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Overcoming recalcitrant transformation and gene manipulation in *Pucciniomycotina* yeasts

Erika P. Abbott · Giuseppe Ianiri · Raffaello Castoria · Alexander Idnurm

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Abstract The red yeasts of the *Pucciniomycotina* have rarely been transformed with DNA molecules. Transformation methods were recently developed for a species of *Sporobolomyces*, based on selection using uracil auxotrophs and plasmids carrying the wild-type copies of the *URA3* and *URA5* genes. However, these plasmids were ineffective in the transformation of closely related species. Using the genome-sequenced strain of *Rhodotorula graminis* as a starting point, the *URA3* and *URA5* genes were cloned and tested for the transformation ability into different *Pucciniomycotina* species by biolistic and *Agrobacterium*-mediated transformations. Transformation success depended on the red yeast species and the origin of the *URA3* or *URA5* genes, which may be related to the high G+C DNA content found in several species. A new vector was generated to confer resistance to nourseothricin, using a native promoter from *R. graminis* and the naturally high G+C nourseothricin acetyltransferease gene. This provides a second selectable marker in these species. Targeted gene disruption was tested in *Sporobolomyces* sp. IAM 13481 using different lengths of homologous DNA with biolistic and *Agrobacterium* transformation methods. Both DNA delivery methods were effective for targeted replacement of a gene required for carotenoid pigment biosynthesis. The constructs also triggered transgene silencing. These developments open the way to identify and manipulate gene functions in a large group of basidiomycete fungi.

Keywords Basidiomycete · *Rhodotorula slooffiae* · *Rhodosporidium kratochvilovae* · β-Carotene · RNAi · T-DNA

Introduction

The *Pucciniomycotina* red yeasts are a paraphyletic assemblage of fungi that are artificially grouped by their morphology, by their capability of producing carotenoid pigments, and by being in the same subphylum within the *Basidiomycota* phylum. The species are free living and prevalent worldwide. They have the potential to serve as models to understand the obligate plant pathogenic rust fungi, which are also members of the *Pucciniomycotina*. Red yeasts in themselves have been proposed as biocontrol agents, sources of novel enzymes, and in lipid production for biodiesel [for a review and phylogeny of this clade, see Aime et al. (2006)]. They can also, in rare cases, cause disease in humans (Tuon and Costa 2008). However, the underlying genetic basis for these interesting properties remains largely unexplored.

Four species of red yeast were used in this study. *Sporobolomyces* sp. strain IAM 13481 was the first *Pucciniomycotina* species with a genome sequenced and was used previously to develop plasmids and approaches for gene manipulation (Ianiri et al. 2011). *Rhodosporidium kratochvilovae* strain LS11 is of interest for biocontrol of fungal pathogens of fruit and degradation of the mycotoxin patulin (Castoria et al. 2003, 2005, 2011; Lima et al. 2005). Its traits underlying biocontrol activity against fungal pathogens of

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E. P. Abbott · G. Ianiri · A. Idnurm (✉)
Division of Cell Biology and Biophysics,
School of Biological Sciences, University of Missouri—Kansas City,
5100 Rockhill Road,
Kansas City, MO 64110, USA
e-mail: IdnurmA@umkc.edu

G. Ianiri · R. Castoria
Dipartimento di Agricoltura, Ambiente e Alimenti,
Università del Molise,
Via Francesco De Sanctis,
86100 Campobasso, Italy

stored fruit have been described at the phenotypic level, but demonstration at the gene level is still missing (Castoria et al. 1997, 2003). *Rhodosporidium* sp. are of additional research interest from the perspective of the evolution of their mating type locus (Coelho et al. 2010). The interest in *Rhodotorula slooffiae* relates to the gene duplication of the light-sensing machinery that has occurred in the *Pucciniomycotina* and *Mucoromycotina* (Idnurm et al. 2010). *Sporobolomyces* sp. strain IAM 13481 shows no overt photosensory response. Screening a collection of red yeasts isolated from the San Juan Islands [Washington State, USA (Fraser et al. 2006)] revealed that pigmentation in *R. slooffiae* strain SJ3 was induced by light. This species is phylogenetically distant from *Sporobolomyces* sp. IAM 13481 and *R. kratochvilovae*, being in a different subclass within the *Pucciniomycotina* (Aime et al. 2006; Fell et al. 2000; Valério et al. 2008). The fourth strain is *Rhodotorula graminis* WP1, isolated as an endophyte from a poplar tree (Xin et al. 2009). This strain fits within a context of genome sequences of a model tree (*Populus trichocarpa*) and its associated basidiomycete fungi that include a pathogen (*Melampsora laricis-populina*), mycorrhizal symbiont (*Laccaria bicolor*), and endophyte (*R. graminis*). The rationale for using this strain was because its genome had been sequenced, which would aid cloning genes or gene fragments for selectable markers, as well as confirming mutations within auxotrophic strains that could act as the recipients for DNA in transformation experiments.

In the study of these yeasts, being able to manipulate their genes is essential for understanding gene functions and engineering beneficial traits. Transformation of these species has rarely been reported. The first report was for *Rhodosporidium toruloides* in the mid-1980s (Tully and Gilbert 1985). We reported methods for transformation of *Sporobolomyces* sp. strain IAM 13481, by biolistic or *Agrobacterium tumefaciens* delivery of the DNA (Ianiri et al. 2011). However, we were unable to achieve successful transformation using the same plasmids and transformation approaches on the related red yeast *R. kratochvilovae* strain LS11, which is also within the *Sporidiobolales* order. Recently, codon-optimized vectors have been used to transform *R. toruloides* (Liu et al. 2012). In this study, we resolve transformation problems in *R. kratochvilovae* and other red yeast species, develop a second selectable dominant marker for transformation, and optimize gene disruption approaches using a species of *Sporobolomyces*.

Materials and methods

Strains and growth conditions

The strains used or that were generated in this study are listed in Table 1. The *R. kratochvilovae* strain LS11 was from a collection housed at the University of Molise, Italy. *R. slooffiae* SJ3 was provided by Drs. Joseph Heitman and James Fraser (Duke University Medical Center, Durham, NC, USA). *Sporobolomyces* strain IAM 13481 and *R. graminis* strain WP1 were provided by the Fungal Genetics Stock Center (Kansas City, MO, USA).

Spontaneous uracil auxotrophs were isolated by growth on yeast nitrogen base (YNB) medium supplemented with uracil (20 mg/L) and 5-fluoroorotic acid (1 g/L) (Boeke et al. 1984). Strains were maintained on yeast extract–peptone–dextrose (YPD) medium and preserved as 15 % glycerol stocks at −80 °C. Media designed for the isolation of lysine and tryptophan auxotrophs were prepared as described previously for *Saccharomyces cerevisiae* (Chattoo et al. 1979; Toyn et al. 2000).

Construction of plasmids carrying selectable markers for fungal transformation

The genome sequence database of *R. graminis* WP1, available on the website of the Joint Genome Institute (US Department of Energy; http://genome.jgi-psf.org/Rhoba1_1/Rhoba1_1.home.html), was searched by BLAST for the homologues of the *S. cerevisiae URA3* and *URA5* genes. The wild-type copies of *R. graminis* WP1 *URA3* and *URA5* were amplified from genomic DNA, purified from agarose gel slices and cloned into plasmids TOPO pCR2.1 (Invitrogen, Life Technologies, Grand Island, NY, USA) and subsequently into pPZP-201BK (Covert et al. 2001), as described below.

