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SHORT COMMUNICATION

Construction of a broad host range expression plasmid vector by Golden Gate cloning

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ABSTRACT

Aims: Expression of recombinant proteins across a range of different host organisms has profound contribution to the advancement in biotechnology. In this study, we aimed to construct a highly versatile broad host range (BHR) expression vector, designated as pYL101C.
Methodology and results: The Golden Gate cloning approach was used to construct pYL101C. Key features of pYL101C include a strong integron promoter (P_{INTc}), a BHR pBBR1 origin of replication (ori), gentamycin resistance gene (Gm^R) as a selectable marker and a multiple cloning site (MCS) downstream of the promoter for easy-cloning purpose. To verify the functionality of pYL101C, we cloned the superfolder green fluorescent protein (*sfGFP*) reporter gene into pYL101C and transferred the resultant recombinant plasmid pYL101C::*sfGFP* into various Gram-negative bacteria. Transformants obtained stably expressed strong green fluorescence under blue light excitation even without selection after four passages.
Conclusion, significance and impact of study: The constructed BHR expression vector, pYL101C and recombinant pYL101C::*sfGFP* are stable and can be used to monitor the presence of Gram-negative bacteria, such as endophytes and pathogens in their hosts and environment.

Keywords: plasmid, expression vector, broad host range, Golden Gate cloning

INTRODUCTION

Development of versatile plasmid vectors have made significant contributions to the advancements in biotechnology and molecular biology in recent decades (Karsi and Lawrence, 2007; Gawin *et al.*, 2019; de Grahl *et al.*, 2020; Toh *et al.*, 2020). In Gram-negative bacteria, most broad host range vector systems are mainly based on pRSF1010 and pRK2 replicons (Graupner and Wackernagel, 2000; Meyer, 2009). Plasmid stability and the efficiency for transcription and translation of gene-of-interest are key requisites for an efficient expression vector. Plasmid stability is mainly dependent on the ori, whereas the efficiency of gene expression is determined by the transcriptional control of the promoter within the expression cassette (Rosano and Ceccarelli, 2014). Integron promoters are desirable due to their relatively strong promoter strength and ability to function in a wide range of Gram-negative bacteria (Papagiannitsis *et al.*, 2009). In this study, we developed a broad host range (BHR) expression vector using Golden Gate cloning approach and designated as pYL101C, consisting of the BHR1 and ColE1 replicons, a strong constitutive integron promoter (P_{INTc}) for heterologous gene expression together with a strong transcriptional terminator downstream of the multiple cloning site (MCS) and a gentamycin resistance gene (Gm^R) selectable marker. Equipped with the pBBR1 ori and P_{INTc}-driven expression cassette, pYL101C can serve multitudinous purposes in molecular biology. We showed application of this vector by stably expressing the superfolder green fluorescent protein (*sfGFP*) gene in several Gram-negative bacteria.

MATERIALS AND METHODS

An overview of the construction for plasmids pYL101C and pYL101C::*sfGFP* is illustrated in Figure 1. Briefly, six DNA fragments were separately amplified by polymerase

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chain reaction (PCR) and subjected to one-pot Golden Gate assembly, utilizing two type IIS restriction enzymes (RE), namely *Aar*I and *Esp*3I, and also T_4 DNA ligase, prior to transformation into competent *Escherichia coli* cells (Engler *et al*., 2008). Resultant Gm^R transformants were screened via colony PCR (Table 1) and positive clones were verified by DNA sequencing. For constructing pYL101C::*sfGFP*, the *sfGFP* gene was amplified by PCR (Table 2), cloned into the *Not*I and *Sal*I sites in the MCS of pYL101C and transformed into competent *E. coli* cells. Recombinant plasmids were extracted from putative transformants and verified by *Xba*I and *Sac*I double digestion.

For testing the stability of plasmid pYL101C, *E. coli* harbouring recombinant pYL101C::*sfGFP* was streaked on agar plates and incubated overnight at 37 °C. This procedure was repeated for four passages without selection. In each passage, the bacterial colonies were observed for green fluorescence visualization under blue light (470 nm) excitation. Plasmid pYL101C::*sfGFP* was subsequently electroporated (2500 V, 0.2 cm cuvette) into electro-competent cells of the Gram-negative bacteria, namely *Agrobacterium tumefaciens*, *Klebsiella pneumoniae*, *Pantoea ananatis* and *P. dispersa* as listed in Table 2. All Gm^R transformants showed strong green fluorescence under blue light (470 nm) excitation (Figure 2).

