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Screening of Growth- or Development-related Genes by Using Genomic Library with Inducible Promoter in *Aspergillus nidulans*

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Using the genomic library constructed at the downstream of the *niiA* promoter, which induces the over-expression of an inserted DNA fragment, we have attempted to screen the genes affecting growth or development by over-expression. The wild-type strain was transformed using the AMA-*niiA*(p) library and cultured on 1.2 M sorbitol media, in which asexual sporulation is induced, but sexual development is repressed. Over 100,000 strains transformed to *pyrG*$^+$ were analyzed with regard to any changes in phenotype. Consequently, seven strains were isolated for further analyses. These strains were designated NOT [*niiA*(p) over-expression transformants] stains. Four of the strains were of the inducible type, and the remaining strains were of the multi-copy suppression type. Two of the inducible-type strains, NOT1 and NOT40, harbored genes which had been inserted in reverse direction, suggesting that the mutant phenotypes had been derived from an excess amount of anti-sense mRNA. Domain analyses of the deduced polypeptides from the DNA fragments rescued from the transformants revealed that NOT1, NOT40 and NOT6 harbored a LisH motif, a forkhead domain, and a $Zn(II)_2Cys_6$ binuclear zinc cluster, respectively.

Key words: *Aspergillus nidulans*, NOT mutants, over-expression library, sexual development

Aspergillus nidulans is considered to be a model organism for the study of the genetic control of developmental processes (Timberlake, 1990; Adams *et al.*, 1998). Upon the release of the genome sequence of *A. nidulans*, it became fairly simple to characterize a mass of genes that were involved in growth and development. Also, a variety of genomic tools, including subtractive hybridization, DNA microarray, and RNAi techniques, are now available for the rapid screening of such genes (Cottrell and Doering, 2003; Ray *et al.*, 2004; Sims *et al.*, 2004; Nakayashiki *et al.*, 2005). In an attempt to exploit the new techniques for high-throughput genomic analysis in the context of a functional genomics approach, Osherov and May (2000) constructed a set of libraries using the AMA1 sequence, which enables the library plasmid to be extra-chromosomally maintained at a high copy number. These libraries are advantageous with regard to their high transformation efficiency and ready recovery of transformed plasmid DNA. In addition, the AMA-*niiA*(p) library, which is available at the Fungal Genetics Stock Center (FGSC), features genomic DNA fragments that are inserted downstream of the promoter of the nitrite reductase gene (*niiA*) in either the (+) or (-) direction (Osherov *et al.*, 2000). The *niiA* promoter has been employed as an inducible promoter, which is induced by nitrate (e.g. 0.6 M sodium nitrate) and repressed by ammonium (0.2 M ammonium tartrate) (Yu *et al.*, 1996). Because the inducible promoter is fused upstream of the inserted genomic DNA fragments, the transformants using this library may exhibit a variety of phenotypic changes under the inducible conditions, whereas phenotypic changes would be less likely to occur under repression conditions.

The development of *A. nidulans* is affected significantly by diverse environmental stresses, including exposure to visible light (Moony and Yager, 1990; Han *et al.*, 2003) or high osmolarity (Han *et al.*, 1990; Han and Prade, 2002). These stresses are known to induce asexual development, and to repress sexual development. In this study, we have attempted to screen the genes involved in the decision of development in response to stresses, using the AMA1-*niiA*(p) over-expression genomic DNA library. The wild-type recipient strain was transformed using the AMA1-*niiA*(p) library and the transformants, which did not develop asexual spores, become fluffy, or develop sexual structures on 1.2 M sorbitol medium, were isolated and

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characterized.

Materials and Methods

Strains, culture conditions, and genetic manipulation

A. nidulans FGSC A4 was utilized as the wild-type strain in this study. A recipient strain for transformation, YBL26 (*pyrG89*; *pyroA4*; *veA*$^{+}$), was isolated via the genetic crossing of FGSC A4 and A773 (*pyrG89*; *wA3*; *pyroA4*; *veA1*). *Aspergillus* complete medium (CM) and minimal medium (MM) with the appropriate supplements were prepared according to the previously described method (Pontecorovo *et al.*, 1953; Käfer, 1977). All of the strains were cultured at 37°C. The standard genetic procedures, including heterokaryosis, diploid isolation, and parasexual analysis, were conducted in accordance with the methods of Pontecorovo *et al.* (1953) and Käfer (1977). The PEG-mediated transformation of *A. nidulans* was performed according to the method established by Yelton *et al.* (1984).

