Construction of Sedoheptulose-1,7-Bisphosphatase (Sbpase) for Manipulation in Guard Cells of *Arabidopsis thaliana* L.

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

Original Research Article

ABSTRACT

Guard cells control the stomata through which exchange of gas takes place in plants by balancing between CO_2 uptake for photosynthesis and water loss through transpiration leading to ultimate plant water use efficiency (WUE). Due to climate change, sustainable agriculture will therefore require a major reduction in plant water use hence stomata have become potential target for manipulation. Understanding the signal mechanisms of stomata is therefore critically important in facilitating an understanding of stomatal regulation. The use of molecular tools and techniques to manipulate chloroplast metabolism specifically in the guard cells are needed to elucidate signals associated with stomatal behaviour towards crop improvement. Ability to assemble multiple or complex DNA molecules containing large number of genetic elements is an essential part of genetic engineering and in order to understand the involvement of guard cell photosynthesis in stomatal function, genetic manipulation of photosynthetic enzymes specifically in guard cells is necessary. This study employed the manipulation and construction of the enzyme Sedoheptulose-1,7-Bisphosphatase (SBpase) by using the golden gate cloning technique and the bioinformatics system- *geneious*. Constructs were designed to alter expression of the SBPase gene in a cell specific manner driven by the guard cell promoter KST1 in the model plant *Arabidopsis thaliana* L. The construct design for the sense plasmid vectors allowed efficient assembly of multiple DNA fragments in a single reaction based on the type IIs restriction enzyme. The potentials of

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manipulating guard cell specific metabolism are therefore enormous and the increase or decrease of photosynthetic genes could be assessed and their impacts on plant development documented accordingly.

1. INTRODUCTION

Genetic manipulation of metabolites involved in metabolism and photosynthetic enzymes in the guard cells themselves could lead to changes in stomatal behaviour which may potentially improve photosynthesis and water use efficiency in plants [1]. In order to understand photosynthesis in stomatal function, genetic manipulation of photosynthetic enzymes specifically in guard cells is necessary [2].

The Calvin cycle is a pathway primarily for carbon fixation in chloroplasts of C3 plants. This process has three stages, where carboxylation is the first stage of accepting CO_2 by an acceptor molecule, ribulose-1,5-bisphosphate (RuBP) and is aided by the enzyme ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco). The second stage is the reduction phase that produces triose phosphate by consuming ATP and NADPH produced by the ETC. The third and final stage is the regenerative phase; in which triose phosphates are used to produce back RuBP. In the cycle, the triose phosphates are key intermediates, and they are also available for allocation to either the starch or sucrose biosynthetic pathway [3,4]. Balance within the cycle is therefore very important in order to avoid exhaustion of these phosphates hence, the catalytic activities of certain enzymes within the cycle are highly regulated [5]. In particular, the activity of sedoheptulose-1,7-bisphosphatase (SBPase) is regulated by the redox potential via the ferredoxin/ thioredoxin system, which modulates the enzyme activities in response to light/dark conditions [6]. In the regenerative phase of the Calvin cycle, SBPase is part of the enzyme that catalyze irreversible reactions [7]. SBPase catalyses the dephosphorylation of sedoheptulose-1,7-bisphosphate in the regenerative phase of the Calvin cycle to sedoheptulose-7-bisphosphate.

Transgenic approaches have demonstrated striking results of the manipulation of the Calvin cycle where energy conversion led to increasing yield potential [8, 9,10,11, 12]. Additionally, other studies have also revealed and shown the importance of SBPase by demonstrating that minimal reductions or the disruption of SBPase and some certain genes impact negatively on carbon assimilation and growth, thereby demonstrating the enzymes control over photosynthetic efficiency [13,14,7, 2,5,15]. No doubt therefore these studies imply that improvements in photosynthesis may be achieved through overexpressing the activity of individual enzymes. Already, evidences supporting this hypothesis from single manipulations have been demonstrated from transgenic tobacco plants (*Nicotiana Tobaccum*) over-expressing SBPase [16] and also the combined multigene approach of over-expressing SBPase and FBPA. These photosynthetic manipulations resulted in increased carbon assimilation, enhanced growth and increased cumulative biomass hence the genetic potentials that lies thereby. It is therefore obvious that number of sense and antisense plants with increased and reduced levels of SBPase have varying photosynthetic capacity and have altered carbohydrate status at the whole leaf level thus, leading to modifications in growth and development.

