Short communication

I-SceI enzyme mediated integration (SEMI) for fast and efficient gene targeting in *Trichoderma reesei*

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A R T I C L E   I N F O

Article history:
Received 13 December 2015
Received in revised form 2 February 2016
Accepted 4 February 2016
Available online 6 February 2016

Keywords:
Hypocrea jecorina
Non-homologous-end-joining
Double strand break
Homologous integration
Ade1

A B S T R A C T

We previously showed that creation of a double strand DNA break (DSB) by expressing I-SceI in an engineered *Trichoderma reesei* (Hypocrea jecorina) strain containing a I-SceI recognition site improved transformation and homologous integration efficiencies. In this study, we further improved homologous integration frequencies by combining I-SceI mediated double strand break with disruption of the *tku70* gene. The inability of the *tku70* mutant to repair a I-SceI mediated DSB via NHEJ was used to force integration of an expression cassette with homologous flanks surrounding the DSB site. Besides expressing I-SceI from a plasmid, we also showed that adding I-SceI enzyme during transformation was successful to generate DSBs. The I-SceI enzyme mediated integration, or SEMI, in combination with a Δ*tku70* mutant has a synergistic effect on homologous recombination efficiencies as 90–100% of the transformants exhibited integration of the expression cassette at the homologous site.

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1. Introduction

Restriction-enzyme-mediated integration has been a useful method for generating non-homologous integration of transforming DNA into the chromosomes of eukaryotic cells (Kuspa and Loomis, 1992; Kuspa, 2006) including filamentous fungi (Sanchez et al., 1998; Sweigard et al., 1998; Thon et al., 2000). Currently, there are four major sequence-specific gene editing technologies, which include (i) meganucleases (Stoddard, 2014), (ii) zinc finger nucleases (ZFNs) (Carroll, 2011), (iii) the transcription activator-like effector nuclease (TALENs) (Jankele and Svoboda, 2014) and (iv) the clustered regularly interspaced short palindromic repeats (CRISPR) with the CRISPR associated (Cas) nuclease (CRISPR-Cas9) (Hsu et al., 2014; Liu et al., 2015; Nedvig et al., 2015). All four nuclease types introduce a site specific double-strand break (DSBs) in the DNA, which is normally repaired by DNA repair mechanisms. Eukaryotic cells have two most prominent mechanisms to repair the broken DNA: (i) the homologous recombination (HR) pathway that involves interaction between homologous sequences for break repair (Heyer et al., 2010; Amunugama and Fishel, 2012) and (ii) the non-homologous end joining (NHEJ) pathway which is predominant in many organisms and repairs the DSB independently of DNA homology (Mao et al., 2008; Davis and Chen, 2013). In fungi, the best-characterized cellular components of homologous recombination system are the RAD proteins which function by coordination upon induction of DNA damage (Boiteux and Juin-Robertson, 2013; Liu and Huang, 2014). The KU heterodimer, which consists of Ku70 and Ku80 proteins, plays an early role in the NHEJ pathway and has a high affinity to DNA ends (Stoddard, 2014). *Ku70* or *ku80* genes deletion lead to significant increase frequency of homologous recombination and has become an important tool to create gene disruption mutants with high frequency in filamentous fungi (Ninomiya et al., 2004; Nayak et al., 2006; Carvalho et al., 2010; Koh et al., 2014).

In our previous study, we have shown that the expression of I-SceI and the subsequent creation of a double strand break (DSB) in a *Trichoderma reesei* strain with an engineered I-SceI restriction sites in its genome stimulates transformation frequency as well as homologous recombination efficiencies (Ouedraogo et al., 2015). In the present study, we combined deletion of *tku70* gene with creating double strand breaks using the *S. cerevisiae* I-SceI meganuclease to further improve targeted integration frequencies. As our previous studies were carried out in the P37Δcbh1pyrG-26 strain
transformation sites. The parental transformed mutants which were used in the transformation mixture. (b and c) Transformation of both strains with the glucosamylase expressing cassette (pJP8) with simultaneous addition of I-SceI enzyme to create DSB. (d) The DSB can be repaired by homologous recombination with the glucosamylase expressing cassette which has homologous regions to the locus containing the I-SceI sites. (e) A correct targeted integration would generate a strain which is resistant to chlorimuron ethyl (aIS), uridine auxotrophic (pyrG) and is expected to express the glucosamylase gene under induction of Pcbh1. Media to grow T. reesei strains and transformations were performed as described (Ouedraogo et al., 2015).

