**Two New Genes Involved in Morphogenesis
in *Neurospora crassa***

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Abstract

The *sbr* (small brown) mutant has a highly restricted, colonial morphology, fails to produce asexual spores, and is ascospore lethal. The *sbr* gene was proposed as a regulatory gene because it encodes a helix-turn-helix (HTH), a putative zinc finger, and a glutamine-rich region. In a protein-DNA binding assay, two SBR target DNA fragments, SBRBD300 and SBRBD600 were isolated (Yan 2001). In this study, these two target DNAs were inactivated using an *in vivo* mutagenesis procedure, repeat induced point mutation (RIP). Mutant strains resulting from the RIP procedure demonstrate striking differences in morphology on minimal media. The mutant strain RIP300-1#, which was isolated from a RIP cross of SBRBD300, exhibited a lower growth rate (0.49mm/hr) than that of wild-type (1.92mm/hr) at RT. RIP300-6# showed more branching than wild-type. Similarly, a RIP cross of SBRBD600 resulted in a mutant strain RIP600-4#, which has a lower growth rate (1.11mm/h). In addition, all of these mutant strains produce fewer conidiospores than wild-type. The results suggested that the effect of inactivation of SBRBD300 and SBRBD600 on morphology and development were dramatic. RT-PCR revealed that the *sbr* gene is expressed at all developmental stages.

Gene prediction of these two DNA fragments identified two hypothetical proteins, and binding site analysis revealed multiple binding sites in these two SBR target fragments. RT-PCR analysis revealed that these two genes are transcriptionally active. The electrophoretic mobility shift assay was used to show that the SBR-DNA binding was specific, and aAAAGc was identified as a possible target sequence for SBR.

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List of Abbreviations

Amp	ampicillin
bp	base pair
BSA	bovine serum albumin
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dH ₂ O	distilled water
DMSO	dimethyl sulfoxide
dNTP	dinucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	N, N-ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
hr	hour
IPTG	isopropyl thio beta-D-galactosidase
kb	kilobase pair
kD	Kilodalton
mw	molecular weight
min	minutes
ng	nanogram
Nt	nucleotides
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCI	phenol:chloroform:isoamyl alcohol (25:24:1 in v/v)
PEG	polyethylene glycol
PTC	40% PEG/50mM Tris, pH8/50mM CaCl ₂
rpm	revolution per minute
RT	reverse transcription
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
Sec	seconds
SSC	standard sodium citrate
STC	sorbitol/tris CaCl ₂ solution
TBE	Tris/ borate/EDTA buffer
TBS	Tris buffered saline
TE	10mM Tris, 1mM EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
μl	microlitre
V	volts
VMM	Vogel's minimal medium
W	watts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

I. INTRODUCTION

1.1. *Neurospora crassa* – a model organism

For sixty years *Neurospora crassa* has served as a model system for exploring what genes do. Work by Beadle and Tatum (1941) with *N.crassa* resulted in the development of the one gene one enzyme hypothesis, the first close connection between genes and biochemical function. As a model organism, it is favored because it is easily manipulated using standard microbiological techniques, has simple nutritional requirements and a relatively small genome. The use of *N.crassa* as a model organism has led to many discoveries in the areas of molecular biology, classical genetics, recombination and cell biology (Perkins, 1992).

1.2. Life cycle of *Neurospora crassa*

Neurospora crassa is classified as a eukaryotic, filamentous fungus belonging to the class Ascomycetes. The life cycle of *N.crassa* encompasses both a vegetative (asexual) stage and a sexual stage (Fig. 1). Initially, vegetative growth of the colony begins with a germinated macroconidial spore or hypha, which gives rise to primary hyphae that branch and grow by apical extension and spread radially along the surface of the substrate (Collinge and Trinci, 1974). The hyphal network forms a mycelium. Mycelia are haploid, although the tubular filaments of the vegetative stage (hyphae) have multiple nuclei in each cell compartments. Conidiation, induced by stress conditions, is characterized by the production of chains of conidia on aerial hyphae. During the asexual cycle, heterothallic species such as *N.crassa* form macroconidia, which have one to several haploid nuclei. The surface of conidia has a loosely adhering, hydrophobic

“rodlet” protein coat, which maintains the hydration of conidia and permits light air movements to disperse them (Davis, 2000). Free conidiospores are easily separated from the chain and are capable of colonizing new substrate to germinate when conditions are suitable for growth. In addition to macroconidia, *Neurospora crassa* produces uninucleate microconidia. These spores form in a different way, being extruded directly and serially from the cells of small, branched microconidiophores (Lowry *et al.*, 1967). Their germination efficiency is lower than that of macroconidia (Maheshwari, 1991).

The sexual reproduction of *N. crassa* requires two strains of different mating type, determined by alternative forms of the genetically complex mating-type region, mat A and mat a. Either strain may present itself as a female parent by forming a multicellular protoperithecium. Usually protoperithecia are induced by reducing nitrogen and carbon (Westergaard and Hirsch, 1954). Following fertilization by conidiospores on hyphae of a strain of the opposite mating type, nuclei are led to the protoperithecium through a specialized hypha (trichogyne). Before karyogamy, nuclei undergo repeated divisions, and premeiotic DNA replication takes place as well (Lyengar *et al.*, 1977). A three-celled crozier is produced after a combination of nuclear division and septum formation. Within the dikaryon compartment, the two nuclei fuse, and complete the fertilization process, resulting in the only diploid stage of the life cycle. Two meiotic divisions and a single mitotic division of the diploid nucleus produce eight haploid nuclei that are linearly arranged within an ascus. From the beaked perithecium, ascospores from each ascus are shot and then await germination. The ascospores germinate upon heat activation and grow thereafter in the presence of suitable nutrients (Pandit and Maheshwari, 1996).

Figure 1 Life cycle of *Neurospora crassa*.

The asexual cycle, the upper sequence, depicts the formation of macroconidia from aerial hyphae and their germination to form a new mycelium. The lower sequence depicts the sexual cycle, originating with a protoperithecium, its fertilization via its trichogyne by a conidium of the opposite mating type (Re-drew from Davis 2000).

1.3. The Genome of *N. crassa*

The *Neurospora* genome consists of seven chromosomes with a total genetic map length of roughly 1000 map units (Perkins and Barry 1977). Recently, the whole genome sequence of *Neurospora crassa* was reported (Galagan *et al.*, 2003). The genome sequence was assembled from deep whole-genome shot gun (WGS) coverage obtained by paired-end sequencing from a variety of clone types. Contigs were assembled into 16 –scaffolds with a total length of 39.9 Mb by using the Arachne package (Batzoglou *et al.*, 2002). A total of 10,082 protein-coding genes were predicted. Genes cover about 44% of the genome sequence with an average gene density of one gene per 3.7 kb. The approximately 1.67 kb average gene length is slightly longer than the 1.4 kb average gene length for both *S.cerevisiae* and *S. pombe*. The greater number of introns in *Neurospora* genes – an average of 1.7 introns per gene is the reason for the difference in gene length (Galagan *et al.*, 2003)

An analysis of repeat sequences longer than 200 bp and with greater than 65% similarity revealed that only 10% of the *Neurospora* assembly consists of repeat sequences (Kellar *et al.*, 2001). Furthermore a tandem array of about 170 copies of a 9.3 kb DNA sequence encoding the three large RNA molecules of the ribosome accounts for most of the repetitive DNA of the organism (Butter and Metzenberg 1989; Krumlauf and Marzluf 1980). Unlike most eukaryotic organisms, the 100 5s rRNA genes of *Neurospora* are dispersed in the genome (Free *et al.*, 1979; Metzenberg, *et al.*, 1985; Selker *et al.*, 1981). The previous investigation suggests that, at least in general, protein coding genes are present as unique sequences in the *N.crassa* genome (Selker, 1990).

1.4. The morphogenesis of *N. crassa*

The extensive genetic investigations suggested that the morphology of fungi is likely to arise from the epigenetic interactions of myriad genes involved in the growth and the response of these genes to environmental stimuli (Vierula, 1996). Among more than 500 mutants described to date, about 25% have altered morphologies. Furthermore, genetic lesions which affect conidiation, vegetative morphogenesis, and development of sexual reproductive structures were found in some of these mutant strains. The pioneers of genetic studies of morphogenesis in *N. crassa* were E.L. Tatum and his colleagues. Their work resulted in the isolation of a large collection of morphological mutants (Garnjobst & Tatum, 1976), which were classified into six categories: a) true colonials, b) spreading colonials, c) semi-colonials, d) spreading morphologicals, e) distinctive morphologicals and f) morphologicals which are dependent on particular environmental conditions to be manifest.

The growth of *N. crassa* is characterized by the highly polarized extension of hyphal tips and the formation of subapical branches. As the tip extends, periodic branches are formed at or near the apex or near the apex of the tip. These branches also extend in a polarized manner as new tips. The two processes of branching and tip growth permit the organism to colonize and efficiently utilize a substrate (Heath, 1995). Genetic analysis of induced and naturally occurring mutants has identified over 100 loci that encode products that can affect tip growth and branching in *Neurospora crassa* (Perkins *et al.*, 1982; Scott 1976). Studies of regional variations in protein and RNA production showed that material for tip extension can come from regions of mycelium at least 12 mm from the colony margin (Zalokar, 1959). Katz *et al.* (1972) proposed that branching would be initiated

when the rate of supply of vesicles outpaces the rate of their incorporation at the growing tip. Genetic investigation in wild-type *N.crassa* suggested that the distribution of branch intervals is likely to be independent of tip extension rate (Wiebe *et al.*, 1992). In contrast, the distribution of branch intervals in colonial mutants (*col-4*, *col-8*, and *col-16* mutants) was dependent on tip extension rate (Watters and Griffiths, 2001).

Conidiation is the major mean of dispersal for *Neurospora crassa*. Relatively few genetic loci have been found that specifically block conidiation. The *fluffy* (*fl*) mutants are unable to produce macroconidia (Lindegren 1933). The single gene responsible for this trait was named *fluffy* (*fl*). The sequence of *fl* revealed that it encodes a Gal4p-type C6 zinc cluster protein. Analysis of several *fl* mutant alleles demonstrated that null mutants are blocked in the budding phase of development required to produce conidiophores (Bailey and Ebbole, 1998). The studies of *fl* expression suggested that FL acts as a developmentally regulated transcription factor required for conidiophore morphogenesis.

The cytoskeleton plays important roles within the cell such as the localization of macromolecules, and the movement and positioning of organelles (Vierula 1996). Morphological mutants *ropy* (*ro*) have proved to be particularly valuable for the genetic characterization of the cytoskeleton. These strains exhibited much more curling and branching than wild-type, with cable-like intertwining of aerial hyphae giving the *ro* colonies a characteristic radial pattern. The genetic studies of several *ro* genes revealed that they encode cytoskeletal components. Two *ropy* mutants, *ro-1* and *ro-3*, deficient in dynein and dynactin respectively, were examined (Riquelme *et al.*, 2000). The study revealed that the Spitzenkörper (Spk) controls hyphal morphology by operating as a

vesicle supply center (VSC). These findings on dynein or dynactin deficiency indicated that the microtubular cytoskeleton plays a major role in the formation and positioning of the Spk, with dramatic consequences on hyphal growth and morphogenesis. In addition, the study in this group of mutants discovered that an actin-related protein, encoded by the *ro-4* gene, is also involved in the microtubule-based motility system (Plamann *et al.*, 1994; Robb, Wilson & Vierula, 1995).

1.5. RIP-Repeat Induced Point Mutation

Hints of instability of repeated sequences in Neurospora

Several observations suggest that the low repetitive DNA content of the *N.crassa* genome is not simply an immediate result of natural selection (Raju and Perkins, 1978). Indeed, the organism probably employs several mechanisms to maintain a streamlined genome. Tandem duplications resulting from integration of transforming DNA by homologous recombination are lost at low but detectable frequencies during vegetative growth (Grant *et al.*, 1984, Selker *et al.*, 1987a). However, *Neurospora* transformants are stable in the asexual stage of the life cycle. On the other hand, a large fraction of transformants of *N.crassa* are unstable in the sexual phase of the life cycle (Case 1986; Selker *et al.*, 1987a; Grant *et al.*, 1984).

This instability was first studied when a set of transformants generated with the plasmid pES174 resulted in a deletion of the entire *am* gene (Selker *et al.*, 1987b). Greater instability of the transformation marker was observed in crosses of the transformant, T-ES174-1, which resulted from integration of the plasmid by homologous recombination. Only up to 16% of the progeny from a cross of an apparently homokaryotic isolate of the transformant and an *am*₁₃₂ strain were *am*⁺, indicating loss or

inactivation of the gene in two thirds of the chromosomes initially carrying the transforming DNA (Selker *et al.*, 1987a). 55% of the asci showed Mendelian segregation of *am* (4+:4-); and 65% of the asci exhibited complete loss of the marker. No progeny were found with two copies of the transforming sequences, suggesting that the transforming DNA was lost by intrachromatid recombination, not by unequal sister chromatid exchange. The consistent loss of the transforming DNA sequences from both meiotic products of the affected tetrads suggested that the recombination event had occurred prior to premeiotic DNA synthesis, and thus prior to karyogamy (Selker 1990).

Investigation of the fate of transforming sequences in crosses of pES174 transformants led to the discovery of RIP. The first clue came from examining progeny derived from crosses of T-ES174-1, the transformant with the local duplication of the flank region (Selker *et al.*, 1987a). Duplications are efficiently detected and altered in a specialized (dikaryotic) tissue formed by fertilization. Because the process occurs between fertilization and nuclear fusion, and affects both duplicated sequences by gene inactivation, changes in the position of restriction sites, and *de novo* methylation, this process was first designated “rearrangement induced premeiotically” (RIP) (Selker *et al.*, 1987a). Further investigation demonstrated that the changed restriction fragments in the duplicated sequence must result from small changes such as point mutation. Heteroduplex analysis of a sequence severely altered by the RIP process showed that the RIP process does not result in gross rearrangements. The sequence analysis of the native and altered regions of the linked duplicated sequence revealed that, all the differences were point mutations, and they were extensively polarized transition mutations of one type; G-C base pairs in the native sequence were replaced by A-T base pairs in the altered

sequence (Cambareri, *et al.*, 1989). Thus, the RIP process results in point mutations, and the name was changed from “rearrangement induced premeiotically” to “repeat-induced point mutation).

Timing of RIP

In the vast majority of asci yielding progeny, the products of sister chromatids displayed common alterations indicating that the changes were determined prior to premeiotic DNA synthesis and therefore prior to karyogamy and meiosis (Selker *et al.*, 1987a). The fact that the 100 or more asci of a perithecium are usually derived from a single maternal nucleus and a single paternal nucleus (Johnson 1976, Nakamura and Egashira 1961), allowed the investigators to determine whether RIP occurred before or after fertilization. The observation of different alterations among progeny of individual perithecia suggests that RIP occurs after fertilization. Finding the same pattern of alterations in multiple isolates from a given perithecium indicated that RIP can occur early during proliferation of the heterokaryotic tissue formed by fertilization (Selker, 1990).

DNA methylation resulting from RIP

It is interesting that methylation is a common feature of sequences altered by RIP, since the bulk of the *Neurospora* genome is unmethylated (Selker and Garrett, 1988). Methylation, where observed, has usually been very heavy; most or all of the sites examined in the duplicated sequence appear methylated in a large fraction of the molecules. Results of restriction analysis suggest that most of the cytosines in the duplication are methylated. No evidence of methylation was found in adjacent sequences, nor in the unduplicated allele (Selker and Stevens 1985; Selker and Stevens 1987).

The observation of methylation of unique sequences resulting from meiotic segregation indicate that sequence duplications initially trigger RIP, but are not required for the methylation. Moreover, a DNA sequence altered by RIP can direct its methylation, *de novo*. When a RIPed region was stripped of its methylation, and then reintroduced into *Neurospora*, it became specifically and reproducibly remethylated. Cambareri *et al* (1991) used a product of RIP generated in the laboratory to produce equivalent results. Therefore, the G:C to A:T mutations somehow render the sequences substrates for DNA methylation.

Limits of RIP

The fact that the *Neurospora* genome is not completely devoid of repeated sequences raised the possibility that different sequences might have different sensitivities to RIP. The possible reasons could be that, the RIP process depends on special sites, some sequences can confer “immunity” to RIP on neighboring DNA, and certain regions of the genome might be protected from RIP. Basically, every sequence of appreciable length (i.e. >1 kb) that has been duplicated in *N.crassa* and tested, has shown signs of RIP. But the small size (<150 bp) and dispersed arrangement of the ~100 5S rRNA genes of *Neurospora* probably protect them from the process (Selker 1990). The tandemly repeated genes encoding the three large rRNA molecules are probably protected because of their particular chromosomal position rather than their sequence. Perkins *et al.*, (1986) noted that a small cluster of rDNA repeats that had been moved from their normal home by two sequential translocations, exhibited abnormally heavy methylation, reflecting the operation of RIP.

