Construction of pDESTR, a GATEWAY vector for gene disruption in filamentous fungi

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Ayumi Abe, Evelyn B. Elegado and Teruo Sone*

Laboratory of Applied Microbiology, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University.
Kita-9, Nishi-9, Sapporo 060-8589, JAPAN

*Corresponding author: sonet@chem.agr.hokudai.ac.jp Tel: +81-11-406-2502, Fax; +81-11-706-4961

Abstract We have constructed pDESTR, a destination vector of Gateway system especially for gene targeting and disruption in filamentous fungi. The vector was constructed by removing multicloning site of pGEM-T easy vector, and inserting hygromycin phosphotransferase gene construct from pCB1004, and a Gateway vector conversion cassette. In order to construct a DNA for gene disruption, only an inverse-PCR amplification of the restricted, target sequence is needed. After the amplification with a 5'CACC-tagged primer and an ordinary primer, the DNA fragment will be inserted into pENTR/D-TOPO vector and then transferred into pDESTR through LR-recombination reaction. The resulting vector has the disruption construct, after being digested with the restriction enzyme used for the inverse-PCR. The effectiveness of this vector was assessed in *Neurospora crassa*. The use of pDESTR will therefore simplify the construction of a targeting vector, where multiple ligation steps are usually needed.

Key words: GATEWAY, Gene targeting/disruption, *Neurospora*, *Magnaporthe*, Hygromycin B resistance

Recently, the number of filamentous fungi whose genome sequence is revealed is increasing [3]. However, there are many hypothetical genes, i. e. "gene-like" sequences which shows similarity with other known genes of *Saccharomyces* or other organisms or whose functions are not clear. In order to know the function of any interesting hypothetical protein encoding genes, gene disruption is inevitable [3].

In the gene disruption analyses in fungi, gene disruption construct which carries substitution of (or a part of) the gene of interest with a marker gene is usually used. Double crossing-over event will substitute the targeted gene with the disruption construct, and disrupt the gene of interest. In order to obtain the disruption construct, several methods are available.

One of those, PCR-aided construction [12, 13,14] is a rapid and efficient method but not always effective especially when the recombination frequency of the host organism is low, because PCR will limit the length of homologous DNA region and the supply of the DNA. Transposon-arrayed gene knockouts (TAGKO) is very effective when the DNA of interest is inserted in a cosmid vector with the transposon [2]. But there is a bias of the integration frequency throughout the cosmid insert, so one must screen the appropriate transposon-tagged clone prior to use for the gene knockout experiment [2]. Inserting a marker gene into the gene of interest by ordinary recombinant DNA technique is the most popular and reliable method. However, it usually needs several ligation steps and is actually time consuming, especially when many knockout experiments are intended.

Ligation with DNA ligase has become the second choice as the method to insert DNA fragments into vectors. TOPO cloning and Gateway system are examples of alternatives for ligation. The topoisomerase I from *Vaccinia* virus covalently bound to the 3' ends of the vector ligates the DNA ends of the vector and the insert, with higher efficiency than bacteriophage T4 DNA ligase. Gateway technology is a DNA strand exchanging system based on the well-characterized lambda phage site-specific recombination system. This technology enables us to make multiple destination vectors for different purposes such as expression in *E. coli* or *S. cerevisiae,* from a single entry vector with inserted gene of interest. This technology was already introduced to fungal reporter vector [10], and thus can be facilitated in the system of fungal gene knockout.

In this paper, we attempted to construct a new destination vector which is specified to be used in fungal gene disruption. The vector will carry the inverse PCR-amplified fragment of the gene of interest, and will be cut with appropriate restriction enzyme prior to use.

Materials and methods

Bacterial and fungal strains. *Escherichia coli* JM109 and TOP10 (Invitrogen, Carlsbad, CA) was used for general DNA manipulation. *E. coli* DB3.1 was purchased from Invitrogen and used for propagation of plasmid which contains *ccdB* gene. *Neurospora crassa* 74-OR8-1a (FGSC988) and *fr* (FCSC102) were obtained from Fungal Genetics Stock Center (FGSC). These strains were maintained on Vogel's 2% glucose agar.

