Identification of a Basic Helix-Loop-Helix-Type Transcription Regulator Gene in *Aspergillus oryzae* by Systematically Deleting Large Chromosomal Segments^{∇†}

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We previously developed two methods (loop-out and replacement-type recombination) for generating large-scale chromosomal deletions that can be applied to more effective chromosomal engineering in *Aspergillus oryzae*. In this study, the replacement-type method is used to systematically delete large chromosomal DNA segments to identify essential and nonessential regions in chromosome 7 (2.93 Mb), which is the smallest *A. oryzae* chromosome and contains a large number of nonsyntenic blocks. We constructed 12 mutants harboring deletions that spanned 16- to 150-kb segments of chromosome 7 and scored phenotypic changes in the resulting mutants. Among the deletion mutants, strains designated $\Delta 5$ and $\Delta 7$ displayed clear phenotypic changes involving growth and conidiation. In particular, the $\Delta 5$ mutant exhibited vigorous growth and conidiation, potentially beneficial characteristics for certain industrial applications. Further deletion analysis allowed identification of the AO090011000215 gene as the gene responsible for the $\Delta 5$ mutant phenotype. The AO090011000215 gene was predicted to encode a helix-loop-helix binding protein belonging to the bHLH family of transcription factors. These results illustrate the potential of the approach for identifying novel functional genes.

Aspergillus oryzae is regarded as an industrially important koji mold utilized in commercial enzyme production and the traditional fermentative manufacture of foods such as sake, soy sauce, and miso in Japan (8). In spite of its industrial significance and recently available genome sequence (10, 12), techniques for gene manipulation in *A. oryzae* have not yet been developed extensively. In particular, gene-targeting efficiency is very low, making gene disruption difficult. Recently, this problem was solved by disrupting the *ku* genes involved in the nonhomologous end-joining pathway (2, 5, 14). Reportedly, the *ku*-disrupted strains markedly enhance the gene-targeting frequency compared to the *A. oryzae* parent strain (19).

Using a *ku70*-disrupted strain, we have developed two methods (loop-out and replacement-type recombination) to efficiently construct large-scale chromosomal deletion mutants of *A. oryzae* (16, 17). Using the two methods, we have successfully constructed aflatoxin cluster-deleted *A. oryzae*. Generally, the replacement-type recombination (direct-deletion) method could be used for single deletions, while the loop-out-type recombination (5-fluoroorotic acid [5-FOA] selection) method enables construction of multiple deletions in a single strain by marker recycling. The potential merits of functional analysis by large-scale deletion include (i) identification of essential genes and construction of an experimental minimal gene set, (ii) detection of phenotypic change by simultaneously deleting multiple genes, (iii) greater convenience and increased effi-

ciency than the single-gene disruption technique, and (iv) the possibility of isolating an industrially useful strain.

Here, we demonstrate a novel approach for identifying functional genes in *A. oryzae* chromosomes. Essential and nonessential chromosomal regions are first determined by analysis of large deletions. Essential (undeletable) regions or deletions leading to clear phenotypic change can then be targeted for successive deletion analysis to gradually isolate the responsible gene(s). We focused on chromosome 7 which contains a large number of nonsyntenic blocks (NSBs). These NSBs were detected by comparison of the *A. oryzae* genome with those of *Aspergillus fumigatus* and *Aspergillus nidulans*, and they contain abundant genes involved in secondary metabolism and *A. oryzae*-specific genes (20). Analysis of chromosome 7 deletions identified a novel gene that has been predicted to encode a basic helix-loop-helix (bHLH) protein on the basis of its conserved motif.

