin resistance cassette, which can be used for *in vivo* homologous recombinations in *E. coli, S. macrospora* and possibly other filamentous fungi. This vector will be useful for transformation of filamentous fungi showing a low rate of homologous recombination, which can only be transformed with the hygromycin B resistance marker.

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