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The Bacterial Transposon Tn7 Causes Premature Polyadenylation of mRNA in Eukaryotic Organisms: TAGKO Mutagenesis in Filamentous Fungi

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ABSTRACT

TAGKO is a Tn7-based transposition system for genome wide mutagenesis in filamentous fungi. The effects of transposon insertion on the expression of TAGKO alleles were examined in *Magnaporthe grisea* and *Mycospharella graminicola*. Northern analysis showed that stable, truncated transcripts were expressed in the TAGKO mutants. Mapping of the 3'ends of TAGKO cDNAs revealed that they all contain Tn7 end sequences, regardless of the transposon orientation. Polyadenylation signals characteristic of eukaryotic genes, preceded by stop codons in all frames, are located in both ends of the bacterial transposon. Thus, TAGKO transcripts are prematurely polyadenylated, and truncated proteins are predicted to be translated in the fungal mutants. Depending on the extent of protein truncation, TAGKO mutations in *HPD4* (encoding *p*-hydroxyphenylpyruvate dioxygenase) resulted in tyrosine sensitivity in the two fungi. Similarly, a particular *M. grisea CBS1* (encoding cystathionine β-synthase) TAGKO cDNA failed to complement cysteine auxotrophy in a yeast CBS mutant. TAGKO, therefore, represents a useful tool for *in vivo* study of truncated gene products in filamentous fungi.

INTRODUCTION

Transposons frequently account for spontaneous mutations that result in natural variations in different organisms (1). They are mobile DNA elements originally discovered by McClintok in maize (2), and have become indispensable tools in the molecular genetics of both prokaryotic and eukaryotic organisms (3, 4). Large-scale transposition in endogenous or heterologous systems has been employed to generate large collections of insertional mutants for gene identification and function analysis (5). A number of *in vitro* transposition systems (based on Tn5, Tn7, Mu, *Himar1*, etc.) have now been developed (6, 7, 8, 9). There are many applications for *in vitro* transposition systems. For example, transposon insertions provide primer sites for sequencing of the recipient DNA. In addition, inserted DNA from whole genome *in vitro* transposition can be introduced to the host for mutant generation when homologous recombination is efficient in a target organism.

The Tn7-based *in vitro* transposition system is highly efficient and useful for analysis of genomes and genes (6). The bacterial transposon Tn7 encodes transposases that directly participate in transposition (10). The notable Tn7 target site preference has been abolished with the use of a mutant form of one of the Tn7 transposases during *in vitro* transposition (6). This feature, together with its innate transposition immunity (11), allows the use of Tn7 to generate random and thorough mutagenic insertions in genomic libraries. MiniTn7 elements with modified end sequences for recovery of derivatized target genes were also described (6). One of the elements has truncated ends that are open in all frames, thus allowing productive fusions with target gene products. The other

elements generate 15 bp (5 amino acid) linker insertions in target genes (proteins) to facilitate the identification of critical base pairs or amino acids.

Recently we described a genome-wide mutagenesis technology, TAGKO (**t**ransposon **a**rrayed **g**ene **k**nock**o**ut), in filamentous fungi using a Tn7 based transposon cassette (12). Basically, only the end segments (Tn7-L, 150 bp; Tn7-R, 90 bp) of Tn7 are required for recognition by the transposition machinery (10). The TAGKO cassette contains a hygromycin phosphotransferase gene (*HPH*) engineered between the transposon ends as a fungal selection marker. Random *in vitro* transposition can be performed on cosmid libraries of fungal genomes with the addition of a Tn7 transposase mix (6, 12, 13). Individual cosmid-based TAGKO clones serve directly as gene disruption vectors and improve the frequency of homologous recombination significantly (12, 14). In addition, a series of mutation alleles in the target genes can be generated with TAGKO, providing a range of phenotypes for gene function analysis.

Transposons insertions can lead to a variety of effects on target gene expression, depending on both the location and the properties of the element (15). In this study, we examine the transcription patterns of different TAGKO alleles in the filamentous fungi *Magnaporthe grisea* and *Mycosphaerella graminicola*, two of the major cereal pathogens in the world. Interestingly, we discovered that Tn7 contains 3'-end formation site information unique to eukaryotic genes, resulting in the generation of truncated transcripts that are chimeric and polyadenylated in the TAGKO mutants. The potential

application of this novel feature of the bacterial transposon Tn7 in fungal gene analysis is discussed.

