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Enhanced gene replacement frequency in *KU70* disruption strain of *Stagonospora nodorum*

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ABSTRACT

To improve the efficiency of gene disruption in *Stagonospora nodorum*, the putative *KU70* gene encoding the Ku70 protein involved in the nonhomologous end-joining double DNA break repair pathway was identified and deleted. The *KU70* disruption strain showed no apparent defect in vegetable growth, conidiation and pathogenicity on wheat and barley compared with the wild-type strain. The effect of the absence of *KU70* on gene replacement frequency was tested by disruption of *TOXA* encoding toxin A and *LIP2* encoding a putative lipase. Frequency of gene replacement for both genes was dramatically increased in the *KU70* disruption strain, compared with the low frequency in the wild-type recipient.

Introduction

DNA double-strand break (DSB) is among the DNA damages inflicted by endogenous and exogenous DNA-damaging agents. DSB is generated when the two complementary stands of the DNA double helix are broken simultaneously at sites that are close to one another. The fact that the DNA ends at a DSB are liable to become physically dissociated from one another makes the strands at DSB prone to inappropriate recombination with other sites in the genome. Cells evolved multiple pathways to repair the DSB. In eukaryotes, two main DSB repair pathways have been identified. The first, homologous recombination (HR), involves interaction between homologous sequences, whereas the second, nonhomologous end-joining (NHEJ), involves direct ligation of the strand ends independent of DNA homology (Wyman and Kanaar 2006). In *Saccharomyces cerevisiae*, the HR system is utilized more frequently, but many filamentous fungi seem to use NHEJ preferentially in DSB repair (Meyer et al. 2007).

Gene disruption (knockout) is a useful technique in studies of gene function in filamentous fungi. It can be used to delete genes entirely, to replace one allele of a gene with another, or to replace a gene's normal promoter with a regulatable promoter, and to tag genes with epitope tags or fluorescent proteins. When constructed foreign DNA enters cells and engages with genomic DNA, the foreign DNA is recognized as DSB and subsequently subjected to DSB repairs. If the foreign DNA carries sequences homologous with the genomic sequences flanking the targeted gene, the cell may utilize HR rather than NHEJ to repair the perceived DSB, and this results in the replacing of the target gene by the foreign DNA. Integration events mediated by NHEJ do not rely on homologous sites and therefore attenuate HR, which results in decreased frequencies of the desired gene disruption in a given transformation experiment with a suitable gene replacement cassette. It is possible to increase the efficiency of gene disruption by blocking the NHEJ pathway to increase the frequency of HR. Among numerous proteins involved in NHEJ, Ku70 and Ku80 form a heterodimer that binds to DNA ends to initiate the NHEJ process. Recent reports indicate that significant improvement in HR frequency can be achieved by disrupting *KU70* or *KU80* in a broad range of fungal species, including important plant pathogens such as *Aspergillus niger* (Meyer et al. 2007), *Botrytis cinerea* (Choquer et al. 2008), *Claviceps purpurea* (Haarmann et al. 2008) and *Fusarium verticillioides* (Choi and Shim 2008).

The necrotrophic filamentous fungus *Phaeosphaeria nodorum* (E. Müller) Hedjarroud [anamorph: *Stagonospora nodorum* (Berk.) Castellani & Germano] causes glume blotch, which is an important disease of wheat worldwide (Eyal 1999). The significance of this pathogen is highlighted by the fact that it was among the first plant pathogens to have its entire genome sequenced (http://www.broadinstitute.org). However, studies of

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gene disruption in *S. nodorum* are rare compared with other important pathogens such as *F. graminearum* and *Magnaporthe grisea*, even though gene disruption is an important strategy for identifying and studying pathogenicity genes. One factor contributing to fewer gene disruption studies in *S. nodorum* is a relatively low frequency of HR during transformation in this fungus. Therefore in the present study we deleted the *KU70* gene from this pathogen and to determine if the efficiency of gene disruption was enhanced in the resulting strain.

Materials and methods

General

The spring wheat (*Triticum aestivum*) cultivar Harvest and winter barley (*Hordeum vulgare*) cultivar Harrington were used as hosts for the pathogenicity tests. Plants were grown and maintained following Feng et al. (in press). *S. nodorum* strain Sn15 was purchased from the Fungal Genetics Stock Center (FGSC, http://www.fgsc.net) and used as the wild-type. The pathogen was maintained on V8 agar (20% V8 juice, 1.5% agar and 0.15% $CaCO_3$) under continuous light at 25 °C.

All chemicals were purchased from Fisher Scientific (Ottawa, ON, Canada) unless otherwise specified. Restriction enzymes and PCR master mix were purchased from Promega (Madison, WI). PCR primers (Table 1) were synthesized by Integrated DNA Technologies (Coralville, IA). When PCR was used for cloning, the master mix was supplemented with 5 units mL^{-1} *Pfu* polymerase (Agilent Technologies, Santa Clara, CA). DNA extraction from fungal mycelia followed Feng et al. (2010) and gel or PCR purification was performed by using a Wizard SV gel and PCR clean-up kit (Promega).

