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Development of molecular tools for the mulundocandin producer *Aspergillus sydowii*: DNA-mediated transformation and reporter gene expression

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Abstract The echinocandin-type antimycotic mulundocandin and its derivatives are produced by the filamentous fungus *Aspergillus sydowii* (strain FH2551). These agents have been considered as a potential drug to treat immunocompromised patients who suffer from severe opportunistic fungal infections. In order to generate strains with a modified mulundocandin biosynthesis, we developed molecular tools for genetic engineering of *A. sydowii* as an alternative to conventional strain improvement procedures. For our experiments, we used strain FH2551, which was discriminated from other *Aspergillus* strains by determining the sequence of the two internal transcribed spacers (ITS1 and ITS2) of the rDNA locus. In addition, the electrophoretic karyotype of *A. sydowii* was established using pulsed-field gel electrophoresis (PFGE), leading to a calculated genomic size of about 40 Mb. For gene mapping, chromosomes were subjected to PFGE either unrestricted or after incubation with rare cutting enzymes and probed with heterologous genes. Using the bacterial hygromycin B phosphotransferase gene as a selectable marker for transformation of *A. sydowii*, we generated transformants with single and multiple copies of plasmid DNA. Subsequently, the heterologous *lac*Z and *gfp* genes were efficiently transferred and expressed in *A. sydowii*. The majority of *lacZ*-transformants showed more than 6 pkat β-galactosidase activity/mg protein, while the control strains had no significant background activity. Fluorescence microscopy of *gfp*-transformants demonstrated that the green-fluorescent protein is present in a stable and active form in the cytoplasm of vegetative hyphae and conidiospores.

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Introduction

Aspergillus sydowii is a filamentous fungus capable of synthesizing a potent antimicrobial substance, the antimycotic mulundocandin. Mulundocandin was identified in a strain that was isolated from soil samples collected in Bangladesh (Roy et al. 1987). The name "mulundocandin" is derived by combining Mulund, a town in India, with "candin", which denotes that this antimycotic is especially active against yeasts of the genus *Candida*. Mulundocandin and the structurally related deoxymulundocandin, which is produced by the same strain (Mukhopadhyay et al. 1992), are members of the echinocandin-type antimycotics. This class of substances was characterized as neutral cyclic peptides with a fatty acid side chain (for review, see Hammond 1993). The cyclic hexapeptide compound of mulundocandin consists of serine, threonine and the following unusual amino acids: 3-hydroxy-4-methylproline, 3,4-dihydroxyhomotyrosine, 4-hydroxyproline and 4,5-dihydroxyornithine (Mukhopadhyay et al. 1987). The polypeptide is probably synthesized by a non-ribosomal peptide synthetase, while the fatty acid side chain is added by another enzyme. The echinocandin-type lipopolypeptides act as 1,3-β-glucan synthesis inhibitors, thus they are well matched for classes of fungi, such as *Candida*, with high amounts of glucans (Sawistowska-Schroder et al. 1984). In fungi, 1,3-β-glucans and chitin are important cell wall components and, therefore, essential for cell viability. The discovery and application of novel antimycotics is of increasingly important medical relevance, since nowadays a growing number of patients show serious fungal infections (review see Mahaguna et al. 2000). Opportunistic fungal infections in immunocompromised patients with cancer, organ transplants or AIDS may lead to candidiasis and aspergillosis. Treatment with antifungal agents such as polyenes and azoles can result in a variety of undesirable and toxic side effects and is often not effective in severe cases. Echinocandins possess a fungus-specific mechanism of action and have a low incidence of unwanted side effects (Hammond 1993).

For efficient industrial production of secondary metabolites from fungi, improvement of the naturally occurring synthetic capacity of fungal strains is a prerequisite. Strain improvement can be achieved by classical mutagenesis and selection techniques. On the other hand, genetic engineering of a producer strain offers the potential to improve not only production yields but also product properties. Engineering of biosynthesis enzymes or the integration of heterologous genes with novel catalytic activities into biosynthesis can give rise to chemical derivatives of the original product. In the case of echinocandins, desirable features are, for example, water solubility, efficient secretion of the product and broader antifungal activity. An example for the genetic modification of an echinocandin-B-producing strain is described for *Aspergillus nidulans*. Amongst other substances, fermentation of this strain also produces significant amounts of the carcinogenic polyketide sterigmatocystin, so that UV mutagenesis combined with appropriate selection methods were used to isolate mutants blocked in the polyketide synthesis (Hodges et al. 1994).