Vectors and DNAs. pGEM-T easy (Promega, Madison, WI) was self-ligated, and extracted from *E. coli* JM 109 culture prior to use. Plasmid pCB1004 [1] was obtained from FGSC. pENTR/D-TOPO cloning system and Gateway vector conversion kit was purchased from Invitrogen Corp., Carlsbad, CA. Oligonucleotide primers were synthesized by Date Concept Co. Ltd, Sapporo, Japan.

DNA manipulation. All plasmids were extracted using Quantum prep plasmid extraction kit (BIO-RAD, Hercules, CA). Restriction enzymes were purchased from Takara Bio Ohtsu, Japan. Blunting and kination reactions was performed with Takara BKL kit (Takara Bio, Ohtsu, Japan). For the ligation, T4 DNA ligase (New England Biolabs, Inc. Beverly, MA) was used. TOPO cloning of PCR fragment into pENTR/D-TOPO and LR clonase (Invitrogen, Corp., Carlsbad, CA) reactions were done following manufacturer's instructions.

PCR and sequencing. For the amplification of pGEM-Hyg, KOD-plus (Toyobo, Osaka, Japan) was used. Expand long template PCR system (Roche Diagnostics, Penzberg, Germany) was used for inverse PCR. All sequencing reaction was done with BigDye Terminator v1.1 cycle sequencing kit and analyzed by ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA). The nucleotide sequence of pDESTR is available from DDBJ/EMBL/Genbank database under accession no. AB218275.

Transformation of *N. crassa*. *N. crassa* was transformed using electroporation, according to the method described by Ninomiya et al. [6], with some modifications. Forty μl of conidia

suspension (10^8 / ml) and DNA (10 μg) were mixed and placed in an electroporation cuvette with 0.2 cm gap (BIO-RAD, Hercules, CA). Electroporation was performed by a charging voltage of 2.0 kV, a resistance of 800 ohms, and a capacitance of 25 μF, using a Gene Pulser apparatus (BIO-RAD, Hercules, CA). After the electroporation, cells were transferred into 1 ml of Vogel's 1.5% glucose liquid medium, and incubated for 2 hrs at 30 °C with gentle shaking. 200 μl of the culture was then inoculated onto a basal agar plate (Vogel's 2% sorbose, with 0.05% glucose and 0.05% fructose) containing 500 μg/ml hygromycin B.

Mutant analysis. Genomic DNA of transformant (2μg) was extracted with the method by Sone et al. [8]. Southern hybridization was performed using Alkphos direct nucleic acid labeling and detection system (Amersham Biosciences, Piscataway, NJ). Microscopic observation was done with Olympus BX 50 microscope equipped with DP 50 digital camera (Olympus, Tokyo Japan).

Results and Discussion

Basic concept of vector designing. Generally, vectors for gene disruption is constructed by inserting a selective marker gene such as drug resistant genes into the center part of the gene to be disrupted. Alternatively, this assembly can be achieved by ligating inverse PCR product of a restriction fragment including the gene of interest into a vector with a selective marker, and digestion with the same restriction enzyme prior to use for transformation (Fig. 1). This alternative method for construction has some advantages comparing with the conventional method of ligating a marker gene into cloned fragment: 1) Cloning of the flanking region is not necessary, even if the information of the sequence of the region is unknown. Southern analysis of the gene of interest is enough to select a suitable restriction enzyme for the fragmentation of the flanking region around the gene, and a little sequence information of the gene of the interest is enough to design a pair of inverse primers. 2) Only one ligation step is

necessary to construct a vector. For this method, however, some important features are required for the vector: 1) The number of restriction sites should be limited. In the final step prior to transformation of target organism, the vector must be linearized by the restriction enzyme used during fragmentation for inverse-PCR. Restriction sites already present in the vector will affect the availability of the number of candidate restriction enzymes to be used in the inverse PCR step. 2) Efficient ligation of the inverse PCR fragment into the vector is ideal for universal usage.

