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Plasmids for Increased Efficiency of Vector Construction and Genetic Engineering in Filamentous Fungi

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Abstract

Fungal species are continuously being studied to not only understand disease in humans and plants but also to identify novel antibiotics and other metabolites of industrial importance. Genetic manipulations, such as gene deletion, gene complementation, and gene over-expression, are common techniques to investigate fungal gene functions. Although advances in transformation efficiency and promoter usage have improved genetic studies, some basic steps in vector construction are still laborious and time-consuming. Gateway cloning technology solves this problem by increasing the efficiency of vector construction through the use of phage integrase proteins and *att* recombination sites. We developed a series of Gateway-compatible vectors for use in genetic studies in a range of fungal species. They contain nutritional and drug-resistance markers and can be utilized to manipulate different filamentous fungal genomes.

Keywords

Genetic engineering; filamentous fungi; Gateway technology; recombination

1. Introduction

Filamentous fungi comprise a large group of biologically relevant organisms. Many filamentous fungal species produce useful metabolites, such as pigments, vitamins, and antibiotics, whereas some other species are infectious and cause disease in humans, other animals, and plants. For this reason, researchers have studied fungal species extensively not only to understand human and plant disease, but also to identify new antibiotics and other relevant metabolites for industrial purposes. Genetic manipulations, such as the creation of

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6. Author Contributions C.K.C created and analyzed the pDONR 221-AfpYrG, pDONR 221-AnpyrG, pDONR 221-BAR, pDONR 221-HPH, pDONR HPH A and B plasmids. T.J.S created and analyzed all other plasmids. T.J.S performed the experiments used as examples in this paper. T.J.S wrote the entire paper with editing from G.S.M. G.S.M supervised the project.

deletion or over-expression strains, are often used when examining virulence factors and/or protein functions.

Plasmids are routinely created for gene deletion and over-expression studies via several methods. One method involves the use of restriction enzymes to isolate a DNA insert and to linearize a destination vector. The two segments of DNA are then ligated, resulting in a final vector whereby the DNA insert has been introduced into the destination vector. This method can be challenging, because the restriction enzymes used must be either identical or compatible for both the insert and vector. Furthermore, if a single restriction enzyme is used to linearize the vector, dephosphorylation of the digested end is required to prevent self-ligation. Even with proper steps and controls, researchers often face low colony yields and false positives.

A second method that can be used for vector construction or direct genetic manipulations involves fusion polymerase chain reaction (f-PCR). In this case, primers are engineered to contain overlaps of different DNA fragments to be joined. For example, the 3' primer for the first DNA fragment would contain a series of nucleotides that are identical to the 5' end of the second DNA fragment, and the 5' primer for the second DNA fragment would contain a series of nucleotides that are identical to the 3' end of the first DNA fragment (Spiliotis, 2012; Szewczyk et al., 2006; Yu et al., 2004). Two separate products are obtained from the first round of PCR and then fused together in the second reaction to create one PCR product. This fused product can either be transformed directly into an organism for genetic studies or can undergo additional cloning methods to create a novel plasmid (Spiliotis, 2012; Szewczyk et al., 2006; Yu et al., 2004). One caveat to using f-PCR to create a deletion construct is the need for multiple fragments to be fused together. Whether gene deletion or placement of epitope tags is the goal, all constructs must have a selective marker plus target DNA fragments. When combining all fragments together, the final product can become quite long, which can result in a decrease in PCR efficiency and thus product yield. Furthermore, to construct a plasmid using an f-PCR product, restriction enzymes and ligases must be employed, which introduces limitations discussed above.

Many of the limitations of the previous methods can be alleviated by the use of recombinatorial cloning systems. Numerous systems exist, including Recombineering, Sequence and Ligation-Independent Cloning (SLIC), Ligation-Independent Cloning (LIC), Mating-assisted genetically integrated cloning (MAGIC), Gateway, DNA assembler, and Cre-*loxP*. Some, such as Recombineering, MAGIC, and DNA assembler, are carried out *in vivo* (Li and Elledge, 2005; Liu et al., 2003; Mosberg et al.; Shao and Zhao, 2009; Sharan et al., 2009), while others, such as Cre-*loxP*, SLIC, LIC, and Gateway can be performed as *in vitro* reactions (Hartley et al., 2000; Li and Elledge; Li and Elledge, 2007; Magnani et al., 2006; Siegel et al., 2004; Yamamura, 2012). Depending on the intended outcome, all systems have their advantages. For instance, MAGIC, which relies on bacterial mating, facilitates the cloning of an insert into multiple destination plasmids (Li and Elledge, 2005). SLIC, Gateway, and DNA assembler have been shown to be efficient for inserting multiple fragments into a single destination vector (Li and Elledge; Li and Elledge, 2007; Magnani et al., 2006; Shao and Zhao, 2009). Recombineering and Cre-*loxP* are frequently used for conditional knockouts in mice, as cloning can be controlled through selective Cre recombinase expression (Liu et al., 2003; Yamamura, 2012). Cre-*loxP* and Gateway recombinatorial cloning are similar in the fact that they both involve site-specific DNA recombination reactions.

Cre recombinase mediates the site-specific recombination of DNA sequences between *loxP* sites (Deng, 2012; Siegel et al., 2004; Yamamura, 2012). The *loxP* site consists of two 13-base pair (bp) inverted repeats separated by an 8-bp nonpalindromic core region. Based on

the orientation and placement of *loxP* sites, DNA fragments can be excised, translocated, or inverted. For example, if a DNA fragment is flanked by *loxP* sites that are oriented in the same direction, the fragment will be excised and circularized. If, however, the DNA fragment is flanked by *loxP* sites that are oriented in opposite directions, the fragment will be inverted. Translocation of DNA fragments is possible if the *loxP* sites are located on different chromosomes. This Cre-*loxP* system has been employed for both rapid vector cloning and *in vivo* genetic techniques, predominantly in yeast and mice (Deng, 2012; Siegel et al., 2004; Yamamura, 2012).

With Gateway technology, phage integrase recombination proteins mediate site-specific recombination reactions. DNA inserts, which are flanked by appropriate recombination sites (e.g., *attB1* and *attB2*), are incubated with phage integrase recombination proteins and donor/destination vectors, which also contain appropriate recombination sites (e.g., *attP1* and *attP2* or *attR1* and *attR2*). Two reactions can be carried out: (BP) *attB* × *attP* → *attL* + *attR*, involving integrase (Int) and integration host factor (IHF), and (LR) *attL* × *attR* → *attB* + *attP*, involving Int, IHF, and excisionase. Orientation of the DNA insert can be controlled because only certain recombination sites will recombine with others; for example, *attB1* will only recombine with *attP1* (Hartley et al., 2000; Magnani et al., 2006).

Gateway technology has been used in numerous laboratories to manipulate fungal genomes. During the past several years, we have created a series of vectors to facilitate genetic engineering in *Aspergillus fumigatus* and *Aspergillus nidulans* using Gateway technology. Other laboratories have also created Gateway-enabled vectors, but the vectors published thus far appear to focus more on protein tagging and fluorescent labeling (Mabashi et al., 2006; Toews et al., 2004). The Gateway-enabled plasmids we have created focus more on gene deletion, gene integration, and high-copy gene expression. Although our laboratory has only used these vectors in *A. fumigatus* and *A. nidulans*, they can potentially be used in a variety of other fungal organisms, including other *Aspergillus sp.*, *Neurospora sp.*, *Fusarium sp.*, *Penicillium sp.*, and other fungal species of agricultural and industrial importance (Abe et al., 2006; Bugeja et al., 2012; Pahirulzaman et al., 2012; Paz et al., 2011). This is due to the fact that the selectable markers we chose are versatile and can be used across different species. Our aim was to create recombinatorial vectors as efficient tools in gene modification techniques in multiple fungal systems.

