Flippase (FLP) recombinase-mediated marker recycling in the dermatophyte *Arthroderma vanbreuseghemii*

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Biological processes can be elucidated by investigating complex networks of relevant factors and genes. However, this is not possible in species for which dominant selectable markers for genetic studies are unavailable. To overcome the limitation in selectable markers for the dermatophyte *Arthroderma vanbreuseghemii* (anamorph: *Trichophyton mentagrophytes*), we adapted the flippase (FLP) recombinase-recombination target (FRT) site-specific recombination system from the yeast *Saccharomyces cerevisiae* as a selectable marker recycling system for this fungus. Taking into account practical applicability, we designed FLP/FRT modules carrying two FRT sequences as well as the *flp* gene adapted to the pathogenic yeast *Candida albicans* (*caflp*) or a synthetic codon-optimized *flp* (*avflp*) gene with neomycin resistance (*nptII*) cassette for one-step marker excision. Both *flp* genes were under control of the *Trichophyton rubrum* copper-repressible promoter (*PCTR4*). Molecular analyses of resultant transformants showed that only the *avflp*-harbouring module was functional in *A. vanbreuseghemii*. Applying this system, we successfully produced the *Ku80* recessive mutant strain devoid of any selectable markers. This strain was subsequently used as the recipient for sequential multiple disruptions of secreted metalloprotease (*fungalysin*) (*MEP*) or serine protease (*SUB*) genes, producing mutant strains with double *MEP* or triple *SUB* gene deletions. These results confirmed the feasibility of this system for broad-scale genetic manipulation of dermatophytes, advancing our understanding of functions and networks of individual genes in these fungi.

**INTRODUCTION**

Dermatophytes are a closely related fungal group of keratinophilic pathogens, which cause the majority of superficial mycoses in humans and animals, leading to poor quality of human life and a heavy economic burden. The molecular mechanisms of host invasion by dermatophytes are not well understood. However, there have been a number of improvements in basic tools for molecular genetics studies of dermatophytes, including the development of efficient genetic transformation methods (Yamada et al., 2009a, b; Alshahni et al., 2011; Grumbt et al., 2011a). In addition, the complete genomes of seven dermatophytes with different host specificities have been sequenced and are available from the Broad Institute (http://www.broadinstitute.org/annotation/genome/dermatophyte_comparative). These advances will facilitate molecular and cellular biological analyses and provide opportunities to extend investigations of their pathogenesis through large-scale reverse genetics studies.
Based on our knowledge of other pathogens, various factors are likely to be involved in infections, which are reflected in differential gene expression patterns at various stages of the invasion process. In many cases, targeting only one of these factors will be unlikely to reveal the whole mechanism. It is often necessary to generate multiple mutations of relevant genes. A good example of these complex biological networks is the secretion of a variety of proteases, which are involved in dermatophytes, although particular functions and roles of each protease remain elusive. Dermatophytes probably have a unique compensatory protease secretion machinery that collectively produces synergistic effects, causing infection. Therefore, it is difficult to elucidate the functions and roles of any given protease in infection through only a single mutation.

Only three dominant selectable markers are currently available for genetic manipulation studies of dermatophytes: hygromycin B, gentamicin (G418) and nourseothricin (Gonzalez et al., 1999; Yamada et al., 2005, 2008; Alshahi et al., 2010; Grumbt et al., 2011b). Using these three markers, it is possible to generate up to triple mutations in a single strain by homologous recombination. However, due to the limited number of available markers, it is impossible to use this strategy to study large protease gene families such as the MEROPS family S8 (subtilisins), M35 (deuterolysins) and M36 (fungalysins) proteases (Rawlings et al., 2012), each of which consists of more than five family members. To overcome this limitation and extend the scale of genetic analyses of dermatophytes, a recycling approach for selectable markers is highly desirable.

The flippase (FLP) recombinase-mediated site-specific recombination system from Saccharomyces cerevisiae (Sadowski, 1995) is the eukaryotic equivalent of the Cre/loxP-mediated site-specific recombination system, which was originally described in bacteriophage P1 (Sternberg & Hamilton, 1981). FLP is naturally encoded within the S. cerevisiae 2 μm plasmid (Broach et al., 1982), does not require cofactors and uses a phosphotyrosine intermediate for energy, similar to Cre recombinase. The FLP actively catalyses specific recombination between two 34 bp FLP recognition targets (FRTs). Four FLP molecules and two FRT sequences are utilized for every FLP-mediated recombination event. The asymmetrical region dictates whether excision (FRT sequences in the same orientation) or inversion (FRT sequences in inverted orientation) of the intervening DNA sequence occurs after recombination. Thus, the FLP/FRT recombination system is available to eliminate selectable markers and has already been successfully applied in a variety of organisms (Dymecki, 1996; Morschhäuser et al., 1999; Schweizer, 2003; Reuß et al., 2004; Raymond & Soriano, 2007; Song & Niederweis, 2007; Ueno et al., 2007; Tan et al., 2013). However, its use in filamentous fungi has been limited to only two filamentous ascomycetes, Penicillium chrysogenum and Sordaria macrospora (Kopke et al., 2010, 2013).

This study was performed to examine applicability of the FLP/FRT recombination system in the dermatophyte Arthroderma vanbreuseghemii (anamorph: Trichophyton mentagrophytes). Three factors were the core of our concern: (1) determination of the codon usage in dermatophytes, (2) identification of dominant selectable markers and (3) determination of the promoter sequence for driving the flp gene. To demonstrate the functionality of our one-step selectable marker recycling system, we constructed a AKu80 mutant strain devoid of any selectable markers. Using this mutant strain as the recipient, we subsequently performed additional rounds of gene disruption to target two or three members of the secreted metalloprotease (fungyalysin) (MEP) and subtilisin protease (SUB) gene families (Rawlings et al., 2012). Successful production of mutant strains with double MEP or triple SUB gene deletions demonstrated the feasibility of our one-step marker recycling system, suggesting the applicability of this powerful tool in broader-scale investigations of the molecular mechanisms underlying host invasion by dermatophytes.