In order to achieve the necessary conditions above, a strategy of construction was made (Fig. 2). For the basic plasmid structure, pGEM-T easy was used. This may be substituted by other pUC-related vectors such as pUC, pBluescript and other pGEM vectors. First, by digestion with *Pvu* II, MCS (multi-cloning sites) were removed. This effectively reduced the number of restriction sites included in the plasmid. On the other hand, the *hph* gene for hygromycin resistance was amplified from pCB1004, with primers HygF (5'-CCGTGGAGGTAATAATTGA-3') and HygR (5'-CGAAGAACGTTTTCCAATGA-3'). The cassette was the modified version of that in pCSN43, with the reduced number of restriction sites [1]. The amplified, blunted-kinated *hph* cassette was ligated into the blunt *Pvu* II site in the pGEM-T easy/ *Pvu* II longer fragment to be named as pGEM-Hyg. A pair of primers (DESTR1, 5'- GATCGGTGCGGGCCTCTTCG-3' and DESTR2, 5'-GTTGCGCAGCCTGAATGGCG-3') was designed to amplify the almost full length of the pGEM-Hyg, and the amplified DNA was blunted, kinated and ligated with the Gateway cassette rfb in the Gateway vector conversion kit, to become pDESTR, the final product. The vector was checked for the ability to transform *N. crassa* wild type strain and *Magnaporthe grisea* Ina168 strain to hygromycin resistant transformants (data not shown).

For the cloning of the inverse PCR fragment, Gateway system is to be utilized, i.e. inverse PCR fragment will be first ligated into entry vector, such as pENTR/D-TOPO or

pCR8/GW/TOPO. For pENTR/D-TOPO, inverse PCR should be done with proofreading enzyme and a pair of primers, one of which with CACC attached at its 5'-end. For pCR8/GW/TOPO, inverse PCR should be performed with non-proofreading enzyme with any primer pair, but *Eco*RI restriction sites at both sides of the cloning site eliminate the possibility to use *Eco*RI as the enzyme the inverse PCR. The constructed entry vector will be used for LR-recombination reaction with pDESTR, and the inversed fragment will be exchanged with chrolamphenicol resistant gene - *ccdB* casette. Finally, disruption construct will be obtained by digesting the resulting plasmid DNA with the restriction enzyme used in the inverse PCR.

Restriction enzymes suitable for the inverse PCR-based gene disruption with pDESTR are listed in Table 1. Most of enzymes present in MCS of most cloning vectors are included. This will help the users to select an appropriate restriction enzyme for the inverse PCR, and is also economically reasonable. In the case one cannot find natural restriction sites flanking the gene of interest, preliminary PCR with a pair of primers with a restriction site of choice at 5' end of each primer can be applied to obtain the inverse PCR product of the gene of interest.

Disruption of *Frost* gene of *Neurospora crassa* using pDESTR system. As a model experiment, *Frost* gene of *N. crassa* was disrupted using pDESTR (Fig. 3A). DNA sequence of *Frost* flanking region was searched from *Neurospora* genome project web site (http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/index.html), using the sequence previously reported for *Frost* gene [9]. *Xho* I-digested 3658 bp fragment is selected, and a pair of primers, Frinv2 (5'-TCGCCAAACCTCGCGTACTC-3') and Frinv3CACC (5'-CACCTGATTGCCAGCCTTCCG-3') was designed for inverse PCR amplification of *Frost*-flanking region. Genomic DNA of *N. crassa* wild type 74-OR8-1a was digested with

Xho I at 0.1, 0.5, 1.0 ng DNA/μl and combined prior to inverse PCR. A clear amplification was observed and amplicon was used for the cloning into pENTR/D-TOPO after the cleanup with the Microspin S-400HR (Amersham Biosciences, Piscataway, NJ). The resulting plasmid, pENTR-fr was used for the LR-recombination reaction, and a destination vector pDESTR-fr was constructed. pDESTR-fr was digested with *Xho* I prior to the transformation of the *N. crassa* wild type. Transformation of *N. crassa* was performed with electroporation. On the hygromycin containing selection medium, hygromycin resistant colonies appeared. Almost half of the colonies showed abnormal, hyperbranching morphology similar to the *frost* mutant (Fig.4). Six colonies with *frost*-like morphology were picked up and their genomic DNAs were extracted. Southern hybridization was performed to confirm that the expected recombination caused the *frost*-like morphology (Fig. 3B). In all 6 transformants, it was revealed that the expected recombination happened at the flanking region of *frost*, and contained *fr*::*hph* structure.

