Construction of a Binary Vector for Knockout and Expression Analysis of Rice Blast Fungus Genes

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We developed an efficient method to analyze gene function and expression of the rice blast fungus. We constructed a GATEWAY binary vector, which generates a gene-targeted disruptant carrying a green fluorescent protein gene under the native promoter of the target gene. Using this method, the knockout efficiency and expression patterns of two hypothetical genes were determined.

Key words: gene knockout; green fluorescent protein (GFP)-tagging; gene expression imaging; Agrobacterium-mediated transformation; Magnaporthe grisea

Magnaporthe grisea is a causal agent of rice blast disease. Conidia are deposited on the plant surface and generate germ tubes and dome-shaped appressoria that are infection-stage specific structures. The fungus forms penetration pegs from the bottom of the appressorium, penetrates into the plant cells, and elongates infection hyphae.

The genome sequence of this fungus has been analyzed,1) and more than 10,000 genes were annotated, but most of the genes are still hypothetical. For functional analysis of the genes, insertional mutagenesis and gene knockout using Agrobacterium-mediated transformation (AMT) have been performed.2,3) Characterizing the expression profile of the gene of interest is also useful in understanding its function. The green fluorescent protein (GFP) gene was used to monitor promoter activity.4) This approach makes possible determination of the spatial and dynamic alteration of gene expression.

We designed a vector system for a GFP-tagging gene knockout (GGKO) method that allows simultaneous production of gene knockout mutants and transformants with GFP under the control of the native promoter of the target gene. We also introduced AMT to achieve higher transformation efficiency, and the GATEWAY cloning system (Invitrogen, Carlsbad, CA) for easier construction of large binary vectors for the GGKO method. Preparation of the knockout vectors consists of two steps: (i) the upstream flanking region (UFR), which includes the promoter region, and the downstream flanking region (DFR) of the target gene are amplified by PCR and cloned into the GATEWAY entry vector, pETHG, to generate pETHG-(target)KO (Fig. 1A), and (ii) the final knockout vector, pCAMBIA-(target)KO, is generated by LR recombination reaction between pETHG-(target)KO and the destination vector pCAMBIA-Bar-RfA (Fig. 1B).

A PtrpC-HPT fragment (hygromycin B phosphotransferase (HPT) under the control of the Aspergillus nidulans trpC promoter) was prepared from pBIG2RHPH2) and cloned into pCR8/GW/TOPO (Invitrogen) to generate an entry vector, pETH. A synthetic GFP (S65T) gene5) was introduced into pETH to construct pETHG. To discriminate gene-targeted disruptants from transformants with the ectopic insertion of the T-DNA region, a PtrpC-Bar fragment (bialaphos resistance gene (Bar) regulated by PtrpC) was prepared from pBIG4MRBrev6) and inserted into the SalI site of pCAMBIA-0380 (GeneBank accession no. AF234290) purchased from CAMBIA (Canberra, Australia) to generate pCAMBIA-Bar. The GATEWAY vector conversion system RfA (Invitrogen) was inserted into pCAMBIA-Bar to construct a destination vector, pCAMBIA-Bar-RfA. The entry vectors constructed were digested with Apol or PvuII to be linearized before the LR reaction with pCAMBIA-Bar-RfA, to increase...
the efficiency of the LR reaction. The resulting knockout vectors were introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation.