MATERIALS AND METHODS

Fungal strains and growth assays

M. grisea strain Guy11 (16) and *M. graminicola* strain PG1 (12) were used as the wild-type (WT) strains in this study. The WT strains and all TAGKO mutants created were maintained on complete medium (CM) agar (17). Growth assay experiments were performed on minimal medium (MM) agar (17) with or without the supplementation of L-tyrosine (Sigma, St Louis, MO) at a concentration of 4 mM.

Generation of TAGKO mutants in *M. grisea* and *M. graminicola*

From our collections of TAGKO clones generated by whole genome *in vitro* transposition previously (12, 14), we selected individual gene disruption vectors with transposon inserted into different locations of the *HPD4* (encoding *p*-hydroxyphenylpyruvate dioxygenase) or *CBS1* (encoding cystathionine β-synthase, 18) genes. Transposon insertion sites were identified by analysis of sequencing data derived from Tn7-end specific primers (12). The selected cosmids were linearized and transformed into the corresponding fungus, following procedures described previously (14, 19, 20). Transformants, selected on hygromycin-containing medium, were isolated and DNA extracted as described (20). To identify TAGKO mutants, primer pairs were designed to flank the transposon insertion sites for PCR screening of homologous recombination events, using the transformant DNA samples as templates (12).

RNA experiments

WT strains and TAGKO mutants were cultured in CM for 4 days before the mycelia were harvested. Total RNA was extracted from lyophilized mycelia with a RNAqueous-Midi kit (Ambion, Austin, TX) following the manufacturer's instructions. Northern blot experiments were performed as described (21). Transcript ends were mapped with a 3' rapid amplification of cDNA (RACE) ends kit (Life Technologies, Rockville, MD), followed by DNA sequencing. Briefly, 4 μg of total RNA was used as the template for first strand cDNA synthesis. Specific cDNA was amplified by PCR using a gene-specific primer that anneals to the known exon sequence and an adapter primer (Life Technologies, Rockville, MD) that targets the poly(A) tail region. The following *HPD4*-specific primers were used for PCR amplification: *M. grisea*, 5'-ATGTCACCCTCTGCCATCAC-3'; *M. graminicola*, 5'-ATCATGGCACCCGGAGCACT-3'.

Yeast complementation analysis of *M. grisea CBS1* TAGKO alleles

CBS1 cDNAs from *M. grisea* WT and TAGKO strains were amplified by PCR using first strand cDNA mixtures as templates with the following primers: CBS5', 5'-CACACAATCTAAAGAATGGC-3', and AUAP (Life Technologies, Rockville, MD), 5'-GGCCACGCGTCGACTAGTAC-3'. The amplified PCR products contained the first start codon (ATG) through the end of the transcripts with the majority of the 5' untranslated region removed. *CBS1* WT and TAGKO cDNAs were cloned into the expression vector pYES2.1/V5-HisTOPO under the control of a *GAL1* promoter (Invitrogen, Carlsbad, CA). A *Saccharomyces cerevisiae* cystathionine β-synthase (CBS)

deletion mutant (strain number 6696, Research Genetics, Inc., Huntsville, AL) was transformed with the different *M. grisea CBS1* expression clones following the manufacturer's instruction (Invitrogen, Carlsbad, CA). The transformants were grown on synthetic complete (SC) medium (Invitrogen, Carlsbad, CA) containing galactose (2% w/v) as the carbon source to induce *CBS1* expression from the *GAL1* promoter. Complementation of CBS mutation was assessed by the growth of the yeast transformants without the supplementation of cysteine in the SC medium.

RESULTS

Expression analysis of *M. grisea* and *M. graminicola HPD4* TAGKO mutants revealed the presence of chimeric mRNA caused by Tn7 end signals

To understand how TAGKO could alter target gene expression, we investigated the transcription events of *HPD4* mutant alleles in *M. grisea* and *M. graminicola*. The *HPD4* genes in the two fungi are both intronless. Two TAGKO alleles with transposon inserted in opposite orientations were selected for analysis in each fungus (Fig. 1A). In the *M. grisea* KO1 mutant, the TAGKO cassette was inserted in the 3'-untranslated region (UTR), while in the KO2 mutant, the transposon was inserted within the coding region. The two *M. graminicola HPD4* mutants have transposon insertions in different locations along the coding region.