While genetic engineering of different species of the genus *Aspergillus* has already been reported, to our knowledge none of the available *A. sydowii* strains have been genetically characterized or successfully used for genetic manipulation. So far, studies on this imperfect fungus have focused on cellulolytic and xylanolytic activities and the cloning of the *sft* gene encoding a fructan biosynthetic enzyme (Olutiola and Cole 1977; Ghosh and Nanda 1994; Heyer and Wendenberg 2001).

This report provides the genetic characterization of a mulundocandin-producing strain of *A. sydowii* using pulsed-field gel electrophoresis to determine the electrophoretic karyotype. DNA-mediated transformation of *A. sydowii* using hygromycin B as a selection marker is described here for the first time. Furthermore, this research succeeded in the expression of different reporter genes as examples for heterologous gene expression and promoter analysis.

Materials and methods

Strains and plasmids

The fungal strain FH2551 is a derivative of strain Y30462 which was classified as *A. sydowii* (Bainier and Sartory) Thom and Church var. nov. *mulundensis* Roy (Roy et al. 1987). *A. sydowii* strains IAM2544, NRRL242, NRRL244, NRRL249, NRRL250 and NRRL254, from the Institute of Applied Microbiology Culture Collection, Tokyo, Japan and the Agricultural Research Service Culture Collection, Peoria, USA, were used for the isolation of ITS sequences. All *A. sydowii* strains were cultivated in liquid PDA medium (2.4% (w/v) potato dextrose broth (Difco), 0.2% yeast extract, pH 5.1) at 27 °C and 180 rpm or on PDA solid media with 2% agar. *Acremonium chrysogenum* strain ATCC 14553 was cultivated as described by Minuth et al. (1982). Plasmids pMW1 (4.3 kb, *pcbC* from *A. chrysogenum* promoter and hygromycin B phosphotransferase gene (*hph*), Kück et al. 1989) and pBC-Hygro (6.8 kb, *cpc1* promoter from *Neurospora crassa* and *hph* gene, Silar 1995) were used for DNA-mediated transformation of *A. sydowii*. Plasmid pSIM9.9 (Menne et al. 1994) contains the *lacZ* gene under the control of the *pcbC* promoter from *Aspergillus chrysogenum*, and plasmid p82.9 carries an EGFP expression cassette (*gpd* promoter and *trpC* terminator from *A. nidulans*, S. Masloff, Ruhr University, Bochum, unpublished data) derived from plasmid pEGFP/gpd/tel (Inglis et al. 1999). EGFP is a modified version of green fluorescent protein with a human codon-usage preference.

Determination of internal transcribed spacer sequences and calculation of phylogenetic tree

Internal transcribed spacer (ITS) sequences of the nuclear rDNA locus from different *A. sydowii* strains were PCR-amplified using primers ITS1 and ITS4 under standard conditions. Primers ITS1 and ITS4 are located in conserved regions of the 18S and 28S rDNA genes (White et al. 1990). Multiple alignment was performed using CLUSTAL X (Thompson et al. 1994), and the program PHYLIP (Felsenstein 1993) was used to generate phylogenetic trees, which were transferred to graphic images with the program TREEVIEW (Page 1996).

Generation of protoplasts

Mycelia of *A. sydowii* were cultivated on solid PDA medium in Petri dishes covered with a cellophane membrane (BioRad). For inoculation, 3 µl of a suspension of conidia were spotted on the membrane every 5–8 mm. After 2 days at 27 °C, five to seven cellophane membranes were incubated in 40 ml of protoplast buffer (0.8 M NaCl, 0.02 M $MgSO_4$, pH 7.5) with 10 mg Novozyme 234 (Novo Industrie AIS)/ml for 2 h at 30 °C and 100 rpm. Protoplasts were separated using a filter funnel (Schott), centrifuged and washed twice with protoplasts buffer. After a final centrifugation, they were resuspended and adjusted to a concentration of 10^9 protoplasts/ml in protoplast buffer. Protoplasts of *Acremonium chrysogenum* were generated according to Walz and Kück (1993).

