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Enhanced Homologous Recombination in *Fusarium verticillioides* by Disruption of *FvKU70*, a Gene Required for a Non-homologous End Joining Mechanism

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Fusarium verticillioides **(teleomorph *Gibberella moniliformis*) is associated with maize worldwide causing ear rot and stalk rot, and produces fumonisins, a group of mycotoxins detrimental to humans and animals. While research tools are available, our understanding of the molecular mechanisms associated with fungal virulence and fumonisin biosynthesis in *F. verticillioides* is still limited. One of the restraints that hampers *F. verticillioides* gene characterization is the fact that homologous recombination (HR) frequency is very low (<2%). Screening for a true gene knock-out mutant is a laborious process due to a high number of ectopic integrations. In this study, we generated a *F. verticillioides* mutant (SF41) deleted for *FvKU70*, a gene directly responsible for non-homologous end-joining mechanism, with the aim of improving HR frequency. Here, we demonstrate that *FvKU70* deletion does not impact key *F. verticillioides* phenotypes, e.g., development, secondary metabolism, and virulence, while dramatically improving HR frequency. Significantly, we also confirmed that a high percentage (>85%) of the HR mutant strains harbor a desired mutation with no additional copy of the mutant allele inserted in the genome. We conclude that SF41 is suitable for use as a type strain when performing high-throughput gene function studies in *F. verticillioides*.**

Keywords **:** functional genomics, *Fusarium verticillioides*, gene knock-out, homologous recombination

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Fusarium verticillioides (Sacc.) Nirenburg (teleomorph *Gibberella moniliformis* Wineland) causes severe stalk rot and ear rot of maize and is found in plant residues in almost every maize field at harvest (White, 1999). Fungal stalk rots, including Fusarium stalk rot, are the most devastating disease of maize in terms of average yield loss. The damage due to stalk rot comes in several different forms, including stalk breakage, lodging, premature death of the plant, and the interruption of the normal grain filling process. In addition, attention to maize diseases caused by *F. verticillioides* has increased due to the fact that the fungus produces fumonisins, a group of carcinogenic mycotoxins, in infested maize ears. Ingestion of fumonisin-contaminated maize by humans and animals has been linked to a variety of illnesses, including leukoencephalomalasia and neural tube defects (Gelderblom et al., 1988; Marasas, 2001; Minorsky, 2002; Missmer et al., 2006). Over the past decade, significant progress has been made in elucidating the molecular genetic mechanisms associated with fumonisin biosynthesis, maize ear rot, and stalk rot in *F. verticillioides* (Sagaram et al., 2006). And in the process, the research community assembled a wealth of publicly available *F. verticillioides* molecular, genetic, and genomic resources. These include a genetic map consisting of 150 biochemical, molecular and morphological markers and 486 AFLP markers (Jurgenson et al., 2002; Xu and Leslie, 1996), the *F. verticillioides* Gene Index at Dana-Farber Cancer Institute (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=f_verticill) and the *Fusarium* Group Database at Broad Institute (http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html).

Despite being equipped with these resources, gene disruption remains as the key experimental procedure to unambiguously determine the functional role of a gene in filamentous fungi. In *F. verticillioides*, protoplast generation methodology was successfully adopted from other fungal systems (Salch and Beremand, 1993; Upchurch et al., 1991) and we now can routinely transform the fungus with gene-disruption vectors (Proctor et al., 1999; Shim and Woloshuk, 2001). In addition, with a double-joint PCR-based strategy gene disruption vector construction is no longer the bottleneck step in *F. verticillioides* gene knock-out experiments (Shim et al., 2006; Yu et al., 2004). Despite these improvements, targeted gene disruption in *F. verticillioides* is a laborious process due to a high number of ectopic integration events that occur during transformation. In filamentous fungi, homologous recombination (HR) is primarily utilized for generating targeted gene replacement mutants, and in *F. verticillioides*, like other fungi, HR frequency is quite low (<2%) (Weld et al., 2006). In

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general, we typically screen hundreds of drug-resistant transformants via PCR and Southern blot analyses before we can verify a true knock-out mutant strain.