The possible mechanisms of RIP

Further studies of RIP have revealed that closely linked duplications are highly susceptible to the RIP process, while unlinked duplications are less susceptible (Singer and Selker, 1995). The RIP process is more probable and more severe with duplications of 1 kb or more, and either both or neither of the two copies involved are altered. But if 3 or more copies are present, the process frequently results in a combination of altered copies (Johnson 1976, Selker and Garrett 1988) suggesting a homologous pairing process (Fincham *et al.*, 1989).

RIP introduces C:G to T:A transition mutations and creates targets for subsequent DNA methylation in vegetative tissue. Two alternative pathways for the enzymatic generation of C to T mutations by a DNA methyltransferase (DMT) have been proposed (Selker, 1990). One hypothetical pathway involves *de novo* DNA methylation of C in paired sequences, followed by catalyzed deamination of some fraction of the resulting 5mC to give T. The second pathway involves direct deamination without release of 5mC-DNA. Freitag *et al* (2002) found that a putative DNMT gene in *Neurospora* is essential for RIP and supports the idea that a DMT or DMT-like enzyme is involved in RIP by one of these mechanisms.

1.6. Electrophoretic Mobility Shift Assay

The principle of EMSA

DNA-binding proteins are involved in cellular processes such as replication, recombination, viral integration, and transcription. In recent years, more and more investigators are interested in the study of these proteins as the advent of recombinant DNA technology has led to the isolation of numerous biologically important genes. Many

researchers are interested in how transcription of these genes is controlled, and have started to characterize the DNA sequences responsible for this regulation. The electrophoretic mobility shift assay (EMSA, also known as the gel shift assay or gel retardation assay) is a technique that is widely used to study DNA-binding proteins (Molly 1999).

Originally, EMSA was developed to study interactions involving RNA (Dahlberg, *et al.*, 1969; Spassky, *et al.*, 1979), but the technique really rose to prominence when it was applied to the study of DNA-binding proteins involved in the regulation of transcription. The principle of EMSA is very straightforward. The DNA-binding protein is mixed with a radiolabelled DNA probe in a suitable buffer and binding is allowed to occur. The equilibrated mixture is subjected to electrophoresis through a nondenaturing polyacrylamide gel. Formation of the DNA-protein complexes usually reduces the electrophoretic mobility of DNA, therefore the migration of DNA-protein complex will be retarded. Following electrophoresis the gel is visualized using autoradiography or phosphor imaging.

Determination of Binding Specificity

DNA-binding proteins often exhibit a strong sequence specificity, the binding of a particular target is far more tight than that of nonspecific DNA. The most common test of specificity is to add unlabelled target DNA to compete for the protein of interest. Specificity of binding is indicated when excess unlabeled target DNA reduces the amount of labeled DNA-protein complex, while excess non-target DNA has no effect (Ausebel, *et al.*, 1993).

Detection of DNA Binding Proteins in Crude Cell extracts

In addition to the study of purified DNA-binding proteins, EMSA can also be performed satisfactorily with mixtures of protein; or even with crude cell extracts. The cell extracts are likely to include many DNA-binding proteins. Therefore, it is important that specific binding is confirmed by performing the experiment in the presence of excess unlabeled specific and non specific competitor DNA.

Generally, EMSA can be used to identify novel DNA-binding proteins (Read, 1995), to check for the presence of previously identified DNA-binding proteins in particular cell extracts (Adler, 1995), or to rapidly test the ability of mutated proteins to bind to a given target sequence.

Critical Parameters

Despite the basic simplicity of EMSA, many parameters may affect the results obtained in this assay, including salt concentrations, detergent concentrations, incubation temperature, pH and time of the binding reaction (Aosebel *et al.*, 1993; Carely 1991). Therefore, for a novel DNA-binding protein, many parameters such as salt concentration, pH of buffer systems, and acrylamide percentage may require optimization (Staudt *et al.*, 1986).

1.7. Epitope tagging

Epitope tagging is a recombinant DNA method for making a gene product immunoreactive to an already existing antibody (Munro and Pelham, 1984). An epitope is any structure or sequence that is recognized by an antibody. Traditionally, researchers have had to raise antibodies to epitopes on naturally occurring proteins to study the function, movement, and modification of the protein *in vivo* and *in vitro* (Evan *et al.*,

1985; Kreis, 1986; Wilson 1984). Many investigations have revealed that a single epitope may occur on (or be inserted into) different proteins and still be recognized by the same antibody (Kolodziej and Young 1999). Therefore, to study proteins that did not normally have an epitope, many investigators began to transfer a small peptide epitope to the protein using recombinant DNA techniques (Cravchik and Matues, 1993; Duden *et al.*, 1991; Field *et al.*, 1988). Subsequently, the epitope-tagged protein could be analyzed and purified with the tag-specific antibody. Epitope tagging provided several advantages over traditional methods. For instance, the same tag-specific antibody will recognize the epitope tag in many different proteins, and also adding a small epitope tag generally does not affect the function of the tagged protein. The first step of epitope tagging is the addition of an epitope peptide to target protein. This could be done by either recombinant techniques or oligonucleotide mediated mutagenesis via the Polymerase Chain Reaction (Wendland, 2003).

Usually locating the tag at the N-terminus or C-terminus of the target protein was preferred for several reasons. Since the first proteins to be epitope tagged were tagged at the termini, therefore when new proteins were tagged it seemed wise to do what worked in the past. Secondly, tagging is often performed using expression vectors that already put the tag at a terminus. Moreover, locating the tag at the termini usually also makes the tag more accessible to antibodies.

An epitope tagging vector must contain the epitope tag sequence, the sequences that allow the vector to be maintained in bacteria (Sambrook, *et al.*, 1989), a drug resistance gene, genetic markers, a multiple cloning site (MCS), a strong eukaryotic promoter (Cravchik and Matus, 1993), a polyadenylation site, appropriate start and stop

codons flanking the tag sequences, and sequences such as the SV40 viral replication origin that allow the plasmid to be replicated independently in a eukaryotic cell (Kain and Ganguly, 1995). The epitope-tagged protein can be easily obtained by adding the coding sequence for the target protein to an existing expression vector that contains an epitope tag.

Commonly used techniques for detecting the epitope tagged proteins include Western blotting, immunoprecipitation, ELISAs, immunofluorescence, immunoelectron microscopy, surface plasma resonance, etc (Harlow and Lane 1988). For example, Western blot analysis allows the detection of epitope-tagged proteins in complex mixtures such as cell or membrane extracts (Canfield and Levenson 1993). When combined with immunoprecipitation, it can reveal information about the interaction of the tagged protein with other cell components (Dietzen *et al.*, 1995). The detection may involve direct detection using one tag-specific antibody, or indirect detection using two antibodies. A wide range of epitope tags have been used to tag proteins including HA tag (peptide from human influenza hemagglutinin protein), FLAG epitope (synthetic peptide), c-myc (human c-myc gene protein), B-tag (VP7 protein of blue-tongue virus), His6 or His10 (polyhistidine), Pk-tag (P/V protein of paramyxovirus SV5), etc (Reviewed by Jarvik and Telmer 1998).

1.8. A new mutant of *N. crassa*

Historically, an important approach to the study of gene function has involved the use of randomly induced mutations. Many *N. crassa* morphological mutants have been produced by chemical or UV mutagenesis. An alternative approach to discovering new

genes and studying their functions is insertional mutagenesis. This technique disrupts the activity of a gene, usually producing a null phenotype, and also tags the mutation, facilitating subsequent cloning and characterization of the gene (Paietta and Marzluf, 1985, Staben *et al.*, 1989). Insertional mutagenesis with transposable elements has been used extensively in both bacterial and plant genetics. However, suitable fungal transposons have not yet been developed. Instead, insertional mutagenesis has been adapted by relying on the propensity of dominant selectable markers to integrate relatively randomly into the genome by non-homologous recombination.

A new mutant was created by insertional mutagenesis with a hygromycin resistance construct in our lab. This mutant has an extreme, colonial morphology (Campsall, 1998). A single colony of this strain forms rough-edged circular mound marked with a glistening, irregular surface. The orange-brown color of the mutant differs from the pink-orange color of wild type. Therefore, the new mutant was named *sbr* (small, brown) (Fig. 2).

The morphology and development of *sbr* are distinct from most other colonial mutants (Campsall, 1998). With the scanning electronic microscope, the hyphae of *sbr* are swollen and spherically shaped and much wider than the wild-type hyphae. Furthermore *sbr* appears to lack conidiophores and macroconidia. Wild-type hyphae are thin and tubular with diameters of 4µm. In contrast, *sbr* colonies consist of fat, rounded hyphae with diameters ranging from 20µm to 80µm. From these tuberous projections arise smaller yeast-like buds (Fig. 2).

Figure 2 Morphology of wild type *N.crassa* and the *sbr* mutant.

- I.** Colony of a) Wild type and b) *sbr* mutant: the mutant shows highly restricted hyphal growth and forms a small colony while the wild type hyphae grow radially over the plate.
- II.** Scanning electron microscopy of hyphae from 3 day old cultures of a-b) wild-type and c-d) *sbr*: wild-type culture shows tubular hyphae (a) and conidiophore (b), the *sbr* mutant shows the swelling spherical hyphae of the mutant. The length of the bar represents 10 μm (Campsall, 1997).

I

II

The *sbr* gene has been cloned previously, and was proposed to be a regulatory gene because it encodes a helix-turn helix (HTH), a putative zinc finger, and a glutamine-rich region (Yan, 2001). During the previous study, the *sbr* gene was sequenced and mapped to linkage group III. The SBR protein was expressed and purified under native conditions. In a protein-DNA binding assay, total genomic DNA digested by restriction enzymes were allowed to bind with SBR, two genomic fragments SBRBD300 and SBRBD600 were isolated from protein-DNA complexes.

1.9.Objectives

Since the previous study suggested that the *sbr* gene codes for a transcriptional activator, the hypothesis being tested here is that the *sbr* gene product localizes to the nucleus. Therefore, one of my goals is to localize the *sbr* gene product by using epitope tagging and immunofluorescent microscopy.

A previous graduate student Yanhua Yan (2001), isolated two DNA fragments from protein-DNA complexes, which were named SBRBD300, and SBRBD600 respectively based on their sizes. The former investigation suggested that these fragments contain SBR protein binding sites. The question being asked here is that does inactivation of any of these regions of the genome results in morphological mutations? The second objective of this study is to inactivate these two segments by RIP process.

The third objective of this study is to narrow down the binding sites of these two DNA segments. Since the size of SBRBD300 and SBRBD600 is too big for the electrophoretic mobility shift assay (EMSA), the shorter sequences of these two fragments will be amplified for EMSA analysis.

Recently more and more information have been deposited in different database and genbank. A more thorough analysis of DNA I studied will be undertaken to search for the related sequences and putative function.

II. MATERIALS AND METHODS

2.1. Culturing of Experimental Organisms

Neurospora crassa

The *sbr* mutant was originally created in 1992 by Sallmen and Vierula (unpublished) using insertional mutagenesis with plasmid vector DNA. All other strains of *N. crassa* will be referred to by their Fungal Genetics Stock Center (FGSC) number (where applicable). The strains used in these experiments are listed in Table 1.

For growth of *N. crassa* on solid medium, hyphal fragments or spores were aseptically transferred to 16x150 mm glass culture tubes containing 5 ml of Vogel's Minimal Medium (VMM) [per litre: 20 ml 50x Vogel's stock (per litre: 127g Na₃citrate ·2H₂O, 250g KH₂PO₄, 100g NH₄NO₃, 10g MgSO₄·7H₂O, 5g CaCl₂·2H₂O predissolved in 25 ml dH₂O added dropwise), 1 ml biotin stock (5 mg biotin in 100 ml of 50% v/v ethanol), 1 ml trace elements (per liter: 50g citric acid·1H₂O, 50g ZnSO₄·7H₂O , 10g Fe(NH₄)₂(SO₄)₂·6H₂O ,2.5g CuSO₄·5H₂O, 0.5g MnSO₄·1H₂O, 0.5g H₃BO₃, 0.5g Na₂MoO₄·2H₂O dissolved in dH₂O), 15g sucrose and 15g agar] solidified on a slant. Tubes were incubated at room temperature (20-25°C) to promote growth.

In order to grow cultures in liquid medium, hyphal fragments recovered from solid media were resuspended in dH₂O (~ 5 ml) and inoculated into 50 ml of VMM liquid media. Cultures were incubated at RT with shaking at 200 rpm for 24 hours to 10 days.

Table 1 *Neurospora crassa* strains used.

Strains with a Fungal Genetics Stock Center (FGSC) number were originally obtained from the FGSC. Strain designations were taken from the Catalogue of Strains supplement to The Fungal Genetics Newsletter no. 43 (1996). na = not applicable

Table 2 Strains of *E. coli* used in experimentation.

Both the genotype and any known antibiotic resistance genes are also indicated.

Description	FGSC number	Mating type	Strain Designation
Wild type	987	A	74-OR23-1A
Wild type	988	a	74-OR8-1a
<i>sbr</i>	na	a	na

Strain	Antibiotic Resistance	Genotype
DH5 α	na	<i>supE44 ΔlacU196 (ϕ80lacZ ΔM15)</i> <i>HsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>
M15[pREP4]	kanamycin	<i>Nal^s, Str^s, Rif^s, Lac⁻, Ara⁻, Gal⁻, Mtl⁻, F⁻, RecA⁺, Uvr⁺, Lon⁺</i>

***Escherichia coli* bacteria strains**

E. coli strains used are listed in Table 2. Bacteria from either a glycerol stock stored at -70°C or from a colony on an LB plate were streaked across the surface of a 90mm LB plate, containing 1.5% agar in 20-25 ml LB media (per liter: 10g NaCl, 10g bactotryptone, 5g yeast extract, pH 7.5), in order to isolate single colonies. The plate was then incubated at 37°C overnight in an inverted position.

To select for colonies with ampicillin resistance, the bacteria were streaked onto LBA plates (LB media supplemented with ampicillin to a final concentration of 100 $\mu\text{g/ml}$ with 1.5% agar).

To grow bacteria in broth, a single colony isolated from a freshly prepared overnight plate culture was inoculated into 2-5 ml LB broth (supplemented with antibiotics when selection of antibiotic resistant bacteria was desired). Cultures were grown overnight (approximately 16 hours) at 37°C while at 250 rpm.

2.2. DNA Analysis Methods

Extraction of *N. crassa* genomic DNA

Liquid cultures (50 ml) of wild-type and mutants were grown at RT for 24-30 hours with shaking at 200 rpm. Mycelia were collected under vacuum by filtering onto 1 MM filter paper lining a Buchner funnel. They were washed with cold d H_2O , dried again by filtration, collected, placed into 15 ml polypropylene tubes, and frozen at -70°C .

Frozen mycelia were ground with 1g of acid-washed sea sand using a cold mortar and pestle. About 3 ml of extraction buffer (0.2 M Tris; pH 8.5, 0.25 M NaCl, 25 mM

EDTA, 0.5% SDS) was added to the ground material. Pronase was added to a final concentration of 0.5 mg/ml and the preparation was then incubated at 37°C for 30 minutes. One volume of phenol:chloroform (1:1) was mixed with the solution and centrifuged at 12,000 rpm in the SM-24 rotor of the Sorvall RC-5B fixed-angle centrifuge, at 4°C for 60 minutes. The upper aqueous layer was transferred to a new tube and RnaseA was added to a final concentration of 300 µg/ml. After incubation at 37°C for 30 mins, 1 volume of chloroform:isoamyl alcohol (25:1) was added, mixed and the solution was spun at 12,000 rpm, at 4°C for 10 minutes. DNA in the upper aqueous phase was precipitated by adding 0.54 volumes of cold isopropanol. The tube was left on ice for 1 hour. DNA was pelleted at 7,500 rpm at 4°C for 10 minutes. The pellet was washed with 70% ethanol and dried before it was resuspended in 500 µl TE (10 mM Tris; pH8.0, 1 mM EDTA) and stored at 4°C. (Raeder and Broda, 1985)

DNA concentration was estimated using the optical absorbance measured at the 260 nm, and 280 nm wavelengths generated by the Hewlett Packard 8452A Diode Array Spectrophotometer.

Extraction of plasmid DNA from *E. coli*

Broth cultures (2 ml) of bacteria containing the desired plasmid were grown overnight at 37°C. The culture was transferred to a 1.5 ml microfuge tube and centrifuged at 14,000 rpm for 2 minutes. The supernatant was discarded and the cell pellet was resuspended in buffer. One of the following methods was used to extract plasmid DNA from contaminating proteins, bacterial DNA and RNA.

