

Multi-Homologous Recombination-Based Gene Manipulation in the Rice Pathogen *Fusarium fujikuroi*

In Sun Hwang and Il-Pyung Ahn*

National Institute of Agricultural Sciences, Rural Development Administration, Jeonju 54874, Korea

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Gene disruption by homologous recombination is widely used to investigate and analyze the function of genes in *Fusarium fujikuroi*, a fungus that causes bakanae disease and root rot symptoms in rice. To generate gene deletion constructs, the use of conventional cloning methods, which rely on restriction enzymes and ligases, has had limited success due to a lack of unique restriction enzyme sites. Although strategies that avoid the use of restriction enzymes have been employed to overcome this issue, these methods require complicated PCR steps or are frequently inefficient. Here, we introduce a cloning system that utilizes multi-fragment assembly by In-Fusion to generate a gene disruption construct. This method utilizes DNA fragment fusion and requires only one PCR step and one reaction for construction. Using this strategy, a gene disruption construct for *Fusarium cyclin C1* (*FCC1*), which is associated with fumonisin B1 biosynthesis, was successfully created and used for fungal transformation. *In vivo* and *in vitro* experiments using confirmed *fcc1* mutants suggest that fumonisin production is closely related to disease symptoms exhibited by *F. fujikuroi* strain B14. Taken together, this multi-fragment assembly method represents a simpler and a more convenient process for targeted gene disruption in fungi.

Keywords : *FCC1*, *Fusarium fujikuroi*, gene deletion, homologous recombination, multi-fragment assembly

Fusarium fujikuroi Nirenberg (teleomorph, *Gibberella fujikuroi* [Sawada] Wollenweber) is a causal agent of bakanae (foolish seedling) disease, one of the most notorious seed-borne diseases affecting rice (*Oryza sativa* L.) fields worldwide. Its major symptom is spindly growth of the host species, which is caused by gibberellin (GA) production by the pathogen within and around the infection site (Wiemann et al., 2013). The fungal species belongs to the mating population (MP)-C of the *Gibberella fujikuroi* species complex, which is composed of more than eleven MPs that are sexually infertile with one another (Bomke et al., 2008). It is known to produce fumonisin (Cruz et al., 2013; Jeon et al., 2013), moniliformin (Desjardins et al., 2000), and beauvericin (Desjardins et al., 2000). In addition to typical bakanae symptoms, a considerable number of strains or isolates trigger stunting in rice (Cruz et al., 2013; Hur et al., 2015; Jeon et al., 2013). The absolute correlation between pathogen genes located in the gibberellin biosynthetic gene cluster (GA cluster) and fungal GA biosynthesis has been determined over the last two decades (Tudzynski, 1999, 2005; Tudzynski et al., 1998, 2003). However, the contribution of GA to symptom development remains to be determined, and the functional characterizations of other genes regulating virulence or pathogenesis is only just beginning. Draft genomes of the strains IMI 58289 and B14, model strains of *F. fujikuroi* known to cause spindly growth and alternative stunting in rice, have been completed in consequence of the recent growth of next generation sequencing technologies for microbial genomes (Jeong et al., 2013; Wiemann et al., 2013). Comparative genome analyses and gene annotation have indicated the presence of more than several hundred genes that should be characterized during the

*Corresponding author.

Phone) +82-63-238-4668, FAX) +82-63-238-4654

E-mail) jinhyung@korea.kr

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interaction between rice and *F. fujikuroi*.

Similar to other biological systems, functional characterization of fungal genes has conventionally been achieved through targeted gene-specific mutation or by using mutants from pools of germplasms harboring T-DNA at random sites within the genome (Bundock and Hooykaas, 1996; Huang et al., 2011; Jeon et al., 2007; Michielse et al., 2005; Piers et al., 1996; Takahara et al., 2004). Although the latter mutant pool has made a huge contribution to the functional characterization of genes present in a limited number of model strains and genes that are conserved across strains, recent findings stress the necessity of target gene-specific gene disruption and deletion: almost all fungal genes resulting in virulence or pathogenicity are located within highly variable regions of the pathogen genome (Schirawski et al., 2010; Wiemann et al., 2013). These strain-specific virulence- and pathogenicity-regulating genes indicate the requirement for novel techniques to generate knock-out constructs efficiently. The ideal structure of a knock-out construct for the disruption of target genes in filamentous fungi is composed of a 0.5–1 kb-long 5' flanking region of coding DNA sequence (CDS); a gene encoding a protein conferring antibiotic-resistance, such as *hygromycin B phosphotransferase (HPH)* or *neomycin phosphotransferase (NPTII)*; and a 0.5–1 kb-long 3' flanking region of CDS. After introduction of this construct into fungal protoplasts via polyethylene glycol (PEG)/CaCl₂ or *Agrobacterium tumefaciens*, the target CDS and both flanking regions of the fungal chromosome are replaced with the cassette *HPH* or *NPTII* by dual homologous recombination between each flanking region. Classical methods for gene knock-out vector construction are almost always dependent on DNA digestion and ligation. These techniques have long been used and remain popular; however, suitable sites for restriction enzyme digestion are frequently unavailable depending on the nucleotide sequence of the target regions, and ligation can be affected by sequence-specific variations in ligation efficiency. To overcome these problems, PCR-based multi-fragment amplification, or double-joint PCR, was designed (Kim et al., 2009; Yu et al., 2004). Although the structure of the final knock-out construct produced using this method is identical to those manipulated by classical methods, this technique is free from the limitations of finding suitable restriction sites and sequences with suitable ligation efficiency. However, a disadvantage of this method stems from the fidelity of Taq polymerase. Taq polymerase almost always integrates at an improper base(s) that is not complementary with the template sequence; the mismatch rate increases exponentially in accordance with the amplicon size and GC ratio. Genome editing techniques employing transcription