Transformation

A. sydowii was transformed according to the method of Walz and Kück (1993) with the following modifications: 100 µl of protoplast suspension were mixed with 5–10 µg of circular or 5 µg of linear plasmid DNA (pMW1 or pBC-Hygro). For cotransformation experiments, 10 µg of pBC-Hygro and 10 µg of pSIM9.9 or p82.9 were used. After 10 min incubation on ice, 200 µl of 25% PEG 600 were added and incubated for 20 min at room temperature. Four ml PDAS top agar (PDA with 20% saccharose and 0.8% (w/v) agar) were mixed with 160 µl transformation mixture and plated on PDAS solid medium. After 24 h, 7 ml of top agar with 150 U hygromycin B/ml were added. Transformants, which appeared after 4–6 days, were transferred to PDA media containing 30 U hygromycin B.

Pulsed-field gel electrophoresis

Intact chromosomes were isolated according to the method of Walz and Kück (1991) with the following modifications. Protoplast suspension was mixed with an equal volume of InCert agarose (45 °C, BioRad). The solidified samples were incubated with detergents and enzymes according to Orbach et al. (1988). The gel electrophoretic separation of chromosomes was done in a CHEF-Mapper apparatus (BioRad) using contour-clamped homogeneous field (CHEF) electrophoresis (Chu et al. 1986). Chromosomes from *Acremonium chrysogenum*, *Schizosaccharomyces pombe* strain 972h$^-$ and *Saccharomyces cerevisiae* strain YNN295 (BioRad) were used as size standards. Chromosomes were electrophoresed in 0.8 or 1% agarose gels (FastLane, BioRad) with 0.5×TBE buffer. Running conditions for intact chromosomes were as follows: constant power of 1.4 V/cm, angle of 120° between the field vectors, 96 h with a 60-min pulse time and 72 h with a 45-min pulse time.

DNA extraction and Southern hybridization

Fungal genomic DNA was isolated as described by Jekosch and Kück (2000). Southern blots were performed according to Sambrook et al. (1989) and were hybridized with the following radiolabeled DNA probes: The *hph*-specific probe was excised from plasmid pMW1 using restriction enzymes *Eco*RI and *Bam*HI. The rDNA probe was derived from plasmid pMY60 containing the rDNA of *Saccharomyces carlsbergensis* (Verbeet et al. 1983).

Quantitative analysis of β-galactosidase activity

β-Galactosidase activity of *A. sydowii* transformants was determined using a fluorometric assay with 4-methylumbelliferyl-β-D-galactoside according to the method of Miller (1972) and Schmitt et al. (2001) with the following modifications. Transformants were grown for 4 days in liquid culture. Mycelia were homogenized using the micro-dismembrator S (Braun Biotech international) with 1 ml extraction buffer and glass beads.

Light and fluorescence microscopy

The cells were observed with a Zeiss axiophot microscope using differential interference contrast light microscopy (DIC) or fluorescence light and the appropriate Zeiss filter combination for GFP and DAPI. Photographs were taken with Ektachrome 64T (Kodak).

Accession numbers

The nucleotide sequences of the 5.8S rRNA gene, ITS1 and ITS2 for the different *A. sydowii* strains are deposited in the EMBL database under the following accession numbers: AJ312219 (strain NRRL242), AJ312220 (strain NRRL244), AJ312221 (strain Y30462/FH2551), AJ312222 (strain NRRL249), AJ312223 (strain NRRL250), AJ312224 (strain NRRL254) and AJ312225 (strain IAM2544). Accession numbers of the corresponding sequences from other fungi used in the phylogenetic analysis are as follows: AJ000933 (*Aspergillus nidulans*), AJ280006 (*Aspergillus niger*), AF176662 (*Aspergillus fumigatus*), AB000533 (*Aspergillus oryzae*) and AF033465 (*Penicillium chrysogenum*).

Results

Growth characteristics and phylogenetic relationship of the *A. sydowii* mulundocandin-producing strain FH2551

For the characterization of the mulundocandin-producing strain FH2551 of *A. sydowii*, several culture conditions were tested. The filamentous fungus was grown on liquid or solid PDA, which encourages fast growth and the generation of conidiospores after 3–4 days. To determine the optimal growth temperature on solid PDA medium, strain FH2551 was grown in a temperature range from 22 to 40 °C for 14 days. Quantitative growth analysis of the strain FH2551 revealed a colony size of 50 cm^2 at 27 °C and a growth reduction of 40% at 22 °C or 60% at 32 °C (data not shown). Retardation of growth was accompanied with a reduction of conidiospore formation or even the loss of conidiogenesis.