MATERIALS AND METHODS

Strains, media, and transformation of *A. oryzae*. *A. oryzae* wild-type strain RIB40 was used as the DNA donor strain, strain RkuN16ptr1 ($\Delta ku70::ptrA \Delta pyrG$) (16) was used for the large-scale deletions in chromosome 7 of *A. oryzae*, and *Escherichia coli* DH5 α was used for DNA manipulation. DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4 , and 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.8) was used for liquid cultivation of *A. oryzae* strains. Czapek Dox (CD) minimal agar medium (0.2% $NaNO_3$, 0.05% KCl, 0.05% NaCl, 0.1% KH_2PO_4 , 0.05% $MgSO_4 \cdot 7H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, and 2% glucose, pH 5.5) was used for the selection of *A. oryzae* transformants. Positive selection of *pyrG*-deficient strains was performed using CD minimal medium plates supplemented with 15 mM uridine and 5-FOA acid (2 mg/ml; Sigma, St. Louis, MO). Malt medium (2% malt extract, 2% glucose, 0.1% polypeptone, and 10 mM uridine) was used as a complete medium. Information on the *A. oryzae* genome was obtained from the Database of the Genomes Analyzed at NITE (DOGAN; National Institute of Technology and Evaluation, Japan; <http://www.bio.nite.go.jp/dogan/Top>). For the sake of simplicity, either the last two or last three digits of the gene identifier (ID) assigned in DOGAN were used to

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TABLE 1. List of large-scale deletion mutants

Mutant	Deletion region ^b	No. of genes	Deletion size (kb)	Frequency of deletion ^c
Δ1	14–58	44	150	5/7
Δ2	60–77	17	45	3/3
Δ3	97–104	7	16	1/8
Δ4	159–204	45	100	3/6
Δ5	204–232	28	65	5/6
Δ6	232–261	29	70	3/3
Δ7	249–285	36	90	2/3
Δ8	294–317	23	50	4/6
Δ9	391–408	18	40	1/2
Δ10	408–419	11	25	2/3
Δ11	724–755	31	75	3/5
Δ12	85–102 (sc206) ^a	24	40	7/8
Total		297	741	

^a Except for the deletion of genes 85 to 102 (sc206), the deletion regions exist in sc011 of chromosome 7. sc, supercontig.

^b Abbreviated gene ID numbers are given for the ranges of the deletion regions.

^c Values indicate numbers of deletants achieved/numbers of transformants.

designate *A. oryzae* genes in this study (e.g., gene 215 represents the abbreviated version of gene model AO090011000215).

A. oryzae transformation was performed as described previously (18), with minor modifications. Conidia of the strains were cultivated in 10 ml DPY liquid medium for approximately 18 to 20 h in a 50-ml Falcon tube. Protoplasts were prepared from the mycelial cultures by Yatalase treatment (Takara, Kyoto, Japan) for 3 h at 30°C and transformed with an amplified PCR product. Transformants were selected using a CD agar medium.

Generation of large-scale deletion cassette by fusion PCR. Deletion cassettes were constructed using fusion PCR as described previously (4). The strategy for construction of the gene 204-gene 232 deletion cassette is shown in Fig. S1-A in the supplemental material as an example. Using the genomic DNA of *A. oryzae* RIB40 as a template, a 2.2-kb *pyrG* gene with primer pair No.33/No.34, and two approximately 1.5-kb DNA fragments outside the targeted region (gene 204 with primer pair No.31/No.32 and gene 232 with primer pair No.35/No.36, which are listed in Table S1 in the supplemental material) were amplified by PCR. PCR amplification was carried out in a GeneAmp 9600 system (Applied Biosystems, Foster City, CA) with KOD plus DNA polymerase (Toyobo, Osaka, Japan). The

sequences from genes 204 and 232 were designed to overlap the sequences of the primer pair No.33/No.34 of the *pyrG* gene, respectively. The three amplified DNA fragments were then mixed, and fusion PCR was performed using the primer pair No.31/No.36. The PCR cycle reaction was as follows: denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 68°C for 5 min. The deletion cassettes generated were used for *A. oryzae* transformation to replace the corresponding targeted genomic regions (see Fig. S1-B in the supplemental material).

Confirmation of large-scale deletion by Southern blot analysis and aCGH. Large-scale deletions in the obtained transformants were identified by PCR, Southern blot analysis, and array competitive genomic hybridization (aCGH). Genomic DNAs of *A. oryzae* strains were extracted as previously described (18). After electrophoresis, the digested genomic DNAs were transferred onto a Hybond N+ membrane (Amersham Biosciences, Amersham, United Kingdom). Southern hybridization was performed as described previously (18). Digoxigenin (DIG)-labeled probes were constructed using a PCR DIG labeling kit (Roche Diagnostics, Mannheim, Germany). Primer pairs used to obtain DIG-labeled probes are listed in Tables S1 and S2 in the supplemental material. Hybridization and detection of signals with the DIG system were performed according to the manufacturer's instructions (Roche Diagnostics). DNA microarrays were purchased from Agilent Technologies. Genomic DNA was prepared and hybridized according to the Agilent array CGH protocol.