activator-like effector nucleases (Aouida et al., 2014) or CRISPR/Cas9 (Laughery et al., 2015; Mans et al., 2015) represent potent, forthcoming alternatives that could replace conventional fungal gene disruption. However, the former has disadvantages in its complicated knock-out vector construction, and the latter requires genome sequencing of the candidate transformants to rule out possible off-targeting artifacts. This situation prompted us to develop an easy, versatile, and time-effective method for the production of knock-out constructs that are less affected by the presence or absence of restriction endonuclease sites, ligation efficiency, and the fidelity of Taq polymerase.

Recently, a novel gene manipulation technique enabling the simultaneous homologous recombination of several DNA fragments was developed (Raman and Martin, 2014). This technique, which is called In-Fusion HD Cloning, is sequence-independent and recombines all components in a designated order with high cloning efficiency. By invoking this technique, we demonstrate an alternative method for the efficient production of knock-out constructs suitable for the disruption or deletion of target genes in filamentous fungi, such as *F. fujikuroi*.

Materials and Methods

Fungal strains and growth conditions. These fungal strains were routinely cultured on oatmeal agar (OMA) at 22°C under continuous fluorescent light and were preserved at –20°C as dried mycelial agar blocks after chopping (Kim et al., 2005).

To assess the morphological characteristics of the mutants, solidified and liquid media were used to assay wild-type (WT) and mutant strains. The fungus grew on culture plates containing OMA and *Fusarium* complete medium (Leslie and Summerell, 2006), respectively. The culture plates were incubated for one week at 22°C, whereas the liquid cultures were incubated for 5 days at 22°C in a rotator shaker.

Primer design for *Fusarium cyclin C1* gene (*FC1*) and *FUM1* deletions. To generate target gene deletion mutants using the In-Fusion[®] HD Cloning system (Clontech Laboratories Inc., Mountain View, CA, USA), primers were designed using a primer design tool developed by In-Fusion[®] HD Cloning. Primers were also designed manually in some cases. To successfully clone any DNA fragment, the primers used must be designed to share an overlap of at least 15 homologous bases between the end of the linearized vector and/or the desired fragment. Thus, a primer will consist of a homologous sequence of 15 to 30 bases at the 5'-end and a gene-specific sequence at

the 3'-end. Depending on the length and sequence of the fragments, the overlap may be longer than 15 base pairs. Primers used for target gene disruption are listed in Table 1.

Construction of *FCC1* and *FUM1* deletion strains.

Construction of targeted gene deletion of *FCC1* and *FUM1* was performed following Changlin's method, with modifications (Fu et al., 2014). Here, we used a pBlue-script sk(-) vector that had been linearized by digestion with *EcoRI* and *HindIII* to obtain the targeted DNA fragment containing the 5' flanking region of the target gene, selection marker and the 3' flanking region of the target gene. The *NPTII* gene, which was used as a selectable marker for the transformation of *F. fujikuroi* by conferring geneticin resistance, was amplified from the pII99 vector. After PCR amplification using specific primer pairs developed by In-Fusion® HD Cloning, the reaction products were analyzed by electrophoresis on a 1% agarose gel to remove non-specific DNA bands and to avoid false-positive clones. A total of 4 purified DNA fragments, including a linearized vector, a 5' target gene flanking region, *NPTII* and a 3' target gene flanking region were combined

in a tube containing 1 µl of 5x In-Fusion reaction mix. For multiple fragment assembly, 100 ng/µl of vector and 250 ng/µl of each insert were used. The reaction tube was incubated for 1 hour at 50°C and transformed directly.

