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Transformation of the fungus *Absidia glauca* by complementation of a methionine-auxotrophic strain affected in the homoserine-acetyltransferase gene

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ABSTRACT

Transformation of fungi by complementation of auxotrophs is generally much more reliable than usage of antibiotic resistance markers. In order to establish such a system for the model zygomycete *Absidia glauca*, a stable methionine auxotrophic mutant was isolated after X-ray mutagenesis of the minus mating type and characterized at the molecular level. The mutant is disrupted in the coding region of the *Met2-1* gene, encoding homoserine O-acetyltransferase. The corresponding wild type gene was cloned, sequenced and inserted into appropriate vector plasmids. Transformants are prototrophs and show restored methionine-independent growth, based on complementation by the autonomously replicating plasmids.

1. Introduction

Over the past two decades, the mucoralean fungus *Absidia glauca* has been developed to an important model for studying genetics and molecular biology of the biotrophic fusion parasitism between the host organism *A. glauca* and the mycoparasite of many *Mucor*-like fungi, *Parasitella parasitica* [1,2]. This type of parasitism is characterized by formation of a cytoplasmic continuum between host and parasites and is, thus, predestined for horizontal exchange of genetic information between only distantly related organisms. Already Burgeff [3] observed that the development of infection structures between *P. parasitica* and *A. glauca* depends on complementarity of mating types between the partners. Exclusively plus mating type strains of *P. parasitica* are able to infect minus mating type strains of *A. glauca*, and minus mating types of the parasites are needed to infect plus type hosts. The mating type dependency of parasitism is determined at the level of recognition between the partners, which is assumed to be mediated by the trisporic acid system, normally being involved in recognition of mating partners [4]. Thus, compatible combinations between hosts and parasites are not a consequence of close phylogenetic relationship. *P. parasitica* is much more related to *Mucor racemosus* and *Mucor circinelloides* than to *A. glauca* [5]. Accordingly, other mucoralean fungi behave differently as hosts; *M. racemosus*, which is often used today for transformation experiments, does not show any mating dependency, and any mating type of the parasite is able to infect both host mating types in this and in interactions with other *Mucor* species [3].

The most intriguing feature of *P. parasitica* infection is the transfer of nuclei from the parasite to the host, followed by establishment of foreign genes in the host. This phenomenon that occurs in frequencies between 10^{-3} and 10^{-2} with respect to the total numbers of uninucleated sporangiospores on the Petri dish used for infection, was proven at the level of plasmid DNA that was passed on from transformed *P. parasitica* [6] to the host and, more elegantly, by complementation of auxotrophies for the amino acids methionine and histidine in *A. glauca* by infection with prototrophic *P. parasitica* [1].

The analysis of the mechanisms behind parasexual gene transfer from *P. parasitica* to its host *A. glauca* requires a reliable genetic toolkit that allows convenient identification of genes and especially the ability to manipulate the recipient of parasexual gene transfer by transformation with individual genes in any vector.

Characterization of the genomic sequences for the zygomycetous species *Rhizopus oryzae* (affinity in Mucorales under discussion) [7], *Mucor circinelloides* (Mucoraceae) and *Phycomyces blakesleeanus* (Phycomycetaceae) turned out to be very helpful. All of these are mucoralean fungi, although from different families than *A. glauca* (Absidiaceae) and *P. parasitica* (unknown affinity in Mucorales). These sequences, although from distantly related species, facilitate considerably the molecular characterization of mutants obtained by conventional mutagenesis. Several auxotrophic mutants of *A. glauca* were isolated after X-ray or nitrosoguanidine treatment and were used for protoplast fusion experiments between the mating types [8]. The molecular characterization of these mutants, in comparison with complementation derivatives obtained by para-recombination, and with re-transformants with the corresponding wild type alleles, helps to understand the complementation process between *P. parasitica* and *A. glauca* in more detail.