The GGKO method was tested with two hypothetical genes, MGG09248 and MGG06594. MGG09248 encodes a chitin-binding module, and MGG06594 a chitinase-like protein. *M. grisea* strain Ina86-137, a Japanese pathogenic isolate from rice (MAFF Gene Bank stock no. MAFF101511) was used. AMT of *M. grisea* was carried out basically according to Tsuji et al., but the co-cultivation step was slightly modified. Conidia were cultured on filter paper on potato dextrose agar medium (PDA, Difco Laboratories, Detroit, MI) for 3 d to form mycelia, before a suspension of *Agrobacterium* was poured onto the filter paper. Transformants were selected by culture on PDA supplemented with 500 mg/ml hygromycin B (Wako Pure Chemical Industries, Osaka, Japan) and 25 µg/ml meropenem (Dainippon Sumitomo Pharma, Osaka, Japan). We obtained 10 to 30 hygromycin B-resistant transformants per filter (90 mm in diameter). Hygromycin-resistant colonies were screened on PDA containing 200 µg/ml of hygromycin B with and without 20 µg/ml of bialaphos (Meiji Seika Kaisha, Tokyo, Japan). Bialaphos was replaced with 0.003% Herbie liquid (5.4 µg/ml of bialaphos, Meiji Seika Kaisha). The knockout of the target gene was checked by PCR, and finally confirmed by Southern analysis (examples with MGG09248 are shown in Fig. 2). Fungal DNA preparation for PCR analysis was carried out with a simple method by Saitoh et al.8) For Southern analysis, DNA was extracted with phenol-chloroform from protoplasts. Southern analysis was performed with a DIG labeling and detection kit (Roche Diagnostics GmbH, Mannheim, Germany).

The 620-bp UFR and 570-bp DFR of the MGG09248 gene were used for homologous recombination. Of 18 hygromycin-resistant transformants from the first screening, 12 transformants were bialaphos-sensitive, and nine of them were confirmed as knockout mutants. For the MGG06594 gene, 637-bp UFR and 624-bp DFR
were used. Of 21 hygromycin-resistant transformants, 16 transformants were bialaphos-sensitive, and 14 of them were knockout mutants. Since the selection marker near the left border of the T-DNA tends to be truncated more frequently than that near the right border,3) a vector with a \( \text{PtrpC-Bar} \) fragment near the right border might increase the knockout efficiency. These mutants exhibited no morphological or pathogenic alterations in rice plants (K. Saitoh and Y. Nishizawa, unpublished data). Using 500–700 bp UFR and DFR, we obtained knockout mutants of several other genes by the GGKO method. Another strain, Guy11, was also applied by the method, but the knockout efficiencies depended on the gene (M. Nishimura, unpublished data).

A knockout mutant generated by the GGKO method expressed GFP regulated by its native promoter region (Fig. 1C), although the junction between UFR and GFP contained several nucleotides derived from the recognition site of the restriction endonuclease used in \( \text{pETHG-(target)KO} \) construction. Microscopy of the knockout mutants of MGG09248 and MGG06594 genes was done during vegetative growth on PDA, appressorium formation (14 h post inoculation), and infectious hyphae development (27 h post inoculation) in onion epidermal cells (Fig. 3). No fluorescence was detected in the MGG09248 knockout mutants during vegetative growth. This fluorescence was restricted during appressorium formation and was low in infectious hyphae. On the other hand, fluorescence in MGG06594 knockout mutants was weakly detected during vegetative growth and strongly detected in infectious hyphae. These data suggest that the MGG09248 gene specifically acts during appressorium formation and that the MGG06594 gene functions in the host cells.

Fig. 2. Knockout of \( M. grisea \) MGG09248 Gene.

A. Maps of genomic DNA of wild-type (WT) and knockout (KO) MGG09248 locus, and ectopically integrated T-DNA region of the knockout construct (ET). The coding sequence (CS), upstream flanking region (UFR), and downstream flanking region (DFR) of MGG09248 gene are shown in black boxes. The recognition sites of \( \text{SalI} \) are depicted. The fragment used to probe the MGG09248 gene is shown by a broken line. B. Southern analysis. Genomic DNA from wild-type and hygromycin B-resistant transformants was digested with \( \text{SalI} \) and probed with the MGG09248 gene fragment.

Fig. 3. GFP Expression of the Knockout Mutants of the MGG09248 and MGG06594 Genes.

Observations were done using BZ-9000 with a BZ filter GFP-BP (Keyence, Osaka, Japan). CO, conidium; AP, appressorium; IH, infection hyphae. Bars, 10 \( \mu \text{m} \).
In conclusion, we developed a new binary vector, that allows simultaneous generation of gene knockout mutants and GFP-tagged lines for gene expression analyses. Since \textit{HPT} and \textit{Bar} have been found to work in many other fungi, our vector system should be a useful tool for other fungi.

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\textbf{References}