Construction of *GGPPS1* deletion strains. To generate a *GGPPS1* deletion mutant, a 2.3 kb fragment of genomic DNA containing a 5' and 3' sequence flanking *GGPPS1* in the *F. fujikuroi* strain was amplified by PCR and cloned into the pGEM-T easy vector (Promega, Madison, WI, USA). Next, a 1,257 bp *ClaI-StuI* fragment of the *GGPPS1* gene in the pGEM-T easy vector was replaced with a 1,020 bp *ClaI-StuI* fragment amplified from pAN7-1 containing the *E. coli* *HPH* gene as a selection marker. This 2.6 kb fragment, which has a 5' sequence flanking *GGPPS1*, was amplified by PCR from the pGEM-T easy vector. Purified DNA was used to transform the B14 *F. fujikuroi* strain. The primers used for *GGPPS1* gene disruption are listed in Table 1.

Fungal genomic DNA isolation. For genomic DNA isolation, strains were grown in 10 ml of *Fusarium* com-

Table 1. Primers used in this study

No.	Primer name	Sequences
1	FCC1(-828)(F1_F)	<u>TCG AGG TCG ACG GTA TCG ATA AGC TAA</u> ACC GGG TTA ACG TTG TG
2	FCC1(-1)(F1_R)	<u>TCT ATC GCC TTC TTG ACG AGT TCT TCT GAT</u> CAA GAT ATG AGA AAT GAG AAA
3	NPTII_F(F2_F)	TCA GAA GAA CTC GTC AAG AA
4	NPTII (F2_R)	<u>GGA ATC GGT ATA TAA CGG TCT ATC AGA AGA TAT</u> GAT TGA ACA AGA TGG ATT G
5	FCC1(UTR+1)(F3_F)	ATC TTC TGA TAG ACC GTT ATA
6	FCC1(UTR+829)(F3_R)	<u>ACT AGT GGA TCC CCC GGG CTG CAG GAA TTG</u> ATC CCA TCA AGA TCG TCA
7	FCC1(-828)_F	AAC CGG GTT AAC GTT GTG
8	FCC1(+829)_R	GAT CCC ATC AAG ATC GTC A
9	FCC1_ORF(89)_F	GAT ATT ATC TCC TGA GTA GCA TCC
10	FCC1_ORF(374)_R	CCA TGG TTG CCA TCC TAC TG
11	NPTII(76)_F	GAA GCG GTC AGC CCA TTC
12	NPTII(373)_R	CGA GCG AGC ACG TAC TC
13	FUM1(-870)(F1_F)	<u>CGG TAT CGA TAA GCT</u> CAC CAA CCG AAG CAC CAC
14	FUM1(-1)(F1_R)	<u>GAC GAG TTC TTC TGA GTT</u> GAA CTT TAA AAT AAT ATA ACT
15	NPTII_F	TCA GAA GAA CTC GTC AAG AA
16	NPTII-FUM1-(F2_R)	<u>TTC TTG TTC AGT TTT</u> ATG ATT GAA CAA GAT GGA TTG
17	FUM1(+1)(F3_F)	AAA ACT GAA CAA GAA GAT AAC A
18	FUM1(+838)(F3_R)	<u>CGG GCT GCA GGA ATT</u> AGG CTG GCT GCT GAA GAT
19	FfGGPPS_-906F	GTT AGG TAG TTA TCA GGA CTG ATG AAT G
20	FfGGPPS_2163R	CAG TTC AGA TGT TAT CCT CGA CTC C
21	ClaI_HPH_1F	ATC GAT ATG CCT GAA CTC ACC G
22	StuI_HPH_1020R	AGG CCT CTA TTC CTT TGC CC

Bold and underlined nucleotides indicate sequences overlapping with the other fragment to be joined.

plete medium at 25°C. After harvesting and lyophilizing the mycelial mass, gDNA was extracted following cetyltrimethyl-ammonium bromide (CTAB)-based methods modified in our laboratory. The lyophilized mycelium was filled with 600 µl of CTAB buffer and beads. These were mixed thoroughly on a Geno/Grinder (SPEX CertiPrep., Metuchen, NJ, USA) for 5 minutes. The samples were incubated for 30 minutes at 65°C; then, chloroform was added and shaken for 1–2 minutes. Tubes were centrifuged for 10 minutes at 12,000 rpm, and the supernatant was collected in a new tube containing ice-cold isopropanol. After centrifugation for 10 minutes, the pellet was washed with ice cold 70% (v/v) ethanol, dried and dissolved in 50 µl of TE buffer (4.5 ml, 10 mM Tris, 1 mM ethylenediamine tetraacetic acid, pH set to 8.0 with HCl). Extracted genomic DNA was verified by agarose gel and stored at –20°C until use.

