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Characterization of the regulatory gene, *mpkB*, by overexpression studies in the model fungus, *Aspergillus nidulans*

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Abstract

The main goal of this research was to understand the function of the mpkB gene (a mitogen activated protein kinases gene, MAPK) in the model fungus *Aspergillus nidulans*. Due to the similarity in DNA sequence with other homologous genes found in other fungi, such as Fus3 and Kss 1 genes in yeast, it is predicted that *mpkB* will also have a similar function. This research was conducted by transforming *A. nidulans* with a plasmid containing the *mpkB* gene attached to a strong inducible promoter, *alcA*. The plasmids also contain an auxotrophic marker for selection purposes, trpC. Results of this study will provide a better understanding of signal transduction pathways in filamentous fungi and how they affect processes such as mating in fungi. Since the genus Aspergillus includes species of great importance in medicine and agriculture, these results could also have a significant impact on these fields.

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The main goal of this research was to understand the function of the mpkB gene (a mitogen activated protein kinases gene, MAPK) in the model fungus *Aspergillus nidulans*. Due to the similarity in DNA sequence with other homologous genes found in other fungi, such as Fus3 and Kss 1 genes in yeast, it is predicted that *mpkB* will also have a similar function. This research was conducted by transforming *A. nidulans* with a plasmid containing the *mpkB* gene attached to a strong inducible promoter, *alcA*. The plasmids also contain an auxotrophic marker for selection purposes, trpC. Results of this study will provide a better understanding of signal transduction pathways in filamentous fungi and how they affect processes such as mating in fungi. Since the genus Aspergillus includes species of great importance in medicine and agriculture, these results could also have a significant impact on these fields.

Introduction

External stimuli cause a cell to tranduce signals that can then trigger necessary physiological responses. Mitogen activated protein kinase (MAPK) cascades are crucial components in this signal transduction (Gustin *et al.*, 1998). This could include, but is not limited to, the ability to direct or influence cell functions necessary for life (Kawasaki *et al.*, 2002). The model filamentous fungus, *Aspergillus nidulans* was used to study the function of the MAPK gene, *mpkB*.

Figure 1. Signal Transduction pathway of Fus3 responsible for pheromone responses.

Due to the similarity in DNA sequence with other homologous genes found in other fungi and because a pattern of similarity has been observed with the basic mechanism of the signaling pathways of different MAPKs, it is predicted that *mpkB* would have a close resemblance to the functions at least two other MAPK genes, Fus3 and Kss 1.

The FUS3 gene can be found in the yeast *Saccharomyces cerevisiae*, where MAPKs responsible for pheromone responses have been studied extensively (Gustin *et al.*, 1998). After a series of actions and responses, the Fus3 gene is activated which is responsible for then activating the transcriptional activator Ste12; a homolog of Ste12 has also been found and characterized in *A. nidulans* (Tedford *et al.*, 1997). Figure 1 describes the signal transduction pathway of Fus3. These series of processes control the expression of genes responsible for the regulation of cell fusion, cell cycle arrest and morphological changes. Without the Fus3 gene, gene pheromones and pheromone receptors involved in mating and cell division would not be able to recognize mating partners or nuclei before meiosis. The absence of this gene would cause the organism to inhibit the organism's reproduction ability. Any disruption in the transduction pathway could lead to undesirable effects (Wei *et al.*, 2003).

Figure 2. Signal Transduction pathway of Kss 1 responsible for filamentation and invasion.

Using the identical transcription factor, Ste 12, the Kss 1 gene is believed to initiate filamentous growth in yeasts (Tedford *et al.*, 1997). Other responsibilities designated to Kss 1 include functioning as a positive regulator of the pseudohyphal response and taking part in the pheromone response pathway (Gustin *et al.*, 1998). Figure 2 describes the signal transduction pathway of Kss 1.

MAP kinases also serve vital roles in other fungi. For example, in *Claviceps purpurea*, a rye pathogen and a homologue of *mpkB*, mutants that have the MAP kinase deleted from its genes demonstrate damaged vegetative behaviors and a drastically decreased virulence (Mey *et al.*, 2002). In *Magnaporthe grisea*, a rice pathogen and another homologue of *mpkB*, MAP kinases directs the formation of appressoria. No matter the organism, MAP kinases, in general, are engaged in many necessary developmental courses, of which include pathogenicity, mating and sporulation (Mey *et al.*, 2002).