RESULTS AND DISCUSSION

In the Golden Gate cloning approach, all PCR-amplified fragments were flanked with recognition sites of *Aar*I or *Esp*3I, which recognize the sequences 5'-CACCTGC-3' and 5'-CGTCTC-3', respectively, and cleave the DNA downstream of their recognition sites. The use of type IIS REs facilitates a scarless cloning as the recognition site would disappear after cloning, leaving no additional RE recognition site in the constructed pYL101C. This cloning approach allows the fast assembly of fragments orderly and simultaneously. The MCS in pYL101C constitutes of several common restriction enzymes recognition sequences, i.e. *Not*I, *Xba*I, *Sal*I, *Spe*I and *Pme*I, allowing facile cloning of gene-of-interest. The MCS is located downstream of P_{INTc} and upstream of *T7* terminator, placing the cloned gene(s) under the control of strong constitutive P_{INTc}, thus providing convenient sites for molecular cloning.

Besides, the P_{INTc} would provide high constitutive expression of the gene insert cloned in the MCS. P_{INTc} is one of the strongest promoter combinations and is known to drive the expression of numerous downstream genes. It is functional in a wide range of bacteria (Papagiannitsis *et al.*, 2009). The constitutive expression system of pYL101C permits the activation of transcription and translation of the desired genes without the need of external molecules as inducers and one of the benefits is permitting the growth rate of the cell and the protein synthesis rate to be balanced at intermediate level, saving up the time lost during the lag phase (Geisel, 2011). However, the constitutive expression of gene-of-insert may cause burden to the host cell and affect the expression of desired protein. The presence of *lac*I gene in pYL101C simplifies the gene expression cassette to be altered into an IPTG-inducible system in the future.

The constructed pYL101C harbours two origins of replication, namely ColE1 replicon and pBBR1 replicon. The former ori permits high copy per cell and easy genetic manipulation in *E. coli*, whereas the latter allows the vector to be replicated and maintained at medium high copy number in a diverse of Gram-negative bacteria, including *Agrobacterium* spp. which play pivotal role in plant genetic engineering. The pBBR1 ori in pYL101C

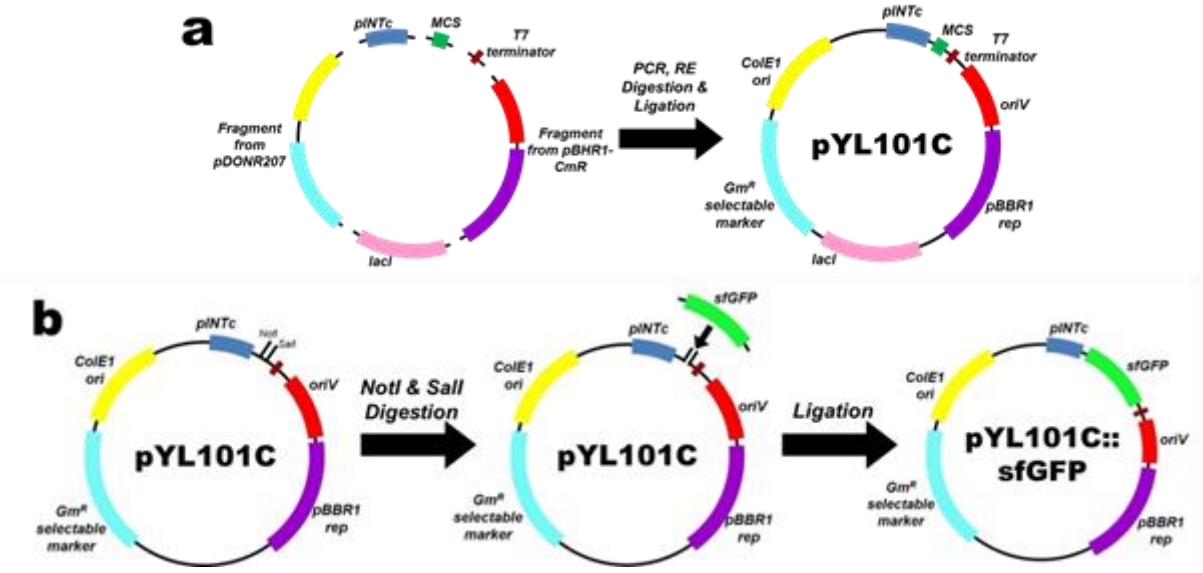

Figure 1: (a) Schematic diagram of the construction of pYL101C (6.0 kb) empty vector. (b) Schematic diagram of the construction of recombinant plasmid, pYL101C::*sfGFP* (6.7 kb).