Rapid plasmid mini-preparations:

The cell pellet was resuspended in 100 μ l TE pH8.0 buffer and incubated at RT for 5 min. Cells were then lysed with 200 μ l of 0.2 M NaOH, 1% SDS solution and left on ice for 5 minutes. The lysis solution was neutralized by adding 150 μ l of ice cold 3M potassium acetate pH 4.8, and vortexed at maximum speed for 2 seconds and returned to ice for 5 minutes. Bacterial DNA was pelleted by centrifugation at 14,000 rpm at RT for 4 min. The pellet was discarded and two volumes of absolute ethanol (900 μ l) were added to the supernatant. DNA was pelleted by centrifuging at 14,000 rpm for 2 minutes, rinsed with 70% ethanol, dried in a desiccator and finally resuspended in 20 μ l TE buffer.

WizardTM mini-preparations (Promega):

When high DNA purity was required for projects such as sequencing, this alkaline-lysis method was used according to the manufacturer's instructions (for details consult that protocol).

Plasmid midi-preparations:

Overnight LB cultures (30 ml) were centrifuged at 4000xg for 5 minutes. The DNA pellet was resuspended in 3ml TE buffer and 5 ml of lysis solution (0.2 M NaOH, 1% SDS) was added and mixed thoroughly (but gently). After 10 min at RT, 3 ml of cold 3M potassium acetate was added and mixed well and the mixture was left for 10 more min on ice before centrifugation at 10,000xg for 15 minutes. The supernatant was filtered through one layer of micro-cloth into a new tube and 0.6 volume of isopropanol was added and mixed. The tube was incubated at RT for 10 min and centrifuged at 10,000xg for 15 min. The pellet was briefly rinsed with 70% ethanol and dried in a fume hood for 10 min before being re-suspended in 500 μ l TE buffer. The DNA solution was transferred into a microfuge tube and re-precipitated with 500 μ l of 1.6M NaCl containing 13% (w/v)

polyethylene glycol (PEG 8000) by centrifuging at 12,000xg for 5 minutes. The supernatant was removed and the pellet was re-suspended in 400 µl TE buffer containing 50 µg/ml Rnase A. After at least 1 hr at room temperature, the solution was extracted with 400 µl PCI followed by 400 µl chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 2 volumes of absolute ethanol in the presence of 1/10 3M sodium acetate (pH 5.2) and dried in a desiccator before being dissolved in 100 µl TE buffer.

Digestion with restriction enzymes

The typical digest with a restriction enzyme involved 1-5 µg of DNA digested with 1 µl of enzyme stock (5-20U/µl) with 1/10 final volume of 10x enzyme buffer, 1/10 final volume of acetylated bovine serum albumin (aBSA), which was only required for some enzymes, in a reaction volume of 20 µl made up with dH₂O. Enzymes were purchased from New England Biolabs (NEB), Gibco/BRL or Invitrogen and the appropriate buffering systems were used according to the manufacturer's guidelines. Reactions were stopped by freezing or adding 6x loading buffer [0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (w/v) sucrose in dH₂O] containing 10mM EDTA.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described by Sambrook *et al.* (2000). The gels varied in agarose concentration from 0.8% to 2.0% depending on the size of the fragment of interest. Agarose (Boehringer Mannheim) was dissolved by heating in 0.5x TBE (45 mM Tris-borate, 1 mM EDTA). After the solution had cooled to < 60°C, 0.05% (w/v) ethidium bromide was added prior to casting the gel. The

electrophoresis unit was filled with enough 0.5x TBE to barely cover the surface of the gel.

DNA samples (0.1 µg-0.5 µg DNA) were mixed with 2 µl of 6x loading buffer and dH₂O to a final volume of 12 µl and the entire volume was loaded into the well of an agarose gel. Commercially-prepared Lambda phage DNA cut with Hind III (NEB) was diluted (0.6 µl of 500 µg/ml stock in 0.9 µl, 10x NEB 2 buffer 7.8 µl dH₂O and 2.7 µl 6x loading buffer containing 10mM EDTA) and loaded into a well to act as a size marker. Voltage was applied to the electrophoresis unit using the Bio-Rad Model 2000/2.0 power supply at approximately 5 V/cm. After the DNA had migrated an appropriate distance, the gel was viewed using 254 nm UV light from a handheld UV wand or on a transilluminator (Fotodyne Inc.) equipped with a Polaroid camera. Band sizes were estimated with the aid of DNAfragTM software 1989 (Schaffer *et al.*, 1981).

DNA recovery from agarose gels

Recovery of DNA from agarose gels was accomplished using the protocol of Boyle and Lew (1995). Agarose gels were viewed using long wave UV light (366 nm) from a hand-held UV wand. The desired bands were excised with a razor blade. The weight of the excised gel slice was measured after transfer to a pre-weighed microfuge tube. The slice was then dissolved in 2.5-3 volumes of 6 M NaI at 50°C. Silica matrix suspension (5 µl) was added, mixed by tapping and left on ice for 5 minutes. The matrix, with bound DNA, was pelleted by centrifugation at 14,000 rpm for 5 seconds in the Eppendorf centrifuge, model 5415 C. The pellet was resuspended in 400 µl of cold wash solution (50 mM NaCl, 10 mM Tris-Cl, 2.5 mM EDTA, 50%(v/v) ethanol), repelleted by centrifugation, and the supernatant was discarded. This was repeated twice before the

DNA pellet was resuspended in 10 µl sterile dH₂O. The DNA was released from the silica matrix by incubation at 50°C for 5 min. The silica beads were pelleted when centrifuged for 30 seconds and the DNA in the supernatant was transferred to a new tube for storage at 4°C.

2.3. Inactivation of SBR binding DNA segments – SBRBD300 and SBRBD600

Cloning SBRBD300 and SBRBD600 into UA vector pDrive

A. PCR amplifying SBRBD300 & SBRBD600 from genomic DNA

Standard PCR reactions were performed using a Perkin Elmer Cetus 480 DNA Thermal Cycler and Invitrogen reagents. Genomic DNA from *N. crassa* wild type strain 988 was used as a template and the sequence specific primers used were as follow, SBR300-f: 5'-TGCCGAGTTGAATGAGACCG-3', SBR300-r: 5'-GGCGYCCTAAATTCTCCGAGC-3', SBR600-f: 5'-ACGGCCCAGTGCTACCTATCT-3', SBR600-r: 5'-GACGACTTGCCATTCCTCTTG-3'. Typical PCR reactions contained: 2.5U of Taq DNA polymerase (added after 95°C denaturation), 5 µl of 10x PCR buffer, 15 µl MgCl₂ (50 mM), 1 µl dNTP (10 mM), primers (25pmoles of each), 1 µl of template DNA, and sterile dH₂O up to 50 µl. After thorough mixing by vortex, a 50 µl overlay of sterile mineral oil was added.

The standard reaction conditions were: 1 min denaturation at 95°C, 1 min annealing at optimal temperature for primer, extension at 72°C for 2 mm. A final 5 min of extension at 72°C was included, totally 35 cycles were run.

B. Cloning of PCR products into pDrive

PCR products were cloned using QIAGEN PCR cloning kit (QIAGEN) following the manufacturer's instructions. 1 μ l of UA cloning vector pDrive (50 μ g/ μ l), 1-4 μ l PCR product, 5 μ l of 2x ligation Master Mix and distilled water up to a final volume of 10 μ l were added to the ligation-reaction mixture. After mixing, the ligation-reaction mixture was incubated at 4-16°C for 30min.

C. Transformation

A transformation protocol from the manufacturer's instructions (QIAGEN) was followed. The appropriate number of tubes of QIAGEN EZ competent cells were thawed on the ice. SOC medium was thawed and warmed to RT. 2 μ l of ligation-reaction mixture was added to each tube of QIAGEN EZ competent cells. The competent cells were incubated at 42°C for 30 sec and then placed on ice for 2 min. The 250 μ l of room temperature SOC medium was added to each tube, and 100 μ l of each transformation mixture was directly plated onto LB plates containing ampicillin. The plates were incubated at RT until the transformation mixture was absorbed into the agar. The plates were inverted and incubated at 37°C for 12-16 hours.

After transformation, white colonies were selected with toothpicks and inoculated in 2 ml LB media containing 100 μ g/ml ampicillin. After incubation at 37°C overnight, plasmid DNA was isolated from *E. coli* cells following the procedure (rapid plasmid mini prep) described above.

Subcloning of SBRBD300 and SBRBD600 into vector pCB1004

Both plasmid DNA which contained insert DNA (SBRBD300 or SBRBD600) and the shuttle vector pCB1004 which contained the hygromycin B resistant gene were

double digested with the restriction enzyme *Bam*HI and *Not*I. After separation by agarose gel electrophoresis, the insert DNA and digested pCB1004 were recovered from an agarose gel. The ligation reaction included 100 ng of insert DNA (SBRBD300 or SBRBD600), 50 ng pCB1004, 2 µl of 5x T4 DNA ligation buffer (NEB), 0.5 µl T4 DNA ligase (5U/µl) and water to final volume of 10 µl, was incubated at 16°C overnight.

Chemical transformation protocols from Sambrook *et al.* (2000) were followed and competent cells were prepared as follows. A single colony of DH5α *E.coli* from a fresh plate culture was inoculated into 2~3 ml of LB broth and grown overnight at 37°C with shaking. A volume of 1 ml of this culture was added to 100 ml LB broth and grown at 37°C (for 90~120min) until the OD₂₆₀ reached 0.5. Cells were then transferred into a cold centrifuge bottle. The bottle was kept on ice for 10 min and then centrifuged at 4000xg in a GSA rotor for 5min at 4°C. The supernatant was discarded and the pellet was suspended in 33 ml of cold buffer (pH5.8) containing 100 mM RbCl, 50 mM MnCl₂·4H₂O, 30 mM potassium acetate, 10 mM CaCl₂·2H₂O and 15% (w/v) glycerol. Cells were kept on ice for 15 min, then centrifuged again at 4000xg for 5min. The pelleted cells were resuspended in 8 ml of cold buffer (pH6.8) which containing 10 mM MOPs, 10 mM RbCl, 75 mM CaCl₂·2H₂O, and 15% glycerol and kept on ice for 15min before being dispensed into cold microfuge tubes (200 µl aliquots) and transferred to a –70°C freezer.

About 50 ng of transforming DNA, in less than 10 µl was added to a 200 µl aliquot of competent cells. The suspension was mixed gently and placed on ice for 30 min. The mixture was then placed in 42°C water in a heating block for exactly 90 sec and immediately transferred onto ice. After chilling on ice for 2 min, 800 µl SOC medium

(2% bactotryptone, 0.5% yeast extract, 100mM NaCl, 25mM KCl, 10mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added and the transformation mixture was cultured at 37°C for 50min. Normally 50-200 µl of the culture were plated on a 90 mm LBC (LB medium with 25 µg chloramphenicol and 1.5% agar) plate containing 15 µl 100 mM IPTG and 50 µl 2% X-gal dissolved in DMSO. Plates were incubated at 37°C overnight and white colonies were selected and inoculated onto another LBC plate for further identification of the correct clone. After the clones containing the expected insert were selected, the plasmid was isolated by rapid plasmid mini prep.

Transformation of *Neurospora*:

A. Preparation of wild type 988 spheroplasts

Conidiospores from wild type *Neurospora* 988 on 8-10 days old plates were washed with 2 ml sterile water and transferred into a 250 ml flask containing 50 ml of VMM media to culture for 7 days. Conidia were harvested from a conidia flask with sterile water and filtered through a nylon stocking. After hydration at 4°C overnight, conidiospores were then concentrated by centrifugation (5,000rpm; 5 min, SORVALL SS-34 rotor) and resuspended in 75 mls of VMM in a 500 ml flask and incubated at 30°C with shaking for 4-6hrs. Then the germinated conidia were pelleted by centrifugation and washed with 75 ml sterile water followed by a wash in 20 ml of 1M sorbitol. The pellet was resuspended in 5 ml of 1 M sorbitol and then 2 ml of 5 mg/ml Novozyme stock solution (in 1 M sorbitol, sterilized by filtration) was added and incubated at 30°C for 2hrs with shaking. The efficiency of spheroplasting was checked by observing the swelling and lysis of the spheroplasts in water by microscopy. Spheroplasts were pelleted by centrifugation at 500g for 5 min and washed 2 times in 15 ml 1M sorbitol, and then

once in 15 ml of STC (50 mM Tris pH 8, 50 mM CaCl_2 , and 1 M sorbitol). Spheroplasts were resuspended in 8 ml of STC and the preparations were completed by the addition of 2ml of PTC (48% PEG, 50 mM Tris pH8, and 50 mM CaCl_2). Aliquots of 1ml were stored at -70°C , according to the protocol of Vollmer & Yanofsky (1986).

B. Transformation of Neurospora

The transformation protocol of Vollmer & Yanofsky (1986) was used. Typically, 5 μl of plasmid DNA (1-2 μg in TE) was mixed with 2 μl of spermidine-3HCl and 5 μl heparin (5 mg/ml in STC). After incubation on ice for a few minutes, the mixture was added to 100 μl spheroplasts, and incubated on ice for 30 min. One ml of PTC was added to the spheroplast suspension followed by 20 min incubation at RT. The spheroplast suspension was then added to 10 ml of molten regeneration agar (per litre: 20 ml 50x Vogel's stock solution, 875 ml water, 182g sorbitol, 15g agar and 50ml 20x SFG sugars), followed by pouring onto a plate containing about 25 ml of bottom agar (per litre: 20ml 50x Vogel's stock solution, 935 mls water, 15ml agar and 50mls 20x SFG sugars). The plates were then incubated at RT.

C. Purification of transformants

After the SFG plate had been incubated for 4-5 days at RT, the colonies which grew well were selected and inoculated onto a second SFG plate containing hygromycin B. Again after 4-5 days incubation at RT, the colonies which grew well were selected and inoculated onto VMM plates containing hygromycin B. After 36-48 hrs, samples of mycelia which had grown faster than others were cut out and inoculated onto a new plate. This step was repeated three times. The selected transformants were then inoculated onto VMM slants.

RIPing process

Crossing procedures were adapted from Pandit and Russo (1993). Wild-type 987A conidiospores were collected from 7-10 day old slants by washing with 2 ml sterile water, and inoculated onto a culture tube containing crossing media (per litre: 0.1% KNO₃, 0.07% K₂HPO₄, 0.05% KH₂PO₄, 0.05% MgSO₄•7H₂O, 0.01% CaCl₂, 0.01% NaCl, 0.01% v/v Biotin, 0.01% v/v Trace elements), using sterile 3MM Whatman filter paper as a carbon source.

The conidiospores were incubated at RT to induce the properithecia. After the properithecia developed, the conidiospores from transformants were collected by washing with 2ml sterile water, and added to the crossing tubes.

After 12 days incubation at RT, ascospores were collected from the wall of the crossing tube, by washing with 1ml sterile water. After heated at 60°C for 1 hour, 20 µl, 40 µl and 100 µl aliquots of ascospores were inoculated on to SFG plates respectively. Germination was allowed by incubating at room temperature for 24 hours, and the plates were examined under dissecting microscope (Olympus, model SZ-CTV). The germinated ascospores were digged up by Pasteur pipette and inoculated onto VMM slants. After 7 days, the phenotype of these progeny was examined.

Measurement of growth rate was performed at room temperature. The race tubes were made by adding VMM medium into 25ml pipettes. The mutants were inoculated onto these slants, and the growth rate was measured every 12 hours.

2.4. Complementation study

The full ORFs of those genes disrupted by RIP were amplified by PCR using specific primers, SBD3-2kF: 5'-GATTGGCAGACTCAACAGCGT-3', SBD3-2kR: 5'-

TCGGGTCAAGTAGAGGCACC-3', SBD6-2kF: 5'-TGTAACACTGCATCGGGCA-3', SBD6-2kR: 5'-CACCGTGCACATCCATATGC-3'. The PCR reactions were performed using a Pekin Elmer Cetus 480 DNA Thermal Cycler and Invitrogen reagents. Hot-started PCR was performed as follows: denaturation at 95°C for 5 min followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at optimal temperature for primer, extension at 72°C for 2min. A final 5min of extension at 72°C was included.

The PCR products were cloned into the TA Cloning vector pCR2.1 using the TA Cloning Kit (Invitrogen) following the manufacturer's instructions. One µl of TOPO cloning vector pCR2.1, 1-4 µl PCR product, 1 µl of ligation buffer, 1 µl salt solution, and distilled water up to 6 µl were added to the ligation-reaction mixture. After mixing, the ligation-reaction mixture was incubated at RT for 30min.

Transformations were performed using the manufacturer's protocol. The appropriate number of tubes of competent cells were thawed on the ice. SOC medium was thawed and warmed to room temperature. Then 2 µl of ligation-reaction mixture were added to each tube of competent cells. The competent cells were incubated for 30 sec on a heating block that preheated to 42°C, and then incubated on ice for 2 min. Finally, 250 µl room temperature SOC medium was added to each tube, and 100 µl of each transformation mixture was directly plated onto LB plates containing ampicillin (100µg/ml). The plates were incubated at RT until the transformation mixture has been absorbed into the agar. The plates were inverted and incubated at 37°C for 12-16 hours.