2. Materials and Methods

2.1. DNA manipulations

We gel purified all PCR and digestion products using a QIAquick gel purification kit (Qiagen, Hilden, Germany) and following manufacturer's instructions. We isolated all plasmid DNA using a PureLink Miniprep kit (Invitrogen, Grand Island, NY) according to manufacturer's instructions. We further purified plasmid DNA by banding on cesium chloride ethidium bromide gradients (Maniatis et al., 1982).

2.2. Construction of vectors

2.2.1. pDONR 221-AnargB, pDONR 221-AnpyrG, pDONR 221-AfpyroA, pDONR 221-AfpyrG, pDONR 221-HPH and pDONR 221-BAR—We amplified the coding region plus short non-coding regions (NCRs) flanking each selective marker using Accuprime *Pfx* DNA polymerase per the manufacturer's instructions. We used genomic DNA from *A. nidulans* 1155 as a template for *AnargB*, *AnpyrG*, and the glufosinate resistance gene (*bar*). We used genomic DNA from *A. fumigatus* Af293 as a template for *AfpyroA* and *AfpyrG*. We used pCB1003 (<http://www.fgsc.net/fgn41/carroll.html>) as a template for the *hygroR* cassette. We constructed primers with *attB* sites to facilitate the use

of the MultiSite Gateway System (Invitrogen, Grand Island, NY). We recombined PCR fragments into pDONR 221 using the BP recombination reaction (Fig. 1A) (Invitrogen, Grand Island, NY). We transformed reaction mixes into TOP10 cells (Invitrogen, Grand Island, NY) via electroporation, as recommended by the manufacturer. We grew transformed cells on lysogeny agar (LA) (1% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 1.5% agar) plus 50 µg/ml kanamycin at 37°C overnight. We picked colonies and transferred them to 2 ml lysogeny broth (LB) (described above for LA, but without agar) plus 50 µg/ml kanamycin to grow overnight in a 37°C shaking incubator. We isolated plasmid DNA and digested it with specific enzymes to verify the correct insertion of each PCR fragment.

2.2.2. pDONR A, pDONR G, pDONR NG, pDONR B, pDONR BAR, pDONR HPH A, and pDONR HPH B—We constructed new Gateway-compatible vectors containing unique nutritional and drug-resistance markers for the complementation of deletion mutants. We used f-PCR to facilitate the integration of the selective markers into pDONR221 (Fig. 1B). For the first reaction, we created two cassettes: a selective marker cassette and a linearized pDONR221 cassette. We obtained both cassettes via PCR using e2TAK DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's recommendations. We used genomic DNA from *A. nidulans* 1155 as a template for *AnpyrG* and BAR. We used genomic DNA from *A. fumigatus* Af293 as a template for the *AfpyrA*, *AfpyrG*, and *AfargB* PCR products. Each selective marker cassette contained a unique 5' SpeI site and a 3' extension identical to the first 30 bps of the pDONR221 fragment. The pDONR 221 cassette contained a 5' extension identical to the last 30 bps of the selective marker cassette and a unique 3' SpeI site.

For the second reaction, we fused both cassettes using the selective marker forward primer and the pDONR221 reverse primer. We amplified a 50-µl reaction containing 50 fmol of each fragment, 0.3 µM of each primer, 500 µM of deoxynucleoside triphosphates, buffer 3 at a 1X concentration, and 1 µl of Expand Long DNA Template Mix (Roche Applied Science, Indianapolis, IN) per the manufacturer's instructions. We digested the PCR fragment with SpeI and circularized it by ligation using T4 DNA ligase (Invitrogen, Grand Island, NY) and manufacturer's recommendations. We transformed the ligation mixture into *ccd*-compatible chemically competent cells (Invitrogen, Grand Island, NY) via heat shock, as recommended by the manufacturer. We grew transformed cells on LA plus 50 µg/ml kanamycin at 37°C overnight. We picked colonies and transferred them to 2 ml LB plus 50 µg/ml kanamycin to grow overnight in a 37°C shaking incubator. We isolated plasmid DNA and digested it with specific enzymes to verify the correct fragment orientation.

We constructed pDONR HPH A and pDONR HPH B via blunt-end ligation, hence plasmid A and plasmid B, representing two orientations of the *hygroR* cassette. We amplified the *hygroR* cassette from pCB1003 using AccuPrime Pfx DNA polymerase (Life Technologies Grand Island, NY) per the manufacturer's recommendations. We digested pDONR 221 with EcoRV and the *hygroR* cassette with HpaI. We then treated pDONR 221 with Antarctic phosphatase (NEB, Ipswich, MA), according to the manufacturer's instructions. We ligated the two fragments using T4 DNA ligase (Invitrogen, Grand Island, NY) per the manufacturer's instructions. We transformed the ligation mixture into TOP10 electrocompetent cells and plated transformed cells on LA plus 50 µg/ml kanamycin at 37°C overnight. We picked colonies and transferred them to 2 ml LB plus 50 µg/ml kanamycin to grow overnight in a 37°C shaking incubator. We isolated plasmid DNA and digested it with specific enzymes to verify the fragment orientation.

2.2.3. pDONR AMA and pDONR AMA/HPH—We created pDONR AMA by ligating the *ccdB* region (*attP1-ccdB*-Chloramphenicol resistance gene [CmR]-*attP2*) from pDONR

221 into pRG3-AMA-NotI. We amplified the *ccdB* region from pDONR 221 using AccuPrime Pfx DNA polymerase (Life Technologies, Grand Island, NY) per the manufacturer's instructions. We engineered primers so the *ccdB* PCR product would have SphI at the 5' end and KpnI at the 3' end. We digested both pRG3-AMA-NotI and the *ccdB* cassette with SphI and KpnI. We then treated pRG3-AMA-NotI with Antarctic phosphatase (NEB, Ipswich, MA) according to the manufacturer's instructions. We ligated the two fragments using T4 DNA ligase (Invitrogen, Grand Island, NY) according to the manufacturer's recommendations. We transformed the ligation mixture into *ccd*-compatible chemically competent cells (Invitrogen, Grand Island, NY) by heat shock according to the manufacturer's instructions. We grew transformed cells on LA plus 75 µg/ml ampicillin at 37°C overnight. We picked colonies and transferred them to 2 ml LB plus 75 µg/ml ampicillin to grow overnight in a 37°C shaking incubator. We isolated plasmid DNA and digested it with specific enzymes to verify the correct fragment orientation.