Conclusion. The gene disruption system using pDESTR, a novel Gateway destination vector reported in this paper gives an efficient way of gene disruption in filamentous fungi. The selection marker of *hph* cassette from pCB1004 was already known to work effectively in other filamentous fungi [5, 11], indicating that pDESTR system will work also in many other fungal strains. A similar Gateway destination vector for gene disruption harboring phosphinothricin resistance gene (*bar*) cassette from pBARKS1 [7] is under construction. This will enable us to disrupt multiple genes. These novel vectors may help the functional characterization of hypothetical genes of genome-sequenced fungi, by combination with non-homologous end joining (NHEJ) – deficient strains [6] and efficient transformation methods [5].

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Figure Legends

Fig.1 Schematic illustration of the inverse PCR-based gene disruption. (1) Selection of an appropriate restriction enzyme (E), which cuts both upstream and downstream of the gene of interest. Then digestion of genomic DNA or larger clone which includes the gene of the interest such as cosmid clone, and self ligation by DNA ligase will be performed. A pair of inverse primers will be designed at the central part of the restriction fragment. The position of these primers will be the junction with the disruption DNA, thus primers should be designed at the desired position for the disruption. (2) Inverse PCR will produce the DNA fragment which connects the upstream and downstream DNA fragments in an opposite order, at the central restriction site (E). (3) Ligation of the inverse PCR fragment into a cloning site (CS) of the vector, which contains a selectable marker. (4) Digestion of the ligated plasmid with the restriction enzyme used for the inverse PCR. This will produce the gene disruption construct.

Fig. 2 The construction of pDESTR. Small arrows indicate primers for PCR. See Results & Discussion for details.

Fig. 3 Disruption of *N. crassa Frost* gene. (A) Maps of genomic DNA of wild type *Frost* locus (WT) and the desired transformant with the disrupted *frost* locus (TF). Disruption construct for the transformation was 7.7 kb *Xho* I fragment and is shown with thick line in TF map. *Frost* gene is indicated with a black solid arrow. *hph*-Amp DNA from pDESTR was indicated with white box. Position of primers for the inverse PCR was indicated with small arrows. The probe for the Southern analysis was indicated with a dotted box. The length of *Eco*R I-*Bam*H I fragments detected in the Southern analysis were indicated. B, *Bam*H I; E, *Eco*R I; X, *Xho* I. (B) Southern analysis of the transformants. Genomic DNA (2μg) was double-digested with *Eco*R I and *Bam*H I and probed with *Frost* gene fragment (left) and *hph*

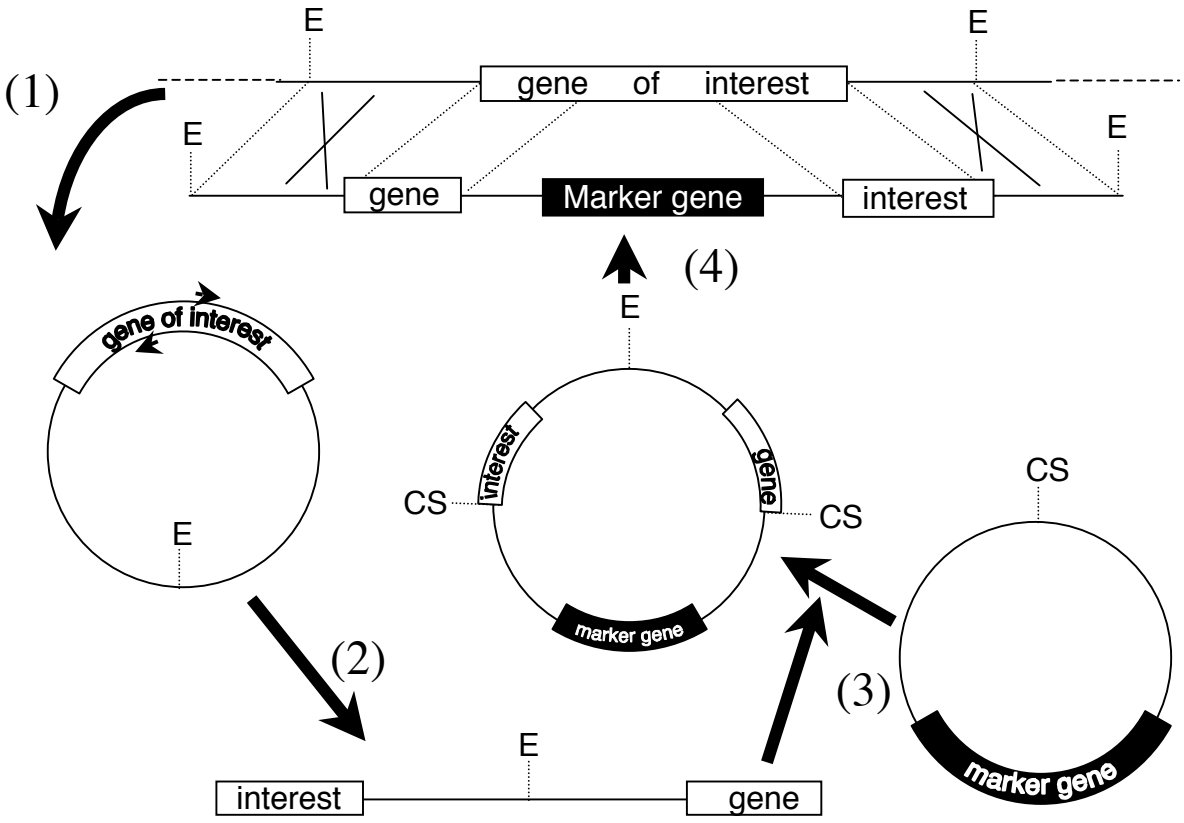
cassette (right). WT: 74-OR8-1a, TF: Six transformants with *frost* phenotype. Sizes are shown in kb.