The spheroplasts from mutants were prepared as described above. The clones containing the gene of interest were co-transformed with the shuttle vector pCB1004 into

mutant spheroplasts using the same procedure as above. Then the transformants were selected on the plates containing hygromycin B.

2.5. Semi-Quantitative PCR

Gene expression of *sbr* was tested using RT-PCR. The PCR reactions were performed using a Pekin Elmer Cetus 480 DNA Thermal Cycler and GIBCO BRL reagents. The cDNA synthesized from wild type mRNA at different developmental stage were used as templates. The primers were used as follows, *sbr* pQEYY: 5'GCGGGA TCCAGGATTCCCTTAA3', yy5Rev: 5'TACCAAGCGGGTGAGGAGTG 3'. The PCR running conditions were the same as described above, but stopped at 25 cycles and 35 cycles respectively.

2.6. Purification of SBR protein under native conditions

Transformation of bacteria

The *sbr* gene was previously cloned into the QIAGEN pQE expression vector (pQE30) in the correct open reading frame (Yan, 2001). Competent cells (M15) had been prepared according to the manufacturer's instructions. The expression vectors which contain the correct *sbr* ORFs were transformed into M15 cells, and the transformed M15 cells were plated on LB plates containing 100 µg/ml ampicillin and 25 µg/ml kanamycin.

Expression of SBR protein

The transformed M15 cells were cultured in 10 ml LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin, at 37°C. Overnight culture (2.5 ml) was added to 50 ml medium and left to continue to growing at 37°C with vigorous shaking until the OD₆₀₀ reached 0.5-0.7 (approximately 30-60 min). IPTG was added to a final concentration of 1 mM and the culture was grown for 5 hours at 37°C. Cells were

harvested by centrifugation at 4000xg for 20 min and the cell pellet was frozen at -20°C for one or a few days.

Protein purification

All purification steps were done at 4°C or on ice. The cell pellet was thawed and resuspended in lysis buffer (50 mM NaH₂PO₄, pH8.0; 300 mM NaCl; 10 mM imidazole) at 5 ml per gram wet weight. The suspension was incubated for 60 min with lysozyme at 1 mg/ml, then sonicated 6x for 10 sec alternating with 10 sec cooling intervals by using a sonicator equipped with a microtip. The lysate was incubated with Rnase A (10 µg/ml) and Dnase I (5 µg/ml) for 15 min before centrifugation at 10,000xg for 30min to pellet the cellular debris. The clear supernatant (soluble crude extract) was collected into a new tube and the pellet (insoluble crude extract) was re-suspended in 5ml lysis buffer. To assess SBR protein solubility, 5 µl of each crude extract was used for SDS-PAGE analysis. Purification of SBR was carried out as follows: 1ml 50% Ni-NTA agarose slurry (QIAGEN) was added to 4 ml of soluble crude extract, and the mixture was incubated for 60 min with gentle shaking on a shaker and occasional hand mixing. The mixture was loaded into a column and the column was then washed with 4 ml wash buffer (50 mM NaH₂PO₄, pH8.0; 300 mM NaCl; 20 mM imidazole), followed by 4 ml wash buffer containing 50% glycerol. The protein was eluted with 4x 0.5 ml elution buffer (50 mM NaH₂PO₄, pH 8.0; 300 mM NaCl; 250 mM imidazole, 50% glycerol) into 4 tubes and then 2.5 µl of each elution was analyzed by SDS-PAGE. For the DNA binding assay, eluted protein was dialysed in 200 ml of dialysis buffer containing the same components without imidazole, at 4°C for two days to reduce imidazole. Protein

concentration was determined by measuring the OD₂₃₀ & OD₂₆₀ and using BSA of known concentrations as standard.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Two 0.75 mm thick polyacrylamide gels were made using the Mini-PROTEAN[®] II Dual Slab Cell (BioRad). The instructions accompanying stacking gel were followed. A 10 well 0.75 mm thick comb was used to make the wells. Five µl of broad-range protein molecular marker (BioLab, New England) were loaded into a well of each gel as a molecular weight standard. The gel was run at 170 V for 50-60 min powered by the Bio-Rad Model 200/2.0 power supply.

Protein gels were stained in 0.1% Commassie Brilliant Blue R-250 in fixative (10% acetic acid, 40% methanol). Background staining was removed with destain (10% acetic acid, 40% methanol). Gels were dried at 80°C for 2 hours in a Model 583 Vacuum Gel Dryer (Bio Rad) while sandwiched between a sheets of cellophane that were equilibrated in destain with 3% (v/v) glycerol.

Western blot and immunostaining

The gel and nitrocellulose membrane (Protran) were equilibrated in transfer buffer (25mM Tris, 192mM glycine, 20% (v/v) methanol) for 30 min. Using the Transblot[®]RSD Semi-Dry paper (the same size as the gel) saturated in transfer buffer were stacked on the anode. The membrane (slightly larger than the gel) was layered next followed by the gel. An additional 3 pieces of filter paper were stacked on top and bubbles were removed at each layer. The cathode was placed on top and transfer was performed at 15V for 36min. The membrane was stored at 4°C in plastic wrap.

The nitrocellulose blots were blocked with 3% BSA in TBS (10mM Tris-Cl, pH7.5; 150 mM NaCl) for 60 min with shaking at RT. Membranes were washed 2x for 10 min in TBS-Tween/Triton buffer (20 mM Tris-Cl, pH7.5; 500 mM NaCl; 0.05% Tween 20; 0.2% Triton X-100) and once in TBS for 10 min, then incubated with a 1:2,000 dilution of mouse RGS-His antibody (QIAGEN) in TBS with 3% BSA at RT for 60 min, washed twice in TBS-Tween/Triton buffer and once in TBS for 10min/ each. The membrane was incubated with 1:20,000 goat anti mouse antibody conjugated with peroxidase in TBS/10% skim milk powder, followed by washing 4X for 10 min in TBS-Tween/Triton buffer.

The Chemiluminescence Blotting System (Roche) was used for detection of membrane-bound protein labelled by antibodies according to the manufacturer's instructions. Membranes were incubated in a mixture of substrate solution A and starting solution (100:1) for 60 sec. The blot was wrapped in plastic wrap. Autoradiographic film was exposed to the membrane for 4 sec to 2 min and developed immediately.

2.7. Electrophoretic Mobility Shift Assay (EMSA)

Preparation of non-specific competitor

The cloning vector plasmid pBSKS+ was used as competitor. Plasmid DNA was digested with *EcoRV* to generate blunt ends, the digestion mixture was processed by gel electrophoresis. Then DNA was recovered from agarose gel.

Preparation of probe DNA

Dephosphorylation of DNA fragments with alkaline phosphatase

A dephosphorylation protocol from Sambrook *et al.* (2000) was followed with minor changes. Probe DNA prepared by PCR were cloned into a vector pGEM-T Easy

(Promega). After plasmid mini preparation, about 1µg of plasmid DNA was digested with *EcoRI* and *NotI*, the digest was analyzed by agarose gel electrophoresis, and the restricted DNA was recovered from agarose gels using the method described above. The following components were added to the reaction mixture: 10~100 pmoles digested DNA, 5µl 10x SAP dephosphorylation buffer, up to 48 µl H₂O, and 2 unit SAP. After mixing, the reaction was incubated at 37°C for 1hour. To inactivate SAP, the reaction was incubated at 70°C for 20 min, and cooled to room temperature. Then the aqueous phase was transferred to a clean microfuge tube, and the DNA was recovered by standard ethanol precipitation in the presence of 0.1 volumes of 3M sodium acetate (pH 5.2). The precipitate was allowed to dry at RT before being dissolved in TE (pH 7.6) at a DNA concentration of 72 nmoles/ml.

End labelling the 5'termini of DNA by forward reaction

In a microfuge tube, the following reagents were added: 10~50 pmoles dephosphorylated DNA, 5ul of 10x bacteriophage T4 polynucleotide kinase buffer, 12 ul (50pmoles) of 10mCi/ml [γ^{32} -p]ATP, 10 units of bacteriophage T4 polynucleotide kinase, and H₂O up to 50ul. Then the reaction mixture was incubated at 37°C for 1 hour. The reaction was terminated by adding 2 µl of 0.5M EDTA (pH 8.0). The total radioactivity in the reaction mixture was measured by Cerenkov counting in a liquid scintillation counter.

Purification of radiolabelled probe

The radiolabelled probe was separated from unincorporated dNTPs by spun-column chromatography through Sephadex G-50 (Sambrook *et al.*, 2000). The bottom of a 1ml disposable syringe was plugged with a small amount of sterile glass wool, and

filled with Sephadex G-50 equilibrated in 1x TEN buffer (pH 8.0). After insertion into a 15ml disposable plastic tube, the syringe was centrifuged at 1600x g for 4 min at RT in a swinging-bucket rotor. More resin was added and centrifuged until the volume of the packed column was ~0.9 ml. One hundred μ l of 1x TEN buffer was added to the columns, and centrifuged at 1600xg for 4 min, this step was repeated twice more. The DNA sample was applied to the column in a total volume of 0.1 ml (1x TEN buffer was used to make up the volume). The spun column was placed in a fresh disposable tube containing a decapped microfuge tube. After centrifugation the effluent from the bottom of syringe (~100 μ l) was collected into the decapped microfuge tube. The syringe which contained unincorporated radiolabelled dNTPs or other small components was removed. The eluted DNA was transferred to a capped, labeled microfuge tube, and stored at -20°C until needed.

Binding reaction

In a 20 μ l reaction, the following was added in order: sterile water, 2 μ l 10x binding buffer (stock: 500 mM Tris-Cl, pH7.5, 1 M NaCl, 100 mM β -mercaptoethanol, 1mM ECTA, 5 mg/ml BSA), competitor DNA (0-10 μ l), probe DNA (1-4 μ l), SBR protein 3 μ l (about 1.5 μ g). Since SBR was stored in 50% glycerol, when less protein was added, the glycerol concentration was adjusted to a final 10-15%. Reactions were mixed and incubated at RT for 20 min.

Electrophoresis

Normally, 4-5% acrylamide gels (stock: 39% acrylamide, 1% bis-acrylamide), were prepared with the running buffer (6.7 mM Tris-Cl, pH7.9, 3.3 mM sodium acetate, 1mM EDTA). After polymerization, the gel was pre-run for 1-2hrs at 10V/cm. The

binding reaction was loaded onto the gel in the cold room and electrophoresed at the 10V/cm for 3-4 hrs. The gel was fixed in 10% methanol and 10% acetic acid for 20 min, transferred to a 3MM paper, covered with plastic wrap, and vacuum dried at 80°C for 1 hour before exposing to Kodak autoradiographic film or phosphor screen.

2.8. Epitope tagging

The whole ORF of the *sbr* gene was amplified by PCR with specific primers, *sbr*-F: 5'GCCTCGAGGACATAGCAGGTGGGCGTCT 3', *sbr*-R: 5'GCGAATTCGCATCTTCACCCAAGCCCT 3'. The PCR reaction was performed using a Perkin Elmer Cetus 480 DNA Thermal Cycler and Invitrogen reagents and genomic DNA from *N. crassa* wild type strain 988 was used as a template. The hot-started PCR was performed under the following conditions: denaturation at 95°C for 5 min followed by 35 cycles of 1min denaturation at 95°C, 1 min annealing at optimal temperature for primer, extension at 72°C for 2 min. A final 5 min of extension at 72°C was included.

The *sbr* gene was then cloned into the UA cloning vector pDrive using a QIAGEN PCR cloning kit (QIAGEN) following the manufacturer's instructions. After the clone containing the *sbr* gene was selected, the plasmid DNA was double digested with restriction enzymes, *Xho*I and *Eco*RI. The digestion mixture was separated by agarose gel electrophoresis, and the restricted *sbr* gene was recovered from the agarose gel. Then the *sbr* gene was subcloned into an expression vector pEpi-3HA, which contains 3HA tags in the correct open reading frame. The construct was co-transformed into *N.crassa* with the shuttle vector pCB1004 which has a hygromycin B resistance marker for selection in *Neurospora*.

After screening on hygromycin B media, the selected transformants were grown on Vogel's minimal medium. To confirm the successful transformation by the tagged *sbr* construct, PCR was performed by using one primer from vector (T7-Univ), and another primer from the *sbr* gene (*sbr* 003).

2.9. DNA analysis with bioinformatic programs

The following bioinformatic programs were used in DNA analysis:

- 1) NCBI BLAST: NCBI'S sequence similarity search tool was designed to support analysis of nucleotide and protein databases:

<http://www.ncbi.nlm.nih.gov/BLAST/>.

- 1) *Neurospora crassa* database at Whitehead Institute: it provides BLAST search for finding similarities to other sequences: [http://www-](http://www-genome.wi.mit.edu/annotation/fungi/neurospora/)

[genome.wi.mit.edu/annotation/fungi/neurospora/](http://www-genome.wi.mit.edu/annotation/fungi/neurospora/).

- 2) MIPS *Neurospora crassa* database (MNCDB): it also provides BLAST search tool sequence similarity analysis specific to *N. crassa*:

<http://www.mips.biochem.mpg.de/proj/neurospora/>.

- 3) NCBI ORF program: The ORF (Open Reading Frame Finder) program is graphical analysis tool which finds all open reading frames of a selectable minimum size in a user's sequence or in a sequence already in the database. This tool identifies all open reading frames using the standard or alternative genetic codes. <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>

- 4) TRANSFAC: the TRANSFAC database is maintained by GBF research group "Bioinformatics" at Braunschweig. It compiles data about gene regulatory DNA sequences and protein factors binding to, and acting through them. These

programs are developed that help to identify putative promoter or enhancer structures and to suggest their features. Transcription factors information and binding sites and related sources such consensus and related publications are also available: <http://transfac.gbf.de/TRANSFAC/>.

- 5) MatInspector V2.2: a TRANSFAC program based on TRANSFAC 4.0, searching for binding sites with different core and matrix scores and detailed information of binding factors are directly associated with TRANSFAC database: <http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl>.
- 6) GENSCAN: a gene identification program developed at MIT. This program predicts the locations and exon-intron structures of genes in genomic sequences from a variety of organisms. This server can now accept sequences up to 1 million base pairs (1Mbp) in length: <http://genes.mit.edu/GENSCAN.html>.
- 7) CGG (computational genomics group) DNA Analysis: a program to identify putative genes. Maintained at the Sanger Center (UK): <http://genomic.sanger.ac.uk/gf/gf.html>.
- 8) Motif search: a collection of different search programs including PROSITE, BLOCKS, ProDom, PRINTS, Pfam is available at address: <http://www.motif.genome.ad.jp/>.

III. RESULTS

3.1. Gene expression of *sbr* during development

The extreme colonial morphology, isometric growth and lack of conidiation of the *sbr* mutant suggest that its developmental programme is altered. In addition, the primary sequence of the SBR protein suggests that it has a regulatory function because it encodes a helix-turn helix (HTH), a putative zinc finger, and a glutamine-rich region (Yan, 2001). It was therefore of interest to determine whether the *sbr* gene was itself under developmental control. In order to analyze expression of the *sbr* gene at different stages during the development, semi-quantitative PCR was performed with gene-specific primers. The cDNA from different developmental stages (conidiospores, germinating conidiospores, conidiation, mycelium, and mating) were used as template. Specific, *sbr* derived, PCR products were observed at all developmental stages even when 25 PCR cycles were performed (Fig. 3A). This result suggests that the *sbr* gene is expressed at all developmental stages (Fig. 3A). The band density of PCR products amplified using cDNA from germinating conidia is significantly higher than other bands. When the expression of *sbr* was normalized to actin however, very similar RT-PCR results were obtained (Fig. 3B). Since the expression level of actin is relatively stable during development, the variation in DNA abundance in Figure 3 can be attributed to differences in concentration of cDNA template in each preparation (Tinsley *et al.*, 1998).

Figure 3 Expression of the *sbr* gene during development assessed by RT-PCR. M. Marker (λ Hind III). **A)** Semi-Quantitative PCR of *sbr* gene: Lane1-5: 25 PCR cycles, Lane 6-10: same templates as lane1-5, but 35 PCR cycles. **B)** Semi-Quantitative PCR of *actin* gene: Lane 1-5: 25 PCR cycles, Lane 6-10: same templates as lane1-5, but 35 PCR cycles.

3.2. Inactivation of SBR Binding DNA Segments by RIP

Generation and isolation of RIP mutants

Previous studies have indicated that SBR is a DNA binding protein with a putative regulatory function (Yan, 2001). Two target genomic DNA fragments (SBRBD300 and SBRBD600) that contain SBR protein binding sites were isolated from protein-DNA complexes. We hypothesized that these DNA fragments are regulatory sequences which respond to SBR activity. To determine whether inactivation of any of these regions of the genome result in morphological mutations, an *in vivo* mutagenesis procedure known as RIP (repeat induced point mutation) was performed. RIP is a process whereby duplicated sequences (such as those that result in ectopic integrations of genes during transformation) suffer cytosine methylation and G-C to A-T transition mutations when transformants carrying duplicated sequences are taken through a cross (Selker, 1990).

