Selection markers for transformation of the sequenced reference monokaryon Okayama 7/#130 and homokaryon AmutBmut of Coprinopsis cinerea

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Abstract

Background: Two reference strains have been sequenced from the mushroom Coprinopsis cinerea, monokaryon Okayama 7/#130 (OK130) and the self-compatible homokaryon AmutBmut. An adenine-auxotrophy in OK130 (ade8-1) and a para-aminobenzoic acid (PABA)-auxotrophy in AmutBmut (pab1-1) offer selection markers for transformations. Of these two strains, homokaryon AmutBmut had been transformed before to PABA-prototrophy and with the bacterial hygromycin resistance marker hph, respectively.

Results: Gene ade8 encodes a bifunctional enzyme with an N-terminal glycinamide ribonucleotide synthase (GARS) and a C-terminal aminooimidazole ribonucleotide synthase (AIRS) domain required for steps 2 and 5 in the de novo biosynthesis of purines, respectively. In OK130, a missense mutation in ade8-1 rendered residue N231 for ribose recognition by the A loop of the GARS domain into D231. The new ade8+ vector pCcAde8 complements the auxotrophy of OK130 in transformations. Transformation rates with pCcAde8 in single-vector and co-transformations with ade8+ selection were similarly high, unlike for trp1+ plasmids which exhibit suicidal feedback-effects in single-vector transformations with complementation of tryptophan synthase defects. As various other plasmids, unselected pCcAde8 helped in co-transformations of trp1 strains with a trp1+ selection vector to overcome suicidal effects by transferred trp1+. Co-transformation rates of pCcAde8 in OK130 under adenine selection with nuclear integration of unselected DNA were as high as 80% of clones. Co-transformation rates of expressed genes reached 26–42% for various laccase genes and up to 67% with lcc9 silencing vectors. The bacterial gene hph can also be used as another, albeit less efficient, selection marker for OK130 transformants, but with similarly high co-transformation rates. We further show that the pab1-1 defect in AmutBmut is due to a missense mutation which changed the conserved PIKGT motif for chorismate binding in the C-terminal PabB domain to PIEGT in the mutated 4-amino-4-deoxychorismate synthase.

Conclusions: ade8-1 and pab1-1 auxotrophic defects in C. cinerea reference strains OK130 and AmutBmut for complementation in transformation are described. pCcAde8 is a new transformation vector useful for selection in single and co-transformations of the sequenced monokaryon OK130 which was transformed for the first time. The bacterial gene hph can also be used as an additional selection marker in OK130, making in combination with ade8+ successive rounds of transformation possible.
Keywords: Adenine auxotrophy, De novo purine biosynthesis, Transformation vector, Para-aminobenzoic acid-auxotrophy, Tryptophan auxotrophy, Hygromycin B resistance, Basidiomycete

Background

Coprinopsis cinerea is a well-known model fungus for studying biological processes in Agaricomycetes. As early as in 1987 and for one of the first fungi of all, protoplast transformation of C. cinerea was successfully established by Binninger et al. [1]. For DNA transformation, protoplasts are usually generated from easy to regenerate single-celled haploid aerial mitotic spores (oidia) and are commonly treated in PEG 4000/CaCl₂-mediated cold-shock transformation with ca. 1 µg plasmid DNA. The protocol is highly efficient in best cases up to several hundreds of transformants per µg DNA [1–4]. Up till today, the protoplasting and transformation protocol of Binninger et al. [1] has not much been changed in the principles. However, the method was later more simplified and specified in details as compared to the original description [2, 3]. Comprehensive troubleshooting tips have been provided to identify and correct possible subconscious while crucial small handling errors in order to ensure reliable transformation [4].

One reason for the very high transformation rates of C. cinerea is that mostly homologous selection markers are used for the complementation of auxotrophies. The bifunctional tryptophan synthase gene trp1+ cloned in the pUC9-based 9.8 kb-sized plasmid pCc1001 [1] is so far most often applied in transformation. More recently, the shorter pBluescript KSⅠ-based trp1+-plasmid pBD5 (7 kb) with higher copy number in Escherichia coli and the trp1+-yeast-shuttle vector pTPtrp1 (9.9 kb) have been established [5]. The two gene halves of trp1+, i.e. trpAP for the Trp1 A domain responsible for the aldo-cleavage of indole-3-glycerol-phosphate (IGP) into indole and trpBP for the Trp1 B domain for the subsequent pyridoxal phosphate cofactor-dependent conversion of indole with serine to tryptophan [5], have been functionally separated into individual yeast-shuttle vectors pYA-dom (8.3 kb) and pYBdom (8.7 kb) to allow successive rounds of transformation into C. cinerea trp1.1,1.6 double mutant strains with first trp1.6 (trpB) and then trp1.1 (trpA) complementation [6].

Two other genes from the tryptophan biosynthesis pathway cloned in vectors for transformation of suitable C. cinerea mutant strains are trp2+ [2] for a trifunctional enzyme with glutamine amidotransferase (GATase; anthranilate synthase component II which releases ammonia from glutamine), phosphoribosylanthranilate isomerase (PRAI) and indol-3-glycerol-phosphate synthase (IGPS) activities [5], and the gene trp3+ [7, 8] for anthranilate synthase component I which uses ammonia and chorismate to produce anthranilate, 2-aminobenzoic acid [5]. Cloned is also a positively selectable mutant gene trp3ar for a dominant 5-fluoroorindole-resistant anthranilate synthase component 1 mutant [9]. pab1+ vectors [3, 10] have been provided for complementation of auxotrophies in para-aminobenzoic acid (PABA) synthesis caused by defects in the bifunctional enzyme Pab1. Conventionally, this fungal enzyme as PABA synthase but more precisely, it is a 4-amino-4-deoxy-chorismate (ADC) synthase. The enzyme consists of an N-terminal PabA domain (37% identity, 53% similarity to E. coli PabA; Fig. 1a) and a C-terminal PabB domain (30% identity, 49% similarity to E. coli PabB; Fig. 1a). PabA presents PABA synthase component II (or better called ADC synthase component II) and has a PabB-dependent GATase function. The PabB domain as PABA synthase component 1 (or more precisely ADC synthase component 1) will aminate chorismate in order to yield ADC as the direct precursor of PABA to be formed by an ADC lyase (PabC) [11, 12]. Regarding further functional C. cinerea selection markers, a cosmid is mentioned in a conference proceeding that could complement an uncharacterized ade8 defect of C. cinerea in transformation [13].

Selection for dominant resistances is another strategy to obtain transformants. A carboxin resistance selection marker (sdi1<sup>R</sup>) has been generated by site-specific mutation of the native C. cinerea sdi1 gene for the iron-sulphur protein subunit (subunit SdhB) of the mitochondrial succinate dehydrogenase (SDH) complex [14]. Flutolanil and carboxin resistance is moreover mediated through a spontaneous point mutation by an allele of the sdhC gene for the SdhC cytochrome b<sub>560</sub> subunit of the SDH complex [15]. The sdi1<sup>R</sup> allele has been cloned behind the heterologous constitutive gpdII promoter of Agaricus bisporus [14] which is highly active in C. cinerea [16]. Transformation rates of such optimized sdi1<sup>R</sup> vectors were then high with >100 transformants/µg plasmid DNA [14]. Transformation rates with the sdhC mutant allele under natural regulatory sequences in contrast were low with 1.0 to 4.8 transformants/10<sup>5</sup> viable protoplasts [15].