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In principle, all identified combinations of auxotrophic mutants and the corresponding wild type alleles may form the basis of novel selection markers for genetic manipulation of *A. glauca*. Introducing DNA into *A. glauca* is a highly efficient process that can be obtained by PEG treatment of protoplasts [9], by electroporation [10,11] or by a biolistic procedure based on DNA bound to tungsten particles [12]. The existing vectors for *A. glauca* rely on the bacterial neomycin-phosphotransferase gene controlled by the homologous promoter for actin or the elongation factor EF1α as dominantly selectable marker. Complementation of auxotrophic mutants with the corresponding wild type allele allows minimisation of false-positive transformants by strong selection for complemented transformants without the problem of physiological adaptation of the fungi also to high concentrations of neomycin. This approach was used for *Mucor circinelloides* [13], *Rhizomucor pusillus* [14] and *Rhizopus niveus* [15] by complementation of *leu1* mutants defective in isopropylmalate isomerase. Other transformation approaches are based on obtaining defined mutants with defects in the orotidine-5′-monophosphate decarboxylase gene for pyrimidine biosynthesis by selecting for growth on 5-fluoroorotic acid containing minimal medium and complementation of these mutants by wild type genes. This approach proved useful for *Mucor circinelloides* [16], *Mortierella alpina* [17] and *Rhizopus oryzae* [18].

In order to add a novel tool to genetic manipulation of *A. glauca*, especially with respect to understanding biotrophic fusion parasitism and horizontal gene transfer, we developed a convenient transformation system based on a stable methionine-auxotrophic *A. glauca* mutant and restoration of prototrophy by transformation with plasmids harbouring the corresponding wild type allele.

2. Materials and methods

2.1. Strains, media and transformation procedure

Absidia glauca mutant strain RVII-324 *met*$^-$ [1,8] was derived from the wild type CBS 101.48 minus type by X-ray mutagenesis, RVIII-249 *met*$^-$ [8] is a X-ray-derivative of CBS 100.48 plus type, and the double mutant RIXNG2-672 *met*$^-$/*lys*$^-$ was obtained from RVII-324 *met*$^-$ by subsequent treatment with nitrosoguanidine. The mutants were used for complementation assays on minimal medium with intermediates of the methionine biosynthesis pathway, and strain RVII-324 *met*$^-$ for fungal transformation. Fungal strains were grown on complete [19] or minimal medium [10], supplemented with the appropriate amino acid or their putative intermediates, homoserine, O-succinylhomoserine, cystathionine and homocysteine (Sigma–Aldrich, Munich, Germany), at final concentrations of 250 μM. Protoplast formation and electroporation conditions were as described [11]. After transformation, spores collected from three successive sporulation cycles on minimal medium were tested for mitotic stability by testing the percent prototrophy of each spore suspensions on minimal (i.e., lacking methionine) and complete medium. The number of colonies on complete medium was used to calculate total viable spore numbers, while counts on minimal medium represent the number of spores containing the wild type *Met2-1* gene. Single spore colonies from the minimal medium were transferred after 3 days onto new minimal medium agar plates and used for subsequent sporulation cycles. *Escherichia coli* strain XL1-blue [20] was used for cloning experiments.

