Sexual development in the industrial workhorse *Trichoderma reesei*

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Filamentous fungi are indispensable biotechnological tools for the production of organic chemicals, enzymes, and antibiotics. Most of the strains used for industrial applications have been—and still are—screened and improved by classical mutagenesis. Sexual crossing approaches would yield considerable advantages for research and industrial strain improvement, but interestingly, industrially applied filamentous fungal species have so far been considered to be largely asexual. This is also true for the ascomycete *Trichoderma reesei* (anamorph of *Hypocrea jecorina*), which is used for production of cellulolytic and hemicellulolytic enzymes. In this study, we report that *T. reesei* QM6a has a *MAT1-2* mating type locus, and the identification of its respective mating type counterpart, *MAT1-1*, in natural isolates of *H. jecorina*, thus proving that this is a heterothallic species. After being considered asexual since its discovery more than 50 years ago, we were now able to induce sexual reproduction of *T. reesei* QM6a and obtained fertilized stromata and mature ascospores. This sexual crossing approach therefore opens up perspectives for biotechnologically important fungi. Our findings provide a tool for fast and efficient industrial strain improvement in *T. reesei*, thus boosting research toward economically feasible biofuel production. In addition, knowledge of *MAT*-loci and sexual crossing techniques will facilitate research with other *Trichoderma* spp. relevant for agriculture and human health.

biofuels | *Hypocrea jecorina* | mating type | female sterility | cellulase

The increasing awareness for the limited supply of fossil fuels, accompanied by the recent rise of energy costs and an imminent climate change has recently led to increased research efforts toward development of biofuels (1). As one of the most prolific cellulase producers, *Trichoderma reesei* represents an ideal model system to study the regulation of plant cell wall degrading enzymes, which play an important role in conversion of cellulosic waste material into glucose, which is then fermented to bioethanol by yeast. Using this approach, lignocellulosic biomass from agricultural byproducts or even municipal solid waste can become an environmentally compatible energy source for the future. However, the efficiency of the respective enzyme mixtures still needs considerable improvement to render this process economically feasible (2, 3).

The enzyme producer *T. reesei* is unique among industrially applied fungi, because it is solely known from the single wild-type isolate QM6a, which was originally isolated from the Solomon islands in World War II because of its degradation of canvas and garments of the US army (4). All strains used nowadays in biotechnology and basic research have been derived from this one isolate. Presently, strain development in this fungus is a major focus of industrial research. Besides the commonly used classical mutagenesis approaches using UV light or mutagenic chemicals, a broad array of genetic engineering techniques and DNA-mediated transformation systems have been developed for *T. reesei* to improve the enzyme production capacity of QM6a-derived strains. However, similar to other industrially important fungi, classical genetic approaches using sexual crossings, as have been established for the model fungi *Aspergillus nidulans* or *Neurospora crassa*, are unavailable.

The genus *Trichoderma*/*Hypocrea* contains several hundred species, some of which only occur as teleomorphs, i.e., in their sexual form, whereas others have so far only been observed as asexually propagating anamorphs (5). In the last decade, the use of DNA-based molecular phylogenetic approaches has succeeded in the identification of anamorph-teleomorph relationships for several fungi (including *Trichoderma* spp.) that were so far believed to occur only in an asexual form. However, only few of these could be mated under laboratory conditions (6). Using gene sequence analysis, Kuhls et al. (7) found that *T. reesei* is indistinguishable from the the pantropical ascomycete *Hypocrea jecorina.* However, despite this in silico evidence, attempts to cross *T. reesei* with wild-type strains of *H. jecorina* failed, giving rise to the assumption that QM6a would be an asexual clonal lineage of *H. jecorina*.

Filamentous ascomycete fungi can have two mating types, *MAT1-1* and *MAT1-2*, and these *MAT* loci occupy the same chromosomal location but lack sequence similarity and are thus termed "idiomorphs" rather than alleles (8). The genes necessary for signal transduction and the formation of sexual reproduction structures for both mating types are present in each genome, but are strictly regulated by the respective *MAT* locus. Ascomycete fungi are haploid during their vegetative life cycle and can either have a heterothallic or a homothallic sexual cycle. Heterothallic fungi need a compatible strain carrying the opposite *MAT* idiomorph for sex (9). Crossing experiments using single ascospore isolates of *H. jecorina* wild-type isolates showed a typical bipolar segregation for mating type, suggesting it to be a heterothallic species (10).