(Outlinedo et al., 2015), a tku70 deletion mutant was made in this background and verified (see Supplementary Figs. 1 and 2 for details). All strains used in this study are listed in Table 1.

Deletion of the tku70 gene in the T. reesei P37Δcbh1pyrG-26 did not reveal apparent growth differences as reported earlier (Guangtao et al., 2009). The effect of deletion of tku70 on homologous recombination (HR) efficiency was tested by deleting the T. reesei ade1 gene (JGI ID: 43662) as the accumulation of a red pigment facilitates easy detection of correctly targeted transformants. Ade1 gene deletion cassettes were constructed (ade1:pyrG) with either 1.0-kb or 0.5-kb flanking regions by PCR as described in materials and methods and Supplementary Fig. 3 and transformed to the tku70 mutant or its parental strain. The homologous recombination (HR) frequencies calculated from the efficiency by which ade1 mutants were obtained go up from 20% (4/20) in the parental to 100% (20/20) in the tku70 mutant when 1.0-kb flanks are used. Shortening the flanking regions to 0.5-kb yielded no ade1 mutants in the wild-type (18 transformants purified) and still 75% (15/20) successful homologous recombination (ade1 mutants) in the tku70 mutant and confirmed that deletion of the tku70 dramatically increases homologous recombination frequencies in T. reesei, similarly as found for other tku70 deletion strains (Guangtao et al., 2009).

We previously engineered a T. reesei strain with a I-SceI restriction site in its genome. The I-SceI restriction site was inserted at a predetermined site (cbh2 locus) using construct (pBPJ6) which is schematically depicted in Fig. 1a and Supplementary Fig. 4. To investigate whether I-SceI mediated creation of a DSB in combination with inactivation of tku70 would further increase and improve homologous recombination frequencies, a PCR amplified linear fragment from pBPJ6 containing the I-SceI restriction site was transformed to the Δtku70 strain (JP11.3.4). Out of seven stable transformants, three transformants contained the complete pBPJ6 cassette integrated into the cbh2 locus and transformant JP12.8 was selected for further analysis (Supplementary Fig. 4).

It was anticipated that the generation of double strand break in the Δtku70 mutant could be harmful to the cells as a DSB cannot be repaired by non-homologous end joining because of the tku70 deletion (McKinney et al., 2013). Indeed, despite several attempts, no transformants were obtained when the pTTT-I-SceI expression plasmid was transformed to JP12.8 whereas transformants were obtained from the same batch of protoplast in control transformations. The results suggest that expression of I-SceI from the cbh1 promoter, even under non-inducing conditions (glucose), is sufficiently high to generate double strand DNA breaks, which are not efficiently repaired in the tku70 mutant, resulting in lethality.

The inability to obtain JP12.8 transformants containing pTTT-I-SceI restricted the possibility to assess whether the HR efficiency in Δtku70 could be further improved by creating a double strand break via controlled I-SceI expression. For this purpose, it was therefore investigated whether addition of recombinant I-SceI during the transformation could improve homologous recombination efficiencies. First, we confirmed that the addition of I-SceI had a positive effect on homologous recombination efficiencies in a parental (functional tku70) background. To test HR frequencies, strain JP7.7 was transformed with linear fragment (pJP8) containing the glucosamylase expression cassette and the aIS selection marker which can integrate via a double cross over event thereby replacing I-SceI/pyrG site for the aIS selection marker (Fig. 1). As shown in Table 2, the addition of I-SceI to protoplast of strain JP7.7 during transformation had a positive effect on obtaining stable transformants and increased homologous recombination efficiencies. The effect of adding I-SceI during the transformation is comparable to
The effect of I-Sce integration on transformation efficiency, stability and homologous integration frequencies (HRF) in tku70+ (JP7.7) and Δtku70+ (JP12.8) T. reesei strains.

| # of primary transformants a | % stable transformants b | pyrC- transformants c | (HRF efficiency) c |
|-----------------------------|-------------------------|-----------------------|-------------------|
| No I-Sce added              |                         |                       |                   |
| 19                          | 22                      | 13/19                 | 18/20             |
| 100                         |                         | 20/20                 | 100%              |
| 5U                          | 31                      | 19/20                 | 20/20             |
| 10U                         | 42                      | 20/20                 | 100%              |
| 25U                         | 49                      | 19/20                 | 20/20             |
| 50U                         | 56                      | 19/20                 | 20/20             |
| 100U                        | 70                      | 20/20                 | 100%              |