For the generation of a construct based on a dominant marker, the nourseothricin acetyltransferase (*NAT*) gene from *Streptomyces noursei* was chosen (Krügel et al. 1993). To drive the expression of this gene, the promoter and terminator of the β-tubulin-encoding *TUB2* gene of *R. graminis* WP1 were used. To this aim, the genome database of *R. graminis* WP1 was searched by BLAST for the homologue of the *TUB2* gene of *S. cerevisiae*. Fragments (790- and 508-bp upstream and downstream) of the opening reading frame (ORF) region of *TUB2* were amplified from *R. graminis* genomic DNA with primers ALID1614–ALID1615, and ALID1616–ALID1617, respectively. The *NAT* gene was amplified from plasmid pCH233 with primers ALID1618–ALID1619. Primer sequences are listed in Table 2. The three products generated by PCR were purified from agarose gel slices and fused together by overlap PCR as previously described (Davidson et al. 2002) to generate the construct P_{TUB2}-*NAT*-T_{TUB2}. The overlap PCR used primers ALID1614–ALID1617. The *NAT* construct was purified from an agarose gel slice and cloned into plasmids TOPO pCR2.1 and pPZP-201BK.

For PCR, *Ex Taq* (Takara, Otsu, Japan) was used to amplify DNA from *Sporobolomyces* sp. This DNA polymerase enzyme mix and the buffer provided did not amplify DNA fragments

Table 1 Strains used in this study

Name	Genotype	Strain background	References
Sporobolomyces sp. IAM 13481	Wild type		Yamazaki and Komagata (1983)
AIS1	*ura3*	IAM 13481	Ianiri et al. (2011)
AIS2	*ura5*	IAM 13481	Ianiri et al. (2011)
EKS1	*car2::URA5 ura5*	AIS2 *Agrobacterium*	This study
EKS2	*car2::URA5 ura5*	AIS2 biolistic	This study
EKS3	*car2::URA5 ura5 car2* unstable	AIS2 *Agrobacterium*	This study
EKS4	*car2::URA5 ura5 car2* unstable	AIS2 biolistic	This study
Rhodotorula graminis WP1	Wild type		Xin et al. (2009)
EKW15	*ura3*	WP1	This study
EKW10	*ura5*	WP1	This study
Rhodotorula slooffiae SJ3	Wild type		Fraser et al. (2006)
SJ3–1A	*ura3*	SJ3	This study
SJ3–2A	*ura5*	SJ3	This study
Rhodosporidium kratochvilovae LS11	Wild type		Castoria et al. (1997)
EKLS1	*ura5*	LS11	This study
EKLS2	*ura5*	LS11	This study
Saccharomyces cerevisiae FY834	*MATα his3Δ200 ura3–52 leu2Δ1 lys2Δ202 trp1Δ63*		Winston et al. (1995)

Table 2 Primers used in this study

Name	Sequence (5'–3')	Purpose/gene amplified
EK001	GGAATTCGGGTGTAGATCGATTCAGAG	*Sporobolomyces CAR2* 5′ flank
EK002	*GGTCTTTCCAGGGAGAGA*CGCATCGCATTCCCAGAG	
EK003	*CAAGTAGAACGAAGGGTT*AGCTTCACACCCTCGTAG	*Sporobolomyces CAR2* 3′ flank
EK004	GGAATTCATCCGCTTACTGTCAAGGAG	
EK005	AACAGGCCACCATACCCTG	*CAR2* 500 bp flanking
EK007	GCGTCGACGGGTCGACCAG	
EK006	CTCCCGACCAAAGCCGAAC	*CAR2* 250 bp flanking
EK008	CTTGATCCGTTTGAATCGG	
EK009	AATCCGTTCTCATCCAGG	Confirming *CAR2* gene replacement
EK010	CTTCGCTTCTCACCAATC	
EK011	CGGAACGAGACAGAAGAAGC	Assessing integration in *URA5* locus
EK012	ATCCCTCGCGTCTGCGAGTG	
ALID0562	TCTCTCCCTGGAAAGACC	*Sporobolomyces URA5*
ALID0564	AACCCTTCGTTCTACTTG	
ALID1164	CACTGCTTTACGCCGACCTG	*R. graminis URA5*
ALID1165	TCGAGAAGGTCGACTTGAGC	
ALID1281	AGCGCCTCGGTGAAAGTG	*R. graminis URA3*
ALID1282	TTCGGGCTCACGACGCTC	
ALID1614	GCGACGACGTGTGGGGCTACC	*R. graminis* β-tubulin promoter
ALID1615	*AAGAGTGGCGGCCGCCAT*CGTGGCTTGCGGGAGTGG	
ALID1616	*AGCATGCCCTGCCCCTAA*GCTTATTCACCTGCACTC	*R. graminis* β-tubulin terminator
ALID1617	TCTCGCGAGCGAGGTTGGC	
ALID1618	ATGGCGGCCGCCACTCTTGACG	*NAT* gene
ALID1619	TTAGGGGCAGGGCATGCTCATG	

Nucleotides are listed 5′ to 3′. The italicized nucleotides indicate regions that overlap other primers. Restriction enzyme sites introduced for subcloning are underlined

from *R. kratochvilovae* strain LS11 or *R. graminis* strain WP1. Takara's *LA Taq*, with GC buffer I to improve PCR from high G+C content DNA, was used for these two species. Amplifications were performed in a Mastercycler PCR machine (Eppendorf, Hauppauge, NY, USA). For restriction enzyme digests, all enzymes were purchased from New England Biolabs (Ipswich, MA, USA).

Genes or gene fragments were amplified, cloned into plasmid TOPO pCR2.1, and transformed into *Escherichia coli* strain DH5α (Invitrogen) cells by heat shock. Transformed *E. coli* cells were selected on Luria–Bertani (LB) medium supplemented with kanamycin (50 μg/ml). Clones that were free from introduction of PCR errors were identified by DNA sequencing. Inserts were subcloned using *Eco*RI restriction enzyme into plasmid pPZP-201BK, an *Agrobacterium* binary vector (Covert et al. 2001). *Agrobacterium* plasmids were transformed into *A. tumefaciens* strain EHA105 (Hood et al. 1993) by electroporation and selected on LB medium containing kanamycin (50 μg/mL).

A plasmid was generated that could be transformed into either *Sporobolomyces* sp. or *S. cerevisiae*. The *URA5* gene of *Sporobolomyces* sp. IAM 13481 was excised from plasmid pAIS2 and subcloned into the *Eco*RI site of plasmid pRS426.

A list of the plasmids used for transformation of red yeasts in the study is provided in Table 3.

Fungal transformation

Transformation of strains followed established protocols for biolistic or *Agrobacterium* delivery of DNA (Ianiri et al. 2011; Idnurm et al. 2004; Toffaletti et al. 1993). Biolistic transformation was directly on YNB+1 M sorbitol for the *URA* markers or onto YPD+1 M sorbitol for the *NAT* marker, followed by 3 h recovery at room temperature and transfer to selective YPD+nourseothricin (200 μg/mL) medium. *Agrobacterium* cocultures were incubated on induction medium (Bundock et al. 1995) for 2 or 3 days prior to transfer to medium to select for transformed fungal strains and cefotaxime to inhibit *Agrobacterium* growth. The lithium acetate transformation protocol followed that of Ito et al. (1983), of DNA into the recipient *Sporobolomyces* strain AIS2 or *S. cerevisiae* strain FY834. Electroporation protocols followed those developed for *Cryptococcus neoformans* (Edman 1992).

Disruption of the *CAR2* gene of *Sporobolomyces* sp.

A set of six plasmids was created for disruption of the *CAR2* gene of *Sporobolomyces* sp. Two of these (pEK627 and pPZP627) were designed to deliver a construct with 1-kb flanking regions, while the others carried constructs with 0.5 kb (pEK5+7 and pPZP5+7) and 0.25 kb (pEK6+8 and pPZP6+8) flanking regions (Table 3).