DNA manipulation and database submission

The pRG3-AMA1-*niiA*(p) library used in this study was purchased from the Fungal Genetics Stock Center (FGSC). Standard methods were employed in the isolation of the genomic DNA of the *A. nidulans* and the plasmids (Sambrook *et al.* 1989; Lee and Tayler, 1990). In brief, ground mycelia were treated with lysis buffer (Lee and Tayler, 1990). After 1 h of incubation at 55°C, the supernatant resulting from the centrifugation was extracted using phenol/chloroform/isoamylalcohol (25:24:1), followed by ethanol precipitation. The pellet was then re-dissolved in TE, and the purified genomic DNA was used to isolate the plasmid DNA. The plasmids were rescued via the transformation of *Escherichia coli* with the isolated genomic DNA. The ampicillin-resistant *E. coli* transformants were then subjected to transformed plasmid DNA purification. The nucleotide sequence was determined with an automatic DNA sequencer (ABI 377, Perkin-Elmer, USA). The *oefA*, *oefB*, *oefC*, and *fhpA* gene sequences were submitted to the GenBank database. The accession numbers of these sequences are AY792355, AY792356, AY792357, and AY792354, respectively (Table 1).

Microscopy and photography

Photomicrographs of the samples were obtained with an Olympus CH30 microscope, fitted with an Olympus C 2000 digital camera (Olympus, USA).

Results and Discussion

Isolation of NOT strains via transformation of the over-expression library

About 100,000 transformants were examined, and more than 50 isolates exhibited altered phenotypes on the 1.2 M sorbitol medium. These over-expression mutants were designated NOT [*niiA*(p) over-expression transformants] strains. Among them, seven were selected for further characterization on the basis of the availability of the rescued plasmids. The phenotypes of these seven transformants are shown in Fig. 1. The six strains, NOT1, NOT2, NOT4, NOT6, NOT9, and NOT26, all manifested 'fluffy' growth, characterized by the formation of undifferentiated aerial hyphae. However, the other strain, NOT40, developed an abundance of Hlle cell aggregates, which are sexual development-specific structures on 1.2 M sorbitol medium, on which no sexual structures were developed by the wild-type strain (Fig. 2). The NOT1, NOT2 and NOT40 phenotypes were visibly altered in the nitrate medium, but not in the ammonium medium, thereby indicating that the observed phenotypic changes had been elicited by the forced over-expression of the inserted

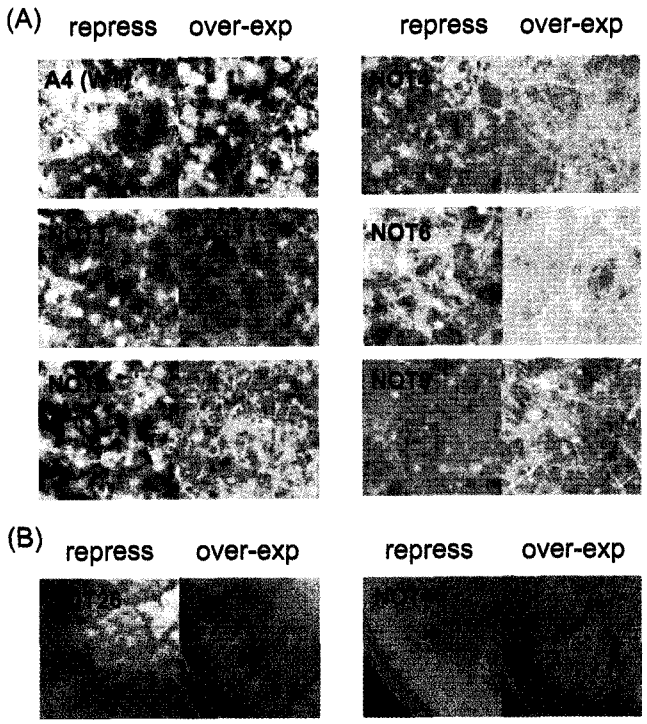

Fig. 1. Morphological changes induced by introduction of the pRG3-*niiA*(p) library. (A) Close-up view of the colony (40X magnification) showed that the NOT1 and NOT2 transformants developed fluffy phenotypes when over-expressed (over-exp), but appeared normal under repression conditions (repress). NOT4, NOT6, and NOT9 transformants produced fluffy hyphae when cultured under repressive conditions, indicating that the introduced multicopy DNA fragments affected cellular development, regardless of whether repression or induction conditions were used. FGSC A4, represented as A4 (WT), was used as a control strain. (B) Plate pictures of NOT26 and NOT40 transformants. NOT26 exhibited a fluffy phenotype under both repression (repress) and induction (over-exp) conditions. The NOT40 strain exhibited a normal phenotype under repressive media conditions (repress), but the sexual development of NOT40 was blocked under overexpression culture conditions (over-exp).