To investigate the phylogenetic relationship of strain FH2551 to other *A. sydowii* strains and different species of the genus *Aspergillus*, highly variable sequences from the genomic rDNA locus were used. ITS1 and ITS2 and the intermediate 5.8S rDNA sequences, which have been used before to study phylogenetic relationships of fungi, were amplified from *A. sydowii* strains using PCR (White et al. 1990). The ITS sequences of three out of six isolates of *A. sydowii* obtained from culture collections were found to be identical, whereas the sequence of strain FH2551 showed significant deviations (data not shown). The phylogenetic tree generated from the sequence data applying the maximum-parsimony method is shown in Fig. 1. Strain FH2551 is grouped separately from the cluster of the other *A. sydowii* strains. This separated position was obtained with a bootstrap value of 100%, indicating its statistical significance. These conclusions were verified by a similar tree, which was generated with the neighbor-joining procedure (data not shown).

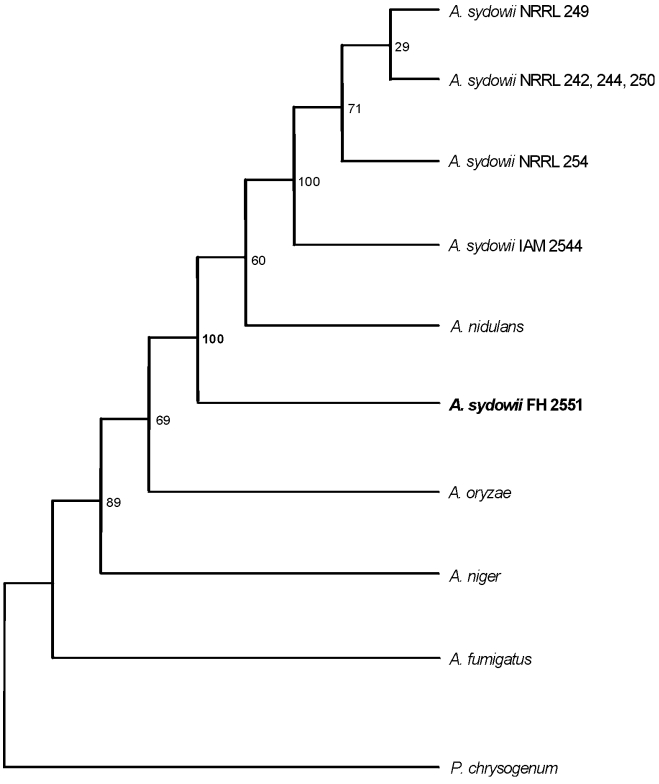

Fig. 1 Phylogenetic relationship of *Aspergillus sydowii* strains based on the nucleotide sequences of the internal transcribed spacers (ITS1, ITS2) and 5.8S rDNA. The tree was generated using the maximum-parsimony method. The sequence from *Penicillium chrysogenum* was used as an outgroup. Accession numbers of sequences are listed in Materials and methods

Pulsed-field gel electrophoresis for karyotype analysis of *A. sydowii*

Genome size and the number of chromosomes are important parameters when genetic engineering of an organism is considered. The application of pulsed-field gel