As functional bacterial resistance genes in C. cinerea, vectors with the E. coli hygromycin B phosphotransferase gene hph [14, 17] and the Streptodoloteichus hindustanus gene ble for a phleomycin binding protein are available [14]. Insertion of a functional intron after the second
codon of the ble gene was essential for successful expression of the gene in C. cinerea behind the A. bisporus gpdII promoter [14]. Regarding expression of hph, presence of an intron was not crucial. However, the entire coding region of hph is required to be inserted behind an active promoter in C. cinerea (native tub1 promoter or heterologous A. bisporus gpdII promoter) [14, 17]. The best-known hph-vector pAN7-1 from transformation in filamentous ascomycetes for example lacks the first two codons for two lysine residues and by this reason did not function in C. cinerea transformation [14] unlike, although at low frequency (1 to 5 transformants/μg plasmid DNA), in the basidiomycetes Hebeloma cylinderum and Crinipellis perniciosa [19].

The obvious advantage of usage of dominant resistance markers for selection is that transformation becomes independent of any auxotrophies that are needed to be generated. Though, using dominant resistance markers for C. cinerea somewhat complicates the transformation procedure. Protoplasts are spread onto regeneration agar but for suppression of unwanted background growth, it requires an extra regeneration agar overlay with antibiotics for selection for positive transformants to grow through this overlay [14, 16]. Handling of complementation of auxotrophies in transformation in contrast is much easier by just plating and then incubating protoplasts on regeneration agar [2–4]. However, through complementation of available auxotrophies and selections for dominant resistance markers, extra rounds of successive transformations in a same background become possible. Such makes strains more versatile for repeated genetic manipulations.

So far, the genomes of two distinct C. cinerea strains, the monokaryon Okayama 7/#130 (short OK130) and the self-fertile homokaryon AmutBmut, have been sequenced by the Broad Institute (Boston, MA) and the JGI (Joint Genome Institute, Walnut Creek, CA), respectively [20, 21]. AmutBmut carries a pab1-1 mutation and is easily be transformed by pab1+ vectors, a feature which is very useful in studying dikaryon-specific growth behavior and fruiting body development in this self-fertile strain, independently of a second genome [22–24]. On the other hand, to the best of our knowledge, strain OK130 with the first C. cinerea reference genome established had not yet been transformed before. This reference monokaryon carries an ade8-1 mutation [8] which we used here in transformation for selection by complementation. Missense mutations in the defective alleles pab1-1 and ade8-1 were identified in this study. In addition, transformants of OK130 were obtained with the dominant bacterial hygromycin resistance selection marker hph.

**Results and discussion**

**Genes pab1 and ade8 in C. cinerea**

Classical mapping of C. cinerea localized gene pab1 0.5 cM upstream and gene ade8 1.3 cM downstream to the bipartite A mating type locus (consisting of Aα and Aβ) on linkage group I [25, 26]. The ca. 20 kb-long A43 mating type allele with all its homeodomain transcription factor genes locates at position Chr_1:2,666,138–2,647,809 in the sequenced OK130 genome [20, 27], pab1 is found at location Chr_1:2,699,078–2,701,362, 32.94 kb apart from the 3’ end of the closest A43α gene a1-1 [20, 27]. pab1+ in OK130 (Broad model CC1G_01849T0) distinguishes from the pab1-1 allele in AmutBmut (JGI ID 414607) by a point mutation in codon 546, with a change from AAG to GAG. This missense mutation resulted in a K546E exchange in the PabB formation in PabA are marked with red, green and blue symbols *, respectively. Other residues affecting enzymatic activities and bonding to PabB are marked with grey squares. The position of a stabilizing residue stretch called oxanion hole is underlaid in light blue, a sequence stretch for chorismate signal transfer in olive [29, 30, 75]. Red letters in PabB mark helical regions, blue letters β-sheets. The conserved PIKGT motif, sequences for interaction with PabA, for signal transfer of chorismate binding, and of a binding pocket for tryptophan implicated in structural stabilization are underlaid in olive, bright yellow, grey and light blue, respectively. The residue K in the PIKGT motif which is mutated in C. cinerea AmutBmut (K546E) is marked in red. Symbols * in red and black mark (predicted) active site residues and Mg2++-binding residues in two chorismate-interacting helices, respectively. Triangles in black indicate residues that contact the bound tryptophan and grey squares further residues where mutations affect functionality [28–31, 76]. b Red, blue, green and magenta letters mark the N, B, A, and C domains of PurD. The positions of the P-loop and the flexible A and B loops in PurD [56] are underlaid in light blue, olive and orange, respectively. Symbols * in black, red, and blue mark residues that recognize the adenine base, ribose and phosphate of the nucleotide, whereas grey squares indicate residues interacting with the ligand PRA [56, 57]. The residue N in the A loop which is mutated in C. cinerea OK130 (N231D) is marked in red. In PurM, symbols * mark (predicted) nucleotide binding residues and triangles (in grey predicted) binding sites of the substrate N-formylglycinamidine ribonucleotide (FGAM) [58].
The recombination rate between pab1 and Aα calculates as \( \geq 66 \text{ kb/cM} \) (\( \geq 70-75 \text{ kb/cM} \) with the whole pab1 gene sequence included \([8, 32]\)). Other studies estimated the average recombination frequency over the C. cinerea genome higher as 27.9 kb/cM \([33]\) and 33 kb/cM \([20]\), respectively. With the same kb/map unit relations, ade8 should then locate about 40 to 100 kb downstream of Aβ. A gene for a bifunctional purine biosynthetic
protein (CC1G_01782T0; Table 1) was found in the
OK130 genome at location Chr_1:2,548,109–2,550,858,
97 kb downstream to the closest A43β gene d1-1 [20, 27],
with a possible recombination rate of 74.6 kb/cM using
1.3 cM for calculation.

Many mutations leading to adenine-auxotrophies
belong directly to the de novo purine biosynthesis path-
way [34–36]. Other indirect mutations include defects
in tetrahydrofolate (THF) cofactor formation, further
folate metabolism and THF-mediated C1-metabolism,
as well as defects in cross-pathway regulation of de novo
purine biosynthesis and syntheses of amino acids (histi-
dine, methionine) mediated by feedback control of cer-
tain metabolites [5’-phosphoribosyl-5-monophosphate
(AICAR)] or shared transcriptional regulators [35, 37–
48]. We screened the OK130 genome for such genes,
using known E. coli and Saccharomyces cerevisiae pro-
teins in tblastn searches. Spread over 7 chromosomes,
genes for all enzymatic functions for de novo purine bio-
synthesis and for other mentioned functions were found
(Table 1). Previously, twelve different ade complementa-
tion groups have been described in C. cinerea, two more
mutants that react to adenine and histidine (ad/his1 and
ad/his2) and another that reacts alternatively to adenine
or methionine (ad/met) [49, 50]. Ten of these genes have
been mapped onto 7 linkage groups [50–52]. Though,
in our analysis only four to possibly seven genes (ade2,
ade8, ade1, ade5, and possibly ade4, ade9, and ade12)
from only four linkage groups could be assigned to spe-
cific positions on sequenced chromosomes (Table 1),
using as additional information their clearly defined
biochemical reactions (cases ade1, ade5 [49]) or approx-
imate positions in the de novo purine biosynthesis path-
way (ade2, ade3, ade4 and ade8 all act prior to imidazole
ring closure [49]) and/or their linkages (ade2, ade3, ade5,
ade8, ade9 and ade12) to other unquestionably identifi-
able gene functions on the classical C. cinerea map ([33,
50–52]; see footnote of Table 1). However, no other con-
vincing candidate for gene ade8 were found in appropri-
ate distance to the A locus on chromosome 1 (Table 1).