This study designed the sense construction of the SBpase enzyme for use in *Arabidopsis thaliana* specifically in the guard cells. This plant model was used because of its usefulness in genetic experiments. Its characteristics features includes short generstion time, small size and prolific seed production. Expression vectors from the cloning strategy Golden gate were used to generate the construct with guard cells specific promotor KST1. YFP tags were also included in several construct to demonstrate cell specificity.

1.1 Golden-gate Modular Cloning Technology

Golden gate cloning technique allows highly efficient directional assembly of multiple DNA fragments in a single reaction [17,18]. The principle of Golden gate cloning makes relies on the type IIs restriction enzyme which makes it possible for constructs or multiple desired genes assembled seamlessly by using the enzyme in a one-pot one-step cloning reaction [11]. Type IIs
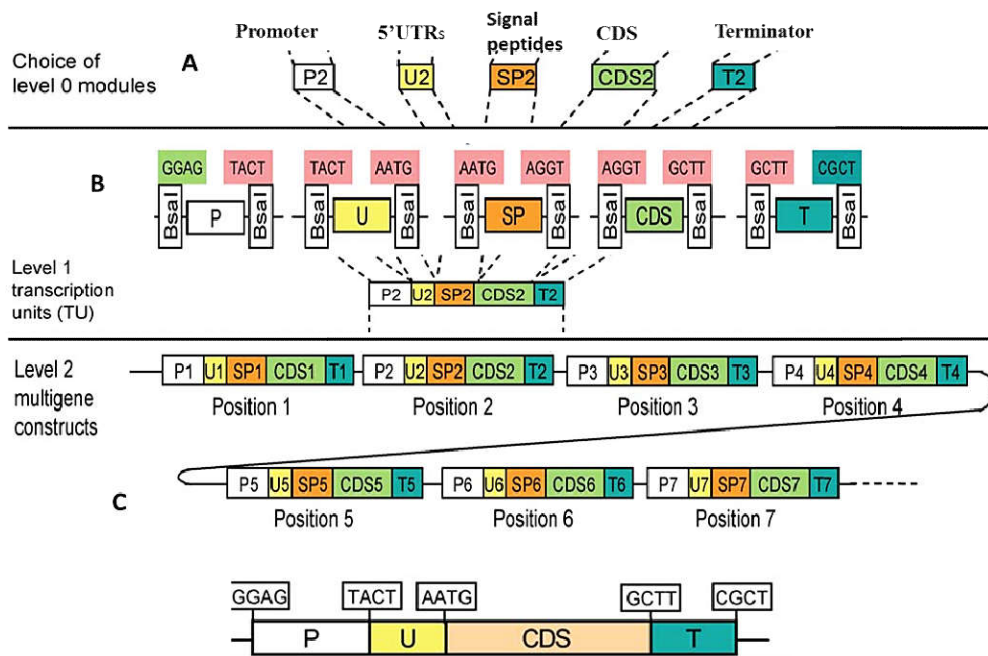
Fig. 1. General assemblage of standardized modular cloning system. (A) level 0 modules of cloned or sequenced genetic elements such as promoters (P), 5 untranslated regions (U), signal peptides (SP), coding sequences (CDS) and terminators (T). (B) level 1 transcription units assembled from level 0 modules using a one-pot one-step cloning reaction. Level one (1) flanked by compatible fusion sites consisting of 4 nucleotides of choice (boxed) flanked by a type IIS enzyme Bsa1. (C) Multigene constructs assembled in a second cloning step from the transcription units. The transcription unit for the cytolic protein was assembled from 4 modules rather than 5, using a CDS module cloned between fusion sites AATG and GCTT. Diagram modified from Weber et al., 2011.

restriction enzyme cut outside of recognition site and fragments ligated easily into a product lacking the original restriction site allowing any DNA fragment of interest from an entry clone to be transferred into an expression vector without any unwanted sequences in the final construct [17]. The Fig. 1 below illustrates the standardized assembly of this unique cloning system.