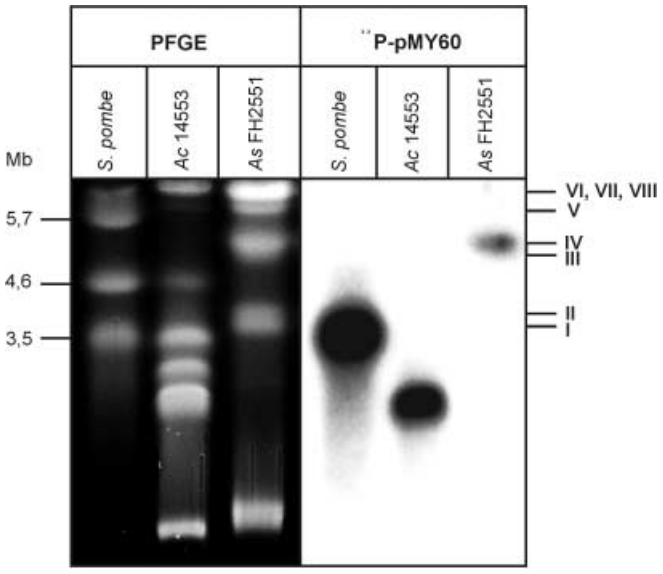

Fig. 2 Pulsed-field gel electrophoresis and Southern analysis of intact chromosomes of *A. sydowii*. Agarose gel of intact chromosomes of *A. sydowii* strain FH2551, *Schizosaccharomyces pombe* and *Acremonium chrysogenum* ATCC 14553. Numbering of *A. sydowii* chromosomes is given on the *right*. Southern analysis using a specific probe for fungal rDNA sequences (pMY60, Verbeet et al. 1983). Sizes (Mb) of *S. pombe* chromosomes are given on the *left*

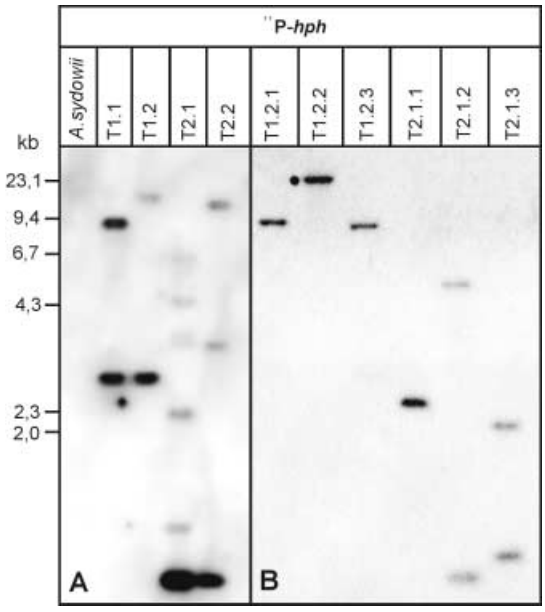

Fig. 3A, B Southern analysis of transformants of *A. sydowii* strain FH2551 harboring plasmids pMW1 (T2.x) and pBC-Hygro (T1.x). Transformation experiments were done with **A** circular and **B** linear plasmid DNA. Thirty µg of genomic DNA were hydrolyzed with *Eco*RI, eletrophoresed and transferred to a nylon membrane. A *hph*-gene-specific probe was used to detect integrated plasmid DNA. *Hin*dIII-hydrolyzed λ-phage DNA was used as a size standard

electrophoresis (PFGE) for the separation of intact chromosomes is an adequate tool in strain improvement for the analysis of chromosomal location of endogenous or transformed genes. In addition, chromosomal translocation, which often occurs during mutagenesis and might affect the biosynthetic capability of a strain, can be documented. We succeeded in separating the intact chromosomes of *A. sydowii* and localized the rDNA locus to chromosome IV (Fig. 2). According to the electrophoretic karyotype, *A. sydowii* possesses eight chromosomes with sizes ranging from approximately 4 Mb to 8 Mb. However, the three largest chromosomes, each of which is greater than 6 Mb, could not be separated, but the intensity of the stained band suggests the presence of three chromosomes. From our data, a genomic size of about 40 Mb was predicted for strain FH2551.

DNA-mediated transformation of *A. sydowii* using the hygromycin B phosphotransferase (*hph*) gene as a selection marker