The aim of this study was to (re)address the question whether the industrial workhorse *T. reesei* QM6a is really an asexual clonal line. We identified the *MAT1-2* mating type locus of QM6a and cloned and characterized the opposite *MAT1-1* locus of a purified, sexually compatible wild-type isolate of *H. jecorina*. Thereby we could also confirm at a molecular level that this species is heterothallic. Our crossing approach also works for strains engineered for enhanced cellulase production. Further, we developed a technique for sexual crossing of *T. reesei* QM6a, the ancestor of all industrially used strains of this species, and we could consequently show that QM6a can be successfully crossed with a *H. jecorina* strain of *MAT1-1* mating type. The presence

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession no. FJ599756) (MAT1–1 locus) or are available at the *Trichoderma reesei* genome database v2.0 (http://genome.jgi-psf.org/Trire2/Trire2.home.html) under protein ID 124341 (*mat1-2-1*).

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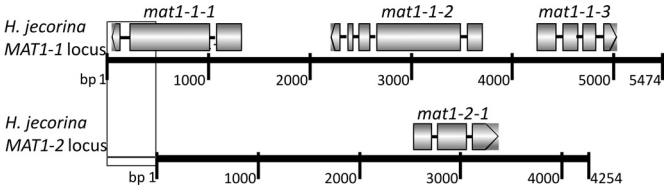

Fig. 1. Mating type idiomorphs of *H. jecorina*. The *MAT1-1* locus has a length of ≈5.5 kb and contains three genes, *mat1-1-1*, *mat1-1-2*, *mat1-1-3*, and *MAT1-2* has a length of ≈4.3 kb and contains one gene, *mat1-2-1*. The exons of genes are shown as boxes, connected by lines that represent the introns. The 3′-end of *mat1-1-1*, present in strains of both mating types, is marked with a gray box. The exon/intron structures are drawn to scale.

of a sexual cycle provides an invaluable tool for classical genetic analyses, and our findings therefore will form the basis to greatly facilitate genetic work and industrial strain improvement with this fungus. In the following, we will use the name of the anamorph *T. reesei* for strain QM6a and mutant strains derived from it, and the name of the teleomorph *H. jecorina* for strains known to propagate sexually.

Results

Analysis of the Mating Type of *T. reesei* QM6a. We screened the *T. reesei* genome database v2.0 (11) for the presence of a mating type locus. Characterization of mutants led to the identification of the *mat1-2-1* gene of the *Neurospora crassa* MAT1–2 idiomorph, which encodes a protein with a high mobility group (HMG) domain, as the main regulator of sexual development in *MAT1-2* strains. In *N. crassa*, *Podospora anserina*, and *Gibberella fujikuroi*, the mating type idiomorphs contain an equal set of genes (9). Tblastn searches of the *T. reesei* genome database with the *mat1-2-1* homologs of these fungi revealed that the sequenced strain QM6a has a *MAT1-2* mating type locus with an intact single ORF encoding an HMG-domain protein (Fig. 1). The corresponding *T. reesei* gene, *mat1-2-1*, denominated following the nomenclature of mating type genes of filamentous ascomycetes suggested by Turgeon and Yoder (12), was found on scaffold 6 and has protein ID 124341 in *T. reesei* genome database v2.0. The finding of a typical *MAT1-2* locus is indicative of a heterothallic sexual lifestyle and therefore successful mating of QM6a would require a strain of the opposite *MAT1-1* mating type.

Isolation of Both Mating Types from *H. jecorina*. To study the potential for sexual reproduction of *T.reesei* QM6a, elucidation of the *H. jecorina MAT1-1* mating type was essential. To find the *MAT1-1* locus of *H. jecorina*, we made use of single ascospore cultures of *H. jecorina* CBS999.97. This strain was reported to be able to form mature stromata (fruiting bodies) on agar plates under laboratory conditions (10). Stromata, the sexual reproduction structures of *Hypocrea* spp. (13) are macroscopical structures consisting of hyphal mass that is pigmented at the surface, into which the actual fruiting bodies, the perithecia, are embedded. In these perithecia, the asci containing the ascospores, derived from the sexual recombination process, grow, mature, and are ejected upon maturation. Lieckfeldt and coworkers (10) had reported that *H. jecorina* isolate CBS999.97 produced stromata without the apparent presence of a mating partner on agar plates, which indicated that this isolate was either homothallic or a vegetatively compatible mixture of both mating types. Because the results of our genome analysis pointed toward a heterothallic lifestyle of *T. reesei*/*H. jecorina*, we tried to obtain strains with a *MAT1-1* and *MAT1-2* mating type from the ascospore progeny of *H. jecorina* CBS999.97. The strain was cultivated under daylight conditions and produced stromata in a regular pattern on the whole agar plate (Fig. S1*A*). Ascospores were collected from the lids of the Petri dishes (Fig. 2*J*), and single ascospore cultures were purified. In contrast to other ascomycota, which can produce stromata independently of a sexual stimulus and need the mating partner only for fertilization of the perithecia, *H. jecorina* cultures derived from single ascospores did not produce stromata anymore when they were grown alone on agar plates (Fig. S1*B*). Instead, in crossing experiments, they formed fertilized stromata in approximately half of the crosses at the interaction zones, indicating a bipolar segregation of mating type (Fig. S1 *C* and *D*). This confirmed that *H. jecorina* is indeed heterothallic, and we now had purified strains with a *MAT1-1* and *MAT1-2* mating type, which we used for further molecular biological studies to elucidate mating in *H. jecorina*. In the following, we will refer to the respective strains, derived from isolate CBS999.97, as *H. jecorina* MAT1–1 and MAT1–2. It should be noted that formation of fruiting bodies was only observed in cultures that were incubated in the presence