Fungal transformation. Fungal gene deletion was performed by homologous recombination as described previously (Kim et al., 2005). Briefly, two primer pairs were used to amplify the 5'- and 3'-flanking regions of the target gene from each construct by PCR. The purified PCR products were used for fungal transformation. To harvest the protoplasts of the B14 strain for transformation, young mycelia were incubated at 25°C for 3 hours in 20% sucrose containing 25 mg/ml Driselase and 10 mg/ml lysing enzyme (L1412; Sigma-Aldrich, St. Louis, MO, USA). Samples were centrifuged, and the resulting protoplast pellets were suspended in STC buffer (20% sucrose, 50 mM Tris-HCl pH 8.0 and 50 mM CaCl₂) and then mixed with a PTC solution (40% PEG 3350 in STC buffer) containing DNA fragments to obtain transformants. The transformed protoplasts were plated in regeneration agar medium (0.3% yeast extract, 0.3% casamino acid, 1.0% glucose, 20% sucrose, and 0.8% agar) with 50 µg/ml geneticin and incubated at 25°C. Hygromycin and geneticin-resistant transformants appeared after 5 to 7 days, and the fungi were grown in potato dextrose agar (PDA; Difco, Sparks, MD, USA) for 3 days for selection. To confirm successful gene deletion, PCR was performed using specific primer pairs (Table 1).

Southern blot. Three micrograms of genomic DNA from WT and mutant fungi was digested at 37°C with *Hind*III (New England Biolabs, Ipswich, MA, USA), which did cut in the mutant but not in the WT probe region. After resolving the digested products on a gel, the DNA was transferred to a nylon membrane (Hybond-N+; Amersham, Piscataway, NJ, USA) by capillary blotting. Hybridization was carried out according to the instructions provided with the DIG High Prime DNA Labeling

and Detection Starter Kit II (Roche Applied Science, Penzberg, Germany). The digoxigenin (DIG)-dUTP labeled 5' flanking region of *FCCI* was used as a probe.

Virulence assays. Healthy cv. Dongjin rice seeds were surface sterilized as previously described (Sahoo et al., 2011) and germinated on half-strength Murashige and Skoog (MS) medium under a 16-hour-light/8-hour-dark cycle at 26°C. The fungal mycelial blocks (0.5 cm in diameter) of *F. fujikuroi* strains grown for 7 days were transferred onto the center of vermiculite soil in the test tube (inner diameter × length = 1.6 × 14.7 cm) and recovered with a 2 cm layer of vermiculite soil. Then, 5-day-old rice seedlings were placed on the upper layer of the soil to prevent direct contact between the inocula and plants. The test tubes were covered with a cap to maintain the humidity for 7 days. Growing roots contacted the inocula within 24–36 hours after inoculation. Five days after inoculation (dai), the seedling height was measured. Seedlings were photographed at 7 dai.

Results

Constructs used for target gene disruption. During the interaction of rice and the *F. fujikuroi* strain B14, B14-infected rice plants showed severe root rot symptoms due to fumonisin production (Hwang et al., 2013). In particular, *FCCI* (*Fusarium cyclin C1*) plays an important role in fumonisin B1 (FB1) biosynthesis (Shim and Woloshuk, 2001). To determine *FCCI* function in the B14 strain, we used a modified gene-disruption method via In-Fusion cloning, which can be used to assemble multiple fragments. This strategy for targeted gene disruption requires a total of three PCR reactions and one linearized vector. Fig. 1A outlines our procedure for gene disruption. First, an amplification containing the 5'- and 3'-flanking regions of the *FCCI* locus was performed with specific primer sets—1/2 and 5/6, respectively—from B14 genomic DNA. In parallel, 795 bp of *NPTII*, a geneticin-resistance gene used as a selectable marker, was amplified from the pII99 vector by PCR using the primer pair 3/4. The observed size of the PCR products resolved by agarose gel electrophoresis corresponded to a predicted size of approximately 800 bp (Fig. 1B). pBluescript sk(-) vector linearized with the restriction enzymes *Eco*RI and *Hind*III was used as the carrier. The purified amplicons and linearized vector were input directly into the In-Fusion cloning system and linked using 15–30 bp overlapping sequences between adjacent fragments. These multiple fragments were mixed in the single tube and incubated at 50°C for 1 hour. The DNA fragments containing the 5'- and 3'- flanking regions of *FCCI* and the

