Bidirectional-Genetics Platform, a Dual-Purpose Mutagenesis Strategy for Filamentous Fungi

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Rapidly increasing fungal genome sequences call for efficient ways of generating mutants to translate quickly gene sequences into their functions. A reverse genetic strategy via targeted gene replacement (TGR) has been inefficient for many filamentous fungi due to dominant production of undesirable ectopic transformants. Although large-scale random insertional mutagenesis via transformation (i.e., forward genetics) facilitates high-throughput uncovering of novel genes of interest, generating a huge number of transformants, which is necessary to ensure the likelihood of mutagenizing most genes, is time-consuming. We propose a new strategy, entitled the Bidirectional-Genetics (BiG) platform, which combines both forward and reverse genetic strategies by recycling ectopic transformants derived from TGR as a source for random insertional mutants. The BiG platform was evaluated using the rice blast fungus Magnaporthe oryzae as a model. Over 10% of >1,000 M. oryzae ectopic transformants, generated during disruption of specific genes, displayed abnormality in vegetative growth, pigmentation, and/or asexual reproduction. In this pool of putative mutants, we isolated insertion mutants with mutations in three genes involved in histidine biosynthesis (MoHIS5), vegetative growth (MoVPS74), or conidiphore formation (MoFRQ) (where "Mo" indicates "M. oryzae"), supporting the utility of this platform for systematic gene function studies.

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genicity-related loci in M. oryzae, a total of 21,070 transformants were generated by ATMT and screened (46).

To improve the efficiency of gene characterization, especially for filamentous fungi with low frequencies of HR, we propose a strategy entitled the Bidirectional-Genetics (BiG) platform. This strategy combines forward and reverse genetic approaches by directing ectopic transformants from TGR to a pipeline for screening random insertion mutants.

We chose the rice blast fungus M. oryzae to evaluate the feasibility and efficiency of this platform. This fungus is regarded as a model phytopathogenic fungus due to its economic impact, suitable features for handling in laboratories, and available genomic data (47–49). Although several gene manipulation tools, such as TGR, REMI, and ATMT, are available for M. oryzae, most predicted genes still remain uncharacterized experimentally. We validated the BiG platform using M. oryzae by successfully characterizing the functions of three genes.

MATERIALS AND METHODS

Fungal strains and culture conditions. M. oryzae wild-type strain KJ201, obtained from the Center for Fungal Genetic Resources (CFGR; http://knrrb.knrc.or.kr/english/index.jsp?rrb=cfg), and its transformants were grown on V8 juice agar (V8A) (80 ml of V8 juice, 310 μl of 10 N NaOH, and 15 g of agar per liter) at 25°C and under constant fluorescent light to promote conidiation. For genomic DNA extraction, the wild type and transformants were cultured in liquid complete medium (10 g of sucrose, 6 g of yeast extract, and 6 g of Casamino Acids per liter) for 5 to 7 days under 25°C in darkness.

Generation of gene knockout constructs and fungal transformation for TGR. Constructs for deleting target genes were created by double-joint PCR (50) with 1- to 1.5-kb-long 5′- and 3′-end-flanking regions of each of the targets and a 2.1-kb region of pBCATPH (51) that contains the hygromycin B phosphotransferase (HPH) gene. The 5′- and 3′-end-flanking regions of each target gene were amplified and fused to the HPH cassette using primers with a 23-bp tail in the reverse primer of the 5′-end-flanking region and the forward primer of the 3′-end-flanking region. The amplified knockout construct was introduced into protoplasts of KJ201. Protocols for protoplast preparation and transformation were adopted from a previous study (52). Selection of hygromycin B-resistant transformants was conducted on TB3 agar (3 g of yeast extract, 3 g of Casamino Acids, 10 g of glucose, 200 g of sucrose, and 8 g of agar per liter) amended with 200 μg ml⁻¹ hygromycin B.