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Table 1: List of primers used in this study.

DNA fragment	Primer	Sequence
1	F-ColE1	GTCACCTGCAGTC**GAGG**TCACTCATGGTGATTTCTCAC
1	R-rrnbTer	GTCACCTGCTATC**GGCT**GAGAGTAGGGAACTGCCAG
2	F-oriV	TGCACCTGCTATT**AGGA**GCTTGCTCTCCGGGCTTC
2	R-oriV	TGCACCTGCTGCA**CGTA**GGAACAGCGCACTTACGG
3	F-pINTc	GTCGTCTCG**AGCC**GAATTAGACGATATAGGCGCCA
3	R-pINTc	GTCGTCTCG**AAAA**CTTGTATGTATACTCCTTC
4	F-T7ter	GTCGTCTCA**CGCT**CACCGCTGAGCAATAACTAG
4	R-T7ter	GTCGTCTCA**TCCT**CGTCCTGTGGATATCCGGA
5	F-*lac*I	GTCGTCTCA**TACG**ATCACGCATCTTCCCGACA
5	R-*lac*I	GTCGTCTCG**CCTC**GTTGTAGGTGGACCAGTTG
6	F-MCS	GGCTACCACCTGCAGTC**TTTT**GCGGCCGCTCTAGAGTCGACACTAGTGTTTAAAC
6	R-MCS	GGCTACCACCTGCAGTC**AGCG**GTTTAAACACTAGTGTCGACTCTAGAGCGGCCGC
Colony PCR	F-col-pINTc	TCTTCCGACTGAGCCTTTCG
Colony PCR	R-col-oriV	GGCTACGGTCTCAGCGTGCTTTGCGTTCCGTTTGC
sfGFP	F-*sfGFP*	TAGGTCTCA**GGCC**ATGTCAAAAGGAGAAGAGCTG
sfGFP	R-*sfGFP*	TTG**TCGA**CTCTAGAGCGGCCGCTTACTTATAAAGCTCATCCATGCCG

*The RE sites are underlined and the sticky end generated by RE digestions are in bold letters.

Table 2: List of plasmids and bacteria used in this study.

Plasmid or bacterial strain	Description	Source
pBHR1	Template for construction of pBHR1-CmR	MoBiTec (2017)
pBHR1-CmR	Template for pBHR1 ori	This study
pDEST17	Template for *T7* terminator	Invitrogen, USA
pDONR207	Template for ColE1 ori and Gm^R selectable marker	Invitrogen, USA
pING	Template for *lac*I gene	Toh, unpublished
pINT	Template for P_{INTc} promoter	Toh, unpublished
pYL101C	BHR expression vector with P_{INTc} promoter	This study
pYL101C::*sfGFP*	Visualization for sfGFP fluorescence	This study
E. coli TOP10	Host for molecular cloning	Invitrogen, USA
A. tumefaciens C58	Host range testing	ATCC 33970
A. tumefaciens LBA4404	Host range testing	Hoekema *et al*. (1983)
K. pneumoniae	Host range testing	ATCC 13883
P. ananatis	Host range testing	Toh *et al.* (2019)
P. dispersa	Host range testing	Toh *et al.* (2019)

was derived from pBHR1, a derivative of BHR plasmid pBBR122 (MoBiTec, 2017). Plasmid pBHR1 harbours the BHR oriV, enabling it to be maintained at medium high copy number in at least 28 Gram-negative microorganisms. This ori is compatible with ColE1 ori as well as commonly used IncP, IncQ and IncW group plasmids. Here, we validated the host range of pYL101C in *E. coli* and *A. tumefaciens* and further expand the list of bacterial hosts by including *K. pneumonia* and *Pantoea* spp. (Figure 2), which have identified as rice pathogens (Toh *et al.*, 2019). In addition, two *A. tumefaciens* strains, C58 and LBA4404, which have different chromosomal background, were successfully tested (Deeba *et al.*, 2014). Therefore, pYL101C is expected to be compatible with most *Agrobacterium* binary vector systems.