**METHODS**

**Strains and culture media.** Escherichia coli DH5α (Nippon Gene) was used for molecular cloning. All A. vanbreuseghemii strains used in this study are listed in Table 1. A. vanbreuseghemii mutant strains TmKu80A49 (Yamada et al., 2009b) and TmL28 (Alshahi et al., 2011), which lack homologues of the human Ku80 or DNA ligase IV, respectively, are deficient in their non-homologous end-joining pathway, allowing efficient homologous recombination. Microconidia formation was induced at 28 °C using modified 1/10 Sabouraud dextrose agar (SDA) (Uchida et al., 2003) supplemented with 500 μg cycloheximide ml⁻¹ (Wako Chemical) and 50 μg chloramphenicol ml⁻¹ (Sigma-Aldrich). For production of secreted proteases, each dermatophyte strain was grown in a liquid medium containing 1.0% (w/v) skimmed milk (Wako Chemical) as the sole nitrogen and carbon source, supplemented with 2 μg cephalosporin sodium ml⁻¹ (Sanofi-Aventis). Flasks containing 50 ml of the medium were inoculated with 2 × 10⁷ microconidia. After incubation at 28 °C for 7 days without shaking, the mycelia were separated from culture supernatant by filtration with a cell strainer (BD Biosciences). To remove cell debris and other impurities, the supernatant was centrifuged at 15 000 r.p.m. at 4 °C for 5 min and then used for measurement of proteolytic activity.

**Total DNA extraction.** Total DNA was extracted according to the method of Girardin & Latge (1994). The growing mycelia from each dermatophyte strain were collected after incubation on SDA for 3 days at 28 °C, frozen and ground twice and cooled on liquid nitrogen with a Multi-Beads shocker (Yasui Kikai) at 2000 r.p.m. for 10 s.

**Generation of a codon-optimized flp gene.** Generation of a synthetic flp gene with a bias for preferred A. vanbreuseghemii codons was ordered to Eurofin MWG Operon (Ebersberg). The nucleotide sequence of the flp gene derived from pSFS2 (Reuß et al., 2004) was used for codon optimization. pSFS2 contains a pathogenic yeast (Candida albicans)–adapted version of the flp (capfl) gene in which the three CUG codons present in the native flp gene from S. cerevisiae were exchanged with another leucine codon (UGL) (Staib et al., 1999). The codon usage bias of A. vanbreuseghemii was predicted based on that of Arthroderma benhamiae, a closely related zoophilic dermatophyte species, taken from the Kazusa Codon Usage Database (http://www.kazusa.or.jp/codon). Codons from highly expressed genes were selected more often, while very rare codons were completely
avoided. Undesired motifs, such as hairpins and direct or inverted repeats, were also avoided. Furthermore, to keep the cloning sites unique, sites recognized by restriction enzymes, such as ApaI, KpnI, SacI, SstI, SphI, and SpeI, were avoided. The resulting synthetic flp gene (avflp) with SacI and SphI sites at its 5' and 3' ends, respectively, was subcloned into pBluescript II SK (+) (Stratagene) and the resulting plasmid was designated pBSK-avflp.

**Primers.** All the primers used in this study are listed in Table S1, available in the online Supplementary Material.

### Construction of transformation vectors for one-step selectable marker excision in dermatophytes.** Two Tmkku80-targeting vectors, pMRV-Tmkku80-T1 and pMRV-Tmkku80-T2 (Fig. 1a), were constructed as follows. The upstream fragment of Tmkku80 (Tmkku80c), *E. coli* neomycin phosphotransferase (*nptII*), *Aspergillus nidulans trpC* promoter (*TrpC*) and *Aspergillus fumigatus* cgrA terminator (*TgrA*) were amplified separately by PCR with the following primers: Tmkku80-5'(F)Spe and Tmkku80-5'(R), Apa PCHR-C104084 (F) and NPT-RB, Apa-FRT-PCHR-C104084 (F) and TrpC25 (R), and nptII-Bfla (F) and TgrAI (R), respectively. pAg1-Lmkku80 and pAg1-Nfrt were amplified from pBluescript II SK (+) (Stratagene) and the resulting plasmid was designated pBSK-avflp.

<table>
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<th>Strain</th>
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<tr>
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<td>Wild-type</td>
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<td>Alshahini et al. (2011)</td>
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**Table 1.** Fungal strains used in this study

A series of AvMEP- and AvSUB-targeting vectors (pMRV-AvMEP3/T, pMRV-AvMEP4/T, pMRV-AvSUB3/T, pMRV-AvSUB6/T and pMRV-AvSUB7/T) were constructed from pMRV-Tmkku80/T2. Approximately 2.0–2.5 kb of the upstream and downstream regions of the AvMEP3, AvMEP4, AvSUB3 and AvSUB6 genes were amplified by PCR with the following pairs of primers: AvMEP3-F1/Spe and AvMEP3-R1/Apa, AvMEP3-F2/BamHI and AvMEP3-R2/KpnI, AvMEP4-F1/Spe and AvMEP4-R1/Apa, AvMEP4-F2/BamHI and AvMEP4-R2/KpnI, AvSUB3-F1/Spe and AvSUB3-R1/Apa, AvSUB3-F2/BamHI and AvSUB3-R2/KpnI, AvSUB6-F1/Spe and AvSUB6-R2/Apa, AvSUB6-F3/BamHI and AvSUB6-R3/KpnI, AvSUB7-F1/Spe and AvSUB7-R2/Apa, and AvSUB7-F3/BamHI and AvSUB7-R3/KpnI. These primers were designed using the draft genome sequence information of the wild-type *A. vanbreuseghemii* strain TIMM2789 (unpublished data). The fragments obtained were subcloned into pUC118 and sequenced. These fragments were excised from each plasmid by SpeI/ApaI or BamHI/KpnI double-digestion and ligated into the corresponding restriction sites of pMRV-Tmkku80/T2, generating a series of pMRV-AvMEP/T and pMRV-AvSUB/T vectors. To construct a vector of the AvMEP*4* gene for genetic complementation of the AvMEP4 deletion mutants, an approximately 4.6 kb fragment...
PCR with a pair of primers, AvMEP4-F5/AvMEP4-R4 containing the coding region of the AvMEP4 gene was amplified by PCR with a pair of primers, AvMEP4-F5/SpeI and AvMEP4-R4/ApaI. After subcloning into pUC118 and sequencing, the fragment was excised from the plasmid by SpeI/ApaI double-digestion and ligated into the corresponding restriction site of pMRV-TmKu80/T vectors. Site-specific recombination between the flanking FRT sequences was carried out by conditional expression of the flipper modules. Replacement of the hygromycin B resistance (hph) cassette was achieved by transformation of the recipient strain TmKu80ΔG (ΔTmKu80) with the FLP/FRT modules from pMRV-TmKu80/T vectors. Site-specific recombination between the flanking FRT sequences was carried out by conditional expression of the flipper genes after transformation with pMRV-TmKu80/T vectors. P, Pst I sites contained in the Amplified fragments were digested with PstI and SpeI and separated by electrophoresis on 0.8 % (w/v) agarose gels. The internal fragments of the hph and nptII genes were amplified with pairs of primers, hph-F1 and R1, and nptII-F1 and R1, respectively. Aliquots of approximately 100 ng of total DNA were used as the PCR template. The wild-type A. vanbreuseghemii strain TIMM2789, the parental strain TmKu80ΔG and the nptII-harbouring strain TmL28 (ΔTmlig4) were used as positive and negative controls. (d) Southern blotting analysis of total DNA samples from transformants. Aliquots of approximately 12 μg of total DNA were digested with PstI and separated by electrophoresis on 0.8 % (w/v) agarose gels.

