CHAPTER – III  

CLONING OF *GMIFS* BY GATEWAY AND CONVENTIONAL CLONING SYSTEMS

3.1 Abstract

DNA manipulation and genetic engineering has become easy due to powerful tools showing exponential growth in applications and sophistication of r-DNA technology. In the present study, *Glycine max Isoflavone Synthase (GmIFS)* gene was cloned into plant expression vector both by Gateway and conventional cloning systems. The *GmIFS* gene was cloned into the pDONR Gateway entry vector by BP clonase enzyme reaction and then further mobilized into the pEARLEY Gate HA 102 and pEAQ-HT Dest3 plant binary vectors by LR clonase enzyme. The gene was also cloned by the conventional restriction–ligation method, using the restriction enzymes *Kpn* I and *Bst* EII into pNut Kan01 expression vector. The plasmids from the transformed *E.coli* DH5α colonies were confirmed by gene specific PCR, restriction digestion and Sanger’s sequencing. The confirmed plasmids were then moved from *E.coli* to *Agrobacterium tumefaciens* strain AGL1 by electroporation for the plant genetic transformation studies.

3.2 Introduction

‘Molecular cloning’ is creation of recombinant DNA molecules has impelled advancement throughout life sciences. Gene cloning is the process in which the identical copies of a particular gene are manufactured by using molecular biology tools. The seclusion of a DNA sequence or a gene from any species and its introduction into a vector for proliferation, without any modification of the original DNA sequence is known as ‘Molecular Cloning’. After the isolation, molecular clones can be exploited to engender
many copies of DNA for the expression analysis of gene sequence or protein and can also be maneuvered in vitro to alter the expression and function of the protein. Recombinant DNA technology involves the insertion of foreign gene to express the desired protein by cloning, which is the first and foremost step in transgenic technology (Pray, 2008).

3.2.1 Gateway cloning

Cloning forms the basis of genetic engineering, where vectors are constructed to express the target gene using traditional restriction- ligation mediated technique, which is a time consuming and laborious process pretensing a snag for the advances in functional genomics or proteomics studies (Earley et al., 2006). The advent of gateway cloning technology provides a high-throughput DNA cloning that exploits the precise, site-specific recombination system utilized by bacteriophage lambda in order to shuttle sequences between plasmids bearing compatible recombination sites (Earley et al., 2006; Hartley et al., 2000; Landy, 1989; Sasaki et al., 2004). Lambda recombination occurs between site-specific attachment (att) sites that serve as the binding site for recombination proteins and have been well characterized (Weisberg and Landy, 1983). Recombination occurs between attB and attP sites to give rise to attL and attR sites. The first step is the introduction of the attB1 and attB2 sites in the desired gene. The gene flanked by attB1 and attB2 sites will then recombine with the entry vector, which harbours the attP1 and attP2 sites where the reaction is mediated by the enzyme BP clonase (Invitrogen). This recombination results in the generation of entry vector with attL1 and attL2 sites, which will further recombine to the final destination expression vector having the attR1 and attR2 sites facilitated by the enzyme LR clonase (Invitrogen), based on the lambda phage excision reaction (Sasaki et al., 2004) (Figure 3.1). In this way, the desired gene is moved from the entry clone to a destination expression clone.
3.2.2 Conventional cloning

Cloning of double-stranded DNA (dsDNA) molecules into plasmid vectors is one of the most commonly employed techniques in molecular biology. The procedure is used for sequencing, building libraries of DNA molecules, expressing coding and non-coding RNA, and many other applications. The basic cloning workflow by the conventional way includes isolation of target DNA fragment, ligation of inserts into an appropriate cloning vector, transformation of recombinant plasmids into suitable host for propagation and screening of hosts containing the intended plasmid. To increase the efficiency of molecular cloning, many specialized tools and methodologies were developed by means of the properties of unique enzymes (Figure 3.2). Restriction cloning can be described in a relatively standard series of steps. The insert is designed with restriction sites that also occur in the vector multiple cloning sites (MCS), but not elsewhere in the insert or vector. The insert and plasmid are digested in separate reactions, using the chosen enzyme(s).
Following digestion, the plasmid is dephosphorylated, then both insert and plasmid were purified to remove all enzymes. The purified digested insert and vector are ligated using the T₄ DNA Ligase and transformed into *E. coli*.