Fungal DNA isolation. Genomic DNA isolation was conducted via two methods. Genomic DNA for Southern blot analysis and inverse PCR was isolated by a standard protocol (53). Genomic DNA of transformants for PCR-based screening was prepared using a quick extraction method (54).

PCR-based mutant screening. Individual hygromycin B-resistant transformants were screened by PCR to determine whether they correspond to a gene deletion mutant or an ectopic transformant. Primer pairs, including HPH_Sf (5′-CAAGGTCTACGACGACATTC-3′)/SF (specific primer upstream of the 5′-end-flanking region) and HPH_Sr (5′-GGCTGTGATCGACGATGC-3′)/SR (specific primer downstream of the 3′-end-flanking region), were used. Genomic DNA extracted via the use of a quick extraction method (54) and a small piece of mycelial tissue from a 36-h-old V8A culture of individual transformants were employed as the templates for routine PCR and direct PCR, respectively.

Phenotype-based screening of ectopic transformants. For rapid phenotypic evaluation, individual transformants were grown on a 24-well plate filled with V8A. To assess vegetative growth, the diameter of each colony was measured at 3 days postinoculation (dpi), and ectopic transformants showing less than 80% of the wild type growth were selected. Pigmentation was observed after 5 to 10 days of incubation, and transformants displaying more or less pigmentation than KJ201 were selected. A microscope at ×50 magnification was used to estimate asexual reproduction visually and qualitatively. The ectopic transformants showing abnormal aerial hyphae (fluffy or smooth), reduced conidiation, and/or abnormal morphology of conidiophore were identified. The putative mutants that were identified via the three methods were transferred to a 6-well plate containing V8A to confirm their phenotypes. At 3 and 5 dpi, vegetative growth was evaluated, and those exhibiting the original growth phenotype were subjected to further characterization. Additionally, since varied sizes of inoculum could affect the colony diameter, some ectopic transformants showing reduced rates of growth (vegetative growth/day) were also selected. Pigmentation was observed at 5 to 10 dpi, and asexual reproduction was examined at 10 dpi.

Characterization of selected ectopic transformants. Genomic insertions in individual ectopic transformants were identified by inverse PCR, followed by sequencing. For inverse PCR, genomic DNA of each ectopic transformant was digested with a restriction enzyme that does not cleave the HPH cassette, and resulting fragments were self-ligated using T4 DNA ligase (TaKaRa Bio Inc., Japan). A pair of primers located in the HPH cassette, HPH_Sf and HPH_Sr, was used to amplify the regions flanking the insertion site. Amplified DNA was directly sequenced via the Sanger sequencing method (3730 DNA analyzer; Applied Biosystems, United States) using a specific primer designed to generate sequences of the flanking region.

In-depth phenotype analysis for gene function characterization. Vegetative growth was measured on modified complete agar (CMA) and modified minimal agar (MMA) (35) at 9 dpi, with three replicates. To test histidine auxotrophy, candidate mutants were grown on MMA and MMA supplemented with 0.5 to 10 mM l-histidine. Quantitative assessment of conidiation was conducted by counting the number of conidia from 10-day-old cultures growing on V8A with and without supplementation with 0.5 to 10 mM l-histidine. Conidia were collected using 3 ml of sterilized distilled water, and the concentration of conidial suspension was measured using a hemacytometer under a light microscope. Conidial germination and appressorium formation were measured on a hydrophobic coverslip. Conidia harvested from 10-day-old V8A cultures were suspended in sterilized distilled water at the concentration of approximately 2 × 10⁶ conidia/ml. Three drops of this conidial suspension (40 μl per drop) were placed onto a coverslip and placed in a moistened box at 25°C. After 24 h of incubation, the percentages of germinated conidia and germinated conidia that formed the appressorium were determined via microscopic examination (three replicates with 100 conidia per replicate). For evaluating pathogenicity, after harvesting of conidia from 7- to 14-dpi cultures on V8A, 10 ml of conidial suspension (approximately 5 × 10⁴ conidia/ml) containing 250 ppm Tween 20 was sprayed onto susceptible rice seedlings (Oryza sativa cv. Nakdongbyeo) at the four- to five-leaf stage. The infected plants were kept in a dew chamber at 25°C for 24 h in darkness and subsequently moved to a growth chamber with a 16-h-light/8-h-dark cycle. Disease severity was measured at 6 to 7 dpi. To assess mycelial morphology, 100 to 200 conidia were spread on a hydrophilic glass slide with 200 μl of diluted liquid complete medium (1 g of sucrose, 0.6 g of yeast extract, and 0.6 g of Casamino Acids per liter). After 2 days of incubation at 25°C, grown mycelia were observed by light microscopy. Neutral red (final concentration at 0.2 mg/ml) was added, and the culture was stained for 1 h to visualize vacuoles in mycelia.