We created pDONR AMA/HPH by ligating an AMA1 cassette into pDONR HPH B. We linearized pDONR HPH B via amplification using e2TAK DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's recommendations. We engineered primers so the linearized pDONR HPH B fragment would contain a HindIII site on both ends. We digested pDONR HPH B with HindIII and treated with Antarctic phosphatase (NEB, Ipswich, MA) according to the manufacturer's instructions. We digested pDONR AMA with HindIII, which separated the AMA1 fragment from the rest of the vector. We ligated the two fragments using T4 DNA ligase (Invitrogen, Grand Island, NY) per the manufacturer's recommendations. We transformed the ligation mixture into *ccd*-compatible chemically competent cells (Invitrogen, Grand Island, NY) by heat shock as recommended by the manufacturer. We grew transformed cells on LA plus 50 µg/ml hygromycin at 37°C overnight. We picked colonies and transferred them to 2 ml LB plus 50 µg/ml hygromycin to grow overnight in a 37°C shaking incubator. We isolated plasmid DNA and digested it with specific enzymes to verify the correct fragment orientation.

2.3. Creation of an *mkkA* deletion mutant using pDONR 221-AfpyrG

To create deletion constructs, we combined the inserts from three pDONR vectors (Table 1) using an LR recombination reaction and pDEST R4-R3 as the destination vector (Invitrogen, Grand Island, NY). The pDEST vector consisted of a 5' NCR, a selective marker, and a 3' NCR (Magnani et al., 2006). We engineered a NotI site into the forward primer of the 5' NCR to create a unique site for digestion to linearize the vector. We transformed LR reaction mixes into TOP10 cells (Invitrogen, Grand Island, NY) via electroporation according to the manufacturer's instructions. We grew transformed cells on LA plus 75 µg/ml ampicillin at 37°C overnight. We picked colonies and transferred them to 2ml LB plus 75 µg/ml ampicillin to grow overnight in a 37°C shaking incubator. We isolated plasmid DNA and digested it with specific enzymes to verify the correct fragment orientation.

We grew *A. nidulans* 1155 (obtained from the Fungal Genetics Stock Center) in malt extract glucose (MEG) (2% malt extract, 0.2% peptone, 1% glucose, trace elements and vitamin mix as modified (Reyes et al., 2006)) supplemented with 5 mM uridine and 10 mM uracil. We performed the transformation as previously described (May, 1989), using pDEST R4-R3-*mkkA* 5' NCR-AfpyrG-*mkkA* 3' NCR. We grew transformants on minimal medium with vitamins (MMV) (1× MM salts [20 mM ammonium tartrate, 7 mM KCl, 2 mM MgSO₄·7H₂O], 1% glucose, 12 mM KPO₄ [pH 6.8], trace elements, vitamin mix as previously described, and 1.25% agar) supplemented with 0.2 M sucrose at 37°C for 3–5 days. We screened for mutants that were prototrophic for uridine and uracil. We prepared genomic DNA from transformant strains (Jin et al., 2004), and we identified deletion mutants via Southern blot analysis (Fig. 2A). We made a DNA probe using the 5' and 3' NCRs of *mkkA*.

2.4. Complementation of the *mkkA* deletion mutant using pDONR A

We obtained the coding region of *mkkA* (flanked by promoter and terminator regions) by PCR using e2TAK DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) according to the manufacturer's recommendations. We used *A. nidulans* 1155 genomic DNA as a template. Primers were constructed with *attB1* and *attB2* sites to facilitate the use of the Gateway System (Invitrogen, Grand Island, NY) (Table 1). We recombined the PCR fragment into pDONR A using the BP recombination reaction (Invitrogen, Grand Island, NY). We transformed the reaction mix into TOP10 cells (Invitrogen, Grand Island, NY) via electroporation according to the manufacturer's instructions. We grew the transformation mix on LA plus 50 µg/ml kanamycin at 37°C overnight. We picked colonies and transferred them to 2 ml of LB plus 50 µg/ml kanamycin to grow overnight in a 37°C shaking incubator. We isolated plasmid DNA and digested it with specific enzymes to verify the correct insertion.

We grew *mkkA* in MEG. We performed the transformation using previously described methods (May, 1989). We grew transformants on MM (as previously described above, but without vitamin mix) supplemented with 0.2 M sucrose at 37°C for 3–4 days. We screened for mutants that were prototrophic for pyridoxine. We prepared genomic DNA from transformant strains (Jin et al., 2004) and verified complementation via Southern blot analysis (Fig. 2B). We made a DNA probe using the coding region of *mkkA*. To verify proper complementation, we grew *mkkA*, *mkkA*(R), and controls on MAG (MEG with 2% agar) at 37°C for 2 days. A *pyrG*⁺ control strain (TS1) was created by crossing 1155 (obtained from the Fungal Genetics Stock Center) with strain 20.1.8 (Wu and Miller, 1997).

2.5. High-copy expression of *gliZ* using pDONR AMA

We obtained the coding region of *gliZ* (flanked by promoter and terminator regions) via PCR using e2TAK DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan) per the manufacturer's instructions. We used *A. fumigatus* Af293 genomic DNA as a template for the reaction. Primers were constructed with *attB1* and *attB2* sites to facilitate the use of the Gateway System (Invitrogen, Grand Island, NY). We recombined the PCR fragment into pDONR AMA using the BP recombination reaction (Invitrogen, Grand Island, NY). We transformed the reaction mix into TOP10 cells (Invitrogen, Grand Island, NY) via electroporation as recommended by the manufacturer. We grew the transformation mix on LA plus 75 µg/ml ampicillin at 37°C overnight. We picked colonies and transferred them to 2 ml of LB plus 75 µg/ml ampicillin to grow overnight in a 37°C shaking incubator. We isolated plasmid DNA and digested it with specific enzymes to verify the correct insertion.

We grew Af293.1 in MEG supplemented with 5 mM uridine and 10 mM uracil. We performed the transformation as previously described (May, 1989), with the following changes. Once protoplasts were formed and incubated for 3 hours, we carried out all the subsequent reactions at half the specified volume. We used 500–750 ng of vector DNA for each reaction. We plated two amounts of protoplasts (20 µl and 50 µl) each in 4 ml of yeast extract glucose (YAG) top agar (0.5% yeast extract, 1% glucose, trace elements and vitamin mix as previously described, 10 mM MgCl₂, and 1% agar) supplemented with 1 M sucrose. These changes were necessary owing to the fact that using the AMA1 construct greatly increases transformation efficiency. Using these two amounts of protoplasts ensured that at least one plate would harbor single colonies, as opposed to an overcrowding of colonies. We grew transformants on YAG (as previously described, but with 1.5% agar) supplemented with 0.2 M sucrose at 37°C for 1–2 days. We screened for mutants that were prototrophic for uridine and uracil. To verify proper plasmid presence, we prepared genomic DNA from transformant strains (Jin et al., 2004). We transformed 1 µl of genomic DNA into TOP10 cells (Invitrogen, Grand Island, NY) via electroporation as recommended by the

manufacturer. We grew the transformation mix on LA plus 75 µg/ml ampicillin at 37°C overnight. We picked colonies and transferred them to 2 ml of LB plus 75 µg/ml ampicillin to grow overnight in a 37°C shaking incubator. We isolated plasmid DNA and digested it with specific enzymes.