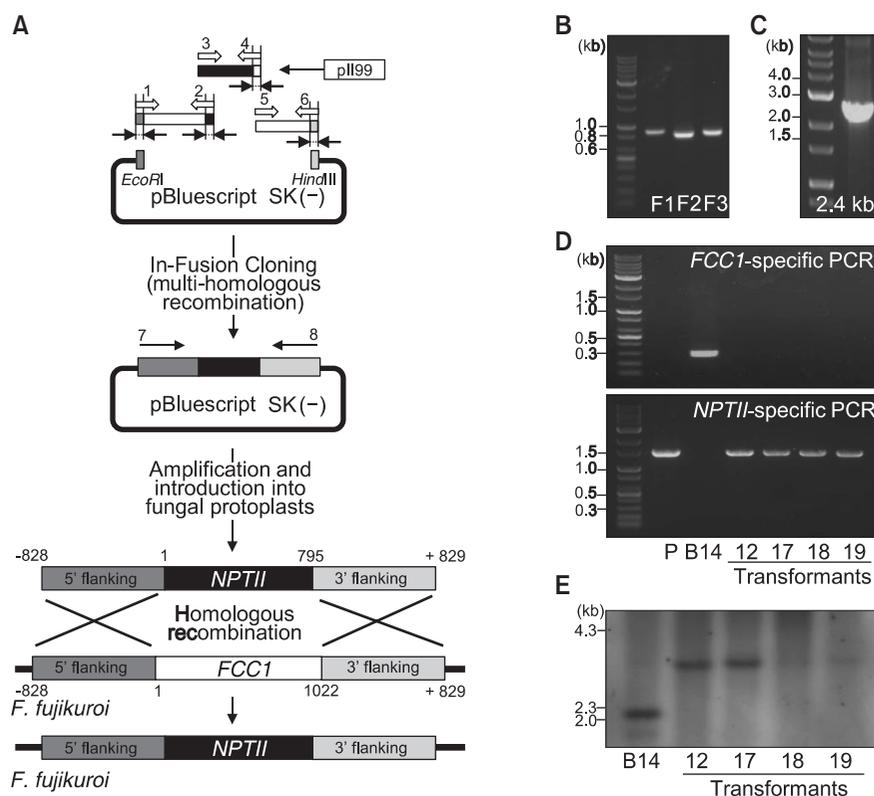


Fig. 1. *Fusarium cyclin C1 (FCC1)* disruption in the *Fusarium fujikuroi* strain B14 using the In-Fusion cloning system. (A) Strategies for *FCC1* disruption by homologous recombination. (B) Amplification of the three primary products: the 5' flanking region of *FCC1*, *neomycin phosphotransferase (NPTII)*, and the 3' flanking region of *FCC1*. (C) Verification of the constructs prior to target gene disruption. (D) PCR screening results from the selected transformants using specific primers. P, positive control. (E) Southern blot analysis of wild-type and *fcc1* mutants.

NPTII gene were amplified from a construct of confirmed sequence and transformed into B14 fungus to delete the *FCC1* gene. Subsequent PCR resulted in a PCR product of approximately 2.4 kb, the expected size of the *FCC1* disruption (Fig. 1C). These results indicated that our homologous recombination-based cloning system generated a construct for the disruption of the *FCC1* gene.

Gene replacement with the *FCC1* gene disruption construct. To disrupt the *FCC1* gene, B14 fungi were transformed with the following disruption cassette: an amplified DNA fragment of approximately 2.4 kb consisting of 5'- and 3'- *FCC1* flanking regions and the *NPTII* gene. In this manner, the *FCC1* gene was disrupted in B14 by homologous recombination, replacing *FCC1* with the *NPTII* gene. This strategy is based on selection for geneticin resistance stemming from the precise replacement of *FCC1* with the *FCC1* gene disruption construct containing an *NPTII* cassette. The *NPTII* selectable marker gene allows the selection of correct transformants using geneticin. Geneticin-resistant transformants were verified by PCR with specific primers (Fig. 1D). Four transformants were confirmed to yield the expected PCR results. These transformants also exhibited the expected restriction enzyme digestion patterns via Southern blot analysis (Fig. 1E).

Phenotypic characterization of *fcc1*-disrupted B14 strains.

The transformants obtained by successful homologous recombination were designated *fcc1* mutants. In terms of the growth rate, the culture broth of the selected *FCC1*-disrupted B14 strains exhibited significantly more red pigmentation at all time-points compared with the WT strain, which showed significantly less coloration. The *fcc1* strains exhibited slow radial growth and decreased conidia production when grown on PDA, suggesting that *FCC1* may be involved in the growth of mycelium and sporulation in this fungus. These mutants also exhibited increased pigmentation compared with the WT strain (Fig. 2A). To determine whether the *fcc1* mutation influences fumonisin production, fumonisin content was measured in the WT and *fcc1* mutants *in vitro*. First, fumonisin production by the *fcc1* mutants and WT strain was tested after growth in PDB for 5 days. As expected, fumonisin-producing ability was repressed by *FCC1* disruption. The *fcc1* mutants produced a lower or undetectable amount of fumonisin compared with the WT strain (Fig. 2B, left panel). Next, we tested whether fumonisin production during interaction with *F. fujikuroi* and rice was affected in *fcc1* mutant-infected rice plants. Significantly reduced fumonisin production was observed in rice infected by *fcc1* mutants compared with WT-infected rice (Fig. 2B, right panel). These findings clearly show that the deletion of *FCC1* has an influence on fumonisin production,