![Restriction-Ligation Mediated Cloning](image.png)

**Figure 3.2 Restriction-Ligation Mediated Cloning (New England Biolabs)**

### 3.3 Materials and Methods

#### 3.3.1 Glycine max isoflavone synthase (GmIFS)

The isoflavone synthase gene which naturally occurs in *Leguminaceae* members was identified and isolated from Soyabean. Soybean *IFS* cDNA bearing Genbank Accession number AF195798 was procured from Dr. Oliver Yu and Dr. Brian Mc Gonigle, Donald Danforth Plant Science Center, USA.
3.3.2 Cloning of GmIFS using gateway technology

3.3.2.1 Gateway compatible primer designing

The gateway primers were designed specifically for the gene of interest (NCBI Acc. No. AF195798) with four guanine residues at 5’ end followed by 25bp sequence of the recombination site, attB1 and then 15-20bp the gene of interest in the forward primer. The reverse primer was also similarly designed but has the attB2 recombination site along with the target gene sequence (Figure 3.3). The details of the primers used are as follows:

IFS-GF#1:

\[
\text{attB1 Kozak} \quad \text{Glycine max ifsl} \\
5’-\text{ggggaagaattgtaaagaaagcagct} \quad \text{cacc atg TTGCTGGAACCTGACTT-3’}
\]

IFS-GR#1:

\[
\text{attB2 Kozak} \quad \text{Glycine max ifsl} \\
5’-\text{ggggaagactttgtacaaagaaagctggt} \text{T} \quad \text{TTAGAAAGGACTTTAGATGCAAC-3’}
\]

Figure 3.3 Primer Designing for the Cloning of GmIFS

3.3.2.2 PCR amplification

PCR was performed using the gateway primers. The forward and reverse primers contained complementary sequences to the gene specific regions along with the attB1 and attB2 sites respectively. Phusion high fidelity DNA polymerase (New England Biolabs, UK) enzyme was used and the reaction mixture was prepared as shown in the Table 3.1.
Table 3.1: PCR reaction mix composition

<table>
<thead>
<tr>
<th>S.No</th>
<th>PCR mix components</th>
<th>For 50 µL reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>5X Phusion HF Buffer</td>
<td>20 µL</td>
</tr>
<tr>
<td>2.</td>
<td>2mM dNTPs</td>
<td>5 µL</td>
</tr>
<tr>
<td>3.</td>
<td>1 pM Forward primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>4.</td>
<td>1 pM Reverse primer</td>
<td>1 µL</td>
</tr>
<tr>
<td>5.</td>
<td>Template DNA</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>6.</td>
<td>Phusion Polymerase</td>
<td>0.4 µL</td>
</tr>
<tr>
<td>7.</td>
<td>Nuclease free water</td>
<td>20.1 µL</td>
</tr>
</tbody>
</table>

Total 50 µL

PCR reaction was performed using the following conditions, initial denaturation at 98°C for 5 minutes followed by 30 cycles of denaturation at 98°C for 30 seconds, annealing at 56°C for 45 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 10 minutes using a MyCycler Gradient PCR machine (BioRad, USA). The PCR products were separated using 1% agarose gel containing ethidium bromide (10 µg mL⁻¹) and the bands were visualized and documented. The cloning strategy employed was as below (Figure 3.4 a & b).

![Cloning Strategy](image)

Figure 3.4 Strategy used for Cloning (Invitrogen)
3.3.2.3 Purification of attB PCR product

PCR products were purified using PCR purification kit (Macheray-Nagel Kit, Germany) following manufacture’s instruction to remove the unbound primers, nucleotides, salts etc. and the DNA quality was checked in 1% agarose gel.