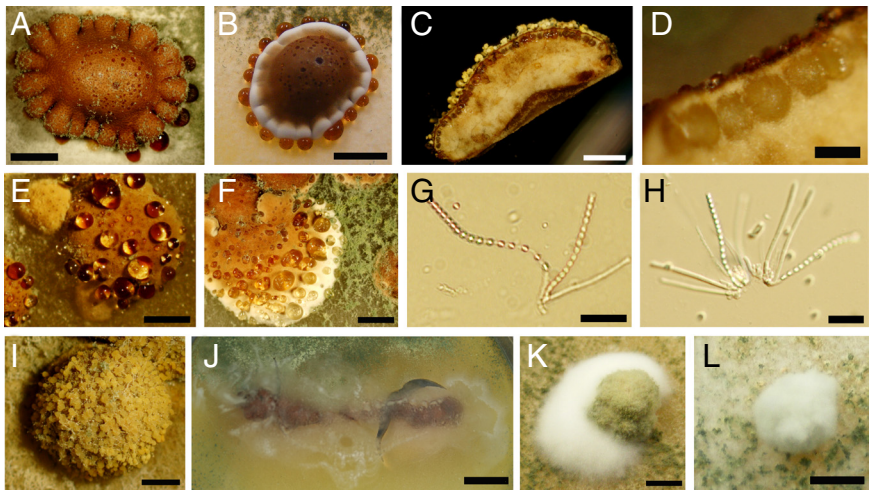

Fig. 2. Morphological characteristics of *H. jecorina* fruiting bodies. (*A*–*F*) Stromata (*A* and *B*) with perithecia embedded in the upper surface (*C* and *D*) and droplets of liquid forming on their surface (*E* and *F*). (*G*–*J*) Asci containing 16 bipart ascospores (*G* and *H*). Ascospores are either squeezed out of perithecia as a yellow substance under dry conditions (*I*) or shot to the lid of Petri dishes where they become visible as a white haze (*J*). (*K* and *L*) Unfertilized fruiting bodies. [Scale bars: 2 mm (*A*–*C*, *E*, *I*, *K*, *L*); 0.5 mm (*D*); 1 mm (*F*); 30 μm (*G* and *H*); 1 cm (*J*).]

of light, whereas no stromata were formed in cultures cultivated in constant darkness, indicating that light is essential for the sexual cycle in *H. jecorina*.

Analysis of the *MAT*-Loci of *H. jecorina*. To clone and analyze the *MAT1-1* locus from purified *H. jecorina* MAT1–1 strains, we first needed to determine the borders and size of the *T. reesei MAT1-2* locus, to be able to design primers binding to its conserved flanking regions because *MAT*-idiomorphs occupy the same genomic region in the respective strains. We therefore compared genomes of *N. crassa* and *Gibberella* species for genes flanking the *MAT*-loci and searched for the corresponding orthologs in the *T. reesei* genome database. The respective genes encode a putative DNA lyase (*T. reesei* protein ID 59147) and a conserved hypothetical protein (*T. reesei* protein ID 76930). In the genome databases of the mycoparasitic fungi *Trichoderma atroviride* IMI206040 and *Trichoderma virens* Gv29–8, which also turned out to have a *MAT1-2* mating type, the same orthologous proteins were detected flanking the *MAT*-loci. From the *H. jecorina* MAT1–1 and MAT1–2 strains the *MAT*-loci of both mating types were consequently amplified (all primers used in this study are listed in Table S1). The obtained 10.7- and 9.4-kb (*MAT1-2*) PCR fragments were subjected to restriction digestions, using three different enzymes, which gave identical patterns for *T. reesei* QM6a and *H. jecorina* MAT1–2, respectively, but different patterns for the MAT1–1 strain. The results indicated that there are no major differences at the *MAT1-2* locus between the sexually and asexually propagating strains (Fig. S2). Further, sequencing of the *mat1-2-1* gene revealed identical sequences for QM6a and *H. jecorina* MAT1–2. The complete *MAT1-1* locus was also sequenced and deposited in DDBJ/EMBL/GenBank, accession number FJ599756. It has a total length of 5.5 kb—compared with 4.2 kb of the *MAT1-2* locus—and contains three genes of which the exact ORFs, including exon/intron structures and 5′- and 3′-untranslated regions were assessed by RACE-PCR and RT-PCR (Fig. 1). In analogy to other heterothallic ascomycetes (9), *mat1-1-1* encodes an alpha-domain protein (380 aa), *mat1-1-2* an A2-domain protein (434 aa), and *mat1-1-3* a HMG protein (205 aa). Further, we detected that 486 bp of the 3′-end of *mat1-1-1* are present in both idiomorphs, but this is unlikely to be a functional gene in the *MAT1-2* locus because no translational start point could be detected for this fragment and the *MAT1-2* sequence adjacent to the shared region contains several stop codons in all reading frames.