The construct carrying 1-kb flanking regions was generated by overlap PCR of three fragments: the 5′ flanking region (primers EK001–EK002), the *URA5* marker (primers ALID562–ALID564), and the 3′ flanking region (primers EK003–EK004). The construct was then purified from an agarose gel slice and cloned into TOPO pCR2.1. After sequencing independent clones, a construct with no errors (pEK627) was generated by subcloning *Spe*I restriction

Table 3 Plasmids used in this study

Name	Features	Reference/source
pAIS1	*Sporobolomyces* sp. *URA3* in TOPO pCR2.1	Ianiri et al. (2011)
pAIS2	*Sporobolomyces* sp. *URA5* in TOPO pCR2.1	Ianiri et al. (2011)
pAIS3	*Sporobolomyces* sp. *URA3* in pPZP–201BK	Ianiri et al. (2011)
pAIS4	*Sporobolomyces* sp. *URA5* in pPZP–201BK	Ianiri et al. (2011)
pEK627	*car2::URA5* overlap in TOPO pCR2.1 (1 kb)	This study
pEK5+7	*car2::URA5* overlap in TOPO pCR2.1 (500 bp)	This study
pEK6+8	*car2::URA5* overlap in TOPO pCR2.1 (250 bp)	This study
pPZP627	*car2::URA5* overlap in pPZP-201BK	This study
pPZP5+7	*car2::URA5* overlap in pPZP-201BK	This study
pPZP6+8	*car2::URA5* overlap in pPZP-201BK	This study
pEKWU3	*R. graminis* WP1 *URA3* in TOPO pCR2.1	This study
pEKWU5	*R. graminis* WP1 *URA5* in TOPO pCR2.1	This study
pPZPWU3	*R. graminis* WP1 *URA3* in pPZP-201BK	This study
pPZPWU5	*R. graminis* WP1 *URA5* in pPZP-201BK	This study
pRS2	*Sporobolomyces* sp. *URA5* in pRS426	This study
pGI1	P_{TUB2}-*NAT*-T_{TUB2} in TOPO pCR2.1	This study
pGI2	P_{TUB2}-*NAT*-T_{TUB2} in pPZP-201BK	This study

enzyme fragments. For *Agrobacterium*-mediated transformation, the insert was subcloned using *Eco*RI into the binary vector pPZP-201BK; the plasmid (pPZP627) was then transformed into *A. tumefaciens* strain EHA105 before coincubation with *Sporobolomyces* sp.

PCR was used to amplify constructs with 0.5 and 0.25 kb flanking the *URA5* gene, off plasmid pEK627 DNA as the template, with primers EK005–EK007 and EK006–EK008, respectively. The products were cloned into TOPO pCR2.1. After sequencing to identify error-free clones, the plasmids were digested with *Eco*RI, the fragments of the inserts were purified from agarose gel slices and subcloned into pPZP-201BK for *Agrobacterium*-mediated transformation. The constructs used for the biolistic transformation were obtained by PCR on pEK627 plasmid DNA, with primers EK005–EK007 and EK006–EK008. The products of the PCR were purified and then used for transformation.

For the transformation experiments aimed at gene replacement of *CAR2*, white colonies were characterized by amplification with primers EK009 and EK010. Gene replacement was further confirmed by Southern blot analysis.

Southern blotting

Two micrograms of genomic DNA samples were cut with restriction enzymes and resolved on 1 % agarose 1× Tris–acetic acid–EDTA gels. Enzymes used were *Xho*I and *Bam*HI for the *URA3* and *URA5* transformants of *R. slooffiae* SJ3-1A and SJ3-2A, respectively; *Eco*RI and *Sac*II for the *URA5* transformants of *R. graminis* EKW10; *Cla*I for the *URA5* transformants of *Sporobolomyces* AIS2; *Sac*II for *URA5* transformants of *R. kratochvilovae* EKLS1; and *Kpn*I for the *NAT* resistant transformants of *R. graminis* WP1 and *R. kratochvilovae* LS11. For the *car2* mutants of *Sporobolomyces* AIS2, genomic DNA was cut with *Pci*I, which cuts once in the middle of the *URA5* gene. DNA was transferred by Southern blotting to Zeta-Probe membrane (Bio-Rad, Hercules, CA, USA). The blots were hybridized overnight in modified Church buffer, with [α-^{32}P] dCTP probes labeled with the RediPrime II kit (Amersham, GE Healthcare, Piscataway, NJ, USA). Blots were washed with 0.5× SSC/0.5 % sodium dodecyl sulfate and stringency at 65 °C, and then exposed to X-ray film. Probes for the *URA* genes were amplified by PCR with universal primers M13F and M13R from the plasmids used for transformation. The probe for *CAR2* was amplified from genomic DNA of *Sporobolomyces* sp. with primers EK001–EK004. The *NAT* probe was prepared by excising it from plasmid pGI1 with *Eco*RI restriction enzyme.

Resource deposition

The plasmids containing the *R. graminis URA* genes and *NAT* cassette, and the *R. graminis* and *R. slooffiae* uracil auxotrophs were deposited with the Fungal Genetics Stock Center, Kansas City, MO, USA (McCluskey et al. 2010; McCluskey and Wiest 2011). Plasmids can be found via direct search of their names listed in Table 3. For the strains, SJ3 is FGSC 10310, SJ3-1A is FGSC 10311, SJ3-2A is FGSC 10312, EKW15 is FGSC 10313, EKW10 is FGSC 10314, and EKS1 is FGSC 10315.

Results

Transformation success is variable in the red basidiomycete yeasts and does not follow phylogenetic boundaries

Plasmids containing the *URA3* and *URA5* genes of *Sporobolomyces* sp. were tested on *R. kratochvilovae* strain LS11. Stable uracil auxotrophs were isolated from LS11 by selection of spontaneous mutants on 5-fluoroorotic acid (5-FOA), and transformation of the auxotrophs was attempted using biolistic, electroporation, and *Agrobacterium* delivery of the *Sporobolomyces URA3* or *URA5* genes. No transformants were obtained with these methods.

After the initial lack of success in transforming *R. kratochvilovae*, these plasmids were tested for transformation of uracil auxotrophs isolated from *R. slooffiae* strain SJ3. Uracil auxotrophs showing resistance to 5-FOA were isolated from strain SJ3 (Fig. 1a). In contrast to *R. kratochvilovae*, transformation was readily achieved for *R. slooffiae* with *Agrobacterium* for two auxotrophs, representing *ura3* and *ura5* mutations and the integration of the DNA confirmed by Southern blotting (Fig. 1b).

Next, transformation was tested in *R. graminis* strain WP1. Uracil auxotrophs were isolated using selection on 5-FOA. We were unable to amplify DNA from *R. graminis* strain WP1 with standard *Taq* DNA polymerase and buffer. DNA amplification was successful using *LA Taq* (Takara) with a buffer optimized for high G+C content DNA. Wild-type copies of *URA3* and *URA5* were amplified from strain WP1, cloned, and error-free clones identified by sequencing. The *URA3* and *URA5* genes were subcloned into the pPZP-201BK plasmid to enable their transformation into fungi by *Agrobacterium*. The *URA3* and *URA5* genes of the WP1 uracil auxotrophs were amplified and sequenced, revealing mutations in both genes (Fig. 2a). The *ura3* mutation is a C–T transition that results in an R–C substitution at position 12. This arginine residue is highly conserved in orotidine phosphate decarboxylase proteins. The *ura5* mutation is a replacement of 4 bp with six Ts, causing a frameshift. The origin of this change is unknown, but may be associated with an 8-bp palindrome GGGCGCCC (Fig. 2a).