3.3.2.4 Culture conditions for entry vector pDONR\textsuperscript{TM}/Zeo

The Gateway compatible entry vector pDONR/Zeo (Life Technologies, USA) was used. The plasmid was moved to One Shot\textsuperscript{®} ccd\textsuperscript{B} Survival\textsuperscript{TM} 2 T\textsuperscript{R}
strain (Life Technologies, USA) by the heat shock method. It was then plated on low salt LB media supplemented with 50 mg L\textsuperscript{-1} Zeocin (Life Technologies, USA) and the plates were incubated at 37\textdegree C overnight. Next day, single colony was inoculated in 10 mL of low salt LB broth with Zeocin (50 mg L\textsuperscript{-1}) and incubated overnight at 37\textdegree C. The plasmid was isolated as per the manufacturer’s protocol (MN Plasmid DNA Isolation Kit, Germany) and used for BP recombination reaction.

3.3.2.5 Generation of entry clones

The purified PCR products were used for generating entry clones in pDONR\textsuperscript{TM}/Zeo (Figure 3.5) using BP recombination reaction kit (Life Technologies, USA). The BP recombination mix was prepared in a microfuge tube (Table 3.2).

Table 3.2: BP Reaction Composition

<table>
<thead>
<tr>
<th>S. No</th>
<th>Components</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>attB- PCR product (100ng/ µL)</td>
<td>2.5</td>
</tr>
<tr>
<td>2.</td>
<td>pDONR\textsuperscript{TM}/Zeo Vector (250ng/ µL)</td>
<td>1.0</td>
</tr>
<tr>
<td>3.</td>
<td>BP Clonase enzyme</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>4.0</td>
</tr>
</tbody>
</table>
The reaction mix was incubated at 25°C for 5-6 hours. After incubation, 1 μL of Proteinase K solution was added and incubated at 37°C for 15 minutes and used for *E. coli* transformation.

### 3.3.2.6 Transformation of recombined entry vector to *E. coli*

The *E. coli* competent cells were prepared according to Sambrook and Russell (2001) with slight variation. Single colony was inoculated in 10 mL LB broth and incubated at 37°C overnight. Inoculum (1%) was transferred to 100 mL of LB and incubated at 37°C in an orbital shaker until it reaches absorbance of 0.6-0.8 at OD$_{600nm}$. The culture was prechilled on ice for 30 minutes. The cells were pelleted by centrifugation at 5000 rpm for 10 minutes at 4°C. The pellet was suspended in 4 mL of ice cold 100 mM CaCl$_2$ and centrifuged at 5000 rpm for 10 minutes at 4°C. The cells were finally resuspended in 1 mL of 100 mM CaCl$_2$ and aliquoted 50 μL each and maintained as stocks and stored at -80°C. To 100 μL of *E. coli* DH5α competent cells, 1
μL of BP recombination reaction mixture was added and the cells were mixed gently by tapping 4-5 times and incubated on ice for 30 minutes and then subjected to heat shock treatment at 42°C for 60 seconds. After heat shock, the cells were immediately placed on ice for 2 minutes and 900 μL of SOC media was added. The tubes were finally incubated at 37°C for 1 hour at 225 rpm in orbital shaker. The cultures were spun down shortly and finally the cells were plated on low salt LB media supplemented with Zeocin having a final concentration of 50 mg L⁻¹ (Life Technologies, USA). These plates were incubated at 37°C overnight for the transformants to grow.

3.3.2.7 Confirmation of recombinants by PCR

Transformed colonies were picked and inoculated in 10 mL of sterile low salt LB broth with zeocin (50 mg L⁻¹) and incubated in orbital shaker at 37°C overnight. The plasmids were isolated from the grown colonies as per the manufacturer’s protocol (MN Plasmid DNA Isolation Kit, Germany) and screened with gene specific primers for GmIFS. PCR reactions were set up using the 2X Emerald AmpR PCR master mix. The PCR components and reaction conditions are given below in Table 3.3. The PCR products were separated using 1% agarose gel.

Table 3.3: PCR Composition and Reaction Conditions

<table>
<thead>
<tr>
<th>S. No</th>
<th>PCR Components</th>
<th>Volume (µL)</th>
<th>Reaction Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2X PCR Master mix</td>
<td>10</td>
<td>Initial denaturation at 94°C for 5 Minutes</td>
</tr>
<tr>
<td>2.</td>
<td>1 pM Forward Primer</td>
<td>1</td>
<td>Denaturation at 94°C for 30 seconds</td>
</tr>
<tr>
<td>3.</td>
<td>1 pM Reverse Primer</td>
<td>1</td>
<td>Annealing at 56°C for 30 seconds</td>
</tr>
<tr>
<td>4.</td>
<td>Plasmid DNA</td>
<td>1</td>
<td>Extension at 72°C for 45 seconds</td>
</tr>
<tr>
<td>5.</td>
<td>Nuclease free water</td>
<td>7</td>
<td>Final Extension at 72°C for 10 minutes</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
<td><strong>Hold at 4°C</strong></td>
</tr>
</tbody>
</table>