Transformation of A. vanbreuseghemii. Protoplast preparation and PEG-mediated transformation of each A. vanbreuseghemii strain were performed according to the method of Yamada et al. (2005) with minor modifications. Aliquots of about 40 μg of transformation vector were digested with KpnI or SpeI/KpnI, collected by ethanol precipitation, resuspended in 20 μl Tris-EDTA buffer (pH 8.0) and used for each transformation experiment. After PEG treatment, the protoplasts were harvested by centrifugation and resuspended in 600 μl of STC buffer. Aliquots of 300 μl of the protoplast suspension were mixed with 10 ml of RYSDA (Yamada et al., 2005) supplemented with 10 μM CuSO4, 7H2O, and poured onto RYSDA plates with the same concentration of CuSO4. 7H2O. The plates were overlaid 24 h later with 10 ml of SDA containing 250 μg geneticin.

Fig. 1. TmKu80 locus targeting and nptII marker excision. (a) Restriction maps of two TmKu80-targeting vectors, pMRV-TmKu80/T1 and pMRV-TmKu80/T2. A, Apal; B, BamHI; C, ClaI; K, KpnI; Sa, SalI; Sc, SacI; Sh, SphI; Sp, SpeI. (b) Schematic representation of the TmKu80 locus before and after excision of the flipper modules. Replacement of the hygromycin B resistance (hph) cassette was achieved by transformation of the recipient strain TmKu80ΔG (ΔTmKu80) with the FLP/FRT modules from pMRV-TmKu80/T vectors. Site-specific recombination between the flanking FRT sequences was carried out by conditional expression of the flipper genes after transformation with pMRV-TmKu80/T vectors. P, PstI sites contained in the Amplified fragments were digested with PstI and SpeI and separated by electrophoresis on 0.8 % (w/v) agarose gels. The internal fragments of the hph and nptII genes were amplified with pairs of primers, hph-F1 and R1, and nptII-F1 and R1, respectively. Aliquots of approximately 100 ng of total DNA were used as the PCR template. The wild-type A. vanbreuseghemii strain TIMM2789, the parental strain TmKu80ΔG and the nptII-harbouring strain TmL28 (ΔTmlig4) were used as positive and negative controls. (d) Southern blotting analysis of total DNA samples from transformants. Aliquots of approximately 12 μg of total DNA were digested with PstI and separated by electrophoresis on 0.8 % (w/v) agarose gels. TIMM2789 and TmKu80ΔG were used as controls. A 645 bp fragment of the TmKu80 locus was amplified by PCR with the pair of primers TmKu80-F1 and TmKu80-R1 and used as a hybridization probe. DNA standard fragment sizes are shown on the left.
**Table 2. Plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristics</th>
<th>Reference or source</th>
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<tr>
<td>pAg1</td>
<td>A streamlined version of the binary vector pBIN19 containing sequences necessary for replication in E. coli and Agrobacterium tumefaciens (oriV, trpA), E. coli neomycin phosphotransferase (nptII) and the transferable DNA (T-DNA) region. A multiple cloning site was placed within the T-DNA region.</td>
<td>Zhang et al. (2003)</td>
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<td>pAg1-TmKa80/T</td>
<td>TmKa80a fragment (position −550 to 1444, with +1 being the start of the TmKa80 ORF), Cochliobolus heterostrophus promoter 1 (Pch), E. coli hygromycin B phosphotransferase (hph), Aspergillus nidulans trpC terminator (TrpC), TmKa80b fragment (position 1533 to 3533)</td>
<td>Yamada et al. (2009)</td>
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<td>pAg1N-trn/T</td>
<td>trn fragment (position −340 to 1339 with +1 being the start of the trn ORF), Pch, nptII, TrpC, trn fragment (position 2101 to 3519)</td>
<td>Yamada et al. (2009)</td>
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<td>pucATPH</td>
<td>Aspergillus nidulans trpC promoter (PtrpC)</td>
<td>Idnur &amp; Howlett (2003)</td>
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<tr>
<td>p500</td>
<td>Aspergillus fumigatus gRA terminator (TgRA)</td>
<td>Vogt et al. (2005)</td>
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<td>pSF52</td>
<td>5’ FRT sequence, C. albicans-adapted flp (caflp), SAT1 flipper, 3’ FRT sequence</td>
<td>Reuß et al. (2004)</td>
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<td>pDTV3</td>
<td>Pch, nptIII, TrpC</td>
<td>Yamada et al. (2008)</td>
</tr>
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<td>TmKa80a fragment, 5’ FRT sequence, Pch, hph, T. rubrum ctr4 promoter (PtrpC), Pch, nptIII, TgRA, PCTR4 caflp3FLAGs, Cryptococcus neoformans trp1 terminator (Trp1), 3’ FRT sequence, TmKa80b fragment</td>
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<td>pBSK-avflp</td>
<td>A. vanbreuseghemii-optimized flp (avflp)</td>
<td>This study</td>
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<td>pMRV-TmKa80/T series</td>
<td>TmKa80c fragment, 5’ FRT sequence, PtrpC, nptII, TgRA, PCTR0 caflp (pMRV-TmKa80/T1) or avflp (pMRV-TmKa80/T2), Trp1, 3’ FRT sequence, TmKa80d fragment</td>
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<td>pMRV-AvMEP/T series</td>
<td>5’ untranslated region of each AvMEP gene, 5’ FRT sequence, PtrpC, nptII, TgRA, PCTR0 avflp, Trp1, 3’ FRT sequence, 3’ untranslated region of each AvMEP gene</td>
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<td>pMRV-AvMEP4/C</td>
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<td>pMRV-AvSUB/T series</td>
<td>5’ untranslated region of each AvSUB gene, 5’ FRT sequence, PtrpC, nptII, TgRA, PCTR0 avflp, Trp1, 3’ FRT sequence, 3’ untranslated region of each AvSUB gene</td>
<td>This study</td>
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ml⁻¹ (Sigma-Aldrich) and 10 μM CuSO₄-7H₂O, and then incubated for 5–7 days. The colonies regenerating on the plates were picked up as putative geneticin-resistant clones, transferred onto solid MOPS-buffered RPMI 1640 medium (RPMI1640A) supplemented with 500 μg cycloheximide ml⁻¹, 50 μg chloramphenicol ml⁻¹, 200 μg cefotaxime sodium ml⁻¹ (SANOFI-AVENTIS) (if necessary) and 10 or 20 μM bathocuproine disulfonate (BCS) (DOJINDO Laboratories), a copper-specific chelator, and passed twice.

