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Flexible gateway constructs for functional analyses of genes in plant pathogenic fungi

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ABSTRACT

Genetic manipulation of fungi requires quick, low-cost, efficient, high-throughput and molecular tools. In this paper, we report 22 entry constructs as new molecular tools based on the Gateway technology facilitating rapid construction of binary vectors that can be used for functional analysis of genes in fungi. The entry vectors for single, double or triple gene-deletion mutants were developed using hygromycin, geneticin and nourseothricin resistance genes as selection markers. Furthermore, entry vectors containing green fluorescent (GFP) or red fluorescent (RFP) in combination with hygromycin, geneticin or nourseothricin selection markers were generated. The latter vectors provide the possibility of gene deletion and simultaneous labelling of the fungal transformants with GFP or RFP reporter genes. The applicability of a number of entry vectors was validated in *Zymoseptoria tritici*, an important fungal wheat pathogen.

1. Introduction

Filamentous fungi are diverse eukaryotic organisms that are important for various reasons in industry, medicine, agriculture, and basic sciences. Many of them are important plant pathogens and cause severe losses in agricultural production. A wide range of filamentous fungi is used in industry for production of commercially valuable proteins and metabolites that are of considerable interest to market. Some of the filamentous fungi like *Aspergillus nidulans* and *Neurospora crassa* are among the first-rate model organisms and have been widely used in fundamental research. The genomes of many filamentous fungi, including plant pathogenic fungi, have been sequenced and are publicly available which opens tremendous possibilities for future functional research of genes and their roles in pathogenesis (Marthey et al., 2008). In addition, advances in genome annotation as well as comparative genomics has revealed an ever-increasing number of interesting and novel genes that require high throughput functional tools for analysis. To date a number of genetics tools has been developed intending to lower the cost of such analyses, addressing biological questions. This requires the construction of vectors to generate knock-out strains, overexpression strains and fluorescently labelled strains to analyse and monitor the function of genes in different biological processes. However, the construction of vectors for fungal transformation has always been an important obstacle slowing down the efficiency of functional analyses. Generating constructs using traditional approaches like digestion/ligation is labour intensive, time-consuming, relatively expensive as it requires several cloning steps. Hence, recently several studies have been conducted to improve or develop new genetic tools for large-scale functional analyses (Paz et al., 2011; Shafran et al., 2008; Zhu et al., 2009). Among these, the Gateway® cloning technology has attracted molecular biologists from different disciplines due to its amenability and robustness (Schoberle et al., 2013). To date, a few methods or constructs have been developed using this technology for the functional analyses of genes in plant pathogenic fungi (Abe et al., 2006; Nakagawa et al., 2007; Paz et al., 2011; Shafran et al., 2008; Zhu et al., 2009). For instance, the One Step

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Construction of Agrobacterium-Recombination-ready-plasmids (OSCAR) has been developed to create deletion constructs for *Agrobacterium tumefaciens* mediated transformation (Paz et al., 2011). Two Gateway vectors, pCBGW and pGWBF, were generated for expression of genes under control of the P*gpdA* promoter and T*trpC* terminator (Zhu et al., 2009). The Gateway RNAi vector was also developed allowing gene silencing in a high-throughput manner (Shafran et al., 2008).

These data indeed confirm the enormous potential of the Gateway cloning strategy and, therefore, new Gateway constructs for different purposes need to be developed. We have generated and described 22 entry vectors based on the Gateway three-fragment vector methodology. They represent a user-friendly tool in the demanding field of molecular biology and will accelerate progress in the functional analyses of genes in plant pathogenic fungi. As an example, the application of a number of entry vectors was validated through the transformation of *Zymoseptoria tritici*, the septoria leaf blotch pathogen that is among the most destructive foliar blights in global wheat production.

2. Materials and methods

2.1. Bacterial, fungal strains and growth conditions

Z. tritici IPO323 (Goodwin et al., 2011) was used throughout this study. The fungus was grown in YGM (1% yeast extract, 3% glucose) in an orbital shaker (Innova 4430; New Brunswick Scientific, Nijmegen, The Netherlands) at 18 °C for five days to produce yeast-like spores, which were collected by centrifugation and subsequently used for fungal transformation or stored at −80 °C (Kema and van Silfhout, 1997). *Escherichia coli* DH5α was used for general plasmid transformation. *E. coli* was grown in or on Luria Bertani (LB) medium amended with appropriate antibiotics. *E. coli* DB3.1 (Invitrogen) was used for propagation of plasmids containing the ccdB gene that is lethal for most *E. coli* strains. *A. tumefaciens* strain AGL-1 was used for all fungal transformations.

2.2. DNA manipulation and analysis

Basic DNA manipulations were according to standard protocols (Sambrook et al., 2001). DNAs were purified using QIA quick PCR Purification. PCR products were extracted from agarose gels and purified using the Illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, Life Sciences). Plasmid DNA was isolated using the plasmid Prep Purification Mini Spin Kit (GE Healthcare, Life Sciences). Fungal genomic DNA of *Z. tritici* IPO323 was prepared from freeze-dried spores using the DNeasy Plant kit (Qiagen). DNA sequences were obtained on an ABI-prism 3100 capillary automated sequencer using the Amerdye terminator reaction mix (GE Healthcare). Primers used in this study are listed in Table 1.