25 cycles
3.3.2.8 Confirmation of recombinants by restriction digestion

The confirmed plasmids with gene specific PCR are further subjected to restriction digestion. Restriction enzyme was selected based on the restriction mapping tool NEB cutter V2.0 and fragment lengths were calculated. Based on the in silico restriction analysis, plasmid DNA was digested using restriction enzyme Nco I (New England Biolabs, UK). The components of the restriction enzyme digestion mix are shown in the following Table 3.4.

**Table 3.4: Restriction Digestion Reaction Composition**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Components</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Plasmid DNA</td>
<td>10</td>
</tr>
<tr>
<td>2.</td>
<td>10X Restriction buffer</td>
<td>1</td>
</tr>
<tr>
<td>3.</td>
<td>Restriction enzyme Nco I(2000U)</td>
<td>1</td>
</tr>
<tr>
<td>4.</td>
<td>Nuclease free water</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>15</strong></td>
</tr>
</tbody>
</table>

Digestion was performed at 37°C for 3 hours. The digested DNA was separated through 1% agarose gel and results were analyzed and documented.

3.3.2.9 Generation of destination clones

The gateway destination vectors pEARLEY Gate 102 HA and pEAQ- HT Dest 3 were kindly provided by Prof. Julian Ma, St. Georges University of London, UK and from Prof. George P. Lomonossoff, John Innes Centre, UK respectively. The target genes in the entry vector pDONR™/Zeo with the recombination site att L1 and att L2 were used for the LR recombination reaction with the plant expression vector pEARLEY Gate 102 HA (Figure 3.6a) and pEAQ-HT Dest 3 (Figure 3.6b), which have att R1 and att R2
sites. The LR recombination reaction mixture was prepared in a microfuge tube as shown below in Table 3.5.

**Table 3.5: LR Recombination Reaction Mix**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Components</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>pDONR Zeo+IFS (45 ng/ µL)</td>
<td>2.25</td>
</tr>
<tr>
<td>2.</td>
<td>pEARLEY Gate 102 Vector (100ng/ µL) and pEAQ Vector (120ng/ µL)</td>
<td>1</td>
</tr>
<tr>
<td>3.</td>
<td>LR Clonase enzyme</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>3.75</strong></td>
</tr>
</tbody>
</table>

The reaction was left at 25°C 5-6 hours after which Proteinase K was added and incubated at 37°C for 15 minutes.

**3.3.2.10 Transformation of recombined destination vector to E. coli**

The recombined pEARLEY Gate 102 HA and pEAQ-HT with IFS after LR reaction were transformed to *E.coli* competent cells as per the protocol described in the chapter 3.3.2.6. Finally the cells were plated on LB media supplemented with kanamycin.
having a final concentration of 75 mg/L. These plates were incubated at 37°C overnight for the transformants to grow.

3.3.2.11 Confirmation of recombinants by PCR and restriction digestion

The transformed colonies were picked and plasmid DNA was isolated. They were confirmed by gene specific PCR as per the methodology described in the chapter 3.3.2.7. Restriction mapping of the recombinant plasmid was carried out and restriction digestion was performed. The insert in pEARLEY GATE 102 HA and pEAQ-HT vectors were further confirmed using combination of Kpn I - Nco I and only Nco I enzymes (New England Biolabs, UK) respectively. The digestion mixes for the reactions were prepared as described previously in the chapter 3.3.2.8 and the digested products were analysed by 1% agarose gel. The plasmid DNA from the destination clones was further confirmed by DNA sequencing at Bioserve Technologies, Hyderabad.