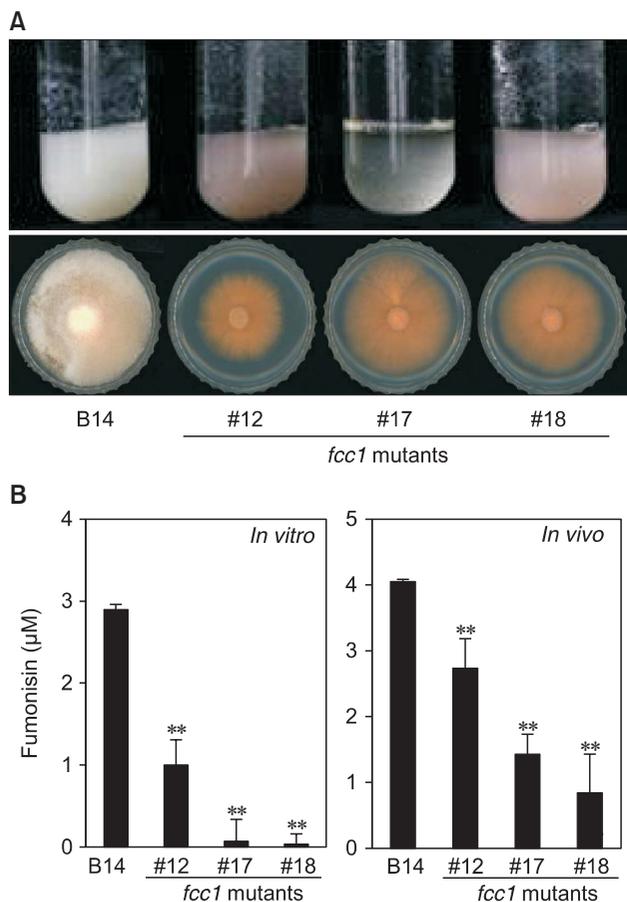


Fig. 2. Phenotypic characterization and fumonisin analysis in the *fcc1* mutant strains compared with the wild-type (WT). (A) Phenotypal analysis of the WT and the *fcc1* mutants in PDB and on PDA for 5 and 7 days. (B) The effect on fumonisin production by *Fusarium cyclin C1* (*FCCI*) disruption *in vitro* and *in vivo*. Error bars are represent as \pm standard deviation. Statistical significance was analyzed by Student's t-test. Asterisks indicate significant differences (** $P < 0.01$).

indicating that *FCCI* is involved in fumonisin synthesis during infection.

Fungal virulence assay on rice. Next, we performed a virulence assay in *fcc1* mutant-inoculated rice seedlings. Seedlings inoculated with the WT strain showed typical B14-mediated disease symptoms, such as root rot and growth restriction. In contrast, seedlings inoculated with fumonisin-deficient *fcc1* mutant strains exhibited a significant reduction in B14-mediated disease symptoms (Fig. 3). Intriguingly, root rot progression was delayed or decreased by *fcc1* mutant infection. This result indicated that the amelioration of disease symptoms by *fcc1* mutants may be related to the ability of these plants to produce fumonisin.

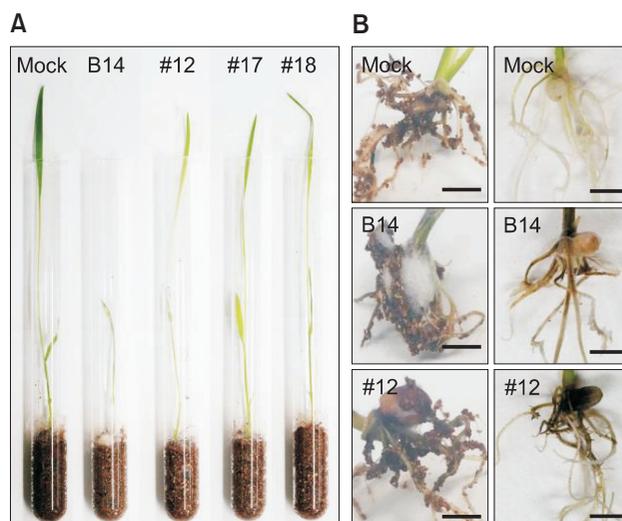


Fig. 3. Virulence assays in wild-type and *fcc1* mutant-challenged rice seedlings. (A) Symptoms in the rice plants inoculated with each strain. (B) Symptoms in the rice roots after infection. Scale bars = 0.5 cm.