The protein encoded by the gene at Chr_1:2,548,109–
2,550,858 has been annotated in GenBank (EUA92737.2)
as Ade1 [Coprinopsis cinerea Okayama 7/#130] which
conflicts the traditional C. cinerea gene nomenclature.
C. cinerea gene ade1 resides on linkage group IV of the
fungus [51, 52] which corresponds to chromosome 5 in
the OK130 genome assembled by comparative sequence
length ([20], Table 1). Moreover, Ade1 of C. cinerea
had been shown in the de novo purine biosynthesis
to function in the 6th step directly after 5-aminomi-
dazole ribonucleotide (AIR) ring closure as phospho-
ribosylaminomimidazole carboxylase in the formation of
5-amino-4-imidazolcarboxamid ribonucleotide (CAIR)
([49], Table 1).

The gene at location Chr_1:2,548,109–2,550,858 has
homologs in other fungi that, by historical naming of
adenine-auxotrophic mutants, are variably known as
ade1 such as in Phanerochaete chrysosporium, ade5 in
Schizopyllum commune, ade2 in Neurospora crassa,
ade5,7 in S. cerevisiae and pur2, pur2,5 and pur2,7 in
Yarrowia lipolytica, Ogataea angusta and Scheffersomy-
ces stipitis, respectively (Fig. 2). Gene ade5+ of S. com-
mune can complement ade1 defects of P. chrysosporium
like the homologous native ade1+ gene and it can com-
plement ade2 defects of the ascomycete N. crassa [53,
54]. All mentioned fungal genes encode bifunctional
enzymes for the de novo biosynthesis of purines, with
an N-terminal glycaminide ribonucleotide synthase
(GARS) domain and a C-terminal aminomimidazole rib-
onucleotide synthase (AIRS) domain (Fig. 1b; Table 1)
which act in the 2nd and the ring-closing 5th step in
de novo purine biosynthesis, respectively [34–36], ade5
of S. commune and ade8 of C. cinerea are conserved in
chromosomal location relative to the position of Aβ,
similar as their pab1 genes are relative to Acr [8, 32, 55].
The gene for a bifunctional GARS-AIRS enzyme identi-
ﬁed here on C. cinerea chromosome I with good likeli-
hood thus presents its ade8 gene.

The N-terminal halves of the fungal bifunctional
GARS-AIRS enzymes correspond to bacterial PurD
enzymes (49% identity, 67% similarity between the C.
cinerea enzyme and E. coli PurD; Fig. 1b) which are gly-
caminide ribonucleotide (GAR) synthases represented
in structure e.g. by the crystalized E. coli PurD protein
(1GSO_A). PurD catalyzes the 2nd step of the de novo
purine biosynthetic pathway, the conversion of phos-
phoribosylamine (PRA), glycine, and ATP to GAR, ADP
(adenosine diphosphate), and phosphate (Pi) ([35, 56,
57], Table 1). The C-terminal halves of the fungal bifunc-
tional GARS-AIRS enzymes are homologous to bacterial
PurM enzymes (55% identity, 67% similarity of the
C. cinerea enzyme to E. coli PurM; Fig. 1b). PurM rep-
resented in structure of the bacterial 1CLI_A is a phospho-
ribosylformylglycinamidine cyclo-ligase that catalyzes
the conversion of formylglycinamidine ribonucleotide (FGAM)
and ATP to AIR, ADP, and Pi, in the 5th step in de novo
purine biosynthesis ([35, 58], Table 1).

The folded bacterial GARSs consist of the three
domains N, A, and C forming the central core of the
enzym and, connected to them by ﬂexible hinges, the
outward-extended domain B [56]. Substrate PRA is
recognized by speciﬁc amino acids in the N, A, and C
domains. The A domain further confers the binding site
for the ligand glycine ([56, 57], Fig. 1b). GARSs are mem-
bers of the ATP-grasp superfamily of enzymes with an
Table 1 Identification of gene functions in de novo purine biosynthesis, formation of folates and THF-mediated one-carbon metabolism in *C. cinerea* OK130

| Steps in de novo purine synthesis and interlinked processes | Enzyme Name, GenBank accession number | *E. coli* | *S. cerevisiae* | Broad model, classic name | Chromosomal location in OK130* |
|---|---|---|---|---|---|
| PRPP to PRA | Glutamine amidophosphoribosyltransferase (GPAT) | PurF, CAA30971 | Ade4, P0406 | CC1G_01222T0, likely Ade2 | Chr_2:1,228,139–1,230,457 |
| PRA to GAR | Glycinamide ribonucleotide synthase (GARS) | PurD, CAA36213 | N-terminal domain of bifunctional Ade5,7, NP_011280 | CC1G_01782T0, N-terminal domain of bifunctional Ade8 | Chr_1:2,548,109–2,550,858 |
| GAR to FGAR | Phosphoribosylglycinamide formyltransferase (GART) | PurN, P08179 | Ade8, NP_010696 | CC1G_04335T0, potentially Ade4 | Chr_1:715,850–716,603 |
| FGAR to FGAM | [Bacterial alternative: formate-dependent phosphoribosylglycinamide formyltransferase] | PurT, NP_416363 | – | – | – |
| FGAM to AIR | Aminomimidazole ribonucleotide synthase (AIRS) | PurM, THH44093 | C-terminal domain of bifunctional Ade5,7, NP_011280 | CC1G_01782T0, C-terminal domain of bifunctional Ade8 | Chr_1:2,548,109–2,550,858 |
| AIR to CAIR | 5-(Carboxyamino)imidazole ribonucleotide synthase + 5-(carboxyamino)imidazole ribonucleotide mutase (AIR carboxylase) | PurK + PurE, NP_415055, NP_415056 | Fused Ade2, P21264 | CC1G_11091T0, fused Ade1 | Chr_5:473,822–471,864 |
| CAIR to SAICAR | Phosphoribosylaminimidazole-succinocarboxamide synthase (SAICARS) | PurC, NP_416971 | Ade1, NP_009409 | CC1G_05887T0 | Chr_7:2,536,570–2,535,540 |
| SAICAR to AICAR | Adenylosuccinate lyase | Bifunctional PurB, THI73349 | Bifunctional Ade13, NP_013463 | CC1G_08733T0, bifunctional Ade5 | Chr_10:936,450–934,462 |
| AICAR to FAICAR | AKAR transformylase | Bifunctional PurH, NP_418434 | Bifunctional Ade16, NP_009409 or isoenzyme Ade17, NP_013839 | CC1G_08365T0 | Chr_7:2,467,163–2,464,958 |
| FAICAR to IMP | IMP cyclohydrolase | Bifunctional PurH, THI73349 | Bifunctional Ade13, NP_013463 | CC1G_08733T0, bifunctional Ade5 | Chr_10:936,450–934,462 |
| IMP to SAMP | Adenylosuccinate synthase | PurA, NP_418598 | Ade12, NP_014179 | CC1G_10027T0 | Chr_2:407,487–405,875 |
| SAMP to AMP | Adenylosuccinate lyase | Bifunctional PurB, THI73349 | Bifunctional Ade13, NP_013463 | CC1G_08733T0, bifunctional Ade5 | Chr_10:936,450–934,462 |
| GTP to DHNTP | GTP cyclohydrolase | FoIe, NP_416658 | FoI2, PS1601 | CC1G_14672T0 | Chr_5:2,160,832–2,161,846 |
| DHNTP and PABA to 7,8-DHP to DHF | Trifunctional dihydropterate synthase/dihydrodroxy- methylypterin pyrophosphokinase/dihydroneopterin aldolase | FoIb + FoIe + FoIP, NP_417530, 3IPQ_A, NP_417644 | FoI1, Foi1, NP_014143 | CC1G_15556T0, fused | Chr_6:783,810–781,706 |
| DHP to DHF | Dihydrofolate synthase/folinylpolyglutamate synthase | FoIC, P08192 | FoI3, NP_013831 | CC1G_00421T0 | Chr_2:3,461,586–3,463,459 |
|  |  |  | Mer7, NP_014884 | CC1G_04580T0 | Chr_5:1,857,755–1,855,944 |
Table 1 (continued)

