World Journal of Microbiology and Biotechnology (2018) 34:26
https://doi.org/10.1007/s11274-018-2409-8

ORIGINAL PAPER

An efficient gene disruption method using a positive–negative split-selection marker and *Agrobacterium tumefaciens*-mediated transformation for *Nomuraea rileyi*

Yu Su[1,3] · Zhongkang Wang[1] · Changwen Shao[2] · Yuanli Luo[3] · Li Wang[1] · Youping Yin[1]

Received: 30 July 2017 / Accepted: 8 January 2018 / Published online: 16 January 2018

Abstract

Targeted gene disruption via *Agrobacterium tumefaciens*-mediated transformation (ATMT) and homologous recombination is the most common method used to identify and investigate the functions of genes in fungi. However, the gene disruption efficiency of this method is low due to ectopic integration. In this study, a high-efficiency gene disruption strategy based on ATMT and the split-marker method was developed for use in *Nomuraea rileyi*. The β-glucuronidase (*gus*) gene was used as a negative selection marker to facilitate the screening of putative transformants. We assessed the efficacy of this gene disruption method using the *NrCat1, NrCat4*, and *NrPex16* genes and found that the targeting efficiency was between 36.2 and 60.7%, whereas the targeting efficiency using linear cassettes was only 1.0–4.2%. The efficiency of negative selection assays was between 64.1 and 82.3%. Randomly selected deletion mutants exhibited a single copy of the *hph* cassette. Therefore, high-throughput gene disruption could be possible using the split-marker method and the majority of ectopic integration transformants can be eliminated using negative selection markers. This study provides a platform to study the function of genes in *N. rileyi*.

Keywords *Nomuraea rileyi* · *Agrobacterium tumefaciens*-mediated transformation (ATMT) · Split-marker · β-Glucuronidase · Negative selection marker

Introduction

Nomuraea rileyi is an important entomopathogenic fungus that can infect lepidopterous pests, especially *Noctuidae* species such as *Spodoptera litura* (Chen et al. 2014), *Anticarsia gemmatalis* (Palma and Del Valle 2015), *Spodoptera frugiperda, Spodoptera exigua, Helicoverpa zea*, and *Heliothis virescen* (Vega-Aquino et al. 2010), making it useful for insect biocontrol.

With the development of high-throughput sequencing technology, the transcriptome and whole genome sequence of *N. rileyi* have recently become available (Song et al. 2013; Shang et al. 2016). These data provide a basis for research on *N. rileyi* gene function. At first, double-stranded RNA was used in *N. rileyi* gene function studies to silence genes (Jiang et al. 2014; Liu et al. 2014; Zhou et al. 2015). However, only transient genetic modifications that are not stably inherited by subsequent generations could be achieved (Shao et al. 2015). Recently, the *Agrobacterium tumefaciens*-mediated transformation (ATMT) system in *N. rileyi* was developed by Shao et al. (2015), and several target genes have been

✉ Youping Yin
ypy128@vip.sina.com

Yu Su
791431633@qq.com

Zhongkang Wang
w-zk@163.com

Changwen Shao
changwen2000@sina.com

Yuanli Luo
823979902@qq.com

Li Wang
lily198213@hotmail.com

[1] School of Life Science, Chongqing University, Chongqing 400030, China

[2] College of Agriculture and Forestry, Linyi University, Linyi 276000, China

[3] Southeast Chongqing Academy of Agricultural Sciences, Chongqing 408000, China

disrupted using this system (Li et al. 2016; Song et al. 2016). The conventional gene disruption strategy for filamentous fungi involves the insertion of two homologous recombination sequences (HRS) on either side of the selected marker gene that fully or partially replaces the target gene via the homologous recombination (HR) pathway (Rothstein 1983; Wendland 2003; Weld et al. 2006). However, low frequencies of detected HR events are achieved in filamentous fungi using this method because the introduction of integrated DNA into the host genome results in a high frequency of ectopic integration via the non-homologous end-joining (NHEJ) pathway (Gauthier et al. 2010).

In order to increase the efficiency of gene targeting, the split-marker technique was developed by Fairhead et al. (1996) for use in yeast and was successfully applied to filamentous fungi by Catlett et al. (2003). This technique can be used to combine many transformation systems, such as protoplast-based transformation (Fairhead et al. 1996), biolistic transformation (Kim et al. 2009), electroporation (electropermeabilization) (Liang et al. 2014), and ATMT (Wang et al. 2010). The system involves the construction of two plasmids or DNA fragments, each containing a HRS with two-thirds of the selection marker gene. The two introduced DNA fragments enable a triple-crossover event between the target gene and the two DNA fragments. Previous studies have indicated that the split-marker technique can increase gene targeting efficiency and it has been used in several gene disruption studies in filamentous fungus (Catlett et al. 2003; Jeong et al. 2007; You et al. 2009; Wang et al. 2010; Liang et al. 2014). However, there are no reports of the split-marker method being applied to gene function research in the entomopathogenic fungi *N. rileyi*.

β-Glucuronidase, encoded by the *gus* gene, hydrolyzes 5-bromo-4-chloro-3-indulyl glucuronide (X-Glue) to a clear blue color and is widely used as a molecular marker in bacteria, fungi, and plant gene manipulation. The *gus* gene can be stably inherited in fungi and the expressed phenotypes of transformants show no differences from wild type (WT) (St. Leger et al. 1995). Therefore, this gene has been used as a negative selection marker for gene targeting in *Magnaporthe grisea* to improve the efficiency of deletion mutant screening (Wang et al. 2009).