DNA-mediated transformation is a principal prerequisite for the genetic engineering of *A. sydowii*. As a first step, the resistance of strain FH2551 to commonly used antibiotics that can be applied for the selection of fungal transformants was assessed. Regeneration of protoplasts and mycelial growth was completely inhibited when a concentration of 30 U hygromycin B/ml or 10 µg benomyl/ml was included in the solid medium. In contrast, 100 µg/ml phleomycin still allowed some mycelial growth, but the regeneration of protoplasts was inhibited at a concentration of 10 µg/ml (data not shown). From these experiments, we concluded that hygromycin B is the most suitable antibiotic to be used for selection in *A. sydowii*. For initial transformation experiments, two plasmids that carry the *hph* gene encoding the bacterial hygromycin B phosphotransferase under the control of two different fungal promoters were used. Plasmid pBC-Hygro contains the strong *cpc1* promoter from *N. crassa* (Silar 1995) and resulted in transformation rates of 40–60 transformants per 10 µg DNA in a total of six independent experiments. Plasmid pMW1, containing the promoter from the *pcbC* cephalosporin C biosynthesis gene of *Acremonium chrysogenum* (Kück et al. 1989), resulted in the generation of 10–30 transformants per 10 µg plasmid DNA. Southern analysis of genomic DNA of the transformants demonstrated ectopic integration of the plasmids into the genomic DNA of *A. sydowii* (Fig. 3). Each transformant had an individual pattern of hybridizing bands, indicating different integration sites for the plasmid DNA. By transforming linearized plas-mids, single-copy integration of both plasmids was obtained in the majority of transformants (e.g. T1.2.1–3, Fig. 3B).

Heterologous expression of *lacZ* and *gfp* reporter genes in *A. sydowii*

To test the efficiency of heterologous gene expression in strain FH2551, two different reporter genes were chosen

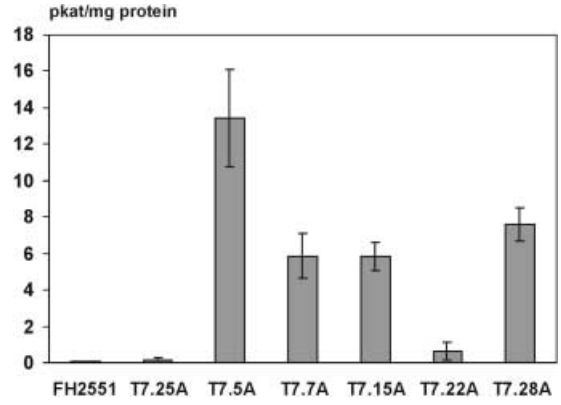

Fig. 4 Quantitative analysis of reporter gene activity of *A. sydowii* recipient strain FH2551 and transformants. Transformant T7.25A contains only the *hph* selection marker on plasmid pBC-Hygro, while the others are cotransformants with plasmids pBC-Hygro and reporter gene plasmid pSIM9.9. β-Galactosidase activity is depicted in pkat/mg protein. *Bars* Standard deviation of four independent experiments.

for analysis. Figure 4 shows the β-galactosidase activity of strain FH2551 and of transformants with plasmid pSIM9.9 containing the *lacZ* gene under the control of the *pcbC* promoter from the β-lactam biosynthesis in *A. chrysogenum*. Transformants were obtained by co-transforming plasmid pBC-Hygro. Southern analysis of the genomic DNA of the transformants detected that transformant T7.25A contained only the plasmid pBC-Hygro with the selection marker but not the reporter gene plasmid pSIM9.9, whereas the other transformants integrated both plasmids into their genomic DNA. Between two (e. g. T7.15A) and seven (e. g. T7.5A) copies of the *lacZ* gene were integrated (data not shown). The β-galactosidase activity varied between 1 and 14 pkat/mg protein. The majority of transformants (>60%) showed more than 6 pkat β-galactosidase activity/mg protein. The activity of the untransformed strain FH2551 and the control transformant T7.25A was nearly zero indicating the absence of endogenous β-galactosidase activity in *A. sydowii*.

Expression of the green fluorescent protein gene (*gfp*) was analyzed using fluorescence microscopy. Figure 5 shows the interference contrast (DIC) and fluorescence microscopic pictures of the untransformed strain FH2551 and of transformant T7.4B carrying multiple copies of the *gfp* gene under control of the constitutive *gpd* promoter from *A. nidulans*. Fluorescence signals resulting from active GFP could be observed in the cytoplasm of hyphae and in the conidiospores, which were still attached to the conidiophore. DAPI staining revealed that each conidiospore contained only one nucleus (Fig. 5). The uneven distribution of GFP fluorescence in the hyphae probably results from cytoplasmic aggregation near septa. In the represented highly vacuolated hyphal compartments the cytoplasm is concentrated at certain spots and GFP fluorescence can be easily detected. This result strongly indicates that GFP is synthesized in *A. sydowii* and is located in the cytoplasm in a stable and active form.

