An efficient shortened genetic transformation strategy for filamentous fungus *Trichoderma reesei*

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The filamentous fungus *Trichoderma reesei* is one of the most important fungi for the production of cellulases and xylanases, which can be used for biofuel production from lignocellulose. We aimed to develop an effective selection marker system for more extensive functional genomic studies in the fungus *T. reesei*, and to construct better industrial transformants for producing cellulases. Here, we present a novel effective G418 selection marker to use a codon-optimized neomycin phosphotransferase II gene *nptII* to transform *T. reesei*. We developed an effective and erasable selection marker, lcNG, and a combined genetic transformation system for gene manipulation in *T. reesei* using a two-*Agrobacterium*-mediated transformation method. This transformation strategy combines two steps in the transformation protocol, which saves 15–30-day’s time. The system could be a useful tool for the genetic engineering of *T. reesei*.

Key Words: G418; optimized codons; *Trichoderma reesei*; two-*Agrobacterium*-mediated transformation

Introduction

The filamentous fungus *Trichoderma reesei* (synonym *Hypocrea jecorina*) is a widely-used organism (Adedokun et al., 2015; Zhong et al., 2012) for investigating the production of cellulolytic and hemicellulolytic enzymes, which are used in broad areas of industry (van den Brink et al., 2014; Wang et al., 2014). Moreover, *T. reesei* has become a potential model organism for evaluating the regulation of cellulase and xylanase expression (Chen et al., 2018). An efficient genetic transformation strategy with an effective selection marker is the key for successful *T. reesei* genetic modification.

Numerous antibiotic resistance genes, such as the hygromycin B resistance gene *hyg* (Ruiz-Diez, 2002) and the phosphinothricin resistance gene *bar* (Zhang et al., 2016a), have been used as selection markers for *T. reesei* transformation. The most commonly used antibiotic selection marker gene in filamentous fungus genetic transformation is *hyg*. We exploited the erasable *hyg* marker LML2.1 (Zhang et al., 2016b) and the erasable *bar* marker lcB (Zhang et al., 2016a, b) for several rounds of genetic transformation in *T. reesei*. However, screening using the lcB marker was very difficult, owing to the high number of spontaneous mutations which contributed to the development of phosphinothricin-resistant false positive colonies (Zhang et al., 2016a). This led to a significant number of false positive colonies after several rounds of screening. Therefore, lcB is not an effective selection marker for several rounds of genetic transformation. To improve the efficiency during continuous rounds of genetic transformation, we need to combine two or more effective selection markers. However, lcB is not a suitable selection marker for this combination.

Geneticin (G418), an aminoglycoside antibiotic that inhibits the elongation step of translation in both prokaryotic and eukaryotic organisms (Yoruk and Albayrak, 2015), is inexpensive and can be manipulated easily. G418 markers have been successfully used in *Trichoderma atroviride* transformation (Gruber et al., 2012), but it has not been used in *T. reesei*.

Therefore, we developed an erasable G418 selection marker, lcNG, for several rounds of gene manipulation in *T. reesei*. Furthermore, we combined LML2.1 (hygromycin B marker) and lcNG (G418 marker) to develop an efficient shortened genetic transformation strategy by combining two steps in one.
Materials and Methods

Strains and media. Escherichia coli DH5a was used as a host strain for the recombinant DNA. T. reesei QM6a (ATCC 13631) and RUT-C30/Δku70 (Zhang et al., 2016b) were used as the hosts for genetic transformation. Agrobacterium tumefaciens AGL-1 was used in the Agrobacterium-mediated transformation system. Lysogeny broth (LB) was used for the growth of E. coli and A. tumefaciens. Mandels’ medium (Xylose or glucose 10 g/l; tryptone 1 g/l; yeast extract 0.5 g/l; (NH₄)₂SO₄ 1.4 g/l; urea 0.3 g/l; KH₂PO₄ 2 g/l; CaCl₂ 0.3 g/l; MgSO₄ 0.3 g/l; FeSO₄·7H₂O 5 mg/l; MnSO₄·H₂O 1.6 mg/l; ZnSO₄·7H₂O 1.4 mg/l; CoCl₂·6H₂O 2 mg/l; pH 5.5) was used for the general fungal culture (Mandels and Weber, 1969).