In this study, a split-marker technique combined with the ATMT transformation system was established to achieve high-efficiency gene disruption in *N. rileyi*. The *gus* gene was introduced as a negative selection marker in the flanking region of the gene disruption vector to facilitate screening of the transformants. This system provides a reliable high-throughput approach for gene disruption in *N. rileyi*.

Materials and methods

Strains, plasmids, and culture conditions

The *N. rileyi* WT Nr01 strain was stored at the Engineering Research Center for Fungal Insecticides, Chongqing, China and was cultured as previously described (Song et al. 2013). *Escherichia coli* DH5α was used to propagate and maintain plasmids following standard procedures. *A. tumefaciens* AGL-1 laboratory stocks were used to transform *N. rileyi*. The WT *A. tumefaciens* AGL-1 was cultured in yeast extract beef (YEB) media with 50 μg mL^{-1} streptomycin. For plasmid-carrying *A. tumefaciens*, 50 μg mL^{-1} of kanamycin was also added to the media. The plasmids pPZP-Hph-Knockout, pPZP-Hph-RNAi and pPZP-Hph-*gus* were kind gifts from Changwen Shao, PhD, Chongqing University.

Construction of split-marker vectors

The split-marker vectors were derived from pPZP-Hph-Knockout described by Shao et al. (2015). The upstream 2/3*hph* cassette and the downstream 2/3*hph* cassette were amplified from the plasmid pPZP-Hph-RNAi using the primers split-marker A–F, A–R, B–F, and B–R (Table 1). Both the PCR products and the pPZP-Hph-Knockout plasmid were purified and digested with *Xho*I and *Xba*I. The two digested PCR fragments were ligated with the purified pPZP-Hph-Knockout backbone. The two new plasmids were named pPZP-split-marker-A and pPZP-split-marker-B (Fig. 1).

To conveniently screen the transformants, the *gus* gene was used as a negative selection marker. The *Ptrpc* promoter was amplified from plasmid pPZP-Hph-Knockout using the primers *Ptrpc*-F and *Ptrpc*-R (Table 1). The *gus* gene was amplified from the plasmid pPZP-Hph-*gus* using the primers *gus*-F and *gus*-R (Table 1). A splice overlap extension PCR (SOE-PCR) (Ho and Horton 1991) was used to ligate the *Ptrpc* promoter and the *gus* gene. The SOE-PCR products and pPZP-split-marker-B plasmid were digested with *Pme*I and ligated with T4 DNA ligase (Takara Biotechnology Inc. Dalian, China). The new plasmid was designated pPZP-split-marker-B-*gus* (Fig. 1).

Construction of *NrCat1*, *NrCat4*, and *NrPex16* gene disruption vectors

A fusion primer and nested integrated PCR (FPNI-PCR) was used to clone the flanking sequence of the target gene *NrCat1* as described by Wang et al. (2011). The products of the FPNI-PCR were cloned into the pMD19-T vector (Takara Biotechnology Inc.) and sequenced by Tsingke