2.3. Construction of entry vectors

The donor vectors, pDONR™-P4-P1R, pDONR™-221, pDONR™-P2R-P3, were used as the backbone to construct the gateway entry vectors (Invitrogen) (Fig. 1). To construct the entry vectors, BP reactions were performed to clone DNA fragments into donor vectors according to the manufactures instructions (Invitrogen). The promoter, pgpdA, was amplified from plasmid pRF-HU2E (Frandsen et al., 2008) using primer pair GW-pgpdA-F1/GW-pgpdA-R1 and inserted into pDONR™-221 generating pRM253. The hygromycin phosphotransferase gene (*Hph*) was amplified from pRF-HU2 (Frandsen et al., 2008) using primer pairs GW-hph-F1/GW-hph-R1 and Hph-P4-F/Hph-P4-R. The resulting PCR products were inserted into pDONR™-221 and pDONR™-P4-P1R, generating pRM250 and pRM247, respectively. The geneticin resistance gene (neomycin phosphotransferase) was amplified from pSM334 (Hou et al., 2002) using primer pairs GW-Gen-F1/GW-Gen-R1 and Gen-P4-F/Gen-P4-R, and inserted into pDONR™-221 and pDONR™-P4-P1R, generating pRM251 and pRM245, respectively. The nourseothricin resistance gene (*Nat*) was amplified from pNR3 (Zhang et al., 2011) using primer pairs GW-Nat-F1/GW-Nat-R1 and Nat-P4-F/Nat-P4-R inserted into pDONR™-221 and pDONR™-P4-P1R, generating pRM249 and pRM246, respectively. To construct entry vectors containing GFP, pSC001 (Armesto et al., 2012) was used as a template to amplify GFP using primer pairs GW-GRFP-F1/GW-GRFP-R1, GRFP-P4-F/GRFP-P4-R and GRFP-P2-F/GRFP-P2-R. The resulting PCR amplicons were inserted into pDONR™-221, pDONR™-P4-P1R, and pDONR™-P2R-P3, generating pRM242, pRM236 and pRM234, respectively. The RFP cassette was amplified from pSC002 using primer pairs GW-GRFP-F1/GW-GRFP-R1, GRFP-P4-F/GRFP-P4-R and GRFP-P2-F/GRFP-P-R and inserted into pDONR™-221, pDONR™-P4-P1R and pDONR™-P2R-P3 generating pRM243, pRM237 and pRM235, respectively.

To construct entry vectors containing the Hph-GFP cassette, pSC001 was used as template to amplify the Hph-GFP cassette using primer pairs Hph-GRFP-P4-F/Hph-GRFP-P4-R and GRFP-P2-F/GRFP-P2-R. The resulting PCR amplicons were inserted into pDONR™-P4-P1R and pDONR™-P2R-P3 generating pRM238 and pRM240, respectively. Likewise, to construct entry vectors containing Hph-RFP, pSC002 was used as template in PCR reactions along with primer pairs Hph-GRFP-P4-F/Hph-GRFP-P4-R, GRFP-P2-F/GRFP-P2-R and GRFP-221-F/GRFP-221-R to amplify the Hph-RFP cassette. The resulting PCR products were inserted into pDONR™-P4-P1R, pDONR™-P2R-P3 and pDONR™-221, generating pRM239, pRM241 and pRM259, respectively.

To generate the geneticin-GFP cassette (Gen-GFP), geneticin and GFP fragments were amplified separately and fused by an overlapping PCR. To this aim, the geneticin resistance gene was amplified from pSM334 (Hou et al., 2002) using primers GW-Gen-F1 and Gen-R1. The GFP fragment was amplified from pSC001 using Gen-GRFP-F1/GW-GRFP-R1. An overlapping PCR was performed using GFP and geneticin fragments (as templates) and GW-Gen-F1 and GW-GRFP-R1 primers. The resulting PCR (Gen-GFP cassette) was purified and introduced into pDONR™-221 generating pRM254. The same procedure was used to generate the Gen-RFP entry vector. Geneticin was amplified from pSM334 using primers GW-Gen-F1 and Gen-R1. RFP was amplified from pSC002 using Gen-GRFP-F1 and GW-GRFP-R1. An overlapping PCR was performed using GW-Gen-F1 and GW-GRFP-R1 and the purified products of RFP and geneticin as template and the resulting PCR (Gen-RFP) were introduced into pDONR™-221 generating pRM257.

To construct the Nat-GFP entry vector (pRM255), an overlapping PCR was used to generate the Nat-GFP cassette. To this aim, the nourseothricin resistance gene was amplified from pNR3 using primers GW-Nat-F1 and Nat-R1. GFP was amplified from pSC001 using Nat-GRFP-F1 and GW-GRFP-R1. The purified products of GFP and Nat were used as a template in a PCR reaction using primer pair GW-Nat-F1/GW-GRFP-R1; and the resulting PCR product (Nat-GFP cassette) was introduced into pDONR™-221, generating pRM255.

2.4. Construction of fungal transformation vectors (FT vectors)

To generate FT vectors, three entry vectors including entry vector derived from pDONR™-P4-P1R, pDONR™-221 and pDONR™-P2R-P3 were used and the LR reaction was performed to recombine the fragments into the binary destination vector, pPm43GW. To generate the FT vector for GFP expression in *Z. tritici*