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Abstract

In *A. nidulans* the *bimG* gene codes for the catalytic subunit of protein phosphatase 1. The wild type *bimG* gene was transformed into *N. crassa* and expressed under the direction of the *alcA* promoter. The heterologous *bimG* mRNA and protein were detected in the transformants by RT-PCR and Western blotting, respectively. However, the transformation did not result in detectable changes in phenotype. This work demonstrates that the *alcA* promoter, a conditional gene expression system widely used in both *Aspergillus* and higher plants, also functions in *N. crassa*.

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Expression of the *Aspergillus bimG* gene in *Neurospora crassa*

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In *A. nidulans* the *bimG* gene codes for the catalytic subunit of protein phosphatase 1. The wild type *bimG* gene was transformed into *N. crassa* and expressed under the direction of the *alcA* promoter. The heterologous *bimG* mRNA and protein were detected in the transformants by RT-PCR and Western blotting, respectively. However, the transformation did not result in detectable changes in phenotype. This work demonstrates that the *alcA* promoter, a conditional gene expression system widely used in both *Aspergillus* and higher plants, also functions in *N. crassa*.

BimG11 was described as a temperature-sensitive recessive mutation that causes the block of mitosis accompanied with the over-phosphorylation of nuclear proteins and distinct morphological changes in *Aspergillus nidulans* (Doonan and Morris, 1989 Cell 57: 987-996). In addition the mutant was defective in cell-wall synthesis at the restrictive temperature (Borgia, 1992 J. Bacteriol. 174: 384-389). Molecular cloning revealed that the *bimG* gene encoded a protein similar to the catalytic subunit of protein phosphatase 1 (PP1c). In agreement with the structural prediction, PP1 activity was reduced in *bimG11* mutants at 40°C (Doonan et al., 1991 J. Biol. Chem. 266: 18889-18894). A single point mutation near the splice-site of intron 2 resulted in the translation of a truncated inactive phosphatase at higher temperature, which was supposed to exert a toxic effect (Hughes et al., 1996 EMBO J. 15: 4574-4583). The rabbit skeletal muscle PP1c cDNA rescued the mutant phenotype, while a *AtPP1b* coding for a PP1c isoenzyme of *Arabidopsis thaliana* affected partial complementation (Arundhati et al., 1995 Plant J. 7: 823-834). The cDNA and gene of PP1c (termed *ppp-1*) has been recently cloned from *Neurospora crassa* in our laboratory (Zeke et al. unpublished results). The predicted amino acid sequence of the PPP-1 protein is highly similar to the *bimG* gene product (96% identity). In order to study the possible application of the *alcA* promoter based conditional expression system in another filamentous fungus, we expressed the wild type *bimG* from *A. nidulans* in *N. crassa*.

We constructed a plasmid (termed pHF3) in which the wild type *bimG* gene was fused at the carboxy-terminus to an influenza haemagglutinin (HA) tag and was placed under the control of the *A. nidulans alcA* (alcohol dehydrogenase) promoter. The construct harbored the *pyr-4* gene of *N. crassa* that was used as a selection marker. A second plasmid (pSRN1) encoding for the AlcR regulatory protein was also used to ensure the efficient gene expression from the *alcA* promoter. A *pyr-4* strain of *N. crassa* (FGSC 4031) was co-transformed with the above two plasmids as described by Vollmer and Yanofsky (1986, Proc. Natl. Acad. Sci. USA, 83: 4869-4873). The transformants were grown at 27 °C for 24 hours in liquid Vogel's minimal medium supplemented either with 1% (v/v) ethanol (inducing the *alcA* promoter) or with 1% (w/v) glucose (repressing condition). Hyphae were harvested by filtration, frozen in liquid nitrogen and stored at -70 °C until use.

The incorporation of the *A. nidulans bimG* gene into the *N. crassa* genome was demonstrated by polymerase chain reaction (PCR). The forward primer: CTTCGTTGGGGGTAAAACGCC hybridized to the 5'-noncoding region of the *bimG* gene, while the reverse primer: ATCTAGAGCGGCCGCACTGAG was specific for the HA tag. One microgram of genomic DNA prepared according to Sambrook et al. (1989, Molecular cloning, 2nd edition, Cold Spring Harbor Laboratories) was amplified with Accu Taq polymerase (Sigma-Aldrich) in 30 cycles according to the following temperature profile: 0.5 min at 94 °C, 1 min at 62 °C, and 1 min at 68 °C. The expected 1444 bp band was detected by ethidium bromide staining in two transformants after agarose gel electrophoresis in 1% (w/v) agarose gels (Fig 1. A).

The expression of the heterologous *bimG* gene was analyzed by PCR after reverse transcription (RT-PCR). Reverse transcription was performed for 45 min at 48 °C with the enhanced avian RT-PCR kit of Sigma-Aldrich. The same primers and temperature profile were used as in the previous PCR experiment for the amplification of 0.3 micrograms of total RNA prepared according to Chomchinsky and Sacchi (1987 Anal. Biochem. 162: 156-159). The 1107 bp *bimG* specific cDNA was visualized with ethidium bromide (Fig. 1. B). Obviously, the control of expression was leaky in the No. 212 transformant, since the mRNA band was detectable even in the presence of glucose. The intensity of the band was elevated under ethanol induction conditions. The *bimG* mRNA was hardly detectable in the No. 214 transformant (not documented) and was absent from the host strain FGSC 4031 (Fig. 1. B).