2.6. RNA dot blot analysis

We performed RNA dot blot analysis on our pDONR AMA-*gliZ* strain to measure RNA levels and gene activity. We grew stationary cultures (5×10^6 spores/ml) in 25 ml of complete medium (CM) (MMV [as previously described], 0.1% yeast extract, 0.2% peptone, 0.1% tryptone, and 1% CM supplement [27 mM adenine HCl, 33.5 mM methionine, 173 mM arginine, and 1.3 mM riboflavin]) at 37°C for 48 hours. We collected total RNA from freeze-dried mycelia using the TRIzol method (Reyes et al., 2006). We mixed total RNA with denaturing solution (50% formamide, 16% formaldehyde, 1X borate buffer [20X borate buffer: 0.4M boric acid, 4 mM EDTA, pH 8.3, with NaOH], and 0.025% bromophenol blue) and incubated for 10 minutes at 65°C. We quenched samples in ice for 10 minutes and added equal volume 20x sodium chloride-sodium citrate (SSC) (3 M NaCl, 0.3 M sodium citrate [pH 7.0]). We equilibrated a nylon membrane in 10xSSC for 10 minutes and placed it in a 96-well dot blot apparatus (Bio-Rad, Hercules, CA) attached to a vacuum manifold. We collected samples in 100-µl volumes via aspiration. Each dot contained 3 µg of RNA. Immediately before and after the samples were collected, we aspirated 50 µl of 10x SSC through the filter in duplicate. This created clean, reproducible binding of the RNA. Once all the samples were collected, we air dried the filter overnight and then baked it in an oven for 2 hours. For prehybridization and hybridization, the filter was sealed in a bag using a heated sealer. We prehybridized the filter at 42°C for 4–6 hours and hybridized the filter at 42°C overnight. We made DNA probes using only the coding region of each gene of interest (*gliA*, *gliZ*, *gliP*, *gliT*, and Actin). We washed the filter and exposed it to a Typhoon 8600 phosphorImager (GE Healthcare Life Sciences, Pittsburgh, PA) overnight. We quantified the intensity of hybridization using ImageQuant 5.1 software (GE Healthcare Life Sciences, Pittsburgh, PA). We quantified Actin mRNA levels as a control and standardized data to Actin, before normalizing to the pDONR AMA empty vector control.

All Gateway-enabled plasmids created in this study will be available at the Fungal Genomic Stock Center.

3. Results

The goal of this work was to create a variety of recombinatorial plasmids containing different marker genes to increase efficiency in vector manipulations used for genetic engineering. The markers chosen are, for the most part, versatile and can be used in multiple biologically relevant fungal organisms. All vectors, selective markers, and primers are listed in Table 1.

3.1. Plasmids for use in gene replacement

We designed several plasmids (Fig. 3A) intended for use with the MultiSite Gateway system (Invitrogen) to join two or more DNA segments (Magnani et al., 2006). We use these vectors to create deletion constructs with a 5' NCR and a 3' NCR flanking the selective marker in a destination vector, such as pDEST R4-R3, and give an example of this below. This can be accomplished in two steps: (1) Clone the 5' NCR and the 3' NCR into donor vectors pDONR P4-P1R and pDONR P2R-P3, respectively, using a BP recombination reaction and (2) Recombine the 5' NCR, the selective marker, and the 3' NCR into a destination vector (e.g., pDEST R4-R3) using an LR recombination reaction to form the final deletion construct. Importantly, different pDONR vectors contain different *attP* sites,

and therefore require different *attB* sites to surround the appropriate DNA insert. For example, pDONR P4-P1R contains *attP4* and *attP1R*, which will only recombine with *attB4* and *attB1R* (Hartley et al., 2000; Magnani et al., 2006). This allows for directionality of the DNA insert in the recombination reactions. When using nutritional markers for gene deletion, we recommend using a selective marker from an organism other than the one being transformed. For example, if we were deleting a gene from an *A. fumigatus* strain, then we suggest using either *argB* or *pyrG* from *A. nidulans*. Using genes from different species decreases the chances of the deletion vector integrating at the nutritional gene locus, thereby increasing the efficiency of homologous recombination at the target locus.

3.2. Plasmids for integrative recombination

We created several pDONR vectors (Fig. 3B) to facilitate the complementation of gene deletions. We engineered these vectors using pDONR 221 as the base vector. Because these vectors still harbor the *ccdB* cassette, they can only be transformed into *ccd*-compatible bacterial strains until a gene of interest is recombined into the *attP* sites. With complementation, using a nutritional marker from the same strain you are working with can be advantageous. For example, if you are complementing a gene deletion created in *A. nidulans*, then it would be beneficial to use a vector that contains a nutritional marker from *A. nidulans*. Therefore, the complementation vector will have a higher chance of integrating at the nutritional gene locus, rather than inserting in a random position that could cause adverse positional effects or change gene expression. For some auxotrophic mutants, the entire nutritional locus has been deleted. In this case, the complementation construct is more likely to integrate randomly in the genome. All nutritional marker genes are flanked by a portion of the 5' NCR and 3' NCR, which contributes to targeted integration of the construct at the nutritional locus. In addition, when creating complementation plasmids, cloning the gene of interest, flanked by at least 1 kb of 5' NCR and 3' NCR, into the complementation vector is advantageous and can increase the likelihood of the construct integrating at a defined locus and not in a random position in the genome.

3.3. Plasmids for high-copy gene expression

We created two pDONR AMA vectors (Fig. 3C) for use in high-copy gene expression and genetic screens. Because these vectors still harbor the *ccdB* cassette, they can only be transformed into *ccd*-compatible bacterial strains until a gene of interest is recombined into the *attP* sites. These plasmids are autonomously replicating circular plasmids that are maintained as such following transformation. This is especially useful for genetic screens because the plasmids are easily recovered from fungal genomes by transformation of the genomic DNA into bacteria (Efimov, 2003; Osherov et al., 2001; Ukil et al., 2008). These plasmids are also valuable in over-expression studies, because there are, on average, anywhere from 10 to 30 copies present per genome (Aleksenko and Clutterbuck, 1997; Gems et al., 1991). Although the use of these vectors results in increased transformation efficiency in many fungal species, they are mitotically unstable and therefore require the use of a selective medium to remain present in the genome. These features are possible based on the presence of AMA1 (autonomous maintenance in *Aspergillus*) from *A. nidulans* (Aleksenko and Clutterbuck, 1997; Gems et al., 1991). The AMA1 cassette consists of a 300-bp central spacer flanked by two inverted copies of a MATE1 (mobile *Aspergillus* transformation enhancer) element. This MATE1 element is a genomic repeat region that is present throughout the genome of *A. nidulans* (Aleksenko and Clutterbuck, 1997; Gems et al., 1991).

We created pDONR AMA by cloning the *ccdB* cassette (*attP1-ccdB*-chloramphenicol resistance gene [*CmR*]-*attP2*) from pDONR 221 into the *KpnI* and *SphI* sites of pRG3-AMA-Not1 (Osherov and May, 2000). This vector contains *pyr4* from *Neurospora crassa*

and complements *pyrG*- mutants of *A. nidulans* and *A. fumigatus*. We generated pDONR AMA/HPH by cloning the AMA1 region from pDONR AMA into pDONR HPH B. This vector behaves as pDONR AMA, but contains a hygromycin resistance cassette (*hygroR*) as a selective marker. Due to the instability of the vector, any strain transformed with pDONR AMA/HPH will require growth on media containing hygromycin to prevent loss of the plasmid. *attB1* and *attB2* are needed for recombination into both of these vectors, since the *ccdB* cassette is from pDONR 221.

