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Quantifiable Downregulation of Endogenous Genes in *Agaricus bisporus* Mediated by Expression of RNA Hairpins

Costa, Ana S. M. B.[1], D. John I. Thomas[1], Daniel Eastwood[1], Simon B. Cutler[1], Andy M. Bailey[2], Gary D. Foster[2], Peter R. Mills[1], and Michael P. Challen[1]*

[1]*Warwick HRI, University of Warwick, Wellesbourne, Warwickshire, CV35 9EF, U.K.*
[2]*School of Biological Sciences, University of Bristol, Bristol, BS8 1UG, U.K.*

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Functional gene studies in the cultivated white button mushroom *Agaricus bisporus* have been constrained by the absence of effective gene-silencing tools. Using two endogenous genes from *A. bisporus*, we have tested the utility of dsRNA hairpin constructs to mediate downregulation of specific genes. Hairpin constructs for genes encoding orotidine 5'-monophosphate decarboxylase (*URA3*) and carboxin resistance (*CBX*) were introduced into *A. bisporus* using *Agrobacterium*-mediated transfection. Although predicted changes in phenotype were not observed *in vitro*, quantitative-PCR analyses indicated unambiguously that transcripts in several transformants were substantially reduced compared with the non-transformed controls. Interestingly, some hairpin transformants exhibited increased transcription of target genes. Our observations show that hairpin transgenic sequences can mediate downregulation of *A. bisporus* endogenous genes and that the technology has the potential to expedite functional genomics of the mushroom.

Keywords: *Agaricus bisporus*, *CBX*, gene-silencing, dsRNA hairpin, RNAi, *URA3*

The white button mushroom *Agaricus bisporus* is a high value horticultural crop of worldwide economic significance, for which transformation technology has recently become available [2, 3, 6, 8, 26]. The most successful approach to transforming *A. bisporus* has been *Agrobacterium tumefaciens*-mediated transformation (Agrotransfection) of excised gill tissue [8]. As gene disruption (knock-out) methods are difficult to apply to this multinucleate heterokaryotic species, functional genomic studies have been hitherto constrained [1, 17, 35]. RNA silencing is locus independent and mediated by a mobile *trans*-acting signal in the cytoplasm, and is a technology proving to have broad utility. Using the exogenous *GFP* reporter gene, we have previously shown that both antisense and hairpin constructs can trigger gene silencing in the model homobasidiomycete species, *Coprinopsis cinerea* (=*Coprinus cinereus*; [20]). Building on our recent advances in the transformation of *A. bisporus* [2, 3, 24], we have tested the utility of hairpin constructs to downregulate endogenous *A. bisporus* genes.

Two genes were selected to model the efficacy of hairpin transformations. The *A. bisporus URA3* gene [6], which encodes orotidine 5'-monophosphate decarboxylase (OMPdecase, E.C. 4.1.1.23) and catalyzes the conversion of orotidylic acid to uridylic acid, during uracil biosynthesis. The *A. bisporus CBX* mutant gene encodes resistance to the fungicide carboxin and results from a mutation in the iron-sulfur protein subunit of succinate dehydrogenase (*SDH*, E.C. 1.3.99.1) [5, 6]. In this manuscript, we report the development of hairpin-mediated gene suppression for *A. bisporus* and quantifiable downregulation of *URA3* and *CBX* transcripts.

Material and Methods

Strains, Culture Maintenance, and Transformation

The *Escherichia coli* strain DH5α was used as the host strain for construction and maintenance of recombinant plasmids. A tissue culture derivative of the commercial A15 strain of *A. bisporus* (Sylvan Inc., U.S.A.) was used for *URA3* silencing experiments. Two carboxin-resistant strains, C54-*carb*.8 and C43-*carb*.9 [5], were used for *CBX* silencing experiments. Mushroom sporophores were produced in the Warwick transgenic mushroom containment facility, using compost culture and standard industry methods [14]. Mycelial cultures were grown at 25°C using malt peptone agar (MPA; [24]) or mushroom minimal medium (MMM; [31]) supplemented with hygromycin (25 mg/ml) and uracil (100 mg/l) as required. *Agrobacterium tumefaciens* strain AGL-1 [22] was used for Agrotransfection of *A. bisporus*, using gill-tissue infiltration [8] and other well-established methodologies [6, 11, 24].

