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`-AACCATGGCCACCCTCGACGACACGGC-3`) and nat1-2 (5`-TAGCGGCCG CTCAGGGGCAGGGCATGCTCATG-3`) using plasmid pHN15 (Werner BioAgents, Jena, Germany) as template. The resulting amplicon is flanked by single *Nco*I and *Not*I 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 *Nco*I and *Not*I 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 pNAT2 (Kück and Godehardt, 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`-GGGCCCTCAGGGGCAGGGCATGCTCATGT AGA-3`) and the pNAT2 plasmid to generate the P*trpC-nat1* amplicon which was inserted into cloning vector pDrive (Qiagen, Hilden, Germany). In this vector, the *nat1* gene is flanked on both sites 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.

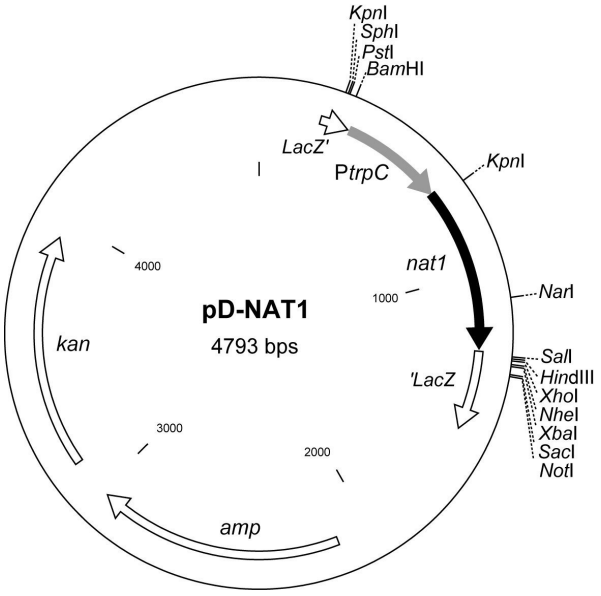

Figure 1. Physical map of vector pD-NAT1. *Kan* and *amp* refer to the kanamycin and ampicillin resistance markers which can be used for selection of *E. coli* transformants. The *nat1* gene is fused with the fungal *trpC* promoter from *A. nidulans* (Mullaney et al. 1985)

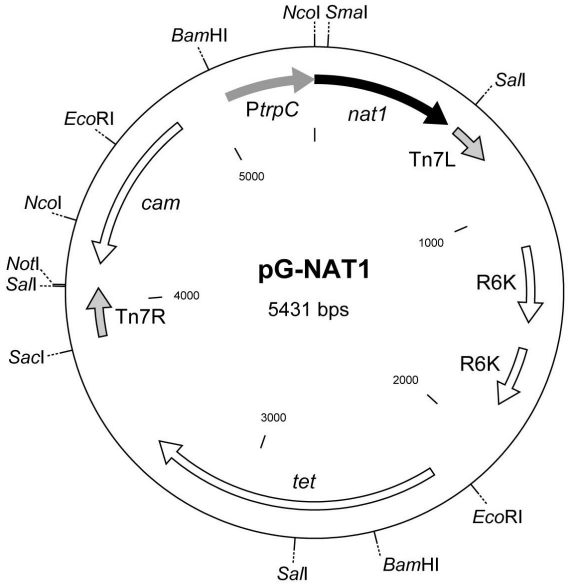

Figure 2. Physical map of vector pG-NAT1. *Tet* and *cam* refer to the tetracycline and chloramphenicol resistance markers which can be used for selection of *E. coli* transformants. The *nat1* gene is fused with the fungal *trpC* promoter from *A. nidulans* (Mullaney et al. 1985). Tn7R and Tn7L indicate the right and left Transprimer™ of transposon Tn7 and R6K marks the *E. coli* R6Kg origin of replication (New England Biolabs, USA)

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 *Eco*RI-*Not*I fragment from pNAT2 was treated with Klenow polymerase to generate blunt ends. This fragment was inserted into the unique *Swa*I 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

Case, M.E., M. Schweizer, S.R. Kushner, and N.H. Giles. 1979. Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. Proc. Natl. Acad. Sci. USA 76: 5259-5263.

Fincham, J.R. 1989. Transformation in fungi. Microbiol. Rev. 53: 148-170.

Goldstein, A.L., and J.H. McCusker. 1999. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast 15: 1541-1553.

Hentges, P., B. Van Driessche, L. Tafforeau, J. Vandenhaute, and A.M. Carr. 2005. Three novel antibiotic marker cassettes for gene disruption and marker switching in *Schizosaccharomyces pombe*. Yeast 22: 1013-1019.

Jadoun, J., Y. Shadkchan, and N. Osherov. 2004. Disruption of the *Aspergillus fumigatus argB* gene using a novel *in vitro* transposon-based mutagenesis approach. Curr. Genet. 45: 235-241.

Krügel, H., G. Fiedler, C. Smith, and S. Baumberg. 1993. Sequence and transcriptional analysis of the nourseothricin acetyltransferase-encoding gene *nat1* from *Streptomyces noursei*. Gene 127: 127-131.

Kück, U., M. Walz, G. Mohr, and M. Mracek. 1989. The 5′-sequence of isopenicillin N-synthetase gene (*pcbC*) from *Cephalosporium acremonium* directs the expression of the prokaryotic hygromycin B phosphotransferase gene (*hph*) in *Aspergillus niger*. Appl. Microbiol. Biotechnol. 31: 358-365.

Lemke, P.A., and M. Peng. 1995. Genetic manipulation of fungi by DNA-mediated transformation. In: Kück U (eds.) The Mycota II Genetics and Biotechnology. Springer-Verlag, Berlin, Heidelberg pp. 109-139.

McDade, H.C., and G.M. Cox. 2001. A new dominant selectable marker for use in *Cryptococcus neoformans*. Med. Mycol. 39: 151-154.

Mullaney, E.J., J.E. Hamer, K.A. Roberti, M.M. Yelton, and W.E. Timberlake. 1985. Primary structure of the *trpC* gene from *Aspergillus nidulans*. Mol. Gen. Genet. 199: 37-45.

Shen, J., W. Guo, and J.R. Kohler. 2005. CaNAT1, a heterologous dominant selectable marker for transformation of *Candida albicans* and other pathogenic *Candida* species. Infect. Immun. 73: 1239-1242.

Stahl, U., P. Tudzynski, U. Kück, and K. Esser. 1982. Replication and expression of a bacterial-mitochondrial hybrid plasmid in the fungus *Podospora anserina*. Proc. Natl. Acad. Sci. USA 79: 3641-3645.

Walz, M., and U. Kück. 1995. Transformation of *Sordaria macrospora* to hygromycin B resistance: characterization of transformants by electrophoretic karyotyping and tetrad analysis. Curr. Genet. 29: 88-95.