The SBRBD300 (1.5kb) and SBRBD600 (1.8kb) fragments were amplified by PCR and cloned into the UA cloning vector pDrive (QIAGEN). Then the inserts were double digested with restriction enzymes *Bam*HI and *Not*I, and subcloned into the shuttle vector pCB1004 which has a hygromycin resistance marker for selection in *Neurospora* (Fig.4). The pCB1004 plasmid derivatives containing putative SBR target sequences were transformed into the wild-type strain of *N. crassa* (FGSC# 988a), and positive transformants were incubated on the plates containing hygromycin B at RT for about 4 days. Transformants were subsequently purified to homokaryons by serial plating on hygromycin B plates three consecutive times and inoculated onto slants containing Vogel's minimal medium to allow the conidiospores to develop. The opposite mating

Figure 4 Constructs for the RIPing process.

The plasmid is composed of the 3.4kb fragment from pBSKS (Bluescript) vector and a 1.4kb altered hygR cassette from pCSN43. The insert is composed of the 1.5kb DNA fragment of SBRBD300 or the 1.8kb DNA fragment of SBRBD600.

strain 74-OR23-1A of *N. crassa* was inoculated onto crossing medium to stimulate the development of properithecia and it was then mated to selected transformants by introducing their conidiospores into the crossing tubes. Twelve days later, ascospores were collected from each cross, and inoculated onto SFG plates. Two day old colonies germinating from these spores were examined under microscope. Approximately 10 mutant strains from each cross were found out of 90 ascospores from each cross examined.

Phenotypic characterization of RIP300 and RIP600 mutants

Among mutant strains from the SBRBD300 cross, the strain RIP300-9# is very different morphologically in comparison with wild-type (Fig. 6d). It has white adherent conidiospores instead of the powdery orange spores, and a dense hyphal plug fills the diameter of the culture tube (Fig. 5B). The growth rate of this strain was similar (1.94mm/hr) to that of wild-type (1.92mm/hr) at RT (Fig. 7). In contrast, another mutant strain RIP300-1# displayed a lower growth rate (0.49mm/hr) than that of wild-type (Fig. 7). This strain produces fewer conidiospores than that of wild-type, and the colour of its colonies are bright yellow instead of pink-orange (Fig. 5B). Among 10 mutants, two mutants grew similar to wild-type, and eight mutants grew slower than wild-type. The colony morphology of these strains is different in comparison with wild-type (Fig. 6).

Mutant strains isolated from the SBRBD600 RIP cross also displayed two different phenotypes. For instance, strain RIP600-4# exhibits a lower growth rate (1.11mm/h) than that of wild-type (Fig. 7). The number of conidiospores of this mutant strain is less than that of wild-type, and the colony displayed a lighter colour than that of wild-type (Fig. 5C and Fig. 6e). Strain RIP600-12# displayed a similar growth rate

Figure 5 Wild-type and RIP mutant strains.

A. Wild-type wta 988. **B.** Mutant strains RIP300-1#, and RIP300-9#. **C.** Mutant strains RIP600-4#, and RIP600-12#.

A

B

C

Figure 6 Comparison of colonies of different mutant strains and wild-type.

All of the colonies have grown on minimal medium for 48 hours at RT. a) wild-type, b) RIP300-1#, c) RIP300-6#, d) RIP300-9#, e) RIP600-4#, and f) RIP600-12#.

Figure 7 Comparison of the growth rate of wild-type 988 and different mutant strains.

Mutant strain RIP300-1#, RIP300-6# and RIP600-4# showed lower growth rates than that of wild-type while RIP300-9# and RIP600-12# exhibited growth rates similar to wild-type.

(2.02mm/h) to that of wild-type (Fig. 7). Also, since conidiospore production is reduced, the colony colour was close to white rather than the usual pink- orange (Fig. 5 and Fig 6).

Scanning electron microscopy revealed that, the mutant strain RIP300-6#, resulting from SBRBD300 RIP cross, exhibited an increase in branching frequency (Fig. 8c). In addition the morphology of hyphae was irregular with light curling. In contrast, the hyphae of the wild-type (Fig. 8a) were more regular, and despite some minor meandering they exhibited long stretches of mostly straight growth. However, the hyphal morphology of RIP300-1# and RIP600-4# are similar to the wild-type (Fig. 8b,d).

Sequence analysis of mutant SBRBD300 and SBRBD600

Genomic DNA of selected putative SBRBD300 and SBRBD600 mutants resulting from RIPing, was used as the template to amplify the endogenous gene by PCR as described in Materials and Methods. The DNA amplification products of four RIP mutants were isolated and cloned into the TOPO cloning vector pCR2.1 (Invitrogen) for sequencing. Nucleotide sequences of the mutagenized SBRBD300 and SBRBD600 DNAs cloned from RIP mutants are reported in Table 3. These (partial) results show that the number of point mutations introduced in each mutant allele varied between 3 and 6, and all mutations were polarized G-A or C-T transitions (Fig. 9, and Fig. 10). Two mutants (RIP300-1#, and RIP600-4#) had C-T transitions, mutant strain RIP600-12# only had G-A transition, and RIP300-9# had A-G and C-T transitions on the coding strand.

The consequences of the mutations in strain RIP300-1# are substitution of Ala with Val at position 188 (Table 3), and the substitution of His with Tyr at position 232, as well as a silent mutation at position 186. Mutant RIP300-9# has one point mutation that

Figure 8 Scanning electron microscopy of RIP mutant and wild-type strains. The fungal cultures have grown for 1-2 days. **a)** wild-type, **b)** RIP300-1#, **c)** RIP300-6#, and **d)** RIP600-4#. The length of the bar represents 100 μm .

lead to amino acid substitutions (Ser 219 > Leu) and three silent mutations at position 21, 77 and 81 (based on the putative open reading frame).

Mutant strain RIP600-4# carries only one significant mutation (Ser 301 > Phe) (Fig. 10, and Fig. 13), as well as a silent mutation at position 123. One amino acid substitution (Arg 222 > Gln) and one silence mutation at position 369 were found in mutant RIP600-12# (base on the cDNA sequence) (Fig. 10, and Fig. 13). These mutations are listed in Table 3, Figure 9, and Figure 10 show their relative positions in the SBRBD300 and SBRBD600.

Complementation Study

To demonstrate that the inactivation of SBRBD300 and SBRBD600 was responsible for the phenotype of RIP mutants, the wild type copy of two putative genes within these two regions were introduced to RIP mutants. Only one transformant was recovered from each transformation but each one showed a normal growth rate and wild type morphology. Although the results are preliminary, they indicate that the lower growth rate and mutant phenotype in RIP300 and RIP600 mutants can be complemented by the introduction of an ectopic copy of the wild-type gene.

Table 3 RIP-induced Mutations of different mutant strains.

Show the positions and types of point mutations in the SBRBD300 and SBRBD600.

Mutant Strain	Number of transition mutations	Significant mutations (nucleotide no.)	Silent mutations (nucleotide no.)	Type
RIP300-1#	4	232 His→Tyr	186	C→T
			188	C→T
			189	C→T
				C→T
RIP300-9#	6	219 Ser→Leu	21	C→T
			77	G→A
			78	C→T
			81	C→T
			82	C→T
RIP600-4#	4	301 Ser→Phe	123	C→T
				C→T
RIP600-12#	3	222 Arg→Gln	369	G→A
				G→A

Figure 9 Multiple alignment of wild-type SBRBD300 and RlPed SBRBD300.
The mutated SBRBD300 DNAs were isolated from mutant strains RlP300-1#, and
RlP300-9#, show the positions of transitions in SBRBD300.

RIP300-1#	CTGCAGGATCGAACTAACAGCTGCAAGGTGAGATGATGGC	40
RIP300-9#	CTGCAGGATCGAACTAACAGCTGCAAGGTGAGATGATGGC	40
SBRBD300	CTGCAGGATCGAACTAACAGCTGCAAGGTGAGATGATGGC	40
Consensus	ctgcaggatcgaactaacag tgcaaggtgagatgatggc	
RIP300-1#	GTTTTGGTGGCTTTTGTAGCGCGGGTTATCACCAGGCT	80
RIP300-9#	GTTTTGGTGGCTTTTGTAGCGCGGGTTATCACCAGATCT	80
SBRBD300	GTTTTGGTGGCTTTTGTAGCGCGGGTTATCACCAGGCT	80
Consensus	gttttggtggcttttgttagcgcggttatcaccag ct	
RIP300-1#	CCGCACAGATTCGGGGCATTTCCTTTTCAGCTAAGTAAGCA	120
RIP300-9#	TTGCACAGATTCGGGGCATTTCCTTTTCAGCTAAGTAAGCA	120
SBRBD300	CCGCACAGATTCGGGGCATTTCCTTTTCAGCTAAGTAAGCA	120
Consensus	gcacagattcggggcatttccttttcagctaagtaagca	
RIP300-1#	TCGCAGACTGCGCAGATCGCTTTTACAAACCCAAACCCCTAA	160
RIP300-9#	TCGCAGACTGCGCAGATCGCTTTTACAAACCCAAACCCCTAA	160
SBRBD300	TCGCAGACTGCGCAGATCGCTTTTACAAACCCAAACCCCTAA	160
Consensus	tcgcagactgcgcagatcgcttttaciaaacccaaaccctaa	
RIP300-1#	GGCACCCCAAGTTGAGTTTCTGTAATTGTTATTGGTAAAGC	200
RIP300-9#	GGCACCCCAAGTTGAGTTTCTGTAATCGCATTTGGTAAAGC	200
SBRBD300	GGCACCCCAAGTTGAGTTTCTGTAATCGCATTTGGTAAAGC	200
Consensus	ggcacccaagttgagtttctgtaat g attggtaaagc	
RIP300-1#	TGTCAGTCAGGCCCTGTGAGCTGAAAACGAAACACCCCGAG	240
RIP300-9#	TGTCAGTCAGGCCCTGTGAGCTGAAAACGAAACACCCCGAG	240
SBRBD300	TGTCAGTCAGGCCCTGTGAGCTGAAAACGAAACACCCCGAG	240
Consensus	tgtcagtcaggccctgt agctgaaaacgaa accccgag	
RIP300-1#	TCCTTTCCGTACCGGTTCCGTGCTCATCAGCTGGCGTCGT	280
RIP300-9#	TCCTTTCCGTACCGGTTCCGTGCTCATCAGCTGGCGTCGT	280
SBRBD300	TCCTTTCCGTACCGGTTCCGTGCTCATCAGCTGGCGTCGT	280
Consensus	tcctttcggtagccggttcctgctcatcagctggcgctcgt	
RIP300-1#	CAACAAGAAAAGCTT	295
RIP300-9#	CAACAAGAAAAGCTT	295
SBRBD300	CAACAAGAAAAGCTT	295
Consensus	caacaagaaaagctt	

Figure 10 Multiple alignments of wild-type SBRBD600 and RIPed SBRBD600
The mutated SBRBD600 DNAs were isolated from mutant RIP600-4#, and RIP600-12#
show the positions of transitions in SBBD600.

RIP600-4#	CTGCAGCAGATGCTGAAAAGCATTGGCTATGATGAAGCAA	40
RIP600-12#	CTGCAGCAGATGCTGAAAAGCATTGGCTATGATGAAGCAA	40
SBRBD600	CTGCAGCAGATGCTGAAAAGCATTGGCTATGATGAAGCAA	40
Consensus	ctgcagcagatgctgaaaagcattggctatgatgaagcaa	
RIP600-4#	GGGAGGATGCATACAGACTCAAAGGCGTCCAGCTCATTGA	80
RIP600-12#	GGGAGGATGCATACAGACTCAAAGGCGTCCAGCTCATTGA	80
SBRBD600	GGGAGGATGCATACAGACTCAAAGGCGTCCAGCTCATTGA	80
Consensus	gggaggatgcatacagactcaaaggcgctccagctcattga	
RIP600-4#	TAGCGTTCGACAAAGCATAAGCCTGTGCGGACCCATCGCT	120
RIP600-12#	TAGCGTTCGACAAAGCATAAGCCTGTGCGGACCCATCGCT	120
SBRBD600	TAGCGTTCGACAAAGCATAAGCCTGTGCGGACCCATCGCT	120
Consensus	tagcggttcgacaaagcataagcctgtgcggacccatcgct	
RIP600-4#	TCTAAGCCTTACCTACGCATTTTGCTGACGAACGCCACAG	160
RIP600-12#	TCTAAGCCTTACCTACGCATTTTGCTGACGAACGCCACAG	160
SBRBD600	TCTAAGCCTTACCTACGCATTTTGCTGACGAACGCCACAG	160
Consensus	tc aagccttacctacgcatttttgctgacgaacgccacag	
RIP600-4#	GCCGGTCAAGACATTCGATACAGCAGCGACCTACTACCAC	200
RIP600-12#	GCCGGTCAAGACATTCGATACAGCAGCGACCTACTACCAC	200
SBRBD600	GCCGGTCAAGACATTCGATACAGCAGCGACCTACTACCAC	200
Consensus	gccgggtcaagacattcgatacagcagcgacctactaccac	
RIP600-4#	ACGTTTCAGAATACCGTTTCCGAGCGCCGAGTACAACCTACG	240
RIP600-12#	ACGTTTCAGAATACCGTTTCCGAGCGCCGAGTACAACCTACG	240
SBRBD600	ACGTTTCAGAATACCGTTTCCGAGCGCCGAGTACAACCTACG	240
Consensus	a gttcagaataacggtttccgagcgccgagtacaactacg	
RIP600-4#	AAGATGTAGCTCTTGCGTCGTTGTTTGTTCATGCAAGGT	280
RIP600-12#	AAGATGTAGCTCTTGCGTCGTTGTTTGTTCATGCAAGGT	280
SBRBD600	AAGATGTAGCTCTTGCGTCGTTGTTTGTTCATGCAAGGT	280
Consensus	aagatgtagctcttgcgtcgttgtttgttgcattgcaagggt	
RIP600-4#	GGAGGATACGATCAAGAAGTCAAGGATGTCCTATGTGCT	320
RIP600-12#	GGAGGATACGATCAAGAAGTCAAGGATGTCCTATGTGCT	320
SBRBD600	GGAGGATACGATCAAGAAGTCAAGGATGTCCTATGTGCT	320
Consensus	ggaggatacgatcaagaagt caaggatgtcctatgtgct	

RIP600-4#	GCGCACAATATCAGGCAGCCCCACGATCAAAGGACTCCGG	360
RIP600-12#	GCGCACAATATCAGGCAGCCCCACGATCAAAGGACTCCGG	360
SBRBD600	GCGCACAATATCAGGCAGCCCCACGATCAAAGGACTCCGG	360
Consensus	gcgcacaaatcagggcagccccacgatcaaaggactccgg	
RIP600-4#	ATGACAAGGTTTCGTCAATTATCTTGTTGCGCATCTACGGT	400
RIP600-12#	ATGACAAGGTTTCGTCAATTATCTTGTTGCGCATCTACGGT	400
SBRBD600	ATGACAAGGTTTCGTCAATTATCTTGTTGCGCATCTACGGT	400
Consensus	atgacaagggttcgtcaattatcttggtg cgatctacggt	
RIP600-4#	GTACCTACTAATATTGACCGCGCAGATGTTTCGATGGTCCA	440
RIP600-12#	GTACCTACTAATATTGACCGCGCAGATGTTTCGATGGTCCA	440
SBRBD600	GTACCTACTAATATTGACCGCGCAGATGTTTCGATGGTCCA	440
Consensus	gtacctactaatattgaccgcgagatggttcgatgggtcca	
RIP600-4#	TCCAAATTCACAGTGGGCCTCGAGCGTCATATTCTGGAGA	480
RIP600-12#	TCCAAATTCACAGTGGGCCTCGAGCGTCATATTCTGGAGA	480
SBRBD600	TCCAAATTCACAGTGGGCCTCGAGCGTCATATTCTGGAGA	480
Consensus	tccaaattcacagtgggcctcgagcgatcatattctggaga	
RIP600-4#	CCATTGGCTTCGACTTCCAGGCTCAGTATCCCCAGAAGTT	520
RIP600-12#	CCATTGGCTTCGACTTCCAGGCTCAGTATCCCCAGAAGTT	520
SBRBD600	CCATTGGCTTCGACTTCCAGGCTCAGTATCCCCAGAAGTT	520
Consensus	ccattggcttcgacttccaggctcagtatccccagaagtt	
RIP600-4#	GCTGATCAAATTACTTCGGAAGATGTTCCCAAAGGGCGAA	560
RIP600-12#	GCTGATCAAATTACTTCGGAAGATGTTCCCAAAGGGCGAA	560
SBRBD600	GCTGATCAAATTACTTCGGAAGATGTTCCCAAAGGGCGAA	560
Consensus	gctgatcaaattacttcggaagatggtcccaaagggcgaa	
RIP600-4#	AAAGGGGACAGTCCCGAGGTCAAGAAGTTCATCAGCGATG	600
RIP600-12#	AAAGGGGACAGTCCCGAGGTCAAGAAGTTCATCAGCGATG	600
SBRBD600	AAAGGGGACAGTCCCGAGGTCAAGAAGTTCATCAGCGATG	600
Consensus	aaaggggacagtcccgaggtcaagaagttcatcagcgatg	
RIP600-4#	CATACGACATGTCCATCGATCTTTACAAGACGTTTGCGCC	640
RIP600-12#	CATACGACATGTCCATCGATCTTTACAAGACGTTTGCGCC	640
SBRBD600	CATACGACATGTCCATCGATCTTTACAAGACGTTTGCGCC	640
Consensus	catacgacatgtccatcgatctttacaagacgtttgcgcc	
RIP600-4#	CTTGAAGCAACCAAGCTT	658
RIP600-12#	CTTGAAGCAACCAAGCTT	658
SBRBD600	CTTGAAGCAACCAAGCTT	658
Consensus	cttgaagcaaccaagctt	

3.3. Sequence Analysis of the SBRBD Binding DNA Fragments

Sequence analysis of SBRBD300

A BLASTN similarity search (NCBI) of SBRBD300 locus in the Whitehead Institute *Neurospora crassa* database identified this sequence in a 77715 bp genomic DNA fragment from *N. crassa* linkage group I. Position 63500 to 63844 contains the SBRBD300 DNA. The sequence from SBRBD300 and 2500 bp downstream or upstream from it was then analyzed for genes that might be responsive to SBR regulation.