T. reesei growth inhibitor scanning. To determine the concentration of G418 which was lethal for mycelia and conidia of T. reesei strains, both agar plugs covered with fungal mycelium (diameter 3 mm) and 10⁵ conidia were mixed, and the mixture was spread evenly on Hybond N (0.45 μm) and deposited onto freshly poured IMAS plates (Lv et al., 2012). After 2 days of co-cultivation at 25∞C, the membrane was transferred onto freshly poured IMAS plates (Amersham Biosciences) and deposited onto freshly poured Mandels’ agar plate plus G418 (0–400 μg/ml).

Plasmid construction. Neomycin phosphotransferase II gene nptII was obtained by PCR using the plasmid pPIC9K (Invitrogen) as the template and the primers G1 and G2 (Table 1). Codon optimized OnptII was designed according to the codon bias of T. reesei and synthesized in vitro by Generay (Shanghai, China). The expression vector pSB903 (Zhang et al., 2016a) was used to express nptII and OnptII to form G418 resistant vectors 9G and 9NG, respectively. The self-excision vectors lcG (for nptII) and lcNG (for optimized OnptII) were constructed according to the protocol reported by Zhang et al. (2016b) by adding the xylose-induced cre recombinase cassette and two loxP sites to 9G and 9NG, respectively. The deletion cassette for the deletion of genes cbh1 (Trire2_123989) and egl1 (Trire2_122081) were constructed using up- and downstream sequences (~1–2 kb) of each gene, obtained by PCR, with the lcNG and LML2.1 cassettes. The genomic DNA of T. reesei was used as the template.

All vectors were constructed using a pEASY-Uni Seamless Cloning and Assembly Kit (TransGen). All primers are listed in Table 1. All constructs (Figs. 1 and 2) were verified by DNA sequencing.

Modified Agrobacterium-mediated transformation protocol for lcG and lcNG. Transformation of the hygromycin B marker cassettes (LML2.1-based plasmids) was performed based on protocols described by Zhang et al. (2016b). The lcG- or lcNG-based plasmids were introduced into T. reesei by Agrobacterium-mediated transformation (Lv et al., 2012) with the following modifications. In brief, the A. tumefaciens strain AGL1 harboring lcG or lcNG cassette and conidia of T. reesei were mixed, and the mixture was spread evenly on Hybond N (0.45 μm, Amersham Biosciences) and deposited onto freshly poured IMAS plates. After 2 days of co-cultivation at 25∞C, the membrane was transferred onto freshly poured Mandels’ medium plates containing 400 μg/ml cefotaxime and 200 or 400 μg/ml G418.

Two-Agro bacterium-mediated transformation protocol. The lcNG- and LML2.1-based plasmids were simultaneously introduced into T. reesei by two-Agro bacterium-mediated transformation. In brief, two A. tumefaciens strains harboring lcNG and LML2.1 based plasmid, respectively, and conidia of T. reesei were mixed, and the mixture was spread evenly on the Hybond N (0.45 μm, Amersham Biosciences) deposited onto freshly poured IMAS plates. After 2–3 days of co-cultivation at 25∞C, the membrane was transferred onto freshly poured Mandels’ medium plates containing 400 μg/ml cefotaxime, 400 μg/ml G418, and 75 μg/ml hygromycin B.