Steps in de novo purine synthesis and interlinked processes

| Substrate—product | Enzymatic function | Enzyme | Name, GenBank accession number | C. cinereok OK130 | Chromosomal location in OK130* |
|-------------------|-------------------|--------|-------------------------------|------------------|--------------------------------|
| DHF to THF        | Dihydrofolate reductase | FoIA, 4GH8_A | Dfr1, P07807                   | CC1G_012670T0, potentially Ade9 | Chr_1:1,571,610–1,572,294 |
| 5,10-Methylene-THF to 10-formyl-THF | NADP-dependent methylene tetrahydrofolate cyclohydrolase, methylene tetrahydrofolate dehydrogenase | Bifunctional FolD, SO22_D | N-terminal domain of trifunctional Ade3, NP_011720 | CC1G_013910T0, N-terminal domain of trifunctional enzyme | Chr_2:1,522,272–1,525,659 |
| 10-Formyl-THF to formate and THF | Formyltetrahydrofolate deformylase | PurU, THH46545 | – | – | – |
| 3-PHP to phosphoserine | O-Phospho-L-serine : 2-oxoglutarate aminotransferase | SerC, THK65673 | Ade9 = Ser1, NP_014827 | CC1G_11497T0 | Chr_2:2,589,569–2,588,293 |
| L-serine to glycine + THF to 5,10-CH2-THF | Glycine/serine hydroxymethyltransferase | SHMT, 3G6M_A | SHM2, NP_013159 | CC1G_10328T0 | Chr_6:1,087,903–1,089,686 |

*Assigning classical linkage groups [50–52] and adenine auxotrophies [49, 50] to the new chromosome classification in OK130 sorted after sequence length [20]: Chromosome 1 = classical linkage group I with A mating type locus, ade8 (with function prior to AIR ring closure [49]) and, 9 cM away from the A mating type locus, ade9 [51, 52] which appears to function as a regulatory enzyme rather than within the direct de novo pathway of purine biosynthesis [49] and might therefore be a dihydrofolate reductase gene for THF production located 752 kb downstream to A43β (recombination rate is then 83 kb/cM) with potential cross-pathway effects between de novo purine biosynthesis and THF-mediated C1, histidine and methionine metabolisms [42, 46]. A gene with potential GART function (step 3 in de novo purine biosynthesis) as one candidate for the unmapped gene ade4 functioning in the pathway prior to imidazole ring closure [49, 52] is present 1932 kb downstream to A43β, closer to the telomere. Chromosome 2 = classical linkage group III with trp1, trp3, ade2 (with function prior to AIR ring closure [49]) and ade12 (0.2 cM apart from ade2 [52]) – an estimated distance of 5.6 to 6.6 kb [20, 33] which could point to CC1G_01221T0 for S-adenosylmethionine synthase at position Chr_2:1,226,385–1,227,850 or CC1G_01223T0 for diadenosine polyphosphate hydrolase and related proteins of the histidine triad (HIT) family at position Chr_2:1,231,397–1,230,670 as potential candidates for ade12). Chromosome 3 = classical linkage group G with trp3 [51, 52], pcc1 [33], and, 16 cM distal to trp2 [51, 52], ade3 unidentified here with a function prior to AIR ring closure [49]. Chromosome 5 = classical linkage group IV with ade1 with CARI synthase function [49]. Chromosome 6 (with a gene for a FGAMS function as another ade6 candidate) and chromosome 7 = classical linkage groups unclear. Chromosome 10 = classical linkage group II with B mating type locus, the bifunctional ade5 with adenylsuccinate lyase function [49], ad/his-1 and ad/his-2 which are likely ade5 alleles with cross-pathway effects on histidine biosynthesis via effects of the regulatory metabolite AICAR [46, 49]. Classical linkage groups V and VI with ade6 and an ad/met locus, respectively [51, 52] = new chromosome numbers unclear.
atypical ATP-binding site (ATP-grasp fold) comprised by the two domains A and B that catch an ATP between them [59]. Accordingly, the A and B domains primarily define the ATP/ADP binding site of GARSs, with distinct residues in domains A and B and also in N contacting the adenine base, ribose and phosphate, respectively ([56, 57], please see Fig. 1b for details). Further, the A domain possesses a flexible specific A loop with a highly conserved unique sequence (DHKRVGDKDTGPNTGGMG in E. coli, see Fig. 1b) which distinguishes GARSs well from all other members of the ATP-grasp superfamily ([56, 57], please see Fig. 1b for details). The E. coli A loop shares 83–89% sequence identity and 94% sequence similarity with the loops in the fungal enzymes analyzed in Fig. 2, with amino acid N231 of wt C. cinerea Ade8=N226 in PurD of E. coli (Fig. 1b). Sequence comparison between the functional ade8+ copy from AmutBmut and the defective ade8-1 allele in OK130 revealed a point mutation that altered codon 231 from AAT into GAT and then, within the flexible A loop in the GARS A domain, the highly conserved amino acid N231 into D231 (Fig. 1b). The D231 mutation in the N-terminal GARS half explains then the former observation that Ade8 acts prior to imidazole ring formation [49] and, more specifically, assigns the loss of the Ade8 function in OK130 to the 2nd step of de novo purine biosynthesis.

The pCcAde8 vector in fungal transformations

The wt genomic sequence with the ade8+ coding region (with 9 exons and 8 introns) and 483 and 569 bp upstream and downstream, respectively were PCR-amplified with chimeric primers Ade8f and Ade8r in order to construct vector pCcAde8 (Fig. 3) by in vivo recombination in yeast with plasmid pRS426 [60]. pCcAde8 was transformed into monokaryon OK130, alone and, using protoplasts from same batches, in parallel co-transformations with other vectors (Table 2). Adenine prototrophic transformants were selected by growth on adenine-free regeneration agar. Diagnosis PCR with amplicon sequencing verified for 25 transformants randomly chosen from group pCcAde8+pYSK-lcc5 (experiment 1 in Table 2,
1st to 4th day of collection) in all cases the presence and function of the ade8⁺ allele.