2.2. Cloning and vector construction

Primer pairs used for cloning of methionine biosynthesis gene fragments and vector construction are listed in Table 1. PCR conditions for amplification with Taq polymerase were as described [2]. Fragments were amplified in a MWG-Biotech thermocycler with the following temperature profile: 5 min – 94 °C, 30 cycles with: 45 s – 55 °C, 60 s – 72 °C, and 45 s – 94 °C. To amplify gene fragments, conserved amino acid motifs were exploited for designing degenerated primer pairs A, B and C, which were used for amplification of parts of the *Met2-1* and *Met2-2* genes, encoding putative homoserine O-acetyltransferases, or *Met3*, encoding the putative γ-cystathionine synthase. Primer pair D was used for inverse PCR with *Hin*dIII digested and ligated genomic DNA of *A. glauca* CBS 101.48 minus wild type strain. The complete *A. glaucaMet2-1* gene was amplified with primer pair E, digested with the restriction enzymes *Bam*HI and *Hin*dIII and cloned into the corresponding restriction sites of pTZ19R, creating pMet2-1. The actin promoter of *A. glauca* was amplified as described [21], digested with *Hin*dIII and *Bsp*HI, and cloned in the *Hin*dIII/*Nco*I sites of pEGFP (Clontech, Palo Alto, USA), creating paEGFP. The neomycin-phosphotransferase gene was amplified by primer pair F with pEBFP (Clontech, Palo Alto, USA) as template. The fragment was digested with *Bsr*GI and *Xba*I and cloned into the appropriate sites of paEGFP. The complete cassette was cut out with *Eco*RI and subcloned in pBluescriptIISK (Stratagene, La Jolla, USA). The cassette was cut out with *Bam*HI and *Hin*dIII, inserted between the *Hin*dIII and the partially restricted *Bgl*II sites of pMet2-1, creating pMet2EGFPNeo. The *A. glauca* actin promoter was amplified with primer pair G, digested with *Bam*HI and *Hin*dIII and cloned in the corresponding sites of YesNTC (Invitrogen, Carlsbad, California, USA) harbouring the coding sequence of the *Mucor mucedo sexM* transcription factor fused to EGFP, a vector constructed for transformation of *Saccharomyces cerevisiae* [22]. The cassette was cut out with *Hin*dIII and *Xba*I and inserted in *Hin*dIII digested pMet2-1. Remaining sticky ends were filled in using Klenow DNA polymerase and the fragments ligated for a second time prior to transformation of *E. coli*. The resulting vector was named pMet2SexMEGFP.

2.3. DNA preparation and Southern blot analysis

Absidia glauca DNAs were extracted as described [21]. DNA probes were amplified using DIG label mix (Roche, Mannheim, Germany) according to the manufacturer's instructions. Primers D_{for} and $D1_{back}$ were used for labelling the *A. glaucaMet2-1* gene fragment, and primer pair H was used for generating a labelled GFP probe with pEGFP as template. Hybridization conditions were as described [23].

2.4. Fluorescence and light microscopy

Microscopic investigations were performed with a Zeiss Axiophot. Differential interference contrast (DIC) was used for light microscopy. For fluorescence microscopy, the following Zeiss filter set was used: BP 485 excitation, FT 510 dichroic and 515–585 emission filter.

3. Results

3.1. Characterization of A. glauca met$^-$ mutants

Growth behaviour of the mutants on minimal medium supplemented with different intermediates of the biosynthesis pathway towards methionine provided the first clues for identifying the function impaired by the mutations (Table 2). The interpretation of these experiments pointed to inactivation of either the gene for homoserine O-acetyltransferase or, alternatively, to a defect in the γ-cystathionine synthase gene, based on a model for methionine biosynthesis established for *Neurospora crassa* [24]. In fungi, O-acetyl homoserine is generally expected as natural product of the latter enzyme. However, the de facto substrate specificity of the *A. glauca* γ-cystathionine synthase has not yet been determined experimentally.

Screening of the three sequenced mucoralean genomes for putative homoserine O-acetyltransferase genes (*Met2*) or γ-cystathionine synthase genes (*Met3*) using the MycoCosm platform [25] reveals two putative *Met2* genes and one putative *Met3* gene in all three genomes.

Table 1
Primer list, restriction sites are underlined.

