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A method for making directed changes to the *Fusarium graminearum* genome without leaving markers or other extraneous DNA

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ABSTRACT

A method is described which allows exact targeted changes to the *Fusarium graminearum* genome, including changes of as little as one particular base pair to gene-size insertions, replacements or modifications. The technique leaves no other DNA in the genome, such as marker genes, and can be used serially to effect multiple complex changes in any desired chromosomal locations. The method is based on our previous finding that after transformation, DNA with homology to *F. graminearum* DNA recombines itself into the genome in a predictable manner involving multiple tandem copies. We designed a cloning vehicle with a built-in hygromycin-resistance marker (*hygB*) which can be used to transform the fungus, and with cloning sites to carry DNA with any desired genomic modifications. To effect a desired genomic change the sequence changes of interest are incorporated between two adjacent borders homologous to *F. graminearum* DNA which will target them to the desired location. This modified DNA is attached within the cloning sites within the vehicle. Transformants are readily obtained in which tandem copies of the vehicle plus insert are inserted between the two genomic border sequence homologues. Progeny of a transformant are subsequently screened for those with a decreased resistance to the antibiotic, and then for those which have completely lost the marker and the entire vehicle, leaving only the desired sequence modifications between the two genomic border sequences which were targeted. This method is demonstrated by exactly replacing the trichodiene synthase (*tri5*) gene coding sequence (CDS) with that of a green fluorescence protein (*gfp*) gene with no other genomic changes. This derivative was then re-engineered to replace the *gfp* CDS with that of the wild type, exactly regenerating the original *F. graminearum* genome.

1. Introduction

DNA sequencing techniques now readily provide large amounts of sequence data, and there is a growing number of fungi and other eukaryotes for which complete genomic sequences are known. At the same time, improvements in DNA *in vitro* synthesis and DNA construction techniques permit scientists to prepare complex DNA molecules with any desired sequence. Together, these advances should allow one to make any genetic modifications to an organism of interest if laboratory-prepared DNA could be placed into the genome in any desired location.

Although there are currently several ways to introduce new DNA sequences into an organism, such as introduction of DNA into protoplasts (Ruiz-Diez, 2002) and *Agrobacterium tumefaciens*-mediated transformation (Frandsen, 2011; Michielse et al., 2005), there are several constraints on the changes that can be made. Transformation inevitably requires that a selectable marker is incorporated into the DNA that is to be introduced into the organism. Usually the marker is an antibiotic resistance gene, although other markers, such as metabolic markers, have been used (Ruiz-Diez, 2002; Weld et al., 2006). Removal of the selectable marker is often desirable, such as to allow marker recycling for serial modifications, or if unmarked genetically-engineered strains are preferred for commercial or environmental applications. Several schemes for marker recycling or exchange have been derived, including selection for recombination between direct repeats (Kaneko et al., 2009; Maruyama and Kitamoto, 2008) and the use of recombinase gene systems to remove segments of DNA bounded by recognition sites that serve as their target substrates (Kopke et al., 2010; Marx and Lidstrom, 2002). Although useful, these approaches require particular mutant recipient strains, or functional plasmid-encoded recombinase genes incorporated into the transforming DNA. Using these approaches, direct repeats or recombinase target site sequences remain incorporated in the genome. Because of these limitations, it is currently not possible, for example, to only change a few base pairs in a chromosome, or to precisely change one gene for another. It is also not possible to make a series of such specific DNA changes to different target sites so that an organisms genome can be successively remodeled.

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In this manuscript we describe a novel approach to making genomic changes to an economically important fungus, *Fusarium graminearum*. *F. graminearum* (teleomorph *Gibberella zeae*), is a fungus which causes Fusarium head blight (FHB) on wheat and ear rot of corn. It produces several mycotoxins, including deoxynivalenol (DON), which is a serious contaminant of grain affecting both humans and animals (Desjardins, 2006). The genome of *F. graminearum* has been sequenced, and this information permits the application of recombinant DNA techniques for manipulating and testing genes of interest. It is readily amenable to transformation, and several fungal transformation methods are available (for reviews, see Fincham, 1989; Mullins and Kang, 2001; Ruiz-Diez, 2002). Introduction of DNA into fungal protoplasts is the most frequently used approach. Since autonomously replicating cloning vehicles are not available for most fungi, the input DNA must be integrated into the genome to be stably retained within the transformed cells. Transformation with input DNA that includes homology to a gene of interest has been used extensively to generate targeted insertions. This approach has usually been applied to the derivation of mutants with genes inactivated by insertional inactivation (gene knockouts) (Cho et al., 2006; Colot et al., 2006; Nayak et al., 2006; Paietta and Marzluf, 1985; Yu et al., 2004). Typically, a selectable marker, such as a hygromycin-resistance gene, is used to interrupt or replace the target gene within the DNA construct before its introduction into the cell, with the intention of replacing the genomic copy by a double-recombination event.