**Screening of the desired transformants.** The desired transformants were finally screened by PCR, Southern blotting analysis and nucleotide sequencing. For PCR, aliquots of 50–100 ng of the total DNA were used as the template. For Southern blotting analysis, aliquots of approximately 12 μg of the total DNA were digested with an appropriate restriction enzyme, separated by electrophoresis on 0.8% (w/v) agarose gels and transferred onto Hybond-N⁺ membranes (GE Healthcare). Southern hybridization was performed using the ECL Direct Nucleic Acid Labelling and Detection System (GE Healthcare) according to the manufacturer’s instructions.

**Assay for proteolytic activity in culture supernatant.** Endoproteolytic activity in culture supernatant of each A. vanbreuseghemii strain was measured as described by Jousson et al. (2004a) using resorufin-labelled casein (Roch Diagnostics) as substrate. The reaction mixture contained 0.4% (w/v) substrate, 20 mM Tris/HCl buffer, pH 7.5, and 100 μl of culture supernatant in a total volume of 500 μl. After incubation at 37 °C for 30 min, the undigested substrate was precipitated by trichloroacetic acid [5% (w/v) final concentration] and separated from the supernatant by centrifugation. An equal volume of 1.0 M Tris buffer (without HCl) was added to the collected supernatant (neutralization step) and the A₅₇₀ of the mixture was measured. For practical purposes, one arbitrary unit (U) of endoproteolytic activity was defined as that producing an absorbance change of 0.01 min⁻¹ ml⁻¹. The inhibitors PMSF and phosphoramidon (Sigma-Aldrich) were dissolved in ethanol and distilled water, respectively. In endoproteolytic assays with phosphoramidon and/or PMSF, each inhibitor was first added to culture supernatant and the mixture was incubated at 25 °C for 10 min before adding the substrate.

**RESULTS**

**Codon adaptation of an flp gene for efficient heterologous expression in dermatophytes**

In a previous study, the synthetic flp gene with P. chrysogenum-optimized codon usage, in parallel with the native sequence, were introduced separately into the recipient strain carrying the drug resistance cassette flanked by the two FRT sequences, although site-specific recombination between...
the FRT sequences was observed only in the transformants harbouring the codon-optimized flp (Kopke et al., 2010). These data suggest that optimization of the codon usage for each amino acid in the flp gene should be a prerequisite for functional FLP/FRT-mediated site-specific recombination in filamentous fungi. Therefore, we generated a synthetic flp gene using codons optimized based on the codon usage bias of a closely related zoophilic dermatophyte, A. benhamiae. The nucleotide sequence of the resulting flp gene (avflp) had a G+C content of 51 %, which was approximately 14 % higher than that of caflp. The two flp genes showed DNA sequence identity of approximately 72 % despite modification of over 70 % of all triplets.

**Construction of FLP/FRT modules composed of the FRT–geneticin (G418) resistance cassette–recombinase gene cassette–FRT**

Together with the codon usage bias of the flp gene, we also investigated selectable markers and promoters driving expression of the flp genes. As dominant selectable markers, three aminoglycoside antibiotics, hygromycin B, geneticin (G418) and nourseothricin, are available for genetic transformation of A. vanbreuseghemii (Yamada et al., 2005, 2008; Alshahni et al., 2010). Of these, the nourseothricin resistance gene (nat1) of Streptomyces noursei, which encodes nourseothricin acetyltransferase, has been used as a resistance cassette in the FLP/FRT recombination system established in yeast pathogens (Morschhäuser et al., 1999; Reuß et al., 2004; Ueno et al., 2007) and filamentous fungi (Kopke et al., 2010, 2013). However, in our previous genetic transformation experiments using vectors with the nourseothricin resistance (nat1) cassette, a high frequency of multiple integration of the cassette into the chromosomes of transformants was often observed. The transformation frequency was also lower than with other resistance gene cassettes. Therefore, the geneticin resistance gene (nptII) from E. coli, which encodes neomycin phosphotransferase, was chosen to generate a resistance gene cassette. By contrast, the neomycin resistance (nptII) cassette had to be excised after transformation for optimal target gene disruption. To accomplish this, the flp genes were placed under the control of a promoter sequence of the T. rubrum copper transporter CTR4 (a homologue of Cryptococcus neoformans CTR4) (P_{CTR4}), which was shown to repress an expression of the downstream gene on medium containing copper ions (Iwata et al., 2012).