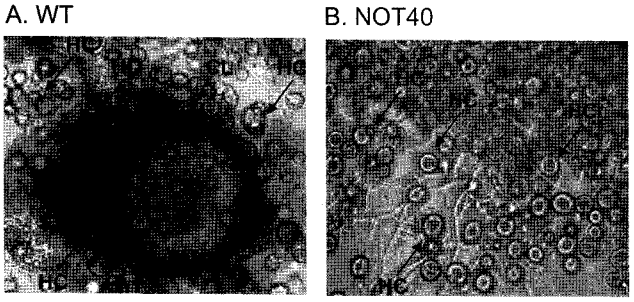

Fig. 2. Impaired sexual development in NOT40 mutant. Wild type strain (A) grown for 3 days at 37°C develops fruiting bodies, i.e. cleistothecia (CL), as well as Hülle cells (HC), specific globular cells associated with sexual development. However, NOT40 mutants (B) grown for a week at 37°C were unable to generate cleistothecia, although the Hülle cells (HC) were produced normally under these conditions.

genes or DNA fragments. However, the phenotypic alteration of the NOT4, NOT6, NOT9, and NOT26 strains was not found to be dependent upon the nitrogen source used, thereby demonstrating that the phenotype alteration was not the result of the over-expression of the insert DNA fragment. Rather, it could be the result of the existence of multiple copies of transforming DNA fragment, which was caused by the AMA1 sequence in the library vector.

Molecular cloning of the oefA, oefB, oefC and fhpA genes

In order to understand the manner in which the inserted DNA fragments induce phenotypic alterations, the transformed library plasmids were rescued from each of the transformants, and then analyzed with regard to their genomic structures. The total DNAs from the transformants were prepared and used to transform the *E. coli* hosts. Both ends of the inserted DNA fragments of the isolated library plasmids were sequenced first, and the total inserted genomic sequences were acquired from the *A. nidulans* genome database, provided by the Broad Institute (http://www.broad.mit.edu/annotation/fungi/aspergillus/).

Analyses of all seven inserted fragments showed that each of the rescued library fragments harbored at least one open reading frame (ORF). All of these ORFs remain, thus far, unidentified. However, due to the difficulty inherent in the analysis of the inserted sequences, we opted to focus on four out of the seven rescued fragments. The genetic characteristics of the four putative genes are summarized in Table 1. The putative polypeptides detected in the genomic DNA fragments recovered from the NOT1, NOT6 and NOT40 transformants were found to harbor a lissencephaly type 1-like homology (LisH) motif, a $Zn(II)_2Cys_6$ fungal zinc finger, and a forkhead domain, respectively, whereas the NOT2 strain harbored no obvious functional domains. We designated the corresponding genes in the NOT1, NOT2, NOT6 and NOT40 strains as *oefA*, *oefB*, *oefC* (over-expressed fluffy), and *fhpA* (forkhead protein), respectively. We then analyzed these four genes in detail, in order to further characterize them.

Genomic and molecular genetic analyses indicated that the *oefA* gene encodes for a 792 amino acid long polypeptide, harboring the LisH domain (Fig. 3A), which is known to be related with the regulation of microtubule dynamics (Emes and Ponting, 2001). Multiple alignment analyses indicated that the LisH domain was highly conserved in both fungi and plants (Fig. 4A). The BLASTP results indicated that this was a homologue (93% identity) of the *Neurospora crassa* SOM1 protein (accession no. AAF75278), although the functional relationship between the two proteins remains to be elucidated. Furthermore, as is shown in Fig. 3A, the DNA fragment harboring the *oefA* gene was inserted downstream of the *niiA* promoter in reversed direction, which might result in the production of the anti-sense mRNA of the *oefA* gene. As the over-expression of the anti-sense mRNA might result in a reduction in the translation level of the corresponding gene, it can be hypothesized that the altered phenotype of the NOT1 strain may have certain similarities to its loss-of-function mutant. In order to evaluate this hypothesis, we deleted the *oefA* gene, and analyzed it in *A. nidulans*. As we had expected, the deletion mutant also exhibited the fluffy growth phenotype, in a manner almost completely identical to that of the NOT1 over-expression mutant (data not shown). The detailed results of these experiments will be published elsewhere.

The oefC gene is a putative $Zn(II)_2Cys_6$ transcription factor

Secondly, the *oefB* gene, which had been isolated from the NOT2 transformant, contained a 648 bp ORF, which was determined to encode for a 216-amino acid polypeptide, which possessed no known functional domains (Fig.