Northern analysis showed that the WT *HPD4* transcripts are approximately 1.7 kb and 1.6 kb in length in *M. grisea* and *M. graminicola*, respectively (Fig. 1B). The *M grisea* KO1 mutant produces a transcript with a similar size to that of the WT, while the

KO2 mutant produces a transcript of only 1.3 kb in length. Similarly, *M graminicola* *HPD*4 KO1 and KO2 mutants produce shortened transcripts of 0.8 kb and 1.5 kb in length, respectively. The transcript lengths for the *HPD4* TAGKO alleles appear to be related to the locations of the transposition event. Thus, shorter transcripts are produced in mutants with TAGKO inserted further upstream along the *HPD4* genes.

The 3' ends of the *HPD4* transcripts from the different TAGKO alleles were mapped using the RACE procedure (Materials and Methods). The 3'-UTRs in the WT *HPD4* transcripts are 208 nt and 128 nt in length in *M. grisea* and *M. graminicola*, respectively (data not shown). All the TAGKO transcripts are chimeric, containing the endogenous *HPD4* sequences up to the insertion site, followed by sequences derived from either the left arm (Tn7-L) or the right arm (Tn7-R) of the transposon cassette (Fig, 1C). Depending on the transposition orientation, 147 bases of Tn-7L or 140 bases of Tn-7R were fused to the 3'-ends of the TAGKO transcripts in both fungi (Fig. 1C). In addition, these transcripts were all polyadenylated. Examination of the Tn7 ends revealed the presence of 3'-site formation signals, ATTAAA in Tn-L and AATAAA in Tn-R (Fig. 2), which are located upstream of the poly(A) tails. Both of the Tn7 end regions also contain stop codons (TAA, TAG, or TGA) in all three reading frames parallel to the direction of the target genes. Thus, fusion gene products truncated at the C-terminus are expected to be translated from the chimeric transcripts in the mutants.

Severity of mutant HPD4 phenotypes correlates to the length of the truncated gene products

We attempted to correlate the mutant phenotypes to the different predicted HPD4 TAGKO proteins. The *M grisea* and *M graminicola HPD4* genes both encode a protein of 419 amino acids in length (Fig. 3A). The *M. grisea* KO1 mutant was expected to produce a WT protein since the TAGKO insertion occurred downstream of the endogenous stop codon. In the *M. grisea* KO2 mutant, the truncated *HPD4* coding sequence is in frame with the first stop codon in the Tn7-L end (Fig. 1C). Thus, the predicted TAGKO protein contains 273 amino acids with a C-terminal extension of 5 amino acids derived from Tn7 (273 + 5 aa; Fig. 3A). Similarly, the *M. graminicola* KO1 and KO2 mutants produce HPD4 TAGKO proteins with 137 + 5 aa and 378 + 17 aa, respectively (Fig. 3A).

Tyrosine and phenylalanine are metabolized through a conserved pathway involving *p*-hydroxyphenylpyruvate dioxygenase (*HPD4* gene) (22, 23, 24). The enzyme converts hydroxyphenlpyruvate, an immediate metabolite of tyrosine, into homogentisate which is further metabolized by other enzymes in the degradation pathway. Disruption of this pathway often leads to the accumulation of toxic intermediates that impair normal growth and development (22). Our growth experiments showed that the WT strains of both *M. grisea* and *M. graminicola* were able to grow on medium supplemented with tyrosine (Fig. 3B). In contrast, all the TAGKO strains expected to produce truncated HPD4 proteins showed limited growth on tyrosine (Fig. 3), indicating that the enzymes were either partially functional or non-functional. The *M. grisea* KO1 mutant was expected to make a full-length HPD4 protein, thus retaining the WT phenotype (Fig. 3).

Yeast CBS mutants can be complemented by *M. grisea CBS1* cDNAs

Cystathionine β-synthase (CBS) is involved in the transsulfuration pathways that allow the inter-conversion of cysteine and methionine (25). *M. grisea CBS1* is a functional and structural homolog of the *S. cerevisiae* CBS gene (18). We have generated three different TAGKO *CBS1* mutants in *M. grisea* (Fig. 4A). Like the *HPD4* mutants, the *CBS1* TAGKO mutants produce 3'-truncated transcripts which are chimeric and polyadenylated (data not shown). In yeast, cysteine biosynthesis occurs exclusively through the pathway involving the enzyme CBS (26). Thus, null mutants of CBS are auxotrophic for cysteine.