Western blotting (Harlow and Lane 1988 Antibodies, Cold Spring Harbor Laboratories) was used to demonstrate the translation of the *bimG* mRNA. Fifty micrograms of protein extracted from the No. 212 transformant was separated by SDS PAGE in a 10% (w/v) gel and transferred onto a HybondTM ECLTM nitrocellulose membrane (Amersham Life Sciences). The blot was blocked, challenged with an anti-HA monoclonal antibody (HA.11, BAAbCO), and visualized by a peroxidase conjugated anti mouse IgG second antibody (Sigma) and ECL technique (Fig. 1.C). In accordance with the RT-PCR results a 36-kDa band of the *A. nidulans* BimG protein was visible in the presence of glucose, and the expression of the protein was more pronounced after the addition of ethanol. The host strain exhibited no

cross-reactivity with the antibody. Our results indicate that the *alcA* based expression system operates in *N. crassa* and is moderately inducible by ethanol at both the mRNA and protein levels. The heterologous expression of the *bimG* gene also implies that splicing signals of the foreign gene were functional in *N. crassa*.

Protein phosphatase 1 (PP1) activity was assayed with ^{32}P -labeled rabbit muscle phosphorylase *a* in the presence and absence of inhibitor-1, a specific inhibitor of PP1 (MacKintosh, 1993 in Protein Phosphorylation, pp. 197-230, Oxford University Press). The PP1 activity in the extract of the control FGSC 4031 strain was estimated to be 1.21 ± 0.23 mU/mg (n=14) in the presence of 1% (w/v) glucose, and 1.30 ± 0.31 mU/mg (n=20) in the presence of 1% (v/v) ethanol. In the No.212 transformant the corresponding values were 1.25 ± 0.16 mU/mg (n=19) in the presence of glucose and 1.25 ± 0.19 mU/mg (n=13) under inducing conditions. Thus the expression of the heterologous phosphatase catalytic subunit had no significant effect on the basal PP1 activity of *N. crassa*. The lack of activity change can be explained either by the relatively low level of expression, or by the operation of a compensatory mechanism eliminating the effect of the surplus *A. nidulans* phosphatase. The transformant exhibited normal growing behavior and no visible changes of morphology were discerned by microscopic observation. In agreement with the latter findings ectopic expression of BIMG phosphatase in *Aspergillus* has no phenotypic consequences for growth or development and does not significantly alter the PP1 activity levels (Doonan, unpublished data).

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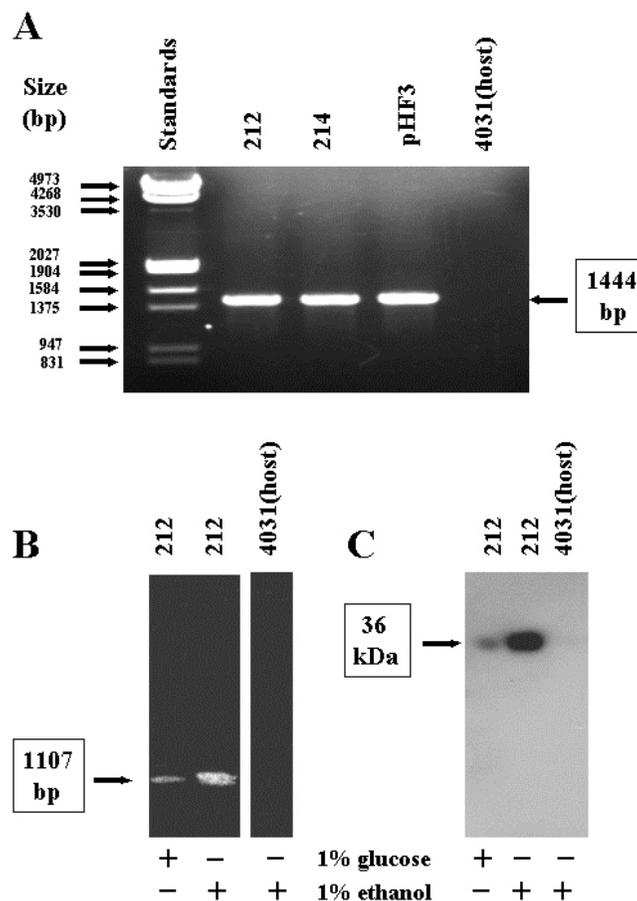


Figure 1. Transformation and expression of the *A. nidulans bimG* gene in *N. crassa*

A. PCR detection of the *bimG* gene in No. 212 and 214 *N. crassa* transformants. The plasmid pHF3 was used as a positive control. **B.** RT-PCR analysis of the *bimG* mRNA in No. 212 *N. crassa* transformant. **C.** Western blot analysis of the BimG protein in the No.212 *N. crassa* transformant. The host strain FGSC 4031 served as a negative control in all experiments. Representative results of three independent experiments are shown.