*Corresponding author
Phone: +44 (0) 24 7657 5078; Fax: +44 (0) 24 76574500;
E-mail: mike.challen@warwick.ac.uk

Table 1. Oligonucleotides used in this study: sequence and purpose.

Primer	Sequence (5'-3')	Description
URA3_497extF	**GACAAGATCTACTAGT**GTAAGCGTGGATGTTACCC	Introduction of restriction sites (bold) to permit directional cloning
URA3_497extR	A**GAGGCGCGCCATTTAAAT**CGGCATCATCTGTGTAC	
URA3_381extF	**GACAAGATCTACTAGT**CGTGGACTCCTATTACTAGC	
URA3_381extR	**AGAGGCGCGCCATTTAAAT**ATACGCATCCCATCCTTC	
CBX_413extF	**GACAAGATCTACTAGT**CCAGCCTTGGCTACAGAAC	
CBX_413extR	**AGAGGCGCGCCATTTAAAT**AAGACCCTTCGGGCAAGTACG	
CBX_368extF	**GACAAGATCTACTAGT**GAAACCCACTCTGCAGTCTTAC	
CBX_368extR	**AGAGGCGCGCCATTTAAAT**CGAGGTCCTTCACAACATACAC	
HYG_F	ATGCCTGAACTCACCGCG	PCR screen: *hph* transgene
HYG_R	TCGGTTTCCACTATCGGC	
GDP-Loop_F	TGCCAACGAACCGGATACCC	PCR screen: sense and antisense hairpin sequences
GDP-Loop_R	GCGGTCGGCATCAGATC6TAC	
Loop-TrpC_F	CCGGTTCGTTGGCAATACTC	
Loop-TrpC_R	TTCCGGTCACATCCACCATC	
SDH_F	TCACGTAAGAGACGCGAACA	RT-PCR screen: confirm absence of gDNA
SDH_R	AACGCAACTCGTGGTACTCA	
q497URA3_F	GCCAGAATGGTTGCGATGTT	Q-PCR: 497 bp *URA3* hairpin transcripts
q497URA3_R	TCGGATTGCTTCAACGTCTTT	
q381URA3_F	GAGGATTTTGATTCCAACCTGATAAA	Q-PCR: 381 bp *URA3* hairpin transcripts
q381URA3_R	TCGGCAAATTTTCTATCTTCGAA	
qCBX_F	CGGCGGCACCCAGAT	Q-PCR: 413 bp & 368 bp SDH transcripts
qCBX_R	ATCAGTACCAGTTCCACATCCTAGGT	
q18S_F	TCGCCGCTCCCTTGGT	Q-PCR: *A. bisporus* 18S rRNA transcripts
q18S_R	GCATCGCCGGCACAA	

Binary vectors were developed using the pGreen002 vector [19] and introduced into *A. tumefaciens* by electroporation. The *A. bisporus URA3* gene was recovered from the C54-*car*b.8 cosmid genomic library [4] by screening for conserved OMPdecase motifs. Specifically, internal fragments were amplified using PCR from the *Schizophyllum commune URA1* [15] and *Phycomyces blakesleeanus* *pyrG* [13] genes and used as hybridization probes to reveal two overlapping cosmid clones (AbLAW5F4, AbLAW32B1). Sequencing of 3,288 bp within these cosmids revealed a 932 bp (1,185–2,116nt) *A. bisporus URA3* ORF region, interrupted by two introns (1,371–1,419nt, 1,531–1,582nt) that encoded a predicted protein of 267 amino acids; database Accession No. FM202068.

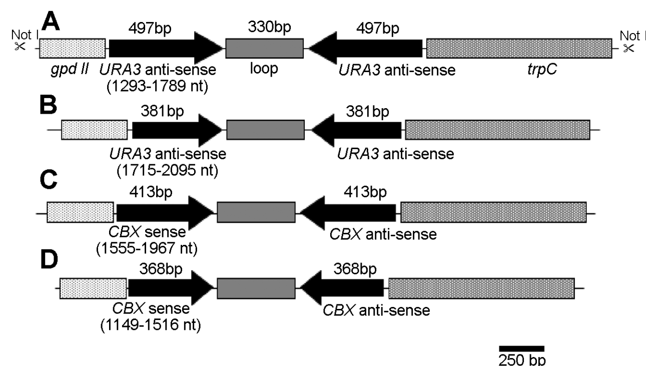

Fig. 1. Schematic representation of hairpin constructs to target *URA3* (**A**. AbURA3_497; **B**. AbURA3_381) and *CBX* (**C**. AbCBX413; **D**. AbCBX368) *A. bisporus* genes.