Mating Types of a Worldwide Collection of *H. jecorina* Isolates. Having assessed the sequences of both mating type loci from *H. jecorina*, we designed mating type-specific primers for diagnostic PCR. We studied the distribution of mating types among a collection of *H. jecorina* isolates from different geographical locations. Of the available 27 *H. jecorina* isolates, 13 had a *MAT1-1* locus and 14 a *MAT1-2* locus (Table 1). Based on the equal distribution of mating types, it can be assumed that these populations propagate sexually in their original habitats.

Morphology of *H. jecorina* Sexual Reproduction Structures. Morphological characteristics of the sexual reproduction structures of *H. jecorina* are shown in Fig. 2. *H. jecorina* stromata are highly variable in size, shape and color. The size can range from a diameter of 3–4 mm up to 2 cm. Stromata have a dark brown pigmentation and perithecia are embedded into their upper surface. Large droplets of liquid can form on and around the stromata during maturation of the fruiting bodies. After 1–2 weeks, mature ascospores are either squeezed out of the perithecia as a yellow substance or, depending on the humidity, ascospores are shot to the lid of the agar plate where they can be seen as a white haze. The asci contain 16-part ascospores, as has been described for *Hypocrea* spp. (13). Occasionally, undifferentiated early forms in fruiting body development can be found on unfertilized *H. jecorina* plates. They appear as fluffy structures and later on become covered with asexual conidia.

Table 1. Mating types of *H. jecorina* isolates

H. jecorina isolate*	Mating type	Origin
South America		
G.J.S. 86–404†	*MAT1–1*	French Guiana
G.J.S. 86–408†	*MAT1–2*	French Guiana
G.J.S. 89–7†	*MAT1–2*	Brazil, Para
G.J.S. 86–410†	*MAT1–1*	French Guiana
G.J.S. 88–6†	*MAT1–2*	Brazil, Para
G.J.S. 97–177†	*MAT1–2*	Brazil, Para
G.J.S. 97–178†	*MAT1–1*	Brazil, Para
AYR 2896, DAOM 220850, 220846†	*MAT1–2*	French Guiana
CTR 72–94†	*MAT1–1*	French Guiana
G.J.S. 84–473, DAOM 220886†	*MAT1–1*	French Guiana
G.J.S. 87–7, CBS 836.91	*MAT1–2*	French Guiana
G.J.S. 86–403†	*MAT1–1*	French Guiana
G.J.S. 97–38, CBS 999.97	*MAT1–1/ MAT1–2*	French Guiana
Macronesia		
G.J.S. 85–249†	*MAT1–1*	Indonesia, Celebes
G.J.S. 85–229, DAOM 220794†	*MAT1–2*	Indonesia, Celebes
G.J.S. 85–230†	*MAT1–2*	Indonesia, Celebes
G.J.S. 85–236†	*MAT1–2*	Indonesia, Celebes
G.J.S. 85–238†	*MAT1–2*	Indonesia, Celebes
CBS 881.96	*MAT1–1*	Papua New Guinea
Micronesia		
G.J.S. 93–23†	*MAT1–2*	New Caledonia
G.J.S. 93–24†	*MAT1–1*	New Caledonia
G.J.S. 93–22†	*MAT1–2*	New Caledonia
CBS 383.78 (= QM6a)†	*MAT1–2*	Solomon Islands
Central America		
G.J.S. 96–401	*MAT1–2*	Puerto Rico
G.J.S. 95–82	*MAT1–1*	Puerto Rico
G.J.S. 95–2081	*MAT1–1*	Puerto Rico
G.J.S. 95–2082	*MAT1–1*	Puerto Rico
G.J.S. 95–123	*MAT1–1*	Puerto Rico

*G.J.S., collection of G. J. Samuels, USDA Beltsville, USA; CBS, Centraalbureau voor Schimmelcultures, Leiden, The Netherlands; CTR, collection of C.T. Rogerson, Botanical Museum of New York, USA; DAOM, Canadian Fungal Culture Collection, Ottawa. Canada.