Table 1 Primers used in this study

Name[a]	Sequence 5′–3′	Amplification purpose
Split marker A–F (Fig. 1)	TACGAATTCCACTCGAGGTCGACAGAAGAATGACA	2/3*hph* cassette
Split marker A–R (Fig. 1)	CTAGTCTAGAGCTGCTCCATACAAGCCAACCACGG	
Split marker B–F (Fig. 1)	CCGCTCGAGCTCGGAGGGCGAAGAATCTCGTGCT	2/3*hph* cassette
Split marker B–R (Fig. 1)	AGCTTGCATGCCTGCAGGTCGACTCTAGACTATTC	
Ptrpc-F (Fig. 1)	AGCTTTGTTTAAACGTCGACAGAAGAATGACATT	*Prtpc* and overlapping PCR
Ptrpc-R (Fig. 1)	ACAGGACGTAACATTCGGTAGATCCACTAGAGCGG	
gus-F (Fig. 1)	GGATCTACCGAATGTTACGTCCTGTAGAAACCCCA	*Gus* and overlapping PCR
gus-R (Fig. 1)	AGCTTTGTTTAAACTTTTTTTTTTCATTGTTTGCCTCCCTGCTG	
hph-F (P1) (Fig. 3)	ACGAGGACATTATTATCATCTGCTGC	Confirm mutants
hph-R (P2) (Fig. 3)	CGGTTTCCACTATCGGCGAGTA	
NrCat1-F (P3) (Fig. 3)	AAAGTTGCAGTCCTTCGACCAC	Confirm mutants
NrCat1-R (P4) (Fig. 3)	TGAACATCACGAGCAGTGTCC	
NrCat1 LF up-F (P5) (Fig. 3)	GTTCCTCGCACCAGTTTCAAGTT	Confirm mutants
NrCat1 LF up-R (P6) (Fig. 3)	AATGTCCTCGTTCCTGTCTGCTAAT	
NrCat1 RF down-F (P7) (Fig. 3)	CTGGACCGATGGCTGTGTAG	Confirm mutants
NrCat1 RF down-R (P8) (Fig. 3)	CGGCCTTGGTGGTGTTATTA	
NrCa4-F (P3) (Fig. 3)	CTCTTCTCCGCAGTAAATGTCGC	Confirm mutants
NrCat4-R (P4) (Fig. 3)	TTGTGCTCTTGTACGGGATGGT	
NrCat4 LF up-F (P5) (Fig. 3)	TCAAAGTTGCGAAAGAGGAT	Confirm mutants
NrCat4 LF up-R (P6) (Fig. 3)	TAGCAGACAGGAACGAGGAC	
NrCat4 RF down-F (P7) (Fig. 3)	GTGCCGATAAACATAACGAT	Confirm mutants
NrCat4 RF down-R (P8) (Fig. 3)	TCAACCCACTGAACAGAAAG	
NrPex16-F (P3) (Fig. 3)	GTCAGCAAGGCGTCTCCTCCAACTC	Confirm mutants
NrPex16-R (P4) (Fig. 3)	CGGCTCTTCGTCCGGTATCTCGTC	
NrPex16 LF up-F (P5) (Fig. 3)	AGCACGCAGAATACGAAACG	Confirm mutants
NrPex16 LF up-R (P6) (Fig. 3)	GCAGACAGGAACGAGGACAT	
NrPex16 RF down-F (P7) (Fig. 3)	GAAATAAAGGGAGGAAGGGC	Confirm mutants
NrPex16 RF down-R (P8) (Fig. 3)	TCAAGCAAGAAGAGAGGCAT	
NrCat1 Probe-F (Fig. 4)	GGCCAAATCACCAGTCACC	Southern probe
NrCat1 Probe-R (Fig. 4)	CCAAGAAAAACGCCGTACGAC	
*NrCat1*LF-F	CCGGAATTCGTTGGGCACAGACAATGGGC	Split marker and linear cassettes
*NrCat1*LF-R	CCGCTCGAGAGATTTCACCACGCTAACAT	
*NrCat1*RF-F	CTAGTCTAGACCGCATCTTCTCCTACCCCG	Split marker and linear cassettes
*NrCat1*RF-R	CCCAAGCTTGAATAGTCACTTTCTTCCAC	
*NrCat4*LF-F	GCCTCCAACACCAACCTCTT	Split marker and linear cassettes
*NrCat4*LF-R	AAAACTGCAGCTATCAATCTTTGTGGCAGTTCTTT	
*NrCat4*RF-F	ACCGAATCACAAAGGATAATGAGAACA	Split marker and linear cassettes
*NrCat4*RF-R	CCGGAATTCTGGAGCAGTACAGGATCGCAAA	
*NrPex16*LF-F	CCCAAGCTTGCTCATCATTCCCGCACCT	Split marker and linear cassettes
*NrPex16*LF-R	GCTCTAGAACACCACCCGCCGCTTA	
*NrPex16*RF-F	CCGCTCGAGGGTGAGGGCGTTGGTCT	Split marker and linear cassettes
*NrPex16*RF-R	CCGGAATTCCGTCCCGACGTGAGGTA	

[a]Text in parentheses indicates positions and generic names of primers in Figs. 1, 3 and 4

Biotech Co. Ltd. (Beijing, China). The flanking sequences of *NrCat4* and *NrPex16* were acquired from the reference genome sequence published by Shang et al. (2016).

The flanking regions of *NrCat1, NrCat4*, and *NrPex16* were amplified using the primers *NrCat1*LF-F, *NrCat1*LF-R, *NrCat1*RF-F, *NrCat1*RF-R, *NrCat4*LF-F, *NrCat4*LF-R, *NrCat4*RF-F, *NrCat4*RF-R, *NrPex16*LF-F, *NrPex16*LF-R, *NrPex16*RF-F, and *NrPex16*RF-R (Table 1). The left flanking region of *NrCat1* and the right flanking regions of *NrCat4* and *NrPex16* were inserted into

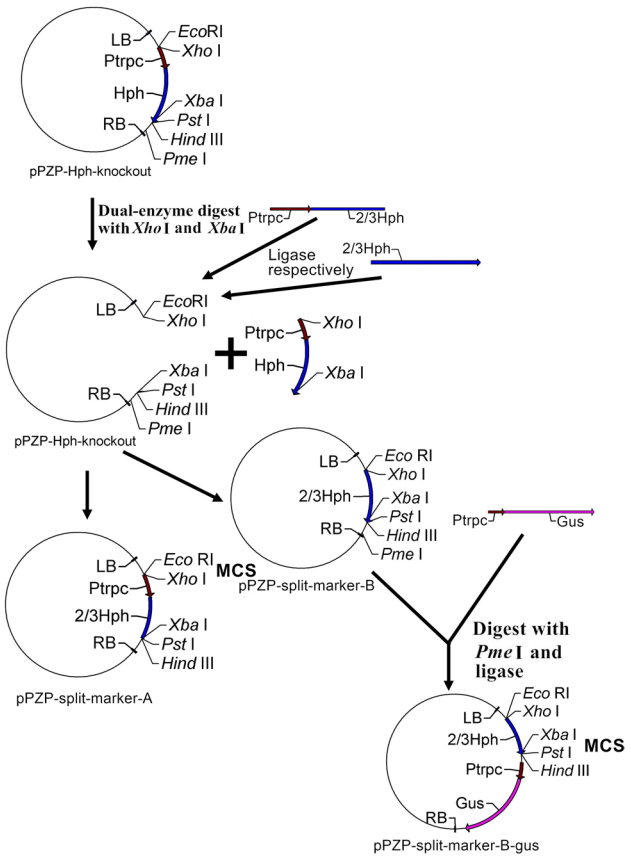

Fig. 1 Multistep construction of vectors for split-marker gene replacement. The vector pPZP-Hph-Knockout was dual-enzyme digested with *Xho*I and *Xba*I. The two 2/3*hph* cassette fragments were amplified from the vector pPZP-Hph-RNAi using the primers split-marker A–F, split-marker A–R, split-marker B–F, and split-marker B–R. The two fragments were ligated with the pPZP-Hph-Knockout backbone and named pPZP-split-marker-A and pPZP-split-marker-B, respectively. The promoter *Ptrpc* was amplified from the vector pPZP-Hph-RNAi using the primers *Ptrpc*-F and *Ptrpc*-R. The *gus* gene was amplified from the vector pPZP-Hph-*gus* using primers *gus*-F and *gus*-R. SOE-PCR was performed to ligate *Ptrpc* and *gus*. The pPZP-split-marker-B was digested with the enzyme *Pme*I and ligated with the *gus* cassettes. The resulting plasmid was named pPZP-split-marker-B-*gus*. The multiple cloning site (MCS) is the insertion site of the flanking sequence