RESULTS AND DISCUSSION

Design of the BiG platform for M. oryzae. In M. oryzae, about 100 to 500 transformants are typically generated in a standard protoplast transformation (56) for TGR, with most of them being ectopic transformants. Assuming that about 200 ectopic transformants are generated from each transformation, only 100 trials of transformation are sufficient for creating a pool of 20,000 insertional mutants. This number is similar to those generated through massive random insertional mutagenesis via REMI and ATMT.
To test if transformants from this platform are suitable for identifying mutants of interesting phenotypes, we screened collected ectopic transformants. In the first round of screening, the ectopic transformants were grown on 24-well plates containing V8 juice agar (V8A) for a few days to identify those showing defects in three phenotypes, including vegetative growth, pigmentation, and asexual development, compared to the wild-type strain KJ201. After the initial screening, the phenotypes of candidate mutants were further evaluated by growing them on V8A in 6-well plates to reevaluate their phenotypes. Only the transformants showing reproducible phenotypes through both screenings were characterized via Southern blot analysis to determine the copy number of the inserted DNA construct. Finally, the insertion site was identified by inverse PCR (58) and sequencing (Fig. 1).

Over 10% of the ectopic transformants screened displayed one or more phenotypic abnormalities. To evaluate the effectiveness of the BiG platform, we screened 180 and 959 ectopic transformants resulting from routine TGR experiments to identify interesting insertional mutants via phenotype-based screening. From a single experiment of transformation, one can obtain both targeted knockout mutants and a large number of random insertional mutants. After PCR-based screening for target gene knockout mutants, ectopic transformants were collected and screened by their phenotypes of vegetative growth, pigmentation, and asexual reproduction. A double screening of mutant phenotypes, first in the 24-well-plate format and then in the 6-well-plate format, decreases the number of false positives. Southern blot analysis demonstrates the single integration of transforming DNA in selected ectopic transformants, and inverse PCR followed by sequencing identifies the location of insertion.

TABLE 1 Number of selected ectopic transformants by their abnormal phenotypes

| Category                                                                 | No. of ectopic transformants (% of total) from TGR for: | Total no. (%) |
|--------------------------------------------------------------------------|----------------------------------------------------------|---------------|
|                                                                          | MGG_09225.7 | MGG_14496.7 |               |
| Total ectopic transformants                                              | 180         | 959          | 1,139         |
| Ectopic transformants with phenotype(s):                                 |             |             |               |
| Vegetative growth                                                       | 17 (9.4)    | 88 (9.2)     | 105 (9.2)     |
| Pigmentation                                                             | 1 (0.6)     | 17 (1.8)     | 18 (1.6)      |
| Asexual reproduction                                                     | 8 (4.4)     | 53 (5.5)     | 61 (5.4)      |
| Vegetative growth and pigmentation                                       | 1 (0.6)     | 14 (1.5)     | 15 (1.3)      |
| Vegetative growth and asexual reproduction                               | 5 (2.8)     | 35 (3.6)     | 40 (3.5)      |
| Pigmentation and asexual reproduction                                    | 0           | 13 (1.4)     | 13 (1.1)      |
| Vegetative growth, pigmentation, and asexual reproduction               | 0           | 12 (1.3)     | 12 (1.1)      |
volved in vegetative growth than pigmentation. Similar frequencies of recovery of putative mutants in two independently generated pools of ectopic transformants support the utility of the BiG platform. Initial screenings of transformants generated by a previous random mutagenesis of KJ201 via ATMT also resulted in 11.3% (2,151/18,968) of putative mutants showing reductions in vegetative growth, abnormal pigmentation, and/or reductions in conidiation (46).

