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Generation of a *Ustilago maydis* *ade2* Mutant

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Abstract

The need exists to create the auxotrophic mutants for basidiomycete *Ustilago maydis* to allow selection of gene transformants in minimal growth media. The ADE2 gene was identified by homology with *Saccharomyces cerevisiae*. Adenine-requiring auxotrophic mutants were effectively created by homologous recombination in protoplasts using a standard plasmid containing the ADE2 locus interrupted by a phleomycin resistance gene, selected on standard complex media. The knockout *ade2* mutant produced grows on minimal, defined medium only if supplemented with adenine.

Keywords: Ustilago; Selectable marker; Auxotrophy, Adenine

Introduction

Phytopathogenic *Ustilago maydis* is a haploid, dimorphic fungal model organism [1] that has been used to characterize genes involved in its infection of maize [2], in DNA recombination, mating determination and various signaling pathways [3,4]. We have used it to generate structural and regulatory genomic mutants of the two histone H3 variant loci using the fungicide carboxin as a selectable marker [5]. Carboxin has been used routinely in suspension cultures and in planta [6-8]. Other antibiotic selection systems have been used but these are not effective in minimal media [9]. In need for new selection alternatives to create additional gene transformants on minimal media in strains that already contained the carboxin-resistance insert, we turned to the auxotrophic mutant option.

Few auxotrophic mutants have been described for *Ustilago*, likely because they cannot be used for selection in planta. An uracil-requiring mutant [10] was created by disrupting the Pr4 gene [11]. The *leu1* mutant [12] cannot be recreated in any desired background. Adenine auxotrophic mutants have been developed by knocking out a putative ADE6 in *Ustilago* strain 521 [13]. This result could not be reproduced in our laboratory.

Based on the published genomic sequence of *Ustilago* [4], we used BLAST searches to identify homologous loci for well-known mutants of *S. cerevisiae*. The 828 amino acid putative anthranilate synthase component II locus (XP_011388676) was identified as a homolog of the yeast TRP1 locus (Ydr007wp). However, in *Ustilago*, this tryptophan synthesis gene is fused to the TRP3 (Ykl211c) gene and thus was not pursued further. We identified homologous genes for the yeast histidine HIS3 loci YOR202w and YNL338w but were unable to obtain knockout transformants that were dependent on histidine in minimal media (results not shown), possibly due to differences in metabolic pathways between *Ustilago* and yeast. Yeast ADE2 is the required purine biosynthetic gene for phosphoribosylaminoimidazole carboxylase (AIR carboxylase). Knockout *ade2* strains produce pink colonies on non-selective media [14]. *Ustilago ade2* knockout strains,

dependent on adenine supplementation of minimal media, were created using a single plasmid, pKO_Ade2_Phleo, available upon request. It consists of a phleomycin-resistance cassette that interrupts the *Ustilago* gene sequence XM_011389059.1, a homolog of yeast ADE2.

Materials and Methods

Ustilago maydis 521 (wild type strain 9021) was obtained from the Fungal Genetics Stock Center (UMKC, Kansas City, MO, USA). Cells were grown at 30°C in defined minimal medium containing 6.7 g Yeast Nitrogen Base without amino acids (Fisher Scientific, BD Difco, Germantown, MD, USA) and 20 g glucose in 1 L water or complex YEPS medium containing 4 g yeast extract (BD Difco, Germantown, MD, USA), 4 g bactopectone (BD Difco, Germantown, MD, USA) and 20 g sucrose in 1 L water with or without 20 g agar (Fisher Scientific). Phleomycin (InvivoGen) and adenine (Sigma-Aldrich) were used at final concentrations of 30 mg/L and 10-25 mg/L, respectively. Phleomycin plates were stored at 4°C overnight before use. Plasmid DNA was isolated from *E. coli* using a Qiagen mini prep Kit (Qiagen, Germantown, MD, USA).

Infusion primers (Invitrogen, Grand Island, NY, USA) were designed using IDT Primer Quest software. Primer 1: Um_7z_Ade2_F: 5'-CGA AAT CGA TAA GCT TTC GAC GCC ACA TCG TTC AGA GG-3'. Primer 2: Um_Ade2_R: 5'-CTC ACT CAC GAC TCG AGC AGT AGT-3'. Primer 3: Um_Ade2_Phleo_F: 5'-CGA GTC GTG AGT GAG AGA TGG CCA CCA TGG CGT GAC A-3'. Primer 4: Um_Phleo_Ade2_R: 5'-TCT CGG CTG CTG CCA CAC TCA GGC CTA TTA ATG CG-3'. Primer 5: Um_Ade2_int_F: 5'-TGG CAG CAG CCG AGA TTC TCA AA-3'. Primer 6: Um_Ade2_7z_R: 5'-CTC CGG ATC CAA GCT CGG CCA ACT CAA GAC GCA GGC TGT AT-3'.

The phleomycin gene was obtained from pMF1-P, kindly provided by Michael Perlin (University of Louisville, Louisville, KY, USA). pGEM-7Zf(+) was used as the vector scaffold using an In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA) to ligate the PCR fragments (Figure 1).