3.4. Deletion and complementation of *mkkA* using pDONR 221-Afp γ R and pDONR A

We created the deletion construct in 6 days from PCR of the 5' and 3' NCRs to transformation of the deletion construct into *A. nidulans*. We generated the deletion mutant by replacing the entire coding region of *mkkA* (AN4189.3) with *Afp γ R*. We screened transformants for proper nutritional prototrophy. Of the colonies screened, 100% were positive for homologous recombination of *Afp γ R* at the *mkkA* locus. The majority of *mkkA* transformants exhibited extremely poor growth, with almost no conidiation. *mkkA* grew best on rich medium supplemented with 1 M glucose as an osmotic stabilizer (Fig. 4A & B). For complementation, we transformed pDONR A-*mkkA* into *mkkA*. We created the complementation vector in 3 days from PCR of the *mkkA* cassette to transformation of the vector into *mkkA*. We screened transformants for the ability to grow in the absence of pyridoxine. Of the colonies we screened, 100% had obtained the *mkkA* coding region via a successful integration event. Upon complementation, we were able to restore conidiation of *mkkA* back to wild-type levels (Fig. 4A).

3.5. High-copy expression of *gliZ*

GliZ is the transcriptional activator of the gliotoxin biosynthetic cluster in *A. fumigatus*. This cluster, like many other secondary metabolite clusters, is under coordinate regulation through GliZ, therefore, induction of *gliZ* results in the induction of all other genes within the gliotoxin cluster (Bok et al., 2006). We amplified *gliZ* (Afu6g09630) with flanking promoter and terminator regions. We created a *gliZ* high-copy vector (pDONR AMA-*gliZ*) in 3 days and transformed pDONR AMA-*gliZ* into Af293.1. We verified the presence of the proper plasmid via digestion analysis. We grew strains in conditions conducive to low gliotoxin cluster gene expression and isolated total RNA. We quantified mRNA levels of multiple genes within the gliotoxin cluster via dot blot analysis. As shown in figure 3, *gliZ* is transcribed at higher than normal levels as a result of the autonomous nature of the pDONR AMA-*gliZ* plasmid. Consequently, other genes within the gliotoxin cluster are being induced to higher than normal levels as well (Fig. 5).

4. Conclusions

Numerous recombinatorial methods have been developed to increase efficiency in genetic manipulations. Some of these methods (recombineering, MAGIC, and DNA assembler) are performed *in vivo* (Li and Elledge, 2005; Liu et al., 2003; Mosberg et al.; Shao and Zhao, 2009; Sharan et al., 2009), while others (Cre-*loxP*, SLIC, LIC, and Gateway) can be carried out as *in vitro* reactions (Hartley et al., 2000; Li and Elledge; Li and Elledge, 2007; Magnani et al., 2006; Siegel et al., 2004; Yamamura, 2012). These methods offer different advantages and disadvantages, depending on the intended outcome. Two methods that have been used quite often, Cre-*loxP* and Gateway, have increase the efficiency of plasmid construction via site-specific recombination events.

The Cre-*loxP* system has been used in conditional knockout studies in different organisms and is advantageous if one is interested in expressing any gene of interest, fluorescent genes, luciferase genes, or mutated cDNAs under the control of the endogenous promoter of a

targeted gene, although the insertion of *loxP* sites around the targeted gene is first required to be able to perform these techniques (Deng, 2012; Siegel et al., 2004; Yamamura, 2012). When *loxP* sites are in place, Cre recombinase is all that is required for the recombination event to occur. One caveat of the Cre-*loxP* system is the lack of directional cloning with wild-type *loxP* sites. DNA fragments that are flanked by these identical wild-type *loxP* sites will insert in either orientation, which can be troublesome if the direction of the insert is important. In addition, if a DNA fragment, flanked by wild-type *loxP* sites, is inserted into a genomic sequence, these *loxP* sites can be targeted by Cre recombinase at any time, which results in unwanted excision of the fragment. To alleviate this problem, several mutated *loxP* sites have been created to facilitate directional recombination and prevent re-excision (Deng, 2012; Siegel et al., 2004; Yamamura, 2012). While these mutant sites have been shown to be stable, they are not commercially available in a multitude of vectors.

The Gateway system has been used in numerous eukaryotic organisms for genetic manipulations. Similar to the Cre-*loxP* system, DNA fragments can be inserted into vectors using recombination sites (*att* sites). These *att* sites are available in a variety of “flavors” and have specific *att* complements. For example, attL2 will only recombine with attR2 and attB1 will only recombine with attP1. This eliminates the need to mutate *att* sites and facilitates directional cloning of any DNA fragment. One of the other factors that make this system highly efficient is the *ccdB* cassette, which is flanked by the *att* sites in donor and destination vectors. This gene encodes a toxin from *Escherichia coli*, which is responsible for post-segregational killing of daughter cells that do not contain the F plasmid (Bernard and Couturier, 1992). The presence of the *ccdB* gene in Gateway vectors ensures that any vectors that do not recombine properly will kill the bacterial strain used for transformation, leaving only recombined vectors containing the insert of interest. For more information on the *ccdB* counter-selection technique, please refer to Bernard and Couturier 1992. Additionally, in contrast to the Cre-*loxP* system, numerous Gateway-enabled vectors are commercially available for different genetic techniques, making the Gateway system a powerful tool for genetic engineering. One caveat to Gateway technology is the need for multiple recombination proteins to carry out reactions, although these proteins are readily available as easy to use kits, which further enhances the efficiency of the reaction.

Taking advantage of recombinatorial cloning techniques, we have created a series of vectors to facilitate gene deletion, gene complementation, and high-copy gene expression in a variety of fungal species. Our aim was to create a set of versatile recombinatorial vectors to be used as efficient tools for genetic manipulations in biologically relevant fungal species. Gateway-enabled vectors have been constructed in other laboratories, but mostly appear to focus on protein fusions and fluorescence tagging (Mabashi et al., 2006; Toews et al., 2004). The benefit of our plasmids is that they are intended to serve as versatile constructs. The specific selective markers we chose have been shown to be compatible with several other fungal species, such as *Aspergillus* species, *Penicillium* species, and *Neurospora* species (Abe et al., 2006; Bugeja et al., 2012; Pahirulzaman et al., 2012; Paz et al., 2011). Therefore, even though our studies focused solely on *A. fumigatus* and *A. nidulans*, we hypothesize that these plasmids will serve as efficient tools in other biologically and agriculturally relevant fungal organisms. We do not claim that the plasmids we have constructed will work ubiquitously, as there are always certain species that are incompatible. For instance, the autonomously replicating plasmids work well with *Aspergillus* species, as hyphae contain septae to separate nuclei. However, the AMA1-containing plasmids may not function as described in fungal organisms that produce coenocytic hyphae.