After the FRT sequence was attached to the 5' or 3' end of the nptII and flp cassettes, the resulting FRT-nptII and caflp- or avflp-FRT cassettes were tandemly arranged at the

![Fig. 2. Production of A. vanbreuseghemii mutant strains lacking double AvMEP genes. (a) Schematic representation of disruption of the Av MEP4 locus in the AvMEP3 gene-disrupted strain. The recipient strain Av1171-3-18-1 (ΔTmKu80 ΔAvMEP3) was transformed with the avFLP/FRT module from pMRV-AvMEP4/T vector. The avFLP/FRT module was then excised by site-specific recombination between the flanking FRT sequences via conditional expression of the avflp gene after transformation. (b) Southern blotting analysis of total DNA samples from the double AvMEP gene-disrupted strains (Av1201-34-12-1 and Av1201-34-24-1). Aliquots of approximately 12 µg of total DNA were digested with BamHI and separated by electrophoresis on 0.8 % (w/v) agarose gels. Strain 1062Av1401 was used as a control. A 569 bp fragment of the AvMEP4 locus was amplified by PCR with the pair of primers AvMEP4-F3 and AvMEP4-R1/Apal and used as a hybridization probe. DNA standard fragment sizes are shown on the left.](http://mic.sgmjournals.org)
**Fig. 3.** Complementation of an *A. vanbreuseghemii* double AvMEP gene-disrupted strain with the AvMEP4 gene. (a) Schematic representation of complementation of the ΔAvMEP3/ΔAvMEP4 mutant strain with the AvMEP4 gene. The recipient strain Av1201-34-12-1 (ΔTmKu80 ΔAvMEP3 ΔAvMEP4) was transformed with pMRV-AvMEP4/C containing the AvMEP4 gene. The avFLP/FRT module was then excised by site-specific recombination between the flanking FRT sequences via conditional expression of the avflp gene after transformation. (b) Southern blotting analysis of a total DNA sample from the revertant strain Av1242-34-4C. Aliquots of approximately 12 μg of total DNA were digested with *Bam*HI and separated by electrophoresis on 0.8% (w/v) agarose gels. Strains 1062Av1401 and Av1201-34-12-1 (ΔTmKu80 ΔAvMEP3 ΔAvMEP4) were used as controls. A 569 bp fragment of the AvMEP4 locus was amplified by PCR with the pair of primers AvMEP4-F3 and AvMEP4-R1/Apal and used as a hybridization probe. DNA standard fragment sizes are shown on the left. (c) Multiplex PCR analysis of total DNA samples from the revertant strain Av1242-34-4C and the recipient strain Av1201-34-12-1. PCR was performed with a mixture of two pairs of AvMEP-specific primers (AvMEP3-F3 and R3, and AvMEP4-F4 and R3). Each fragment of the AvMEP3 and AvMEP4 loci in the control strain 1062Av1401 was amplified separately. Molecular masses of the amplified fragments are shown on both sides.
corresponding sites within transformation vectors, generating the FLP/FRT modules (Fig. 1a).

**Development of the functional FLP/FRT recombination system in dermatophytes**

We previously produced a 

Ku80Δ strain TmKu80Δ49 as a recipient host for efficient homologous recombination in A. vanbreuseghemii (Yamada et al., 2009b). TmKu80Δ49 was used to test the FLP/FRT recombination system. As shown in Fig. 1(b), the TmKu80 locus of this strain had been inactivated by integration of the hygromycin B resistance (hph) cassette via homologous recombination. To exchange the hph cassette with the FLP/FRT module, two TmKu80-targeting vectors (pMRV-TmKu80/T1 and pMRV-TmKu80/T2) were constructed (Fig. 1a). The FLP/FRT modules containing the caflp or avflp gene were flanked by the upstream and downstream 1.5 kb TmKu80 homologous fragments as well as two same-directional FRT sequences. Each 7.1 kb disruption fragment containing the caFLP/FRT or avFLP/FRT module was introduced into TmKu80Δ49 via protoplast/PEG-mediated transformation, respectively. To minimize the leaky expression of the caFLP or avFLP recombinase, screening of transformants was performed on copper-containing selective medium. Colonies regenerating on the selective medium were transferred onto solid RPMI1640 medium (RPMI1640A) supplemented with BCS, a copper-specific chelator, incubated for 4 days and then roughly screened for the TmKu80 locus. Direct colony PCR analysis with the specific primers for the hph gene (Alshahni et al., 2009) suggested that of the 16 strains transformed with the caFLP/FRT module, three lost the hph cassette due to the correct integration (data not shown). An identical result was also obtained in two of 16 strains transformed with the avFLP/FRT module (data not shown).

These five transformants carrying the avFLP/FRT or caFLP/FRT module were then subcultured twice using BCS-containing RPMI1640A plates to induce caFLP- or avFLP-mediated recombination, and characterized for the TmKu80 locus by PCR, nucleotide sequencing and Southern blotting analysis to verify the functionality of the FLP/FRT systems in A. vanbreuseghemii. For these analyses, we used total DNA samples from these strains. As shown in Fig. 1(c), the 1062Av0101 and 1062Av1401 strains transformed with the avFLP/FRT module showed no bands in PCR with either the hph- or the nptII-specific primers. We confirmed by nucleotide sequencing that after replacing the hph cassette, the avFLP/FRT module was completely missing from the TmKu80 locus in these strains (data not shown). These results were correlated with those of Southern blotting analysis. As shown in Fig. 1(d), the TmKu80-specific probe detected a single hybridization band of about 3.9 kb in both strains, indicating that the avFLP/FRT module of about 7.1 kb was excised after integration into the TmKu80 locus by homologous recombination. These results clearly demonstrated that the codon-optimized flp gene could be fully functional in A. vanbreuseghemii. Using the codon-optimized flp gene, we have produced recipient strains for efficient homologous recombination that are free of any selectable markers. By contrast, completely different results were obtained in three strains carrying the caFLP/FRT module. As shown in Fig. 1(c), all strains showed a specific band only in PCR with the nptII-specific primers, suggesting that the caFLP/FRT module remained even after integration into the TmKu80 locus by homologous recombination. The presence of the caFLP/FRT module within the TmKu80 locus of these strains was then confirmed by nucleotide sequencing (data not shown). For one strain harbouring caflp (1171Ca0602), Southern blotting analysis with the TmKu80-specific probe was further performed, and a single hybridization band of about 7.9 kb including the caFLP/FRT module was detected as expected (Fig. 1d). These data showed that the caflp gene failed to function in A. vanbreuseghemii.