Expression constructs containing *CBS1* cDNA isolated from different *M. grisea* strains were transformed into a yeast CBS null mutant (Fig. 4B). As shown in Fig. 4C, growth of the yeast mutant in the absence of cysteine was rescued by the expression of WT, KO1 and KO2 *CBS1* cDNAs from *M. grisea*. However, KO3 failed to reverse the mutant phenotype in yeast, indicating that the CBS1 TAGKO protein was not functional. In fact, almost 200 endogenous amino acids were removed from the C-terminus in the KO3 TAGKO protein. The gene product of *CBS1* KO2 (Fig. 4D) was predicted to be identical to that of the wild type since the transposon insertion occurred downstream of the endogenous stop codon. The KO1 TAGKO protein (Fig, 4D), which was truncated by 65 endogenous amino acids at the C-terminus, was still functional. Interestingly, a human CBS protein with 145 amino acids truncated at the C-terminus was also catalytically active (26). The C-terminal domain of human CBS inhibits the enzyme

activity, which is regulated positively by S-adenosylmethionine (26). Our findings suggest that a similar functional domain may be present in the *M. grisea* CBS1 protein.

DISCUSSION

Transposons have been conveniently and widely used as insertional elements, providing physical markers, and transcriptional and translational fusions to target genes (6). In this study, we uncovered a novel feature in Tn7 through the analysis of TAKGO alleles in *M. grisea* and *M. graminicola*. TAGKO insertion affects the expression of target genes in the fungi by generating 3'-end truncated transcripts that are chimeric and polyadenylated. Interestingly, the TAGKO transcripts contain cryptic eukaryotic polyadenylation signals that are derived from the Tn7 ends (Tn 7-L, ATTAAA ; Tn7-R, AATAAA). A number of eukaryotic transposons are known to contain 3'-site formation signal that causes premature polyadenylation of target gene transcripts, e.g. gypsy (*Drosophila*, 27), Mu1 (maize, 28), and Fot1 (*Fusarium oxysporum*, 15). As a result, truncated transcripts are often detected for the interrupted genes. To our knowledge, this study represents the first report of a bacterial transposon carrying cryptic sequence information for 3'-end formation in eukaryotic transcripts.

Polyadenylation is an essential step in the maturation of mRNA in eukaryotic cells. Following transcription, the 3' ends of mRNAs are processed by endonucleolytic cleavage and addition of a poly(A) tail. The precise mechanisms of mRNA 3'-end formation in filamentous fungi are yet to be defined (29, 30). Nevertheless, it is generally believed that polyadenylation promotes the initiation of translation and the export of

mRNA from the nucleus (31). Polyadenylation also confers stability upon mRNA and its removal precedes the degradation of certain mRNA species (31). The poly(A) tails are frequently located 10 to 30 nucleotides downstream of the polyadenylation signal "AAUAAA". While the hexanucleotide is not an absolute feature, consensus or related sequences are present in a number of filamentous fungal genes (29). In our studies, the sequence "AAUAA" is present 12 nucleotides upstream of the poly(A) tail in the *HPD4* transcript of *M. graminicola* (data not shown). Similarly, the sequence "AUAAA" was found 33 nt upstream of the poly(A) tail in the *HPD4* transcript of *M. grisea* (data not shown). Thus, we reason that the cryptic poly(A) signals identified in the Tn7 ends are compatible with the endogenous mechanisms for mRNA 3'-site formation in filamentous fungi.