Regulatory sequences are common in all constructs: *A. bisporus gpdII* promoter (277 bp) and *A. nidulans trpC* terminator (711 bp). Inverted repeats are separated by 330-bp nonfunctional sequences from the *E. coli gusA* gene (loop).

Hairpin constructs were based on the pRNAiDE001 vector [14, 20] where self-complementary sense and antisense sequences are separated by a 330-bp nonfunctional sequence (loop) isolated from the *E. coli gusA* gene. Expression of hairpin sequences was regulated by the *A. bisporus gpdII* promoter [18] and *A. nidulans trpC* terminator [30]. Target hairpin sequences were amplified by PCR using primers that introduce appropriate restriction sites to permit sequential cloning in sense (*Swa*I-SpeI) and antisense (*Asc*I-BglII) orientations. Specific primers used to clone *URA3* and *CBX* fragments are described in Table 1. Two *URA3* (AbURA3_497, AbURA3_381) and two *CBX* (AbCBX_413, AbCBX_ 368) hairpin constructs were produced with differing hairpin sequences (Fig. 1). Silencing cassettes were introduced into the pGreen_hph1 [14], which contain the hygromycin resistance gene and fungal regulatory sequences from pAN7-1 [30]. The hairpin expression cassette was cloned in two orientations with respect to the hygromycin marker, as unidirectional or divergently transcribed sequences.

PCR and Quantitative RT-PCR Analysis (Q-PCR)

Fungal DNAs were prepared using a Chelex® 100 Resin (Bio-Rad Laboratories, CA, U.S.A.) miniprep method [7]. Presence of hairpin

transgene sequences in genomic DNA was detected using PCR and primers annealing with either the promoter or terminator and noncoding *gusA* loop sequences (Table 1). Total RNA was extracted from freeze-dried mycelium using TRI Reagent® (Sigma Genosys, Sulfolk, U.K.) according to the manufacturers, instructions. Total RNA (1 μg) was treated with DNase (Promega Corporation, Madison, WI, U.S.A.) and the cDNA synthesized using random hexamers and SuperScript® II reverse transcriptase (Invitrogen, Paisley, U.K.). Three independent RNA extractions were performed for each strain/transformant tested. Absence of genomic DNA in cDNA preparations was confirmed using primers that span an intron of the *A. bisporus SDH* gene (Table 1). Transcription of the target genes was quantified using SYBR green Q-PCR as previously described [20], and the primers are identified in Table 1. RNA samples were normalized against the *A. bisporus* 18S rRNA.

Phenotypic Analysis

The *ura3* phenotype was tested by growing transformants on MMM with or without uracil. Sensitivity to carboxin was evaluated by growth on MMM agar with or without carboxin (15 μg/ml). Controls were non-transformed A15, carboxin-resistant mutants C43-*carb*.9 and C54-*carb*.8, or the C63-ura (*ura3*) mutant [6]. Phenotype experiments were performed twice.

RESULTS AND DISCUSSION

RNAi-mediated gene suppression is becoming increasingly pervasive as a tool for functional genomics, and has recently proved applicable to homobasidiomycete fungi [10, 12, 20, 28, 34]. In this study, we have modeled hairpin-mediated gene suppression in the premier cultivated mushroom *A. bisporus* using two endogenous genes (*URA3* and *CBX*). Two different target regions and stem-lengths were exploited for each gene (497, 381 bp for *URA3*; 413, 368 bp for *CBX*). Hairpin expression units were cloned in two transcriptional directions (unidirectional and divergent) with respect to the hygromycin expression cassette and introduced into *A. bisporus* gill tissue using Agrotransfection.

Transformants were recovered using each of the different binary vectors. Transformation efficiency was assessed as the ratio of the number of transformants and the number of gill tissue plated [24]. The recovery of transformants differed substantially between host mushroom strains, *e.g.*, using the carboxin-resistant mutants C54-*carb*.8 and C43-*carb*.9, transformation efficiencies were ranged between 3% and 23%, respectively. Variation in the recovery of transformants from different host strains of *A. bisporus* has been previously observed [23, 32]. However, to ensure that these observations were not partly due to transforming with hairpin vectors, all host strains (A15, C54-*carb*.8, and C43-*carb*.9) were transformed with the control binary pGreen_hph1. Again, the efficiency of transformation was much lower using C54-*carb*.8 (3%) than either C43-*carb*.9 (52%) or strain A15 (37%), indicating that C54-*carb*.8 has a low affinity for Agrotransfection.