the pPZP-split-marker-A vector. The right flanking region of *NrCat1* and the left flanking regions of *NrCat4* and *NrPex16* were inserted into the pPZP-split-marker-B-*gus* vector. The split-marker disruption vectors obtained were named pPZP-split-marker-A-*NrCat1*, pPZP-split-marker-B-*gus*-*NrCat1*, pPZP-split-marker-A-*NrCat4*, pPZP-split-marker-B-*gus*-*NrCat4*, pPZP-split-marker-A-*NrPex16*, and pPZP-split-marker-B-*gus*-*NrPex16*.

The linear cassettes of *NrCat1*, *NrCat4*, and *NrPex16* were also constructed for use as controls by inserting flanking regions into the pPZP-Hph-Knockout plasmid and were named pPZP-Hph-Knockout-*NrCat1*, pPZP-Hph-Knockout-*NrCat4*, and pPZP-Hph-Knockout-*NrPex16*.

Agrobacterium tumefaciens-mediated transformation (ATMT)

We transformed *N. rileyi* using the method described by Shao et al. (2015) with a modified blastospore preparation method. The conidia were inoculated on Sabourand maltose agar yeast extract (SMAY) media. The yeast-like blastospores were harvested by flooding with sterile distilled water (Thakre et al. 2011), washed twice with distilled water, and suspended in induction medium (IM) at a concentration of 10^7 blastospores mL^{-1}. The two *A. tumefaciens* strains carrying the 2/3*hph* split-marker cassettes and the blastospore suspension were mixed at a ratio of 1:1:1 for co-transformation. The transformation procedure was performed according to the method of Shao et al. (2015).

Screening for deletion mutants

Gus assay

Transformants were inoculated on SMAY plates containing 450 μg mL^{-1} hygromycin B and 400 μg mL^{-1} cephalosporin and incubated for 4–7 days at 25 °C. Resistant transformants were transferred to fresh SMAY plates containing hygromycin B and cephalosporin and cultured for five generations. The transformants were then transferred to fresh SMAY plates without antibiotics and cultured for five generations to determine their morphological stability. Negative screening was performed using a *gus* assay kit (Huayueyang Biotechnology, Beijing, China). The hygromycin B-resistant transformants that exhibited no *gus* expression activity were considered to be putative mutants (Fig. 2).

PCR analysis of mutants

Chromosomal DNA was isolated from putative deletion mutants using a genomic DNA isolation kit (Axygen Bio Inc., Union City, CA, USA). To verify mutants, PCR was performed using two pairs of primers, P5 and P6, and P7 and P8 that detected the absence of targeted gene fragments, as shown in Fig. 3a. After sequence verification analyses, those transformants containing amplicon (c) and amplicon (d) sequences were considered to be gene knockout mutants.

Southern blotting

Mycelia were collected from 100 mL of SMY liquid media and 5–10 μg of chromosomal DNA was isolated from each sample using the cetyltrimethylammonium bromide (CTAB) method. The chromosomal DNA was digested with *Nco*I and