Transcripts containing premature stop codons are liable to translate to defective proteins with potentially deleterious effects. In both prokaryotes and eukaryotes, mRNA transcribed from genes with nonsense mutations are degraded rapidly. Such "nonsense-mediated mRNA decay" could lead to significant reduction in the abundance of specific mRNA species (32). Preceding the putative polyadenylation signals, the Tn7 transposon ends have stop codons in all three reading frames (Fig. 2). However, the steady state levels of the TAGKO transcripts, as revealed by northern analysis (Fig. 1), did not appear to be significantly lower than those of the WT transcripts. We reason that nonsense-mediated decay is likely to be hindered or prevented by the premature polyadenylation of the TAGKO transcripts. Such mechanism could avoid the presence of aberrantly long 3'UTRs that would otherwise lead to rapid mRNA degradation (33). Thus, *in vivo*

studies of truncated gene products are possible in the TAGKO mutants, presenting a powerful tool for dissection of gene functions. For example, an allelic series of TAGKO mutants can be generated to identify functional domains by studying mutant phenotypes (Fig. 3). Truncated transcripts can be isolated from the mutants and analyzed in yeast complementation assays (Fig. 4), and *in vitro* activity and binding assays. In addition, leaky mutations could be resulted from truncation of gene products and are useful for the analysis of lethal genes.

Our TAGKO system generates truncated proteins with C-terminal Tn7 derived extensions. At present we do not have conclusive evidence to suggest whether these amino acid extensions would affect protein functions. At least in the case of *M. grisea CBS1* KO1, the 9-aa Tn7-R derived extension appears to be compatible with the enzyme function. The stop codons in Tn7-R are relatively close to the terminal end and the resulting extensions in all frames contain less than 10 amino acids. On the other hand, the longer Tn7-L derived extension (17 aa) in the *M. grisea* HPD4 KO2 mutant might have inhibited the function of the TAGKO protein in which 41 endogenous amino acids were removed (Fig. 3). In fact, truncated proteins in frame with the third codon in Tn7-L (Fig. 2) would have the longest C-terminal extension (22 aa).

Biery et al. (6) found that a miniTn7 element composed of only the terminal 70-bp Tn7-R fragment at both ends are sufficient to allow active *in vitro* transposition reactions. Importantly, the cryptic polyadenylation signal and the 3 stop codons are all located in this region (Fig. 2). In addition, nucleotide substitutions could be made to the

miniTn7 end sequences without affecting transposition efficiencies (6). Therefore, to refine our TAGKO system for protein function analysis in filamentous fungi, it is possible to engineer the stop codons closer to the terminal ends in a miniTn7 element to generate truncated proteins with minimal amino acid extensions.

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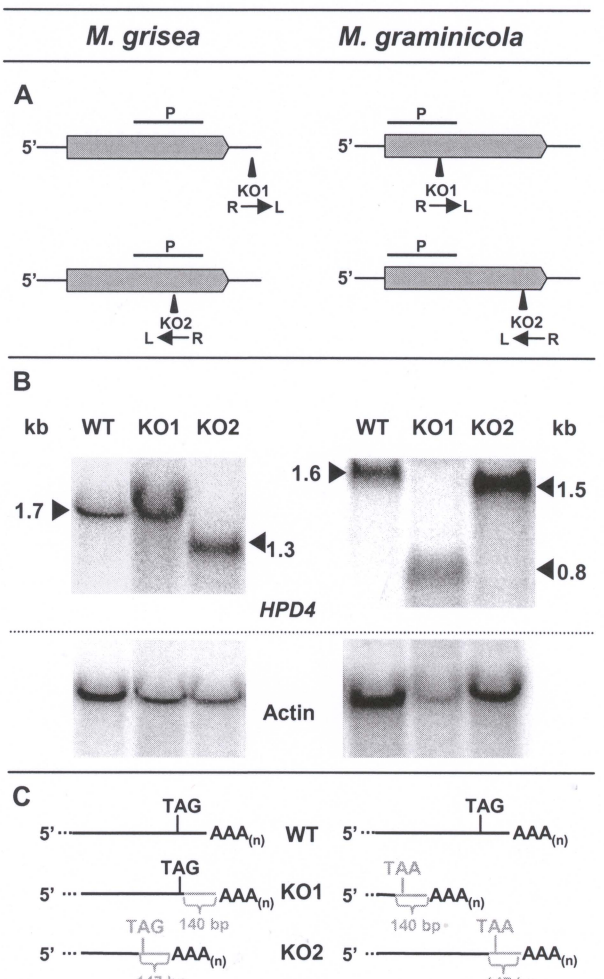
FIGURE LEGENDS