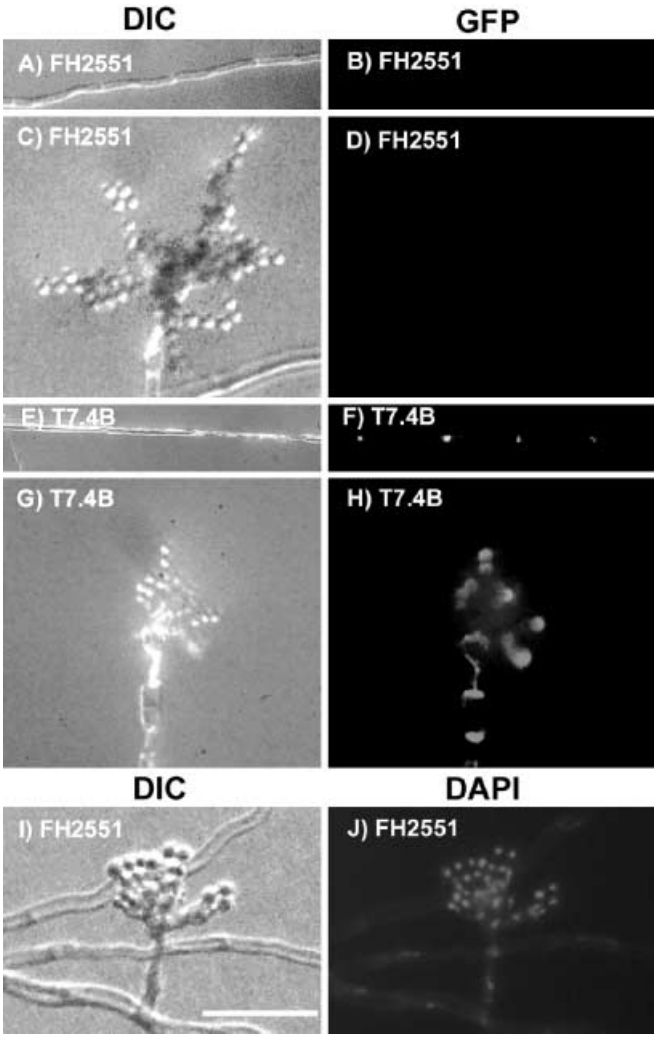

Fig. 5A–J Fluorescence microscopy of *A. sydowii* transformants expressing the *gfp* reporter gene, and DAPI staining of conidiophores. **A–D** Hyphae and conidiophores of untransformed strain FH2551, **E–H** *gfp*-expressing transformant T7.4B. **I–J** DAPI staining of a conidiophore from *A. sydowii* strain FH2551; the nuclei can be seen. *Bar* 12.5 μm

Discussion

This is the first report of the genetic characterization of the filamentous fungus *A. sydowii* to which different molecular techniques were applied. The main objective of the study was to assess the suitability of this fungus for molecular engineering. We used strain FH2551, which is known to be the producer of the antimycotic mulundocandin. Considering morphology and culture characteristics, this strain was previously classified as *A. sydowii*. However, Roy et al. (1987) described the abundant hulle cells and the production of mulundocandin as non-typical for *A. sydowii* and, consequently, classified the strain as a new variant of *A. sydowii* (var. nov. *mulundensis* Roy). In addition, Roy et al. (1987) reported a favored growth temperature of 27 °C for strain Y30462, whereas for other *A. sydowii* isolates the optimal growth

temperature was determined to be 37 °C (Pitt et al. 1988).

Here, the first molecular evidence is provided for the mulundocandin-producing strain FH2551 of *A. sydowii* as not being closely related to other *A. sydowii* strains (Fig. 1), based upon the ITS sequences, which reveal significant differences. The separate position of the *A. sydowii* strain FH2551 in the phylogenetic tree is consistent with the observation that no mulundocandin biosynthesis was described for other *A. sydowii* strains. Thus, deviations in the ITS sequences can be used for the unambiguous identification of the mulundocandin-producing strains of *A. sydowii*.