Transformation rates of OK130 to ade8⁺ prototrophy in single-plasmid and two-plasmid transformations were in ranges of about 40 to 60 clones each (Table 2). Gene ade8⁺ therefore might not confer any significant feedback inhibition on the de novo purine biosynthesis pathway in C. cinerea. On the contrary, the trp1⁺ selection marker of C. cinerea can cause suicidal feedback inhibition on tryptophan biosynthesis with loss of affected clones by a sudden overflow of the amino acid from more expressed trp1⁺ copies [5, 6]. This adverse effect on clone viabilities is greater with the single-plasmid transformation than when using mixtures of two plasmids, because singular plasmids in transformation without competition are likely to integrate into twice as many spontaneous DNA breaks per nucleus [5, 6]. As in our previous work with trp1.1.1.6 monokaryons [5, 6], reduced amounts of tryptophan prototrophs were obtained in only trp1⁺-vector pDB5 transformations of strains FA2222 and PG78 as compared to any co-transformations (Tables 3 and 4). pCcAde8 was newly tested in such co-transformations. Numbers of total transformants under trp1⁺ selection were about 1.5–2.5 times higher in the co-transformations with pCcAde8 than in the single-vector transformation, similar to results of co-transformations with other plasmids (Tables 3 and 4). In co-transformations of monokaryon PG78 with pab1⁺-vector pPAB1-2 for selection for PABA-prototrophy, total transformation rates were slightly higher with pCcAde8 (1.9 × and 1.3x) as compared to other plasmids and in single-plasmid transformation (Table 4). PABA is an intermediate in the biosynthesis of folate [61] which in turn is required in steps of de novo purine biosynthesis for the cofactor THF (Table 1). Co-transforming pab1⁺-vector pPAB1-2 with pCcAde8 might have an initial promoting effect on protoplast regeneration and clone numbers. Typically in transformations of C. cinerea with selection schemes other than adenine, we add adenine sulfate as optional supplement to regeneration agar (50 or 100 mg/l) [3, 4] because this can stimulate protoplast regeneration [advice by late L.A. Casselton kindly given to UK].

Co-transformation of a selectable vector together with one or more other plasmids is an efficient means to introduce and find non-selectable genes in transformed C. cinerea clones [62]. Because we have a deeper interest in laccase functions and applications [16, 63–68], several vectors used here in co-transformations contained either C. cinerea laccase genes for enzyme overexpression or were antisense constructs designed for laccase gene silencing (Tables 2 and 3). Most C. cinerea monokaryons in fungal cultures have some background laccase activities through expression of Lcc1 and Lcc5 and possibly other enzymes, with the exception of the laccase-free strain FA2222 [16, 64, 65]. Co-transformation to laccase production in monokaryon FA2222 can therefore potentially be easily followed up on regeneration agar by enzymatic conversion of the colorless 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) into a blue-greenish product seen as well-stained halos around growing clones [16]. Accordingly, co-transformation rates of strain FA2222 with lcc1 expression vector pYSK7 in this study were 34% and 35%, respectively (Table 3) and were in the range of ratios (25 to 43%) obtained in other C. cinerea co-transformation experiments [5, 6, 16]. Each 20 clones were randomly selected for liquid fermentations from the pBD5 and the pBD5+pYSK7 transformants, respectively. All selected pBD5 transformants showed no enzymatic activity whereas enzymatic activities for the staining pBD5+pYSK7 transformants were between 0.3 U/ml and 3.4 U/ml. Co-transformation rates of strain FA2222 with pCcAde8 with three different laccase overexpression constructs were similar like in the FA2222 co-transformations described above. Co-transformations of monokaryon OK130 led in 26% to 42% of all clones to phenotypically increased enzyme activities, from background laccase activities in OK130 and pCcAde8 control transformants of around 0.1 U/ml to 0.6–3.1 U/ml for lcc1 and 2.0–7.5 U/ml for lcc5 and lcc9 transformants as determined by activity tests in liquid fermentation and further shown in native-PAGE by strongly increased staining activity of those bands which was characteristic for the respective laccase gene used in transformation. Only one clone from single-pCcAde8 transformation produced sizeable amounts of laccase (2.3 U/ml) by over-expression of both Lcc1 and Lcc5 which was probably caused by an unknown mutation in the clone (experiment 1, Table 2).

In experiment 2 in Table 2 performed with lcc9-antisense constructs, co-transformation rates were determined by integrated DNA from 66 randomly selected OK130 clones, through PCR amplification from genomic DNAs of lcc9-antisense fragments linked with A. bisporus gpdI promoter and lcc1 terminator sequences using primers PF and PR (Table 5). Accordingly, 80 and 72% of the obtained clones were co-transformants of both plasmids. Functionality of inserted DNA in lcc9-silencing was then tested in co-cultivation of transformants in SAHX medium according to Pan et al. [65] with the
Fig. 3 Physical map of the yeast-E. coli shuttle vector pCcAde8 with the cloned C. cinerea gene ade8+

Table 2 Transformations of C. cinerea OK130 (ade8-1) with ade8+-vector pCcAde8 alone or, using same batches of protoplasts, in combination with various pYSK7 laccase gene derivatives

| Plasmid(s)                      | ade8+-selected transformants collected on* | Total transformants* |
|---------------------------------|-------------------------------------------|----------------------|
|                                 | 1st day | 2nd day | 3rd day | 4th day |                       |
| Experiment 1: Laccase overexpression |         |         |         |         |                       |
| pCcAde8                         | 17 (1)  | 15      | 7       | 2       | 41                     |
| pCcAde8 + pYSK7                 | 26 (8)  | 20 (13) | 7 (3)   | 7 (1)   | 60 (25)                |
| pCcAde8 + pYSK-lcc5             | 14 (2)  | 27 (8)  | 25 (5)  | 10 (5)  | 76 (20)                |
| pCcAde8 + pYSK-lcc9             | 10 (4)  | 23 (10) | 23 (5)  | 8 (0)   | 64 (19)                |
| Experiment 2: Laccase silencing |         |         |         |         |                       |
| pCcAde8                         | 17      | 20      | 12      | 6       | 55                     |
| pCcAde8 + pYSK-lcc9-antisense-1 | 5 (2)   | 7 (2)   | 12 (6)  | 6 (4)   | 30 (14)                |
| pCcAde8 + pYSK-lcc9-antisense-2 | 2 (1)   | 9 (5)   | 17 (12) | 8 (6)   | 36 (24)                |

*Data in brackets of experiment 1 indicate number of clones with > sixfold increased levels of laccase as detected by activity assay in liquid fermentation and native-PAGE; data in brackets of experiment 2 indicate clones with 2- to 11-fold (2^ΔΔCT) decreases in lcc9 mRNA transcriptional levels as detected by qRT-PCR
fungus Gongronella sp. w5 which induces lcc9 expression in OK130 [65, 67]. Using cDNAs from co-cultivated OK130 transformants and qRT-lcc9-F and qRT-lcc9-R as primers (Table 5), qRT-PCR analysis revealed silencing ratios of lcc9 in 47% and 67% of all transformants for the two lcc9 antisense constructs, respectively.