**Production of dermatophyte mutant strains lacking multiple protease genes by avFLP recombinase-mediated marker recycling**

The experiments described above demonstrated that the avFLP/FRT recombination system could be fully functional for one-step selectable marker recycling in dermatophytes, allowing multiple rounds of targeted gene disruption through sequential gene deletions. Using this system, we performed multiple rounds of disruption of A. vanbreuseghemii genes encoding major keratinolytic proteases of the metalloprotease (fungalysin) (MEP) and the subtilisin (SUB) families. Recent whole-genome sequencing of seven dermatophyte species revealed that most dermatophytes harbour five MEP genes and 12 SUB genes in each genome (11 SUB genes in Microsporum canis and 13 in Trichophyton tonsurans) (Martinez et al., 2012). It was reported in A. benhamiae that some selected members of the SUB and MEP gene families were specifically activated during *in vitro* growth on keratin-soy protein medium or during infection to the skin of guinea pigs (Staib et al., 2010). Zhang et al. (2014) also pointed to the importance of the MEP family in pathogenicity of T. mentagrophytes. With reference to these data, we selected the MEP3, MEP4, SUB3, SUB6 and SUB7 homologues (AvMEP3, AvMEP4, AvSUB3, AvSUB6 and AvSUB7, respectively) as target genes. For the AvMEP gene family, disruption of AvMEP3 gene was first performed. The 9.04 kb disruption fragment for the AvMEP3 locus was excised from pMRV-AvMEP3/T by SpeI/KpnI digestion, and introduced into the marker-free recipient strain 1062Av1401 (Fig. S2a). Colonies regenerating on selective medium supplemented with CuSO₄ were picked up and roughly screened for the AvMEP3 locus. On direct PCR analysis with primers specific for the AvMEP3 locus, two of the 11 colonies analysed showed the correct integration of the disruption fragment into the genome (data not shown). Of these transformants, one (Av1171-3-18-1) was further chosen, passaged twice on BCS-containing medium and screened again for the AvMEP3 locus. Southern blotting analysis using the AvMEP3-specific probe yielded a single hybridization band of the expected molecular mass (about 2.63 kb) in this
transformant (Fig. 2b). The results of nucleotide sequencing analysis indicated that after integration into the corresponding site of the genome of Av1171-3-18-1, the avFLP/FRT module was removed by site-specific recombination between the flanking FRT sequences. Next, disruption of the AvMEP4 gene in the ΔAvMEP3 strain Av1171-3-18-1 was performed using pMRV-AvMEP4/T (Fig. 2a). Thirty-five colonies, which were regenerated on CuSO4-containing selective medium, were roughly screened for the AvMEP4 locus by direct PCR analysis; two of these colonies showed the correct integration of the disruption fragment into the genome (data not shown). These transformants (Av1201-34-12-1 and Av1201-34-24-1) were transferred onto BCS-containing medium to induce avFLP-mediated recombination, and then screened again for the AvMEP4 locus. On Southern blotting analysis, the AvMEP4-specific probe detected a single hybridization band of the expected molecular mass (about 1.83 kb) (Fig. 2b). Furthermore, for one strain Av1201-34-12-1, nucleotide sequencing and multiplex PCR analysis (Fig. 3c) were performed, and deletion of the AvMEP4 gene, together with the AvMEP3 gene, in this strain was confirmed. According to the same strategy, multiple rounds of disruption of AvSUB genes were also performed. Disruption of AvSUB6 gene in the host strain 1062Av1401 was performed using pMRV-AvSUB6/T (Fig. S3a). As the result of rough screening of the AvSUB6 locus by direct PCR analysis, five of 11 colonies, which were regenerated on CuSO4-containing selective medium, showed the correct integration of the disruption fragment into the genome (data not shown). Of these transformants, one (Av1101-6-7-4) was further chosen, transferred onto BCS-containing medium and screened again for the AvSUB6 locus. Successful excision of the avFLP/FRT module in the resulting transformant, Av1101-6-7-4, was confirmed by Southern blotting analysis (Fig. S3b) and further nucleotide sequencing. Next, disruption of the AvSUB3 gene in the ΔAvSUB6 strain Av1101-6-7-4 was performed using pMRV-AvSUB3/T (Fig. S4a). As the result of rough screening of the AvSUB3 locus by direct PCR analysis, all five colonies regenerated on CuSO4-containing selective medium showed the correct integration of the disruption fragment into the genome (data not shown). Of these transformants, one (Av1121-36-5-1) was further chosen, transferred onto BCS-containing medium and screened again for the AvSUB3 locus. Successful excision of the avFLP/FRT module in the resulting transformant, Av1121-36-5-1, was confirmed by Southern blotting analysis (Fig. S4b) and further nucleotide sequencing. Finally, disruption of the AvSUB7 gene in the ΔAvSUB3,6 strain Av1121-36-5-1 was performed using pMRV-AvSUB7/T (Fig. 4a). As the result of rough screening of the AvSUB7 locus by direct PCR analysis, six of 20 colonies, which were regenerated on CuSO4-containing selective medium, showed the correct integration of the disruption fragment into the genome (data not shown). Of these transformants, one (Av1131-367-11-5) was further chosen, transferred onto BCS-containing medium to induce avFLP-mediated recombination and screened again for the AvSUB7 locus. On Southern blotting analysis, the AvSUB7-specific probe detected a single hybridization band of the expected molecular mass (about 2.08 kb) (Fig. 4b). The results of nucleotide sequencing and multiplex PCR analysis (Fig. 4c) confirmed deletion of the AvSUB7 gene, together with the AvSUB3 and AvSUB6 genes, in this strain.