†Strains indicated have been published previously (7, 10). All other strains were identified as *H. jecorina* by ITS1 and two sequence analysis using TrichOKey.

Sexual Development of *T. reesei* QM6a. After the finding that *T. reesei* QM6a has a *MAT1-2* locus, mating experiments were set up between strains QM6a and *H. jecorina* MAT1–1 using different growth conditions and taking into account the requirement of light for sexual reproduction in *H. jecorina* (see above). We were able to obtain stromata after 7–10 days at the respective interaction zones of *T. reesei* QM6a and *H. jecorina* MAT1–1 in all cases, independent of the growth medium or the temperature used (Fig. 3*A*). Microscopical analysis confirmed that the stromata were fertilized, the fruiting bodies had the same appearance as upon mating between *H. jecorina* MAT1–1 and MAT1–2 (Fig. 3 *B* and *C*), the frequency of ascospore germination was completely normal. To confirm that sexual recombination had occurred, a *T. reesei* mutant strain containing a single copy of the

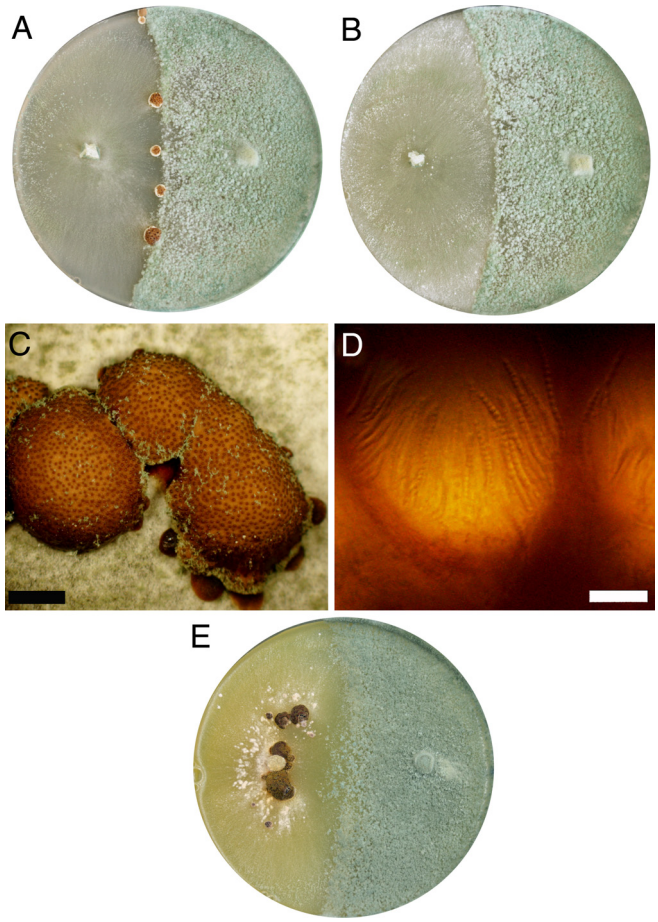

Fig. 3. Mating with *T. reesei* QM6a. (*A*) *H. jecorina* MAT1–1 was crossed with QM6a on agar plates, and the formation of stromata was observed at the interaction zone of the fungal colonies. (*B*) No stromata were formed upon mating of QM6a with *MAT1-2* strains. (*C*) The stromata have a typical appearance for *H. jecorina* and perithecia, which can be seen as small dark dots, are embedded into their upper surface. (Scale bar: 2 mm.) (*D*) Fertilized perithecia contain asci with 16 part-ascospores. (Scale bar: 2 mm.) (*E*) In crossings of *T. reesei* QM6a with the unpurified *H. jecorina* CBS999.97 isolate, often no stromata were formed.

hygromycin-phosphotransferase gene *hph* (14) was used for crossing experiments. Approximately 50% of the progeny were resistant to hygromycin B, hence confirming that mating between the two strains had occurred. As expected, no formation of stromata was detected upon mating of QM6a with *MAT1-2* strains (Fig. 3*D*).

It should be noted that in crossings of *T. reesei* QM6a with the unpurified *H. jecorina* CBS999.97 strain, also often no stromata were observed at the interaction zone (Fig. 3*E*), suggesting that sexual reproduction occurred preferably between *H. jecorina* wild-type strains in the unpurified isolate than between CBS999.97 and QM6a. This could possibly be due to a lower or absent expression of genes related to the sexual cycle in QM6a, which might impair the efficient induction of the sexual cycle between CBS999.97 and QM6a.