Table 3 Transformations of C. cinerea FA2222 (trp1.1,1.6) with plasmid pBD5 alone or, using same batches of protoplasts, in combination with other non-directly selectable vectors

| Plasmid(s) | trp1⁺-selected transformants collected on | Total transformants | Ratio of clones |
|------------|------------------------------------------|---------------------|----------------|
|            | 1st day | 2nd day | 3rd day | 4th day | 5th day |                      |                  |
| Experiment 1 |                     |                      |                  |            |            |                  |                  |
| pBD5       | 13      | 8       | 12      | 3       | 2       | 38               | 1.0              |
| pBD5 + pYSK7* | 30 (8) | 20 (13) | 32 (7)  | 9 (2)   | 4 (2)   | 95 (32)          | 2.5              |
| pBD5 + pDB3 | 32      | 13      | 25      | 6       | 3       | 79               | 2.1              |
| pBD5 + pPAB1-2 | 18      | 17      | 17      | 7       | 2       | 61               | 1.6              |
| pBD5 + pCcAde8 | 34      | 27      | 11      | 4       | 2       | 78               | 2.1              |

*Date in brackets indicate clones expressing laccases as deduced from stained halos around their colonies. Non-producers of laccase did not stain the agar. Random subsets of unstained pBD5 and of staining pBD5 + pYSK7 clones from both experiments were further tested in liquid fermentations.

Table 4 Transformations of C. cinerea PG78 (trp1.1,1.6, pab1-1) with either trp1⁺ plasmid pBD5 or pab1⁺ vector pPAB1-2 alone or, using same batches of protoplasts, in combination with other non-directly selectable vectors

| Plasmid(s) | Transformants collected on | Total transformants | Ratio of clones |
|------------|----------------------------|---------------------|----------------|
|            | 1st day | 2nd day | 3rd day | 4th day | 5th day | 6th day | 7th day |                      |                  |
| Experiment 1: trp1⁺ selection |                     |                      |                  |            |            |            |            |                  |                  |
| pBD5       | –       | –       | –       | 21      | 26      | 14      | 6       | 67               | 1.0              |
| pBD5 + pYSK7 | 10      | 16      | 31      | 31      | 14      | 4       | 0       | 106             | 1.6              |
| pBD5 + pDB3 | 2       | 4       | 0       | 50      | 69      | 15      | 12      | 152             | 2.3              |
| pBD5 + pCcAde8 | –       | –       | –       | 45      | 67      | 20      | 16      | 148             | 2.2              |
| pab1⁺ selection |                     |                      |                  |            |            |            |            |                  |                  |
| pPAB1-2    | 40      | 18      | 14      | 8       | –       | –       | –       | 80              | 1.0              |
| pPAB1-2 + pYSK7 | 40      | 13      | 40      | 11      | –       | –       | –       | 104             | 1.3              |
| pPAB1-2 + pDB3 | 53      | 11      | 31      | 8       | 4       | –       | –       | 107             | 1.3              |
| pPAB1-2 + pBD5 | 14      | 6       | 19      | 13      | 15      | 3       | –       | 70              | 0.9               |
| pPAB1-2 + pCcAde8 | 59      | 32      | 49      | 9       | 3       | –       | –       | 152             | 1.9              |

Experiment 2: trp1⁺ selection

| pBD5       | 20      | 21      | 20      | 15      | 7       | –       | –       | 83              | 1.0              |
| pBD5 + pYSK7 | 26      | 42      | 31      | 13      | 13      | –       | –       | 125             | 1.5              |
| pBD5 + pDB3 | 34      | 38      | 29      | 13      | 7       | –       | –       | 121             | 1.5              |
| pBD5 + pCcAde8 | 18      | 27      | 49      | 16      | 12      | –       | –       | 122             | 1.5              |
| pab1⁺ selection |                     |                      |                  |            |            |            |            |                  |                  |
| pPAB1-2    | 25      | 29      | 50      | 19      | 13      | –       | –       | 136             | 1.1              |
| pPAB1-2 + pYSK7 | 40      | 19      | 37      | 37      | 12      | –       | –       | 145             | 1.1              |
| pPAB1-2 + pDB3 | 33      | 46      | 33      | 26      | 17      | –       | –       | 155             | 1.1              |
| pPAB1-2 + pBD5 | 7       | 30      | 37      | 25      | 18      | –       | –       | 117             | 0.9               |
| pPAB1-2 + pCcAde8 | 37      | 32      | 54      | 38      | 18      | –       | –       | 177             | 1.3              |
The bacterial hph gene in OK130 transformations

We also used vector pCRII-hph with an integrated anti-sense-lcc9 fragment for transformation of monokaryon OK130 under hygromycin B resistance selection. Transformation rates in 5 rounds of experiments were not as efficient, with only between 7 to 15 transformants per 1 μg plasmid DNA. After re-screening on new plates containing 200 mg/l hygromycin B, 40 of a total of 70 transferred clones (=57%) failed to grow. Noteworthy, the tolerance of OK130 to hygromycin B varied among different batches of experiments. Screening under a constant hygromycin B concentration of 200 mg/l in the overlay on regeneration agar plates did not always work, leading sometimes to high proportions of false-positive transformants. Of the 30 remaining hygromycin B-resistant clones tested positive by PCR for hph integration, 12 (=40%) were silenced for laccase Lcc9 production as determined by qRT-PCR analysis of cDNAs from transformants co-cultured with Gongronella sp. w5. In summary, hph selection and transformation efficiencies were inferior to the ade8+ selection and transformation efficiencies in OK130 with vector pCcAde8 while lcc9 silencing frequencies in co-transformants were nearly as good.

Conclusions

In this work, we have constructed pCcAde8 as a new selection vector for transformations of C. cinerea strains with ade8 auxotrophies, such as the sequenced reference monokaryon OK130. Co-transformation rates of genes expressed from unselected vectors transformed with pCcAde8 were between 26 and 67% in ranges as observed in co-transformations with other selection markers in other strains. Using gene ade8+ for selection, this had no recognizable negative feedback effects on reducing numbers of viable transformants, similar as when using the pab1+ selection marker of C. cinerea for pab1 complementations and unlike as experienced with the trp1+ selection marker in trp1-auxotrophic C. cinerea strains. pab1+ can be used to complement the pab1-1 defect in the also sequenced homokaryon AmutBmut. Defects in the mutated ade8-1 and pab1-1 alleles in the two sequenced C. cinerea reference strains were defined as missense mutations in the N-terminal GARS domain of the bifunctional GARS-AIRS enzyme from the de novo purine biosynthesis pathway and in the C-terminal PabB domain of the bifunctional 4-amino-4-deoxychorismate synthase in the PABA biosynthesis pathway, respectively. We have used lcc9-antisense constructs in co-transformation of strain OK130 with pCcAde8 in order to suppress native laccase production at high frequency in resulting transformants. Other attempts of lcc9 silencing were made with a single vector carrying an hph selection marker and in addition cloned lcc9-antisense sequences for gene silencing. This second selection system is independent of a gene defect in a host strain. It is in principle also working, but was less efficient in transformation rates than using the pCcAde8 vector in single-vector transformation and in co-transformation. By its better transformation efficiency, ade8+ selection would thus be the first choice for transformation of the C. cinerea reference monokaryon OK130. Nevertheless, when further rounds of transformations in the same strain background are required, hph selection offers extra possibilities after a complementation of the ade8-1 defect in OK130 by transfer of ade8+.