Growing mycelia of the double AvMEP4 gene-disrupted strains Av1201-34-12-1 and Av1201-34-24-1 (ΔTmKu80 ΔAvMEP3 ΔAvMEP4) and the triple AvSUB gene-disrupted strain Av1131-367-11-5 (ΔTmKu80 ΔAvSUB3 ΔAvSUB6 ΔAvSUB7) were examined by light microscopy, and no remarkable morphological differences were detected among these strains (data not shown).

Phenotypic analyses of protease gene mutants

Similar to other ascomycetes, dermatophytes secrete a variety of endo- and exoproteases in medium containing protein as a sole nitrogen and carbon source (Jousson et al., 2004a, b; Monod et al., 2005). The effects of multiple rounds of gene disruptions in A. vanbreuseghemii on secreted endoproteolytic activity were examined by using resolufin-labelled casein as substrate (Fig. 5). A. vanbreuseghemii AvMEP and AvSUB mutants as well as the recipient host strains 1062Av1401 and TmKu80ΔA49 were grown in liquid medium containing skimmed milk to promote protease secretion. Endoproteolytic activities in culture supernatants of the AvMEP3 gene-disrupted strain Av1171-3-18-1 and the triple AvSUB gene-disrupted strain Av1131-367-11-5 were similar to those of both recipient host strains 1062Av1401 and TmKu80ΔA49 used for multiple gene disruption. In contrast, AvMEP4 disruption in the ΔAvMEP3 mutant showed a dramatic effect by almost totally abolishing endoproteolytic activity (on average, up to around 98% reduction). These results are consistent with those obtained by measuring endoproteolytic activities in the presence of protease inhibitors in the culture supernatants. A. vanbreuseghemii was totally inhibited by phosphoramidon, a specific inhibitor of Zn²⁺ metalloproteases, and was not altered by adding PMSF, which specifically inhibits serine proteases. From the above results, MEP4 appeared to be the major endoprotease secreted by A. vanbreuseghemii in skimmed milk. The importance of MEP4 in fungal extracellular proteolytic activity was confirmed by the generation of a revertant strain (Av1242-34-4C), which was produced by complementation of the ΔAvMEP3,4 strain Av1201-34-12-1 with the AvMEP4 gene (Fig. 3). The endoproteolytic activity of the fungus was restored to around the levels of those of recipient host strains (Fig. 5). Furthermore, similar to both double AvMEP gene-disrupted strains, the AvMEP4 gene-disrupted mutant strain Av1241-4-27 (Fig. S5) showed dramatically decreased endoproteolytic activity (Fig. 5), suggesting that there is no functional redundancy between AvMEP3 and AvMEP4 genes.

DISCUSSION

Despite the advances that have been made in development of basic tools for molecular genetics studies of dermatophytes
Fig. 4. Production of an *A. vanbreuseghemii* mutant strain lacking triple *AvSUB* genes. (a) Schematic representation of the *AvSUB7* locus before and after excision of the avFLP/FRT module. Disruption of the *AvSUB7* gene was achieved by transformation of the recipient strain Av1121-36-5-1 (ΔAvSUB3/ΔAvSUB6) with the avFLP/FRT module from the pMRV- *AvSUB7*/T vector. The avFLP/FRT module was then excised by site-specific recombination between the flanking FRT sequences via conditional expression of the avflp gene after transformation. (b) Southern blotting analysis of a total DNA sample from the transformant Av1131-367-11-5 (ΔAvSUB3 ΔAvSUB6 ΔAvSUB7). Aliquots of approximately 12 μg of total DNA were digested with XhoI and separated by electrophoresis on 0.8 % (w/v) agarose gels. Strain 1062Av1401 was used as a control. A 525 bp fragment of the *AvSUB7* locus was amplified by PCR with the pair of primers AvSUB7-F3 and AvSUB7-R3 and used as a hybridization probe. (c) Multiplex PCR analysis of total DNA sample from strain Av1131-367-11-5. PCR was performed with a mixture of three pairs of *AvSUB*-specific primers (AvSUB3-F3 and R3, AvSUB6-F4 and R3, and AvSUB7-F3 and R4). Each fragment of the *AvSUB3*, *AvSUB6* and *AvSUB7* loci in the control strain 1062Av1401 was amplified separately. Molecular masses of the amplified fragments are shown on both sides.
Yamada et al. (2009a, b; Alshahni et al., 2011; Grumbt et al., 2011a), two significant limitations remain. The first is the lack of conditional promoters to control expression of genes of interest and the other is the limited number of available selectable markers. Recently, we found that the T. rubrum CTR4 promoter (PCTR4), a copper-repressible promoter, was fully functional as a controlled on/off switch of gene expression in dermatophytes (Iwata et al., 2012). However, there remains a pressing need to simultaneously resolve the problem of limited selectable markers.

The present study was performed to establish an efficient and reliable selectable marker recycling system mediated by the FLP recombinase in A. vanbreuseghemii. To accomplish this, it was necessary to control the time point of selectable marker excision mediated by the FLP recombinase. Therefore, we constructed FLP/FRT modules carrying two FRT sequences as well as the flp genes with a neomycin resistance cassette (Fig. 1a). To obtain better regulation of the flp genes, we placed them under the control of PCTR4. In the presence of copper ions, PCTR4 was fully functional as a repressible promoter to prevent spontaneous loss of the selectable marker cassette during the process of transformant selection. Expression of the flp genes in the transformants was easily restored using the copper chelator, BCS, which led to successful removal of the selectable marker and also to the resulting production of a Ku80Δ49 mutant strain (1062Av1401) free of any selectable marker (Fig. 1). KU proteins have been reported to be involved in DNA double-strand break repair through the NHEJ pathway (Critchlow & Jackson, 1998), and their deletion leads to marked increases in targeted gene disruption frequencies via homologous recombination. Accordingly, the 1062Av1401 strain is a valuable recipient that allows rapid and efficient production of multiple gene deletion strains by this marker recycling system.