The modular cloning strategy allows systematic assembly of complete transcription units and of multigene constructs from basic standardized modules as exemplified above.

2. MATERIALS AND METHODS

2.1 Primer Design

Primers were designed based on the sequences of the gene of interest Sedoheptulose 1,7-bisphosphatase (SBPase)) in Arabidopsis. SBPase was flanked by BsaI and BpiI restriction sites and using PCR amplification of its coding sequences. Fusion sites overlapping with coding

sequences have a start codon with AATG at the 5 ends and GCTT at the 3 end so as to minimize changes to encoded proteins [18]. Therefore, AATG is included in the forward primer while AAGC is included in the reverse primer. Standardized transcription units of all the level were flanked by specific fusion sites as described by (Weber, Engler, Gruetzner, Werner, & Marillonnet, 2011). In addition to the geneious tool used for designing primers, Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and the NCBI tool (http://www.ncbi.nlm.nih.gov/tools/primerblast/index.cgi?ORGANISM=9606&INPUT_SEQUENCE=NM_001618.3&log$=seqview_box_primer) were also utilised.

2.2 Cloning Sedoheptulose-1, 7-bisphosphatase (SBPase) Gene Using Golden Gate Technology

The sequences of the genes of interest SBPase (AT3655800) *Arabidopsis thaliana* was retrieved

from the TAIR database and primers designed as previously described. The plasmid vectors used for the plant transformation were constructed using the golden gate cloning and the Moclo system [17,18]. The construct design for the sense plasmid vectors allowed efficient assembly of multiple DNA fragments in a single reaction based on the type IIs restriction enzyme that cut outside of recognition site allowing fragments to be ligated into products lacking the original restriction site and leaving no unwanted sequences in the final constructs [17]. This made the assembly of constructs of each transcriptional units to be expressed hence multiple desired genes were assembled seamlessly in a one-pot one-step cloning reaction. The golden gate assembly enabled promoter (PU), coding sequences (SC) and terminator (T) assembled together seamlessly and expression driven by the highly specific guard cells promoters KST1 (NP001275475) [19,20, 21]. All DNA fragment of interest from an entry clones were transferred into their expression vector pAGM4723 without any unwanted sequences in the final construct. All sequences of expression vectors were confirmed by sequencing (samples analysed by source bioscience) and the assembled construct was transformed into 20 µl of competent *E. coli.*

2.3 Single Colony PCR Screening of *E. coli*

Single colony PCR screening of *E. coli* was carried out where DNA was released by dipping into the PCR mix a tip dipped in the middle of a fresh colony obtained from the transformed *E.coli* colonies to the PCR reaction. PCR reactions were performed in 0.2 ml PCR tubes using 10X DreamTaq 0.5 µl of 20 mM stock per each 25 µL PCR reaction (Thermo Scientific), 1.5 µL of 10 pmol µl^{-1} stock primer solution per each PCR reaction,1–2 µl of DNA and dNTPs 0.5 µl of 10 mM stock per each 25 µl PCR reaction (Thermo Scientific), and the recommended amount of enzyme and buffer with water were added. The volume of the reactions was made up to 15 µL. Cycle conditions were mostly at 35 depending on the size of the fragment to be amplified. Gel electrophoresis was used to determination fragment sizes afterward.

2.4 Agarose Gel Electrophoresis of Nucleic Acids

Nucleic acids were separated for analysis using gel electrophoresis. 1% agarose gels were prepared and ran at 100 volts for 40 min (150 ml gels). Tris-borate buffer (TBE: 89 mM Tris, 89 mM Boric acid, 2mM EDTA) or Tris-acetate buffer (TAE: 40 mM Tris ultrapure, 20 mM Acetic acid glacial, 1 mM EDTA.Na2) was used for the preparation of the gels and as running buffer in the tanks respectively. Safe view nucleic acid (NBS Biologicals) was added to the gel for nucleic acid visualization under UV or blue light respectively.

