

A self-replicating vector for efficient transformation and screening of gene libraries in *Chryso sporium lucknowense*

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Due to growth characteristics and relatively low transformation efficiencies, fungal systems were as yet unsuited for high throughput screening (HTS) using robotics. At Dyadic International, we have identified a derivative of *Chryso sporium lucknowense* C1 which has a morphology that allows cultivation in microtiter plate wells and transfer of individual transformants using robotic liquid handling equipment (Fig 1). Here we describe the development and optimization of an efficient DNA transfer and activity screening protocol for *C. lucknowense*.

C1 Transformation and Expression System

Initially, a transformation procedure was developed for integrative vectors based on protoplasts and a CaCl₂/PEG treatment. For this purpose specific mutant strains in the uridine biosynthetic pathway of C1 were generated. The corresponding genes encoding orotate-P-ribosyl transferase (*pyr5*) and for orotidine-5'-P- decarboxylase (*pyr4*) and their *Aspergillus nidulans* orthologs, respectively *pyrE* and *pyrG*, were successfully used for transformation. Also dominant selection markers were developed. The transformation frequency was comparable, if not higher, than those reported for other fungi.



Fig 1: The Zymark Allegro library seeding, compression and replication station (left) and Staccato assay station (right) at DNL facility in Zeist.

Construction of a C1 Self-Replicating Vector

For the construction and screening of expression libraries, much higher transformation frequencies were needed. We tested, in a vector that contained the *pyrE* gene as selection marker, two elements that are reported to promote autonomous replication in some fungi:

- i) two inverted copies of a human telomere sequence
- ii) the AMA1 sequence of *A. nidulans*.

Increased transformation frequencies were observed only for the telomere based vector (up to 13,000 transformants per microgram of DNA). In more than 90 % of these primary transformants, the vector was maintained as a non-integrated linear DNA molecule as shown by Southern analysis. Although the telomeric vector was capable of autonomous replication, the linear plasmids tended to integrate at the end of C1 chromosomes after prolonged cultivation, as was shown by Southern analysis. During this integration, portions of the expression cassette were lost in a polar fashion; genetic elements closer to the selection marker were retained more frequently. To overcome the problem of insert loss, the telomeric vector was redesigned in such way that the expression cassette was flanked by two selection markers (Fig 2). Additionally, two *NotI* restriction

sites were introduced in order to facilitate reisolation of the expression cassette from transformants.

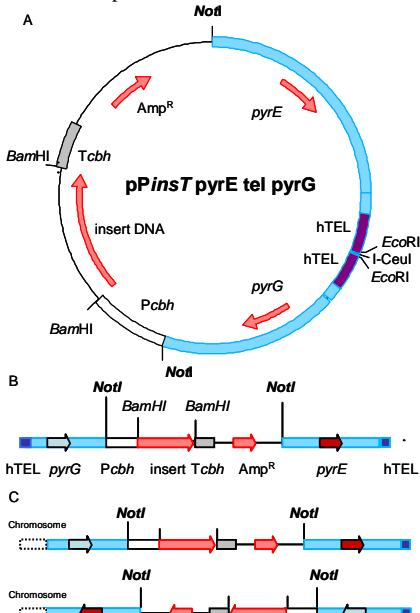


Fig 2. Schematic representation of circular transformation vector (A), the derivative after in vivo linearization (B) and after integration at the telomeric ends of C1 chromosomes (C).

Analyses showed that more than 90 % of transformants of this new vector had one complete copy of the vector integrated. This led to more consistent gene expression levels than what has been observed in vectors leading to random integration (Fig 5).

Integration with Dyadic's FHTS technology

To demonstrate the concept for fungal HTS the laccase encoding gene from *Pycnoporus cinnabarinus* was inserted in the double marker vector between the C1 expression signals yielding pPlacT *pyrE* tel *pyrG*. Propagules of a Lac⁺ transformant were mixed with the propagules of a transformant with the empty expression vector.

In this proof-of-concept experiment, transformants were seeded into the wells of microtiter dishes using the Allegro system (Fig 1). After suitable cultivation, the cultures form propagules (Fig 3). These propagules were transferred robotically to fresh production medium. The culture supernatants from the resulting daughter cultures

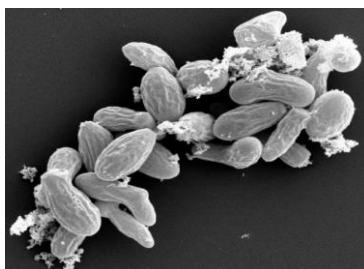


Fig 3. C1 propagules by scanning microscopy.

were assayed for enzymatic activity using the Staccato robotic system (Fig 1). After hit identification (Fig 4) the activity was verified in a second screening.

Genomic DNA isolated from a verified positive hit was treated with *NotI* and self-ligated, and the ligation mixture was introduced into *E. coli*. Plasmid DNA from ampicillin-resistant colonies was analyzed for the presence of the laccase gene. The recloned plasmid was also reintroduced into C1 to verify the expression of the laccase gene (Fig 5A). This recloned vector can directly be used to generate overproducing strains (Fig 5B).

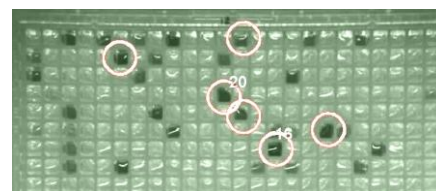


Fig 4: Screening assay plate for laccase activity (ABTS oxidation). C1Δ*pyr5*Δ*pyr4* [pPT *pyrE* tel *pyrG*] and C1Δ*pyr5*Δ*pyr4* [pPlacT *pyrE* tel *pyrG*] were mixed such that one well in eight was expected to have a positive hit.

Together, these data indicate that the individual steps for FHTS – transformation, identification of positive clones, and recovery of corresponding expression constructs from positive hits – is technically feasible.

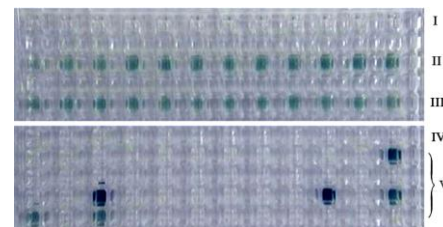


Fig 5. Laccase activity assay on various transformants of C1 host strains. Upper panel: individual transformants of C1Δ*pyr5*Δ*pyr4* strain transformed with negative control (I) the recloned laccase vector (II) and the original laccase vector (III) Lower panel: individual transformants of C1Δ*pyr5* strain transformed with *pyr5* (IV) and *pyr5* + recloned laccase vector (V)..

Conclusions

+ A new double marker transformation vector was designed that results in:

- Generation of complex libraries.
- Reduced variation in the expression levels between independent transformants.

+ Full integration of our complete screening protocol with Dyadic's robotic system.

+ Shortened development time between screening and product development.

For further information

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