One of our major future goals is to apply the FLP/FRT recombination system to investigate functions and roles in pathogenicity of large gene families such as secreted proteases in dermatophytes. Recent whole-genome sequencing efforts have revealed that dermatophytes contain particular large families of secreted proteases such as subtilisins (SUBs) and metalloproteases (MEPs) (Martinez et al., 2012). Several high-throughput genetic and proteomic analyses indicated that dermatophytes selectively activate appropriate members of each secreted protease gene family in response to various growth conditions during infection (Giddey et al., 2007a, b; Zaugg et al., 2009; Staib et al., 2010; Sriranganadane et al., 2011). These findings suggest that

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**Fig. 5.** Secreted endoproteolytic activity of each A. vanbreuseghemii strain. The fungi were grown in 1.0% (w/v) skimmed milk medium at 28 °C without shaking. Endoproteolytic activities in their culture supematants were measured as described in Methods. ΔMEP3, Av1171-3-18-1; ΔMEP4, Av1241-4-27; ΔAvMEP3,4-1, Av1201-34-12-1; ΔAvMEP3,4-2, Av1201-34-24-1; RevAvMEP4 (AvMEP4 revertant), Av1242-34-4C; ΔAvSUB3,6,7, Av1131-367-11-5. The results were obtained from three independent trials.
individual members of these protease gene families have their own special roles, and their synergistic effects allow the high adaptability of dermatophytes to host tissues or other environments. Selectable marker recycling via the FLP/FRT system can be useful when studying complex networks among genes encoding such redundant products, which require multiple gene deletions for functional dissection.

Phenotypic analysis of the mutant strains lacking multiple protease genes revealed that the quasi-total secreted endoproteolytic activity of *A. vanbreuseghemii* in a medium containing skimmed milk as sole nutrient was due to MEPS (Fig. 5). No detectable activity appeared to be mediated by any SUB. This result is comparable to our previous observation that the secreted endoproteolytic activity in this fungal species was essentially due to MEPS in contrast to other dermatophyte species such as *A. benhamiae* or *Microsporum canis* where 30% of total secreted endoproteolytic activity was due to SUBs (Jousson *et al.*, 2004a; our unpublished results). Of important note, the growth abilities of mutant *A. vanbreuseghemii* strains lacking the double *AvMEP* genes and the triple *AvSUB* genes, respectively, were unaffected when grown on media containing skimmed milk (data not shown), indicating the necessity of more intensive *in vitro* and *in vivo* exploration of proteases produced from dermatophytes by generating relevant multiple mutants. In parallel, further disruptions of selected members of different protease gene families are also now in process in our laboratory. In both of these cases, the same marker cassette was repeatedly used for construction of consecutive mutations in the same host, but the presence of multiple chromosomal FRT sequences may lead to undesirable secondary effects, such as inversions or deletions between these sites. Therefore, the combined use of the FLP/FRT system and other available site-specific recombination systems, such as Cre/loxP (Sternberg & Hamilton, 1981) and phiC31 (Belteki *et al.*, 2003), will provide new perspectives for constructing multiple gene deletions in this fungus.

As shown in Fig. 1(c,d), when the pathogenic yeast *C. albicans*-adapted *flp* (*caflp*) gene and the codon-optimized *flp* (*cafvflp*) gene were separately introduced into *A. vanbreuseghemii*, site-specific recombination between the FRT sites was observed only in the transformants harbouring the codon-optimized *flp* gene. Similar results were obtained in the ascomycetes filamentous fungi *P. chrysogenum* (Kopke *et al.*, 2010), suggesting that one of the keys for successful FLP-mediated site-specific recombination in filamentous fungi may be the optimization of codon usage of the *flp* gene to the pattern of the host species. However, little information is currently available on the mechanisms by which codon optimization improves FLP-mediated site-specific recombination efficiency in these fungi. In general, codon usage is considered one of the critical factors for efficient expression of heterologous genes (Gustafsson *et al.*, 2004). Studies of heterologous gene expression through codon optimization have reported improved heterologous protein production in several filamentous fungi (Gouka *et al.*, 1997; Te’o *et al.*, 2000; Cardoza *et al.*, 2003; Nelson *et al.*, 2004; Koda *et al.*, 2005; Gooch *et al.*, 2008; Tokuoka *et al.*, 2008). Codon frequencies are closely related to the amounts of cognate tRNAs within the cell. tRNAs that read rare codons are often present at low levels, and unfavourable codon content can cause slow elongation of the nascent peptide or premature termination of translation due to a shortage of available cognate tRNAs in heterologous hosts, decreasing the levels of protein production. Thus, codon optimization of the *flp* gene to *A. vanbreuseghemii* and *P. chrysogenum* would probably reduce the translational inefficiency of the transcripts with unfavourable codons, resulting in increased levels of FLP recombinase expression in these fungi. By contrast, codon optimization may also improve the transcript stability of the *flp* gene in these fungi. Transcripts can be destabilized by permanent or temporary translational arrest due to stop codons or high contents of rare codons, leading to their degradation (Caponigro *et al.*, 1993; Jacobson & Peltz, 1996). It has also been reported in several *Aspergillus* species (Gouka *et al.*, 1997; Tokuoka *et al.*, 2008) that poor expression of some heterologous proteins was due to the addition of cryptic polyadenylation signals to the mRNA within the coding region of the native genes, leading to aberrant mRNAs that are rapidly degraded. In both of these cases, codon optimization resulted in increased G+C content and removal of the cryptic polyadenylation sites in the coding regions of the transcripts. Similarly, the G+C contents of the codon-optimized *A. vanbreuseghemii* and *P. chrysogenum* *flp* genes were 51 and 57 mol%, respectively, which were significantly higher than that of the *caflp* gene (37 mol%). Therefore, we also postulated that codon optimization of the *flp* gene may reduce production of the unstable aberrant transcripts attributed to premature polyadenylation, resulting in increased transcript levels in these fungi.

In conclusion, to our knowledge, this is the first report of a practical selectable marker recycling system mediated by the FLP recombinase for use in dermatophytes. The reliability and applicability of this system established in *A. vanbreuseghemii* were demonstrated by production of mutant strains free of any selectable markers or lacking multiple protease genes (*AvMEP3* and *AvMEP4* or *AvSUB3*, *AvSUB6* and *AvSUB7*) through sequential deletion. This system will extend the potential of genetic manipulation in dermatophytes.

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