| Name | Oligo sequences (5’ to 3’) |
|------|-----------------------------|
| G1   | GACATCACATGTCGACCGTG(Tmpl)ATGAGCCATATTCAACGGGAAAC          |
| G2   | GTTGGTGGTTGCGCCTCCGAC(GauB)TTAGAAAACTTCATGCACTAATT            |
| nptII_confirm-1 | CTCCTCCGACCATCAACATT cities for the construction of plasmids by the Assembly Kit. |
| nptII_confirm-2 | TCACCGAGGAGATTTCCATAG                                    |
| OnptII_confirm-1 | CGAGATGGTCCGCTCAACT                                      |
| OnptII_confirm-2 | CGACGGCTATCCTGGGACAACG                                   |
| hyg_confirm-1 | CGGGAAGATCCTGCTCAATACA                                  |
| hyg_confirm-2 | TACACGACCCTGGTCACG                                       |
| bar_confirm-1 | CACCATGTCACCACTACATC                                      |
| bar_confirm-2 | CGGAGTCCGGTCGCTGAAG                                     |
| Del-cbh1-1 | GATATCGAGAATTGTCGAACGAGACATCAACATCAGTC                    |
| Del-cbh1-2 | TGCTATACGAAATTGTCGAACGAGACATCAACATCAGTC                   |
| Del-cbh1-3 | ACTATATGCAGCGTTCACTGGAGAACTTCATG                        |
| Del-cbh1-4 | AGTCGCCAGATCCATCCATCTGCTCCATCAATACATC                      |
| Dcbh1_confirm1 | GATGGTGGTGGAGACAAAT                                       |
| Dcbh1_confirm2 | TCTGGAGACATATGTCGTTGAG                                  |
| Del-egl1-1 | GATATCGAGAATTGTCGAACGAGACATCAACATCAGTC                    |
| Del-egl1-2 | TGCTATACGAAATTGTCGAACGAGACATCAACATCAGTC                   |
| Del-egl1-3 | ACTATATGCAGCGTTCACTGGAGAACTTCATG                        |
| Del-egl1-4 | AGTCGCCAGATCCATCCATCTGCTCCATCAATACATC                      |
| Degl1_confirm1 | TGACTGGAACCTACCCCTGGA                                  |
| Degl1_confirm2 | GTGATGATGTTGGAAGATC                                        |

*The overlapped sequences in primer is underlined which is used for construction of plasmids by the Assembly Kit.
Efficient transformation of *T. reesei*

Characterization of the transformants. For each vector transformation, 6–12 transformants were collected, and we used diagnostic PCR (nptII-confirm-1/nptII-confirm-2, OnptII-confirm-1/OnptII-confirm-2, Dcbh1-confirm1/Dcbh1-confirm2, or Degl1-confirm1/Degl1-confirm2 pairs) to verify that the cassettes were successfully integrated into the genome and that the target genes were interrupted (Supplementary Materials Fig. S1).

Excision of the lcNG and LML2.1 markers. The excision protocol was performed following the protocol reported by Zhang et al. (2016b) by xylose-induced cre recombinase. The verification medium was Mandels’ agar plate containing G418 antibiotics for 7 days of cultivation. B. Construction of G418 marker cassette lcG. P, promoters; nos T., nos terminator (GenBank: KF499077.1); cre cassette, Cre recombinase expression cassette; :loxP, a 34-bp target DNA sequence; Tru., truncated; syn2, xylanase II encoding gene.

Results

Sensitivity of wildtype *T. reesei* to geneticin G418

To determine the lethal concentration of G418 against *T. reesei* wild-type strain QM6a, agar plugs covered with fungal mycelium (diameter, 3 mm) and $10^7$ conidia were placed on Mandels’ agar plate plus G418 ranging from 0 µg/ml to 200 µg/ml. Mycelial growth was completely inhibited by 200 µg/ml of G418. Conidial germination was completely blocked by over 150 µg/ml of G418 (Fig. 1a). Based on these results, 200 µg/ml G418 was used for the selection of *T. reesei* transformants.

Construction of G418 selection marker in *T. reesei*

To develop the *Agrobacterium*-mediated transformation system using G418 as the selection marker, lcG (Fig. 1b) was constructed and introduced into *T. reesei* using a modified *Agrobacterium*-mediated transformation protocol. Several *T. reesei* clones with G418 resistance survived after lcG transformation and selection on G418 plates. Eight visible colonies were randomly selected from the plates. Two of the eight clones were able to grow on G418 plates in the second-round cultivation, which were confirmed to have nptII integrated in their genomes by PCR-amplified nptII gene from their genomes and mitotic stability for G418 resistance after three rounds of single spore isolation. The transformation experiment was repeated three times to get two, four, and five accurate transformants,
respectively, from eight visible clones. The result of lcG transformation of T. reesei is similar to the report of Gruber et al. (2012), who established the nptII gene as a novel selection marker for the transformation of Trichoderma atroviride. Our results demonstrate for the first time that G418 marker has been successfully applied in T. reesei transformation.