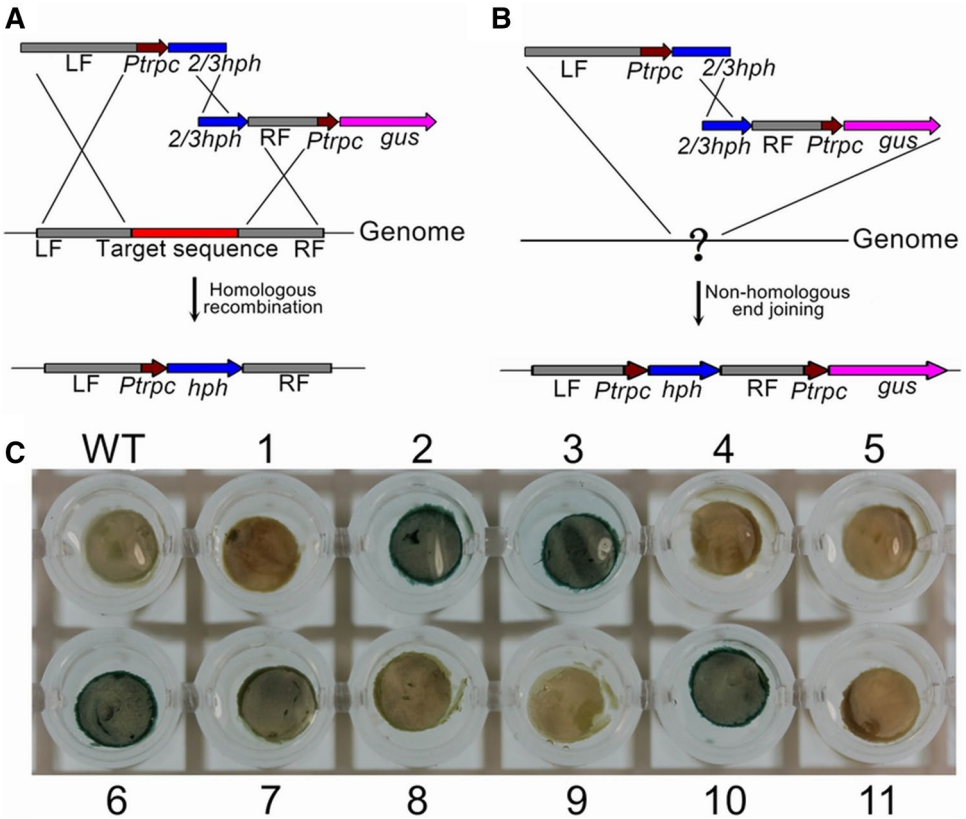

Fig. 2 **a, b** Transformation and integration into the host genome. The transfer DNA of pPZP-split-marker-A and pPZP-split-marker-B-*gus* were transformed into the *N. rileyi* Nr01 strain by ATMT. **a** If the two transfer DNAs overlap and integrate with the host genome by HR as shown, the target sequence of the transformant is disrupted and only *hph* is expressed. **b** If the crossover event takes place between the two transfer DNAs and the transfer DNAs integrate into the host genome at a random location by ectopic insertion as shown, the targeted sequence will be not replaced by the *hph* cassette, and *hph* and *gus* will both be expressed. The mutants can initially be screened by negative-selection using the *gus* assay, and the transformants that do not demonstrate *gus* activity are further identified by PCR analysis. **c** *Gus* assay of putative transformants. Agar plugs are removed from *N. rileyi* cultures. Column WT, wild type; columns 1–11, putative transformants; columns 2, 3, 6, 7, and 10, *gus* positive transformants; columns 1, 4, 5, 8, 9, and 11, *gus* negative transformants

separated by electrophoresis on 0.8% agarose gels. The gel was then blotted onto a Hybond-N+ membrane (Amersham Biosciences, Little Chalfont, UK). Part of the left flanking sequence was amplified using the primers *NrCat1* Probe-F and *NrCat1* Probe-R (Fig. 4). The PCR-amplified product was digoxigenin (DIG)-labeled for use as a specific probe for signal detection. Probe hybridization and immunological detection were performed according to the manufacturer's protocol (Roche, Mannheim, Germany).

Results

Construction of split-marker vectors and *NrCat1*, *NrCat4*, and *NrPex16* gene disruption vectors

The modified gene disruption system employing the Hph-split-marker transformation method used in this study is described in Fig. 1. The pPZP-split-marker-A plasmid contained the upstream two-thirds of the *hph* cassette and the pPZP-split-marker-B plasmid contained the downstream two-thirds of the *hph* cassette. There was 649 bp of overlap in the center of the *hph* cassette. SOE-PCR was used to fuse the *Ptrpc* promoter and the *gus* gene. The *gus* cassette was inserted into the pPZP-split-marker-B vector near the right border for use as a negative selection marker. The MCS served as insertion sites for the HR sequences.

The flanking sequences of the *NrCat1* gene were identified using FPNI-PCR (Wang et al. 2011). The PCR products ranged in size from 0.37 to 0.8 kb.

Transformation and gene disruption efficiencies were compared between the split-marker and linear deletion cassettes methods. For *NrCat1*, 1.5 kb of left flanking sequence and 1.3 kb of right flanking sequence were used. For *NrCat4*, 1.0 kb of left flanking and 1.2 kb of right flanking sequence were used. For *NrPex16*, 1.7 kb of left flanking sequence and 1.5 kb of right flanking sequence were used. HR sequences were inserted into