Construction of plasmids for deletion analysis by using In-Fusion PCR cloning. We first amplified an approximately 4.2-kb fragment for deletion of the region between gene 204 and gene 232 by fusion PCR (see Fig. S1 in the supplemental material) with LA *Taq* polymerase (Takara). The primers used in the study are listed in Table S2 in the supplemental material. The amplified PCR product was inserted into pCR2.1-TOPO with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The generated plasmid was linearized by PCR with primers (see Table S2 in the supplemental material) which hybridized to the end of the *pyrG* marker and gene 232 (see Fig. 5A). Approximately 1.0-kb sequences from targeted genes (genes 210, 215, 220, 225, and 230) were amplified with primer sets listed in Table S2 in the supplemental material and inserted into the end of the *pyrG* marker of the linearized cloning vector gene with the In-Fusion PCR cloning kit (Takara) according to the manufacturer's protocols, because the designed PCR primers of the inserts have 16 bases of homology with the terminal sequences of the linearized cloning vector. Finally, plasmids for the deletion of genes 204 to 210, 204 to 215, 204 to 220, 204 to 225, and 204 to 230 were constructed for *A. oryzae* transformation. The plasmids for deletion of genes 211 to 215, 212 to 215, 213 to 215, and 214 to 215 and the 215 promoter were constructed by the same method.

Construction of the 215 deletant. To replace the open reading frame of 215 (ORF 215) with the *pyrG* marker, a deletion vector was constructed as follows.

FIG. 1. Identification of the essential and nonessential regions of chromosome 7 (Chr. 7) by a series of large-scale chromosomal deletions. Each number indicates a chromosome 7 gene ID based on DOGAN (e.g., 14 represents gene ID AO090011000014).

FIG. 2. Deletion analysis by aCGH. Data analysis of aCGH of the $\Delta 5$ mutant (A) and the $\Delta 7$ mutant (B). Left panels, scatter plots of the DNA microarray result. All the dots below the dotted line correspond to the genes that were deleted in the corresponding mutant. Right panels, data analysis of aCGH of chromosome 7 (Chr. 7). Each bar represents the $\log_2$ ratio of normalized signal intensity for each gene from this mutant, relative to its parent strain. WT, wild type.

An approximately 3.0-kb fragment containing ORF 215 and promoter and terminator regions was amplified by PCR with primer pair No.25/No.26 (see Table S2 in the supplemental material). This product was inserted into pCR2.1-TOPO. The generated plasmid was linearized by PCR with primer pair No.27/No.28 and then fused to the *pyrG* marker similarly amplified by PCR with primer pair No.29/No.30 (see Table S2 in the supplemental material) using the In-Fusion PCR cloning kit. Using the generated plasmid as a template and the primer pair No.25/No.26, the DNA segment encompassing the *pyrG* marker gene as well as the noncoding regions upstream and downstream of ORF 215 was PCR amplified. The PCR products were used to transform the RkuN16ptr1 strain. Genome extraction and Southern blot analysis of the obtained transformants were performed as described above.

Restoration of the 215 deletant by introducing 215. For introduction of 215 into the 215-disrupted strain, an approximately 3.0-kb fragment including ORF 215 was amplified by PCR with primer pair No.25/No.26 (see Table S2 in the supplemental material). PCR products were used to transform the 215 deletant, and transformants were grown on CD medium with 15 mM uridine. The obtained conidia (10^6 to 10^8) were placed on CD medium containing 5-FOA and uridine to select *pyrG*-deficient transformants. The *pyrG*-deficient strains were isolated 4 days later. The chosen transformants were confirmed by PCR and Southern blot analyses.