3. RESULTS

3.1 Level 0 Modules

Level 0 destination vectors confer a spectinomycin resistance (Sp^R) and are based on a pUC19 backbone. SBPase, the gene of interest consisted of level 0 fragments in the SC position of the destination vectors, pL0M-SC-SBPase while promoter KST1 (pL0M-PU-KST1) in the PU position of destination vectors and terminator, pL0M-T-HSP. The positions in the destination vectors were created to allow the possibility of creating or cloning two or more genetic standardized element as a single module (PU instead P, U, and SC instead of S,C). All level zero (0) were synthesized and cloned by the PCR-amplification of the designated sequences. Additional level 0 construct were designed to determine guard cell specific expression, with YFP (pICSL80014). These were all systematically assembled into level 1 constructs with the incorporated guard cell specific promoter.

3.2 Level 1 Modules

Level One (1) module consisted of the backbones, promoters, coding sequences and terminators created by the assembly of compatible sets of sequenced level 0 described and assembled into a level 1 destination vector. The backbone for level 1 modules confer an ampicillin resistance with fusion sites compatible from one vector to the next so that multiple level 1 modules were directionally cloned together and into a level 2 destination vector. A bioinformatic software called geneious was used in designing the constructs or plasmid maps and also to determine the exact fragment sizes in base pairs of all the constructs. All the level one constructs were also sequenced and the expected inserts confirmed for all with the correct sequences at the cloning junctions. Additional level 1 constructs were designed to determine guard cell specific expression, with YFP (pICSL80014 pL0M-SC-YFP).

(a)
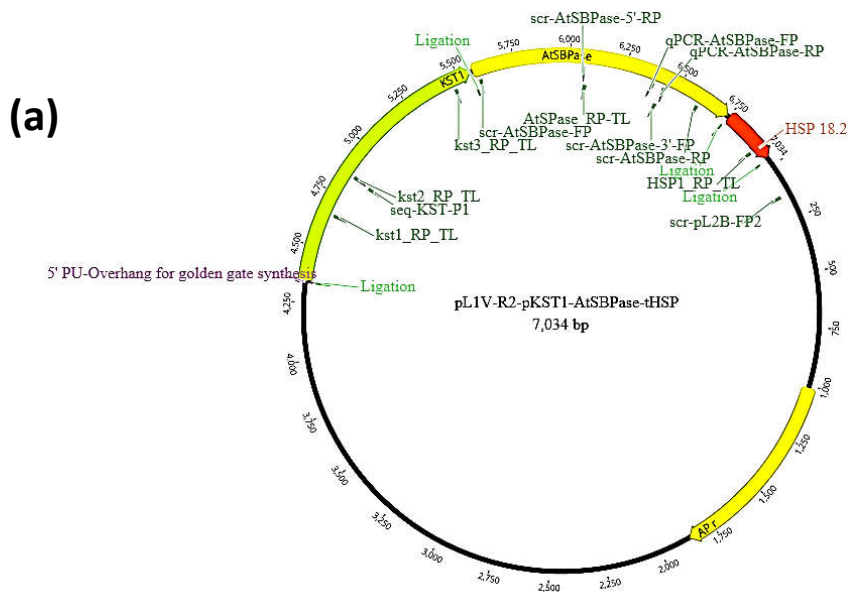
Fig. 2. Construct map for level one plasmid pL1M-R2-pKST1-AtSBPase-tHSP for over-expression of SBPase in plants. (a) Standardized modules of promoter (pL0M-PU-KST1), coding sequence (pL0M-SC-AtSBPase and terminator (pL0M-T-HSP) assembled into a level one backbone EC47811 (pL1V-R2). Plasmid consists of 5 PU overhang for golden gate synthesis fusion sites with 4 nucleotide sequences. Primers for synthesis annealed to specific sites for efficient cloning. Multiple alignments were performed using Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and geneious software (www.geneious.com.)