Pair	Direction	Nucleotide sequence	Used for
A	For	5′GGGGATCCGCNGAYGTNGARGAYTGG3′	*Met2 - 1*
	Back	5′GGGTCGACTARCARTTNGCRTTCRAA3′	
A1	For	5′GGGGATCCATGGGNGGNATGCA3′	*Met2 - 1, 2 - 2*
B	For	5′GGGGATCCGGNTGGTGGGARGAYTT3′	*Met2 - 2*
	Back	5′GGGTCGACATYTCYTTYTGYTGCCA3′	
C	For	5′GGGGATCCAARCARTTYTGGCARCA3′	*Met3*
	Back	5′GGGTCGACGGRAANCCRAARCANAC3′	
D	For	5′GGGTCGACGTTGGTCTCTCAGCTGCAAG3′	*Met2* - 1, *A.g.*
	Back	5′CCGGATCCAAGACTTGCATACCACCCATGG3′	
D1	Back	5′CCCTGCAGCACCATTGCGTAATGTCCACC3′	
E	For	5′GGAAGCTTTGACAGCTGTTTCATTGATG3′	*Met2* - 1, *A.g.*
	Back	5′TCGGATCCCTTGAAGCCAGGTACGATAATGTC3′	
F	For	5′CTGTACACAATTGAACAAGATGG3′	Neo^R
	Back	5′CTCTAGAAAGAACTCGTCAAG3′	
G	For	5′GGAAGCTTCGCAAAAGGGAGAATGCTAC3′	Actin promoter
	Back	5′GGGGATCCCATAGACATGATGATGTGG3′	
H	For	5′CCACCATGGTGAGCAAGGGC3′	EGFP
	Back	5′CTTGTACAGCTCGTCCATGCC3′	

Table 2
Metabolic characterization of *A. glauca* mutants. Strains were grown on minimal medium containing the indicated supplements.

Intermediate of methionine biosynthesis	RVIIING-249 plus	Growth RVII-324 minus	RIXNG2 - 672[a] minus
No addition	–	–	–
Homoserine	–	–	–
O-Succinyl - homoserine	–	–	–
Cystathionine	+	+	+
Homocysteine	+	+	+
Methionine	+	+	+

[a] For growth of the double mutant RIXNG2 - 672 lysine was always added to the medium.

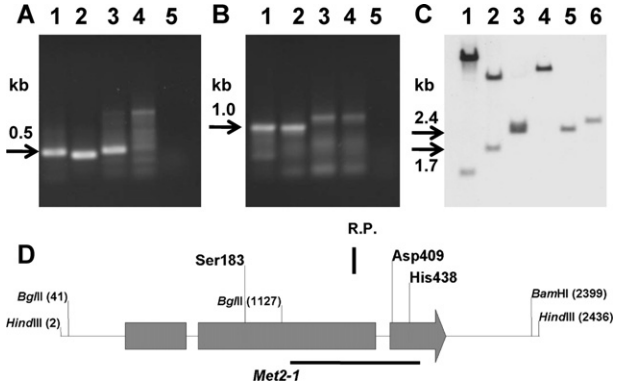

Fig. 1. Molecular characterization of the *Absidia glauca met*⁻ mutant RVII-324 compared with the parental wild type minus strain CBS 101.48 by PCR and hybridization with the corresponding wild type *Met2 - 1* gene. (A and B) PCR with genomic DNAs of *Mucor circinelloides* R7B (lanes 1), *Parasitella parasitica* CBS 412.66 (lanes 2), *Absidia glauca* CBS 101.48 (lanes 3), RVII-324 (lanes 4) and without DNA as control (lanes 5), obtained with primer pair $A1_{for}$ / A_{back} (A) or primer pair B (Table 1), amplifying *Met2 - 1* or *Met2 - 2* gene fragments. (C) Hybridization of RVII-324 genomic DNA (lanes 1–3) or CBS 101.48 DNA (lanes 4–6) with the *A. glaucaMet2 - 1* wild type gene fragment (underlined part in D). DNAs were restricted with *Bam*HI (lanes 1, 4) *Hin*dIII (lanes 2, 5) or *Nco*I (lanes 3, 6). (D) Map of the sequenced *A. glaucaMet2 - 1* wild type gene. Positions of important amino acids forming the putative catalytic triad are shown. The recombination point disrupting the open - reading frame in the mutant is marked by R.P.

The *Met2* copies differ to a higher extent within a single species than between different species when comparing each of the two types between species. This indicates an ancient gene duplication event and supports the assumption that wild type *A. glauca* contains only a single active gene for homoserine O-acetyltransferase.