Methods

Strains, transformation and growth conditions

Monokaryons Okayama 7/#130 (short name in literature OK130 [8]; ATCC MYA-4618, FGSC 9003; genotype: A43, B43, ade8-1), FA2222 (DSM 28333; A5, B6, acu1, trp1.1,1.6 [69]) and PG78 (DSM 28337; A6, B42, pab1-1, trp1.1,1.6 [69]), and the self-fertile homokaryon AmutBmut (FGSC 25122; genotype: A43mut, B43mut, pab1-1 [69]) were routinely cultivated on YMG/T medium at 37 °C [3]. Oidia per fully grown plates were harvested in sterile water, filtered through sterile glass wool, washed, protoplasted and transformed as described before [3, 4]. For fungal transformation, plasmid DNA with bacterial RNA was isolated from 3 ml E. coli XL1-Blue (Agilent,
Table 5  Primers used in this study

| Name                  | Sequence (5'-3')                                                                 | Purpose                                                                 |
|-----------------------|---------------------------------------------------------------------------------|------------------------------------------------------------------------|
| ade8_f                | GAATGGTGACGGGCGCCTGCGGACTGCACTGGCCGTTGATA GCGATGTG (sequence upstream of the HindIII-site in pRS426 in italic, sequence upstream of ade8* in normal letters) | Cloning of ade8* in pCCade8                                           |
| ade8_r                | GCCGCTCTAGACTATGGATGGTCCGGGGTGCTGACCTGTTCCATGG TCACAT (sequence downstream of the EcoRI-site in pRS426 in italic, sequence downstream of ade8* in normal letters) | Cloning of ade8* in pCCade8                                           |
| Lcc5-fwd              | CTCCCATCTACACAACAAAGCTTATGGCCATGTCCTGAGAAAT GGCCGATGGCATG (A. bisporus Gpd II sequence is in italic, lcc5 sequence in normal letters) | Cloning of lcc5 for overexpression in pYSK-lcc5                        |
| Lcc5-rev              | CCACCGGCCCTGTCGCACTATAATATATATTAGGGTAGGACAGAG (A. bisporus P_gpd sequence is in italic, lcc5 sequence in normal letters) | Cloning of lcc5 for overexpression in pYSK-lcc5                        |
| Lcc9-fwd              | CTCCCATCTACACAACAAAGCTTATGGCCATGTCCTGAGAAAT GGCCGATGGCATG (A. bisporus Gpd II sequence is in italic, lcc9 sequence in normal letters) | Cloning of lcc9 for overexpression in pYSK-lcc9                        |
| Lcc9-rev              | CCACCGGCCCTGTCGCACTATAATATATATTAGGGTAGGACAGAG (A. bisporus P_gpd sequence is in italic, lcc9 sequence in normal letters) | Cloning of lcc9 for overexpression in pYSK-lcc9                        |
| Lcc9-antisense 1-fwd  | CTCCCATCTACACAACAAAGCTTATGGCCATGTCCTGAGAAAT GGCCGATGGCATG (A. bisporus Gpd II sequence is in italic, lcc9 antisense 1 sequence in normal letters) | Cloning of lcc9-antisense fragment 1 in pYSK-lcc9-antisense-1         |
| Lcc9-antisense 1-rev  | CACCTGCCCCTGTCGCACTATAATATATATTAGGGTAGGACAGAG (A. bisporus P_gpd sequence is in italic, lcc9 antisense 1 sequence in normal letters) | Cloning of lcc9-antisense fragment 1 in pYSK-lcc9-antisense-1         |
| Lcc9-antisense 2-fwd  | CTCCCATCTACACAACAAAGCTTATGGCCATGTCCTGAGAAAT GGCCGATGGCATG (A. bisporus Gpd II sequence is in italic, lcc9 antisense 2 sequence in normal letters) | Cloning of lcc9-antisense fragment 2 in pYSK-lcc9-antisense-2         |
| Lcc9-antisense 2-rev  | CACCTGCCCCTGTCGCACTATAATATATATTAGGGTAGGACAGAG (A. bisporus P_gpd sequence is in italic, lcc9 antisense 2 sequence in normal letters) | Cloning of lcc9-antisense fragment 2 in pYSK-lcc9-antisense-2         |
| P_gpd-F               | GATATCGAAGAAGAATTCAGAGGATCGCAAGTA (A. bisporus P_gpd sequence, EcoRI site underlined) | Cloning of A. bisporus gpdII promoter for pCRII-hph-lcc9 vector construction |
| P_gpd-R               | AAGTGGTGCCGGAATAGCTTTGGTTGGTAGATGG (A. bisporus P_gpd sequence is in italic, lcc9 antisense 2 sequence in normal letters) | Cloning of A. bisporus gpdII promoter for pCRII-hph-lcc9 vector construction |
| Lcc9-antisense-hphF   | GCTATCGGCCGGAACACTTTCCTCTGGGGGCA (A. bisporus P_gpd sequence is in italic, lcc9 antisense 2 sequence in normal letters) | Cloning of lcc9-antisense fragment 2 for pCRII-hph-lcc9 vector construction |
| Lcc9-antisense-hphR   | TGCTATCGACTCTCATGTCAGGACTACCGATG (Tlcc9 sequence is in italic, lcc9 antisense 2 sequence in normal letters) | Cloning of lcc9-antisense fragment 2 for pCRII-hph-lcc9 vector construction |
| T_lcc9-F              | ACCATGAGCGACTCATACATGCACTGCCGACACAT (Tlcc9 sequence is in italic, lcc9 antisense 2 sequence in normal letters) | Cloning of C. cinerea lcc9 terminator for pCRII-hph-lcc9 vector construction |
| T_lcc9-R              | GCCGCCCTCAAGAGGATCGCAGGCTTGGGATA (Tlcc9 sequence, Apd site underlined) | Cloning of C. cinerea lcc9 terminator for pCRII-hph-lcc9 vector construction |
| DPf                   | ATGTGACGATCCTACACTTACTTXTTCTC (sequence of ade8 from start codon onwards) | Diagnosis PCR for nuclear ade8* insertion                               |
| DPPr                  | ATCCCAAGGCGAGGATCAGTG (sequence of ade8 with its last triplets for amino acids) | Diagnosis PCR for nuclear ade8* insertion                               |
| PF                    | ACATCCACATTCCTGTGTTCCCAT (A. bisporus P_gpd sequence) | PCR of OK130 co-transformants of lcc9-antisense-constructs             |
| PR                    | TGACTATAGGCGCTTACCTACCTG (Tlcc9 sequence) | PCR of OK130 co-transformants of lcc9-antisense-constructs             |
| qRT-lcc9-F            | ATGGTGAGAAGAATCTTTGCTCCTCG (lcc9 sequence + 1 to + 25) | qRT-PCR of lcc9                                                         |
| qRT-lcc9-R            | ATGGTGAGAAGAATCTTTGCTCCTCG (reverse complementary lcc9 sequence of +79 to +101) | qRT-PCR of lcc9                                                         |
Böblingen, Germany) overnight LB (amp) cultures by a modified Birnboim-Doly method [4]. Per transformation sample and per plasmid, 1 µg plasmid DNA was used. When required for testing laccase activities in transformants, 0.5 mM ABTS was added to regeneration agar [16]. Prototrophic transformants appeared at first on regeneration agar 3.5–4 days after plating (= 1st day of picking clones reported in Tables 2, 3, 4). Day by day, all new clones were counted and collected from regeneration agar onto minimal medium with suitable supplements [3, 4]. Using in experiments the same protoplast batches, ratios of transformants were calculated by dividing the total number of clones obtained by a co-transformation by the total number of clones obtained from the single-vector transformation under the same scheme of selection. For selection for hygromycin B resistance after transformation, an extra 5 ml of regeneration agar (low melting point agar, 1%) containing 200 mg/l hygromycin B were overlaid after protoplast plating on regeneration agar. Individual hygromycin B-resistant transformants which appeared on these plates were re-screened by culturing again on regeneration agar containing 200 mg/l hygromycin B. hph-transformants were further verified based on PCR amplification of a gpdII promoter-lcc9 antisense-lcc9 terminator fragment with their genomes as templates and P_gpd-F and T_lcc9-R as primers (Table 5). OK130 transformants for lcc9 silencing were cultured in SAHX medium using sucrose as the carbon source and cocultivation with Gongronella sp. w5 for lcc9 induction according to Pan et al. [65]. qRT-PCR analysis using qRT-lcc9 antisense-2 and transformants’ cDNAs as substrate was performed to further evaluate their silencing ratios [72]. For laccase activity tests in fermentation, clones were grown in YMG medium and supernatants of the culture broth were withdrawn every 12 h for activity assay and native-PAGE was performed as previously described [65]. Lcc1, Lcc5 and Lcc9 can be well distinguished in native-PAGE by differential migration patterns [64, 65].