We analyzed ectopic transformants showing abnormalities in three phenotype categories by Southern blotting. Though over 50% of transformants showed single- or double-copy insertions of transforming DNA in TGR of both MGG_09225.7 and MGG_14496.7, proportions of single-copy insertions were different. Single- and double-copy insertion rates were 52.9% (9/17) and 5.9% (1/17) in MGG_09225.7 TGR and 17.8% (18/101) and 33.7% (34/101) in MGG_14496.7 TGR, respectively. These results provide direction for further research to investigate the insertion event by NHEJ with different types of transforming DNA.

Characterization of MoHIS5, MoVPS74, and MoFRQ. To demonstrate that the BiG platform can be used to identify genes of interesting phenotypes, we identified and characterized three genes in the selected ectopic transformants. One mutant, named MoHIS5BiG, where “Mo” indicates “M. oryzae,” was selected based on its defects in vegetative growth, pigmentation, and asexual reproduction. Genomic characterization revealed that a single copy of the introduced DNA construct disrupted the MoHIS5 (MGG_14904.7) gene, which encodes a putative histidinol-phosphate aminotransferase (Fig. 2A). It is similar to Saccharomyces cerevisiae HIS5, which plays a role in the seventh step of histidine biosynthesis (59). MoHIS5BiG was a histidine auxotroph and could not grow on modified minimal agar (MMA). Although MoHIS5BiG could grow on modified complete agar (CMA), its growth was reduced compared with that of KJ201. An intact copy of the MoHIS5 gene was reintroduced to MoHIS5BiG, and resulting transformants grew normally on both MMA and CMA (Fig. 2B). Although the application of external L-histidine enabled its growth on MMA, the restoration of growth was still partial (Fig. 2C). It was possibly due to (i) the insufficient and limited capacity of histidine uptake to fully supplement the growth requirement, (ii) an adverse effect of the biosynthetic intermediate that accumulates in the mutant, and/or (iii) an additional function of MoHIS5 that is required for vegetative growth. Moreover, MoHIS5BiG could not produce conidia on V8A, while its conidiation was partially recovered when histidine was added (Fig. 2D). Although conidia of MoHIS5BiG normally germinated and formed the appressorium, pathogenic growth in rice leaves was impaired (Fig. 2E and F). Because the environment inside the plant cell was expected to resemble the minimal medium, MoHIS5BiG, an auxotrophic mutant, probably could not grow well. Similarly, Sweigard et al. reported that one of the reduced-virulence mutants generated via REMI showed partial histidine auxotrophy. In this mutant, insertion occurred in the gene encoded by MoHIS5BiG (MoHIS5/MoHIS5) on CMA and MMA with or without exogenous L-histidine. (C) Vegetative growth of KJ201 and MoHIS5BiG on MMA supplemented with 0 to 10 mM L-histidine. (D) Quantitative analysis of conidiation on V8A supplemented with 0 to 10 mM L-histidine. (E) Assessment of the conidial germination rate (CG) and appressorium formation (AF) of KJ201 and MoHIS5BiG. (F) Lesions formed on rice leaves infected with conidia of KJ201 and MoHIS5BiG.
ing imidazole glycerol phosphate dehydratase, an enzyme required for the sixth step of histidine biosynthesis (40).