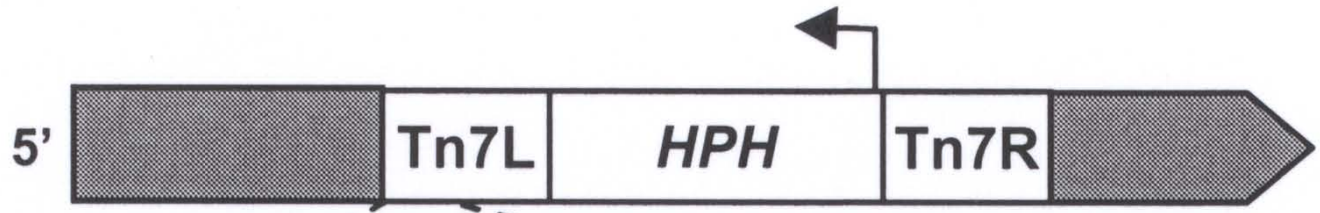
FIGURE 1. Chimeric, polyadenylated *HPD4* transcripts in *M. grisea* and *M. graminicola*. A. Two *HPD4* mutant alleles, KO1 and KO2, are presented in each fungus. Block arrows represent the *HPD4* coding regions. The orientation of the TAGKO transposon cassette is shown in arrows (Tn7-R, R → Tn7-L, L). P1 and P2 denote PCR-derived probes used in northern hybridization experiments. B. Northern analysis of *HPD4* gene expression. Approximately 10 µg of total RNA was used for each strain. Actin genes served as positive controls for gene expression in the two fungi. C. Mapping of the 3'-ends of *HPD4* cDNAs. TAGKO transcripts are chimeric and polyadenylated. Endogenous *HPD4* sequences are shown in dark. Sequences derived from Tn7 transposon ends are shown in grey, and stop codons in frame with the truncated *HPD4* coding sequences are indicated. Numbers in parenthesis represent the first (1) or second (2) stop codon identified in the respective Tn7 end.

FIGURE 2. Examination of transposon end sequences in the TAGKO cassette. An *HPH* gene was engineered between the transposon ends (Tn7-L and Tn7-R). Two possible orientations of transposon insertion into a gene (block arrow) are presented. Arrows indicate the direction of the *HPH* gene. Tn7-L (147 bp) or Tn7-R (140 bp) sequences that appeared in the 3'-ends of the TAGKO cDNAs (Fig. 1C) are shown below each diagram. Putative polyadenylation signals are in bold face, and stop codons in three different reading frames are underlined.

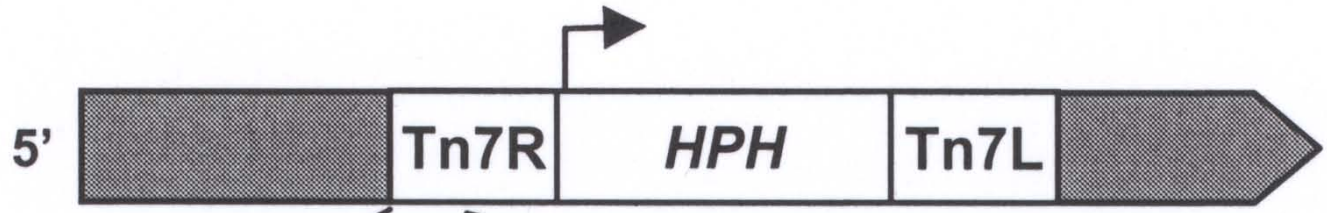
FIGURE 3. Phenotypes of *HPD4* TAGKO mutants. A. Predicted *HPD4* gene products. Numbers in parenthesis indicate the length of truncated HPD4 protein. Sequences derived from Tn 7 transposon ends in the TAGKO proteins are shown in grey. B. Plate images showing the growth of *M. grisea* (after 5 days) and *M. graminicola* (after 8 days) strains, on MM with 4 mM of tyrosine. Note the limited growth in *M. grisea* KO2, and *M. graminicola* KO1 and KO2 mutants. *M. grisea* KO1 mutant showed WT phenotypes.