3.3.2.12 Transformation of recombined plant expression vector construct to Agrobacterium using electroporation

3.3.2.12.1 Preparation of electrocompetent cells

Agrobacterium tumefaciens strain AGL1 was inoculated in 5 mL of YENB medium containing 60 mg L⁻¹ rifampicin and incubated at 28°C for two days. 1 mL culture was transferred to 100 mL of YENB medium + 60 mg L⁻¹ rifampicin and incubated at 28°C for 15-20 hours with continuous shaking (250 rpm) until OD₆₀₀nm reaches 0.6-0.7 absorbance. The cells were then chilled on ice for 15 minutes and spun down by centrifugation at 4,000 rpm for 5 minutes at 4°C. The supernatant was decanted and the cells were washed with 10 mL of sterile distilled water. This step was repeated 3
times and finally the pellet was resuspended in 500 μL of sterile 10% (v/v) glycerol. Aliquots of 100 μL suspensions were dispensed into pre-chilled microcentrifuge tubes and frozen immediately in liquid nitrogen and stored at -80ºC.

3.3.2.12.2 Electroporation

*A. tumefaciens* AGL1 competent cells were mixed with 1 μL of DNA (recombined destination vector) and added to the pre-cooled electroporation cuvette. The mixture was electroporated at 200 ohms, 25 μFD and 2.4 kV using Micro-pulser electroporator apparatus (Bio-Rad, USA). After electroporation, 1 mL of YENB medium was added and incubated at 28ºC for 2 hours in a shaker. The cells were pelleted by centrifugation at 8000 rpm for 2 minutes and supernatant was discarded. Cells were resuspended in 100 μL of LB broth and plated on LB agar medium containing rifampicin 60 mg L⁻¹ and kanamycin 75 mg L⁻¹. The cells were incubated at 28ºC for 2-4 days.

3.3.3 Cloning of *GmIFS* by restriction and ligation

3.3.3.1 Selection of restriction enzymes and designing of primers

Restriction enzymes, which do not have any sites in the gene but have single recognition site in the multiple cloning region of the vector, were selected based on the restriction mapping tool NEB cutter V2.0. The two restriction enzymes chosen were *Kpn* I and *Bst* EII at the forward and reverse ends of the gene to clone into pNUT Kan01 vector (Figure 3.7a). The restriction enzyme recognition sites were incorporated in the primers, which were designed for the target gene. The details of the forward and reverse primers are as follows (Figure 3.7b),
Forward Primer

5’ CGCGTG \textbf{GGTACC} ATG TTG CTG GAA CTT GCA CTT 3’
\textit{Kpn I}

Reverse Primer

5’ CGTCGT \textbf{GGTGACC} AA TTA AGA AAG GAG TTT AGA TGC AAC 3’
\textit{Bst EII}

3.3.3.2 \textit{PCR amplification and purification}

PCR was performed using the conventional primers. The forward and reverse primers containing the restriction recognition site sequences along with the gene specific regions. The reaction mixture was prepared as shown in the Table 3.1 and the conditions were as described in 3.3.2.2. The amplified products were resolved on 1% agarose gel with ethidium bromide. The amplified fragments were gel eluted using the protocol suggested by the manufacturer of Axygen gel elution kit. The concentration of the PCR gel eluted product was checked using the NanoDrop, Thermo Scientific, USA.
### 3.3.3.3 Restriction digestion of purified product and vector

The eluted PCR amplified product and the binary expression vector pNut Kan01 are subjected to restriction digestion with the enzymes *Kpn* I and *Bst* EII (New England Biolabs, UK) in two steps. The reaction mixture for the restriction enzyme digestion is mentioned in the **Table 3.6**.

**Table 3.6: Restriction Digestion Composition**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Components</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Plasmid DNA/ PCR Product</td>
<td>20</td>
</tr>
<tr>
<td>2.</td>
<td>10X Restriction buffer</td>
<td>3</td>
</tr>
<tr>
<td>3.</td>
<td>Restriction enzyme <em>Kpn</em> I(2000U)</td>
<td>2</td>
</tr>
<tr>
<td>4.</td>
<td>Nuclease free water</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>30</strong></td>
</tr>
</tbody>
</table>