a Number of primary transformants on transformation plate. Numbers of primary transformants are shown for the same protoplast batch to be able to compare efficiencies. Results for a typical experiment are shown.
b Primary transformants were purified on selective medium (TrMM + uridine + alS substrate). Stable transformants grow well on these plates. Abortive transformants (not stable) do not grow.
c Stable transformants were tested for the pyrC- phenotype by inoculating spores of each transformant on TrMM with uridine or TrMM without uridine. The efficiency of targeted integration was determined by scoring transformants which have lost the pyrC selection marker (Supplementary Fig. S5). To confirm the targeted integration of the glucoamylase cassette, transformants were further analyzed by Southern blot and for glucoamylase production as described (Ouedraogo et al., 2015).d Protoplast transformation which included the addition of I-Sce (Thermo scientific) were performed as follows: to 200 μL of protoplasts, different amounts of I-Sce enzyme (5–100 Units) plus 4 μL of linearized pJP8 cassette, 30 μL (1× final concentration) enzyme buffer and water was added to give a final volume of the protoplast mixture of 300 μL which was incubated for 15 min at room temperature followed by a 20 min incubation on ice. After the 35 min of incubation, the protoplast mixture was plated out on transformation plate containing 10 mM uridine and 5 μg/mL of chlorimuron ethyl (alS substrate) (Ouedraogo et al., 2015).

In a similar experiment, the Δtku70 mutant (JP12.8) was transformed with the homologous recombination cassette (pJP8) in the presence of increasing amounts of I-Sce. Even when no I-Sce was added, analysis of the transformants revealed that a substantial percentage of transformants (28%) were likely to be the result from a targeted integration event as these transformants showed a pyrC- phenotype (Table 2). The addition of I-Sce during transformation had a further positive effect of HR efficiencies in tku70 mutant leading to 90–100% homologous recombination frequencies when adding low concentrations of I-Sce (5 or 10 Units I-Sce per transformation). Higher amounts of I-Sce addition (25 Units of I-Sce per transformation or higher) turned out to be harmful to the tku70 protoplasts as the number of transformants obtained dropped significantly (Table 2). However, the stable transformants obtained are likely to be the result from a targeted integration event as all transformants showed a pyrC- phenotype.

To analyze if the pyrC- transformants obtained after transformation, contain the glucoamylase expression cassette at the intended locus via a HR event, 18 pyrC- transformants were randomly selected from the different transformation experiments and analyzed for glucoamylase production and integration patterns. As shown in Fig. 2, in all 18 pyrC- transformants, comparable levels of glucoamylase activity was measured in the culture fluid. In 18 randomly selected pyrC- transformants, only four transformants produced glucoamylase with variable levels (Fig. 2).

Ten pyrC- transformants and five pyrC+ transformants randomly taken from the transformant analyzed for glucoamylase activity showed high homogeneity of glucoamylase activity compared to random integration transformants (pyrC- transformants). The targeted (n=18) and random (n=18) integrated transformants are pyrC- and pyrC+ phenotype, respectively. The pyrC- transformants were selected randomly from the different transformation experiments with the I-Sce enzyme. Plots were created using GraphPad Prism 6 (column scatter graph). The horizontal bars represent the mean values with standard deviations.
production (see above) were analyzed by Southern blot to determine the integration pattern of the glucoamylase cassette. As shown in Supplementary Fig. 5 all pyrG- transformants analyzed had the glucoamylase gene targeted integrated at the predefined locus. The pyrG- transformants have the cassette randomly integrated into the genome according to the differences in integration pattern between the transformants.

This study demonstrated the successful use of I-SceI enzyme to improve the transformation efficiency and targeted integration frequencies of the DNA cassette in both wild-type and tku70 mutant strains. The approach of using I-SceI enzyme is a variant of the restriction enzyme-mediated insertional (REMI) approach that has been used in several studies to increase the frequency of insertion mutagenesis in fungi (Manivasakam and Schiestl, 1998; Sanchez et al., 1998; Sweigard et al., 1998; Yaver et al., 2000). However, a major advantage of I-SceI enzyme mediated integration (SEMI) over REMI is that I-SceI cuts only once in the genome at a predetermined site. The SEMI gene targeting system is therefore well suitable for high throughput screening of enzyme variants or gene libraries in T. reesei and readily applicable to other fungal expression hosts.

Acknowledgements

We thank Bob Schepers for his helpful contributions to the project and Dr. Jaap Visser for helpful discussions. This project was carried out within the research programme of the Kluiver Centre for Genomics of Industrial Fermentation, which is part of the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2016.02.012.

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