pCCade8 vector construction

Chimeric primers ade8_f and ade8_r (Table 5) were designed from the AmutBmut genome for PCR amplification of the wt ade8⁺ gene from chromosomal DNA using Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific Inc., Darmstadt, Germany). The amplified DNA fragment was transformed into the Δura3 yeast strain RH 1385 [70] together with the HindIII-EcoRI double-digested E. coli-yeast shuttle ura3⁺-vector pRS426 [60] for in vivo plasmid construction by homologous recombination [71]. Plasmids were isolated from prototrophic yeast clones and further amplified in E. coli XL1-Blue. Proper fragment insertion was confirmed by sequencing as described [6]. Diagnosis PCR for insertion of pCCade8 in nuclear DNA of transformants was performed with primers DPf and DPr (Table 5) which amplify the complete ade8 coding region. Sequencing of the amplicons from 25 randomly selected transformants verified insertion of ade8⁺ copies by presence of either a wt A (1x) or a mixture of an A and a mutant G (24x) at position 691 in codon 231 of the gene.

Other plasmids

trp1⁺-vector pBD5 and trp3⁺-vector pDB3 are described in [5] and [7], respectively. pPAP1-2 is a pTZ18R-based pab1⁺ selection vector [3]. Plasmid pYSK7 is a pRS426 [60] derive containing the C. cinerea laccase gene lcc1 cloned behind the A. bisporus gpdII promoter and with its own terminator [16]. pYSK-lcc5 and pYSK-lcc9 were generated through in vivo recombination in yeast [71] of PCR-amplified OK130 cDNA (for primers, please see Table 5) with BamHI and Hpal linearized plasmid pYSK7. Similarly, pYSK-lcc9-antisense-1 and pYSK-lcc9-antisense-2 were constructed by amplifying lcc9 sequences with primers Lcc9-antisense 1/2-fwd and Lcc9-antisense 1/2-rev (see Table 5) from strain OK130 and inserting the resulting fragments (lcc9-antisense 1 is from bp +305 to +514 of lcc9; lcc9-antisense 2 is from bp +752 to +1032 of the gene) into BamHI and Hpal linearized plasmid pYSK7 through in vivo recombination in yeast [71]. The lcc9-antisense 2 plasmid pCRII-hph-lcc9 was constructed based on the pCRII-TOPO derivative pCRII-hph which contains in the vector TOPO TA-cloning site a 1.0 kb β-tubulin promoter and a 0.5 kb terminator sequence of Trametes hirsuta AH28-2 and the bacterial hph gene in between [72]. Briefly, a 281 bp reverse complementary sequence cloned from cDNA of laccase gene lcc9 (bp + 752 to + 1032) was joined to the A. bisporus gpdII promoter sequence (277 bp) and the C. cinerea lcc9 terminator sequence (500 bp) by overlapping PCR using the primer pairs of P_gpd-F and P_gpd-R, and T_lcc9-F and T_lcc9-R listed in Table 5. The fused sequences were then digested with EcoRV and Apal and inserted into the EcoRV and Apal polylinker sites of pCRII-hph.

Sequence analyses

The published genomes of monokaryon Okayama 7/130 (https://mycocosm.jgi.doe.gov/Copci1/Copci1.home.html) and homokaryon AmutBmut (https://mycocosm.jgi.doe.gov/Copci_AmutBmut1/Copci_AmutBmut1.home.html) on the JGI Mycocosm side were used for defining chromosomal loci of genes of interest and obtaining relevant DNA and protein sequences. Protein sequences from E. coli and S. cerevisiae (Table 1) were used in tblastn searches. Homologous protein sequences retrieved from the JGI homepages and from NCBI were...
aligned by ClustalX 2.0 [73] and the MEGA 6.0 software was used with 1000 bootstrap values for constructing a neighbor-joining tree [74].

Abbreviations

ABTS: 2,2’-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); ADC: 4-Amino-4-deoxychorismate; ADP: Adenosine diphosphate; AICAR: 5’-Phosphoribosyl-5-monophosphate; AIR: Aminomimidazole ribonucleotide; AIRS: Aminomimidazole ribonucleotide synthase; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; CAIR: 5-Amino-4-imidazolecarbonamide ribonucleotide; DHF: Dihydrofolic acid; DHNTP: 7,8-Dihydroxypterin 3’-triphosphate; DHP: Dihydropyroteroate; FACAR: S-Formalimidazolide-4-carboxamide ribotide; FGAM: Formylglycaminde ribonucleotide; FGAMS: Formylglycaminde ribonucleotide synthase; FAGAR: Phosphoribosyl-N-formylglycinamide; GAR: Glycinamide ribonucleotide; GARS: Glycinamide ribonucleotide synthase; GART: Phosphoribosylglycinamide formyltransferase; GATase: Glutamine amidotransferase; GPAT: Glutamine amidophosphoribosyltransferase; GTP: Guanosine-5’-triphosphate; HIT: Histidine triad; IGP: Indole-3-glycerol-phosphate; IPG: Indol-3-ylglycerol-phosphate synthase; IMP: Inosine monophosphate; NAD: Nicotinamide adenine dinucleotide; NADP: Nicotinamide adenine dinucleotide phosphate; NADPH: Nicotinamide adenine dinucleotide phosphate; NSP: Nicotinamide adenine dinucleotide phosphate synthase; P450: 15-Pheophytin a oxidase; P448: Pheophytin a oxidase; P530: Pheophytin a oxidase; PABA: P-benzoyl-2 alaninamide; PABA synthases. J Basic Microbiol. 2002;42:91–103.

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