Table 1. Summary of information about NOT mutants and the corresponding genes

Strain name	Gene name	ORF (bp)	Protein (aa)	Linkage group	Domain	Genbank Accession No.	Annotated locus[a]
NOT1	*oefA*	2,376	792	IV	LisH	AY792355	AN9087.2
NOT2	*oefB*	648	216	III	None	AY792356	AN9451.2
NOT6	*oefC*	1,911	637	VI	$Zn(II)_2Cys_6$	AY792357	AN3075.2
NOT40	*fhpA*	2,010	670	III	Forkhead	AY792354	AN4521.2

[a]The hypothetical gene locus name which is analyzed and annotated by Broad Institute (http://www.broad.mit.edu/annotation/fungi/aspergillus/).

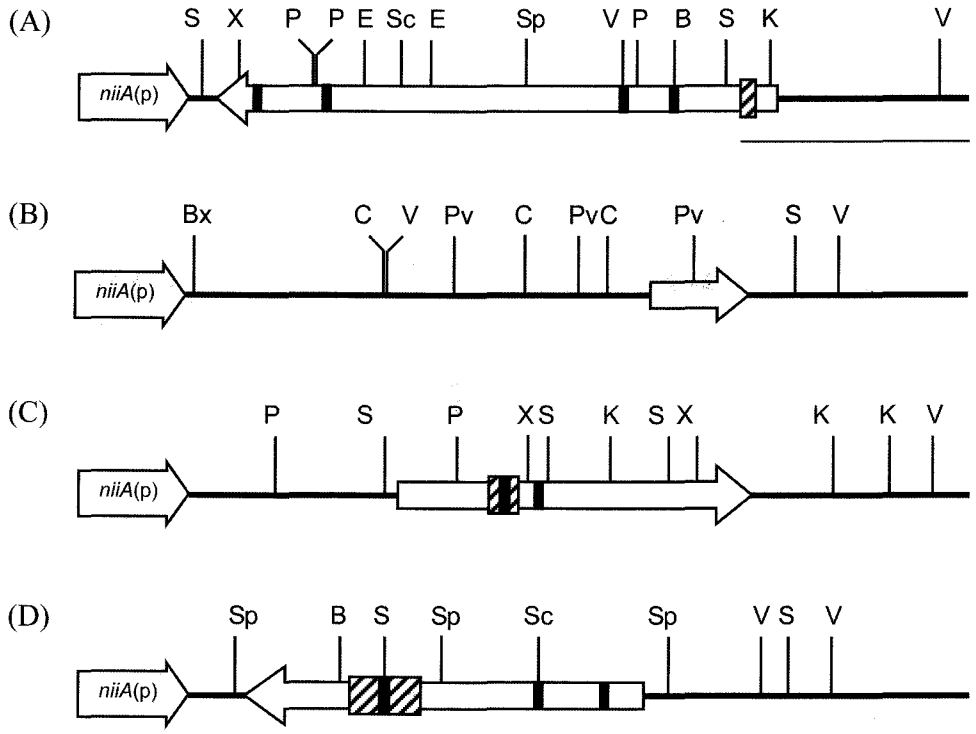

Fig. 3. Restriction enzyme maps and gene structures of the rescued library plasmids. The plasmid rescued from NOT1 (A) harbored an ORF of the *oefA* gene with four introns (black bar) and a conserved LisH domain (dashed box) with the *niiA* promoter in reversed orientation. The arrow box indicates the size and direction of the ORF, and the bar represents 1 Kb. Plasmids from NOT2 (B), NOT6 (C), and NOT40 (D) harbored *oefB*, *orfC* and *fhpA* genes, respectively. The dashed boxes in (C) and (D) represent the conserved GAL4 type zinc finger and forkhead domains, respectively. S, *Sal*I; X, *Xho*I; P, *Pst*I; E, *Eco*RI; Sp, *Sph*I; V, *Eco*RV; B, *Bam*HI; K, *Kpn*I; Bx, *Bst*XI; C, *Cla*I; Pv, *Pvu*II; Sc, *Sac*I.