### 3.3 Multigene Constructs or Level Two

In the same way level 1 modules were cloned from standardized modules, multiple transcriptional units of level 1 modules were directionally cloned into a level 2 destination vector. Level 2 constructs were designed in such a way that the upstream fusion sites are compatible with the upstream fusion site of a corresponding level 1 module. This reduces the need for re-cloning of the same transcription unit for different positions [18]. However, the downstream fusion site is unique to level 2 destination vectors (GGGA) because of the addition of end-linkers (pELE-n) which connect the GGGA fusion site with the fusion site of the last assembled transcription unit in the DNA fragment. All level 2 constructs confer a kanamycin resistance and encode a red colour selectable marker. The end linkers plasmid however, confer an ampicillin resistance like the level 1 constructs but flanked by BpiI sites. Therefore, multigene level 2 constructs are assembled with BpiI from the chosen level 1 modules, a matching end-linker and a level 2 destination vector. Two steps of digestion and ligation can therefore be replaced by a single restriction-ligation step [18]. The plasmid maps

constructed by the *geneious* for level 2 constructs determines the exact fragment sizes to be expected in base pairs of all the construct. This information was used to check using PCR and the gels.

All constructs were used as templates in order to amplify the desired fragment which were transformed in TOP 10 competent *E. coli*. The construction of the level two construct was successfully transformed in TOP 10 competent *E. coli* and evidence shown on Fig 5 in colony PCR/gel respectively.

3.4 Transformed *E. coli* Colony Selection

The efficiency of golden gate cloning technique was demonstrated by the positive growth of transformed *E. coli* colonies in (Fig. 4). Growth yielded more of white colonies than orange colonies indicating high and efficient transformation. All white colonies picked and tested by PCR showed positive which affirmed successful transformation. Fig. 4 shows the transformation efficiency indicated by colour selection. Few orange colonies indicates non-transformed construct.

(a)

(b1)