PCR screening of 25 *URA3* and 21 *CBX* hygromycin, resistant mycelial colonies established a pool of 17 *URA3* and 11 CBX transformants, in which the presence of the hygromycin resistance transgene was confirmed. Of the 17 *URA3* transformants, 14 yielded both sense and antisense hairpin PCR amplicons. Nine of these were recovered using the AbURA3_497 (T1, T2, T3, T5, T7, T18, T19, T27, and T35) and the remaining five (T8, T26, T31, T32, and T45) using the ABURA3_381 construct.

All of the 11 *CBX* transformants (T13, T22, T41, T57, T58, T59, T74, T80, T82, T85, and T88) yielded the appropriate sense and antisense hairpin amplicons in PCR screens. Five *CBX* transformants were obtained with the AbCBX_413 construct (T22, T41, T57, T85 and T88) and six (T13, T58, T59, T74, T80 and T82) using AbCBX_368.

To determine whether transcription of the endogenous genes was affected, Q-PCR was performed using primers exclusively designed outside the hairpin regions. Transcripts were initially quantified in 14 *URA3* hairpin transformants, and these exhibited diverse levels of *URA3* transcription

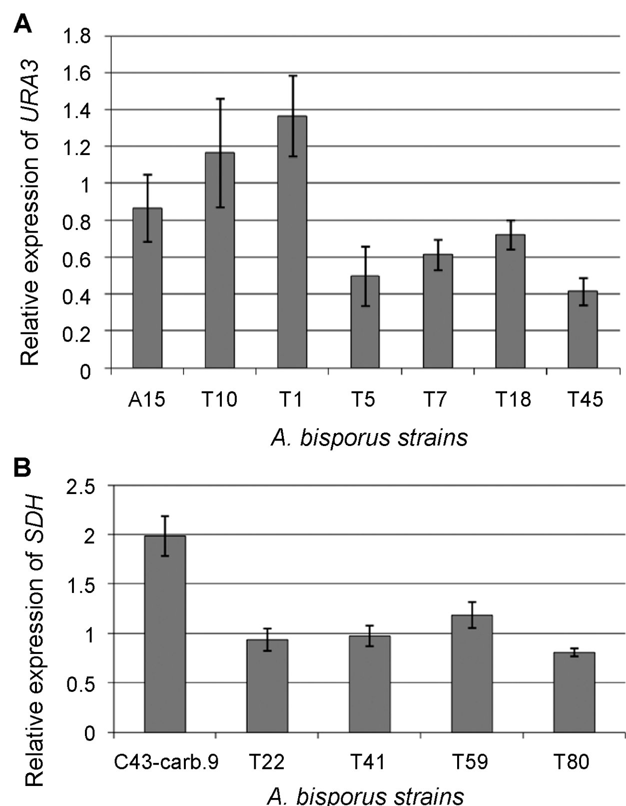

Fig 2. Quantitative RT-PCR analysis of *A. bisporus* hairpin transformants.

A. Four *URA3* hairpin transformants (T5, T7, T18, T45) exhibited a range of *URA3* downregulation compared with the nontransformed host (A15) and control transformant (T10); **B**. Four *CBX* transformants show a significant decrease of *SDH* mRNA compared with the nontransformed host C43-*carb*.9. Transcripts were normalized against 18S rRNA. Standard error bars are from 2-4 biological replicates.

when compared with nontransformed A15 and the transformed control T10. Eight *URA3* hairpin transformants did not differ from the controls. In two transformants, *URA3* transcripts were significantly increased, and four transformants exhibited reduced transcripts. The efficiency of downregulation was relatively low with the two *URA3* constructs tested; 22% of AbURA3_497 transformants were downregulated, as were 40% of the AbURA3_381 transformants. In a highly replicated Q-PCR analysis of five *URA3* hairpin transformants, one transformant (T1) exhibited significant upregulation of *URA3* transcripts, and in three transformants (T5, T7, and T45), transcription was significantly reduced (Fig. 2A). Two *URA3* transformants did not differ significantly to the controls.