Genes

The *KU70* gene of *S. nodorum* was identified by BLASTing the *S. nodorum* genome database using protein sequences of *KU70* genes from other fungal species, including *A. niger* (GenBank accession number: XP-001396808), *B. cinerea* (XP-001545469), *C. purpurea* (CAM56211) and *F. verticillioides* (FVEG04235). After the *KU70* disruption strain (ΔKu70-1) had been developed, two other genes were used to confirm the HR frequency of ΔKu70-1: *TOXA* (SNOG_16571; Friesen et al. 2006) and *LIP2* (SNOG_06502), which is the ortholog of *FGL1* of *F. graminearum* (Voigt et al. 2005). These genes were selected because they may function as virulence factors in this pathogen.

Vector construction

For *KU70* disruption, a fusion PCR method (Szewczyk et al. 2006) was used to generate transforming DNA (Fig. 1). The fungal transformation vector pBarKS1 (Pall and Brunelli 1993) was purchased from FGSC. A 1.4-kb fragment corresponding to the bialaphos resistance gene (*bar*) was cut from pBarKS1 with *Spe*I and *Xba*I, and then cloned into the same restriction site of pBluescript II KS (Agilent Technologies). The *bar* gene in the resulting vector was amplified using the primer pair 1–2 (Table 1). Using primer pairs 3–4 and 5–6, the 5′ and 3′ flanking sequences of *KU70* were amplified. Primers 4 and 5 were tailed with sequences that reverse complemented to the sequences of primers 1 and 2, respectively. The three purified PCR products (from primer pairs 3–4, 1–2, and 5–6) were mixed at a molar ratio of 1:1:1 and used as template in a PCR using primer pair 3–6 with an annealing temperature at 50 °C. The resulting 2.3-kb fragment was gel purified, confirmed by PCR using primer pairs 3–2 and 1–6, and used for fungal transformation.

The vectors for disruption of *TOXA* and *LIP2* were constructed as described by Feng et al. (in press). Briefly, a genomic fragment of the corresponding gene, which included the open reading frame (ORF) and its flanking sequences, were cloned to pBluescript II KS. In the resulting vector, the ORF was replaced by a hygromycin resistance gene that was either PCR amplified or cut from pGC1-1 (Rikkerink et al. 1994). The constructed vectors were used for transformation. The 5′ and 3′ flanking sequences were 0.6 and 0.9 kb for *TOXA* and 1.2 and 1.3 kb for *LIP2*.

Fungal transformation

Transformation of *S. nodorum* followed Feng et al. (in press). For each transformation, 2.5×10^9 protoplasts suspended in 200 μL STC buffer (1 M sorbitol, 50 mM Tris–HCl pH 7.5, 50 mM $CaCl_2$, sterilized with a 0.22-μm filter) were supplemented with 5 μg PCR product (for *KU70*) or 30 μg unlinealized plasmid DNA (for *TOXA* and *LIP2*). The selection media was YDAS agar (Ahuja and Punekar 2008) containing 100 μg mL^{-1} glufosinate (Sigma–Aldrich, St. Louis, MO) for *KU70* or 4% potato dextrose agar (PDA) containing 75 μg mL^{-1} hygromycin for *TOXA* and *LIP2*. The regenerated transformants were transferred from the selection media within 14 days after transformation onto fresh V8 agar plates containing the same concentration of the corresponding antibiotic as in the selection media.

Confirmation of gene disruption strains

Primer pairs 7–8 and 1–2 (Table 1) specific to the ORF of *KU70* and the *bar* gene cassette, respectively, were used to confirm the *KU70* disruption strains. The presence of *bar* and absence of *KU70* indicated that *KU70* had been replaced by *bar* and the strain was considered as a *KU70* disruption strain. If both fragments were present, the strain was considered to be an ectopic integration strain. The putative *KU70* disruption strains identified in this way were further confirmed by the primer pair 9–10, which were specific to the regions outside the *KU70* 5′ and 3′ sequences and could amplify a 3300-bp and a ~2600-bp fragment from the wild-type Sn15 and the *KU70* disruption strains, respectively. *KU70* disruption strains were transferred to and re-isolated from V8 agar containing 100 μg mL^{-1} glufosinate three times and then reconfirmed by PCR before being used in the growth assay and pathogenicity tests. To confirm the gene replacement strains of *TOXA* and *LIP2*, a similar PCR strategy was applied using the primer pairs 11–12 and 13–14.

Growth assay and pathogenicity test

Colonies of the gene disruption strains and the wild-type Sn15 were grown on V8 agar or V8 agar containing 100 μg mL^{-1} glufosinate by transferring a 1-cm diameter plug of fungal culture onto fresh media. The plates were incubated under continuous light at 25 °C for 5 days and then photographed for growth assessment. Sporulation was assessed based on the numbers of conidia produced by Sn15 or ΔKu70-1 on ten V8 agar (without glufosinate) dishes after 10 days of incubation. The pathogenicity of each strain was assayed on seedlings of wheat and barley using both spray- and point-inoculation methods with an inoculum concentration of 3.75×10^6 conidia mL^{-1}. Collection of conidia, inoculation, and plant maintenance after inoculation followed Feng et al. (in press). The disease severity was assessed on the first leaves of 20 wheat plants at 7 days after spray-inoculation using a 0–5 rating scale (Feng et al. 2004).

Results

Identification and disruption of KU70

A BLAST search of the *S. nodorum* genome database with the protein sequences of known *KU70* from other fungal species revealed