The electrophoretic karyotype established for *A. sydowii* is another reliable molecular tool to genetically characterize strain FH2551. The electrophoretic conditions were adopted from other filamentous fungi, such as *N. crassa* (Orbach et al. 1988) or *Acremonium chrysogenum* (Walz and Kück 1991), in order to distinguish its large chromosomes. The calculated genome size of approximately 40 Mb and the number of chromosomes (8) for *A. sydowii* is consistent with other aspergilli. In *A. nidulans*, *A. niger* and *A. oryzae*, eight chromosomes each were detected, with genome sizes ranging from 31 to 38.5 Mb (Brody and Carbon 1989; Debets et al. 1990; Sakaguchi et al. 1992). Electrophoretic karyotyping and the localization of genes on specific chromosomes can be used to monitor chromosomal rearrangements during classical and molecular strain improvement. Such rearrangements have occurred for example in fungal antibiotic production strains (Smith et al. 1991; Walz and Kück 1991; Fierro et al. 1993).

The main target of our study was the successful DNA-mediated transformation and the subsequent expression of heterologous genes in *A. sydowii*. We obtained vital transformants using the *hph* gene as a selection marker. Southern analysis showed that all putative transformants contained the *hph* gene, indicating that this selection marker is indeed suitable for *A. sydowii* as was already suggested by the resistance tests. However, the transformation efficiency largely depends on the plasmid used. Observed differences are probably caused by other regulatory regions upstream of the *hph* gene that mediate gene expression. The plasmid pBC-Hygro with the *cpc1* promoter from *N. crassa* resulted in up to 6 transformants/µg DNA and was more efficient than plasmid pMW1. The latter plasmid contains the *pcbC* promoter from *A. chrysogenum* and resulted in 10,000 transformants/µg plasmid DNA when transforming *Aspergillus niger*. Most probably, these high transformation rates are a result of the 100-fold higher regeneration rate of protoplasts of *A. niger* compared to *A. sydowii* (Kück et al. 1989).

The ectopic and multi-copy integration of plasmid DNA into the genomic DNA that was observed in Southern hybridization experiments (Fig. 3) is commonly found in filamentous fungi (review see Fincham 1989). To transform reporter genes in the recipient strain, co-transformations were performed resulting in about 30% of transformants carrying both plasmids (data not shown). Cotransformants harboring the *lacZ* gene, encoding a functional enzyme of 1,021 amino acids, showed reproducible β-galactosidase activity. The *lacZ* gene has often been used as a reporter gene in fungi since its first expression in yeast and *A. nidulans* (Rose and Botstein 1983; van Gorcom et al. 1985). The different β-galactosidase activities in the transformants of *A. sydowii* (Fig. 4) can be explained by position effects due to ectopic integration and variations in the copy number of the reporter gene. The transformant showing the highest activity (T7.5A) contains seven copies of the *lacZ* gene. However, there is no strict correlation between copy number and β-galactosidase activity. Improvement of the expression level can probably be achieved by using strong homologous promoter sequences for gene expression in *A. sydowii*.

The *gfp* gene was the second reporter gene expressed in transformants of strain FH2551. GFP encompasses 238 amino acids and is widely used as a cellular marker in prokaryotic and eukaryotic hosts (Chalfie et al. 1994). However, expression of the wild-type *gfp* sequence in fungi did not result in the emission of green fluorescence when fungal transformants were irradiated with blue light (Lorang et al. 2001). To overcome this problem, different derivatives of the GFP protein showing a range of different excitation and emission peaks were tested and, in some cases, the codon usage was optimized for eukaryotic hosts. In our study, we used the EGFP derivative, with an excitation peak of 489 nm and an emission peak of 508 nm (Patterson et al. 2001), in a version in which silent mutations have generated a human-codon usage preference (Inglis et al. 1999). Similarly, a plant-adapted *gfp* gene version has been successfully expressed in fungi such as *Ustilago maydis* and *A. nidulans* (e.g. Spellig et al. 1996; Suelmann et al. 1997; Fernandez-Abalos et al. 1998). The successful application of the *gfp* gene as a reporter in *A. sydowii* should prove invaluable in the study of cellular localization of GFP-tagged proteins such as the mulundocandin biosynthesis enzymes.

In summary, the *A. sydowii* strain FH2551 seems to be particularly suitable for genetic engineering using the above-described molecular tools. The eukaryotic *gfp* gene, the bacterial *hph* gene and the *lacZ* gene were successfully expressed using three heterologous fungal promoters. Further work will be aimed at the construction of genetically engineered strains with modified biosynthesis genes in order to significantly improve the production yields of mulundocandin.

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