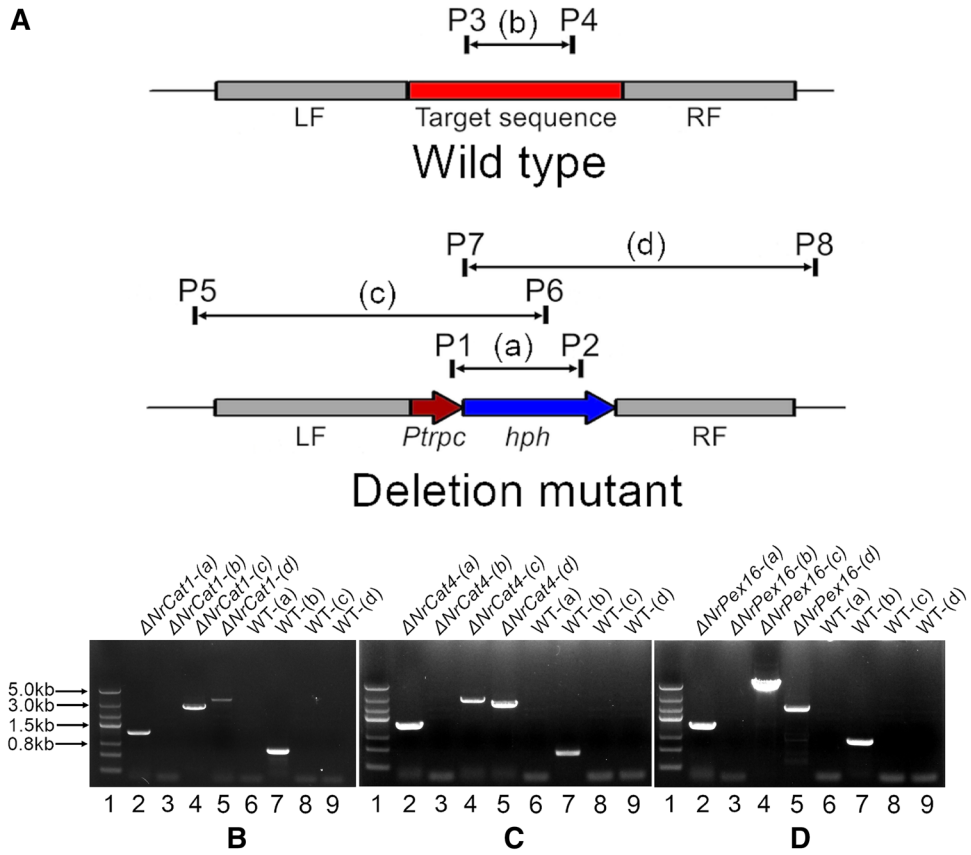

Fig. 3 PCR verification of deletion mutants. **a** Predicted DNA structure of target sequences in WT and deletion mutants. P1 and P2 were extracted from the sequence of the *hph* cassette. P3 and P4 were extracted from the target sequence. P5 and P8 were extracted from the sequence beyond the flanking regions used for HR. P6 and P7 are located in the *hph* cassette and, therefore, no PCR products are expected for products (a), (c), or (d) for WT. **b**–**d** Results of PCR amplification from Δ*NrCat1*, Δ*NrCat4*, Δ*NrPex16*, and WT. Lane 1 shows the positions and sizes of the fragments of Marker III DNA Ladder. Lanes 2–5 show the deletion mutants from which products (a), (b), (c), and (d) were amplified. Lanes 6–9 show the WT from which products (a), (b), (c), and (d) were amplified

the pPZP-split-marker-A, pPZP-split-marker-B-*gus*, and pPZP-Hph-Knockout vectors.

Efficiency of ATMT

Linear deletion cassettes were used to determine the ideal transformation parameters to be applied to ATMT in *N. rileyi*. The yeast-like blastospores obtained after 72 h were incubated at 25 °C on SMAY media at a concentration of 10^6 cells per plate, which was typically suitable for transformation. The highest transformation efficiencies were obtained on co-cultured plates incubated for 72–96 h at 26 °C.

Transformation efficiencies of linear deletion cassettes were approximately 11 times higher than split-marker deletion cassettes. A total of 630 hygromycin B-resistant transformants were obtained from 15 plates using linear deletion cassettes with an average of 42 transformants per plate. In contrast, 229 hygromycin B-resistant transformants were obtained from 60 plates using split-marker cassettes with an average of 3.8 transformants per plate.

Screening mutants and gene disruption efficiencies

In our experiments, 127 linear deletion cassettes and 76 split-marker *NrCat1* transformants, 96 linear deletion cassettes and 84 split-marker *NrCat4* transformants, and 96 linear deletion cassettes and 69 split-marker *NrPex16* transformants were selected to verify the efficiency of HR for the two methods. PCR products (a), (c), and (d), but not (b), were obtained from deletion mutants (Fig. 3). In contrast, only PCR product (b) was obtained from the WT strain (Fig. 3). PCR verification results showed that, using the linear deletion cassettes, the HR rates for *NrCat1, NrCat4*, and *NrPex16* were 1.6, 4.2, and 1.0%, respectively. Meanwhile, from the *gus* assay used for preliminary screening, 42, 62, and 39 putative split-marker transformants of *NrCat1, NrCat4*, and *NrPex16* were obtained, respectively. These transformants grew on media containing hygromycin B, and they had no *gus* activity (Fig. 2). PCR identification confirmed that the HR rates for the *NrCat1, NrCat4*, and *NrPex16* genes were 81.0, 82.3, and 64.1%, after negative screening (Table 2). These results indicated that the transformation efficiency of the split-marker method was lower, but the HR rates were approximately 21 times higher than those of the linear deletion cassette.

In order to assess copy number and integration events of the split-marker method used in this experiment, six *NrCat1* mutants were selected and assessed using Southern blotting (Fig. 4). Only a single copy of the deletion cassette was found in the selected six mutants. No mutant showed evidence of extra ectopic integration.

