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A method to synthesize cDNA constructs by homology based recombination cloning

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ABSTRACT

We introduce a homology-based recombination approach for generating a cDNA construct. This method depends on amplifying several exon fragments and their fusions by the homology-based recombination. This method provides a way to generate the cDNA sequence of any gene without any need for its mRNA. The paper describes the strategy by assembling cDNA of the *MYB*1 and *MYB*2 genes of *Arabidopsis thaliana*.

KEYWORDS

Homology Based Recombination Cloning; cDNA Assembly

1. INTRODUCTION

In several instances, the cDNA of a gene is required for its expression in heterologous or transgenic systems. Traditionally cDNA is synthesized by using mRNA as a template. In some instances, availability of mRNA is limited due to a narrow time window of expression, tissue specificity or lack of information. In such instances, the cDNA sequence can be assembled by chemical synthesis of DNA or by recursive PCR, followed by its cloning in appropriate vectors. In recent years, Homology Based Recombination (HBR) [1] has been used to assemble recombinant molecules. In the present work, we have used HBR cloning to assemble cDNA fragments. This involves amplification of exon fragments with overlapping homology. Reverse primers used in amplification of exons, are designed to have at least 15 base pair (bp) homology sequence with the adjacent exon fragment. By HBR cloning, the amplified exons of the target gene are linked to each other via the regions of overlap to generate the cDNA sequence which then can be cloned in a suitable plasmid vector using the same strategy.

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2. MATERIALS AND METHODS

2.1. Polymerase Chain Reactions

All PCRs were carried out in a total volume of 50 µl with Phusion high fidelity DNA polymerase (Thermo Fisher Scientific, USA) amplification kit. 100 ng of genomic DNA isolated from *A. thaliana* was used for amplification of the exons.After an initial denaturation at 98˚C for 5 minutes, 30 cycles of denaturation at 98˚C for 30 s, annealing at temperatures according to T_m of primer pairs for 30 s and extension at 72˚C for 1 min was carried out. This was followed by a final extension for 8 min at 72˚C. The PCR product was then size-fractionated on a 1% Agarose gel in TAE buffer. The corresponding band was excised and DNA purified for further assembly. Sequences of primers used in amplification of *MYB*1 and *MYB*2 genes are summarized in **Tables 1** and **2**, respectively.

2.2. Homology Based Recombination Cloning Reaction

After amplification of exons, In-Fusion reactions were performed using In-Fusion® HD Cloning Kit (Clonetech Laboratories, Inc.). The recommended protocol was modified for assembling two exon fragments in a vector (Three-way assembly) and for three exon fragments ina vector (Four-way assembly). In both types of assembly, each exon fragment was used in ten-fold molar excess to the vector. 70 to 100 ng of vector was used in a 20 µl reaction volume consisting of exon fragments, digested vector DNA and 4 µl of Infusion HD Enzyme Premix as supplied in the kit against a recommended use of 2 µl of

Table 1. *MYB*1 primers.

Primer Name	Primer Sequence
Primer 1	5' TGGAGAGGACCTCGAGACGAGACTCAACGCTTTTACAGT 3'
Primer 2	5' AGCCTGATCCTCTACCT CAGTAAATGAATTGCGTATAAG 3'
Primer 3	5' AGGTAGAGGATCAGGCTATCATC 3'
Primer 4	5' AGGATCCCCGGGTACC TTATGTGGACAGGACATTGGT 3'

Table 2. *MYB*2 primers.

Primer Name	Primer Sequence
Primer 1	5' TGGAGAGGACCTCGAGAATCCACAAAACCATTCACACC 3'
Primer 2a	5' CCAGTTCGCTTTAGCCCAGAGGAACGAGCGATGTG 3'
Primer 3	5' GGCTAAAGCGAACTGGTAAG 3'
Primer 4a	5' CGCAATCTTCGACCACCTATTGCCCCAAAGAGAATG 3'
Primer 5	5' GTGGTCGAAGATTGCGCA 3'
Primer 6	5' AGGATCCCCGGGTACCCCTTAATTATACGAATACGATGTCG 3'
Primer 2b	5' CAGAGGAACGAGCGATGTG 3'
Primer 4b	5' CTATTGCCCCAAAGAGAATG 3'

the premix per reaction. The mix was incubated at 37˚C for 15 min followed by incubationat 50˚C for 15 min. 5 µl of the reaction mixture was used for transformation of *E. coli*.

3. RESULTS AND DISCUSSION

We present examples of developing cDNA constructs for two genes of *Arabidopsis thaliana*.