FIGURE 4. Yeast complementation assays for *M. grisea CBS1* TAGKO cDNAs. A. Mutant alleles of the *M. grisea CBS1* gene. Transposition orientations are indicated by arrows. Stop codons in frame with the truncated *CBS1* coding sequences are indicated, and numbers in parenthesis represent the first (1) or third (3) codon identified in Tn7-R. B. *S. cerevisiae* CBS mutant (Sc 6696) was transformed with different CBS expression clones (MgCBS). *CBS1* cDNAs, isolated from WT and TAGKO *M. grisea* strains, were cloned into yeast expression vectors. Sc 6696 transformed with the indicated vector was grown on yeast peptone dextrose (YPD) medium. C. The yeast transformant strains were grown on cysteine-depleted (-CYS) medium. Sc 6696 is a cysteine auxotroph. MgCBS WT, KO1, and KO2 rescued the yeast mutant phenotype, while MgCBS KO3 did not have any effect. D. Predicted *CBS1* gene products. Numbers in parenthesis indicate the length of truncated CBS1 protein. Sequences derived from the Tn7 ends are shown in grey.


M. grisea
M. graminicola
A
P
5'
KO1
R → L
KO2
L ← R
B
kb
WT
KO1
KO2
1.7
1.3
1.6
1.5
0.8
HPD4
Actin
C
TAG
AAA(n)
WT
KO1
KO2
TAA
140 bp
147 bp



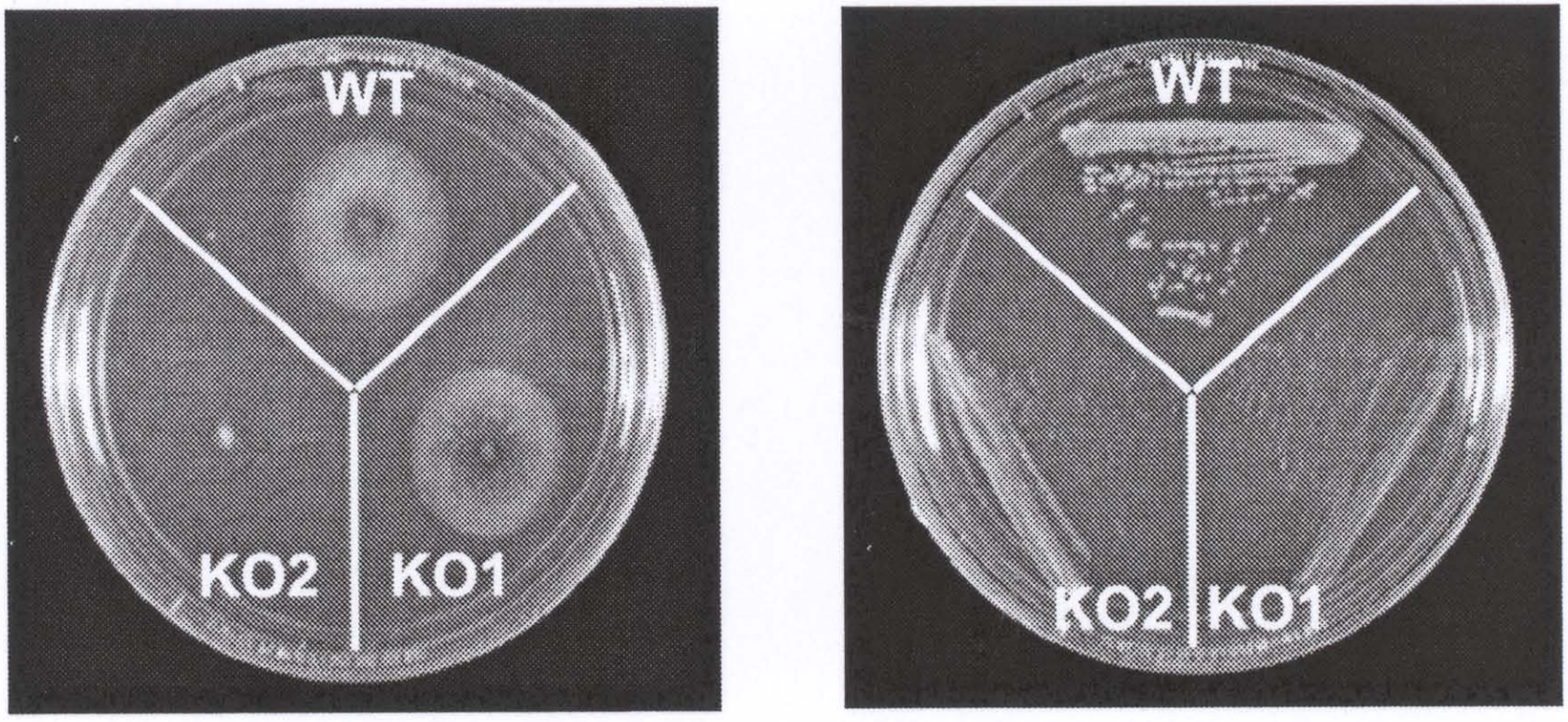
5′ TGTGGGCGGACAAAATAGTTGGGAACTGGGAGGGGTGGAAATGG
AGTTTTTAAGGATTATTTAGGGAAGAGTGACAAAATAGATGGGAAC
TGGGTGTAGCGTCGTAAGCTAATACGAAA**ATTAAA**AATGACAAAAT
AGTTTGGAACTaaaaaaaaaa$_{(n)}$