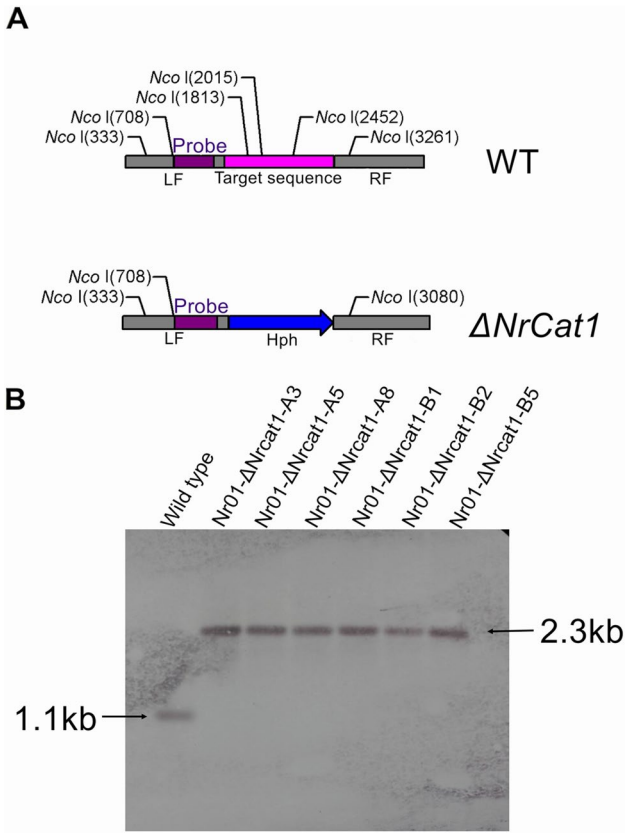

Fig. 4 Southern bolt analysis for confirmation of *NrCat1* mutants. Genomic DNA samples from *N. rileyi* Nr01 WT and six Δ*Nrcat1* samples were digested with *Nco*I, fractionated on a 0.8% TAE (Tris base, acetic acid, and EDTA) agarose gel, and probed with a 1.1 kb *Nco*I–*Nco*I fragment (Nr01-wild-type) and a 2.3 kb *Nco*I–*Nco*I fragment (six Δ*Nrcat1* mutants)

Discussion

Previously, gene targeting in *N. rileyi* was achieved via a one-step gene disruption technique developed by Rothstein (1983). However, the gene targeting efficiency of this method was extremely low. For example, Song et al. (2016) demonstrated that gene targeting efficiencies for *Nrhog1* and *Nrslt2* knockouts were only 2.5 and 6.7%, respectively. In this study, we developed a modified split-marker gene knockout strategy for use in *N. rileyi* with a much higher gene targeting efficiency than that achieved with conventional linear deletion cassettes. In our experiment, *NrCat1, NrCat4*, and *NrPex16* were knocked out using both the modified split-marker strategy and linear gene deletion cassettes and the results showed that gene targeting efficiencies of split-marker were much higher than those of linear deletion cassettes. We also successfully disrupted two other *N. rileyi* genes, *NrSod1* and *NrSod3*, using the split-marker method with an integration frequency between 33 and 78% (unpublished data). The split-marker method has also been reported to achieve a high rate of HR in other fungi (Catlett et al. 2003; Jeong et al. 2007; Liang et al. 2014).

Although the split-marker method allows for high-efficiency gene targeting, it had a transformation efficiency rate that was, on average, 11-fold lower than linear deletion cassettes, in our study. Similarly, in *Grosmannia clavigera*, transformation efficiencies of linear deletion cassettes are approximately 20-fold higher than the split-marker method (Wang et al. 2010). The reason for this is that transformants cannot grow on selective media after integration of incomplete selectable marker genes. However, transformants can grow on selection media when HR occurs between the overlapping regions of the selectable marker gene. As the frequency of ectopic integration decreases, higher frequencies of gene targeting can be achieved. The transformation efficiency also decreased because of the integration of incomplete selectable genes.

Although the split-marker method can achieve higher gene targeting efficiencies, a number of ectopic integration transformants are still obtained. To eliminate ectopic integration transformants, the *gus* gene was introduced as a negative selection marker. As shown in Fig. 2, split-marker cassettes were able to be integrated through the HR pathway, allowing for replacement of the target gene by *hph*. Thus, the *hph* gene was expressed, whereas the *gus* gene was lost

Table 2 Camparation of gene disruption efficiency between split-marker and linear cassette vectors

Target gene	Cassettes	Total transformants	Putative mutants	Identified mutants	PCR screening efficiency (%) after	
					Positive[a]	Positive–negative[b]
NrCat1	Split cassettes	76	42	34	44.7 (34/76)	81 (34/42)
	Linear cassette	127	–	2	1.6 (2/127)	–
NrCat4	Split cassettes	84	62	51	60.7 (51/84)	82.3 (51/62)
	Linear cassette	96	–	4	4.2 (4/96)	–
NrPex16	Split cassettes	69	39	25	36.2 (25/69)	64.1 (25/39)
	Linear cassette	96	–	1	1.0 (1/96)	–

[a]Positive selection, hygromycin B-resistant transformants, mean total transformants

[b]Positive selection, hygromycin B-resistant but not with *gus* ativity transformants, mean putative mutants

during the integration process. However, the transformants showed both hygromycin B resistance and *gus* activity when integration occurred via the NHEJ pathway, indicating that the majority of ectopic integration transformants were eliminated using a *gus* assay (Table 2). Previously, negative selection markers have been used with both linear deletion cassettes and the split-marker method, including studies using the *HV-tk* gene (Gardiner and Howlett 2004), the *GFP* gene (Xu et al. 2014), and the *neo* gene (Liang et al. 2014). However, use of the *gus* gene is both more convenient and faster compared with these genes.

The described technique, based on split-marker HR cassettes with dual selection and the ATMT system is a simple, reliable, and extremely efficient gene-disruption system for use in *N. rileyi*. This technique offers several advantages over traditional linear deletion cassettes, including potential use in dual- or even triple-gene knockout studies.