There are two main paths in finding the function of the *mpkB* gene. The gene can either be deleted from the genome, or it can be over-expressed. In both cases, observations of phenotypical changes should reveal clues indicating the gene function. This research specifically deals with studying the effects of over-expressing the *mpkB* gene in *A. nidulans*.

The *A. nidulans* alcA promoter [*AlcA*(p)], functioning as a key factor in the over-expression of the *mpkB* gene, is known to be reliable and controllable. This explains why AlcA(p) is extensively used in this fungus for over-expression studies. *AlcA(p)* allows a gene to be scrutinized at numerous phases of growth by taking into advantage the ability to turn on and off gene expression through alterations in environmental conditions. For example, *it* can be strongly induced by ethanol and threonine, and in the absence of glucose as carbon source. On the other hand, complete repression takes place in the *alcA* gene when glucose is present (Romero *et al*, 2003).

Experimental Procedures

Fungal strains and growth conditions - *Aspergillus nidulans* strain used was RDIT 55.27, an auxotroph to tryptophan, pyrodoxin, and methionine (*pyroA4; meth-; trpC801, veA*+). The strain was grown on glucose minimal medium (GMM) in addition to the necessary supplements corresponding to the auxotrophic markers (Calvo *et al*, 2001). Incubation temperature was at 37°C, unless specified otherwise, in continuous dark light.

Construction of the mpkB overexpression strain - Overexpression was achieved using similar procedures as described in Shimizu *et al*, 2001. A 2 kb dsDNA fragment, corresponding to the *mpkB* gene coding region, was obtained by PCR using cosmid 11 which contains *mpkB* (from an A. nidulans genomic library purchased from the Fungal Genetics Stock Center, FGSC) as template and the the following primer set: primer #66 5'-CATTTTACCTCGCATGCGTGC-3' (containing an artificial *Sph* I site, underlined) and #67 5'-GCGCAGTTCATGCTCGAGCA-3' (containing an artificial *Xho* I site, underlined). The information necessary to design these primers was obtain from the A. nidulans genome database. The *Sph* I and *Xho* I restriction sites

were engineered in the PCR primers to facilitate the ligation procedure. The 2 kb fragment was integrated at the *Sph* I and *Xho* I restriction sites of pCN2 using sticky ended ligation. The product of this ligation was the *mpkB* coding region fused into the *alcA(p) (alcA(p)::mpkB)*, generating the transformation vector pMMS1. This newly formed plasmid was then introduced into the *A. nidulans* RDIT 55.27 by fungal transformation following a procedure described by Miller *et al*, 1991. The colonies were then single-spored to obtain pure cultures. This gave rise to TMMS1.1, a tryptophan prototroph that was isolated containing the *alcA(p)::mpkB* construct. The parallel transformation of pCN2 into RDIT 55.27 served as an isogenic control strain. Doing this will be beneficial during future phenotypic studies for comparison purposes.

Verification using PCR - The integration of pMMS1 into the fungal strain was checked using the primers #86 5'-GGGAGCAACCGTCACTG-3' and #87 5'-CTCTCCGTGCGGACATC-3'. These primers were designed to anneal at the first half of the trpC gene and also at the *alcA* promoter. Genomic DNA from TMMS1.1 AND was used as template in a PCR test. First the DNA was extracted from conidial suspension by a rapid boil/freeze method. The extracted DNA was first freezed in liquid nitrogen, then boiled for five minutes and briefly vortexed. The DNA was then freezed, boiled, and vortexed following the same procedure two more times. After the third time, the sample was centrifuged for five minutes. Then the supernatant containing DNA was diluted 2, 5, 10, 25, and 50 fold and 1 µl from these dilution was added to the PCR. The results of these PCRs were 800 bp DNA fragments. Data not shown.