A BLASTX similarity search with 1.5kb of sequence spanning SBRBD300 that was disrupted by RIP retrieved a gene, B24P7.300, which encodes a 138 amino acid hypothetical protein. Three other hypothetical genes were also identified by a BLASTX (NCBI) search of DNA 2500bp downstream and upstream of SBRBD300. The hypothetical gene B24P7.300 is located from 150bp upstream of SBRBD300 to 750 bp downstream. The second ORF in this region is the hypothetical gene, B24P7.310, encoding a protein of 116 amino acids which is located 1093bp downstream of SBRBD. The third hypothetical gene codes for a 248 amino acid protein related to cytosolic Cu/Zn superoxide dismutase, and it is located 2006bp downstream of SBRBD300 (Fig. 11). The fourth hypothetical protein is related to hxB protein [MIPS] *N. crassa*. It encodes 632 amino acids, a homology of hydroxylase, and is located 2362 bp upstream of SBRBD300. Since the second, third, and fourth hypothetical proteins are located more than 1000 bp away from the SBRBD300, and only the B24P7.300 is located within the disrupted region (SBRBD300-1.5k), this is probably the gene which has been disrupted by RIP. Translation analysis of this gene revealed two introns in this putative gene (Fig. 13).

Sequence analysis of SBRBD600

Similarly, a BLASTN search in the *Neurospora crassa* database retrieved a 137571 bp genomic DNA fragment (contig 3.230) from *N.crassa*, position 114806-115463 which contains the sequence of SBRBD600. Sequences 2500 bp downstream or upstream of SBRBD600 were also analyzed for genes that might be responsive to SBR regulation.

Only two ORFs were identified in the 5 kb region flanking SBRBD600. The first one codes for a hypothetical protein XP_323848.1 of 442 amino acids. It is located from 200bp upstream of SBRBD600 to 646 bp downstream. The second OFR is located 1891 bp upstream of SBRBD600 and it encodes a hypothetical protein of 315 amino acids (Fig. 12). Since the first protein is located within the disrupted region (SBRBD600-1.85k), and overlaps with the SBRBD600, it is most likely to have been disrupted by RIP. Furthermore, two cDNA clones, b4c04np.r1 and b4c04np.f1, were found in *Neurospora crassa* perithecia cDNA database which have high similarity (bits: 183, E-value: 5e-48, and bits: 154, E-value: 2e-39) to this gene. However, no poly A tail was found in either of these two cDNA. A comparison of cDNA and SBRBD600 sequences revealed two introns within this ORF (Fig. 14).

3.4. Binding sites in SBRBD300 & SBRBD600

The bioinformatics program MatInspector V2.2 (a TRANSFAC program based on TRANSFAC4.0) was used in the binding site search. Many candidate binding sites were found for the SBRBD300 and SBRBD600; those with the highest matching score and common to each isolated DNA fragment were listed in table 4. All binding sites listed in table 4 had a score of 1 (100% match) to the core sequence. Two binding sites

Figure 11 Map of the genome flanking SBRBD300 showing the putative genes.

1. Hypothetical protein B24P7.300-*Neurospora crassa*. 2. Hypothetical protein B24P7.310-*N.crassa*. 3.Hypothetical protein (related to cytosolic Cu/Zn superoxide dismutase-*N. crassa*. 4. Related to hxB protein [MIPS] *N. crassa*

Figure 12 Map of the genomic region of SBRBD600.

The predicted genes at the SBRBD600 locus are: 1. Hypothetical protein (XP_323848.1), and 2. Hypothetical protein (XP_323847.1).

Figure 13 The sequence of the putative gene which overlaps the SBR binding target DNA fragment, SBRBD300.

The putative gene encodes a hypothetical protein of 138 amino acids. Three introns were found in this gene and shown in lower case letters. The amino acid substitutions are shown in bold below the wild-type sequence. The asterisks indicate stop codons. The potential binding sites were underlined.

1 aggggacggctggcaaaggtcatgaatcttggaaatgtggaagcaacaagcgcttcgagg
61 ctgagagtgagaattttattgtcagttcctttccattgggaccaccacttcatggca
121 cactcacagttgaagtgaggcagaaggaaggaaccacccgatagactgcaggatcgaact

181 aacagctgcaaggtgagatgCGCTTTTGGTGGCTTTTGTTAGCGCGGgttatcacca
M A F W W L L L A R
241 ggccctccgcacagattcggggcatttcctttcagctaagtaagcatcgcagactgcgcag

301 atcgcttttacaacccaaaccctaaggcacccaagttgagtttctgtaatcgccattggg
T T
361 aaagctgtcagtcagGCCCTGTCAGCTGAAAACGAACACCCCGAGTCCTTTTCGGTACCGG
A L S A E N E H P E S F R Y R
L Y
421 TTCCGTGCTCATCAGCTGGCGTCGTCAACAAGAAAAGCTTGCACCTTCCATGTGCGGAAG
F R A H Q L A S S T R K A C T F H V R N
481 tgggaagctgcgacccgacccttttcctgtgttaccaaccgcgttgcggtgccggtgatgagc

541 gagtaagactttaagggacacagCATAGCCCTTGTTCCTTCTCGAAGTTTGGTGTCCG
I A L V C L L E V W C P
601 TCAGCGAACAATATTCTCAGCCGAGgttaggagttggattttggtagtttctcctatgttc
S A N N I L S R
661 tgtcagcagacaagatatctcaagctatttccaagttcctgccagtcctgtagagctagc

721 ttgagacatggatctgcgcagGAAAACCTCCGATTCGATAAAATAGCTATCTTTCCTCCCA
G K L R F D K I A I F P P
781 AATCGCCAGCTGTCAAATGCCAAAGCTCATGGACGCGAAAGGCAGGCAAGCCAGCGCAAC
K S P A V K C Q S S W T R K A G K P A Q
841 TTGGCTGGGATATTGACCGATTACAGCTCGGAGAATTTAGGACGCCAAAACGTCAAGACA
L G W D I D R L Q L G E F R T P K R Q D
901 TGGCTGCTGAGCTTAGAAACATCAACTTCGTGAAGCCGGCGCTCAAGAGCAGAGCCAAGT
M A A E L R N I N F V K P A L K S R A K
961 gaaatgaagttgcagttgcggatgaagaagcattggaacgcccagtcctgaccgagggga
*
1021 cctatcttatgctgttcttcgaatcgtagccatcggttaatgagctaacttgaaactgg
1081 caggtgcctctacttgaccga

Figure 14 The putative gene which overlaps the SBR binding target DNA fragment, SBRBD600.

The putative gene encodes a hypothetical protein of 243 amino acids. Two introns were found in this gene and shown in lower case letters. The amino acid substitutions are shown in bold below the wild-type sequence. The asterisks indicate stop codons. The potential binding sites were underlined

1 aaatctccgagtcggaatggcttgacatcccgagtcggaatggcttgacatccggc
61 cgagtgcccttccaccggatagcaaccgaaccaacgcatggcataaaatgggatccgc
121 gaggtacggcccagtgctacatctacccgactttaatagactccttatcgattccgg
181 tgggcgctgtgacgttgatgagatatctggatagtgacgagggaagagtaaccggaaca
241 gcaatgatgggaatttgagaatggaaatggaaaaggattcggaatcgagacagagggga
301 ggcacgggaggtcggtattgtggaggcccaagggggaattggaggcacagagcggggccc
361 attagcgcccacggcccagtggttatcttatcaggcggttaggtgcacggttcacccaaa
421 ttgttaccagctccaaccggaccagtgctcattgtggacagcggcgcgctgcaagcaa
481 cccctcgcaattggcgctcgcaaggttgcaactcggttcccttccctacccattttcatt
541 ctgctcgacatctttcaccatctacgccccgacctcgaaacgaaagcgaccctctcgag
601 accttctgggattacactggcagcaaaagtttcaacttgccgttccacattcaggcg
661 ccgaacctcgactgcgagctctaaacccatccatttcgagcgctcagaacgattcccga
721 tttccaacaagggtcgctcccaacgcatgacgacgggctctatgccactcctcccaa

781 ccgattctagccaagtcatctgccaaacttccccaGCACCGAGCATACCAACTGAGCC
M A P S I P T E P

841 GGCCTCGAAACGCAACGGCGTCAATGACCATGAAGCGGTCCCGATAGGGCCTTCCCCAGG
A S K R N G V N D H E A V P I G P S P G
T SBRBD600-001F→
901 GCTTTTCGTCCAACCCGCCACGCTACATCTCGGAACAGACGCTGCAGCAGATGCTGAAAAG
L S S N P P R Y I S E Q T L Q Q M L K S
S
961 CATTGGCTATGATGAAGCAAGGGAGGATGCATACAGACTCAAAGGCGTCCAGCTCATTGA
I G Y D E A R E D A Y R L K G V Q L I D

1021 TAGCGTTTCGACAAAGCATAAGCCTgtgcggaacccatcgcttccaagccttacctacgcatt
S V R Q S I S L

1081 tttgctgacgaacgccacagGCCGGTCAAGACATTTCGATACAGCAGCGACCTACTACCAC
P V K T F D T A A T Y Y H
A
1141 AGGTTTCAGAATACGGTTTCCGAGCGCCGAGTACAACCTACGAAGATGTAGCTCTTGCGTGC
R F R I R F P S A E Y N Y E D V A L A S
Q T -SBRBD600-001R
1201 TTGTTTGTGTCATGCAAGGTGGAGGATACGATCAAGAAGTCCAAGGATGTCCTATGTGCT
L F V A C K V E D T I K K S K D V L C A
SBRBD600-002F→ P
1261 GCGCACAATATCAGGCAGCCCCACGATCAAAGGACTCCGGATGACAAGgttcgtcaatta
A H N I R Q P H D Q R T P D D K
a
1321 tcttgttgccgatctacgggtgtaccatctaattgaccgcgagATGTTTCGATGGTCCA
M F D G P

1381 TCCAAATTCACAGTGGGCCTCGAGCGTCATATTCTGGAGACCATTGGCTTCGACTTCCAG
S K F T V G L E R H I L E T I G F D F Q

1441 GCTCAGTATCCCCAGAAGTTGCTGATCAAATTACTTCGGAAGATGTTCCCAAAGGGCGAA
A Q Y P Q K L L I K L L R K M F P K G E

1501 AAAGGGGACAGTCCCGAGGTCAAGAAGTTCATCAGCGATGCATACGACATGTCCATCGAT
K G D S P E V K K F I S D A Y D M S I D

←SBRBD600-002R

1561 CTTTACAAGACGTTTGCGCCCTTGAAGCAACCAAGCTTTCCGCTTGTGATGGCAAATTCTT
L Y K T F A P L K Q P S F P L V M A I L

1621 GAACTAACAGTCCTCCTTACGGGAATGGGTGGAGATCATATCCCACAGTTCTATGCCGAC
E L T V L L T G M G G D H I P Q F Y A D

1681 CGGTATCCGGCTAGGAAAGGCTGTGTTCTCGAGGTCATGTTGGATCTAATGGATCTCTAC
R Y P A R K G C V L E V M L D L M D L Y

1741 ACACAATTTCCAAAGTCCACCAAAGTCGGCAGCAGATACGATCTTAACAAGTTGATGGAC
T Q F P K S T K V G S R Y D L N K L M D

1801 GTTAAAATTGACATCAACAAGATGCTATTGGGGAATCGGTACCACCGCCACTTTGCATGG
V K I D I N K M L L G N R Y H R H F A W

1861 TGCGACAAGTGTGCCAAGACAGCTCGGATGCTCTTTCCGTCACGCCGGGTCTGTGATC
C D K C A Q D S S D A L S V T P G S V I

1921 TCGCCTGCGACTAACAGCTCGCTACCTGGAAATATCACTGTCAAGAGGAATGGCAAGTCG
S P A T N S S L P G N I T V K R N G K S

1981 TCTGAAGGAACCTTGCGATTTGTCTTCGACCATGAAGACGCCCGCTTCGAGCGGGAAACA
S E G T L R F V F D H E D A R F E R E T

2041 GTGGCGGAATATTTGATGATGTCTACGAGGAAATCGAAGAGGAGGTGAGGAAAGAATC
V A E Y F D D V Y E E I E E E V E E R I

2101 CCAGATGAACCACGGCACTCGAGCCGGAGTGCTCATCACTCGCATTTCGTCACATCGAAAT
P D E P R H S S R S A H H S H S S H R N

2161 CACACTGACCATGGCTGGAGTCCATATTCGCGAAGTCGGCATGGCGGCCACAACAGTGAC
H T D H G W S P Y S R S R H G G H N S D

2221 CGACACAAGGGGCGTAAAAGTCACGGTTACTATTGATcactgtcgcctctctgtcattct
R H K G R K S H G Y Y *

2281 ccttggtgggctatggggcatatggatgtgcacggtgtttgcgaggtttgtcttttacggt
2341 ggttaccctaagttcagcggctaaggagtttacggctacttgatgataccagtcctata
2401 gcacatctggatgggaatgattggacgcgttcgcgtccttggaagacggtg
2461 ctttcgataatgacaattggatcccaaagactcaacgttgtgaaccaattgactctgcga
2521 ggggtgtgccggaatgtcaccatgcttgaactgtcttctcactttatgatccgaacacccat
2581 ggaccaaggcagccaggc

for the fungal transcription factors, NIT2 site and StuAp site, are present. NIT2 is an activator of nitrogen-related genes (Fu and Marzluf 1990), and StuAp is a sequence-specific transcription factor that regulates developmental complexity in *Aspergillus nidulans* (Dutton *et al.*, 1997). A few binding sites from other species were also found with very high matching scores. DFD proteins are deformed (Dfd) homeotic genes encoding transcriptional regulatory proteins in *Drosophila* (Egger *et al.*, 1992). DOF proteins are a family of transcription factors in plants (Yanagisawa and Schmidt, 1999). GATA binding factors are widely found in vertebrates, insects and fungi (Ko and Engel, 1993; Lowry and Atchley 2000). AP1 is activator protein 1 which belongs to the family of MADS box proteins found in vertebrates, plants and yeast (Karin *et al.*, 1997). Among these motifs the binding site of DOF proteins (AAAG) is common to both of the segments that specifically bind to SBR. The positions of these motifs are labeled on the SBR binding sequences (SBRBD300 and SBRBD600) in figures 14 and 15.

Table 4 Examples of potential binding sites in SBRBD300 & SBRBD600.

Example sequences show good match to the consensus sequences. Four of the binding factors were reported in fungi. Analysis was performed by MatInspector.

Transcription Factor	Score (Core)	Score (Matrix)	Consensus	Example (core in uppercase)
NIT2	1.000	0.967	TATCDH	<u>TATC</u> ac
STUAP	1.000	0.902	WWCGCGWNM	Ag <u>CGCG</u> ggt
DFD	1.000	0.964	ATTAMY	<u>ATT</u> Aca
DOF	1.000	0.986	WAAAGC	a <u>AAAG</u> c
GATA	1.000	0.950	WGATAR	t <u>GATA</u> a
API	1.000	0.935	RSTGACTNMNW	Cc <u>TGAC</u> tgaca

+ forward stand, - reverse strand, D: A or G or T, H: A or C or T, M: A or C, R: A or G, S: C or G, W: A or T, Y: C or T
consensus sequences were underlined

Figure 15 Binding motifs on SBR target sequences showing the positions of motifs.