The PCR patterns for *Met2-1* differ between *A. glauca* wild type and the auxotrophic mutant RVII-324 (Fig. 1A). No differences in PCR patterns between mutant and wild type were found for *Met2-2* (Fig. 1B). Southern blot analysis using the cloned *A. glauca* wild type *Met2-1* copy (Acc. No. JQ898110) as probe reveals large rearrangements at the *Met2-1* locus in the chromosomal DNA of the RVII-324 mutant (Fig. 1C), which was found to be due to a translocation with one end point in the open-reading frame. This gene disruption event leads to two independent gene fragments located in different genomic regions as shown by restriction analysis. Analysing the mutant locus by hybridization with probes derived from upstream or downstream regions with respect to the recombination point (Fig. 1D) shows only a single hybridization signal, thus excluding the possibility for a gene duplication event. Disruption of the open-reading frame separates the conserved amino acid motifs, which are assumed to form the putative catalytically active triad (Fig. 1D), as shown for other fungal homoserine O-acetyltransferases [26]. The role of the second *Met2* hybridization signal in *A. glauca* is unclear. It is, however, not able to complement the mutant phenotype, otherwise the methionine auxotrophy would not have been recognized following the initial mutagenesis experiment.

Southern blot analysis with a *Met3* gene fragment as hybridization probe does not reveal differences between RVII-324 and wild type *A. glauca* (data not shown). It is, therefore, highly improbable that X-ray treatment has affected the gene for γ-cystathionine synthase in addition to the *Met2-1* gene for homoserine acetyltransferase.

As expected, the double mutant RIXNG2-672, derived from RVII-324 by a second mutagen treatment, shows identical rearrangements at *Met2-1*.

3.2. Complementation of the mutant by transformation with the Met2-1 wild type allele

The *A. glauca* wild type *Met2-1* gene was inserted in several vectors useful for transformation of *A. glauca*. The plasmid pMet2-1 allows selection of transformants on minimal medium without methionine, and pMet2GFPNeo (Fig. 2A) carries as additional markers the gene for enhanced green fluorescent protein (EGFP) and the neomycin-phosphotransferase gene under the control of the *A. glauca* actin promoter. The third vector, pMet2sexMGFP (Fig. 2B), contains the open-reading frame for the *sexM* transcription factor gene of *M. mucedo* [22].

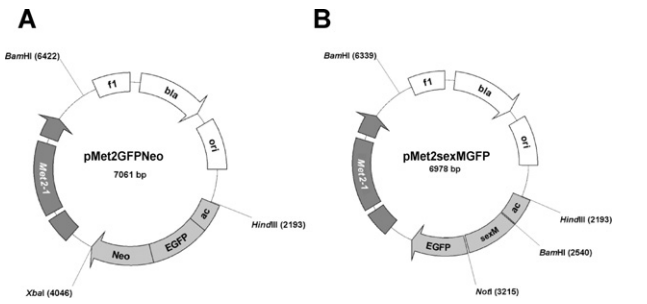

Fig. 2. Map of transformation vectors. (A) pMet2GFPNeo carries the gene for enhanced green fluorescent protein (EGFP) and the neomycin-phosphotransferase gene (Neo) under the control of the *A. glauca* actin promoter (ac). (B) pMet2sexMGFP contains the open-reading frame for the sexM transcription factor gene (*sexM*) of *Mucor mucedo* fused to EGFP.

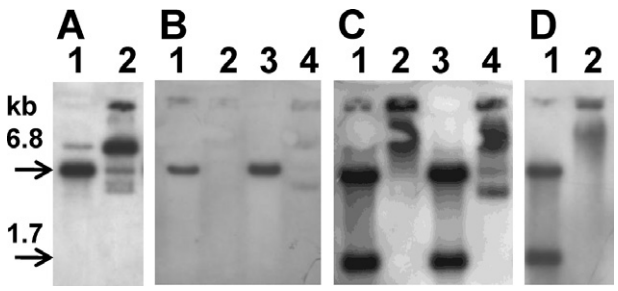

Fig. 3. Hybridization analysis of *A. glauca*, transformed with pMET2sexMGFP. (A–D) DNAs were restricted with *Hind*III (lanes 1 and 3) or loaded without digestion (lanes 2 and 4). (A) Vector DNA was loaded and hybridized with a labelled EGFP probe. (B and C) Genomic DNAs of transformants 1 (lanes 1 and 2) and 2 (lanes 3 and 4) were hybridized with the EGFP probe (B) or the *Met2-1* gene fragment (C). (D) Control DNA of the untransformed RVII-324 mutant was hybridized with *Met2-1*.