The *sfGFP* reporter gene, which produces bright green fluorescence under blue light excitation, was cloned into pYL101C by RE digestions of *Bsa*I on its 5'-end and *Sal*I at the 3'-end to produce complementary overhangs to the linearized pYL101C. The recombinant plasmid was verified by visualization of green fluorescence under blue light and subsequently digested with *Xba*I and *Sac*I. The expected banding pattern was obtained, indicating the *sfGFP* insert was successfully cloned. The *sfGFP* gene was chosen as the reporter gene in this study because it allows non-invasive monitoring and requires no substrate for visualization. The sfGFP protein is well-known for its brightness, improved folding ability and enhanced folding kinetics, even when fused to poorly folded proteins, leading to higher tolerance to chemical denaturants.

The constructed pYL101C::*sfGFP* enables transformed pathogenic Gram-negative bacteria to emit strong green fluorescence under blue light excitation. Hence, this can be useful for pathogenicity-related study. For instance, pYL101C::*sfGFP* can be used to visually monitor the infection pathway of *Pantoea* spp. on rice

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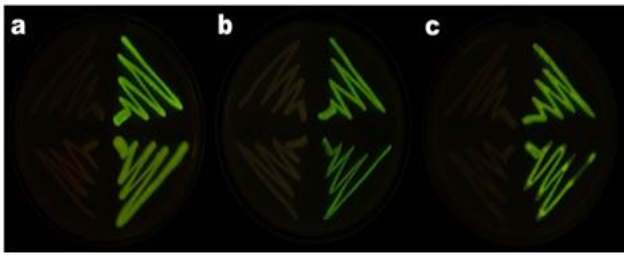

Figure 2: Visualization of sfGFP fluorescence in various Gram-negative bacterial hosts harbouring plasmids, pYL101C empty vector (left) and the recombinant pYL101C::*sfGFP* (right), under blue light (470 nm) excitation. Both plasmids were tested for plasmid stability in (a) *E. coli* TOP10 strain (top) and were able to replicate in (a) *K. pneumoniae* (bottom), (b) *A. tumefaciens* C58 strain (top), *A. tumefaciens* LBA4404 strain (bottom), (c) *P. ananatis* (top) and *P. dispersa* (bottom).

plants and thus aids in elucidating its molecular mechanism of pathogenicity and its respective counteractions. The strong constitutive expression of sfGFP reporter protein afforded by the P_{INTc} promoter enables it to provide real-time observation and localization of these bacteria.

In addition, pYL101C could be used as a shuttle vector between *E. coli* and *A. tumefaciens* and utilized in *Agrobacterium*-mediated transformation (AMT). During AMT, a desired transgene is inserted into a binary vector and co-transformed *Agrobacterium* cells together with a disarmed Ti plasmid. The transgene is subsequently transferred and incorporated into host plant chromosome via *Agrobacterium* type IV secretion system (Wise and Binns, 2016). However, plant transformation efficiency of many crops remained low, thus impeding the application of advanced genome engineering technologies. The virulence genes which are responsible for AMT mostly reside on the *Agrobacterium* chromosome or the large Ti plasmid. Low expression of these virulence genes is a bottleneck for efficient plant transformation. The 6-kb pYL101C with medium copy number may boost virulence genes expression, thus improving plant transformation efficiency.

CONCLUSION

In a nutshell, we showed the ease of using Golden Gate cloning approach in constructing the plasmid vector pYL101C which is a BHR expression vector that can be used in a diverse of Gram-negative bacteria. The strong *sfGFP* expression from the recombinant pYL101C::*sfGFP* reflected the functionality of the expression system in these bacterial strains, including *A. tumefaciens,* which is widely used for plant transformation. This plasmid vector may be used in research that involves manipulation of the genetics of Gram-negative bacteria, such as *Agrobacterium,* for improving plant transformation and elucidation of molecular mechanism of pathogenicity of *Pantoea* spp.

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