5′ TGTGGGCGGACAATAAAGTCTTAAACTGAACAAAATAGATCTAA
ACTATGAC**AATAAA**GTCTTAAACTAGACAGAATAGTTGTAAACTGA
AATCAGTCCAGTTATGCTGTGAAAAAGCATACTGGACTTTTGTTAT
GGCTaaaaaaaa$_{(n)}$

M. grisea *M. graminicola*

A

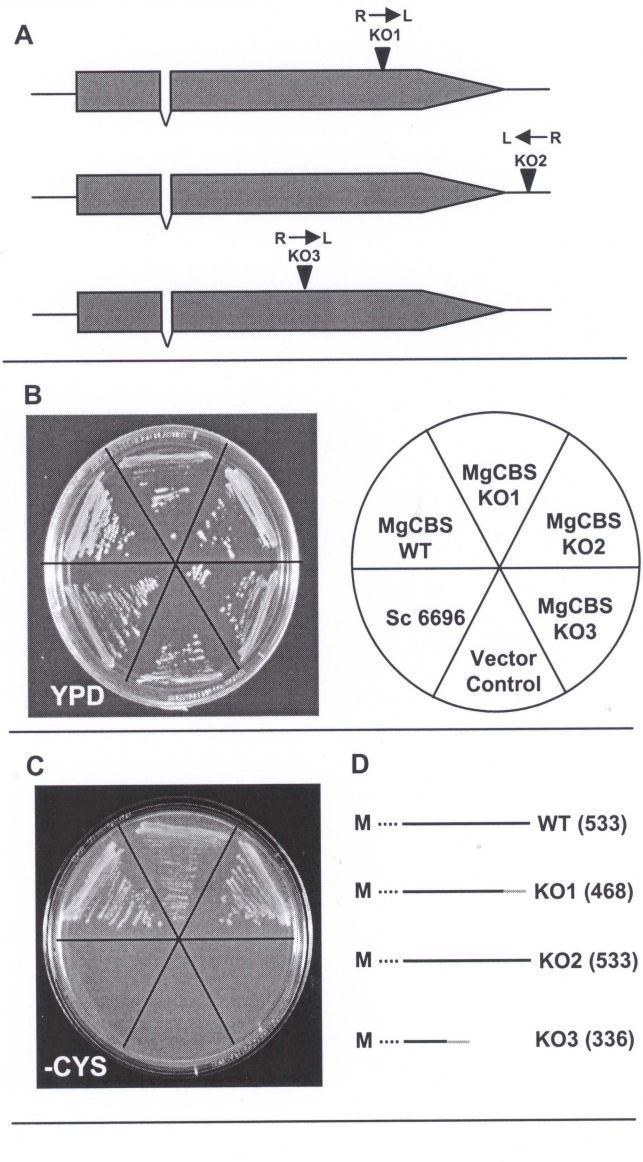


B

M ···——— WT (419 aa) M ····——— WT (419 aa)

M ···——— KO1 (419 aa) M ····——— KO1 (154 aa)

M ····——— KO2 (278 aa) M ····——— KO2 (383 aa)


A
R→L
KO1
L←R
KO2
R→L
KO3
B
YPD
MgCBS KO1
MgCBS KO2
MgCBS KO3
Vector Control
Sc 6696
MgCBS WT
C
-CYS
D
M
WT (533)
KO1 (468)
KO2 (533)
KO3 (336)