Microscopy and image analysis. Conidial morphology under different culture conditions was analyzed using a microscope (MZ FL III; Leica) with a digital camera (PowerShot 580; Canon). Images were edited with Adobe Photoshop 5.0 software (Adobe Systems Inc.).

RESULTS

Identification of essential and nonessential regions in chromosome 7. In our previous research, we have successfully developed two recombination methods (loop-out and replacement type) for deleting large chromosomal DNA segments.

Using the replacement-type recombination method, we attempted to identify the essential and nonessential regions in chromosomes by generating large-scale chromosomal deletions. To exemplify the utility of our approach, we focused on chromosome 7 regions, which contain a large number of NSBs. Chromosome 7 consists of two supercontigs, sc011 and sc206, and the NSBs are concentrated mostly near the telomere of one arm (sc011). These regions of chromosome 7 were targeted for deletions.

Large-scale deletion cassettes were constructed by fusion PCR utilizing short overlapping sequences at terminal regions of three amplified DNA fragments to connect them together. As an example, the construction of the gene 204-gene 232 deletion mutant ($\Delta 5$ in Table 1) is shown in Fig. S1 in the supplemental material. The constructed gene 204-gene 232 deletion cassette was integrated into the *ku70*-disrupted (RkuN16ptr1) strain (see Fig. S1-B in the supplemental material), which has been reported to markedly enhance the gene-targeting frequency (19). Two transformants obtained were verified by PCR using primer pair No.31 and No.36 to amplify a 4.7-kb DNA fragment (compared to no band amplification with the parent strain) (see Fig. S1-C in the supplemental material). Simultaneously, Southern analysis revealed a 5.4-kb band with the parent strain, whereas the 204-232 transformants showed a 3.9-kb band (see Fig. S1-D in the supplemental material). This result indicated that the range between 204 and 232 was completely removed by replacement-type recombina-

FIG. 3. Comparison of growth between the deletion mutants and the parent strain. (A) The $\Delta 5$ and $\Delta 7$ mutants showed clear phenotypic change compared to the parent strain (WT). Deletion mutants and the parent strain were cultivated on malt and CD media at 30°C for 3 days. The two mutants displayed fewer conidia at 30°C, and the $\Delta 5$ mutant produced more conidia than the parent strain at 37°C on malt agar medium. (B) The $\Delta 5$ mutant produced more conidia under some stress conditions. These strains were point inoculated on agar media, and growth rate and conidiation were observed after 6 days.

tion. All deletion mutants were confirmed by the same method. Thus, among the deletions of 25 large chromosomal segments covering most regions of chromosome 7, 12 mutants ($\Delta 1$ to $\Delta 12$) harboring deletions that spanned 16- to 150-kb segments of chromosome 7 were successfully constructed (Fig. 1; Table 1). The deletable regions are distributed mainly in NSBs, as expected, while most of the undeletable regions belong to or partially include syntenic blocks (SBs). In contrast, the region of genes 77 to 79 belonging to NSBs could not be deleted; conversely, some regions partly including SBs were removed without difficulty (Fig. 1).

The large deletion mutants obtained and the frequency of deletion for each mutant are listed in Table 1. Mutants of all deletable regions were efficiently obtained, suggesting that this recombination technique may be broadly applicable for genomic engineering. Summing up the length of these 12 deleted regions, at least 741 kb of 2.93 Mb in chromosome 7 may be regarded as nonessential. As for the other undeletable regions, all transformants obtained were confirmed by Southern blot analysis to be heterokaryons that consist of two genetically distinct types of nuclei. The same result was obtained even though the transformation experiments were repeated three times.

To ensure that only intended changes occurred in the genome, further analysis by aCGH and pulsed-field gel electrophoresis was done to compare these mutants to the parent strain. The results of the aCGH experiments with the $\Delta 5$ and $\Delta 7$ mutants unequivocally demonstrate that the expected chro-

mosomal regions were completely deleted and that unintended chromosomal modifications, such as extraneous deletions or amplifications, did not occur in these mutants (Fig. 2). Likewise, the pulsed-field gel electrophoresis patterns show that the deletions of these large chromosomal DNA segments did not give rise to visible chromosomal rearrangements (data not shown).