Table 3
Stability of the prototrophic phenotype of single spore isolates from pMet2sexMGFP transformant no. 2. The primary colony starts from a multinucleated protoplast containing an unknown number of nuclei, at least one of which is transformed, whereas in sporulation cycles 1 and 2, the colony grows up from a single uninucleated spore. Aliquots of the total spore progeny were plated on complete medium for counting total progeny and on minimal medium to determine the number of prototrophs after sporulation cycles. Prototrophic rates between sporulation cycles are comparable within a factor of 2, as the second cycle is started with a prototrophic uninucleated spore of the first one.

Sporulation cycle	Total number of plated spores	Number of prototrophic colonies	Ratio of prototrophic colonies
Primary colony	8×10^6	37	4.6×10^{-6}
1	1.2×10^5	86	7.2×10^{-4}
2	2.4×10^5	93	3.9×10^{-4}

This high mobility group transcription factor gene is fused in frame to EGFP and is transcriptionally controlled by the *A. glauca* actin promoter. The *sexM* gene was identified in *P. blakesleeanus* and proposed to represent the major transcription factor gene for control of sexual reaction in the minus mating type [27].

Transformants growing methionine-independently on minimal media were obtained with all three vectors. Protoplasts obtained from germinating mycelia from 10^7 to 10^8 spores and 5–10 μg vector DNA yielded between 1 and 10 transformants, depending on the individual experiment. Differences in transformation efficiency between the plasmids were not observed. In accordance with previous observations with transformation of zygomycetes, some of the transformants show an unstable, abortive phenotype, and the sporangiospore progeny fails to develop colonies on minimal medium. Other transformants give rise to prototrophic single spore isolates for several sporulation cycles (Table 3). The term sporulation cycle describes the development, ideally, from a single spore colony to a completely grown and sporulated Petri dish culture. In a syncytial organism without delimited individual cells like the zygomycete *A. glauca*, it is not possible to measure stability of genetic traits in subsequent mitotic divisions. A reasonable, convenient technique to assess genetic stability makes use of the progeny of mitotic sporulation cycles. One cycle gives rise to approximately 10^9 uninucleated sporangiospores. It is widely accepted to base stability calculations on sporulation cycles, as the increase in sporangiospores is the only parameter that can reliably be measured. The mycelium itself contains of course additional nuclei, but as these are not passed on to the subsequent growth cycle, these must not be taken into account in this simplified but reliable calculation approach. Based on this consideration, the increase in spore number from 1 to 10^9 (approximately 2^{30}) corresponds to 30 subsequent mitotic divisions. This simplification for nuclear divisions, an analogy to growth behaviour of single cells, permits comparable calculations.

3.3. Southern blot analysis of transformants

Two derivatives of RVII-324 obtained by transformation with pMet2sexMGFP that express the gene for EGFP were chosen for characterization by Southern type hybridization experiments (Fig. 3). DNAs from transformants and the mutant strain were digested with *Hind*III and compared with the restriction pattern of the vector. Blots were hybridized with a probe for EGFP (Fig. 3A and B), followed by hybridization with the *A. glaucaMet2-1* probe (Fig. 3C and D). The *Hind*III hybridization signals with EGFP of the transformant DNA match with the linearized vector. Several weak signals are found in experiments with undigested bulk DNA (Fig. 3B, lane 4), which points towards autonomous replication of the plasmid, the normal situation in *A. glauca* transformation experiments. The genomic *Met2-1* hybridization pattern does not differ between transformants and the parental mutant RVII-324, indicating that the *Met2-1* locus was not affected by recombination events following transformation.