A. Binding motifs on SBRBD300. **B.** Binding motifs on SBRBD600

A. Binding motifs on SBRBD300

CTGCAGGATCGAACTAACAGCTGCAAGGTGAGATGATGGCGTTTTGGTGG
CTTTTGTTAGCGCGGGTTATCACCAGGCCTCCGCACAGATTCGGGGGCATTC
CTTTTCAGCTAAGTAAGCATCGCAGACTGCGCAGATCGGCTTTACAAACCCA
AACCTAAGGCACCCAAGTTGAGTTTCTTGTAATCGCCATTGGTAAAGCTG
TCAGTCAGGCCCTGTCAGCTGAAAACGACACCCCGAGTCCTTTCGGTACC
GGTCCG TGCTCATCAG CTGGCGTCGT CAACAAGAAA AGCTT

B. Binding motifs on SBRBD600

CTGCAGCAGATGCTGAAAAGCATTGGCTATGATGAAGCAAGGGAGGATGCA
TACAGACTCAAAGGCGTCCAGCTCATTGATAGCGTTCGACAAAGCATAAGC
CTGTSCGGACCCATCGCTTCCAAGCCTTACCTACGCATTTTGCTTGACGAACGC
CACAGGCCGGTCAAGACATTCGATACAGCAGCGACCTACTACCACAGGTTCA
GAATACGGTTTCCGAGCGCCGAGTACAACCTACGAAGATGTAGCTCTTGCGTC
GTTGKTGTTGCATGCAAGGTGGAGGAKACGATCAAGAAGTCCAAGGATGTC
CTATGTSCTGCGCACAATTATCAGGCAGCCCCACGATCAAAGGACTCCGGATG
ACAAGGTTTCGTCAATTATCTTGTTGGCGATCTACGGTGTACCTACTAATATTG
ACCGCGCCAGATGTTTCGATGGTCCATTCCAAATTSACAGTGGGCCTCGAGCGT
KCATATTCTGGAGACCATTTGGCTTTCGACTTTCAGGCTCAGTTATTCCCCA
GAAGTTGCTGATTBAACATTACTTCGGAAGATGTTCCCAAAGGGCGGGAAAG
GGGACAGTCCCGAGGTCAAGAAGTTCATCAGCGATGCATACGACATGTCCAT
CGATCTTTACAAGACGTTTGCGCCCTTGAAGCAACAAGCTT

3.5. Gene Expression of SBRBD300 and SBRBD600 in *sbr* Mutant

In order to determine whether the *sbr* gene regulates the genes adjacent to the SBR binding region, RT-PCR analysis was performed. For this experiment mRNA isolated from the *sbr* mutant and wild-type were used as templates for oligo(dT) primed reverse transcription and gene specific primers were used as described in Materials and Methods for PCR. Interestingly, the sizes of the PCR fragments amplified from mRNA were smaller than those obtained from genomic DNA in both cases (Fig 16A). This result indicated that there are introns within these genes which is consistent with the result of the sequence analysis of SBRBD600 (Fig. 14). To analyze the expression level of SBRBD300 and SBRBD600, a semi-quantitative PCR was performed on total cDNA. No bands were detected with the primer pair designed from SBRBD300 when cDNA synthesized from *sbr* mRNA was used as template (Fig. 16B). A very faint band was observed after 35 PCR cycles with the same primer pairs when cDNA from wild-type mRNA was used as template. However a Northern blot detected the expression of the putative gene in SBRBD300 region in both *sbr* mRNA and wild-type mRNA (Fig. 16C). In contrast, when primers designed from SBRBD600 were used for PCR, bands were detected after 25 cycles with both *sbr* cDNA and wild-type cDNA (Fig.16B).

3.6. Expression and Purification of SBR Protein

The *sbr* gene was previously cloned into pQE32 (QIAGEN) in the correct reading frame (Yan, 2001). Subsequently, M15 cells containing this construct were cultured in LB medium (100ug/ml ampicillin, and 25µg/m kanamycin). After the OD₆₀₀ of this

Figure 16 RT-PCR of putative genes in SBR binding regions with mRNA from wild-type and *sbr* mutant strain.

A: PCR of putative genes in SBRBD300 and SBRBD600 regions. Lane1~3. RT-PCR of the putative gene in SBRB D300 region. Lane 4~6. RT-PCR of the putative gene in SBRBD600 region. Lane 7~9. RT-PCR of *actin* as a control. **B:** Semi-quantitative PCR of these two putative genes with *sbr* cDNA and wt cDNA. Lane1~6: Using primers designed from SBRBD300. Lane7~12: Using primers designed from SBRBD600. **C:** Northern blot of the putative gene in SBRBD300 region with *sbr* mRNA and wt mRNA.

A

B

C sbr wt

bacterial culture reached to 0.5-0.6, IPTG was added to the culture, and incubation was continued for 5hrs to induce the overexpression of the SBR fusion protein contains 6x-His tag. The purification procedure was done by following the manufacturer's instructions (QIAGEN). The crude extract was loaded into a column containing 50% Ni-NTA agarose slurry for affinity purification of His tagged SBR protein.

The expression efficiency and specificity were examined by SDS-PAGE and Western blots with anti-6xHis antibody. Proteins were lightly expressed in the control without IPTG (Fig. 17). The solubility of SBR proteins in M15 cells was examined by comparing the sample of total proteins with the sample pelleted after native lysis. Very little SBR protein remained in the pellet, indicating that the SBR protein was soluble under native conditions (Fig. 17). The six-His tagged SBR proteins were purified under native conditions with Ni-NTA agarose matrix and assessed by SDS-PAGE (Fig. 17). Very little contamination by other proteins was detected.

3.7. Electrophoresis Mobility Shift Assay (EMSA)

Smaller segments of SBRBD300 DNA were amplified by PCR, and the PCR products were named SBRBD300-1 (100bp), SBRBD300-2 (101bp), and SBRBD300-3 (130bp), respectively. The PCR products were cloned into the TOPO-pCR2.1 vector (Invitrogen). After restriction digestion, the probe DNA from each of the clones was radio-labelled with $\gamma^{32}\text{PdATP}$ as described in materials and methods. The radioactive probe DNA was allowed to react with the tagged, purified SBR protein. Since the plasmid DNA pBluescript showed non-specific binding with SBR, it was used as the competitor DNA. After the binding reaction, the reaction mixture was electrophoresed on a 5% acrylamide gel.

Figure 17 SDS-PAGE of the fusion SBR protein expressed in *E. coli*.

A: M. Marker, 1. Control cell extracts without IPTG (non-induced), 2. Extracts of cells induced with IPTG, 3. Soluble proteins, 4. Flow-through, 5. First wash, 6. Second wash.

B: E1. Elution 1, E2. Elution 2, E3. Elution 3, E4. Elution 4. Gels were stained with Commassie Blue R-250.

A

B

The results showed that the SBR protein bound to all of the three short sequences. After 25- fold competitor DNA was added, the DNA-protein complex of SBRBD300-2 disappeared, indicating non-specific binding to this fragment. On the other hand, the other bands of DNA-protein complex of SBRBD300-1, and SBRBD300-3 did not disappear. Interestingly, two bands of DNA-protein complexes were observed in lane 9; one of the complexes moved slower than other bands, indicating multiple binding sites in SBRBD300-3 (Fig. 18).

Shorter sequences of SBRBD600 (600-1, and 600-2) were also cloned and radio labelled with $\gamma^{32}\text{PdATP}$ by end labelling. After a binding reaction, the EMSA (electrophoretic mobility shift assay) was performed. The gel showed bands of DNA-protein complex of both probes even after 50 folds of competitor was added (Fig. 19A).

SBRBD600-1, and 600-2 DNAs were digested with restriction enzyme and the restricted fragments (600-A, 600-B, 600-C, and 600-D) were then radio labelled with $\alpha^{32}\text{PdCTP}$ as described in materials and methods. After EMSA analysis, DNA-protein complexes were observed from 600-B, 600-C, and 600-D. In comparison to the results with the 600-1 and 600-2 probes, the DNA-protein complexes of 600-B, 600-C, and 600-D migrated faster than that of 600-1, and 600-2 (Fig. 19B).

The RIPed SBRBD300 was amplified from mutants RIP300-1# and RIP300-9# by PCR, and cloned into vector pCR2.1. After restriction digestion, the RIPed SBRBD300 DNA was radio labelled with $\alpha^{32}\text{PdCTP}$. The EMSA analysis revealed that the SBR protein still can bind to SBRBD300 amplified from RIP300-1# but not RIP300-9#.

Figure 18 EMSA analysis of SBRBD300.

SBRBD300-1, SBRBD300-2, and SBRBD300-3 were used as probe. Arrows indicate the DNA-protein complexes, F indicates free DNA probe.

	SBRBD300-1			SBRBD300-2			SBRBD300-3		
probe DNA	+	+	+	+	+	+	+	+	+
SBR	-	+	+	-	+	+	-	+	+
competitor DNA	-	-	25x	-	-	25x	-	-	25x

Figure 19 EMSA analysis of SBRBD600.

A: Results of EMSA using SBRBD600-1 and SBRBD600-2 as probe. Lane 1-4, EMSA of SBRBD600-1. Lane 5-8, EMSA of SBRBD600-2. **B:** Results of EMSA using 600-A, 600-B, 600-C, and 600-D as probe. Arrows indicate DNA-protein complexes, F indicate free DNA probe.

A

B

The RIPed SBRBD600-1 and 600-2 were also amplified from mutants RIP600-4# and RIP600-12# and analyzed using EMSA. The bands of DNA-protein complexes were observed from both mutant strains even after addition of 50x competitor DNA. This result suggests that the SBR protein can bind to RIPed SBRBD600.

3.8. Epitope tagging of SBR protein in *Neurospora crassa*

The full ORF of the *sbr* gene was amplified by PCR and cloned into vector pDrive. After double digestion with restriction enzymes *Xho*I and *Eco*RI, the *sbr* gene was subcloned into an expression vector pEpi-3XHA, which contains 3 HA (hemagglutinin) tags in the correct open reading frame (Fig.20). The construct was co-transformed into *N. crassa* with the vector pCB1004, which contains hygromycin B resistant gene. After screening on hygromycin B media, the selected transformants were grown on Vogel's minimal medium. To confirm the successful transformation by the tagged *sbr* construct, PCR was performed by using one primer from vector (T7-Univ), and another primer from the *sbr* gene (*sbr* 003). In total three transformants (sbr3HA 3#, sbr3HA 4#, and sbr3HA 6#) were found to contain that construct (Fig. 21). However, the tagged protein was not detected by Western blotting of cell extracts from the transformants.

Figure 20 Cloning strategy for epitope tagging the SBR protein.

The plasmid is composed of the 3.4kb fragment of pBluescript SK+, and the 3xHA tag in the multiple cloning sites. The *sbr* gene was cloned into the vector, and the 3xHA tag was located at the N-terminal of the SBR.

Figure 21 PCR of pEpi-sbr3HA transformants.

The PCR results showed that the same PCR products were amplified from transformant 3, transformant 4, transformant 6, and the vector which transformed into *N. crassa*.

IV. DISCUSSION

4.1. Expression of the *sbr* gene during development of *N.crassa*

The *sbr* mutant has a highly restricted, colonial morphology under all growth conditions tested. This mutation impacts upon all stages of the life cycle including vegetative hyphal growth, conidiation and the germination of sexual spores. The *sbr* strain does not produce hyphae, conidiophores or conidiospores. Growth is more reminiscent of yeast cells which grows isotropically and produce spherical buds at random (Campsall, 1998). The fact that *sbr* does not produce free conidia suggests that there is a defect in conidial separation. It is possible that the defect may occur even earlier in the developmental pathway.

Sequence analysis revealed that the *sbr* gene encodes a helix-turn helix (HTH), a putative zinc finger, and a glutamine-rich region (Yan, 2001). Therefore based upon the phenotype and genotype, it is possible that the *sbr* gene encodes a regulatory protein that functions during development.

To determine whether the expression of the *sbr* gene is itself developmentally regulated, a semi-quantitative PCR experiment was performed using cDNA from different developmental stages as templates. The results suggest that the *sbr* gene is expressed at different developmental stages such as in conidiospores, during germination or conidiation, mycelium, and mating (Fig. 3A).

When the expression of *sbr* was normalized to actin, as shown in Figure 3, the abundance of *sbr* mRNA was found to be much lower than of actin by RT-PCR. A 2-fold higher band intensity of *sbr* PCR products was observed at conidiol germination, but a similar pattern was also obtained for actin amplification. Therefore, this difference is

most likely caused by a difference in the concentration of cDNA template rather than a difference of expression since the expression level of actin is known to be relatively stable during development.

4.2. Phenotypic characterization of RIP Mutants and genetic evidences

The *sbr* gene was proposed to be a regulatory gene, and two target genomic DNA fragments containing SBR binding sites were isolated from protein-DNA complexes in a protein-DNA binding assay. It was reasoned that if SBR is a regulatory protein, inactivation of downstream genes may also affect morphogenesis directly. To address whether inactivation of any of these regions of the genome results in morphological mutations, RIP (repeat induced point mutation) was utilized to generate transition mutations *in vivo*. The technique of RIP was developed, and shown to be successful, for the *in vivo* generation of mutants in non-essential genes of *N. crassa* (Metzenberg and Groteleuschen, 1992). A variation of the basic procedure, sheltered RIP, can be used to mutagenize essential genes (Harkness *et al.*, 1994). Despite the observation in the present study that the genes in these regions are not essential, the method was also useful for isolating mutations in these genes.

RIP mutants demonstrate striking differences in morphology and development

Under standard growth conditions, the filamentous ascomycete *Neurospora crassa* grows by continuous hyphal elongation and branching, which results in the formation of spreading radial colonies. Hyphal growth is a complex process which requires the proper orchestration of a multitude of events (Perkins *et al.*, 1982).

The mutants resulting from RIP in this study display striking morphological differences in comparison with wild-type *N. crassa*. Most of the mutant strains grow at a slower rate than wild-type and exhibit a decrease in the production of conidia.

Among 10 mutant strains isolated from the RIP cross of SBRBD300, 8 mutant strains had slow growth rates and reduce production of conidiospores. For example, after incubation for 48 hrs on minimal medium at RT, mutant strains RIP300-1# and RIP300-6# (Fig. 6b and c) exhibit a morphology resembling spreading colonial mutants (Garnjobst and Tatum, 1967). These strains begin as a colony, but growth does not remain restricted; it slowly spreads over the surface of a plate. For example, although it has this spreading mode of growth, strain RIP300-1# developed slowly and produced few aerial hyphae. Then the colony filled in behind the growing front, resulting in a characteristic layered appearance. In addition, RIP300-6# displayed more branches, a dense colony, and a low growth rate, 1.03mm/h (Fig. 6, and Fig. 7).

Interestingly, mutant strain RIP300-9# exhibited a very different morphology in comparison with wild-type and other mutant strains (Fig. 6d). For example, other mutant strains grow slower than wild-type, but this strain has a similar growth rate with wild-type at RT. It resembles to a mutant strain called plug (*pl*) which is characterized by dense hyphae which fill the diameter of a 10mm tube (Perkins, 1959). Since it has white adherent conidiospores instead of the powdery orange spores, the color of this strain is off-white rather than yellow or orange.

In total, of the eleven mutant strains isolated from the RIP cross of SBRBD600, eight mutant strains display lower growth rates than that of wild-type, while three mutant

strains display a growth rate similar to wild-type. All of these mutant strains produce less conidiospores than wild-type and exhibit a lighter color than wild-type (Fig 5, and Fig 6).

After inoculation for 48 hrs on plates containing minimal medium at RT, mutant strain RIP600-4# exhibits a morphology resembling to spreading mutants (Garnjobst and Tatum, 1967), which begin as a small colony and gradually produces hyphae and conidiophores with very small amounts of conidia. On the other hand, mutant strain RIP600-12# displays a hyphal morphology similar to wild-type, but because of the decrease in the production of conidia, it has a lighter color than wild-type. Based on their morphologies, strains RIP300-1# and RIP300-6# have been tentatively named *spco-15* and *spco-16*.

In summary, the effects of inactivation of SBRBD300 and SBRBD600 on morphology and development were dramatic. Mutant strains produced by RIP mutagenesis of both genes had a distinctive morphologies on minimal media (Fig 5, and Fig. 6), implying both genes were repped. The primary effect appeared to be on the control of tip elongation since the rate of hyphal extension at the apex was greatly decreased in most of the mutants. This change could account for the observed growth phenotype of slow spreading of mutant strains across an agar surface. Defects in apical growth might also restrict the ability of the organism to form conidia.

These phenotypic characteristics for mutations in putative downstream genes are consistent with the hypothesis that SBR is a morphological regulatory gene. If *spco-15* and *spco-16* function downstream of *sbr*, their phenotypes could be less severe. On the other hand, mutations in many different pathway are known to impinge on morphogenesis.