Acknowledgements This research was financially supported by the National Natural Science Foundation of China (Grant No. 31570073).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Catlett NL, Lee BN, Yoder OC et al (2003) Split-marker recombination for efficient targeted deletion of fungal genes. Fungal Genet Newslett 50:9–11

Chen H, Yin YP, Feng EY et al (2014) Structure and expression of a cysteine proteinase gene from *Spodoptera litura* and its response to biocontrol fungus *Nomuraea rileyi*. Insect Mol Biol 23(2):255–268

Fairhead C, Llorente B, Denis F et al (1996) New vectors for combinatorial deletions in yeast chromosomes and for gap-repair cloning using 'split-marker' recombination. Yeast 12(14):1439–1457

Gardiner DM, Howlett BJ (2004) Negative selection using thymidine kinase increases the efficiency of recovery of transformants with targeted genes in the filamentous fungus *Leptosphaeria maculans*. Curr Genet 45(4):249–255

Gauthier GM, Sullivan TD, Gallardo SS et al (2010) SREB, a GATA transcription factor that directs disparate fates in *Blastomyces dermatitidis* including morphogenesis and siderophore biosynthesis. PLoS Pathog 6(4):e1000846

Ho SN, Horton RM (1991) Method for gene splicing by overlap extension using the polymerase chain reaction. US 5023171 A

Jeong JS, Mitchell TK, Dean RA (2007) The *Magnaporthe grisea* snodprot1 homolog MSP1 is required for virulence. FEMS Microbiol Lett 273(2):157–165

Jiang SS, Yin YP, Song ZY et al (2014) RacA and Cdc42 regulate polarized growth and microsclerotium formation in the dimorphic fungus *Nomuraea rileyi*. Res Microbiol 165(3):233–242

Kim MS, Kim SY, Yoon JK et al (2009) An efficient gene-disruption method in *Cryptococcus neoformans* by double-joint PCR with NAT-split markers. Biochem Biophys Res Commun 390(3):983–988

Li Y, Wang ZK, Liu XE et al (2016) Siderophore biosynthesis but not reductive iron assimilation is essential for the dimorphic fungus *Nomuraea rileyi* conidiation dimorphism transition resistance to oxidative stress pigmented microsclerotium formation and virulence. Front Microbiol 7(440)

Liang LQ, Li JQ, Cheng L et al (2014) A high efficiency gene disruption strategy using a positive-negative split selection marker and electroporation for *Fusarium oxysporum*. Microbiol Res 169(11):835–843

Liu JJ, Yin YP, Song ZY et al (2014) NADH: flavin oxidoreductase/NADH oxidase and ROS regulate microsclerotium development in *Nomuraea rileyi*. World J Microb Biotechnol 30(7):1927–1935

Palma L, Del Valle EE (2015) The fungus *Nomuraea rileyi* growing on dead larvae of *Anticarsia gemmatalis* associated with soybean plants (*Glycine max*) in Esperanza (Argentina). Rev Argent Microbiol 47(3):277–278

Rothstein RJ (1983) One-step gene disruption in yeast. Methods Enzymol 101(6):202–211

Shang YF, Xiao GH, Zheng P et al (2016) Divergent and convergent evolution of fungal pathogenicity. Genome Biol Evol 8(5):1374–1387

Shao CW, Yin YP, Qi ZR et al (2015) *Agrobacterium tumefaciens*-mediated transformation of the entomopathogenic fungus *Nomuraea rileyi*. Fungal Genet Biol 83:19–25

Song ZY, Yin YP, Jiang SS et al (2013) Comparative transcriptome analysis of microsclerotia development in *Nomuraea rileyi*. BMC Genom 14(1):411

Song ZY, Zhong Q, Yin YP et al (2016) The high osmotic response and cell wall integrity pathways cooperate to regulate morphology microsclerotia development and virulence in *Metarhizium rileyi*. Sci Rep-UK 6:38765

St. Leger RJ, Shimizu S, Joshi L et al (1995) Co-transformation of *Metarhizium anisopliae* by electroporation or using the gene gun to produce stable *GUS* transformants. FEMS Microbiol Lett 131(3):289–294

Thakre M, Thakur M, Malik N et al (2011) Mass scale cultivation of entomopathogenic fungus *Nomuraea rileyi* using agricultural products and agro wastes. J Biopestic 4:176–179

Vega-Aquino P, Blanco CA, Sanchez-Pea SR (2010) Activity of oil-formulated conidia of *Nomuraea rileyi* and *Paecilomyces tenuipes* against *Spodoptera Heliothis* and *Helicoverpa larva*e and pupae. J Invertebr Pathol 103:145–149

Wang JY, Zhang Z, Du XF et al (2009) Dual screening for targeted gene replacement mutant in *Magnaporthe oryzae* with *GUS* as negative marker. Chin J Biotechnol 25(1):129–138 (**Chinese**)

Wang Y, Diguistini S, Wang T et al (2010) *Agrobacterium*-meditated gene disruption using split-marker in *Grosmannia clavigera* a mountain pine beetle associated pathogen. Curr Genet 56(3):297–307

Wang Z, Ye SF, Li JJ et al (2011) Fusion primer and nested integrated PCR (FPNI-PCR): a new high-efficiency strategy for rapid chromosome walking or flanking sequence cloning. BMC Biotechnol 11(1):109

Weld RJ, Plummer KM, Carpenter MA et al (2006) Approaches to functional genomics in filamentous fungi. Cell Res 16:31–44

Wendland J (2003) PCR-based methods facilitate targeted gene manipulations and cloning procedures. Curr Genet 44:115–123

Xu C, Zhang X, Qian Y et al (2014) A high-throughput gene disruption methodology for the entomopathogenic fungus *Metarhizium robertsii*. PLoS ONE 9(9):e107657

You BJ, Lee MH, Chung KR (2009) Gene-specific disruption in the filamentous fungus *Cercospora nicotianae* using a split-marker approach. Arch Microbiol 191(7):615–622

Zhou GL, Song ZY, Yin YP et al (2015) Involvement of alternative oxidase in the regulation of hypha growth and microsclerotia formation in *Nomuraea rileyi* CQNr01. World J Microb Biotechnol 311:1343–1352