The reaction mixture was incubated at 37°C for 3 hours and then heat inactivated at 80°C for 10 minutes. The digested products were resolved on 1% agarose gel electrophoresis and the fragments were gel eluted using the protocol given in Axygen gel elution kit. The purified DNA fragments after digestion with *Kpn* I was subjected to restriction digestion with the second enzyme *Bst* EII (New England Biolabs, UK). The reaction composition was same as described in **Table 3.6** except for the restriction enzyme *Kpn* I, *Bst* EII was added. This reaction mix was incubated at 60°C for 3 hours and then resolved on 1% agarose gel.
3.3.3.4 Ligation of the purified double digested PCR product

The resolved DNA fragments were then gel eluted after the double digestion with respective restriction enzymes using the protocol given in Axygen gel elution kit. The double digested purified PCR product and vector were then joined together by T₄ DNA ligase. The Ligation reaction mixture components are given as follows (Table 3.7):

**Table 3.7: Ligation Reaction Mix**

<table>
<thead>
<tr>
<th>S. No</th>
<th>Components</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10X buffer</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Digested pNut Kan01 Vector (80ng/µL)</td>
<td>1</td>
</tr>
<tr>
<td>3.</td>
<td>Digested PCR Product (29.4 ng/µL)</td>
<td>3</td>
</tr>
<tr>
<td>4.</td>
<td>T₄ DNA ligase</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>5.5</strong></td>
</tr>
</tbody>
</table>

The ligation mixture were then incubated at 16°C overnight in a thermocycler and then transformed into *E. coli*. The product was stored in -20°C till further use.

3.3.3.5 Transformation of the ligated product to *E. coli*

The *E. coli* competent cells were prepared according to Sambrook and Russell (2001) with slight variation. Single colony was inoculated in 10 mL LB broth and incubated at 37°C overnight. Inoculum (1%) was transferred to 100 mL of LB and incubated at 37°C in an orbital shaker until it reaches absorbance of 0.6-0.8 at OD₆₀₀nm. The culture was prechilled on ice for 30 minutes. The cells were pelleted by centrifugation at 5000 rpm for 10 minutes at 4°C. The pellet was suspended in 4 mL of ice cold 100 mM CaCl₂ and centrifuged at 5000 rpm for 10 minutes at 4°C. The cells were finally resuspended in 1mL of 100 mM CaCl₂ and aliquoted 50 µL each and
maintained as stocks and stored at -80°C. To 100 μL of *E. coli* DH5α competent cells, 1 μL of ligated mixture was added and the cells were mixed gently by tapping 4-5 times and incubated on ice for 30 minutes and then subjected to heat shock treatment at 42°C for 60 seconds. After heat shock, the cells were immediately placed on ice for 2 minutes and 900 μL of SOC media was added. The tubes were finally incubated at 37°C for 1 hour at 225 rpm in orbital shaker. The cultures were spun down shortly and finally the cells were plated on LB media supplemented with kanamycin having a final concentration of 75 mg L⁻¹ (Life Technologies, USA). These plates were incubated at 37°C overnight for the transformants to grow.

### 3.3.3.6 Confirmation of recombinants by PCR

Transformed colonies were picked and inoculated in 10 mL of sterile LB broth with kanamycin (75 mg L⁻¹) and incubated in orbital shaker at 37°C overnight. The plasmids were isolated from the grown colonies as per the manufacturer’s protocol (MN Plasmid DNA Isolation Kit, Germany) and screened with gene specific primers for *GmIFS*. PCR reactions were set up using the 2X Emerald Amp<sup>R</sup> PCR master mix. The PCR components and reaction conditions are the same as mentioned in **Table 3.3**. The PCR products were separated using 1% agarose gel.

### 3.4 Results and Discussion

#### 3.4.1 GmIFS gene sequence

The *GmIFS* cDNA was procured from Dr. Oliver Yu, Donald Danforth Plant Science Center, USA bearing the Gen Bank Accession number AF195798. The complete gene sequence with the coding region is given below (**Table 3.8**):
Table 3.8: *GmIFS* Gene Sequence

