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P. G. Mullin
Cornell University

B. G. Turgeon
Cornell University

O. C. Yoder
Cornell University

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Abstract

Transformation systems for most filamentous fungi are based on selection for drug resistance. This strategy is advantageous becasue wild-type strains, including isolates collected directly from the field, can be used as recipients in transformation experiements.

Complementation of *Cochliobolus heterostrophus trp*- mutants produced by gene replacement

P.G. Mullin, B.G. Turgeon and O.C. Yoder - Department of Plant Pathology, Cornell University, Ithaca NY 14853

Transformation systems for most filamentous fungi are based on selection for drug resistance. This strategy is advantageous becasue wild-type strains, including isolates collected directly from the field, can be used as recipients in transformation experiements. Drug resistance as a selection strategy is limited for those fungi which are insensitive to most drugs since the number of selectable markers available for sequential indroduction of genes is reduced. An alternative approach is complementation of an auxotroph with a cloned gene encoding the missing enzyme, followed by selection for prototrophic growth. Auxotroph complementation is widely used with genetically developed fungi, but most economically important species lack the requisite auxotrophic strains. In this study, we produced an auxotroph by mutating the native copy fo the tryptophan synthase, TRP1 (Turgeon et al. 1986 Gene 42:79-88), of the plant-pathogenic fungus *Cochliobolus heterostrophus*. The resulting trp- strain was readily transformed to prototrophy using TRP1. This adds auxotroph complementation to the drug resistance [hygromycin B and the *hygB* gene (Turgeon et al. 1987 Mol. Cell. Biol. 7:3297-3305), bialaphos and the *bar* gene (Straubinger et al. 1992 Fungal Genet. Newsl. 39:82-83)] and substrate utilization [acetamide and the *amdS* gene (Turgeon et al. 1985 Mol. Gen. Genet. 201:450-453)] systems available for transformation of *C. heterostrophus*.

The sequence of *C. heterostrophus* TRP1 has been deposited (EMBL Data Library Accession Number X70035). There is an open reading frame (Figure 1) extending from bp 544 to bp 2989 (including the stop codon), interrupted by an apparent intron (140 bp) from position 863 to 1002. At the amino acid level, the gene has 62% and 64% identity to the corresponding genes from *Aspergillus nidulans* (*trpC*) and *Neurospora crassa* (*trp-1*).

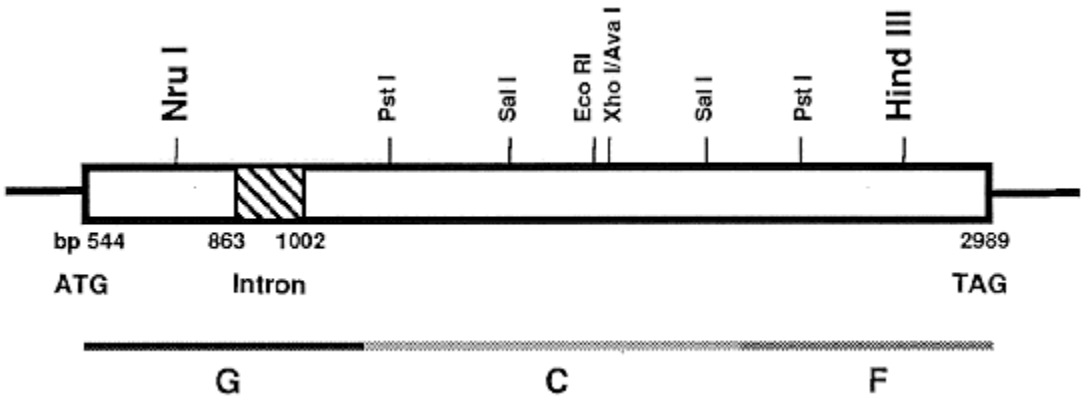

Figure 1. Structure of the *C. heterostrophus* TRP1 gene, which encodes a trifunctional polypeptide that performs three separate enzymatic conversions in the tryptophan biosynthetic

pathway (G=step 1, glutamine amidotransferase; C=step 4, indole glycerol phosphate synthase; F=step 3, phosphoribosyl anthranilate isomerase); the direction of transcription is from left to right. The TRP1 sequence is similar to that of *trpC* from *Aspergillus nidulans* and *trp-1* from *Neurospora crassa*, except that only the *C. heterostrophus* gene contains an intron. Six-base-pair restriction enzyme recognition sites are shown; large bold letters indicate sites of introduced frameshift mutations. Base pair numbering refers to the sequence as listed in the EMBL database (Acc. no. X70035)

Sequence and restriction enzyme site data were used to construct pTK2 (Figure 2), a vector designed for introduction of a point mutation into TRP1. It is based on plasmid pGB2 (Churchward et al. 1984 Gene 31:165), which has no homology to pBR322 and can be used when interaction with pBR322-based vectors is to be avoided. pGB2 was digested with *Sma*I and a 3.8 kb *Bam*HI-*Pvu*II fragment containing TRP1 from pChTrp24B (Turgeon et al.1986 Gene 42:79-88) was inserted, eliminating the *Sma*I site. The new plasmid, pETE3, was digested with *Nru*I, which cuts once in the coding region (bp 734, in the G domain) of TRP1; *Sma*I linkers were added and the plasmid religated. Insertion of linkers into the *Nru*I site introduced a frame-shift mutation, which was confirmed by restriction mapping and sequence analysis. This plasmid, pTKG, was then partially digested with *Hin*dIII and end-filled. Restriction anayisis identified a plasmid, pTKGF, which contained a mutation in the *Hin*dIII site at the 3' end of the coding region (bp 2653) of TRP1 (Fig. 2). pTKGF was digested with *Hind*III, blunt-ended and ligated to *Nru*I digested pDH25 (Cullen et al.1987 Gene 57:21-26). The final construction, pTK2, contained the hygromycin B phosphotransferase gene from *Escherichia coli* with 5' and 3' regulatory sequences from *A. nidulans trpC* and the *C. heterostrophus trp1* gene with frame shift mutations in the 5' and 3' regions of the ORF.