A comparative study of the efficiency of cloning systems. Compared with restriction enzyme- and ligase-dependent cloning strategies for fungal gene deletion, multiple fragment assembly using the In-Fusion cloning system is simple. To generate a construct for *GGPPS1* disruption in *F. fujikuroi*, a 2.3 kb fragment of genomic DNA containing a 5' flanking *GGPPS1* region (0.8 kb), *GGPPS1* (1.2 kb), and a 3' flanking *GGPPS1* region (0.8 kb) from *F. fujikuroi* is required. Either naturally occurring restriction enzyme sites or engineered sites introduced during PCR amplification are essential for the insertion of the *HPH* gene for targeted gene replacement (Fig. 4A). However, construction of the *FUM1* gene disruption cassette, which can delete the *FUM1* gene (8.1 kb in total), required only a single PCR amplification and one reaction to assemble multiple fragments (Fig. 4B). Screening by PCR using *NPTIII* gene specific primer pairs showed that this method reached over 90% efficiency. The final cloning efficiency by enzyme digestion and sequence analysis dropped to approximately 60–70% due to some mutations such as deletions or mismatches. However, these clones contained a correctly assembled insert of the 5' flanking sequence, *NPTIII*, and the 3' flanking sequence. These clones were base perfect, suggesting that multi-fragment assembly by the In-Fusion cloning system can be easily applied for fungal gene disruption due to its high efficiency (Fig. 4C).

Discussion

In *F. verticillioides*, the *FCCI* is involved in fumonisin production by inhibiting the transcription of *FUM5*,