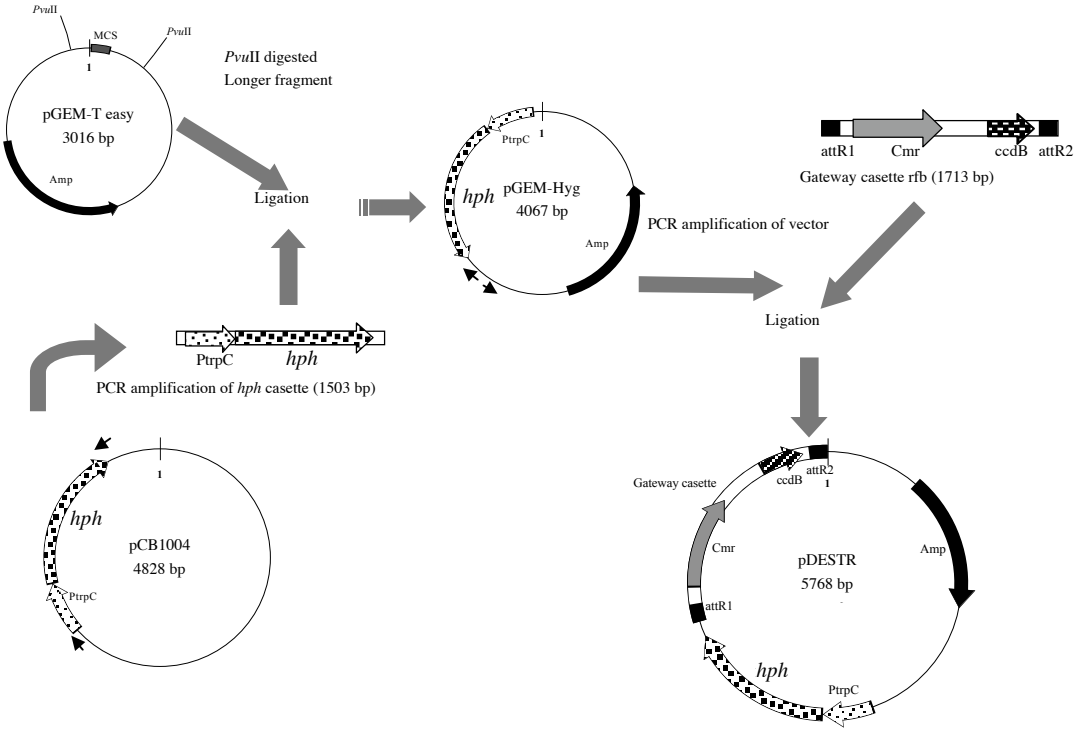
Fig 4 Morphology of *fr*::*hph* disruptant. Bar = 0.1mm.