The vectors derived from *Sporobolomyces* sp. and *R. graminis* were used in all combinations for transformation

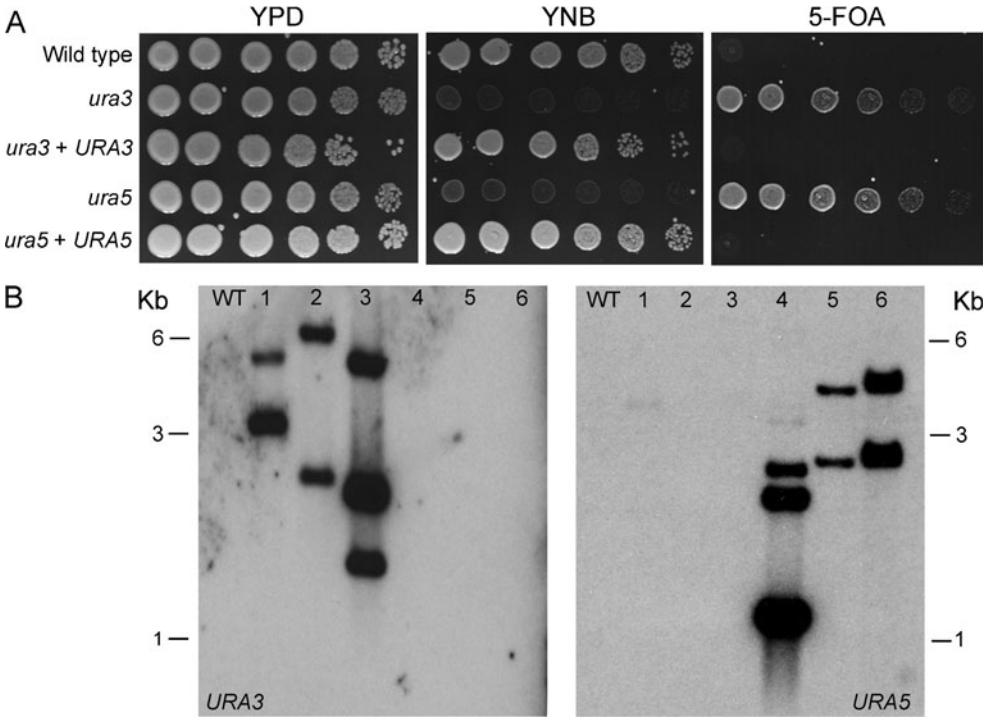

Fig. 1 Transformation of *R. slooffiae* with genes from *Sporobolomyces* sp. **a** Phenotypes of wild-type, uracil auxotrophs SJ3-1A and SJ3-2A, and the same auxotrophs transformed with *Agrobacterium* T-DNA with cloned *URA3* or *URA5* genes from *Sporobolomyces* sp. Strains were tenfold serially diluted and spotted onto YNB±uracil±5-FOA. **b**. Southern blots on DNA from untransformed auxotroph (*WT*), three *ura3*+*URA3* transformants (1–3), and three *ura5*+*URA5* transformants (4–6), probed with the *URA3* gene (*left*) or *URA5* gene (*right*). The probes were amplified from the full length fragments used in the plasmids for transformation. DNA was digested with *Xho*I for the *URA3* transformants and with *Bam*HI for the *URA5* transformants. Both enzymes cut in the middle of the delivered DNA molecule

into *Sporobolomyces* sp. IAM 13481 and *R. graminis* WP1 by biolistic and *Agrobacterium* methods. *R. graminis* could be transformed by biolistic or *Agrobacterium* delivery of DNA, but only with its copies of the *URA3* or *URA5* genes and not those from *Sporobolomyces* (Fig. 2; Table 4). Southern blot analysis was performed on genomic DNA extracted from untransformed strain EKW10, and eight randomly selected *URA5* transformants generated with *Agrobacterium*-mediated transformation. DNA was digested with *Eco*RI (Fig. 2c, left), which cuts at both borders of the T-DNA, and with *Sac*II (Fig. 2c, right), which does not cut. As expected, a strong 1,603-bp hybridization band corresponding to the delivered T-DNA-*URA5* was obtained for all *Eco*RI-digested transformants and not in the untransformed strain (Fig. 2c, left). A weak signal to high molecular weight DNA was also detected, presumably corresponding to the native *URA5* locus. Hybridization to *Sac*II-digested DNA revealed a common 2,102-bp hybridization band of the native *URA5* locus for all tested strains, including the untransformed auxotroph. Moreover, for *URA5* transformants, additional hybridization bands ranging from 3 to 12 kb, corresponding to the number of T-DNA insertions, were detected (Fig. 2c, right).

In contrast to *R. graminis*, the *Sporobolomyces* sp. was amenable to transformation by genes from either *R. graminis* or *Sporobolomyces* sp. and with both methods (Fig. 3; Table 4) (Ianiri et al. 2011). Phenotypically, *URA5* transformants and the *ura5* auxotroph AIS2 showed the ability to grow on selective media YNB and 5-FOA, respectively (Fig. 3a). For molecular confirmation, Southern blot analysis was performed on genomic DNA of the untransformed *ura5* AIS2 and 11 randomly selected *URA5* transformants generated with both biolistic (lanes 1–7) and *Agrobacterium*-mediated (lanes 8–11) transformations (Fig. 3b). DNA was digested with *Cla*I, which does not cut in the *URA5* gene of *R. graminis* WP1. While for the untransformed strain AIS2, there is no hybridization signal; in all selected transformants, a single hybridization band is detected, confirming the success of the transformation using as a marker the *URA5* gene of *R. graminis* WP1.

The lithium acetate-PEG heat shock method is commonly applied to ascomycete yeasts (Ito et al. 1983); however, *Rhodosporidium toruloides* is reported as resistant to this transformation approach (Tully and Gilbert 1985). To test this method again, we generated a plasmid (pRS2) that could be transformed into both *Sporobolomyces* and *Saccharomyces*. Identical growth conditions and transformation at the same time were used on uracil auxotrophs of both species. Colonies were obtained for *S. cerevisiae*. No