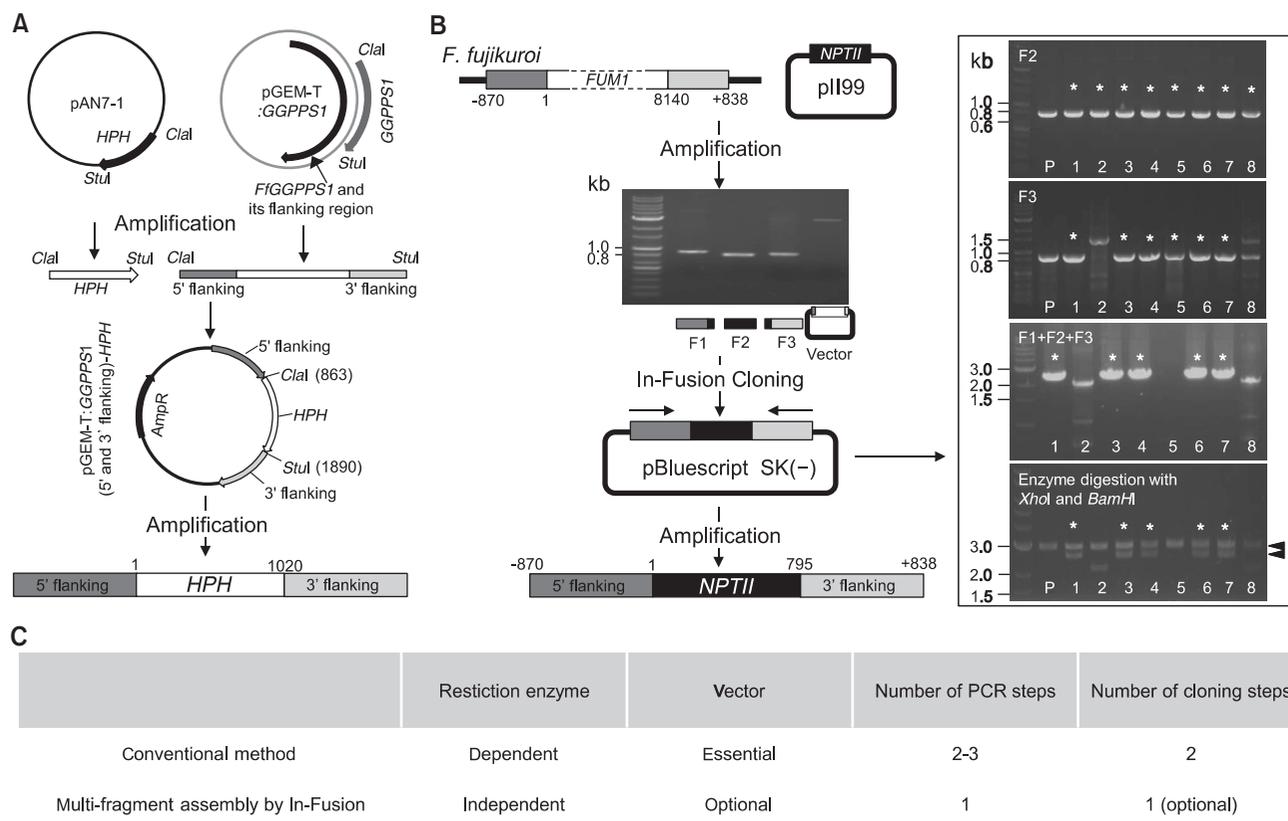


Fig. 4. Schematic representation of the construction of a gene replacement cassette. (A) Construction of a disruption and replacement cassette using restriction enzyme- and ligase-dependent cloning. *HPH*, *hygromycin B phosphotransferase*. (B) Construct used for *FUM1* (8.1 kb) disruption in *Fusarium fujikuroi* created using the In-Fusion cloning system. *NPTII*, *neomycin phosphotransferase*. *The marks indicate clones exhibiting expected results. (C) Comparison of the construction systems used in these experiments for targeted gene deletion in fungi.

which encodes a polyketide synthase necessary for FB1 biosynthesis (Shim and Woloshuk, 2001). This gene exhibits high homology across different *Fusarium* species, and *FCCI* of the IMI58289 and B14 strains shares 100% sequence identity. In this study, we introduced a simple method to generate constructs for *FCCI* gene disruption in the *F. fujikuroi* strain B14 in order to identify this gene's function. Although restriction enzyme- and ligase-dependent cloning strategies for constructing gene targeting vectors are the most commonly used methods, they are at times inconvenient due to the need for unique restriction enzyme sites. In addition, it can be difficult to clone large fragments; sometimes, fungal genomic DNA has over 10 kb, including the 5'- and 3'- flanking regions necessary for homologous recombination. As mentioned, the conventional method requires complicated steps and takes several days to several weeks, depending on the success of the restriction enzyme digests necessary to insert a selectable marker cassette. Recently, strategies for vector construction that do not require multiple cloning steps have been developed to overcome these issues

(Frandsen, 2011). Yu et al. (2004) reported a double-joint PCR method that does not require restriction enzyme sites for cloning steps. Similarly, construction by fusion PCR has been widely used for gene deletion in fungi (Kuwayama et al., 2002). However, these methods typically require multiple PCR steps to generate the final amplicon and can be complicated by low efficiency caused by the presence of repetitive DNA sequences. To get through these difficulties, many strategies have been developed for directional cloning and/or multi-gene/fragment assembly by homologous recombination. In *Saccharomyces cerevisiae*, cloning method by homologous recombination with high efficiency is also employed for single and multiple fragment assembly (Joska et al., 2014).

In this report, we introduced a simple method for the generation of a targeted gene disruption construct in *F. fujikuroi*. This method, which is termed multi-fragment assembly by In-Fusion cloning, requires only one standard PCR reaction and one fragment assembly reaction (Zhu et al., 2007). To generate a construct for *FCCI* deletion, we first performed PCR using specific primers containing

15–30 bp overlapping sequences and then incubated 3 fragments and the linearized vector digested by a restriction enzyme at 50°C for 1 hour. To obtain high levels of the final DNA construct containing 5' and 3' *FCCI* flanking regions and the *NPTII* gene, we selected a strategy that is compatible with any linearized vector; multi-fragment assembly offers single-step construction regardless of the presence of a vector. This one-step method is simple, includes primer design, and is faster than restriction enzyme- and ligase-dependent cloning systems and/or any other time-consuming methods used to generate constructs for fungal gene deletion. Using this strategy, we successfully created constructs for *FCCI* deletion in a short period of time. We used these constructs to determine the relationship between fumonisin production and B14-mediated disease symptoms. Correct deletion of *FCCI*, which regulates fumonisin synthesis, in B14 resulted in compromised fumonisin production, leading to weakened disease symptoms during infection. This result demonstrates that fumonisin promotes non-typical bakanae symptoms in rice seedlings. In addition, our results showed that *fcc1* mutants produce small amounts of fumonisins *in planta* during their interaction compare with when *fcc1* mutants were grown in media, suggesting that *F. fujikuroi* produces numerous secondary metabolites such as fumonisins that may be not essential for life *in vitro* but are indispensable for survival under natural conditions, including during fungal-plant interaction.

In conclusion, we herein report the function of *FCCI* via gene disruption in the *F. fujikuroi* strain B14. We have obtained several mutants that exhibited reduced fumonisin production or different growth patterns *in vitro*. Furthermore, we demonstrated the excellence of multi-fragment assembly. This method is a highly efficient process that is not dependent on restriction enzyme sites and/or ligation. Multi-fragment assembly by the In-Fusion cloning system can be easily controlled and modified for gene deletion in fungi, regardless of which restriction enzyme sites are needed, the size of the gene and its 5'- and 3'- flanking regions, or how many fragments are necessary for gene disruption. We expect that multi-fragment assembly will provide a wide range of possibilities for functional analysis through gene deletion in fungi.

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