Previously, we have analyzed the genomic structure of DNA integrated into the genome of *F. graminearum* transformants, and found that their structure is not consistent with that expected by classical models of double-recombination such as described by Szostak et al. (1983). Instead, we proposed a model by which two DNA repair processes, non-homologous end joining (NHEJ) (Friedberg et al., 2006b) and homologous recombination (HR) (Friedberg et al., 2006a), interact to integrate DNA at a target site specified by the homologous DNA segments (Watson et al., 2008). Linear DNA introduced into a cell is first made into a circle by the NHEJ system, often with some trimming of the two ends before joining (Daley et al., 2005; Roth et al., 1985; Roth and Wilson, 1986; Ruley and Fried, 1983). Next, the circular DNA is copied into the target site by a replication process which is coupled with, or perhaps an integral part of, the HR system. Replication begins in one of the two genomic homologous borders and shifts to its homolog in the circularized input DNA. Replication proceeds around the circular DNA an indeterminate number of times making tandem copies. The process ends when replication transfers back to the genome, again through one of the homologous borders. When there are two homologous borders in the input DNA there are four possible structures which may arise in the genome, depending on which borders are used to initiate the copy process and to resolve it. We have referred to these as types I, IIa, IIb and III. The type I recombination event is usually desired, since it results in the original target region between the two homologous borders being replaced by tandem copies of the input DNA. Types IIa and IIb retain the target region and include the tandem copies within one or the other of the two bordering homologues. Type III structures have a duplication of the target region, with the two copies separated by the tandem repeats. Ectopic transformants are a fifth type of transformant, but these do not involve the target region. They appear to be caused by the NHEJ system alone joining the linear input DNA directly into other sites in the genome.

Tandem copies of the input DNA have been frequently reported in fungal transformants (Brown et al., 1998; Itoh and Scott, 1997; Proctor et al., 1995). This phenomenon was previously ascribed to successive recombination events due to homology between integrated and nonintegrated DNA (Itoh and Scott, 1997; May, 1992; Proctor et al., 1995), though that explanation is not consistent with our results. In most other studies, the possibility that tandem DNA is present has not usually been tested for, and requires suitable hybridization probes or PCR primers.

In our previous study we also noted that some transformants exhibited characteristics suggesting that their tandem DNA copies were able to undergo recombination between copies. Using our recombination model and the observed propensity of the tandem repeats for recombination, we have developed a powerful method to engineer the Fusarium genome. In this communication we describe this technique and demonstrate its application by preparing a *F. graminearum* strain in which the ATG to TAA coding sequence (CDS) of the *tri5* (trichodiene synthase) gene is exactly replaced by the CDS of a green fluorescent protein (*gfp*) gene, with no other genomic alterations or other added DNA. We then applied the method again to the modified strain, this time reintroducing the *tri5* CDS to replace that of the *gfp* gene and exactly regenerating the original wild-type genome.

2. Materials and methods

2.1. Strains and media

The *F. graminearum* strain used was DAOM180378, from the Canadian Collection of Fungal Cultures, Agriculture & Agri-Food Canada, Ottawa. *Escherichia coli* DH10B was used for all cloning and plasmid manipulations. *F. graminearum* was grown on SNA or V8-juice agar plates (Burgess et al., 1994). Hygromycin B was included in V8-juice agar plates at 50 μg/ml for selection and growth of transformants. Plate-to-plate transfers of the fungus were made by pulling agar plugs containing the fungus using Transfertubes (Spectrum Laboratories Inc.) and inserting them into plug holes prepared in the receiving plate. Liquid culture medium used was SN (SNA without agar). The fungus was grown at 28 °C. Dilution of SN cultures for plating of macroconidia for single colonies was done in Phosphate Buffered Saline (NaCl, 8 g/L; KCl, 0.2 g/L; Na_2HPO_4, 1.44 g/L; KH_2PO_4, 0.24 g/L; pH 7.4). V8-juice with oxgall plates (V8 juice, 335 ml/L; Oxgall, 10 g/L; Agar, 10 g/L; pH 6.0) were used to grow single colonies of *F. graminearum* from macroconidia dilutions.

2.2. Plasmid and strain constructions

Plasmid constructions were done using standard PCR and cloning techniques (Sambrook and Russell, 2001). PCR parameters have been described previously (Watson et al., 2008). Sequences of the primers used are given in Table 1. Usually, PCR fragments were joined using restriction sites incorporated into the tails of PCR primers. Frequently, *Sap*I sites were used in constructions; the recognition sequence is nonpalindromic, and the enzyme cuts outside of the recognition sequence on one side to generate a three-nucleotide single-stranded end with any sequence.

Plasmid cloning vehicle pCV40 was constructed using pCV9 (GenBank accession no. EU284107) and pCV12 (EU284108) (Watson et al., 2008), and plasmid pBR322 (J01749). It also includes the cauliflower mosaic virus (CaMV) polyadenylation signal segment (from L. Robert, Agriculture and Agri-Food Canada) as a terminator A segment of pCV9 containing the replication (rep) region and tetracycline resistance gene (*tetA*) were amplified by PCR using primers fcv1 and fcv2. A segment of pBR322 containing the beta-lactamase gene (*bla*) were amplified using PCR primers amp5 and amp6. These four primers contain *Asc*I or *Not*I sequences in the tails, so the two fragments could be ligated into a circle after cleavage and ligation. This was used for transformation, producing an intermediary plasmid giving ampicillin- and tetracycline-resistance phenotypes. The plasmid also contains two back-to-back