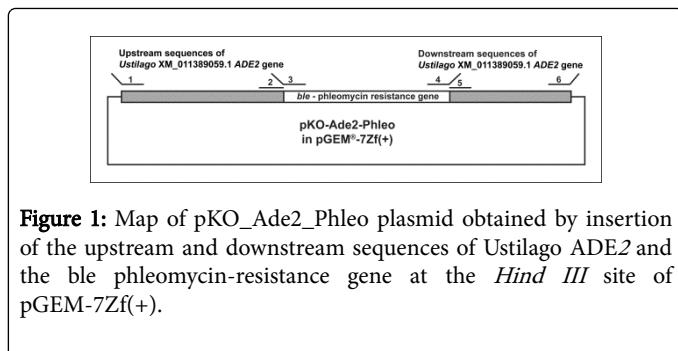


Figure 1: Map of pKO_Ade2_Phleo plasmid obtained by insertion of the upstream and downstream sequences of *Ustilago ADE2* and the *ble* phleomycin-resistance gene at the *Hind III* site of pGEM-7Zf(+).

The plasmid construct was transformed into *E. coli* DH5 α cells and named pKO_Ade2_Phleo. *Ustilago* was transformed as described [5]. Genomic DNA was isolated from putative *ade2* colonies by the glass bead technique described [5] and used to confirm homologous recombination by colony PCR, followed by amplification and sequencing of the insert.

Results and Discussion

The yeast *ADE2* gene (Open Reading frame YOR128C, 1716 bp, intron-free) on chromosome 15 codes for the 484 amino acid phosphoribosylaminoimidazole carboxylase. BLAST searching identified its homolog on chromosome 2 of the *U. maydis* genome [4] as the 1905 bp XM_011389059.1 locus. It codes for a putative 635 amino acid phosphoribosylaminoimidazole carboxylase. This locus was positively identified with an E-value of $9e-22$ with 72% identity and 30% coverage for protein. The next best matched locus had an E-value of 0.40 and 1% coverage.

The In-Fusion method was selected to create a knockout cassette with at least 1 kb flanking sequences required for site-specific homologous recombination in *Ustilago* [15]. Primers were designed to produce PCR fragments that contained approximately 1 kb upstream of the transcription start site and 458 bp coding sequence followed by 682 bp downstream of the stop codon. The *ble* phleomycin resistance gene cassette was amplified by PCR from the pMF1p vector to replace the central coding region of the XM_011389059.1 locus (Figure 1). The three amplified fragments were ligated into the pGEM-7Zf(+) vector using an In-Fusion method and transformed into *E. coli* DH5 α cells. Transformation of *U. maydis* protoplasts was performed using the plasmid named pKO_Ade2-Phleo as reported previously [5]. In short, 5 μ L plasmid DNA (1 mg/mL) was used to transform 106 protoplasts in 50 μ L and plated in complex YEPS agar. Yeast extract contains free adenine [16], so transformants were selected by addition of phleomycin to 30 μ g/mL. Selected colonies of putative auxotrophic mutants were plated on defined minimal medium supplemented with adenine to 25 μ g/mL and replica-plated on plates without adenine. Of the initial 210 phleomycin-resistant colonies, 67 (32%) grew on both plates. These were likely transformed heterokaryons, as observed previously [5], in which untransformed nuclei supported wild type-like growth in the absence of adenine. The remaining 143 colonies (68%) were repeatedly replica-plated and subjected to eight to ten rounds of selection on minimal media with and without adenine to select for homogeneous homokaryon auxotrophic *ade2* knockout mutants (Figure 2).

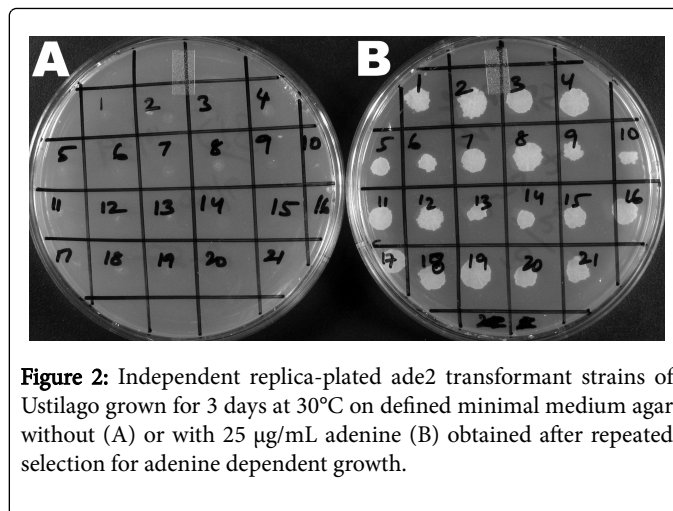


Figure 2: Independent replica-plated *ade2* transformant strains of *Ustilago* grown for 3 days at 30°C on defined minimal medium agar without (A) or with 25 μ g/mL adenine (B) obtained after repeated selection for adenine dependent growth.

Initially these colonies were pale pink, as seen in *ade2* mutants in yeast and *Candida albicans* [14,17], but this accumulation of pink colour was lost during the repeated rounds of selection. This experimental result demonstrates a simple method to produce adenine-dependent auxotrophic mutants of *Ustilago* using plasmid pKO_Ade2_Phleo. Note that application of CRISPR-cas9 technology, recently described for *Ustilago* [18] with an efficiency of 70%, similar to that reported here, provides an alternative strategy to create *ade2* knockout and other auxotrophic mutants.

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