Both of the *CBX* constructs tested were equally effective at initiating downregulation. Using AbCBX413, *SDH* transcripts were downregulated in 60% of the transformants, and with AbCBX368 50% of transformants had transcripts that were significantly lower than the controls. The relative expression of *SDH* transcripts for C43-*carb*.9 transformants is shown in Fig. 2B; five transformants (T22, T41, T57, T59, and T80) were downregulated compared with the nontransformed host.

The phenotype of the hairpin transformants was evaluated on appropriate agar media. If *URA3* was fully silenced, the hairpin transformants would be expected to behave as OMPdecase auxotrophs and unable to grow on minimal medium. However, despite some minor changes in colony morphologies, all *URA3* hairpin transformants tested were able to grow uninhibited on MMM. To ensure that uracil was not limiting recovery of transformants with the mutant phenotype, experiments were also performed where uracil was incorporated into selective media at a rate that permits growth of the C63-ura mutant on MMM. Transformants recovered with and without uracil supplementation behaved identically. Experiments to silence the mutant *CBX* gene were more of a “long shot” and were initially designed to evaluate the specificity of hairpin transformations. If our hairpins specifically silenced the mutant *CBX* gene conferring carboxin resistance, then transformants of our C54-*carb*.8 and C43-*carb*.9 host strains might exhibit a carboxin-sensitive phenotype. Alternatively, if silencing was nonspecific, then both mutant *CBX* and wild-type *SDH* alleles would be silenced and hairpin transformations could prove lethal. Although we were unable to differentiate between the two alleles using Q-PCR, there was no evidence for either event in our *CBX* hairpin experiments. All transformants recovered proved capable of growth on carboxin amended agar medium, despite substantial reductions in *SDH* transcription, and there was no obvious reduction in the recovery of transformants that might be associated with a lethal event. As with the *URA3* hairpin transformants, it seems likely that low-level transcription of the target *CBX* gene was sufficient to permit maintenance of the resistance phenotype.

The *A. bisporus URA3* and *SDH* genes were successfully downregulated using Agrotransfection and several different hairpin sequences of the target gene. The arrangement of the hairpin sequences within the T-DNA, with respect to the hygromycin resistance selectable marker, did not affect transformation rates. The relative proportions of transformants exhibiting downregulation varied between experiments, but further work would be required to determine whether the hairpin stem length, the target region, or if the specific gene used was having significant effects in *A. bisporus*. In plants, the incorporation of intron sequences into hairpin vectors can lead to improved efficacy [33], and the inclusion of intron sequences has also proved effective in some fungi [29]. We have not observed concomitant increases in efficacy when using a vector that replaces the *gusA* loop sequence of pRNAiDE001 with an intron from *A. bisporus* (Sergeant *et al*., unpublished data). Although some hairpin transformants exhibited reduced transcripts, concomitant changes in phenotype were not observed. Similar results were obtained when targeting the *C. cinerea URA3* gene with hairpin sequences; although *URA3* transcripts were downregulated, transformants could grow on minimal medium [9]. The use of antisense constructs to attempt suppression of another uracil biosysnthesis gene, *PYR3* (dihydroorotase), in the heterobasidiomycete *Ustilago maydis* also failed to yield mutant phenotypes [21]. Likewise in *Neurospora crassa*, hairpin transformants targeting the *PAN2* gene (pantothenic acid biosynthetic pathway) also exhibited reduced transcripts but did not induce the defective phenotype [16]. Collectively, these experiments demonstrate that although substantial downregulation of mRNAs can be achieved through hairpin transformations, very low-level transcription of key biosynthetic genes may be sufficient to permit growth of the organism on selective media.

The substantial upregulation of *URA3* and *CBX* transcripts in some transformants is an interesting observation. We routinely observe upregulation as a result of hairpin transformation [9, 14]. Although the reason for this phenomenon is not clear, reports of increased target gene transcripts in RNAi transformations has been reported in other systems [25]; elevated expression may prove equally useful for functional gene characterization.

The approaches described here can be used to modify expression of specific genes in the mushroom *A. bisporus* and are likely to have utility in other edible mushrooms. We are now using Agrotransfection with hairpin sequences to alter the expression of genes involved in mushroom post-harvest development [14] and those expressed in response to pathogen attack [27].

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