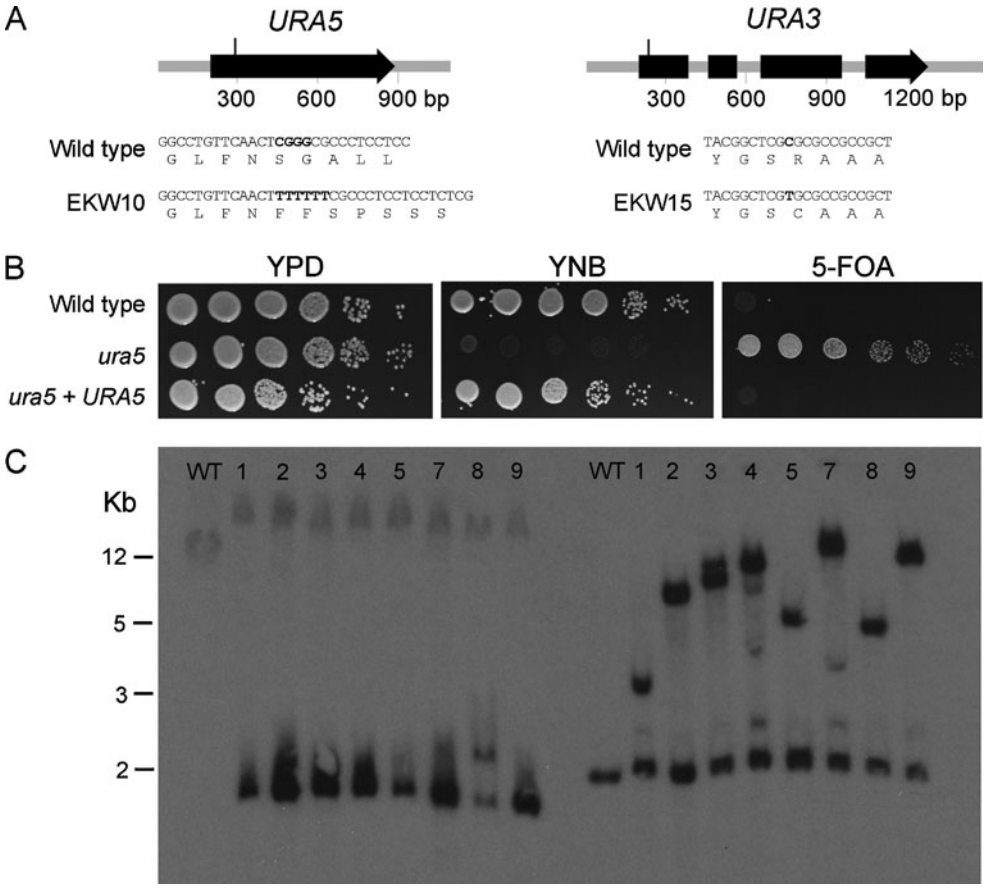

Fig. 2 Transformation of *R. graminis* with genes from *R. graminis* using uracil auxotrophs as recipient strains. **a** Position (*black line*) and type of mutation in the *ura3* and *ura5* strains, EKW15 and EKW10, isolated from strain WP1 that were used for transformation. The altered DNA sequence is indicated below in bold. **b**. Phenotypes of wild-type, *ura5* uracil auxotroph, and a transformed strain. Strains were tenfold serially diluted and spotted onto YNB±uracil±5-FOA. **c** Southern blot of auxotroph EKW15 (*WT*) and nine strains transformed with *Agrobacterium* T-DNA. Genomic DNA was digested with *Eco*RI (*left*), which cuts at the borders of delivered DNA, and *Sac*II (*right*), which does not cut within the T-DNA. The probe was the full length *URA5* fragment cloned into the transformation plasmid. The common band of 1,603 bp obtained for *Eco*RI-digested DNA is the exogenous *URA5* inserted by the transformation. Weak hybridization with high molecular weight DNA indicates low efficiency transfer during blotting of the fragment containing the endogenous *URA5* gene. The common band of 2,012 bp obtained for *Sac*II-digested DNA is the original *URA5* locus

colonies were obtained for *Sporobolomyces*. Thus, this method is also ineffective on *Sporobolomyces*.

Successful transformation of *R. kratochvilovae* strain LS11

With the insight gained from transformation of *R. graminis*, we returned to explore transformation of *R. kratochvilovae* strain LS11. Two uracil auxotrophs of this strain were tested with *Agrobacterium* and biolistic delivery of the *URA3* and *URA5* genes from *Sporobolomyces* or *R. graminis*. As observed previously, the *URA3* or *URA5* gene of *Sporobolomyces* could not transform this strain. In contrast, the *URA5* gene from *R. graminis* WP1 was able to transform two uracil auxotrophic strains using biolistic or *Agrobacterium*-

Table 4 Summary of transformation results

	DNA source/transformation method			
Strain	*Sporobolomyces* sp./biolistics	*Sporobolomyces* sp./*Agrobacterium*	*R. graminis*/biolistics	*R. graminis*/*Agrobacterium*
Sporobolomyces sp.	Yes	Yes	Yes	Yes
R. slooffiae	Yes	Yes	No	No
R. graminis	No	No	Yes	Yes
R. kratochvilovae	No	No	Yes	Yes

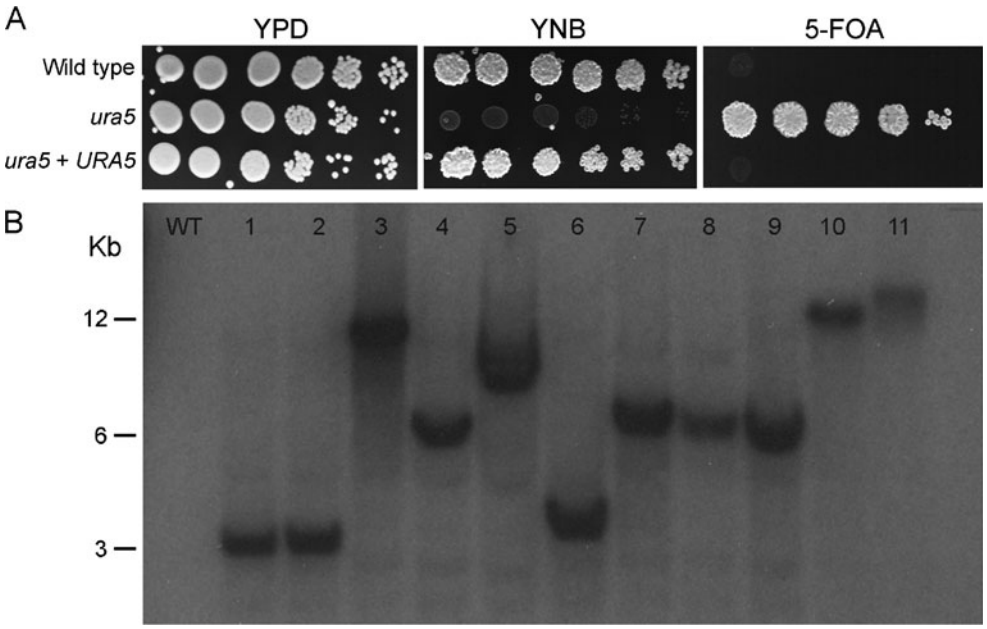

Fig. 3 Transformation of *Sporobolomyces* sp. with the *R. graminis URA5* gene. **a** Phenotypes of the wild type, *ura5* uracil auxotroph and a transformed strain. Strains were tenfold serially diluted and spotted onto YNB±uracil±5-FOA. **b** Southern blot of auxotroph AIS2 (*WT*) and 11 transformed strains DNA samples cut with *Cla*I, which does not cut in the delivered DNA. The probe was the fragment including *URA5* cloned into the plasmid for transformation. *Lanes 1–7* are strains generated with biolistics, and *lanes 8–11* are strains generated with *Agrobacterium*-mediated transformation

mediated delivery of the plasmid or T-DNA (Fig. 4). The strains EKLS1 and EKLS2, which presumably have mutations within the *URA5* homologue, showed sensitivity to YNB and also 5-FOA resistance. Conversely, the derived *URA5* transformants were able to grow only on YNB. In Fig. 4a, the phenotypes observed for strain EKLS1 are shown. For Southern blot confirmation, genomic DNA of the untransformed *ura5* auxotroph and 14 randomly selected *URA5* transformants, of which eight were obtained with biolistics (lanes 1–8) and six with *Agrobacterium*-mediated transformation (lanes 9–14), was digested with *Sac*II. There is no *Sac*II site in the *URA5* gene of *R. graminis* WP1. Hybridization bands ranging from 3 to 15 kb were detected, confirming the success of the transformation. For all tested strains, including the untransformed *ura5* auxotroph, two faint bands of 1,200 and 600 bp were also detected (WT