Mating of Strains Engineered for Enhanced Cellulase Production. Having identified a mating partner for *T. reesei* QM6a, we now tested whether two strains mutated for enhanced cellulase production, RUT-C30 and QM9414, and a uridine auxotrophic strain frequently used for genetic transformation, TU-6, had maintained the ability to mate with a *MAT1-1* strain. Although the common ancestor of these strains is QM6a, strains RUT-C30 and QM9414 were obtained independently during different mutation programs. We could confirm that crossing indeed was successful with all of them, despite the harsh treatments used during mutagenesis of these strains and hence that this method is feasible for use with industrial mutants to engineer superior production strains.

Conversion of Mating Types in *H. jecorina* and *T. reesei*. To be able to cross industrial strains—all of them are derived from QM6a and thus have a *MAT1-2* locus—it would be convenient to be able to switch their mating type. We therefore proposed to replace the *MAT1-2* locus of *T. reesei* QM6a with the entire *MAT1-1* locus amplified from the respective CBS999.97-derived strain. As a first step, it was necessary to assess whether it is possible at all to change the mating type in *H. jecorina*. We used the *H. jecorina* MAT1–2 strain that was isolated in this study and transformed it with a linear 10.7-kb fragment of the *MAT1-1* locus including 3.3 kb and 1.9 kb of the upstream and downstream flanking regions, respectively, in a cotransformation with a DNA-fragment that contains a hygromycin B resistance cassette (15). Hygromycin B-resistant transformants were checked by crossing them with *H. jecorina* MAT1–1, *H. jecorina* MAT1–2, and *T. reesei* QM6a. Thereby, transformants were obtained that indeed had their mating type converted from *MAT1-2* to *MAT1-1* (which was confirmed by PCR) and therefore now formed fruiting bodies upon contact with *H. jecorina* MAT1–2 and *T. reesei* QM6a but not with *H. jecorina* MAT1–1. Replacement of *MAT1-2* with *MAT1-1* was also attempted in *T. reesei* QM6a and QM9414. The *MAT1-1* locus was successfully integrated into the genome of these strains and consequently a switched mating behavior was achieved, i.e., fruiting body formation occurred with *H. jecorina* MAT1–2. However, none of the respective strains was able to develop fruiting bodies upon mating with the native *T. reesei* QM6a. We thus hypothesize that *T. reesei* QM6a is able to act as male mating partner, but that QM6a cannot produce the female fruiting bodies and is thus female sterile.

Genes Involved in Sexual Development. To possibly find an explanation for the observed female sterility, we analyzed the genome of *T. reesei* QM6a (http://genome.jgi-psf.org/Trire2/Trire2.home.html) for genes with a reported role in sexual development in *Aspergillus*, *Neurospora*, or yeasts (16, 17). Although putative orthologs for the respective genes in *Aspergillus* and *Neurospora* were mostly detected, we could not find them for *N. crassa asd-1* and *asd-3* (ascus development-1 and -3; NCU05598.3 and NCU05597.3): These genes are in immediate vicinity to each other in the *N. crassa* genome (http://www.broad.mit.edu/annotation/genome/neurospora/Home.html), but unfortunately the respective genomic region is not syntenic to *T. reesei*, and thus we cannot test whether these genes have been specifically lost in *T. reesei* QM6a but might be present in *H. jecorina* strains. However, because *N. crassa* mutants in these genes are still able to initiate sexual development (18), we consider it unlikely that these genes are the cause for the female sterility of *T. reesei*.

Discussion

Sexual reproduction is the prevalent reproduction form of eukaryotes. It is essential for the long-term population persistence of most eukaryotic species despite the fact that it requires more time and energy and is sometimes even termed less efficient than asexual reproduction (19). The evolutionary benefits that promote sexual reproduction are DNA maintenance and repair during meiosis as well as the production of increased genetic variation among offspring, which allows more efficient natural selection and elimination of deleterious mutations (19). Fungi differ from this general scheme insofar because they

exhibit a huge variation in their reproduction strategies. Although most basidiomycota are obligatory sexual, and sexual development is common in ascomycota, sexuality has never been observed in other groups, most of them combined in the deuteromycota (fungi imperfecti), and they reproduce by production of asexual spores. The advent of molecular biological methods in the analysis of fungal taxonomy and population biology has, however, shown that many of these "asexual" fungi (anamorphs) show genetic evidence of recombination and corresponding sexual forms (teleomorphs) have been identified based on genomic sequence patterns (20, 21).

Fungi comprise some of the most important organisms used in biotechnology for the production of enzymes and secondary metabolites (22). Although many of the fungi applied in industry are considered to reproduce asexually and fungi of medical or agricultural relevance molecular biological studies have been performed to establish anamorph-teleomorph relationships, this information has not been available so far for any biotechnologically important species. The lack of sexual recombination in these fungi is a major limiting factor for industrial strain improvement by classical strain crossing approaches. In the case of recombinant fungal strains containing resistance markers against antibiotics, the possibility of crossing would allow for straightforward removal of marker genes and hence considerably facilitate work with those fungi.