Verification using Southern Analysis – High quality genomic DNA was extracted from mycelial samples from the isolated transformants (TMMS1, TCN2.X and TCN2.X) obtained from an over-night stationary cultures of liquid GMM plus 5g/L of yeast extract inoculated with conidia.

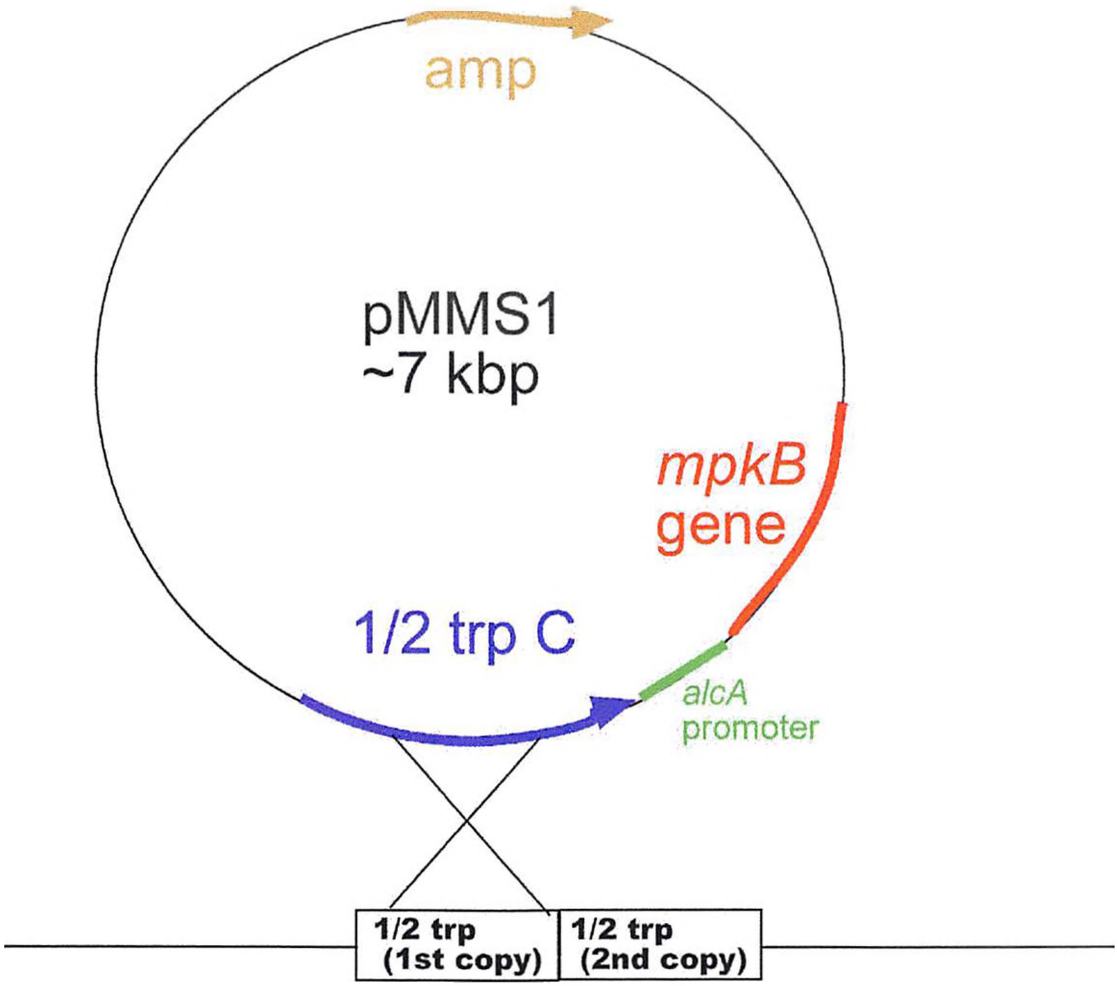

Figure 3. Recombination at the trp C gene.

The genomic DNA samples were digested for five hours with Hind III . Digested DNA was separated by electrophoresis in a 1% agarose gel. The DNA was then blotted to nylon membrane (Hybond) by capillarity using 4x SSC solution over-night. The membrane containing DNA was then cross-linked using a UV Crosslinker at an intensity of 1200 x 100 μ J/CM^2.