Our laboratory has used the pDONR 221 vectors for numerous gene deletions, and the pDONR vectors for gene complementation. In the present study, we demonstrated the competence of the pDONR 221-*AfpyrG* vector and the pDONR A vector with the *mkkA*

deletion mutant and *mkkA* complemented strain, respectively, in *A. nidulans*. Construction of the pDEST R4-R3 deletion vector and pDONR A complementation vector was quick and efficient. We have used most of the Gateway-enabled vectors in our own experiments and have not encountered any predicament with recombination or gene expression within the fungal strain being used. To our knowledge, the presence of the *att* sites does not cause any setbacks with gene expression or growth. We have also used both pDONR AMA and pDONR AMA/HPH in high-copy expression experiments and have given an example in the present study with the *gliZ* high-copy strain in *A. fumigatus*. Recombination experiments have been rapid and successful for both pDONR AMA vectors, usually only taking 1 day for recombination and an additional 2 days for insert verification. They behave as autonomously replicating plasmids and the presence of the *att* sites do not interfere with gene expression. Although AMA1-containing plasmids have been used successfully in numerous *Aspergillus* species and even a few *Penicillium* species, one caveat to using AMA1-based plasmids is their mitotic instability (Efimov, 2003; Fierro et al., 1996; Fierro et al., 2004; Gems et al., 1991; Osheroev et al., 2001). This can pose problems, as the AMA1-based plasmid has a higher chance of being lost in progeny than an integrative plasmid. This instability is not constant in all fungal species that have been studied using these autonomously replicating plasmids. For instance, mitotic stability of AMA1-based plasmids is higher in *P. nalgiovense* than in *P. chrysogenum* (Fierro et al., 1996; Fierro et al., 2004). In our lab, we have used AMA1-based plasmids frequently with great success and find that as long as strains are grown on selective medium, the incidence of plasmid loss decreases.

As our results demonstrate, using Gateway-enabled vectors expedites the process of vector construction for genetic techniques. Not only is Gateway technology widely available and easy to use, but it also has many advantages over traditional vector construction, such as increased effectiveness and built-in screening. The variety of different *att* sites is ideal for directional cloning of DNA fragments and can be used to fuse multiple DNA fragments together or simply attach a tag. We have constructed a series of Gateway-enable vectors containing various selective markers to streamline genetic manipulation. These vectors are meant to be used as versatile genetic tools, as they can be used in a variety of fungal species.

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Highlights

- We created a series of Gateway-enabled plasmids for use in filamentous fungi
- These plasmids can be used in gene deletion, gene integration, and high-copy gene expression
- The plasmids contain a variety of nutritional and drug-resistance markers that are functional across multiple fungal groups

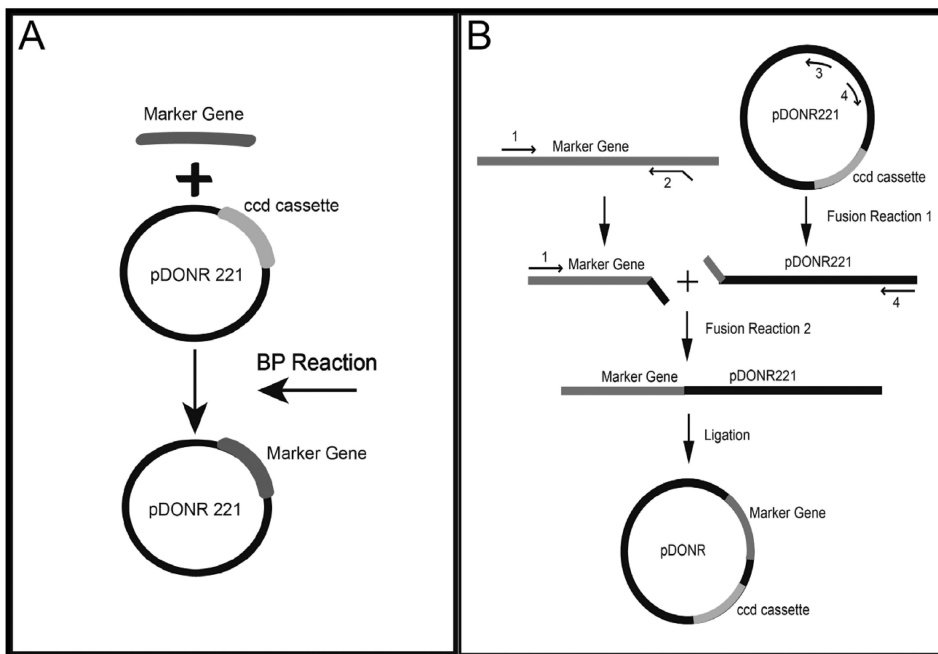


Figure 1. Schematic illustration of pDONR221 and pDONR vector construction
 (a) Schematic of the BP reaction used to create the plasmids for gene deletion. (b) Schematic of the f-PCR used to create the plasmids for gene integration. All gene integration plasmids were created by this methods, except pDONR HPH A and pDONR HPH B, which were created using restriction digest and ligation.

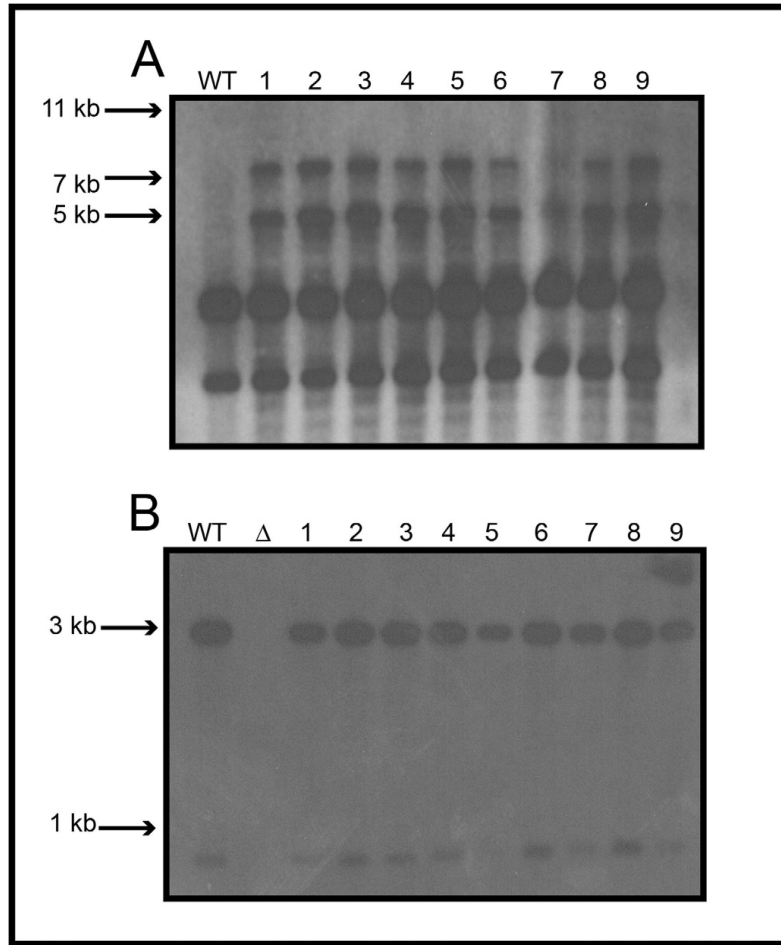


Figure 2. Southern hybridization analysis for the *mkkA* and *mkkA(R)* strains
 (a) Southern hybridization for the *mkkA* deletion mutant. WT is the wild-type strain. WT should display a band at 11909 bps and the deletion mutants should display bands at 7161 bps and 4965 bps. Note that there are two extra bands in all lanes. We used the deletion construct as the probe and the extra bands are possibly due to hybridization of part of the pDEST R4-R3 plasmid. (b) Southern hybridization for the *mkkA(R)* strain. WT is the wild-type strain and Δ represents the *mkkA* mutant used in the study. WT and complemented strains should display two bands at 2999 bps and 865 bps, while the *mkkA* deletion strain should have no band.

