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NOTES

Inducible RNA Interference of *brlA*β in *Aspergillus nidulans*▽

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An inducible RNA interference (RNAi) construct composed of inverted repeating *alcA* promoters flanking the developmental regulatory gene *brlA*β was tested in *Aspergillus nidulans*. On inducing medium, the RNAi strains failed to sporulate and lacked *brlA*α and *brlA*β expression. RNAi was specific for *brlA*β, but not *brlA*α, silencing, indicating *brlA*α regulation by *brlA*β.

Aspergillus nidulans has been a filamentous fungal model for RNAi using inverted repeat transgenes (10, 11) and short interfering RNAs (siRNAs) (15). *A. nidulans* reproduces asexually by developing radial hyphae and aerial conidiophores whose tips differentiate into vesicles, primary and secondary sterigmata (metullae and phialides, respectively), and uninucleate conidia (3, 6, 18). *bristle* (*brlA*) mutants maintain normal hyphal extension, but conidiophores are elongated and fail to develop viable conidia (9). *brlA* null mutations result in the complete obliteration of conidiophores, and leaky mutations may form vesicles and sterigmata but not viable conidia (3, 9). The leaky mutants are thought to have partial *brlA* expression, and variability between phenotypes may be a result of a dose effect (3, 23). *brlA* has two overlapping transcripts, *brlA*α and *brlA*β (Fig. 1A). The *brlA*β transcript begins at −851 and has an intron between −99 and +293 and extends to +2085. The *brlA*α transcript begins within the *brlA*β intron at +1 and extends to +2085 (23).

Our RNAi construct (Fig. 1B) flanks *brlA*β with inverted repeats of an inducible alcohol dehydrogenase promoter that is repressed on glucose but strongly induced on threonine (1). Double-stranded RNA is transcribed, triggering the RNAi mechanism to silence *brlA*β. To engineer the construct, *alcA* promoters were cloned as inverted repeats into EcoRI and NotI sites of a plasmid containing the *brlA*β sequence (−2902 to −404) cloned into the BamHI restriction site on pBluescript KS(+). An *argB* nutritional selection gene was cloned into the SacI site (14), and the final construct was sequenced for verification.

A. nidulans strains RMS11 (*pabaA1 yA2 argB*::*trpC veA1 trpC801*) and LR191 (*pyrG pabaA1 yA2 argB pyroA veA1*) were transformed with the construct as previously described (26), except that germlings were digested with Driselase (Sigma) instead of Novozyme234. Eight primary transformants were obtained, and two of these RNAi strains, JB1 and JB3, were chosen for further analysis. The integration of the construct into the genomes was verified with PCR and Southern blot analysis (data not shown) of genomic DNA. Southern blots also verified that the native *brlA* gene was not disrupted by homologous recombination.

In submerged culture, *A. nidulans* conidiophore development is suppressed, but spores develop vegetatively to produce mature mycelia. Conidiophore development begins in a synchronized manner when the mycelia are plated and exposed to air (5, 6). The wild-type, JB1, and JB3 spores were inoculated in minimal medium (22) broth and shaken for 24 h at 250 rpm. Mycelia were harvested by filtration, washed thoroughly with sterile water, and plated on glucose and threonine (Fig. 2A). After 24 h of developmental induction, the wild type developed yellow-pigmented conidia on both glucose and threonine, but the RNAi strains developed conidia only on glucose (Fig. 2A). On threonine, JB1 displayed a *brlA* null phenotype, but JB3 displayed a *brlA* leaky phenotype that develops rudimentary vesicles and sterigmata (Fig. 2B and C). This indicated that *brlA* is silenced to various degrees when the RNAi strains develop on threonine, but it is not silenced on glucose.

In *brlA* expression analysis, RNA was isolated with Tri reagent (Sigma-Aldrich) from mycelia 24 h after being plated. Northern blots and reverse transcription-PCR (RT-PCR) showed a drastic reduction of both *brlA*α and *brlA*β on threonine compared to that on glucose (data not shown). In order to quantify the level of change (*n*-fold) of the *brlA*α and *brlA*β transcripts, real-time RT-PCR on an Applied Biosystems 7500 real-time PCR system was employed (Fig. 3A). For analysis, the threshold cycle (C_T) value was set at 1.5, and $\Delta\Delta C_T$ calculations were performed relative to values for actin and the mean ΔC_T. The changes (*n*-fold) in gene expression were derived from $10^{(-\Delta\Delta^{CT}/\ln 10/\ln 2)}$, where the primer efficiency (ln10/ln2) was calculated experimentally for each primer set. In the wild type, *brlA*α and *brlA*β expression was not reduced on threonine. In JB1, *brlA* expression on glucose is approximately equal to that of the wild type on glucose, but there is only a slightly detectable abundance of *brlA*α and *brlA*β on threonine. JB3 had a large amount of *brlA*α and *brlA*β on glucose, nearly fivefold more than the wild type, but *brlA*α and *brlA*β expres-