This high frequency of ectopic integration in filamentous fungi is perhaps attributable to the nonhomologous end-joining (NHEJ) pathway that is activated in the event of double-strand break (DSB) (Hefferin and Tomkinson, 2005). When exogenous gene disruption DNA constructs are introduced into protoplasts, they are recognized as DSB and subsequently subjected to DSB repairs in the fungal nucleus. Two independent pathways are involved in DSB repair: (1) HR which depends on sequence similarity, and (2) NHEJ which integrates exogenous DNA fragments into the genome independent of sequence homology (Ninomiya et al., 2004). NHEJ is the predominantly active mechanism for repairing DSB in filamentous fungi, whereas HR is the dominant strategy in *Saccharomyces cerevisiae* (Meyer et al., 2007). The mechanism of DSB repair via NHEJ involves binding of Ku proteins (Ku70-Ku80 heterodimer) to foreign DNA fragment (Hefferin and Tomkinson, 2005; Ninomiya et al., 2004). Recent reports demonstrate drastic improvement in HR frequency in filamentous fungi by disrupting Ku70 or Ku80 (Goin et al., 2006; Haarmann et al., 2008; Krappmann et al., 2006; Meyer et al., 2007; Nayak et al., 2006; Ninomiya et al., 2004; Ueno et al., 2007). In this study, we hypothesized that *F. verticillioides* HR frequency can be improved by disrupting the NHEJ mechanism. To test this hypothesis, we generated a gene deletion mutant of *F. verticillioides KU70* homolog (*FvKU70*) and analyzed HR frequency in the mutant strain (SF41). We also investigated the impact of *FvKU70* deletion on key *F. verticillioides* phenotypes, e.g., development, secondary metabolism, and virulence, in SF41.

Materials and Methods

Fungal strain and culture media. The wild-type *F. verticillioide* strain 7600 (also designated M-3125, Fungal Genetics Stock Center, Kansas City, KS) was stored in 30% glycerol at −80°C. The fungus was grown on V8 agar (200 mL of V8 juice, 3 g of $CaCO_3$, 20 g of agar per liter) for inoculum. For genomic DNA preparatioin, a Erlenmyer flask containing 100-ml YEPD medium (Difco, Detroit, MI, USA) was inoculated with 1×10^7 conidia and incubated on a rotary shaker (150 rpm) at 24°C for 48 h before harvesting fungal tissue.

Nucleic acid isolation and manipulation. Bacterial plasmid DNA and fungal genomic DNA were extracted with Wizard miniprep DNA purification system (Promega, Madison, WI, USA) and OmniPrep Genomic DNA Extraction kit (G Biosciences, St. Louis, MO, USA), respectively. Southern analysis was performed as described previously (Sagaram et al., 2006). The probes used in all hybridization experiments were ^{32}P-labeled with Prime-It random primer labeling kit (Stratagene, La Jolla, CA, USA).

Polymerase Chain Reaction (PCR). All primers used in this study are listed in Table 1. PCR amplifications were

Table 1. Primers used in this study

Number	Name	Primer sequence (5'-3')
1	KU-che-F	GATAAGCACCAGATCCTTCTGACCCTTGG
2	Gene-R	GAGAACCTGCGTGCAATCCATCTTGTTC
3	KU70-A	GAGTACTGAGGATATTGTGTGAATGCGACAG
4	KU70-B*	TCACTGGCCGTCGTTTTACAAGATACGATTGATGTCTGTTGCTCTTAGTG
5	KU70-C*	CATGGTCATAGCTGTTTCCTGGAATTGATCGAGAGAATCGAGG
6	KU70-D	GTACTCCCATCGGTTGAGCTGATTGTCG
7	KU70-nest-F	CTCGGCGTTTTGGTATTCAGACCACTTTCGG
8	KU70-nest-R	CGATGTTGTTTAGGCTCTCGAGTGTCCC
9	CPP-F	CACATAGACCTTCCATTGAAGG
10	HPH-R2	CTGAAAGCACGAGATTCTTCGC
11	CMG-F	CAAGATCGACGAGATGCTCATTGTTCC
12	HPH-R2	CTGAAAGCACGAGATTCTTCGC
13	CPE-F	GTGGCCCATAGCTCTTACCAAGG
14	HPH-R	TGTAGAAGTACTCGCCGATAG
15	ORD-F	GAGAGCGAGCTTCCATTCTTGG
16	HPH-R	TGTAGAAGTACTCGCCGATAG

*M13-Forward and M13-Reverse primer sequences (underlined) were incorporated for double joint PCR application (Yu et al., 2004)

performed in a GeneAmp PCR system 9700 thermocycler (PE Applied Biosystems, Norwalk, CT, USA). PCR of DNA (except double-joint PCR) was performed in 25 μL total volumes with *Taq* DNA polymerase (Promega). The PCR conditions were 2 min of pre-denaturation at 94°C followed by 30 cycles of 45 sec denaturation at 94°C, 45 sec annealing at 54-57°C and 2 min extension at 72°C, unless specified otherwise. Double-joint PCR was performed using Expand Long Polymerase (Roche, Indianapolis, IN, USA) using the manufacturer's suggested protocol. For reference, *F. verticillioides* β-tubulin gene (*TUB2*) (GenBank U27303) was used.