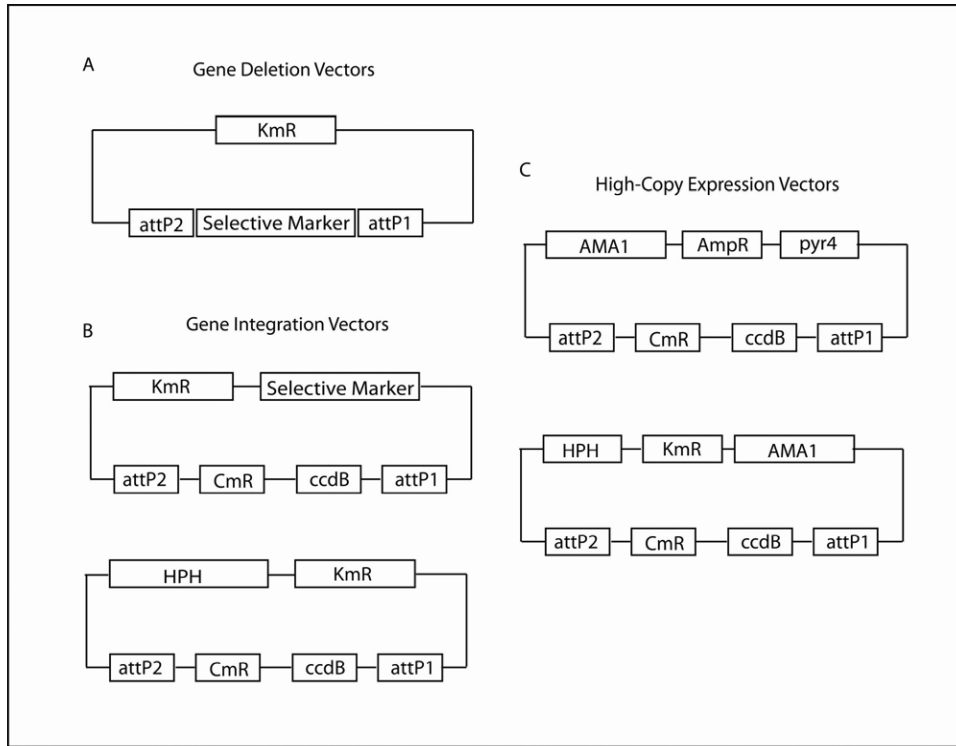


Figure 3. General structure of vectors created

(a) All gene deletion vectors have a selective marker that was inserted in between the *attP* sites. The *ccdB* region has been removed. (b) All gene integration vectors have a selective marker and a *ccdB* region flanked by *attP* recombination sites. Of note, in pDONR HPH A and pDONR HPH B, the *hph* resistance marker is in a different spot in the vector than the selective markers in the other pDONR vectors. The pDONR 221 and pDONR vectors are kanamycin resistant in bacteria. (c) Both high-copy expression vectors contain AMA1 from *A. nidulans*. pDONR AMA has the *ccdB* region, flanked by *attP* sites, which was cloned into the pRG3-AMA-Not1 vector. This vector contains the *pyr4* selective marker from *N. crassa* and is ampicillin resistant in bacteria. pDONR AMA/HPH has an *hph* resistance marker and the *ccdB* region flanked by *attP* recombination sites. This vector is kanamycin resistant in bacteria. Empty pDONR and pDONR AMA plasmids are also chloramphenicol resistant, until they lose the *ccdB* region through recombination.

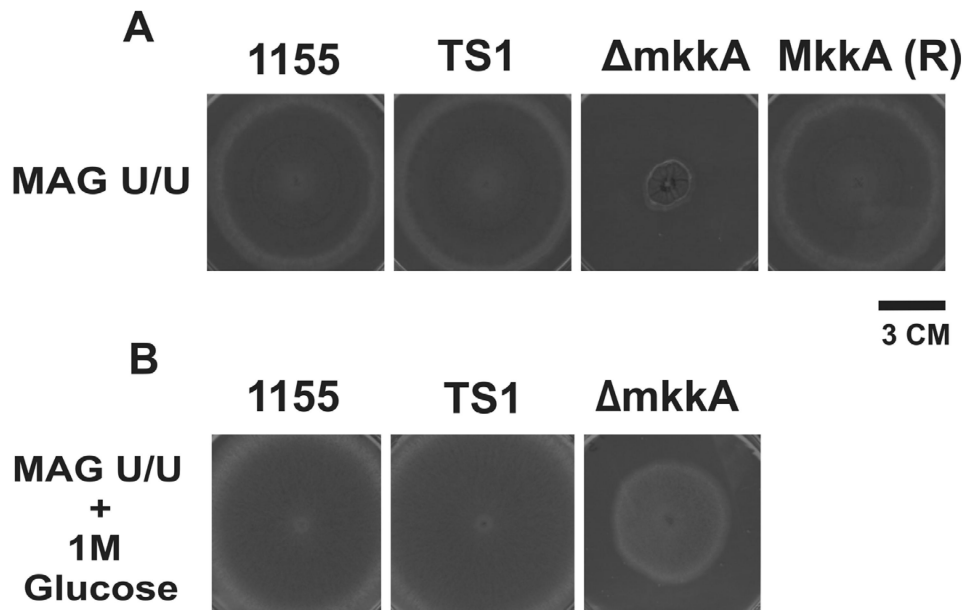


Figure 4. Plate growth of *mkkA* and *mkkA* (R)

(a) The *A. nidulans mkkA* deletion mutant and complemented strain *mkkA*(R) grown alongside An1155 and TS1 (controls). The use of pDONR A-*mkkA* as an integration vector restores *mkkA* growth to wild-type levels. (b) The *mkkA* deletion mutant is severely defective in growth, but growth is partially restored in the presence of 1 M glucose as an osmotic stabilizer.

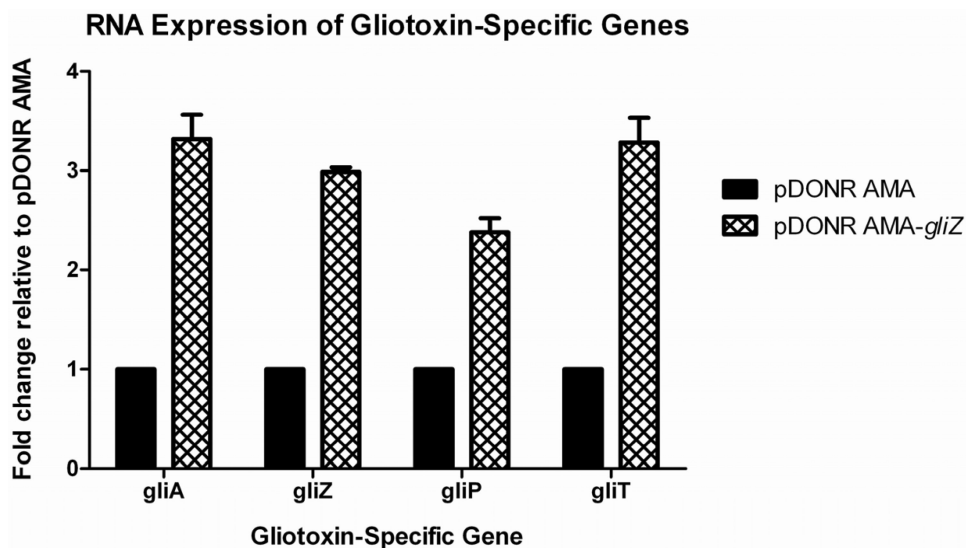


Figure 5. Transcription of gliotoxin-specific genes in a *gliZ* high-copy strain

The presence of extra copies of *gliZ* causes an increase in RNA levels of multiple genes within the gliotoxin biosynthetic cluster. We collected total RNA and quantified mRNA levels of designated genes by RNA Dot Blot Analysis. Data are expressed as fold change relative to a pDONR AMA empty vector control. All data was standardized to Actin before normalizing to the empty vector control.