Growth phenotype of large-scale deletion mutants. To examine growth effects of the deletions, mutants and the parent strain were cultivated on CD and malt media for several days. All exhibited parental-like phenotypes, except for two mutants, $\Delta 5$ and $\Delta 7$. Both produced fewer conidia relative to the parent strain when spot inoculated on malt agar medium at 30°C, and the $\Delta 5$ mutant produced more conidia on CD medium (Fig. 3A and B). The $\Delta 7$ mutant also exhibited a growth rate faster than that of the parent strain on CD medium at 30°C. Conidiation of the two mutants increased when their conidia were spot inoculated on malt medium at 37°C. In particular, the $\Delta 5$ mutant produced many more conidia than the parent strain under the same culture conditions (Fig. 3A). In order to gain further insights regarding the characteristics of the $\Delta 5$ mutant, we analyzed its conidiation behavior under different stress conditions. We observed increased conidiation in the $\Delta 5$ mutant under certain types of stress (37°C, 1 M NaCl, and 5 mM H_2O_2 ; Fig. 3A and B).

Accordingly, the $\Delta 5$ mutant was examined in more detail. First, the $\Delta 5$ mutant and parent strain were cultivated on malt medium for several days with spot, spread, or streak inocula-

FIG. 4. Growth phenotype of the $\Delta 5$ mutant. (A) Observation of the $\Delta 5$ mutant phenotype. The $\Delta 5$ mutant and parental strain (WT) were cultivated on malt medium at 30°C for several days with spot, spread, or streak inoculation. The photomicrographs (magnification of $\times 10$ and $\times 40$) show production of conidia by the $\Delta 5$ mutant that is denser than that by the parent strain when spread and streak cultured. The white thick line is 1 mm in length. Comparison of conidial numbers between the parent strain and $\Delta 5$ mutant after cultivation on malt medium at 30°C for 2 to 7 days by (B) spot inoculation and (C) spread culture. White bar, conidial production of the parental strain; gray bar, conidial production of the $\Delta 5$ mutant.

tion. This mutant formed conidia more densely at 30°C when cultivated on malt medium with spread or streak inoculation than with spot inoculation (Fig. 4A). To verify whether $\Delta 5$ produced more conidia, this mutant and the parent strain were cultivated on malt agar medium at 30°C for 2 to 7 days with spot and spread inoculation, and the conidia were counted (Fig. 4B and C). When the conidia were spot inoculated on malt medium, the parent strain produced more conidia until the 5th day, but this situation gradually reversed, with the conidial number of the $\Delta 5$ mutant increasing and even exceeding that of the parental strain. In contrast, when the conidia were spread cultured on the entire surface of the malt medium, the $\Delta 5$ mutant produced more conidia from the beginning, on average producing 60% more conidia than the parental strain.

Identification of the gene responsible for $\Delta 5$ mutant phenotype. To identify the gene(s) responsible for the $\Delta 5$ mutant phenotype, we constructed a series of plasmids for further deletion analysis (Fig. 5A). The fragments for deletion of genes 204 to 210, 204 to 215, 204 to 220, 204 to 225, and 204 to 230 were amplified by PCR and transformed into an *A. oryzae* *ku70*-disrupted (RkuN16ptr1) strain. The transformants were

selected on CD medium. Thereafter, the obtained transformants were studied by PCR and Southern blot analyses (data not shown). Confirmed deletion mutants were streak cultured on malt agar medium, and their phenotypes were compared with those of the parent strain and the $\Delta 5$ mutant. By observation and comparison, all mutants except the $\Delta 204$ -to-210 mutant displayed phenotypes similar to that of the $\Delta 5$ mutant. We therefore concluded that the gene responsible for the $\Delta 5$ mutant phenotype exists between genes 210 and 215 (Fig. 5B).

Next, using the 210-to-215 deletion plasmid as a vector, we replaced 210 by amplifying an approximately 1.0-kb fragment upstream of 211, 212, 213, 214, and 215 using In-Fusion PCR cloning as described above. Using the constructed plasmids for deletion of genes 211 to 215, 212 to 215, 213 to 215, and 214 to 215 and the 215 promoter as templates, the deletion fragments were amplified and used to transform the parent strain, and the phenotypes of the obtained transformants were observed. All the deletion mutants showed a phenotype similar to the $\Delta 5$ mutant, suggesting that the 215 promoter deletion ($\Delta 215$ -215) leads directly to dense conidiation (Fig. 5C).