Optimization of G418 selection marker in T. reesei

The number of transformants of lcG transformation was much lower (only 2–6 small colonies per plate after 4 days of cultivation in our study) than that of LML2.1 transformation (50–100 colonies per plate after 4 days of cultivation). The number of lcG transformants increased to more than 40 when the incubation time was prolonged to ten days. The significant difference between the growth rates of lcG- and LML2.1-transformants hinders the possibility to combine two transformation steps into one step; however, lcG can be applied in T. reesei transformation independently.

The nptII gene was cloned from E. coli. The production level of neomycin phosphotransferase (NPTII) encoded by nptII may be insufficient, because its encoding gene contains six of the rarest codons (“TTA” coding leucine; http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=51453) of T. reesei (Supplementary Materials Fig. S2). Therefore, we optimized the codon usage of NPTII using the preferred codons of T. reesei and synthesized its encoding gene (named as OnptII) to construct lcNG (Fig. 2).

We successfully screened the transformants using lcNG. Consequently, the number of lcNG transformants was 40–70 colonies per plate using 10⁶ T. reesei conidia for 4 days of cultivation, of which more than 96% of the transformants were positive for OnptII gene integration. The efficiency and accuracy of lcNG were higher than those of lcG. The results demonstrate that codon optimization improves heterologous protein production in T. reesei, and the codon optimized OnptII can be more easily translated to the protein NPTII in T. reesei than the natural nptII sequence, and that the improved production of NPTII enhances the growth rate of lcNG transformants on G418 plates.

To verify the feasibility of using lcNG for several rounds of genetic transformation, we transformed lcNG, lcG, lcB, and LML2.1, respectively, into T. reesei QM6a and per-
formed self-excision three times. Eight to ninety-six clones were randomly selected from plates and positive clones were confirmed by the PCR-amplification of resistance genes. Then, the positive clones were cultivated in xylose-based medium for marker self-excision for the next round of transformation.

We transformed lcNG into *T. reesei*, which then excised itself. We used the same protocol to transform it twice more consecutively. Similarly, the other transformations were performed using lcG, LML2.1, and lcB. The efficiencies of the three rounds of transformation using lcNG, lcG, lcB, and LML2.1 are shown in Table 2. This indicates that lcNG is an effective selection marker for several rounds of genetic transformation, similar to the hygromycin resistance marker LML2.1 and higher than that of lcG. This also indicates that lcB is not suitable for several more rounds of genetic transformation as they exhibited a positive rate below 5% at the third round of transformation.

**Combining two steps in one**

To save time during continuous rounds of genetic transformation, we attempted to combine two genetic manipulations into one step using G418 and hygromycin B markers simultaneously. We found that hygromycin-resistant *T. reesei* LML2.1-transformants had a characteristic colony morphology in media containing 200 μg/ml G418 (Fig. 3a). However, G418-resistant *T. reesei* lcNG-transformants could not grow in media containing 75 μg/ml hygromycin B. Hygromycin B and G418 are both aminoglycoside antibiotics (Martinelli and Sheikh, 1991) and their resistance genes share a certain degree of cross-resistance in our study. Hence, we could not screen out positive transformants using 200 μg/ml G418 plates when the host is hygromycin-resistant and could not use hygromycin B and G418 markers in tandem, or simultaneously, to develop an efficient genetic transformation strategy combining two steps into one. To address this, we made a modification.

Because the hygromycin-resistant strain cannot grow on 400 μg/ml G418 plates, we increased the concentration of G418 from 200–400 μg/ml. *T. reesei* lcNG transformants can grow on 400 μg/ml G418 plates and show a similar growth rate as on 200 μg/ml G418 plates. Therefore, we chose 400 μg/ml G418 and 75 μg/ml hygromycin B for selection of two-*Agrobacterium*-mediated transformation.