Attempts to successfully mate *T. reesei* QM6a with wild-type isolates of *H. jecorina* had previously failed despite considerable efforts, although its teleomorph *H. jecorina* was determined by in silico analysis more than 10 years ago. Therefore, in agreement with the "slow decline" hypothesis (23), this strain was regarded to represent a lineage having lost fertility (7). An alternative hypothesis, suggested by Ekelund and Ronn (24), which states that there may be no need for sexual development if the natural habitat provides constant environmental conditions to which the organism is adapted, can be rejected for *T. reesei* QM6a because the habitats of sexually propagating *H. jecorina* strains such as CBS999.97 show similar characteristics as the Solomon islands from which QM6a was isolated. Therefore, the so far clandestine sexual development of *T. reesei* may be due to inappropriate cultivation conditions or the lack of compatible mating partners under experimental conditions. The availability of a (partially) functional sexual cycle in *T. reesei* is of utmost significance for industrial applications and classical genetics—also for other members of the genus, which includes *Trichoderma* species involved in plant symbiosis (25), biocontrol, and mycoparasitism (26): In the genomes of *T. atroviride* (http://genome.jgi-psf.org/Triat1) and *T. virens* (http://genome.jgi-psf.org/Trive1), which are used in agriculture as biological control agents, a *MAT1-2* mating type locus is also present. Although the sequenced strains propagate asexually under laboratory conditions, for these species, the corresponding teleomorphs, *H. atroviridis* and *H. virens*, have already been described. The identification of the second mating type of a member of the genus *Hypocrea*/*Trichoderma* provides us with a means to investigate important questions for other *Hypocrea* spp.: What is the impact of sexual recombination of strains on the biocontrol efficiency of *Trichoderma*/*Hypocrea* spp.? Further, similar issues can now be addressed in opportunistic human pathogenic species of the genus *Hypocrea*, *H. orientalis*/*T. longibrachiatum* (27–29): Does sexual development play a role in virulence (30)? Because of the considerable antibiotic resistance (29, 31) and potential lethality of the strains analyzed so far, detailed investigations on their mechanism of virulence are indispensable for developing appropriate treatment strategies.

To facilitate crossing of industrial strains, we attempted to alter the mating type of *T. reesei* QM6a from *MAT1-2* to *MAT1-1*. Changes in the reproductive mode of fungi have already been carried out in different species. Targeted gene deletion of MAT-locus genes in the homothallic *Gibberella zeae* rendered this fungus heterothallic (32). For *Cochliobolus heterostrophus*, conversion from heterothallic to homothallic lifestyle, albeit with reduced fertility, was achieved (33). In *N. crassa*, transformants with a replaced mating-type locus from *MAT-A* (*MAT1-1*) to *MAT-a* (*MAT1-2*) grew normally and were fertile upon crossing with *MAT-A* strains, but transformant strains with an ectopic integration of the *MAT-a* mating type locus resulted in a vegetative incompatibility reaction (34). Direct switching of a fungal strain from *MAT1-2* to *MAT1-1* by genetic engineering had not been reported previously. We were able to successfully change the mating type in *H. jecorina* MAT1–2 and in *T. reesei* QM6a, because the obtained transformants, containing the *MAT1-1* locus, could be crossed with the original MAT1–2 strain. However, upon crossing with *T. reesei* QM6a, only the *H. jecorina*$^{\text{MAT1–2}\rightarrow\text{MAT1–1}}$, but not the respective QM6a$^{\text{MAT1–2}\rightarrow\text{MAT1–1}}$ transformants, were able to produce fruiting bodies. We conclude from these findings, that QM6a is—independent of its mating type—not able to produce female reproduction structures and is thus female sterile.

Several mutations causing female sterility have been reported (35), but there are two possibilities why these mutations could have occurred to *T. reesei* QM6a: First, after its collection from the Solomon islands, QM6a was subcultivated and maintained for decades under laboratory conditions, which could have caused the loss of female fertility. However, the theory of Leslie and Klein (36) provides an alternative explanation: They suggest that in a heterothallic haploid ascomycete capable of sexual and asexual reproduction, loss-of-meiosis mutations accumulate during vegetative reproduction, which result in female sterile mutants. For these mutants, the advantage not to spend metabolic resources for production of female structures, which may never be fertilized, can even be beneficial during asexual reproduction (35). Based on the relative frequency of female fertile strains, an estimation of the relative amounts of sexual and asexual reproduction occurring in a given population is possible (36). The extent of female sterility within a population varies widely. It is tempting to speculate that female sterility of *T. reesei* QM6a led to efficient asexual growth, prevalence in its natural habitat, and eventually to its isolation.