Results & Discussion

The original pCN2 plasmid contains only the first ½ half of the *trpC* gene. This ½ *trpC* gene fragment will only remediate the tryptophan auxotrophy caused by a point mutation in the first ½ half of the *trpC* in the RDIT 55.27 by exclusive recombination at the *trpC* locus of this

strain's genome. This molecular tool leads to desirable directed integrations of transformation cassettes at a particular locus. In this selection system, the only way colonies can grow on plates that do not include tryptophan is if a "good full copy" of the tryptophan gene was generated by this integration into the fungal genome, making those colonies tryptophan prototrophs. This step thus concludes that the recombination did occur at the tryptophan gene as desired.

Precisely, this case of recombination is illustrated in Figures 3 and 4. The mutated trpC gene is still present, but is recessive to the wild type copy of the trpC gene. Recombination at the trpC locus also resulted in the integration of the *mpkB* gene directly following the *alcA* promoter, accomplishing our goal of construction an *mpkB* over-expression strain.

After constructing a plasmid with the desired features and transforming it into the chosen fungal strain, verification of the integration pattern (presence of the transformation vector and integration copy number) and the location of incorporation (if more than one copy has been integrated) as a result of the recombination must be checked. This was done by using a Polymerase Chain Reaction (PCR) and Southern Blotting.

The results of the PCR showed positive outcomes with the samples showing a band of about 800 bp PCR product in TMMS1.1, TCN2.3, and TCN2.4. The primers used in this PCR were designed to anneal at locations where both the *alcA* promoter and a tryptophan gene were present. Since the constructed plasmid is the only piece of DNA in the transformant genome that includes both of these components on the same DNA region, the 800 bp PCR product could only be generated if the transformation vector were going to be integrated in the fungal genome. This verifies that the constructed plasmid was successfully transformed and integrated into the *A. nidulans* strain. However, the specific location (if more than one copy is integrated) and the

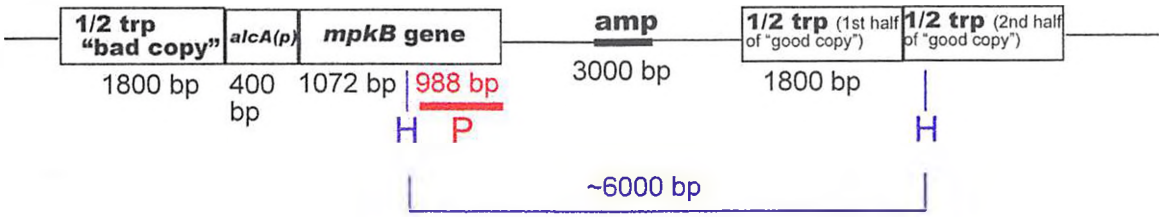

Figure 4. Gene order after recombination at the tryptophan gene. This is the desired order of genes because the mpkB gene directly follows the alcA promoter. Here, "H" signifies the location where the Hind III restriction enzyme should cut. "P" represents the location where the probe is expected to bind.

number of copies of integration cannot be confirmed using PCR, thus leading to the performance of Southern Analysis.

Southern Analysis helps to verify the location of integration and the number of copies present in the transformant. Both transformants, TMMS1.1, TCN2.3, and TCN2.4, were digested. The DNA used in probe is designed to anneal at the second half of the *mpkB* gene (see figure 4). Depending on the number of bands present, the conclusions as to the location of integration and the number of copies present can be made. It is predicted that if a 6 kbp is detected in the hybridization/phosphor-imager analysis, then the recombination occurred at the tryptophan locus as desired. On the other hand, the presence of bands that are 6 kbp and 3 kbp will signify that recombination occurred at both the *mpkB* and the trpC gene.

This is an ongoing, continuous study of the *mpkB* gene in *A. nidulans* that could have considerable impact on the future of our health and agriculture. Especially since related Aspergillus species are of great importance in medicine and agriculture, and considering that

regulatory pathways tend to be conserved in fungi, this study could contribute to the control of the beneficial and detrimental effects of fungi.

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