To verify a combined genetic transformation system, we took the *T. reesei* RUT-C30Δku70 strain (Zhang et al., 2016b) as the host for its high efficiency of gene targeting, which is nearly 100% (Zhang et al., 2009). As shown in Fig. 4a, 60 days were needed for two rounds of continuous gene deletion using only one selection marker. First, the construct LML2.1- cbh1 (Fig. 4d) was introduced into *T. reesei* RUT-C30Δku70 using *Agrobacterium*-mediated transformation (Zhang et al., 2016b), which takes 15 days to screen for positive hygromycin B resistant transformants, and deletion of *cbh1* was confirmed by diagnostic PCR using primer pairs *cbh1*-confirm1/2. Second, the marker cassette LML2.1 was self-excised by xylose-induced cre recombinase to generate RUT-C30Δku70Δcbh1 strains with no resistance gene (Zhang et al., 2016b), allowing for the use of the LML2.1 marker again to delete another gene, egll. Third, the construct LML2.1- egll (Fig. 4d) was introduced into the RUT-C30Δku70Δcbh1 strains using the *Agrobacterium*-mediated transformation protocol to screen for positive hygromycin B resistant transformants and deletion of egll was confirmed by diagnostic PCR using primer pairs.
Degl1-confirm1/2. Finally, the marker cassette LML2.1 was self-excised again to generate RUT-C30Δtku70Δcbhl2egl1 strains.

Here, we developed a combined genetic transformation system in T. reesei. As shown in Fig. 4b, first, the construct LML2.1- cbhl1 (Fig. 4d) was introduced into T. reesei RUT-C30Δtku70 using Agrobacterium-mediated transformation (Zhang et al., 2016b) to generate RUT-C30Δtku70Δcbhl1 strains with hygromycin B resistance gene and deletion of cbhl1 was confirmed by diagnostic PCR. Second, the construct lcNG-egl1 (Fig. 4d) was introduced into the RUT-C30Δtku70Δcbhl1 hygromycin B resistant strains using the Agrobacterium-mediated transformation protocol to screen for positive G418 and hygromycin B double resistant transformants and deletion of egl1 was confirmed by diagnostic PCR. Third, the hygromycin B and G418 markers were self-excised simultaneously using xylose-induced excision protocol (Zhang et al., 2016b). In total, 15 days are saved by the proposed method (Figs. 4a and b).

Adding to the convenience of the method, the vectors LML2.1- cbhl1 and lcNG-egl1 can be simultaneously introduced into T. reesei using a two-Agrobacterium-mediated transformation protocol. The positive transformants can be screened for by a one-step screening on agar containing both hygromycin B and G418 (Fig. 4c). The two markers were additionally shown to have been excised simultaneously. In total, 30 days are saved by using this protocol (Fig. 4c).

The homologous integration and self-excision frequencies of the three solutions A, B, and C are shown in Table 3. The efficiency of homologous integration in solutions A and B is approximately 100%, which is in accordance with previous research on T. reesei Δtku70 strains (Zhang et al., 2009, 2016b). However, the efficiency of homologous integration in solution C is approximately 50% lower than that of solutions A or B. Another 50% of transformants from solution C showed only one gene knocked out (cbhl1 or egl1), which indicates that two-Agrobacterium-mediated transformation would increase the workload for screening the positive transformants with two-gene homologous integration. However, a success rate of 50% is still acceptable. The self-excision frequencies in solution A are similar with the reported 70–90% from the original study (Zhang et al., 2016b). However, the self-excision frequencies in solutions B and C are 50–60% lower than that of solution A because two marker genes are needed to excise in one step in solutions B and C, which reduces the self-excision frequency.

### Discussion

In this study, we developed a novel, erasable G418 selection marker for several rounds of gene manipulation in T. reesei and an efficient genetic transformation strategy by combining hygromycin B and G418 markers, thereby reducing the time needed to complete these manipulations.

Vector lcG (Fig. 1b) is the first G418 marker successfully applied in T. reesei transformation. We can get 2–8 small T. reesei colonies per plate using 106 conidia in four days after lcG transformation. Gruber et al. (2012) did not mention the number of colonies per plate using nptII transformation in T. atroviride, but the number of transformants of lcG transformation is typically lower than that of LML2.1 transformation (50–100 colonies per plate in four days after transformation). The low number of colonies with lcG transformation could be a result of low growth rate, and prolonging the cultivation time could produce more colonies. However, the significantly different growth rates between lcG- and LML2.1-transformants hinders the strategy of combining two genetic manipulations into one step.