3.1. cDNA Assembly of *MYB*1 Gene from *A. thaliana*

The transcribed region of the *MYB*1 gene (Acc. no. NC_003074), is 1.9 Kb long and carries a single intron of 494 bp. The aim was to assemble the cDNA and clone it downstream to a CaMV35S promoter available in the vector pRT101 [2]. In order to amplify the exons, two sets of primers were designed. Primer sets 1, 2 and 3, 4 (**Table 1**) were designed to amplify exons 1 and 2 respectively. Further 5' ends of primer 1 and 4 had 15bp overhangs which were homologous to the ends of *Xho*I - *Kpn*I digested vector molecule, where the cDNA sequence had to be inserted. Similarly primer 2 had a 15 bp overhang homologous to 5' end of exon 2 as shown in **Figure 1**. While exon 2 could be easily amplified (**Figure 2(A)**), exon 1 could not be amplified using primer 1 and 2 in spite of several modifications in the PCR conditions. We felt that the 15 bp overhang of primer 2 complementary to the 5' end of exon 2 was leading to unproductive annealing (**Figure 1**). A similar situation was also observed in our second example (**Figure 3**), which is described later.

This problem was circumvented by first amplifying the complete *MYB*1 gene using primers 1 and 4, followed by its cloning into pRT101 vector by HBR cloning reaction. By using the restriction enzyme *Xmn*I, exon 2 was deleted from the construct and exon 1 was then amplified using primers 1 and 2 (**Figure 2(B)**). A proper amplicon was observed in this reaction. This showed that primer 2 was properly designed and its homology with exon 2 led to unproductive annealing in the earlier instance.

The amplified exon 1, exon 2 and the digested vector were finally assembled by HBR cloning reaction. cDNA synthesis of *MYB*1 gene was confirmed by restriction digestion of plasmids isolated from several colonies. The size of the products observed on 1% agarose gel electrophoresis was in agreement with the predicted sizes (**Figure 2(C)**). Further the assembled cDNA from several clones was sequenced and found to carry no errors.

3.2. cDNA Assembly of *MYB*2 Gene from *A. thaliana*

In a second example, the cDNA of *MYB*2 (Acc no. NC_003071.7) gene of *A. thaliana* was assembled. This gene is 1.3 Kb long and carries two introns of 92 bp and 102 bp. Three sets of primers 1, 2a; 3, 4a and 5, 6 (**Table 2**) were designed for amplification of three exons of *MYB*2 gene similar to the initial strategy of example 1. While exon 3 could be amplified using genomic DNA as a template, with primer 5 and 6, exons 1 and 2 could not be amplified with their respective primers (**Figure 3**). This showed that the 15 bp overhang in the primer 2 and

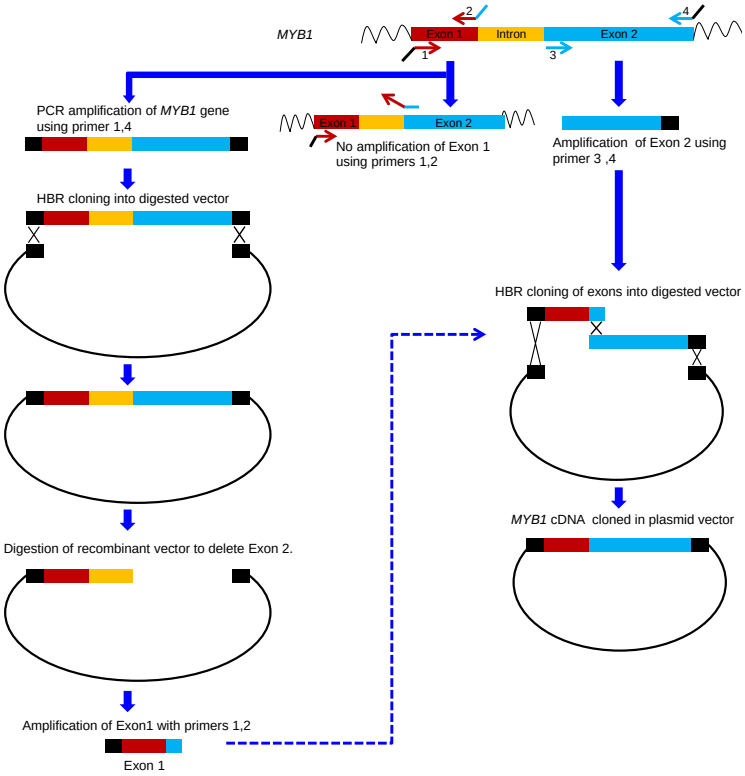

Figure 1. Outline of the strategy to assemble and clone the cDNA sequence of the *MYB*1 gene from *A. thaliana*.