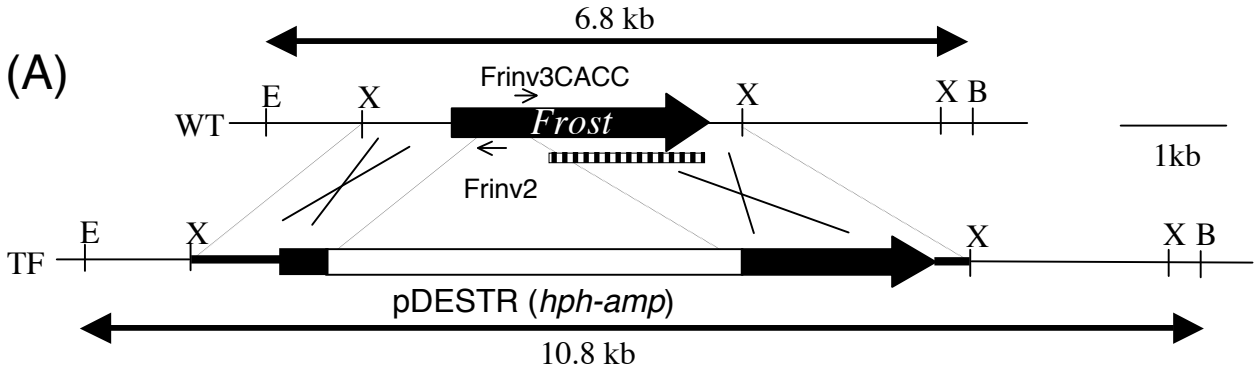
Table. List of restriction enzymes suitable for the inverse-PCR based gene disruption using pDESTR*

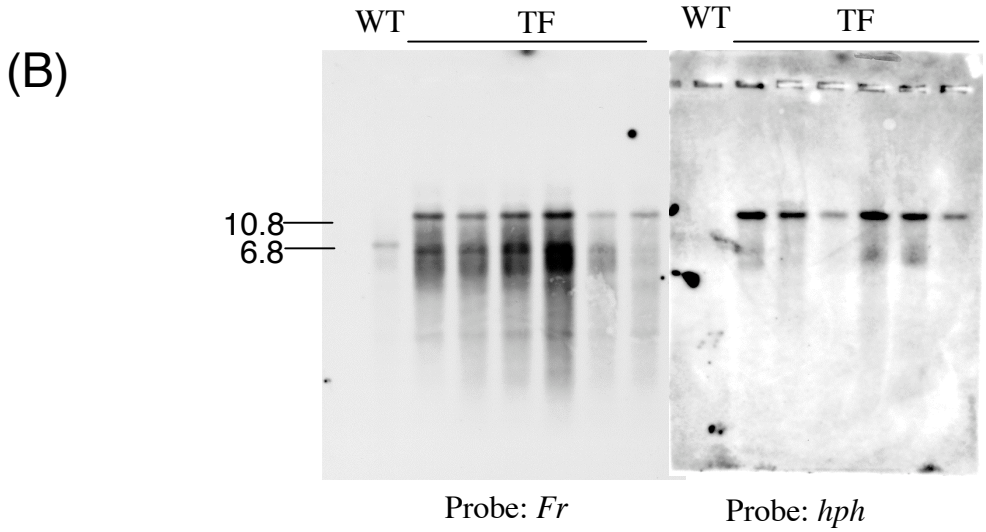
**Aat* I/*Eco*147 I/*Pce* I/*Sse*B I/*Stu* I, *Aau* I/*Bsp*1407 I/*Bsr*G I/*Ssp*B I, *Acc*65 I/*Asp*718 I,
Acv I/*Bbr*P I/*Eco*72 I/*Pma*C I/*Pml* I, *Afe* I/*Aor*51H I/*Eco*47 III/*Fun* I,
Age I/*Asi*A I/*Bsh*T I/*Csp*A I/*Pin*A I, *Ahl* I/*Bcu* I/***Spe*I**, *Apa* I, *Asc* I,
Asu II/*Bpu*14 I/*Bsi*CI/*Bsp*119 I/*Bsp*T104 I/*Bst*B I/*Csp*45 I/*Lsp* I/*Nsp* V/*Sfu* I, *Asu*NH I/*Nhe* I,
Avr II/*Bln* I/*Bsp*A2 I/*Xma*J I, *Bal* I/*Mls* I/*Msc* I/*Msp*20 I, ***Bam*H I**,
Ban III/*Bsa*29 I/*Bsc* I/*Bse*C I/*Bsi*X I/*Bsp*106 I/*Bsp*D I/*Bsp*X I/*Bsu*15 I/*Bsu*TU I/***Cla* I**/*Zho* I,
Bbe I, *Bbv*C I, *Bcl* I/*Bsi*Q I/*Fba* I/*Kso*22 I, *Bgl* II, *Bmg*B I/*Btr* I, *Bsi*W I/*Pfl*23 II/*Psp*L I/*Sun* I,
*Bse*P I/***Bss*H II**/*Pau* I, *Bsp*68 I/*Nru* I, *Bsp*120 I/*Psp*OM I, *Bss*H I/*Pae*R7 I/*Sfr*274 I/*Sla* I/*Tli* I/***Xho* I**,
*Bss*NA I/*Bst*Z17 I/*Bst*1107 I, *Bst*SN I/*Eco*105 I/*Sna*B I, *Cci*N I/***Not* I**, *Cfr*9 I/*Psp*A I/*Vma* I/*Xma*C I,
*Cfr*42 I/***Sac* II**, *Eci*36 II/*Eco*ICR I, ***Eco*R I**/*Fun* II, ***Eco*R V**/*Eco*32 I, *Eco*T22 I/*Mph*1103 I/*Nsi* I/*Zsp*2 I,
Ege I/*Ehe* I/*Sfo* I, *Fse* I, ***Hin*d III**, *Kas* I, ***Kpn* I**, *Mfe* I/*Mun* I, *Mlu* I, *Mly*113 I/*Nar* I, *Mss* I/*Pme* I, *Pac* I,
Pst* I**, *Pvu* II, *Psp*124B I/Sac* I**/*Sst* I, ***Sal* I**, *Sbf* I/*Sda* I/*Sse*8387 I, ***Sma* I**, *Smi* I/*Swa* I, *Srf* I, ***Xba* I**