During *sbr* development, the colony radius reaches a limited size and then grows by isotropic expansion, and repeated budding increases the density and height of the colony. Although the 'buds' are capable of germinating, the sexual spores (ascospores) from a cross with *sbr* are not viable. Ascospores produced from a mating between wild-type and *sbr* produced perithecia and ascospores that were normal in morphology, but the germination frequency of the spores was poor (Campsall, 1998). Therefore, based on the phenotype of *sbr* mutant and mutants which resulted from inactivation of SBRBD300 and SBRBD600, it is possible that the *sbr* gene and genes in the regions of SBRBD300 and SBRBD600 share the same pathway, or SBR may down regulate these genes during development.

Molecular nature of RIP mutants

Partial sequence analysis of four of the RIP- induced mutants (RIP300-1#, RIP300-9#, RIP600-4#, and RIP600-12#) showed that they all carry a limited number of point mutations, indicating that the RIP process has occurred. The number of point mutations introduced in each allele varied between two and six; all mutations were polarized G-A or C-T transitions, representing a loss of 0.4%-2.7% of the cytosine and guanosine content of coding strand. This result is similar to the previous results obtained with the *am* gene. In a survey of eight defective alleles of the *am* gene that resulted from the operation of RIP on an unlinked duplication of a 2.6 kilobase segment, 0.6-12% of the G:C pairs were mutated in a single passage through the sexual cycle (Singer *et al.*, 1995).

The frequency and severity of damage by RIP varies, however, and depends only partly on obvious variables such as length and position of the duplicate elements. By chance, a duplication may slip through a cross unscathed, be lightly mutated, perhaps by

one cycle of RIP, or be heavily mutated and show evidence of more than one round of RIP (Watters *et al.*, 1999). The mutant strains obtained in this study resulted from a single RIP cross, and shown to be lightly mutated.

4.3. DNA-protein binding study

EMSA (electrophoretic mobility shift assay) is a powerful tool for studying the binding activity of transcription factors to specific DNA sequences. The EMSA technique relies on the ability of a protein to bind to a radiolabelled DNA fragment *in vitro*, followed by electrophoretic separation of DNA-protein complexes from the unbound DNA on non-denaturing polyacrylamide gels (Ausubel *et al.*, 1989; Carey, 1991).

Since a previous study suggested that SBR is a regulatory protein with putative DNA binding domains (Campbell, 1998), two genomic fragments bound to SBR were isolated (Yan, 2001). To test the binding specificity of SBR, EMSA was performed. The key factor in evaluating the binding specificity is using unlabeled competitor DNA (Garner and Revzin, 1981; Fried and Crothers, 1981). The competitor DNA should not contain any copies of the specific binding sites for the protein. Phage λ DNA, plasmid DNA, *E.coli* genomic DNA and calf thymus DNA have all been used as non-specific competitors (Shanblatt and Revzin, 1984; Varshavsky 1987). Unnatural polynucleotides with simple repeat sequences such as poly d(I-C), poly d(G-C), poly d(A-T) are favored in many EMSA studies, because it increases sensitivity in identifying binding protein from crude nuclear extract (Carthew *et al.*, 1985; Singh *et al.*, 1986). In this study, since the sequence of the binding site for SBR is unknown the pBluescript plasmid alone was used as a competitor because it showed non-specific binding to SBR. Previous investigation reported that competitors were not able to compete specific protein-DNA

bindings until they were present at 25- to 100-fold higher levels, depending on the binding protein and the competitor used (Yan 2001).

Previous work showed that the SBR protein specifically binds to the sequence of SBRBD300, and the results suggested that more than one binding sites was involved (Yan, 2001). To narrow down the binding sites in SBRBD300, shorter sequences (about 100 bp) were amplified by PCR, and incubated with the EMSA-binding buffer in the presence of SBR. These results indicate that SBR specifically bound to SBRBD300-1, and SBRBD300-3 (Fig. 18). In addition two distinct bands of DNA-protein complex were observed with the probe SBRBD300-3, and one of the bands migrated much slower than the free probe, indicating multiple binding sites on this DNA fragment. Additional experiments such as EMSA of smaller fragments of these DNA targets (about 30-50bp), and DNase I footprinting are required to determine the precise binding sites on SBRBD300. To determine whether the SBR protein is present in the observed complex, the 'super gel shift assay' technique with the specific antibodies against SBR can be employed (Kristie, 1986).

The binding specificity of SBRBD600 was also determined by EMSA analysis (Fig. 19). Short sequences (SBRBD600-1 and SBRBD600-2) were amplified by PCR and incubated with the binding buffer in the presence of the SBR. The bands of DNA-protein complex were observed from both sequences. The SBR-DNA binding was not competed by the cloning vector pBluescript even after 50-fold competitor DNA was added in both cases. This demonstrated specific binding of SBR protein to both sequences SBRBD600-1 and SBRBD600-2. The migration speed of these DNA-protein complexes was much slower than the free probe, implying more than one binding site in these probes. This is

consistent with the finding that many promoters have multiple binding sites for a regulatory factor. For instance, in *N.crassa* the regulatory sequence of the major nitrogen-regulatory protein NIT-2 was found to have multiple NIT-2 binding sites at both directions on the two strands. (Fu and Marzluf, 1990a).

To narrow down the binding sites in SBRBD600, SBRBD600-1 and SBRBD600-2 were restriction digested into four short fragments which were named 600-A (90bp), 600-B (300bp), 600-C (180bp), and 600-D (240bp) respectively. After incubation with binding buffer containing SBR, the bands of DNA-protein complex were observed in 600-B, 600-C, and 600-D (Fig. 19B). Two distinct bands of complex were found with probe 600-B and 600-D. In addition to the shifted complexes similar to that found in 600-C, additional less mobile ones were observed with 600-B and 600-C, indicating multiple binding sites within these two DNA probes.

The EMSA is a rapid, extremely sensitive, and widely used method for investigating nucleic acid-protein interaction based on the observation that binding of protein to DNA or RNA fragments usually leads to a reduction on the electrophoretic mobility of the fragment in non-denaturing gels. Several parameters which might influence complex formation and stability such as binding buffer, temperature, duration of complex formation, non-specific competitor, gel concentration and composition, gel running buffer and temperature have been reported previously (Roder and Schweizer, 2001). In the case of EMSA analysis of SBRBD300 (Fig. 18), no migration of probe DNA was observed when no competitor was added. This is an indication of multiple SBR molecules binding nonspecifically with the probe molecule (Taylor *et al.*, 1994). After

non-specific competitor and 0.5% NP-40 were added to binding buffer, the mobility of the complex was improved (Fig. 18).

The binding specificity of mutated SBRBD300 and SBRBD600 resulting from RIP were also tested with EMSA. The DNA-protein complexes were observed with probe prepared from mutant strains RIP300-1#, RIP600-4#, and RIP600-12#. However, the complexes formed displayed an increased mobility when compared to those with probe from the wild-type *N.crassa* strain. Furthermore, they were more diffuse indicating higher instability. These results indicated that the RIP procedure did not disrupt all of the binding sites. This is consistent with the results from sequence analysis of the mutated SBRBD300 and SBRBD600.

4.4. Gene prediction of SBR target DNA fragments

Gene prediction of SBRBD300

If SBR is a regulatory protein, a gene should be associated with each target DNA sequence isolated from specific SBR-DNA complexes. In total, four putative genes were identified by a BLASTX (NCBI) search of DNA 2500bp downstream and upstream of SBRBD300 (Fig. 11). However, two of them, a hypothetical protein related to cytosolic Cu/Zn superoxide dismutase and hypothetical protein related to hxB protein, were located more than 2kb upstream and downstream from SBRBD300 respectively. Probably they are outside the regulation region of SBRBD300. A hypothetical protein B24P7.300 identified by BLASTX (NCBI) is located from 150bp upstream of SBRBD300 to 750bp downstream. A 333bp ORF found in this region is located 1093bp downstream of SBRBD300. The transcription start site could be a 30 to 600bp or away from the starting site of the predicted genes. (Bruchez *et al.*, 1993a) A regulatory binding site could be as

far as 1.2kb upstream to a transcription site (Fu and Marzluf, 1990a). Therefore, the SBRBD300 sequence could be in the regulatory region of these possible genes. SBR could bind to a promoter and /or an enhancer since enhancers can be located upstream, downstream, or even within a transcription unit (Hatzopoulous *et al.*, 1988).

Interestingly, RT-PCR using specific primers designed from SBRBD300 template cDNA from wild-type *N. crassa* 988 resulted in a band of about 220bp (Fig 14A). In comparison, the PCR product amplified using genomic DNA is about 300bp (Fig. 14A), indicating an intron within this region. This result confirms the existence of a real gene in this region, and the transcription of this gene.

Gene prediction of SBRBD600

Two ORFs were identified in a BLASTX (NCBI) search of DNA 2500bp downstream and upstream of SBRBD600 (Fig. 11). The putative gene located 1891bp upstream of SBRBD600 encodes a hypothetical protein (XP_323847.1). Another putative gene located from 200bp upstream of SBRBD600 to 646bp downstream encodes a hypothetical protein XP_323848.1. In addition a cDNA clone (b4c04np.r1) was found in *Neurospora crassa* perithecia cDNA database matching with this gene.

Furthermore, RT-PCR using sequence specific primers from SBRBD600 and template cDNA from a wild-type strain of *N. crassa* (FGSC# 988a). The PCR product amplified from cDNA is about 500bp (Fig. 16A). In contrast, the PCR product amplified from genomic DNA is about 600bp. This is consistent with our finding using BLAST that an intron is located within SBRBD600 (Fig. 14). Therefore, these results indicate that this putative gene is a real gene and has been transcribed during the development of perithecia.

4.5. Does SBR regulate the putative genes?

Since two putative genes were found in the regions of SBR target sequence SBRBD300 and SBRBD600, it was hypothesized that SBR may regulate these two genes. If SBR controls the expression of these two genes, it was hypothesized that changes in the levels of mRNA transcribed from these two genes may be detectable. This was tested initially using the technique of Reverse transcript PCR (RT-PCR). PCR amplification of specific cDNAs derived from the *sbr* mutant strain with primers from the SBRBD300 DNA failed to generate the predicted product (Fig. 16A). A very faint band was observed in the RT-PCR with wild-type mRNA using the same primer. Similarly, semi-quantitative PCR showed that no band was detected in the RT-PCR using *sbr* mRNA even after 35 PCR cycles (Fig 16B). A very faint band was found after 35 PCR cycles in the RT-PCR using wild-type mRNA. These results indicate that even in wild-type the putative gene encoding hypothetical protein B24P7.300, is expressed at a very low level. Northern blot analysis has revealed the expression of this gene in mRNA from the *sbr* strain suggests that it is expressed in *sbr* mutant at levels which are comparable to wild-type (Fig. 16C).

Semi-quantitative RT-PCR of the putative gene which overlaps SBRBD600 was also performed with mRNA isolated from *sbr* mutant and wild-type (FGSC# 988a). In both cases the same specific primers designed from SBRBD600 were used. The results suggest that the putative gene encoding hypothetical protein XP_323848.1 was expressed in both the *sbr* mutant strain and wild-type *N. crassa* (Fig. 16B). In addition, semi-quantitative RT-PCR also revealed that the intensity of DNA band resulting from 25 PCR

cycles with wild-type mRNA is brighter than the band amplified from *sbr* mRNA suggesting lower expression level of this gene in the *sbr* mutant than in wild-type.

However, to determine whether the SBR protein regulates the putative genes found in the regions of SBR target binding sites, further experiments such as Northern blotting and a precise calculation of the band's intensity needs to be done.

4.6. SBR target binding sites analysis

Transcription factors usually bind to multiple target sequences and regulate multiple genes. Thus, the intrinsic specificity of transcription factors is rather low compared with prokaryotic DNA-binding proteins, probably because the synergistic action of multiple transcription factors on the same promoter may be the strategy for the complex regulation of gene expression (Werner and Burley, 1997). Explosive amounts of sequence information and numerous transcription factors have been identified. Several bioinformatic programs have been developed to exploit this data and can be used to predict binding sites and identify promoters/enhancers (Frech *et al.*, 1993, 1997a, 1997b; Quandt *et al.*, 1995; Heinemeyer *et al.*, 1998; Klingenhoff *et al.*, 1999).

Identifying the functional elements encoded in a genome is one of the principal challenges in modern biology. Recently a comparative analysis method based on high-quality draft sequences of related species has been developed for direct identification of genes and regulatory motifs. The motif analysis in the yeast *Saccharomyces cerevisiae* automatically identified 72 genome-wide elements including most known regulatory motifs and numerous new motifs (Kellis *et al.*, 2003). Currently, the sequence-based method is the most commonly used method for the target site prediction. It relies on sequence information obtained from known binding sequences. Usually, consensus

sequence patterns or weight matrices are used to scan the database. Prediction of binding sites using matrix databases is usually considered to be of a higher quality. However, there are a limited number of matrices available and many binding sites are not represented by a weighted matrix.

The sequences of SBR target fragments, SBRBD300 and SBRBD600, were used to search for potential transcription factor binding sites with the program MatInspector2.2 as described in Material and Methods. Both the matrix and the core of the consensus sequence are provided. The core sequence is the binding sequence without exception for different binding assays. It usually indicates the most critical part for the protein binding. The calculation of matrix similarity includes the information content of individual positions within the matrix (Quandt *et al.*, 1995). For instance, if the matrix contains a position where only an A occurs in all training sequences and the test sequence features a T at the corresponding position, the overall score of this region will be dramatically reduced.

In the two SBR target fragments, the consensus of six transcription factors have been found with core score 1 (100% match) and matrix score >0.9 (Table 4). In *N.crassa*, the NIT2 protein and GATA factor proteins such as WC1 and WC2 contain a Cys4 type DNA binding domain. STUAP containing a helix-loop-helix binding domain has been reported in *Aspergillus nidulans* (Dutton *et al.*, 1997). The DOF binding domain contains a specific single zinc finger which has a cysteine in the intervening sequence between zinc ion binding cysteines (two C-X₂-C). Since the putative zinc finger of SBR appears to be involved in the DNA binding activity (Yan, 2001), its binding to any of these zinc finger target sites is more likely than the non-conventional helix-turn-helix domain which

did not bind to either of the target sequences. In addition, only DOF and AP1 consensus sequences were found at multiple positions in the SBRBD300 and SBRBD600 DNAs. Moreover, the EMSA results suggest multiple-molecule binding to these fragments. However, AP1 belongs to MAD box family of proteins which have no similarity to the SBR protein. Therefore the target consensus sequence (AAAGC) for DOF single zinc finger is the most likely candidate as an SBR binding sequence.

Not only can two factors of the same family of binding domain bind to a consensus sequence, two factors with no similar binding domains can bind to an identical sequence. For instance, transcription factors CTF/NF1 and C/EBP both can bind to an identical sequence, the CAAT box. On the other hand, many transcription factors have the ability to bind to several dissimilar sequences using the same DNA binding domain. For example, the glucocorticoid receptor binds to a specific DNA sequence in genes that are induced by glucocorticoid and to a distinct DNA sequence in genes which are repressed by this hormone, the same binding domain of the protein being used in each case (Sakai *et al.*, 1988).

In summary, the binding sites analysis found multiple consensus sites in each target DNA fragment implying that each fragment contains a regulatory response sequence. A single transcription factor binding site often does not encode a transcription function of its own because biological function usually requires cooperation of two or more transcription factors (Jones *et al.*, 1988; Carey, 1998). In fact, other consensus sites with high matching scores were also found in each fragment. Ultimately, concrete evidence, such as DNase I footprinting will be required to confirm any of these speculations of possible SBR binding sites.

4.7. Epitope tagging

To localize the SBR protein in *Neurospora crassa*, the full ORF of the *sbr* gene was cloned in an epitope tagging vector pEi-3XHA (Fig. 18), and co-transformed into *N. crassa* with the shuttle vector pCB1004. PCR revealed that the construct of epitope tagging was successfully transformed into *N. crassa* (Fig. 19). Unfortunately, Western blotting of cell extracts from these transformants was performed using tag specific antibodies, but only non-specific bands were detected. The most likely explanation is that the tagged protein is expressed at very low levels, and may not be detectable using the available antibody. Therefore, the tagged protein may need to be concentrated by immunoprecipitation (Harlow and Lane, 1988).

4.8. Conclusion

Mutant strains resulting from the RIP procedure demonstrate striking differences in morphology on minimal media (Fig. 6), suggesting that the effect of inactivation of SBRBD300 and SBRBD600 on morphology and development were dramatic. The hypothesis that SBR is a regulatory protein is supported by the following experiments. The results of EMSA indicate the specific binding of SBR to the SBR target DNA fragments, SBRBD300 and SBRBD600. Gene prediction of these two DNA fragments identified two hypothetical proteins, and binding sites analysis revealed multiple binding sites in these two SBR target fragments. This strongly suggests that the target fragments SBRBD300 and SBRBD600 contain multiple potential regulatory elements.

EMSA with shorter DNA (30-50 bp) and other types of competitor (such as synthetic polymer) is needed. Moreover, DNase I footprinting with short DNA fragments is required to further confirm DNA binding and clarify the binding sites sequence.

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