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Abstract
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Application of the nourseothricin acetyltransferase gene (nat1) as dominant marker for the transformation of filamentous fungi

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Here, we report the construction of two transformation vectors, pD-NAT1 and pG-NAT1, carrying the nat1 gene encoding the nourseothricin acetyltransferase. The nat1 gene is expressed under the control of the Aspergillus nidulans trpC promoter and thus can be used as a dominant drug-resistance marker for the DNA-mediated transformation of filamentous fungi. The successful application of both vectors was demonstrated by transforming the homothallic ascomycete Sordaria macrospora as well as the β-lactam producer Acremonium chrysogenum. For both fungi and for both vectors, transformation frequencies were between 10 and 40 transformants per 10 µg of plasmid DNA.

Since the first successful DNA-mediated transformation of filamentous fungi (Case et al. 1979, Stahl et al. 1982) several marker genes have been developed for selection of transgenic fungal strains (Fincham 1989, Lemke and Peng 1995). This includes the hph gene encoding the hygromycin B phosphotransferase which is applicable in transformations without the need for constructing appropriate recipient strains. In order to generate an alternative dominant marker gene that does not exhibit cross-resistance to hygromycin B, we have chosen the nat1 gene conferring resistance to nourseothricin. The nat1 gene product is the nourseothricin acetyltransferase from Streptomyces noursei (Krügel et al. 1993) which has been successfully used in the transformation of several yeast species including Saccharomyces cerevisiae, Schizosaccharomyces pombe, Candida albicans and Cryptococcus neoformans (Goldstein and McCusker 1999, McDade and Cox 2001, Hentges et al. 2005, Shen et al. 2005).

Here, we present the construction of two transformation vectors, carrying the commercially available nat1 gene (Werner BioAgents, Jena, Germany) that are suitable for the transformation of filamentous fungi. As a first step in establishing the nat1-based transformation system, sensitivity against the antibiotic nourseothricin (Werner BioAgents, Jena, Germany) was tested for A. chrysogenum and S. macrospora. For A. chrysogenum and S. macrospora, growth was inhibited on solid media at nourseothricin concentrations of 25 µg and 50 µg/ml, respectively. In the following, the construction of two transformation vectors, which can be used for different experimental purposes, is described.

First, the bacterial nat1 gene was fused to a fungal promoter that allows the expression of the nat1 gene in a wide range of filamentous fungi. The nat1 gene was amplified with primer pair nat1-1 (5’-AACCATGGCCACCTCGACGACACGGC-3’) and nat1-2 (5’-TAGCCGGCC CTCAGGGGCAGGGCATGCTCATG-3’) using plasmid pHN15 (Werner BioAgents, Jena, Germany) as template. The resulting amplicon is flanked by single NcoI and NotI restriction sites which were used for insertion into the corresponding sites of vector pHAN (Kück, unpublished). pHAN is an expression vector that is suitable for use in filamentous fungi. This vector carries a cloning site with NcoI and NotI recognition sequences that are flanked 5’ and 3’ by the A. nidulans trpC promoter and terminator, respectively (Mullaney et al. 1985). The resulting plasmid was named pHNAT2 (Kück and Godedhardt, unpublished) and used as a source for the trpC promoter-nat1 fusion. The following construction describes two vectors carrying this gene fusion. We found out that the trpC terminator sequence is not necessary for efficient expression of the nat1 gene in the fungal hosts.

Vector pD-NAT1 (Fig. 1) was constructed using primers nat1-3 (5’-GAATTCAACTGA TATTGAAGGAGCA-3’) and nat1-4 (5’-GGGCCCTCAGGGCCAGGGCATGCTCATGT AGA-3’) and the pHNAT2 plasmid to generate the TrpC-nat1 amplicon which was inserted into cloning vector pDrive (Qiagen, Hilden, Germany). In this vector, the nat1 gene is flanked on both sides by multiple cloning sites that can be used for directed insertion of fungal genomic sequences to construct gene disruption strains by homologous recombination. Using previously reported transformation procedures for A. chrysogenum and S. macrospora, the transformants were kept without selection for 24 hours (Kück et al. 1989, Walz and Kück 1995). The germinated protoplasts were subsequently overlaid with top agar containing nourseothricin concentrations of 25 µg/ml (Ac) and 50 µg/ml (Sm). After 7 (Ac) or 2 (Sm) days, transformants were transferred on solid rich medium with the above mentioned nourseothricin concentrations. We obtained with vector pD-NAT1 frequencies of about 20 to 40 transformants per 10 µg of DNA, which is comparable to the rather low frequencies that are obtained with the hph gene (Kück et al. 1989, Walz and Kück 1995). Most importantly, the transformants did not show cross-resistance neither to hygromycin B nor to phleomycin.
To allow the nat1 gene to be used in further applications, for example, disruption of genes that can be used to generate fungal knock-in strains, plasmid pG-NAT1 (Fig. 2) was generated. This vector carries a modified Transprimer™ element derived from pGPS2.1. This plasmid is part of the GPS™-1 Genome Priming System (New England Biolabs, USA) which provides a simple in vitro method for generating a population of recombinant plasmids (or cosmids) with randomly inserted Transprimer™ elements. The resulting disrupted genes are flanked by large border sequences that promote homologous recombination in fungal recipient strains.

For construction of pG-NAT1, a 1.3 kb EcoRI–NotI fragment from pNAT2 was treated with Klenow polymerase to generate blunt ends. This fragment was inserted into the unique SmaI site within the Transprimer-2™ element of plasmid pGPS2.1 resulting in the generation of pG-NAT1. In addition to the nat1 gene for selection in filamentous fungi, pG-NAT1 also carries the cam and tet genes as selectable markers for E. coli transformation. Successful application of this plasmid was shown when the Transprimer-2™ element of pG-NAT1, carrying the nat1 gene, was randomly inserted into cosmid clones carrying about 40 kb of fungal genomic sequences (Dreyer, Engh and Kück, unpublished data). The resulting plasmids, isolated from E. coli, had a size of about 50 kb and were successfully transformed into A. chrysogenum and S. macrospora at a frequency of about 20 transformants per 10 µg DNA. A similar plasmid to pG-NAT1 was recently described by Jadoun et al. (2004) and instead of the nat1 gene, it carries the pyr4 gene, which can be used for transformation of the appropriate uracil auxotrophic recipient strains. In the case of pG-NAT1, any fungal recipient strain showing sensitivity against nourseothricin can be used for DNA-mediated transformation.

In conclusion, the two vectors described here will provide useful alternatives when other than the commonly used resistance marker genes will be required for DNA-mediated transformation of filamentous fungi. For example, multiple tagged or deleted strains can be constructed and in the case when simultaneous imaging of multiple proteins is desirable, several reporter genes can be introduced.

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References