* Enzymes often present in multi cloning sites of cloning vectors are listed with **bold** letters. Enzymes with ambiguous recognition sequence (for example, *Nci* I (CCSGG)) were not included in this list because such restriction fragments were not always self-ligated. **Isoschizomers are shown with "/".

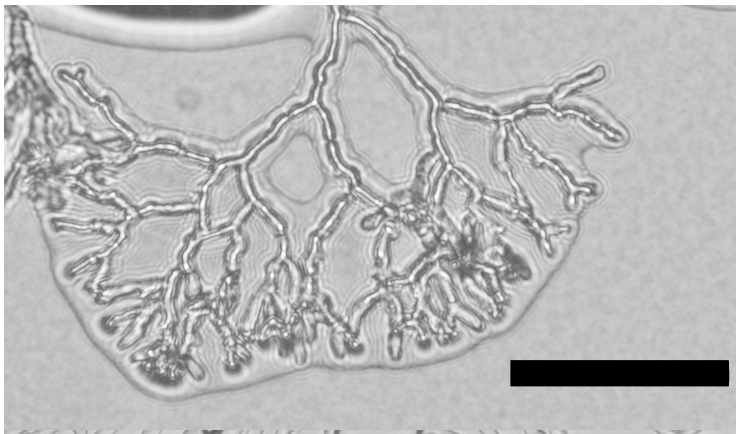

(1)
E
E
gene of interest
E
E
gene
Marker gene
interest
(4)
E
gene of interest
E
CS
interest
gene
CS
marker gene
CS
(2)
(3)
E
interest
gene
marker gene


PvuII
MCS
PvuII
1
pGEM-T easy
3016 bp
Amp
PvuII digested
Longer fragment
Ligation
PtrpC
hph
pGEM-Hyg
4067 bp
Amp
1
attR1
Cmr
ccdB
attR2
Gateway casette rfb (1713 bp)
PCR amplification of vector
Ligation
PtrpC
hph
PCR amplification of hph casette (1503 bp)
hph
pCB1004
4828 bp
PtrpC
1
Gateway casette
ccdB
attR2
1
Cmr
pDESTR
5768 bp
Amp
attR1
hph
PtrpC


(A)
6.8 kb
Frinv3CACC
Frost
WT
E
X
X
X B
1kb
Frinv2
TF
E
X
X
X B
pDESTR (hph-amp)
10.8 kb


(B)
WT
TF
WT
TF
10.8
6.8
Probe: Fr
Probe: hph

fr (FGSC102)



fr:: *hph*
disruptant