***F. verticillioides* transformation.** *F. verticillioides* protoplasts were generated using the protocol described by Shim and Woloshuk (2001), except that Mureinase (2 mg/ml) was replaced with Drieselase (5 mg/ml) (Sigma, St. Louis, MO, USA). Gene disruption vector (DKU1) containing a geneticin (G418) -resistance gene (*GEN*) as a selectable marker was constructed via double-joint PCR strategy as described previously (Sagaram et al., 2006; Yu et al., 2004). First, 950-bp 5' and 1000-bp 3' flanking regions of *FvKU70* were amplified from *F. verticillioides* strain 7600 genomic DNA using *Taq* DNA polymerase. The primers KU70-A and KU70-B were used to amplify 5' flanking region and primers KU70-C and KU70-D were used to amplify 3' flanking region. Simultaneously, *GEN* was amplified from plasmid vector pBS-G with M13-F and M13-R primers using Expand Long Polymerase, which has proof-reading activity (Sagaram et al., 2007). Subsequently, the three amplicons were mixed in a single tube in a 1:3:1 (5' fragment: marker: 3' fragment) molar ratio and joined by PCR without using any primers as described previously (Sagaram et al., 2006; Yu et al., 2004). Finally, nested primers KU70-nest-F and KU70-nest-R were used to amplify the 4.0-kb DKU1 construct. *F. verticillioides* transformation was performed as described (Sagaram et al., 2007), and the geneticin-resistant transformants were screened for *FvKU70* gene deletion by PCR and Southern blot analyses.

Fungal physiology, pathogenicity, and fumonisin B_1 analyses. *F. verticillioides* strains were grown on potato dextrose agar (PDA, Difco) and V8 agar to observe morphology and growth (Choi and Shim, 2008a). Carnation leaf agar was used to quantify *F. verticillioides* conidia production (Sagaram and Shim, 2007). Stalk rot assays were performed on 8-week-old plants of the B73 corn lines as described previously (Sagaram et al., 2007). Briefly, internodal regions of 8-week-old B73 maize stalks were punctured with sterile needle and 10^4 spores were inoculated into the wound, and disease symptoms were observed by splitting stalks open longitudinally after a incubation for 14 days (25°C, 40% humidity). For fumonisin production analysis, fungal strains were grown on cracked corn (B73