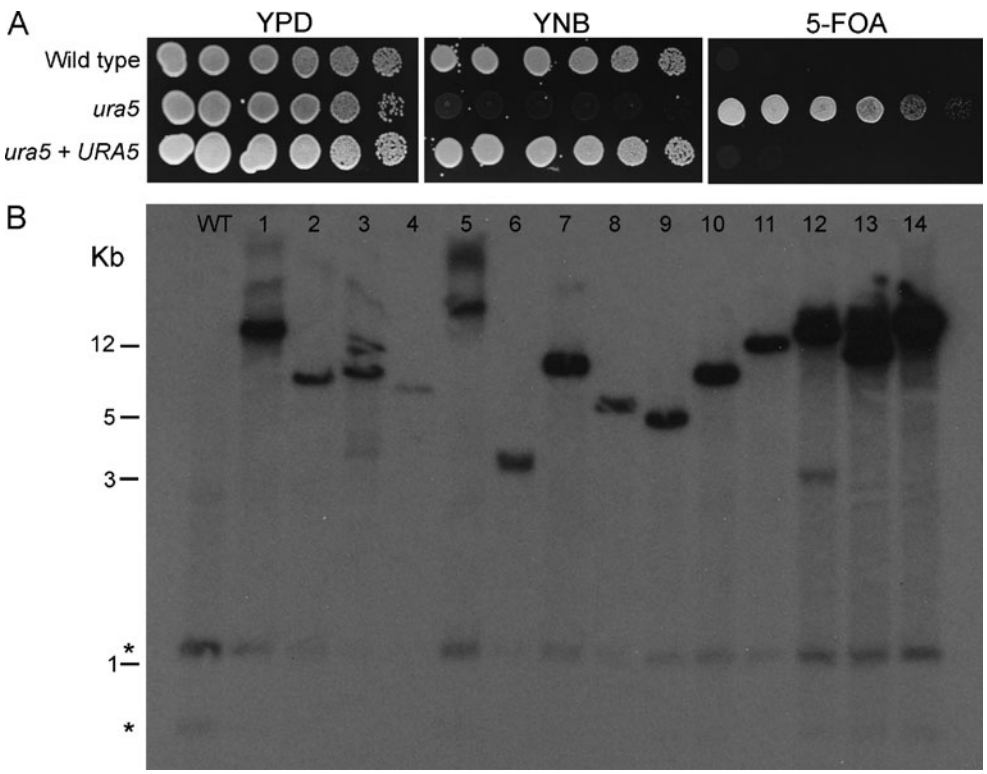

Fig. 4 Transformation of auxotroph EKLS1 of *R. kratochvilovae* with the *R. graminis URA5* gene. **a** Phenotypes of the wild type, *ura5* uracil auxotroph and a transformed strain. Strains were tenfold serially diluted and spotted onto YNB±uracil±5-FOA. **b** Southern blot of auxotroph EKLS1 (*WT*) and 14 transformed strains. DNA samples were digested with *Sac*II, which does not cut within the delivered DNA. *Lanes 1–8* are strains generated with biolistics, and *lanes 9–14* are strains generated with *Agrobacterium*-mediated transformation. The two common faint bands of 1,200 and 600 bp, marked by the *asterisks*, may correspond to the native *ura5* gene of *R. kratochvilovae* because the *URA5* probe hybridizes to them in the untransformed strain (*WT*)

lane in Fig. 4b). We hypothesize that those two bands correspond to the native *URA5* gene of *R. kratochvilovae*, which presumably has a *Sac*II site within the ORF.

Development of a new selectable marker for transformation

One disadvantage in relying on uracil auxotrophs for transformation is the availability of only a single selectable marker. Although the 5-FOA/*ura* selection system can be recycled, it would be more convenient if a second selectable marker were available. Auxotrophs in the tryptophan and lysine biosynthetic pathways were sought using methods established for *S. cerevisiae*, with the toxic molecules 2-amino-5-fluorobenzoic acid and 2-aminoadipic acid, respectively (Chattoo et al. 1979; Toyn et al. 2000). However, the red yeast strains gained unstable resistance or grew on these media.

The *Sporobolomyces* sp. strain IAM 13481 also acquires resistance at a high frequency to drugs used for transformation selection in other fungi (Ianiri et al. 2011). However, this was less evident for *R. kratochvilovae*, *R. graminis*, and *R. slooffiae*. Thus, a new vector for expression of nourseothricin acetyltransferase (*NAT*) was constructed, using the *R. graminis* WP1 β-tubulin promoter, *NAT*, and β-tubulin terminator (Fig. 5a). *NAT* from the *Actinobacteria* species *S. noursei* is naturally high in G+C content (Krügel et al. 1993). Consistent with the results obtained with the *URA* genes of *R. graminis* WP1, this vector was successfully used to transform both strains LS11 and WP1 (Fig. 5b, c), but failed in transforming *R. slooffiae*. For Southern blot analysis, genomic DNA from the wild-type strains LS11 and WP1 and from nourseothricin-resistant transformants was digested with *Kpn*I, which cuts twice in the T-DNA and generates an 868-bp fragment (Fig. 5a). As expected, this fragment was detected in all transformants of LS11 (Fig. 5b) and in seven out of nine transformants of WP1 (Fig. 5c). In the two remaining transformants of WP1, probably a deletion at the right border of the T-DNA occurred during integration to remove a *Kpn*I site.

Factors influencing targeted gene replacement of the *CAR2* gene in *Sporobolomyces*

Targeted gene replacement is a powerful tool to assess the function of a specific gene in the biology of an organism. Previously, the *LEU1* gene of *Sporobolomyces* IAM 13481 was disrupted by biolistic delivery of a DNA fragment containing 1.5 and 1.0 kb of DNA specific to the gene placed on either side of the *URA3* gene used as the selectable marker (Ianiri et al. 2011). However, in that study, just one transformant was obtained, and it was a *leu1* replacement strain. Thus, the efficiency of homologous recombination in relation to DNA length and DNA transformation method was unknown.

The *CAR2* gene was chosen for gene replacement in *Sporobolomyces* sp. *CAR2* encodes a bifunctional protein with two domains, catalyzing phytoene synthase and lycopene cyclase as the first and third steps in the biosynthesis of β-carotene and other carotenoid molecules (Klassen 2010). These pigments give the colonies of the red yeast fungi their characteristic pink, orange, or red color. The rationale for using this gene was the ability to screen for strains with mutations in *CAR2* based upon their white color due to loss of carotenoid pigment production.

Three constructs were generated. These had 1,002 bp upstream and 1,001 bp downstream, 508 bp upstream and 498 bp downstream, or 255 bp upstream and 248 bp downstream of homologous sequence flanking the *URA5* gene (Fig. 6a). The constructs were cloned into plasmid pCR2.1 TOPO, and clones without errors identified by DNA sequencing. Each was excised with *Eco*RI and subcloned into the *Eco*RI site of plasmid pPZP-201BK enabling their transformation into a *Sporobolomyces ura5* mutant with *Agrobacterium*.

The use of two transformation methods, i.e., biolistic and *Agrobacterium* delivery of the DNA, and constructs with different flanking region sizes compared the efficacy of the methods on this organism as measured by the frequency of isolation of white colonies (Fig. 6b). The number of transformed cells is consistently higher with *Agrobacterium* than with biolistics. For the *Agrobacterium* transformants, from the construct with 0.5-kb flanking regions, only 22 white colonies grew on a total of >2,700 colonies. Moreover, from the construct with 0.25-kb flanking regions, only one white colony grew on a total of >5,800 colonies screened, and it was unstable in phenotype. The highest frequencies were achieved with the constructs carrying the 1-kb flanking regions. The percentage of white colonies versus the total number of colonies was 6 %. Similarly, for transformants obtained with biolistic transformation white colonies occurred in 14.5 % of cases. White transformants that were stable in phenotype were further characterized by PCR and Southern blot analysis, confirming that these are gene replacement strains (Fig. 6c, d).

Although both methods of transformation worked for targeted gene replacement, many of the colonies obtained on selective medium that were initially white were unstable or not complete loss of function. These produced colonies with a half pigmented–half white pattern or were pale pink (Fig 6b). PCR and Southern blotting showed that these were not gene disruption strains since the native *CAR2* gene was still present (Fig. 6c, d).