3.4. Localizing EGFP expression

After transformation with the GFPNeo construct, GFP expression becomes visible in the cytoplasm as expected (Fig. 4A and B) The usefulness of the selection for methionine prototrophy in combination with GFP as additional reporter gene becomes even more obvious if GFP is expressed as fusion protein with the *M. mucedo* transcription factor sexM. This fusion protein stays essentially in the cytoplasm. The fluorescence seems to be slightly increased near the cell membrane (Fig. 4C). This novel vector allows for the first time to follow the fate of proteins in mucoralean fungi, here *A. glauca*.

4. Discussion

The new transformation system based on selection for prototrophic growth after transformation of auxotrophic mutants with the corresponding, homologous gene is a major improvement of previous approaches that relied completely on the bacterial Neo^R marker. The most obvious advantage is the stronger selection for growth of transformants that avoids the high proportion of false-positives that are normal for selection on neomycin or other aminoglycoside antibiotics. The model zygomycete *Absidia glauca* generally establishes incoming plasmids as autonomously replicating genetic elements [9,11], which is the general rule for any zygomycete. This observation holds also true for the new vectors harbouring the *Met2-1* selective marker. Recombination with chromosomal DNA after transformation has been shown [10], but is extremely rare in *A. glauca*, and permanent homologous integration has never been observed. Also in this respect, the *Met2-1* gene does not behave differently. Mitotic stability

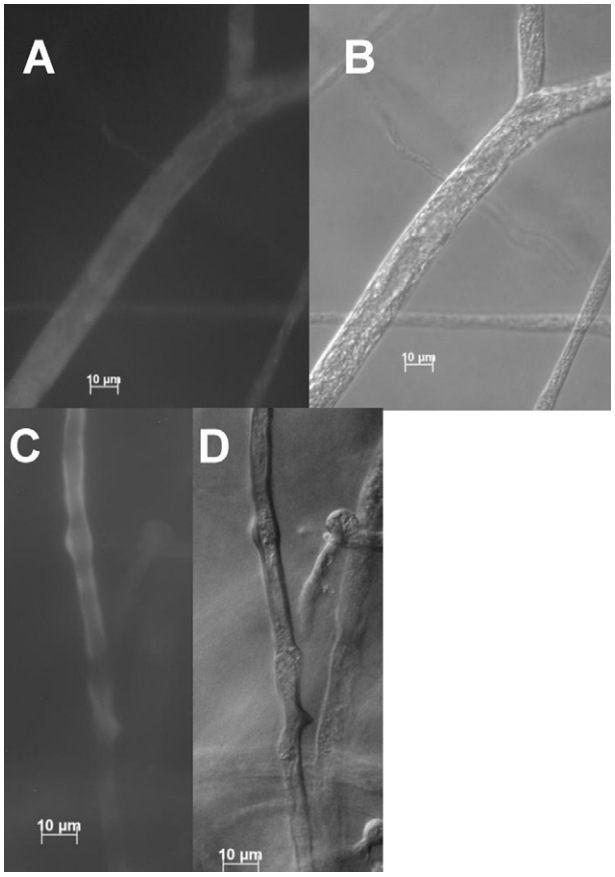

Fig. 4. EGFP expression in *A. glauca* transformants. (A and B) Mycelium of transformant expressing the GFPNeo cassette. (C and D) Mycelium of a SexMGFP expressing transformant. (B and D) Images obtained by differential interference contrast, (A and C) fluorescent images using the following filter combination: excitation: 485 ± 20 nm, emission: 515–585 nm.

of transformants is however sufficiently high to be used for complementing mutants and for using the GFP marker.

The new transformation approach together with the set of versatile vectors described in this communication becomes especially attractive due to the lack of reversion for the mutation leading to methionine auxotrophy in the minus mating type. X-ray treatment leads very often to chromosomal single- and double-strand breakage, and subsequent repair processes often initiate considerable rearrangements. The minus mating type methionine auxotrophic mutant RVII-324 fits into this general scheme. Our analysis reveals a translocation event with one break point in the coding region of the *Met2-1* gene. This situation explains the low reversion frequency below the detection level and stability of the mutant phenotype. The mutant phenotype can, however, be restored completely by transformation with the wild type allele, proving clearly that the other necessary enzymes towards methionine biosynthesis are functionally active in the mutant.

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