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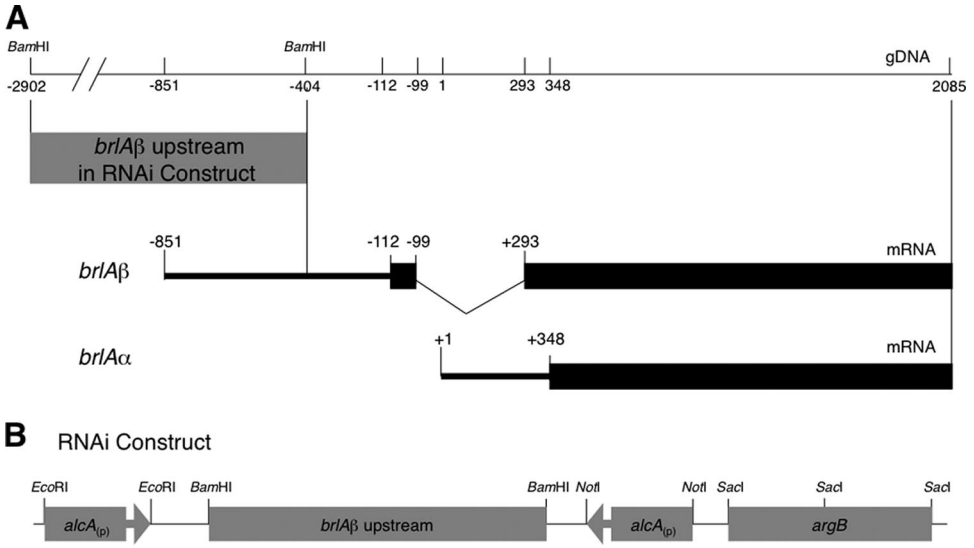

FIG. 1. *brlA* locus and RNAi construct. (A) The *brlA* locus consists of two overlapping transcriptional units, *brlA*α and *brlA*β. The top line represents the *brlA* genomic DNA (gDNA). The portion of the locus flanked by BamHI sites (−2902 to −404) used in the RNAi construct included only *brlA*β. The line of the transcripts (mRNA) represents untranslated RNA, and the box represents translated mRNA. *brlA*β has one intron (−99 to +293) where *brlA*α transcription begins. (B) The RNAi construct consists of inverted repeats of inducible *alcA* promoters [$alcA_{(p)}$] flanking *brlA*β in a unique BamHI site. The RNAi construct also contains an *argB* marker for the nutritional selection of transformants.

sion on threonine was three- to fourfold less abundant than that on glucose. These experimental results imply that the expression of *brlA* is silenced in the RNAi strains on threonine. The silencing in JB1 is more drastic than the silencing in JB3, which corresponds to the respective *brlA* null and leaky phenotypes.

With a few rare exceptions, RNAi is sequence specific but not locus specific in fungi (20). *A. nidulans* contains two RNA-dependent RNA polymerases that could amplify the RNAi signal through transitive RNAi, which has been shown to occur in *Caenorhabditis elegans* (4), but transitive RNAi has been absent in previous *A. nidulans* silencing experiments (11). Furthermore, if the RNAi signal were amplified, all RNAi strains would have null *brlA* phenotypes.

To investigate RNAi specificity, Northern blot analyses were performed with ^{32}P-UTP-labeled RNA probes constructed with a Maxiscript T7 kit (Ambion). In antisense Northern blots (data not shown), a band specific for *brlA*β was present in the RNAi strains on threonine, but antisense RNA specific for *brlA*α was undetectable. For siRNA analysis (Fig. 3B and C), sodium carbonate-treated probes (7) were used with a mirVana microRNA detection kit (Ambion). siRNAs specific for *brlA*β (Fig. 3B) were present in the RNAi strains on threonine but not in the wild type or on glucose. No siRNAs were detected for the overlapping portion of *brlA*α and *brlA*β (Fig. 3C). siRNAs were more abundant in JB1 than JB3, corresponding to the respective *brlA* null and leaky phenotypes. These blots verified that RNAi mecha-