There are several reasons that may explain the low growth rate of lcG transformants. Selection marker genes derived from bacteria are poorly translated in eukaryotes owing to the differences in codon utilization patterns (Sun et al., 2014). The encoding gene nptII contains six of the rarest codons (“TTA” coding leucine) of T. reesei (Fig. S2). Weak promoters used to express the resistance gene nptII could be another reason for the low growth rate. In

### Table 3. Homologous integration and self-excision efficiencies of deleting cbhl1 and egl1 genes.

| Solutions | Agrobacterium-mediated transformation steps | Homologous integration frequencies | Self-excision frequencies |
|-----------|------------------------------------------|-----------------------------------|--------------------------|
|           |                                          | Positive colonies       | Percentage | Positive colonies       | Percentage |
| A         | 1                                        | 8(8)                  | 100%       | 87(96)                  | 90.6%      |
|           | 2                                        | 8(8)                  | 100%       | 83(96)                  | 86.5%      |
| B         | 1                                        | 8(8)                  | 100%       | 56(96)                  | 58%        |
|           | 2                                        | 8(8)                  | 100%       | 51(96)                  | 52%        |
| C         | 1                                        | 4(8)                  | 50%        |                         |            |

**A-1**: Generate hygromycin B-resistant transformant using the LML2.1-cbhl1 cassette by Agrobacterium-mediated transformation.

**A-2**: Generate hygromycin B-resistant transformant using the LML2.1-egl1 cassette by Agrobacterium-mediated transformation.

**B-1**: Generate hygromycin B-resistant transformant using the LML2.1-cbhl1 cassette by Agrobacterium-mediated transformation.

**B-2**: Generate hygromycin B and G418-resistant transformant using the lcNG-egl1 cassette by Agrobacterium-mediated transformation.

**C**: Generate hygromycin B and G418-resistant transformant using the LML2.1-cbhl1 and lcNG-egl1 cassettes by two-Agrobacterium-mediated transformation.
lcG, the nptII gene was under the control of the truncated xyN2 promoter from T. reesei, whose transcriptional level in glucose-based media is low (Zhang et al., 2016a). However, the advantage of the truncated xyN2 promoter is that it is short, only 200 bp in length, which is helpful in constructing a compact G418 marker cassette.

Therefore, we synthesized a new NPTII gene, Onp112, with optimal codon usage of T. reesei to construct lcNG (Fig. 2). The growth rates of lcNG-transformants significantly improved, and the number of lcNG transformants was 40–70 colonies per plate using 10^6 T. reesei conidia after 4 days of cultivation, which is similar with that of LML2.1 transformation. Codon optimization has been demonstrated to be useful for improving heterologous protein production in T. reesei.

We made the novel finding that a hygromycin resistance gene showed a certain degree of cross-resistance for G418 (Fig. 3a). Therefore, we chose a higher G418 concentration, 400 μg/ml, with 75 μg/ml hygromycin B for selection of two-Agrobacterium-mediated transformations. We chose Δku70 strains as hosts for transformation, in which the efficiency of cbh1 and eggl targeting is over 95% (Zhang et al., 2009). Only in the C model of two-Agrobacterium-mediated transformations (Fig. 4c), the efficiency of cbh1 and eggl simultaneously targeting is about 50%, lower than 95%. However, this is still acceptable, as it saves 30 days in the protocol. Of course, the self-excision frequency of the B and C models is lower than that of the A model (Table 3) for simultaneously excising two marker genes in one step.

In conclusion, we developed an effective selection marker, lcNG, and a combined genetic transformation system for gene manipulation in T. reesei using a two-Agrobacterium-mediated transformation method. Our future work will concentrate on further developing more convenient genetic manipulation techniques in T. reesei for more extensive functional genomics studies.

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Supplementary Materials

Supplementary figures are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

Research Involving Human Participants and/or Animals

Not applicable.

Author Contributions

Wei Wang initiated, designed, and coordinated the study and reviewed the manuscript. Chuan Wu planned and carried out experiments and measurements. Junmei Chen interpreted experimental data. Xiaoxue Huang, Shishuai Sun, Jinnan Luo, and Zhiwen Lu cultured and counted fungi strains. Yushu Ma supported the research funding. All authors have read and approved the final manuscript.

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