Discussion

In this study, we address the challenges in transforming different members of the *Pucciniomycotina*. The difficulties

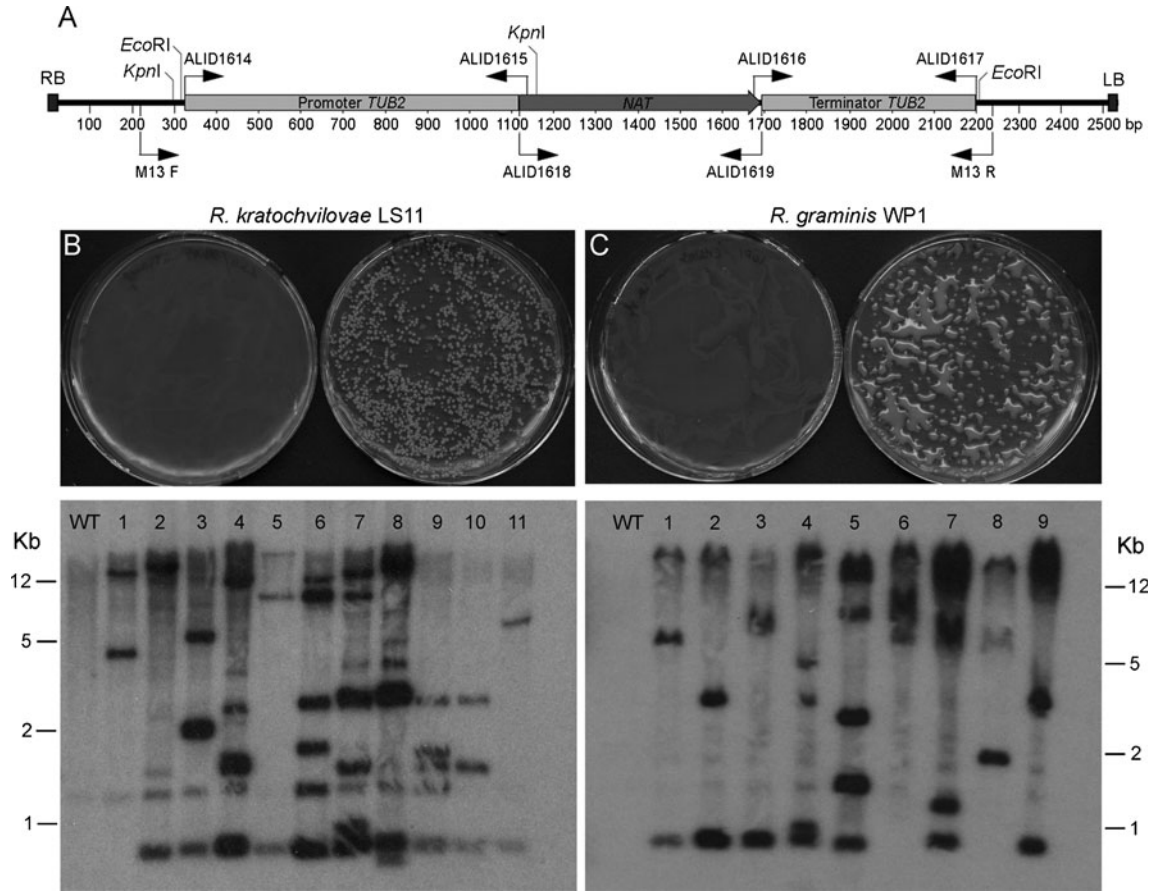

Fig. 5 Development of a new selectable marker for transformation of *R. kratochvilovae* and *R. graminis*. **a** Map of the P_{TUB2}-*NAT*-T_{TUB2} construct in the T-DNA. **b**, **c** Transformed plates of *R. kratochvilovae* strain LS11 (**b**) and *R. graminis* strain WP1 (**c**) on YPD+nourseothricin. The *left plates* are the untransformed controls. Southern blots of *R. kratochvilovae* and *R. graminis* wild-type (*WT*) and *Agrobacterium* T-DNA transformants. Genomic DNA was digested with *Kpn*I, which cuts twice in the T-DNA and generates an 868-bp fragment that is detected with the probe that spans the entire *NAT* construct

in this technique have meant that, to date, just three reports of transformation exist for the red yeasts within this subphylum (Ianiri et al. 2011; Liu et al. 2012; Tully and Gilbert 1985) and an unreported number of failures. In our preliminary studies, multiple attempts of transformation were carried out for *R. kratochvilovae* strain LS11. Negative results were obtained using the genes or their regulatory regions of the *Agaricomycotina* species *C. neoformans* and *Ustilagomycotina* species *Ustilago maydis* (Idnurm et al. 2004; Marchand et al. 2007; Walton et al. 2005). Surprisingly, *R. kratochvilovae* strain LS11 could not even be transformed with genes from *Sporobolomyces* sp. strain IAM 13481 that is also a member of the *Pucciniomycotina*. On the other hand, plasmids generated from genes cloned from strain IAM 13481 (in subclass *Microbotryomycetes*) functioned as selectable markers for transforming *R. slooffiae* (which is in subclass *Cystobasidiomycetes*). It was thus peculiar that the same plasmids were unable to transform *R. kratochvilovae*, and also *R. graminis*, that are classified within the same order (*Sporidiobolales*).

Our results indicate that at least two factors restrict transformation success in *Pucciniomycotina* species. The first is the DNA itself, and the second is the recipient species. The *URA3* and *URA5* genes of *R. graminis* WP1 could transform three strains: *R. graminis* WP1, *R. kratochvilovae* LS11, and *Sporobolomyces* sp. IAM 13481. Conversely, the *URA3* and *URA5* genes from *Sporobolomyces* sp. could not transform the *R. graminis* or *R. kratochvilovae* strains. We hypothesize that this is a reflection of G+C content for these genes. The promoter regions are sufficient to drive expression in a species (i.e., *R. slooffiae*) in another subclass and *R. graminis* WP1 is intron-rich like *Sporobolomyces*, excluding transcription or splicing as factors. However, the G+C content is dramatically different between these organisms, at 53.8 % for *Sporobolomyces* sp. IAM 13481 and 67.8 % for *R. graminis* WP1. The G+C content of strain LS11 is unknown. The G+C contents reported for *R. kratochvilovae* strains range from 64.7 to 71.3 % (Hamamoto et al. 1986; Sampaio et al. 2001). Moreover, we cloned and sequenced part of the *URA3* gene and surrounding regions from strain