To verify whether gene 215 is responsible for the phenotype,

FIG. 5. Identification of the gene responsible for the $\Delta 5$ mutant phenotype. (A) Plasmid construction for deletion analysis by the In-Fusion PCR cloning method. The designed PCR primers of the inserts have 16 bases of homology with the terminal sequences of the linearized cloning vector. Black bar indicates genes 210, 215, 220, 225, or 230. (B) Identification of the gene range responsible for the $\Delta 5$ mutant phenotype. The 204-to-210, 204-to-215, 204-to-220, 204-to-225, and 204-to-230 deletion mutants were constructed. Except for the 204-to-210 mutant, all other mutants have phenotypes similar to the $\Delta 5$ mutant, indicating that the responsible gene exists between 210 and 215. (C) Identification of the gene between 210 and 215 responsible for the $\Delta 5$ mutant phenotype. The phenotype of all deletion mutants is similar to that of the $\Delta 5$ mutant, suggesting that the 215 promoter deletion ($\Delta 215$ -215) directly caused the phenotype of dense conidiation.

we deleted ORF 215 (Fig. 6A). Southern blot analysis indicated that a band shift occurred predictably between the obtained transformants and parent strain, suggesting that ORF 215 was deleted in the transformants, as expected. Comparative analysis indicated that the 215 deletant was similar to the $\Delta 5$ mutant exhibiting dense conidiation and sparse sclerotia relative to the parent strains (Fig. 6B). Additionally, the 215 deletant displayed high levels of conidiation under some kinds of stress conditions (37°C, 1 M NaCl, and 5 mM H₂O₂; data not shown). Taken together, these results suggest that the phenotype of the $\Delta 5$ mutant can be conclusively attributed to a single gene, 215 (AO090011000215). The restoration of the parental phenotype by introducing gene 215 into the 215-deleted strain also supported this conclusion (Fig. 6B). Gene 215 was predicted to encode an unknown protein of 302 amino acids. Alignment of the deduced amino acid sequence of *A. oryzae* 215 shows 64.2%, 50.8%, and 42.3% overall identity

with putative proteins from *A. niger*, *A. fumigatus*, and *A. nidulans*, respectively (<http://www.broad.mit.edu/annotation/fungi/aspergillus/> and <http://www.tigr.org/tdb/e2k1/afu1/>) (Fig. 7). On the other hand, the gene 215-predicted protein also shares relatively high similarity to a *Penicillium chrysogenum* protein, but extremely low similarity to hypothetical proteins in other sequenced filamentous fungi (such as *Neurospora* and *Trichoderma*), yeast, and higher eukaryotes, such as mammals. The conserved sequences from amino acids 210 to 268 formed a putative bHLH motif, suggesting that gene 215 belongs to the bHLH family of transcription factors. Further analysis of gene 215 is in progress.

DISCUSSION

We here described a new approach for identifying novel functional genes in the fungus *A. oryzae* RIB40 by systematically deleting large chromosomal segments. We previously developed two methods (replacement type and loop-out) to efficiently create large-scale chromosomal deletions (16, 17). The loop-out method (intrachromosomal recombination) allows marker recycling and therefore consecutive rounds of deletions, but it is more sensitive to the effect of genome structure and gives lower recombination frequencies relative to the replacement-type method (16, 17). In addition, the construction of deletion cassettes for replacement-type recombination by fusion PCR is faster and more efficient than the loop-out method. Therefore, in this study, the replacement-type recombination method was used to systematically identify essential and nonessential regions in chromosome 7 of *A. oryzae*.