Table 1

Vectors, selective markers, and primers used in this study.

| Vector | Selective marker | Primers |
|---------------------------------|--|--|
| pDONR 221-AnargB | <i>argB A. nidulans</i> | ArgB1- <u>ggggacaagttgtacaaaaaacaggcctaagcttatttcgcggttt</u> ArgB2- <u>ggggaccactttgtacaagaaagctgggtgacgtacacccattggg</u> |
| pDONR 221-AnpyrG | <i>pyrG A. nidulans</i> | attB1-AnpyrG5F- <u>ggggacaagttgtacaaaaaacaggcctcctatgccagtcattgagcaatg</u> attB2-AnpyrG3R- <u>ggggaccactttgtacaagaaagctgggtcagtgagcgaaacattctctc</u> |
| pDONR 221-AfpyroA | <i>pyroA A. fumigatus</i> | attBpyroA1- <u>ggggacaagttgtacaaaaaacaggcctaagttgatcgtgatgaatgacattc</u> attBpyroA2- <u>ggggaccactttgtacaagaaagctgggtctaagggcaccagcatgagtgac</u> |
| pDONR 221-AfpyrG | <i>pyrG A. fumigatus</i> | attB1-AfpyrGF- <u>ggggacaagttgtacaaaaaacaggcctcctcaaacatgctcttcaac</u> attB2-AfpyrGR- <u>ggggaccactttgtacaagaaagctgggtattctgtctgagaggagcacc</u> |
| pDONR 221-HPH | HPH <i>Escherichia coli</i> | attB1-HPH F- <u>ggggacaagttgtacaaaaaacaggcctcaaacatgctcttcaac</u> attB2-HPH R- <u>ggggaccactttgtacaagaaagctgggtgaattcgtcagcttaactgc</u> |
| pDONR 221-BAR | BAR <i>Streptomyces hygroscopicus</i> | attB BAR 1- <u>ggggacaagttgtacaaaaaacaggcctcaaacatgctcttcaac</u> attB BAR 2- <u>ggggaccactttgtacaagaaagctgggtcagagagattgaaactggagc</u> |
| pDONR A | <i>pyroA A. fumigatus</i> | pyroA F-GCGCGCACTAGTgcatcccaagggaaggtagc pyroA FR- ctagcgttaacgcgagagtagggaactgc cctcaagccaccagcatgagtg pDONR221 FFA- cagttgacggcactcatgctgggtcctta ggcagttccctactctcgcgt pDONR221 R1-GCGCGCACTAGTaatgctgtgctcactgccc |
| pDONR G | <i>pyrG A. fumigatus</i> | pyrG F-GCGCGCACTAGTatgctcttcaaccccttctcgcg pyrG FR- ctagcgttaacgcgagagtagggaactgc ccctgagaggagcactgatcgcg pDONR221 FFG- ttggcatcagcactcagtcctctcagggcagttccctactctcgcgt pDONR221 R1-GCGCGCACTAGTaatgctgtgctcactgccc |
| pDONR NG | <i>pyrG A. nidulans</i> | AnpyrG F-GCGCGCACTAGTctatgccagcattgagcaatg AnpyrG FR- gagtagggaaactgc cagtgagcgaaccaattctct pDONR221 FFG-AN- ttggctcctcacc ggcagttccctactctcgcgt pDONR221 R1-GCGCGCACTAGTaatgctgtgctcactgccc |
| pDONR B | <i>argB A. fumigatus</i> | AfargB F-GCGCGCACTAGTaaactgacgatgagcgtgat AfargB FR- gagtagggaaactgc ccaacgggtcttggctggaagg pDONR221 FF-AfargB- agccaagaacccgttt ggcagttccctactctcgcgt pDONR221 R1-GCGCGCACTAGTaatgctgtgctcactgccc |
| pDONR HPH A pDONR HPH B | HPH <i>Escherichia coli</i> | HPH F-ACTAGTgaattcgtcagcttaactgc HPH FR- ctagcgttaacgcgagtagggaactgc ccgtcagcttaactggttccc |
| pDONR BAR | BAR (Bernhard Straubinger 1992) <i>Streptomyces hygroscopicus</i> | BAR F-GCGCGCACTAGTaaagccagatgagaaccgac BAR FR- gagtagggaaactgc ccgagagagattgaaactggagc pDONR221 FF-BAR- ggttcaactctctcc ggcagttccctactctcgcgt pDONR221 R1-GCGCGCACTAGTaatgctgtgctcactgccc |
| pDONR AMA | <i>pyr4 Neurospora crassa</i> | ccd F-GGGGCATGccccaaataatgattttattt ccd R-GGGGGTACCaaataatgattttatttgaactga |
| pDONR AMA/HPH | HPH <i>Escherichia coli</i> | pDONR HindIII F-GCGCGCAAGCTTaaagcgaagagcgcaccaatac pDONR 221 R3-GCGCGCAAGCTTggcggtaatacggttatccacagaatcag |
| pDONR P4-P1R- <i>mkkA</i> 5' FR | | mpkka 1- <u>ggggacaactttgtatagaaaagttgaa</u> GCGGCCGTCaatatattcgaactcaga mpkka 2- <u>ggggactgctttttgtacaaacttgc</u> ggcgagactgttttgcctctc |
| pDONR P2R-P3- <i>mkkA</i> 3' FR | | mpkka 3- <u>ggggacagcttttgtacaaagtggaa</u> actctttggtctttgacgctta mpkka 4- <u>ggggacaactttgtataataaagtgc</u> acttcccgggtctactctgt |
| pDONR A- <i>mkkA</i> CR | | mkkA C1 - <u>ggggacaagttgtacaaaaaacaggcctaagc</u> ggcggcagccagccagccatgatcag mkkA C2- <u>ggggaccactttgtacaagaaagctgggtc</u> tacagcctgcaagcttttcag |
| pDONR AMA- <i>gliZ</i> | | gliZ attB 1- <u>ggggacaagttgtacaaaaaacaggcctc</u> gacccagcagctgattggag gliZ attB 2- <u>ggggaccactttgtacaagaaagctgggtc</u> gattccctttgtccgcc |

attB sites are underlined. All primers are listed 5' to 3'. Bolded sequences are extension regions for f-PCR. Uppercase regions are SpeI site in all primers, except ccd F (SphI), ccd R (KpnI), pDONR HindIII F (HindIII), pDONR R3 (HindIII), and mpkka 1 (NotI). All nutritional markers are flanked by their respective promoter and terminator regions. HPH is preceded by the *A. nidulans trpC* promoter region (<http://www.fgsc.net/fgn41/carroll.html>), and BAR is flanked by the *amdS* promoter region from *A. nidulans* (with increased expression from I9 and I66 mutations) and the glucoamylase terminator from *A. niger* (Bernhard Straubinger 1992).