The second mutant, termed MoVPS74BiG, was selected based on its defect in vegetative growth and pigmentation. In this mu-

FIG 3 Functional characterization of MoVPS74BiG. (A) Ectopic integration disrupted the promoter region of the MGG_00839.7 locus, which encodes a homolog of vacuolar protein sorting-associated protein 74 (VPS74) of *S. cerevisiae*. (B) Vegetative growth of KJ201, MoVPS74BiG, and the complemented strain (MoVPS74BiG/MoVPS74) on CMA and MMA. (C) MoVPS74BiG formed swollen hyphal tips during vegetative growth. Bars = 50 μm. (D) Vacuole shapes in vegetative mycelia were observed by staining with neutral red. Bars = 20 μm. (E) Assessment of conidial germination rate (CG) and appressorium formation (AF) of KJ201 and MoVPS74BiG. (F) Lesions formed on rice leaves inoculated with KJ201 and MoVPS74BiG.
tant, an insertion occurred in the putative promoter region of MoVPS74 (MGG_00839.7) (Fig. 3A). MoVPS74 is a homolog of vacuolar protein sorting-associated protein 74 (VPS74) of S. cerevisiae, which was shown to be important for proper vacuole function (60). MoVPS74<sup>BIG</sup> showed abnormal mycelial shapes with swollen hyphal tips and strikingly reduced radial growth. To demonstrate that these phenotypic changes were caused by disruption of MoVPS74, genetic complementation was conducted, which generated transformants that are indistinguishable from KJ201 (Fig. 3B and C). Vacuole staining also exhibited dramatic differences between KJ201 and MoVPS74<sup>BIG</sup>. Vacuoles of MoVPS74<sup>BIG</sup> were smaller than those of KJ201, and only the circular type were observed in the mutant (Fig. 3D). Additionally, MoVPS74<sup>BIG</sup> could germinate and form the appressorium on hydrophobic surfaces but showed a reduced virulence, which probably was caused by its retarded mycelial growth (Fig. 3E and F). These observations suggest that vacuole functions under the control of MoVPS74 can affect vegetative growth, cell maintenance, and virulence, while conidiation, conidial germination, and appressorium formation were not affected by this gene product.

The last mutant, MoFRQ<sup>BIG</sup>, showed abnormal morphology during conidiophore development. Genomic characterization revealed that MoFRQ (MGG_17344.7) was disabled by insertion (Fig. 4A). MoFRQ is a homolog of the N. crassa Frequency protein, a key regulator of the circadian rhythm (61). MoFRQ<sup>BIG</sup> was indistinguishable from KJ201 in vegetative growth (Fig. 4B), conidial germination, appressorium formation, and pathogenicity (data not shown). MoFRQ<sup>BIG</sup> produced relatively short conidio- phores and held conidia densely. Genetic complementation was performed to confirm that disruption of MoFRQ was responsible (Fig. 4C). Since MoFRQ is a homolog of a circadian clock–related protein, our data suggest a potential relationship between the cir- cadian clock and conidiophore morphology.

**Conclusions.** Application of the BiG platform to M. oryzae led to the discovery of three genes involved in vegetative growth, pig- mentation, asexual development, and/or pathogenicity. Our data suggest that this novel approach compensates for the low efficiency of gene disruption by TGR by reusing undesirable ectopic transformants as a source for identifying novel mutations. In this study, we found novel functions associated with MoVPS74 and MoFRQ in the maintenance of cell shape and conidiophore elongation, respectively. This platform will help researchers generate useful forward and reverse genetics data simultaneously. Compared to ATMT, in which random integration of T-DNA prefers promoter regions (62), the BiG platform can potentially increase the randomness of integration due to the generation of ectopic transformants using various types of transforming DNAs. Analysis of many more insertion sites will be necessary to test this idea. In conclusion, this platform offers a novel and efficient method for elucidating the molecular functions of both known and novel genes, thus facilitating rapid genetic research in filamentous fungi, even if their TGR efficiency is very low.

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