To determine the essential and nonessential genes, various approaches have been reported (3, 6, 7, 9, 13, 22, 24). One method is to assemble a theoretical minimal genome by comparing a variety of different but closely related microbial genomes. This method assumes that all essential genes exist in homologous form in all genomes and all nonessential genes will be absent in one or more cases. Based on this theory, we focused mainly on NSBs, which were identified by comparison of the *A. oryzae* genome with those of *A. fumigatus* and *A. nidulans* (20). We predicted that the deletion of these *A. oryzae*-specific regions would have no significant impact on growth rate and other phenotypic changes. Aside from NSBs, we also constructed some deletion cassettes to eliminate SBs. However, we failed to delete almost all these regions, whereas the deletable regions were distributed mainly in the NSBs as expected (Fig. 1). It is conceivable that the undeletable regions could be due to essential genes in the area, dramatic changes in chromosome stability in these regions after deletion, or the failure to efficiently separate homokaryon from heterokaryon. Another possibility is that deletion of more than one nonessential gene within a region can also lead to synthetic lethality. As mentioned in our previous papers, deletion efficiency may be influenced by the length of the deletion, the location of the deletion region, and other effects, such as the instability of telomeric regions (16, 17). However, all deletions on chromosome 7 in this report were achieved by precise homologous recombination, and illegitimate deletion was not observed (Fig. 2). Additionally, on chromosome 7, the location and deletion length had no obvious effect on the deletion

FIG. 6. Confirmation that ORF 215 is the gene responsible for the $\Delta 5$ mutant phenotype. (A) Disruption of ORF 215 and Southern blot analysis. The 1.0-kb fragment upstream of gene 215 was used as a probe. Genomic DNAs of the parent strain and transformants were digested with BamHI and hybridized with a ^{32}P -215 probe. In comparison with the gained 5.0-kb band in the parent strain, the 1.2-kb hybridization band in the transformants indicates the occurrence of homologous recombination. pro, promoter; ter, terminator. Lane 1, wild type (WT, parent strain); lanes 2, 3, and 4, ORF 215 disruptants. The ^{32}P probe was constructed as described in Materials and Methods. DIG labeling was used. (B) Observation of the phenotype of the 215-disrupted strain. The 215 disruptant ($\Delta 215$), $\Delta 5$ mutant, parent strain, and 215-restored strain ($\Delta 215$ [215]) were streak cultured on malt agar medium at 30°C for 3 days.

frequency. For instance, the 150-kb deletion of genes 14 to 58 ($\Delta 1$ in Table 1), which is located near the telomere, was achieved with a relatively high frequency. Accordingly, we mapped the deletable and undeletable regions of most of chromosome 7 of *A. oryzae* (Fig. 1). Although this map could not provide more details, it can be used to outline the essential and nonessential regions. We have no doubt that this map will prove to be very valuable and offer necessary information for further identifying functional genes.

Two mutants displayed phenotypes different from the others; the $\Delta 7$ mutant produced less conidia, and the $\Delta 5$ mutant displayed a dense conidiation when its conidia were streak or spread cultured on malt medium. However, no gene involved in conidiation has been reported in the deletion regions of the two mutants. Generally, blocking of conidiation occurs when a gene function related to conidiation is lost (1, 23), and the $\Delta 5$ mutant was therefore of some interest.

Further deletion analysis indicates that gene 215 is the most

direct factor affecting the $\Delta 5$ mutant phenotype, despite the loss of a 65-kb sequence in this mutant. The 215 protein is a potential transcription factor since it contains a conserved bHLH motif. The HLH family is a group of eukaryotic transcription factors that are involved in the regulation of neurogenesis, myogenesis, cell proliferation and differentiation, cell lineage determination, sex determination, and other essential processes in organisms ranging from yeasts to mammals (11, 15, 21). Although *A. oryzae* possesses 13 predicted HLH proteins, none of the *A. oryzae* HLH factors has been characterized to date. This 215 gene, may possibly play an important role in negatively regulating conidiation.