>gi|6979519|gb|AF195798.1| Glycine max isoflavone synthase 1 (ifs1) mRNA, complete cds (67-1632)

```
GTAATTAACTCACTCAAAAATCGGGATCACAGA
AACCAACAACAGTTCTTGCACTGAGGTTTCACG
ATGTGCTGGAACTTGCACTTGGTTTTGTGTTAGCTTTTCTGCACCTTGCTGCCACCAACCAAGTGCAAAA
ATCAAAAGCATTCTGCCACCTCACAACAAACCTCCAAGGCCAACAAAGCTCCTCTCTCCCTCTCCCTCCAAAC
CCACGAGGCAACTTCCCTTACCAAAACAGGTTCCTCCACATAAGGGAAGGCTTCTCTACAAATGCAAGACCAAC
ATCAAAAGGACAGAAGGGAATCCTTCCTTCAATGGCAGGTTCGCTCTCTACAAATGCAAGACCAAC
```

3.4.2 Cloning of *GmIFS* using gateway technology

Firstly, the primer annealing temperature was optimized for the *GmIFS*. Among the temperatures tested, 56ºC was found to be optimum. The PCR conditions were optimized using equal primer concentrations (forward primer- 1 pM, external forward,
reverse primer- 1 pM). The PCR reaction was initiated which facilitated the addition of attB1 sites at the 5’ end and attB2 site at the 3’ end. The triumph of cloning for gene expression studies mainly depends on generating proof read error free PCR products for which Phusion DNA polymerase (New England Biolabs) was used. The co-expression of novel Pyrococcus-like enzyme with a processivity-enhancing domain is responsible for high efficiency of the enzyme. The phusion DNA polymerase generates products with the error rate of 4.4x10\(^{-7}\), which is approximately 50 fold lower than Thermus aquaticus DNA polymerase and 6-fold lower than that of Pyrococcus furiosus DNA polymerase. Phusion DNA polymerase also possesses 5’→3’ DNA polymerase activity and 3’→5’ exonuclease activity, so that it generates blunt ends in the amplification products. The PCR products were separated through 1% agarose gel and the amplicon size was as expected (Figure 3.8).

![Image of agarose gel](image.png)

**Figure 3.8 PCR Amplification of GmIFS**

### 3.4.3 Generation of entry clones and confirmation by PCR and restriction enzyme digestion

The gateway cloning method was used in the current study due to its advanced technology involving recombination reactions, accuracy and rapidness of cloning than the
usual conventional cloning methods. The purified PCR products (GmIFS) were first cloned in the entry vector pDONR™/Zeo by BP reaction. The BP reaction involves the transfer of gene of interest flanked by attB1 and attB2 sites into a donor vector, which has attP1 and attP2 sites that yielded an entry or master clone with attL1 and attL2 sites and is catalyzed by the BP Clonase mix that contains the combination of the bacteriophage λ Integrase (Int) and Excisionase (Xis) proteins along with E. coli Integration Host Factor (IHF). These enzymes bind to specific sequences (att sites), bringing together the target sites, cleave them and covalently attach the DNA. The recombined products were transferred to E. coli DH5α cells after the BP recombination reaction. Colonies were selected randomly for plasmid isolation. PCR was done using IFS gene specific primers to validate the plasmid for positive colonies. A band at the expected size of 1500 bp proved the presence of target gene in entry vector (Figure 3.9).

![Figure 3.9 PCR Confirmation of GmIFS Clones](image)

The Plasmid DNA isolated was further subjected to restriction digestion to confirm the orientation of target gene (GmIFS) in the entry vector. The restriction
mapping was carried out using online tool Neb cutter. \textit{Nco} I restriction enzyme was used to confirm the presence of insert in the entry vector (Figure 3.10).

![Figure 3.10 Restriction Digestion of \textit{GmIFS} –pDONR/Zeocin Construct with \textit{Nco} I enzyme](image)

