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A high efficiency gene disruption strategy using a positive–negative split selection marker and electroporation for *Fusarium oxysporum*

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ABSTRACT

The *Fusarium oxysporum* species complex consists of fungal pathogens that cause serial vascular wilt disease on more than 100 cultivated species throughout the world. Gene function analysis is rapidly becoming more and more important as the whole-genome sequences of various *F. oxysporum* strains are being completed. Gene-disruption techniques are a common molecular tool for studying gene function, yet are often a limiting step in gene function identification. In this study we have developed a *F. oxysporum* high-efficiency gene-disruption strategy based on split-marker homologous recombination cassettes with dual selection and electroporation transformation. The method was efficiently used to delete three RNA-dependent RNA polymerase (*RdRP*) genes. The gene-disruption cassettes of three genes can be constructed simultaneously within a short time using this technique. The optimal condition for electroporation is 10 μF capacitance, 300 Ω resistance, 4 kV/cm field strength, with 1 μg of DNA (gene-disruption cassettes). Under these optimal conditions, we were able to obtain 95 transformants per μg DNA. And after positive–negative selection, the transformants were efficiently screened by PCR, screening efficiency averaged 85%: 90% ($RdRP_1$), 85% ($RdRP_2$) and 77% ($RdRP_3$). This gene-disruption strategy should pave the way for high throughout genetic analysis in *F. oxysporum*.

1. Introduction

The fungal species complex *Fusarium oxysporum* is an economically important soil-borne pathogen that causes vascular wilt disease on more than 100 cultivated species throughout the world (Armstrong and Armstrong 1981). Strengthening research on *F. oxysporum* physiological mechanisms, therefore, has important theoretical significance for effective blight control. Large-scale target gene knockouts, based on known genome sequences, have become a very common molecular biology tool for studying gene function, with the advent of the era of functional genomics. The first filamentous fungus to be transformed was the saprobe *Neurospora crassa* (Case et al. 1979); The first plant pathogenic fungus transformed was *Cochliobolus heterostrophus* (Turgeon et al. 1985). Gene-disruption systems for other plant pathogenic fungi were established on this basis.

The basic procedure for knocking out genes contains three major parts: construction of homologous gene-disruption cassettes, transformation, and regeneration on selective media. Gene-disruption cassettes can be obtained using two methods. The traditional method is to construct a vector carrying two flanking DNA fragments from the targeted locus to promote homologous recombination (HR) (Hamer et al. 2001). However, constructing gene-disruption cassettes requires several tedious and time-consuming cloning steps. Alternatively, fusion PCR techniques avoid the challenging cloning steps, and include only two rounds of PCR. The flanking region sequences of the targeted gene

Abbreviations: AMT, *Agrobacterium*-mediated transformation; CTAB, cetyltrimethylammonium bromide; GFP, green fluorescent protein; HR, homologous recombination; *hyg*, Phosphotransferase B gene conferring phosphinothricin resistance; MOPS, 3-(N-morpholino) propanesulfonic acid *neo* neomycin resistance gene; *RdRP*, RNA-dependent RNA polymerase.

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and the selectable marker are amplified in the first round of PCR, and then fused together in the second round. The final PCR product is a linear cassette. The 5′-end and 3′-end of the linear cassette is the 5′-flanking region sequence, and 3′-flanking region sequence of the targeted gene; and the center section of the linear cassette is the selection marker gene. This method was used for the pathogenic fungi *Cryptococcus neoformans* (Davidson et al. 2002) and *Candida albicans* (Noble and Johnson 2005). Split-marker cassettes are based on PCR fusion yet are more efficient than traditional fusion PCR. This method was initially developed for the model organism *Saccharomyces cerevisiae* (Fairhead et al. 1996, 1998). Split-marker cassette technology has becoming more widely used in filamentous fungi because of its high-efficiency. Split-marker techniques have previously been used for *C. heterostrophus* (Catlett et al. 2003), *Gibberella zeae* (Goswami et al. 2006), *Cryptococcus neoformans* (Fu et al. 2006; You et al. 2009; Kim et al. 2009), *Cercospora nicotianae* (You et al. 2009), but not yet for *F. oxysporum*.

Transformation of gene-disruption cassettes has been accomplished by three different ways thus far: PEG-mediated protoplast transformation, electroporation-mediated protoplast transformation, and *Agrobacterium*-Mediated Transformation (AMT). AMT avoids the tedious steps of producing protoplasts, and is very efficient (de Groot et al. 1998), but often results in high-frequency ectopic integration. Conventional PEG-mediated protoplast transformation is associated with high-frequency HR, but has low transformation efficiency. Recently electroporation has been used to transform protoplasts from the fungus *Mucor circinelloides* (Gutierrez et al. 2011), and the result showed that the efficiency and reproducibility were higher than traditional PEG-based methods (Van Heeswijk and Roncero 1984). Integration of foreign genes is considered a rare event in filamentous fungi and presumably influenced by a nonhomologous end joining mechanisms (Ninomiya et al. 2004). Thus, identification of the desired mutants often requires a large number of transformants to be screened (You et al. 2009). To identify the desired mutants rapidly positive-negative selection was developed for *Cryptococcus neoformans* (Chang and Kwon-Chung 1994). In this method another negative marker is added in the flanking region of the gene-disruption vector.