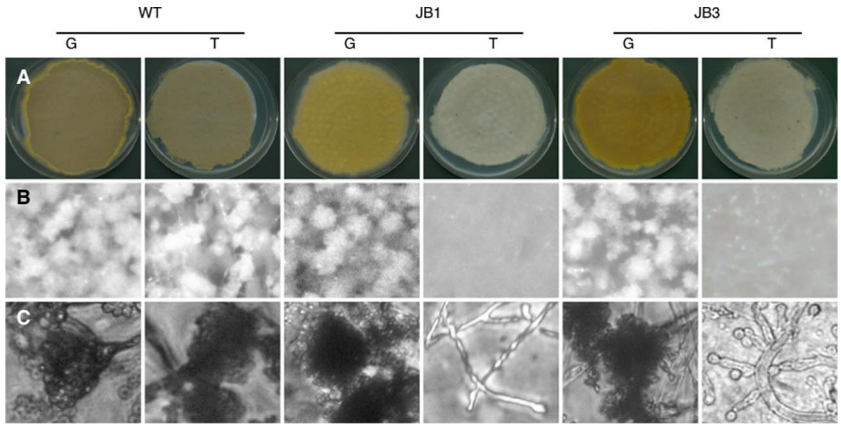

FIG. 2. Phenotypes of RNAi strains. (A) Plates that contained either glucose (G) or threonine (T) as the sole carbon source were scanned with a Microtek ScanMaker 4700. (B) Plates were photographed with a Canon Powershot A620 under a Nikon SMZ-U stereomicroscope at ×60 magnification. (C) Tape mounts were taken from the plates and photographed under a Nikon TMS microscope at ×1,000 magnification. These images show the wild type (WT) under both conditions and the RNAi strains (JB1 and JB3) on glucose with mature conidiophore development and viable conidia. The RNAi mutants on threonine show *brlA* phenotypes.

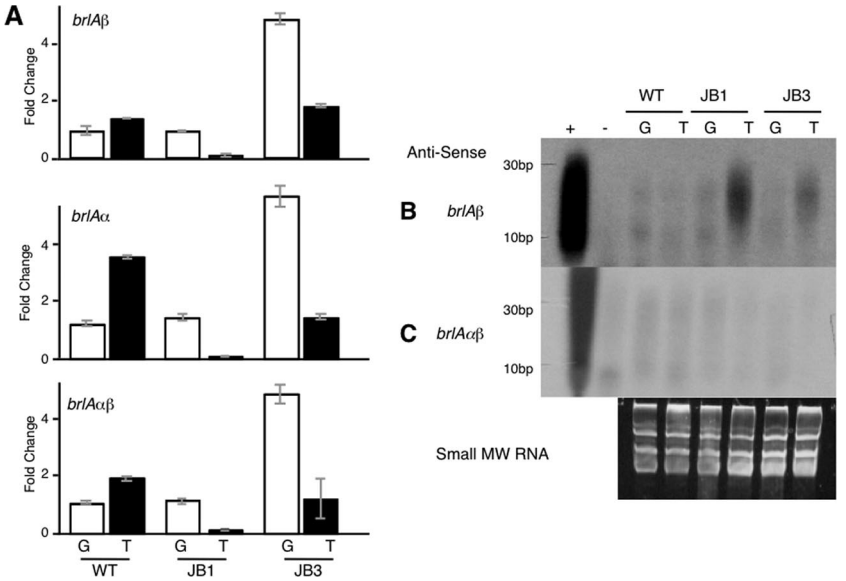

FIG. 3. *brlA* expression and siRNA analysis. (A) Primers used in real-time RT-PCR were specific for *brlA*α, *brlA*β, and the overlapping portion of *brlA*α and *brlA*β (*brlA*αβ). The wild type (WT) had normal expression of *brlA* on both glucose (G) and threonine (T). JB1 had normal *brlA* expression on glucose but almost undetectable expression on threonine. JB3 had high levels of *brlA* expression on glucose but reduced expression on threonine. (B) siRNAs specific for *brlA*β in the RNAi strains on threonine were detected. No siRNAs were detected in the wild-type or RNAi strains on glucose. Untreated probes (+) or probes treated with RNaseA/T1 (−) were used as controls. (C) No siRNAs were detected using a probe specific for the overlapping portion of *brlA*α and *brlA*β. This indicates that the RNAi mechanism is specifically targeting for *brlA*β, not *brlA*α. Low-molecular-weight (MW) RNA is shown to verify its integrity.

nisms are the direct cause of *brlA*β silencing and that *brlA*β, not *brlA*α, is the target of RNAi.

brlA regulates developmental genes such as *abaA* (2, 19), *wetA* (16), *rodA* (8, 24), and *stuA* (17). The most current models suggest that *brlA*β initiates asexual development, since it is present in small quantities in vegetative cells, and *brlA*α continues the development through a feedback mechanism with *abaA* (3, 12, 13). Furthermore, in a *brlA*β knockout mutant, *brlA*α expression was not detected after 12 h of developmental induction (23), and the overexpression of *brlA*β induces the expression of *brlA*α even when *abaA* is not present (12). This evidence and our results strongly suggest that *brlA*β plays a key role in regulating the expression of *brlA*α.

The novel RNAi construct serves as a powerful genetic tool, because RNAi is easily induced and any gene of interest may be cloned into the single BamHI site in either orientation. Problems associated with lethal knockouts of essential genes is circumvented, because these strains are isolated on glucose and then characterized by RNAi induction on threonine. Additionally, the one-step cloning of a gene in either orientation is advantageous over the currently popular use of inverted repeat transgenes, where the gene must be cloned twice in specific orientations to produce double-stranded hairpin RNA (10, 11, 21, 25).

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