(b2)
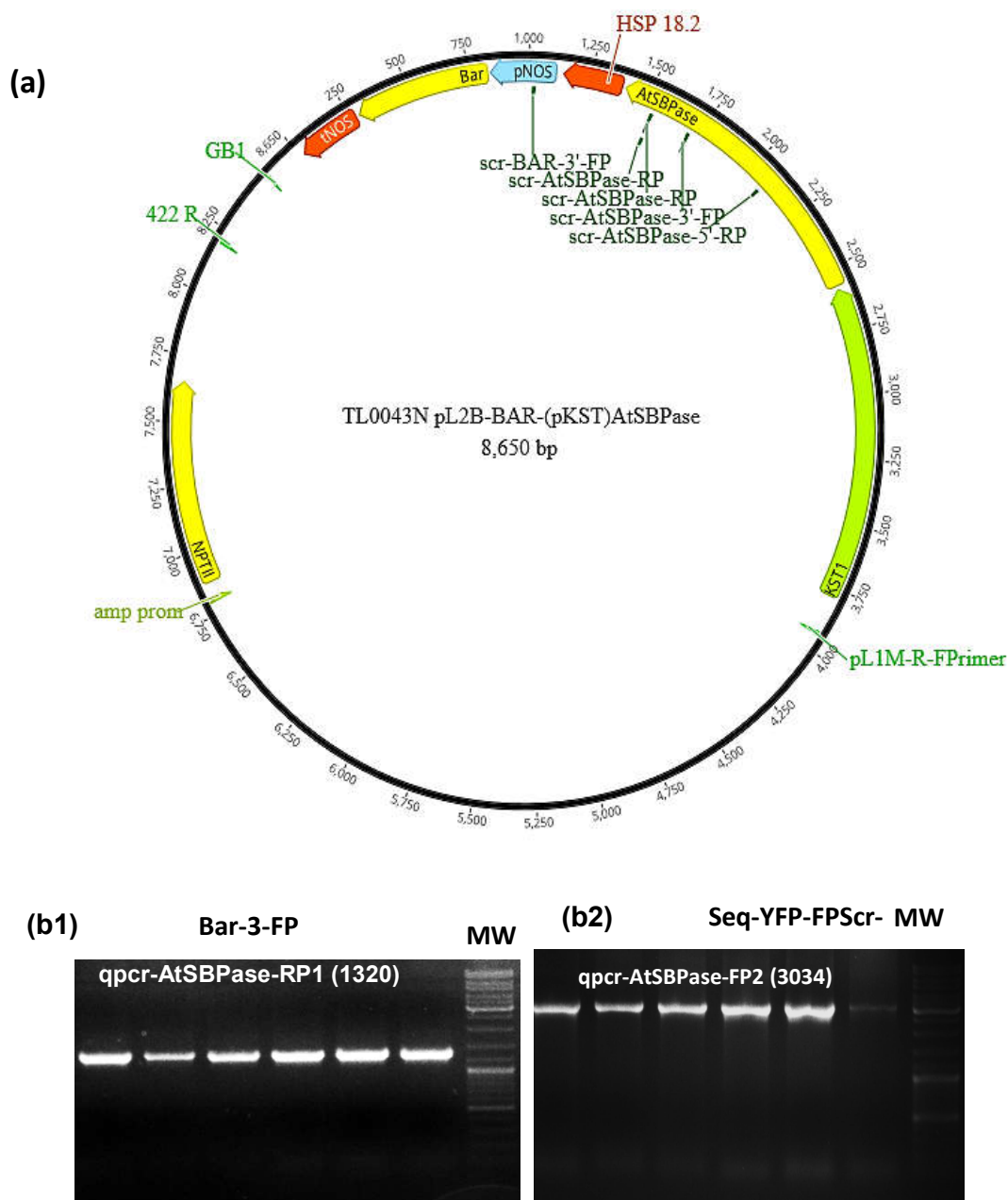
Fig. 3. Construct map for level two plasmid (pL2B-BAR-(pKST1)-AtSBPase-(pKST1)YFP) for expression of SBPase in plants. (a) Plasmid contains the genetic modules of level one assembled together of KST1 promoter, AtSBPase coding sequences, bar gene with its respective promoter and terminator for herbicide selection and the YFP together with its promoter and terminator for the yellow fluorescence protein expression in guard cells. (b1) First reaction with Scr-Bar-3-FP as the forward primer and qpcr-AtSBPase-RP1 as the reverse primer yielding 1320bp as expected of the construct. (b2), second reaction using Seq-YFP-FP and qpcr-AtSBPase-FP2 for forward and reverse primers respectively yielding exact DNA fragment size of 3034bp. The expression vector produced also carries the NPTII genes for kanamycin selection in bacteria. Construct designed by geneious www.geneious.com
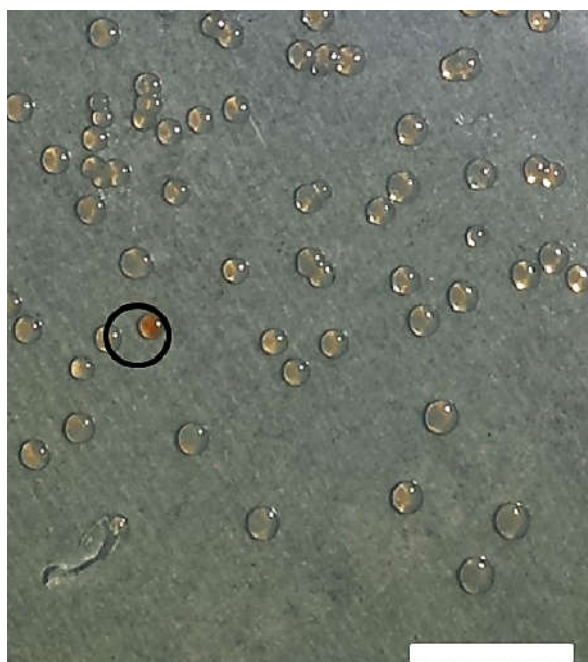
Fig. 4. Selection on plates showing cloning efficiency of level two construct pL2B-BAR-(pKST)AtSBPase-(pKST)YFP of an overnight luxuriant growth of transformed *E .coli* cells. Majority of colonies (white) indicates transformed cells while few (orange colonies) indicates untransformed cells. The selection of colonies were performed on plate incorporated with kanamycin (50 μg/mL) and grown overnight at 37°C. Scale bar 2cm
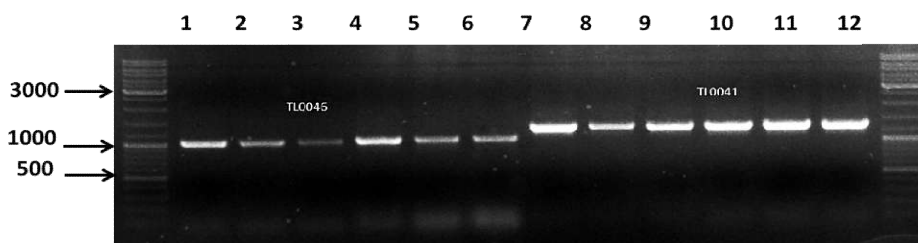
Fig. 5. Colony PCR analysis of *E. coli* in Arabidopsis construct. The presence of the fragments of interest were cloned and checked using the forward primer Sc-Bar-3-FP and reverse primers for amplification of fragments. The fragments of interest (in two constructs above) yielded bands as expected in all the colonies selected per construct. TL0046 (1-6)1039bp and TL0041 (7-12) 1320bp. PCR products run alongside molecular weight markers (DNA generular ladder mix from thermoscientific) MW in base pairs