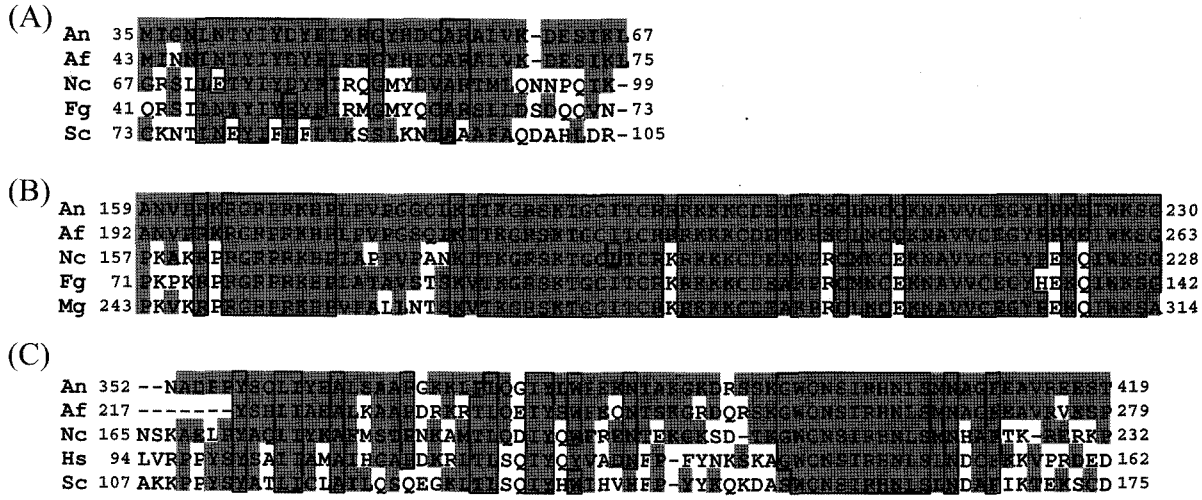

Fig. 4. Multiple alignments of conserved domains found in the rescued genomic DNA fragments. All multiple alignment analyses were conducted using ClustalW, and displayed using the SeqVu 1.1 program. Grey regions indicate homologous residues, and boxed regions indicate identical residues. Numbers represent the amino acid positions of each protein. (A) shows the conserved LisH domain in the OefA gene product. An, *A. nidulans* OefA; Af, *A. fumigatus* SOM1 (Afu7g02260); Nc, *N. crassa* SOM1 (NCU05791.1); Fg, *Fusarium graminearum* FG02650.1; Sc, *S. cerevisiae* Flo8. (B) and (C) represent conserved $Zn(II)_2Cys_6$ domain and a forkhead domain, respectively. In (B), An, *A. nidulans* OefC; Af, *A. fumigatus* Afu3g09670; Nc, *N. crassa* NCU02307.1; Fg, *F. graminearum* FG10812.1; Mg, *Magnaporthe grisea* MG10422.4. (C) An, *A. nidulans* FhpA; Af, *A. fumigatus* Afu2g03050; Nc, *N. crassa* NCU08634.1; Hs, *Homo sapiens* FOXI1; Sc, *S. cerevisiae* Hcm1.

3B). This polypeptide was located downstream of the *niiA* promoter, but was oriented in the correct direction, which indicates that the over-expression of the functional *oefB* gene product should be responsible for the fluffy phenotype expressed by the NOT2 strain. The *oefC* gene, which had been isolated from the NOT6 strain, encodes for a

637-amino acid polypeptide, which harbors a $Zn(II)_2Cys_6$ zinc binuclear domain in its N-terminal region. This domain was strongly conserved, particularly in fungal proteins, including the recently identified RosA in *A. nidulans*, and PRO1A in *N. crassa* (Masloff *et al.*, 2002; Vienken *et al.*, 2005). Interestingly, the phenotypic change occurring in the NOT6 strain was consistently observed, even in cases in which the *niiA* promoter had been repressed (Fig. 1A). This clearly revealed that the fluffy phenotype of the NOT6 strain had been caused by the presence of additional multiple copies of the gene, rather than by the over-expression of the *oefC* gene.

Forkhead domain protein might be involved in sexual development

Finally, the *fhpA* gene product, which consists of 670 amino acids, was determined to harbor the conserved forkhead DNA binding domain in its C-terminal region (Carlsson and Mahlapuu, 2002). Recent studies have shown that the forkhead domain proteins are important to both stress response and sexual development in fungi (Schmitt *et al.*, 2004; Shapira *et al.*, 2004; Szilagyi *et al.*, 2005). The FhpA protein harbors an abundance of serine and threonine residues throughout the polypeptide (12% and 5% respectively), some of which appears to be strongly phosphorylated by protein kinases, including cAMP-dependent protein kinase A (PKA). As the orientation of the *fhpA* gene in the rescued library derived from the NOT40 strain was reversed in direction, much like the *oefA* in the NOT1 strain (Fig. 3D), the phenotype in the over-expression condition, in which sexual development was inhibited, could also be generated via disruption of *fhpA* gene expression by excess anti-sense mRNA. To the best of our knowledge, this is the first evidence that the forkhead domain protein influences sexual development in the filamentous fungus, *A. nidulans*.

Acknowledgements

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