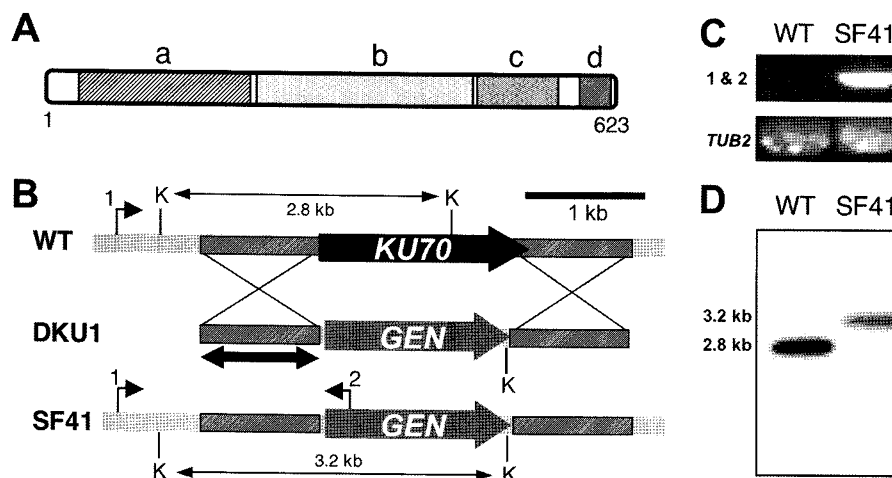

Fig. 1. Molecular characterization of *Fusarium verticillioides FvKU70* gene. (A) Schematic representation of the putative 623-amino acid (aa) FvKU70 protein. Box a represents a Ku70/Ku80 N-terminal alpha/beta domain, box b represents a Ku70/Ku80 beta-barrel domain, box c represents a Ku70/Ku80 C-terminal arm, and box d represents a SAP (SAF-A/B, Acinus and PIAS) domain. (B) WT is a representation of *FvKU70* locus in the wild-type strain. DKU1 is the *KU70* disruption construct harboring a geneticin-resistance gene (*GEN*) as the selectable marker. SF41 is a schematic representation of *KU70* locus in the knock-out strain after the homologous recombination event. Shaded box represents the region where homologous recombination occurred. The double arrow on DKU1 indicates the fragment used as ^{32}P-labeled probe in the Southern blot. The numbered arrows indicate the location of primers used for PCR assays. K: *Kpn*I restriction site. (C) PCR analysis of *KU70* disruption using the primers described in Fig. 1A. WT: wild type, SF41: *FvKU70* knock-out mutant. The number on the left (upper panel) indicates primer combinations for PCR amplification. Beta-tubulin gene PCR was used as a control. (D) Southern blot analysis of wild-type (WT) and SF41 strains. Fungal genomic DNA was digested with *Kpn*I, and the blot was hybridized with ^{32}P-labeled DNA probe (shown in Fig. 1A). Molecular sizes are indicated on the left.

line; 1 g dry weight) medium for 10 days. Fumonisin B_1 (FB_1), the major fumonisin produced by *F. verticillioides*, was extracted and analyzed by HPLC following the method described by Sagaram et al. (2007).

Results and Discussion

F. verticillioides FvKU70 sequence was identified by screening the *Fusarium Group* Database. The search revealed that supercontig 4, specifically sequence 2,393,440 to 2,395,474, harbors a 2,035-bp *FvKU70* gene sequence (FVEG_04235.3). Conceptually translated FvKU70 protein shares significant similarity with *Neurospora crassa* MUS-51 (E value = 0.0) and *Claviceps purpurea* Ku70 (E value = 0) (Haarmann et al., 2008; Ninomiya et al., 2004), and harbors four key domains that are characteristics of Ku70 proteins, i.e., a Ku70/Ku80 N-terminal alpha/beta domain, a Ku70/Ku80 beta-barrel domain, a Ku70/Ku80 C-terminal arm, and a SAP (SAF-A/B, Acinus and PIAS) domain (Fig. 1A).

Primers KU-che-F and Gene-R, designed to produce an amplicon only in the event of HR, were used to confirm the gene replacement (Fig. 1B). Of the 45 transformants screened, one isolate designated SF41, produced the expected band (2.1 kb) whereas no amplicon was observed in the wild type (Fig. 1C). Southern blot analysis further confirmed that the *GEN* gene replaced *FvKU70* in SF41. The 950-bp ^{32}P-labelled DNA probe hybridized to a 2.8-kb band in the wild-type, whereas to a 3.2-kb band in the SF41 strain (Fig. 1D). We concluded that *FvKU70* gene was completely replaced with *GEN* marker in the SF41 strain.

Subsequently, we analyzed the impact of *FvKU70* deletion on key *F. verticillioides* phenotypes using methods described previously (Sagaram et al., 2007). We first assessed whether the mutation impacts growth and development by growing the wild type and SF41 on PDA and V-8 agar. We did not observe any difference in colony morphology or growth rate (Fig. 2A and 2B). The number of conidia harvested from carnation leaf agar cultures showed no significant difference in their ability to reproduce asexually.