Though the techniques for disrupting fungal genes have matured, deleting targeted gene for *F. oxysporum* needs to be optimized because of the great amount of variation within the species complex. Furthermore, conventional PEG-mediated transformation is quite inefficient in *F. oxysporum*. Therefore, we have developed a homologous disruption approach based on dual selection (positive and negative) and split-marker recombination by electroporation.

RNA-dependent RNA Polymerases (*RdRPs*) play important roles in the RNA silencing mechanism of many organisms. They are involved in the production of double-stranded RNA (dsRNA) molecules that initiate silencing mechanisms as well as in the amplification of the silencing signals (Calo et al. 2012). There are three *RdRP* genes in *F. oxysporum*. The lengths of these *RdRP* genes are 4.2 kb (*RdRP*$_1$), 3.9 kb (*RdRP*$_2$) and 4.2 kb (*RdRP*$_3$). Their special biological function in *F. oxysporum* are unknown. In our study these three *RdRP* genes were efficiently deleted, this will provide the mutants materials for further researching their biological function. Additionally, three electroporation factors, capacitance, resistance, and field strength, affecting the efficiency of target gene disruption were evaluated. The results of this work suggest that the strategy is an efficient method for improving target gene disruption efficiency in *F. oxysporum*, and possibly applicable to improve HR frequencies in other filamentous fungi. Furthermore, the mutants we produced in this work exhibited stable inheritance. This gene-disruption strategy should pave the way for high-throughput genetic analysis of *F. oxysporum*.

2. Materials and methods: protocols

2.1. Strains, media, and growth conditions

Fusarium oxysporum f. sp. *conglutinans* wild-type Fox-A8 strain was obtained from Beijing Academy of Agriculture and forestry Science (native to Italian) and stored as a conidial culture at −80 °C until used. Growth reactivation was done by dropping 1 μl conidial culture on the center of 1% water/agar supplemented with irradiated cabbage leaf-pieces (Fisher et al. 1982). The strain Fox-A8 was used as the transformation recipient for targeted gene disruption in this study. Complete medium (CM, consisting of 0.6% yeast extract, 0.3% enzyme hydrolysis casein, 0.3% acid hydrolysis casein, 1% sucrose, and 1.5% agar) supplemented with 200 μg/ml hygromycin B (Roche, Branchburg, NJ, USA) or 200 μg/ml neomycin (Amresco, Solon, OH, USA) was used to screen hygromycin B-resistant and neomycin-resistant transformants. Potato dextrose agar (PDA, consisting of 20% potato, 2% dextrose, and 1.8% agar) supplemented with 50 μg/ml ampicillin, 200 μg/ml streptomycin, and 0.35% lactic acid was used for routine culturing, and supplemented with 100 μg/ml hygromycin B additionally for subculturing the *RdRP*-deletion mutants (Δ*RdRP*) to investigate its genetic stability. All cultures were grown at 28 °C.

2.2. Construction of gene-disruption cassettes

The entire methodology of our overlap PCR and gene-disruption technique is summarized in Fig. 1. All of the primers that we used are shown in Table 1. The plasmid pKOV21 was used as the template for amplifying the *neo* and the *hyg*, as shown in Fig. 1(A). Sufficient amounts of the *hy* (5′ truncation of *hyg*), *yg* (5′ truncation of *hyg*) and *neo* sequences were pre-amplified, purified, and stored at −20 °C. The genomic sequence of the wild Fox-A8 strain was previously sequenced by the Beijing Genomics Institute (BGI), and the gene date is being processed by our group, so the data has not yet been released. The genomic DNA was extracted using CTAB method.

2.2.1. First round PCR

The individual components were amplified by two separate standard PCR protocols in the first PCR round. The 5′-flanking region amplicons of the three *RdRP* genes contain 19 base-pairs complementary to 3′-end of the *neo* in their 5′-end, and 22 base-pairs complementary to the 5′-end of the *hy* fragment in their 3′-end. The 3′-flanking amplicons of the three *RdRP* genes contain 23 base-pairs complementary to 5′-end of the *yg* fragment in their 3′-end. The PCR conditions were carried out following standard procedure. The PCR products were checked by 1.2% agarose gel-electrophoresis, and cleaned-up with a general PCR-cleanup kit.

2.2.2. Second round PCR

In the second round of PCR, the individual components were diluted with ddH_2O 50 times, and combined the ingredients as listed in Table 2 to produce final products using the overlap PCR method as per standard procedure. The three, the *neo*, the 5′-flanking region, and the *hy* fragment amplicons, were mixed together in a 1:3:1 molar ratio and fused together using double-joint overlap PCR. The elongation time, 5 min 30 s, was set according to the size of the desired final product (1 min/kb). And the two, the 3′-flanking region and the *yg* fragment amplicons, were mixed together in a 1:1 molar ratio, and then fused together using single-joint overlap PCR. The elongation time was 2 min 30 s. The final PCR products (*neo* + *hy* + *RdRP* 5′-flanking region, region (a) and *yg* + *RdRP* 3′-flanking region, region (b)) were checked by 1.2% agarose gel-electrophoresis and purified with a general DNA gel extraction kit.