3.5 Selection and Determination of Fragment sizes in *E .coli* Colonies

The colony PCR results showed exact fragment sizes transformed and analyzed by gel electrophoresis as the plasmid construct. All six colonies selected per construct showed positive. The fragments of interest were amplified using DNA polymerase and bands of interest yielded exact base pairs as expected.

4. DISCUSSION

With recent technologies, desired phenotypes are generated by multiple combinations of various coding sequences which do not necessarily need to operate at genome level. This method allows generation of constructs containing enough genes for pathway engineering [18].

This work has utilized optimal efficiency of constructing raw pieces of DNA that allowed the assembly of its discrete functional genetic materials as evidently shown. The Golden gate technique has also simplified the assemblage of these constructs coupled with minimal number of cloning steps and times required.

The construction design was based on the principles of having a set of compatible overhangs, specific colour selection indicating presence of successful transformation and specific antibiotic selection markers which have all yielded successful transformation. These have all yielded the desired results as shown on Figs 2 to 4 for the level one and two constructs indicated by the assembling of the modules into transcription units of the level one and level one modules into level two. The efficiency of the cloning Fig. 4 where white successful colonies dominated the untransformed ones agreeing with Engler et al. [17] confirming the high efficiency of this technique. The modular cloning system(MoClo) engineers novel biological functions that allows high throughput assembly of multiple genetic elements. Unlike the conventional methods where cloning strategies were designed requiring multiple cloning steps performed manually in a slow and inefficient process and which could easily not be automated in order to obtain the desired construct.

Overall, the colony PCR of the construct indicated the DNA fragment sizes as expected. The presence and size of the bands of all the constructs have further confirmed the functionality of this construct which will be transformed in the plant system. This will be used in the production of transgenic homozygous Arabidopsis and tobacco plants and their characterization exploited. This will also present the potentiality of these plants for the future advances exploiting guard cell functions.

5. CONCLUSION

Synthetic biology is seen to have progressed at a rapid pace and with the recent generation of organisms containing synthetic genomes. In this work, the plasmid vectors used for plant transformation were constructed seamlessly using the golden gate cloning or the Moclo system. All DNA fragment of interest from an entry clones were transferred into their expression vector without any unwanted sequences in the final construct and the confirmation of the sequences of expression

vectors. This work has further confirmed that the assemblage of complete genomes is now possible. The challenge of designing gene networks that encode biological functions even at a small scale is now possible through the use of the Moclo system that allows the assembly of multiple DNA fragments in a one-pot reaction.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
https://www.sdiarticle4.com/review-history/72312