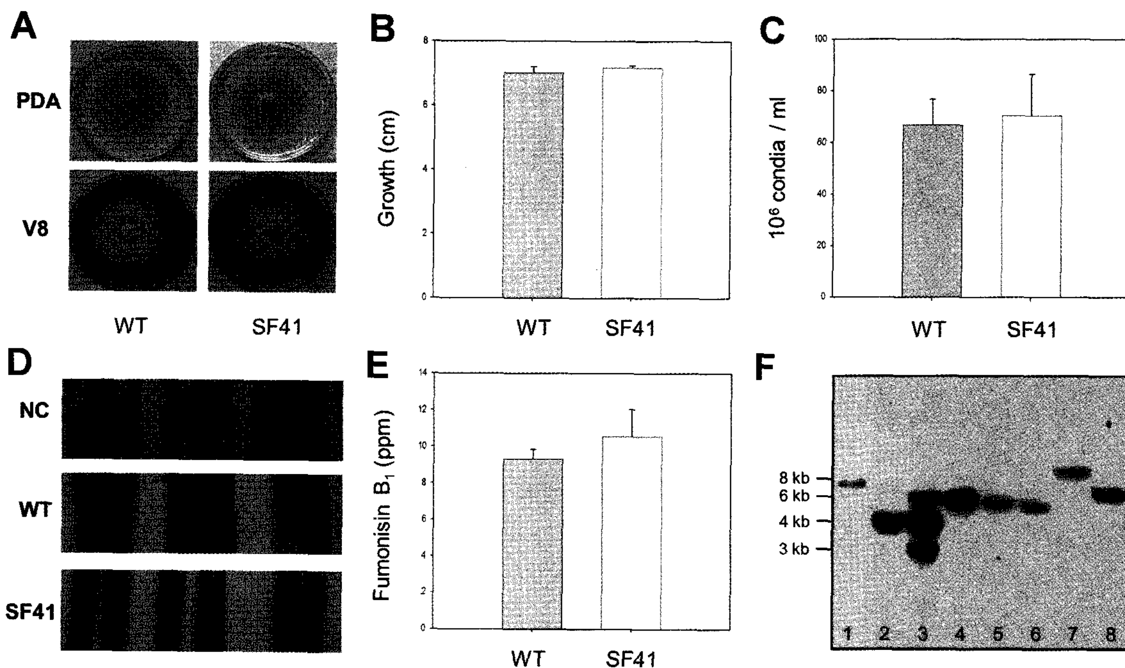

Fig. 2. Phenotypic analysis of SF41 strain. (A) Colony morphology of the wild-type (WT) and SF41 strains grown on 0.2×PDA and V8 plates. Strains were point inoculated with an agar block (0.5 cm in diameter) and incubated for 7 days at 25°C under a 14 h light/10 h dark cycle. (B) Radial growth of strains grown on 0.2×PDA for 7 days was measured and presented as a bar graph. Results are means of three biological replications. (C) Conidia production was measured by harvesting conidia from 7-day-old carnation leaf agar as described previously (Sagaram et al., 2007). Mean number of conidia obtained from three biological replications is presented. (D) Fusarium stalk rot assay was performed as described by Shim et al. (2006). After incubation for 14 days, infected maize stalks were split longitudinally to assay the development of stalk rot. Three independent repetitions are shown. NC: negative (water) control. (E) Sterile cracked corn (1 g) was inoculated with wild type and SF41, incubated for 14 days, and fumonisin B_1 (FB_1) was analyzed by HPLC (Sagaram et al., 2007). All values represent means of three biological replications with standard deviations shown as error bars. (F) Southern blot analysis of eight randomly selected HR mutants with SF41 genetic background (Table 2). Lanes 1-2: *CPP1*-deletion strains, Lanes 3-4: *CMG1*-deletion strains, Lanes 5-6: *CPE1*-deletion strains, Lanes 7-8: *ORD1*-deletion strains. Molecular sizes are indicated on the left. Genomic DNA (10 μg) samples were digested with *Eco*RV and were subjected to electrophoresis in 1.0% agarose gel. Blot was hybridized with ^{32}P-labeled *HPH* marker.

We also successfully performed a sexual cross using strain 7598 (Fungal Genetic Stock Center) as the opposite mating type (data not shown). Next, we tested whether SF41 is affected in virulence and fumonisin production (Sagaram et al., 2007). These results led us to conclude that deletion of *FvKu70* does not influence stalk rot virulence in *F. verticillioides* (Fig. 1D). Fumonisin assays were performed as described previously (Shim and Woloshuk, 2001). HPLC analysis of three biological replications revealed that there is no significant difference in fumonisin B1 production in wild type and SF41 (Fig. 1E). Taken as a whole, we concluded that SF41 holds the same phenotypic traits as the wild-type strain and therefore suitable for gene characterization studies as an alternative type strain.