3.4.4 Generation of destination clones

LR recombination reactions were carried out for generating destination clones. Recent literature suggested that competence of the LR recombination reaction was 30\% and the most favorable efficiency was shown when super coiled \textit{att} \textit{L} with linear \textit{att} \textit{R} sites were used for the reaction. The transformants were screened using LB + kanamycin and LB + chloramphenicol plates where the colonies were observed only in LB + kanamycin but not in LB + chloramphenicol plates indicating a successful LR recombination reaction due to the recombination of transgene to the destination vector in place of \textit{CmR} gene. High efficiency LR recombination products are resulted when equimolar concentration of entry clone and pEARLEY vector was used. If the ratio is not equimolar, it leads to co-integration of the destination vector, which may react with the pEARLEY vector and entry clone resulting in a higher background. pEAQ destination
vector was also used for transient expression of the IFS in a short span of time. The main advantage of pEAQ vector is the high expression of target genes due to presence of p19 sequence and CPMV-HT in the T-DNA region of vector. There is no requirement for co-infiltration with a separate suppressor-expressing construct as the suppressor of silencing (p19) and the target gene are in a single plasmid. The presence of 5’ and 3’ UTR region also called as hyper translation (HT) region and p19 have the ability to enhance the recombinant protein expression (Voinnet et al., 2003). Finally plasmids were confirmed for the presence of *GmIFS* with gene specific PCR (Figure 3.11) and orientation of the target gene by using the combination of restriction enzymes *Kpn* I - *Nco* I and *Nco* I only for pEARLEY and pEAQ destination clones respectively (Figure 3.12).

**Figure 3.11 PCR Confirmation of Destination Clones**
3.4.5 Confirmation of the destination clones by sequencing

The plasmids from the destination clones were aliquoted along with the gene specific primers for \textit{GmIFS} and sequenced at Bioserve technologies, Hyderabad. The chromatopherograms obtained after sequencing were read using codon code aligner software version 5.0.2 (USA) (Figure 3.13). The forward and reverse sequences were merged after editing into the FASTA format. Using the NCBI Nucleotide BLAST, these sequences was analysed for the 100% similarity with the available sequences of organisms by the search hits (Figure 3.14).
Figure 3.13 Chromatopherogram of sequenced samples

Figure 3.14 NCBI Nucleotide BLAST for Destination Clone Sequence
3.4.6 Transformation of recombined destination vector to *Agrobacterium* by electroporation

Plant expression vector pEARLEY 102 harboring *GmIFS* gene was mobilized to *Agrobacterium* AGL1 by electroporation. Electroporation is the use of short high-voltage pulses, which helps to beat the capacitance of the cell membrane inducing reversible breakdown of the membrane. This transient, permeabilized state of the membrane allows the passageway of the DNA into the cell (Gehl, 2003). After electroporation, transformed *Agrobacterium* colonies were selected with 75 mg L\(^{-1}\) kanamycin and 60 mg L\(^{-1}\) rifampicin and confirmed by colony PCR (Figure 3.15) using the gene specific primers. Single colony was selected and subcultured and used for the genetic transformation studies in onion and green leafy vegetables.

![Figure 3.15 Colony PCR of Agrobacterium Clones](image)

Lane 1: Positive control
Lane 2-9: *Agrobacterium* clones
Lane 11: DNA Marker
Lane 10: Negative control

**Figure 3.15 Colony PCR of Agrobacterium Clones**
3.4.7 Restriction-Ligation cloning of GmIFS

Apart from the advanced techniques of cloning, the usual laborious restriction-ligation cloning was also employed to construct the expression vector harboring GmIFS gene. The GmIFS cDNA was amplified with the primers having restriction sites for the enzymes Kpn I and Bst EII (Figure 3.16a). The resulting PCR product with the recognition sites for the enzymes and the pNut Kan01 vector was digested with the enzymes, so that the products will generate cohesive sticky ends at 5’ and 3’ ends (Figure 3.16b). The sticky ends will enable high efficiency joining of the phosphate group to the hydroxyl groups. T4 DNA ligase enzyme catalyzes the joining of two strands of DNA between the 5’-phosphate and 3’-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt ended configuration. It does not facilitate the joining of single stranded DNA fragments. The ligated product was transformed into E.coli. The plasmid isolated from the transformed colonies was confirmed by gene specific PCR using the GmIFS primers (Figure 3.16c).

3.5 Conclusion

In conclusion, GmIFS was cloned in plant expression vectors pEARLEY GATE 102 HA and pEAQ-HT Dest 3 using the gateway cloning technology and the expression vector was moved to Agrobacterium tumefaciens AGL1. By using the traditional restriction-ligation based cloning the GmIFS was also cloned in the pNut Kan01 vector. The gateway-cloned vectors were further used for the stable and transient expression of the gene in onion and green leafy vegetables.
Figure 3.16 Conventional Cloning of *GmIFS*