Nevertheless, such a possibly beneficial mutation is a major issue in direct application of our study to industrial strain improvement, but can be circumvented with only minor additional efforts. From crossings of *H. jecorina* MAT1–1 with industrial strains, the progeny could be screened for the capability of sexual development and retaining of production efficiency. At the same time, elimination of unintended random mutations in the industrial mutants detrimental to, for example, growth, by complementation with the wild-type background can be beneficial even without further crossings with other production strains. For identification of altered loci in this isolate and to consequently overcome this deficiency by genetic engineering, a comparison of the complete genome sequence of QM6a with that of the fertile *H. jecorina* strains will be essential.

With respect to crossing of strains engineered for enhanced production efficiency, a further issue should be considered: Repeat induced point (RIP) mutation, which efficiently detects and mutates duplicated sequences and only acts during the sexual cycle (for a review see ref. 37) and was therefore not relevant in *T. reesei* so far. Now this process should be considered in strain improvement using our crossing approach, especially when working with strains bearing multiple copies of certain genes. Although the introduction of multiple cellulase genes or regulators is not frequently used anymore, for example additional alleles bearing point mutations or genes duplicated during strain improvement might be subject to RIP. Reduction in duplicated genes in *T. reesei* (11) points at a functional RIP mechanism. Hence, effects similar to *N. crassa*, for which this process has been studied in detail, can be expected. However,

because it has been shown for *N. crassa* that deletion of a single gene (*rid-1*) (38), which has a homolog in *T. reesei*, is sufficient to abolish RIP, this mechanism should not significantly interfere with application of crossing approaches to industrial strain improvement with *T. reesei*.

Materials and Methods

Microbial Strains and Culture Conditions. Fungal strains QM6a (13631; ATCC), G.J.S. 97–38 (CBS999.97; obtained from the Centraalbureau of Schimmelcultures, Utrecht ,The Netherlands), QM9414 (26921; ATCC), RutC30 (56765; ATCC), and TU6 (MYA-256; ATCC) were maintained at 28 °C on malt extract agar plates, supplemented with 10 mM uridine for TU-6 (39), and 0.1% Triton X-100 for single spore isolation, and stock cultures were kept at −80 °C. The most favorable conditions for induction of sexual development turned out to be malt extract agar (3% wt/vol; Merck), 20–22 °C incubation temperature in a SANYO MIR-154 incubator (Sanyo Europe Ltd) set to a 12-h light–dark cycle or in daylight—both light conditions gave equal results. Sexual development was also analyzed upon growth on potato dextrose agar (PDA; Difco, BD Biosciences) and Mandels Andreotti medium (40) with glucose (1% wt/vol; Merck) as a carbon source. Single spore cultures were obtained by inoculating malt extract agar plates containing 0.1% Triton X-100 with a diluted spore solution and picking cultures grown from single spores after 1–2 days of incubation at 28 °C.

Microscopic Analyses. For microscopic analysis of samples, we used differential interference contrast optics on an inverted T300 microscope (Nikon) and a M420 Photomacroscope (Wild; Leica). Images were captured with a Nikon DXM1200F digital camera and digitally processed using Photoshop CS3 (Adobe).

Manipulation of Nucleic Acids and Analytical PCR. Strains were grown on malt extract agar plates overlaid with cellophane (#700–2101; VWR). Mycelia were ground to a fine powder in liquid nitrogen, and DNA was isolated as described in refs. 41 and 42, and for RNA, the guanidinium thiocyanate method (43) was used. Mating-type loci were amplified using primers in the conserved up- and downstream regions and the Long Template Expand PCR kit (Roche). Analytical PCR of the mating types of *H. jecorina* isolates was carried out using the GoTaq system (Promega). RACE-PCR was carried out as described by Seidl and coworkers (44), using the Creator SMART cDNA library construction kit (Clontech) followed by PCR with adapter primers from the kit and gene specific primers. Primers used in this study are listed in Table S1. PCR fragments of the *MAT*-loci were digested with *Pst*I, *Hin*dIII, and *Sac*I (Fermentas). DNA sequencing of a PCR fragment comprising the *MAT1-1* locus of CBS999.97 was carried out at Eurofins MWG Operon (Ebersberg, Germany).

Transformation of *H. jecorina*. Protoplast preparation and DNA-mediated transformation of *T. reesei*/*H. jecorina* strains was essentially done as described in ref. 39. The 10.7-kb *MAT1-1* fragment was used for a cotransformation together with the circular plasmid pRLMex30 (15). After transformation protoplasts were stabilized and regenerated on malt extract medium containing d-sorbitol (1 M) and 50 μg/mL hygromycin B. Colonies were transferred to malt extract agar and purified by single spore isolation on selection medium.

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