To analyze the impact of *FvKU70* deletion on *F. verticillioides* HR frequency, we selected four genes, *CPP1* (FVEG_09543.3), *CMG1* (FVEG_12106.3), *CPE1* (FVEG_11127.3), and *ORD1* (FVEG_07201.3), for targeted gene disruption in the wild-type and SF41 strains. *CPP1* encodes a protein phosphatase 2A catalytic subunit that plays a role in fumonisin biosynthesis regulation (Choi and Shim, 2008a). *CMG1*, *CPE1*, and *ORD1* encode putative cell membrane protein, serine-type carboxypeptidase F precursor and oxidoreducatase, respectively. These genes were identified via *F. verticillioides* microarray and proteomics studies (Choi and Shim, 2008b; Pirttilä et al., 2004). Primers for double-joint PCR were designed and subsequently utilized to amplify gene-disruption constructs (primers not shown), harboring a hygromycin B phosphotransferase (*HPH*) gene as the selectable marker, as previously described (Choi and Shim, 2008a; Sagaram et al. 2007; Yu et al., 2004). The wild-type and SF41 protoplasts were transformed with these constructs and selected for hygromycin-resistant transformants. We then used PCR to determine the HR frequency in these transformants (Choi and Shim, 2008a; Sagaram et al., 2007). Primers for *CPP1* (CPP-F and HPH-R2), *CMG1* (CMG-F and HPH-R2), *CPE1* (CPE-F and HPH-R), and *ORD1* (ORD-F and HPH-R) would give rise to an amplicon only in the event of HR by gene-disruption by the constructs. These results demonstrated that the HR frequency in *F. verticillioides* was drastically increased by the deletion of *FvKU70*. In SF41 strain, we observed HR frequency of 30-60%, which is a significant increase from 0.5-5% observed in the wild type (Table 2); notably, we were able to increase *CMG1* HR frequency up to 61% by using SF41, a dramatic increase from 1% in the wild-type strain. Subsequently, we randomly selected eight HR gene-disruption mutants, i.e., two strains selected from each gene knock-out group (Table 2), and these strains were tested by Southern analysis to determine gene-disruption construct presence in the genome. Significantly, a high percentage of the mutants (7 out of 8) showed a single disruption-construct integration via HR (Fig. 2F). Also, we would like to note that four genes we tested are located on different chromosomes (I, IV, VIII, and IX), suggesting that the increased HR in SF41 is not restricted to certain loci. One negative effect of *FvKU70* deletion that we observed in this process was an overall decrease in the number of transformants. However, this was not surprising, and perhaps anticipated, since *FvKU70* deletion is not directly associated with improving HR process *per se* but rather eliminating or reducing NHEJ events that leads to frequent ectopic integration of disruption constructs during transformation (Hefferin and Tomkinson, 2005).

In summary, we demonstrated that HR frequency in *F. verticillioides* can be improved significantly by using a nonhomologous end-joining-deficient mutant strain SF41. Published reports show that HR frequency of 70-100% can occur in other filamentous fungi, e.g., *N. crassa*, *Aspergillus fumigatus*, *A. nidulans*, *Cryptococcus neoformans*, *Sordaria macrospora*, and *Magnaporthe grisea* (Goin et al., 2006; Krappmann et al., 2006; Nayak et al., 2006; Ninomiya et al., 2004; Villalba et al., 2008). While the level of HR frequency was not as high as these filamentous fungi, increased HR frequency in SF41 is a drastic improvement from that of wild type. And in fungi where targeted gene disruption is considered difficult, such as *F. verticillioides* and *Claviceps purpurea* (Haarmann et al., 2008), HR frequency of 30-50% may have huge implications in future systemic gene function studies. It is

Table 2. Homologous recombination frequency in the wild-type and SF41 strains

	Wild type			SF41		
Genes	Transformants[a]	Mutants[b]	HR %[c]	Transformants[a]	Mutants[b]	HR %[c]
CPP1	180	2	1.1	13	5	38.5
CMG1	236	2	0.85	13	8	61.5
CPE1	40	2	5	12	4	33.3
ORD1	44	1	2.27	12	5	41.7

[a]Total number of transformants screened for the homologous recombination (HR) event
[b]The number of HR (knock-out) mutant isolates determined via PCR
[c]HR percentages were calculated by dividing the number of HR mutant isolates with total number of transformants screened.

unclear why we still observe relatively high percentage of NHEJ events in *F. verticillioides* SF41 strain. Recent report by Iwabuchi et al. (2006), which describes a Ku70-independent NHEJ mechanism mediated by 53BP1 pathway in chicken cells, suggests that there are alternative pathways to repair DSB events in eukaryotes. However, alternative NHEJ pathways has not been identified nor characterized in filamentous fungi (Haarman et al., 2008). SF41 strain is not impaired in key *F. verticillioides* phenotypes, such as development, secondary metabolism, and pathogenicity, which render SF41 suitable for high-throughput gene function studies in *F. verticillioides*. We are currently in the process of incorporating *FvKu70* deletion into the opposite mating type strain 7598 and replacing *GEN* in SF41 with *GFP* marker to further exploit SF41 strain in molecular genetic and genomic research.

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