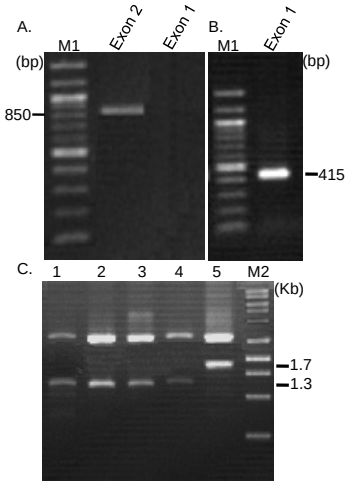

Figure 2. (A) Amplification results of exon 2 and exon 1 of the *MYB*1 gene with primer set 3, 4 and 1, 2, respectively using genomic DNA as a template; (B) Amplification of exon1 with primer set 1, 2 using pRT101:*MYB*1 with deleted exon 2; (C) Analysis of clones with *MYB1* cDNA in the vector pRT101 (lanes 1 - 4) and its comparison with a *MYB*1 genomic DNA clone (lane 5). Lanes M1 and M2 represent 100 bp and 1 Kb size markers, respectively.

4, complementary to 5' end of exon 2 and 3 were leading to unproductive annealing.

In this example, we circumvented the problem by designing two reverse primers 2b and 4b (**Table 2**) for exon 1 and 2 respectively. These primers were similar to 2a and 4a except that they did not carry the 15 bp overhang complementary to 5' end of the adjacent exon. Exon 1 and exon 2 were first amplified using primers 1, 2b and 3, 4b respectively. The amplicons thus obtained were again amplified with primers 1, 2a and 3, 4a, respectively to introduce the region of homology in exons 1 and 2 (**Figure 3**).

The amplified exon 1, exon 2, exon 3 and the vector were finally assembled by the homology-based recombination reaction. The cDNA synthesis of *MYB*2 gene was confirmed by restriction digestion of plasmids isolated from several colonies. Further the assembled cDNA from several clones was sequenced and found to carry no errors.

In summary, this is the first report of a method to synthesize cDNA sequence of any intron containing gene, as well as its cloning in a suitable plasmid vector molecule by the homology-based recombination cloning. It is a simple, efficient and error free method.

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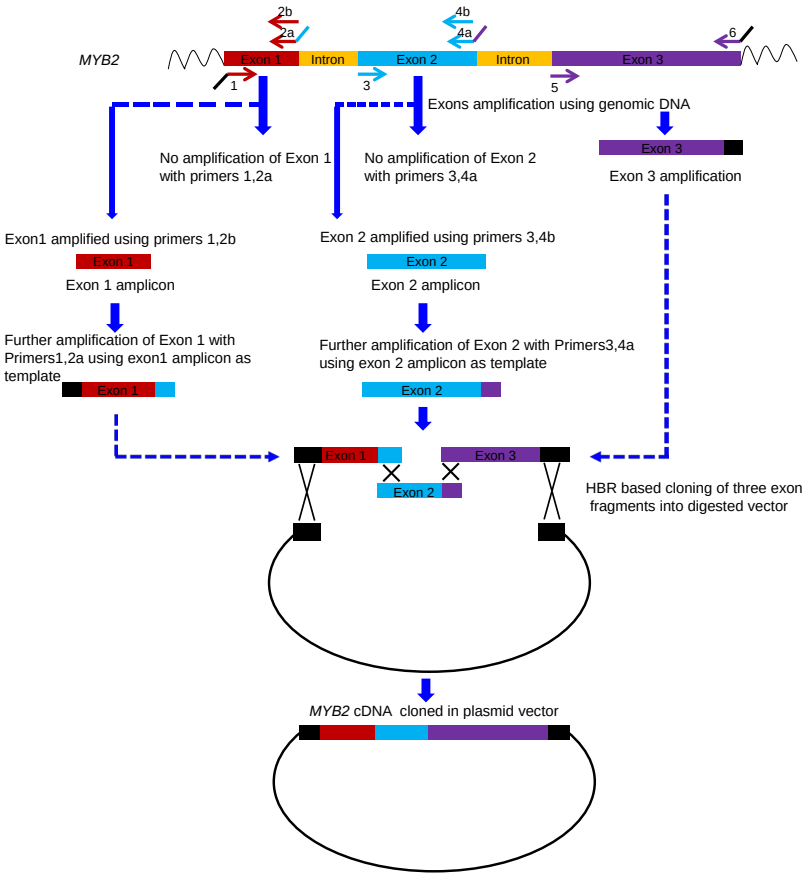

Figure 3. Outline of the strategy to assemble and clone the cDNA sequence of the *MYB*2 gene from *A. thaliana*.

Industrial Research (CSIR), Delhi, India.

REFERENCES

[1] Sleight, S.C., Bartley, B.A., Lieviant, J.A. and Sauro, H.M. (2010) In-fusion biobrick assembly and re-engineering. *Nucleic Acids Research*, **38**, 2624-2636. http://dx.doi.org/10.1093/nar/gkq179

[2] Topfer, R., Matzeit, V., Bruno, G., Schell, J. and Steinbiss, H.H. (1987) A set of plant expression vectors for transcriptional and translational fusions. *Nucleic Acids Research*, **15**, 5890.