In addition to gene 215, this procedure is applicable to the identification of essential genes in the undeletable regions shown in Fig. 1. This method can be used for further constructing the minimal gene set and could be also extended to comprehensive identification and characterization of a number of essential genes and genes possessing important genetic func-

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Aor_AO090011000215  ---MAYTRTDPSFTLSDDERMYSMSHSPMHRPYDTFAAPKGPDPISANWNYDSAIDLFSL
Ang_An14g02540      ---MAYPRPD-SFSLDDERMYSMSHPSPLTRPNDTFK--GPDPLSANWSYDNAIDLFSL
Afm_AFUA_4G03460    MAYNNRPDASTAFTFDNDRFVNQQPK-----GPDPLSANWSYDSAIDLFSL
Ani_AN7170.2        -----MSPDPLSANWSYDSAIDLFSL
                        .*****.***.*****

Aor_AO090011000215  NTMPENFALDVPNEPMGVDPKDFPADFFAPPPDISGFTISNHSGEDAG---SITSDES
Ang_An14g02540      NTMPETTFPLEMSNEMMNLDPKDFPADFFAPPPDISAFTISNHSGEDAASCGLSSDLDS
Afm_AFUA_4G03460    NTMLPETFPLDIPN-DMLLDPKDFPTDLFAPPADISGFAIS-HSGEDSLS-----SDQES
Ani_AN7170.2        NTMIPEPFPLDIPN-DMLWDAKELPADFFAAPT DINGFTVSHDESLSDDQ-----ES
                        ***:** *.*::.* * *.*::*:**.*.*.*::* ... .. : *

Aor_AO090011000215  DDQSWSPTYAAP-----AEMLPAPGRQSTRKTTPAVKRETTWSSSPELAPQ
Ang_An14g02540      DDQSWSPTCRVSPLEPIHME LPKPAARTSRTSTRKTASQPKPREVTATRWSSSPEITPQ
Afm_AFUA_4G03460    EDQPWSPAYRVSSLDSTTPDAPT VSPRSTEKPTR--RRTTTQKREQATRWSSSPEMTPQ
Ani_AN7170.2        DDQSFSPNNFMAS TPPSASTLDLPSMAMP MNIKTEPQT TINP---CSTQSQQPKRKAS
                        **:.*:** . . . : *.*: ..

Aor_AO090011000215  EYPAHTS---PQTTPSPVNRKMTRTTSVDSNASTGQTTTATTTSGRNAAKRAAHNII
Ang_An14g02540      DYPATSVS---PPPAPSSPAANNTARKTTRSLSSDSNASTGQAQTTTGRNAAKRAAHNII
Afm_AFUA_4G03460    EYPVTSQATTSPAPAPVSPPAASTSRKNSRSLSTD-----QTATGRNAAKRAAHNII
Ani_AN7170.2        MISDSSSRYSPELSPQQPSPPRQTTTGRRSRAANATNGDENTARGRNAAKRAAHNII
                        . : * :*. .: * : *: *****

Aor_AO090011000215  EKRYRTNMNAKFVALEKAMCG-----GVQKSNKSGS--ASLKKSEILTNAIAYMQ
Ang_An14g02540      EKRYRTNMNAKFVALEKAMCG-----GVQKPTKGS--ASLKKSEILTNAITFMQ
Afm_AFUA_4G03460    EKRYRTNMNAKFVALEKAMSGS-----GVQKPTKGGSGPASLKKSEILTNAIAYMQ
Ani_AN7170.2        EKRYRTNMNAKFVALEKAMSVKTSGVSKAVSSTNSSNGVKSSASLKKSEILSNAITYMQ
                        *****. ....:***:***

Aor_AO090011000215  ELQEENKALQKELALFKQNMVPSGMWRHTKGAETFRA
Ang_An14g02540      ELQEENKVLQKELAMLKQSMVPNGMWRHSGSEAFHA
Afm_AFUA_4G03460    ELQDQNAALQKELALLKQNLPGGLWRHNKENDKFRT
Ani_AN7170.2        ELQEMNERLRKEVAALTRERRANAYC-----
                        ***: * *.*:* :.. ...

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FIG. 7. Comparison of the deduced amino acid sequence of 215 with other eukaryotic proteins. The enclosed regions indicate a putative bHLH binding domain. Aor, *A. oryzae*; Ang, *A. niger*; Afm, *A. fumigatus*; Ani, *A. nidulans*.

tions as well as genes conferring morphological traits. Our methodology therefore opens new avenues for global identification and characterization of functional genes, especially in *A. oryzae*.

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