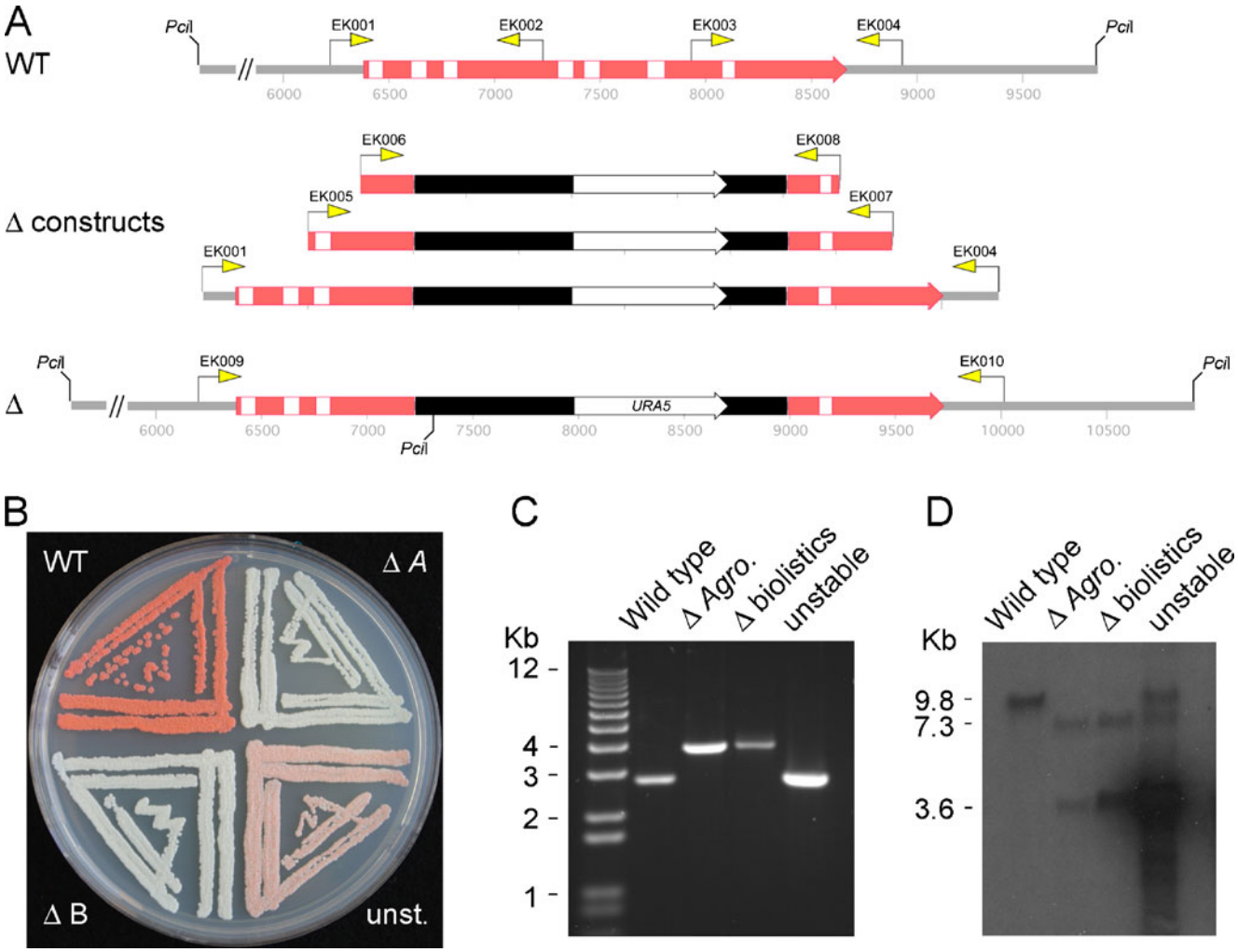

Fig. 6 Disruption of the *CAR2* gene of *Sporobolomyces* sp. **a** Diagrams of the native locus of *CAR2*, disruption cassettes, and situation after gene replacement. *CAR2* exons are in *orange*. The URA5 ORF is the *white arrow*, with surrounding DNA in *black*. Primers used for creating the constructs are drawn as *arrows*. **b** Pigmentation phenotypes of wild type (*WT*), two *car2* replacement strains via *Agrobacterium* (*Δ A*) and biolistic (*Δ* B) transformations, and unstable strain EKS4 (*unst.*) growing on YNB medium. **c** PCR analysis with primers EK009–EK010 of wild type, two gene disruption strains from biolistic and *Agrobacterium* delivery of the DNA, and one transformed strain showing a silencing phenotype. **d** Southern blot of DNA isolated from the four strains. DNA was digested with *Pci*I and probed with the wild-type *CAR2* gene amplified with primers EK001–EK004

LS11: This was 64.9 % G+C over 3.2 kb of sequence (unpublished data). Thus, it is reasonable to hypothesize that *R. kratochvilovae* strain LS11 also has a high G+C content genome.

The second factor controlling transformation is the species. The *Sporobolomyces* strain accepted DNA that was high in G+C content (i.e., from *R. graminis*), but *R. graminis* could not use the *Sporobolomyces* genes. Thus, the G+C content by itself is not a restriction for transformation success in all species, but is important within the overall genome context of specific strains or lineages within the *Pucciniomycotina*.

Our findings corroborate those made recently on *R. toruloides* (Liu et al. 2012). Transformation was initially unsuccessful for these authors. Liu et al. observed a skew in C nucleotides in the wobble codon position of the *GPD1* gene encoding glyceraldehyde phosphate dehydrogenase and modified two genes to account for this bias. Use of the *GPD1* promoter and a codon-optimized version of the gene encoding hygromycin phosphotransferase enabled successful transformation. Similarly, no expression of the green fluorescent protein from the standard coding DNA was achieved, whereas it was with a codon-optimized version.

We compared the codon usage in the two *URA* genes in both species from which they were cloned. For *R. graminis* WP1, C is used in position 3 57.8 % of the time, while G is used 35.4 %. In contrast, for *Sporobolomyces* sp. IAM 13481, C is used 36.2 % and G is used 26.7 %. The nourseothricin acetyltransferase gene of *S. noursei* that functions in the red yeasts has C used 56.8 % and G

38.0 %. Thus, in the *Pucciniomycotina*, whether the limitation in transformation and expression is a reflection of codon usage or G+C content, or both, remains to be determined by experiments designed specifically to address these two criteria.

Targeted gene disruption is a vital tool, available by transformation and homologous recombination, to replace DNA regions with selectable markers. The efficiency of sizes of flanking regions was tested in *Sporobolomyces* sp. IAM 13481 for targeted gene replacement vs. ectopic integrations. Accurate gene replacements were obtained with ~500 or 1 kb of *CAR2* DNA regions on either side of the *URA5* selectable marker, whereas none were obtained with ~250 bp of homologous sequence. Of note, the efficiency of gene disruption may be reduced by the targeting of the *URA5* gene to its endogenous locus (Fig. S1), as well, in the case of *Agrobacterium*, delivery of the DNA leading to a high proportion of ectopic integration.

Both the biolistic and *Agrobacterium* methods were effective at gene replacement in *Sporobolomyces* sp. *Agrobacterium* has the advantage of not requiring specialized equipment, unlike the use of a biolistic delivery system. Ascomycete species are amendable to gene replacement by *Agrobacterium* transformation, but this is not a universal feature of all fungi. For example, the basidiomycete *C. neoformans* does not integrate T-DNA by homologous recombination (McClelland et al. 2005). The disruption of *CAR2* in *Sporobolomyces* sp. represents the first example using *Agrobacterium* for gene replacement in the *Basidiomycota*.

A surprising outcome of the gene disruption experiments of *CAR2* was the isolation of pale and/or unstable white strains from both transformation approaches. The silencing of transgenes has been reported in other fungi, with parallels between the unstable white transformants of *Sporobolomyces* with the silencing of the *al-1* and *al-3* genes in *Neurospora crassa*, leading to the discovery of "quelling" mediated by RNA interference (Catalanotto et al. 2002; Romano and Macino 1992). RNA silencing is used in basidiomycetes as an experimental system to repress gene expression, including in the *Pucciniomycotina* (Lawrence et al. 2010). There are few reports of inadvertent silencing of transformed genes in basidiomycete species (Wang et al. 2012). While the mechanisms that control silencing in *Sporobolomyces* are currently undefined, this trait may represent a useful property for experimental studies to silence expression of genes in this or other red yeasts species.

For *R. kratochvilovae*, a strain is now available with two selectable markers for transformation. We predict that the recently developed P_{GPD1}-*hpt-3* construct would also function as a third selectable marker to confer resistance to hygromycin (Liu et al. 2012). Strain LS11 is also fertile to the extent of producing filaments and teliospores with a strain of opposite mating type, potentially providing the experimental benefits of Mendelian genetics (Sampaio et al. 2001).

The fungal species that are best studied at the molecular level can have their DNA manipulated with ease. Many other species have never been tested for transformation or are a challenge, thereby limiting the feasibility for experiments. The approaches presented here, i.e., identification of an auxotroph and use of the original gene sequence introduced by a variety of transformation methods, may be more widely applicable to these species.

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