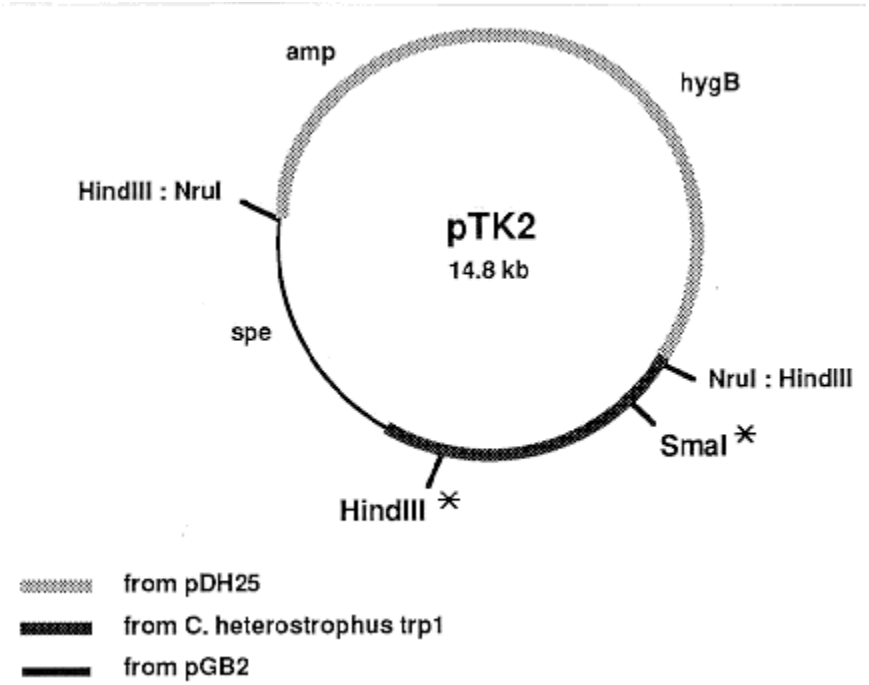

Figure 2. Structure of pTK2, constructed as described in the text. Asterisks indicate positions of point mutations introduced into the TRP1 ORF. *amp* and *spe*: *E. coli* genes for resistance to

ampicillin and spectinomycin; *hygB*: *E. coli* gene for resistance to hygromycin B, fused to *A. nidulans trpC* initiation and termination signals for selection in fungi.

A *trp1* mutant strain of *C. heterostrophus* was constructed by two-step gene replacement. Protoplasts of wild-type strain C3 (tox1-, MAT1-2; ATCC 48330) were transformed (Turgeon et al. 1987 Mol. Cell. Biol. 7:3297-3305) with pTK2. Seventy two hygromycin B resistant transformants were purified by single conidiation and screened for ability to grow on complete medium in the absence of supplemental tryptophan. Nearly 20% were unable to grow on unsupplemented complete medium, but grew normally (as compared with the untransformed strain C3) on medium containing either tryptophan (4 mM final concentration) or indole (25 ug/ml). These Trp- transformants retained their resistance to hygromycin B. Southern analyses of genomic DNAs from the transformants revealed both homologous and ectopic integrations. Transformants with signals which matched those predicted for homologous integration of a single copy of pTK2 at TRP1 (which resulted in a recombinant chromosome with two copies of *trp1*: one carrying the 5' mutation and one carrying the 3' mutation, separated by vector sequences) were crossed to *C. heterostrophus* strain C2 (alb1 Tox1+ MAT1-1; ATCC 48329); the goal was to evict one copy of trp1 and the invervening vector DNA. Mating medium was supplemented with indole (25 ug/ml); no viable ascospores were found when mating medium was not supplemented or contained 4 mM tryptophan. One of the trp1- hygBR transformants, pTK2.4.4.4, yielded progeny that were predominantly parental but also included progeny which were trp1-, hygBS. Southern analysis of one of these ascospore isolates, D1.51 (alb1, hygBS, MAT1-1, Tox1+, trp1-) confirmed that one copy of *trp1* and all vector sequences had been evicted. The chromosome of this isolated was identical to that of wild type except that the 3' *Hin*dIII site in the F domain of TRP1 was missing, as expected.

The *trp1*- mutant of *C. heterostrophus* was transformed to prototrophy with TRP1. Protoplasts from TMHS24 (a pigmented backcross progeny derived from D1.51) were treated with plasmid DNA carrying TRP1 (e.g. pChTRP24B). Southern analyses of genomic DNAs isolated from transformants showed both homologous and ectopic integrations of transforming plasmid resulting in prototrophy.

Availability of two-step gene replacement for *C. heterostrophus* means that it should be possible to assess the biological function of any cloned single-copy gene from this fungus by replacing the wild-type allele with a copy that has been specifically altered in vitro. In addition, complementation of the *trp1*- auxotroph with the cloned TRP1 gene provides an alternative transformation strategy for *C. heterostrophus*. Approximately 70% of all integration events are the result of homologous integration of the transforming DNA at TRP1. For comparison, homologous integration with promoter 1 from *C. heterostrophus* (Turgeon et al.1987 Mol. Cell. Biol. 7:3297-3305) is approximately 85% when 1 kb of homologous DNA is carried on the transforming plasmid. Random integration occurs when the transforming plasmid contains no *C. heterostrophus* DNA (Turgeon et al. 1985 Mol. Gen. Genet. 201:450-453).